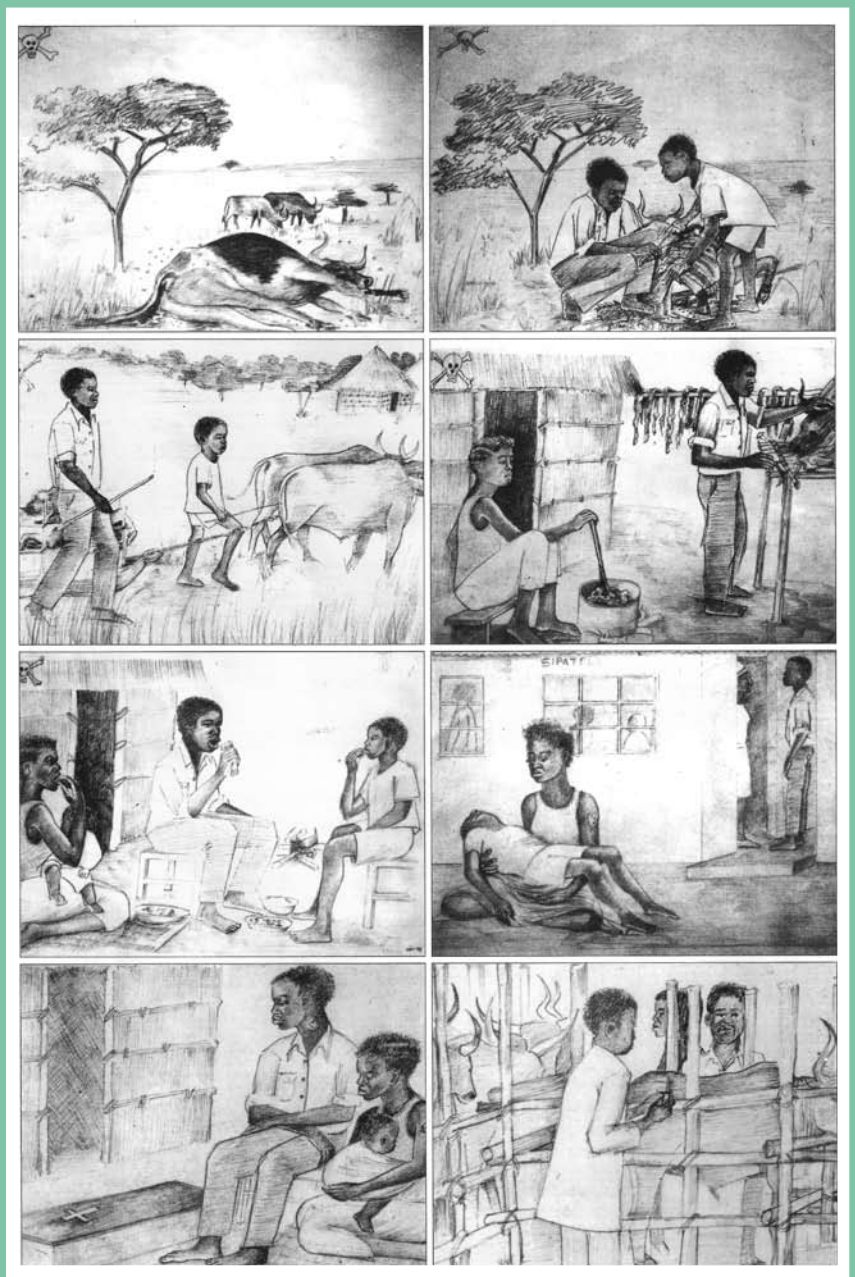
FOURTH EDITION

Anthrax
in humans and animals



World Organisation for Animal Health

World Health Organization

Food and Agriculture Organization of the United Nations

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Front cover illustration: The pictures are from a book, and also featured on a poster, conveying the story of the problems the fictional Liseli family suffered following the death of their cows from anthrax. The message to farmers/owners of livestock is that they should not handle and butcher the carcasses of animals that have died unexpectedly or eat meat from such carcasses, but rather that they should call a veterinary official to supervise correct disposal of the carcass. (Source: Dietvorst, 1996b.)

Back cover photograph: Scanning electronmicrograph of spores of *B. anthracis* isolated from an elephant (LA[1]25.04.91RD) that had died of anthrax in 1991 in the Etosha National Park, Namibia (Turnbull et al., 1998b). The long axis of the spore is just over 1 µm (0.001 mm).

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[1] The executive editor has signed a declaration of interest in accordance with WHO policy and no conflict of interest was declared. The declaration of interest is available on request.

[2] We deeply regret the untimely death of Dr Regula Leuenberger. She contributed to the successful completion of this project and her dedication and commitment to her work will be greatly missed by her colleagues.

Development of these guidelines

This fourth edition of the anthrax guidelines encompasses a systematic review of the extensive new scientific literature and relevant publications up to end 2007, as well as the conclusions of an expert meeting[1] held in Nice, France, in 2003. Draft versions of this new edition have undergone two rounds of a rigorous peer-review process. For many sections of the document, contributions from scientists with specific areas of expertise were actively sought and the draft versions were sent to these contributors for their corrections and approval.

WHO is planning to develop a training package to support Member States in applying this guidance. Although new scientific information on anthrax is expected to continue to become available, this document should remain a relevant source of information for the management of anthrax in humans and animals for at least the next five years. WHO therefore plans to review these guidelines within the next five to seven years.

Despite a process of wide consultation and extensive peer review, it is recognized that there is always room for improvement when developing guidance of this nature. Feedback from users in the form of comments on the scientific contents, usefulness and accessibility, and suggestions for improvements, are especially welcome.

[1] *Improving public health preparedness for and response to the threat of epidemics: anthrax network*. Report of a WHO meeting, Nice, France, 29–30 March 2003. Geneva, World Heath Organization, 2003 (document WHO/CDS/CSR/GAR/2003.9; http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_CSR_GAR_2003_9/en/).

Preface

Anthrax essentially ceased to be regarded as a disease of major health or economic importance after the enormous successes of Max Sterne's veterinary vaccine developed in the 1930s, and subsequent analogs in the former Soviet Union, in dramatically reducing the incidence of the disease in livestock throughout the world in the ensuing two decades. The 1980s saw a resurgence of interest in the disease, partly stimulated by a renewed focus on *Bacillus anthracis*, the agent of anthrax, as a potential agent for a biological weapon, after the largest reported outbreak of human inhalational anthrax that took place in 1979 in the city then called Sverdlovsk (now Ekaterinburg) in the former Soviet Union, and partly because of increasing recognition that anthrax had by no means "gone away" as a naturally occurring disease in animals and humans in many countries.

The production of the first edition of what has now become familiarly referred to as the "WHO anthrax guidelines" was written by Howard Whitford in 1987,[1] reflecting the new interest in anthrax in the 1980s. It was not formally published but was a highly regarded and timely reference document. The same new focus on anthrax in the 1980s led to the formation of a WHO working group on anthrax,[2] and the second edition of the "WHO anthrax guidelines" in 1993[3] was the direct result of a resolution by the working group to update Dr Whitford's edition. The second edition again was not formally published and, with no decline in the global interest in anthrax, it was again updated and on this occasion formally published in 1998 as the third edition.[4] By this time, the World Wide Web was well established, and the third edition has been accessible electronically[5] as well as in printed form.

The present fourth edition was commissioned in April 2001, initially as a simple update and expansion of the highly popular third edition. It was well on its way to completion when the notorious "anthrax letter events" took place in the USA in September to December that same year. The result of these events was a massive surge in critical analyses of detection, diagnostic, epidemiological, decontamination, treatment and prophylaxis procedures for anthrax, accompanied by a rapid and equally massive rise in the number of associated research activities receiving unprecedented levels of funding. The fourth edition was consequently put on hold until the results of these new analyses and research activities had become available.

Thus the fourth edition is now being issued seven years after it was commissioned, but it is certainly greatly improved as a result of the new information that emerged in the 3–4 years after the anthrax letter events. Formally dedicated to Max Sterne, whose remarkable work in the 1930s made naturally occurring anthrax a controllable disease, it is also dedicated to the 22 persons who contracted anthrax as a result of the anthrax letter events, and particularly to the memory of the five who regrettably died from their infections, as well as to the unknown number of people that become sick and die of the naturally occurring disease, mainly among the poor in developing countries.

Peter Turnbull
Executive editor

[1] *A guide to the diagnosis, treatment, and prevention of anthrax, 1987* (WHO/Zoon./87.163).
[2] *Report of a WHO consultation on anthrax control and research, 1991* (WHO/CDS/VPH/91.98).
[3] *Guidelines for the surveillance and control of anthrax in humans and animals*, 1993 (WHO/Zoon./93.170).
[4] *Guidelines for the surveillance and control of anthrax in humans and animals*, 1998 (WHO/EMC/ZDI./98.6).
[5] http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/.

This work is dedicated to the memory of Max Sterne (1905–1997).
He knew much of what we subsequently discovered and his wisdom is still a guiding light for those addressing the issues and problems of anthrax.

Summary

1. The disease and its importance

The natural disease

Anthrax is primarily a disease of herbivores. Humans almost invariably contract the natural disease directly or indirectly from animals or animal products.

Deliberate release

Bacillus anthracis has always been high on the list of potential agents with respect to biological warfare and bioterrorism. It has been used in that context on at least two occasions, prepared for use on several other occasions and been the named agent in many threats and hoaxes.

2. Etiology and ecology

Etiology

Anthrax is caused by the bacterium *Bacillus anthracis*.

Cycle of infection

The infected host sheds the vegetative bacilli onto the ground and these sporulate on exposure to the air. The spores, which can persist in soil for decades, wait to be taken up by another host, when germination and multiplication can again take place upon infection. Flies appear to play an important role in large outbreaks in endemic areas.

Humans acquire anthrax from handling carcasses, hides, bones, etc. from animals that died of the disease.

Influencing factors

The cycle of infection is influenced by (i) factors that affect sporulation and germination, such as pH, temperature, water activity and cation levels; and (ii) factors related to the season, such as available grazing, the health of the host, insect populations and human activities.

3. Anthrax in animals

Infectious dose in animals

LD_{50}s range from < 10 spores in susceptible herbivores to > 10^7 spores in more resistant species when administered parenterally. However, *B. anthracis* is not an invasive organism and by inhalational or ingestion routes, LD_{50}s are in the order of tens of thousands, even in species regarded as susceptible. The relationship between experimentally determined LD_{50}s and doses encountered by animals acquiring the disease naturally is poorly defined.

Global incidence in animals

National programmes have resulted in a global reduction of anthrax, although this is counteracted by the failure of more recent generations of veterinarians, farmers, etc., through lack of experience, to recognize and report the disease, and the abandonment of vaccination. The disease is still common in some Mediterranean countries, in small pockets in Canada and the USA, certain countries of central and South America and central Asia, several sub-Saharan African countries and western China. Sporadic cases and outbreaks continue to occur elsewhere.

Transmission in animals

- It is a long-held belief that animals generally acquire anthrax by ingestion of spores while grazing or browsing. However, anomalies in the epizootiology of the disease often arise that are hard to explain in terms of simple ingestion of spores.
- Flies appear to play an important role in explosive outbreaks.
- Inhalation within dust may be important on occasion.
- Direct animal-to-animal transmission is believed to occur to an insignificant extent, excluding carnivores feeding on other victims of the disease.
- Human activities in the form of trade have long

been responsible for the spread of the disease globally.
- The age, sex and condition of animals may all influence the incidence of the disease at any one site.
- Carriage or latent infection are unproven states but have not been disproved either.
- Many anomalies and unknowns remain in our understanding of how animals acquire anthrax and how it is transmitted.

Deliberate release

Examples exist where alleged or suspected deliberate infection of animals with anthrax has been carried out for bioaggressive purposes. There is one instance on record of accidental release of anthrax spores from a microbiology laboratory resulting in deaths in livestock downwind.

Clinical manifestations

These vary somewhat from species to species, presumably reflecting differences in susceptibility. First signs in the more susceptible livestock species are one or two sudden deaths within the herd or flock with retrospective recall of preceding mild illness. In more resistant species, local signs such as swellings of the oral and pharyngeal region are seen. In wildlife, sudden death is the invariable sign, often (but not always) with bloody discharges from natural orifices, bloating, incomplete *rigor mortis* and the absence of clotting of the blood.

Diagnosis

For most circumstances the simplest, quickest and best on-site diagnostic method is the one established in the early 1900s – examination of a polychrome methylene blue-stained blood smear for the capsulated bacilli supported, where possible, with culture back-up. Modern on-site anthrax-specific antigen tests have been devised but remain to be developed commercially. Genetically-based confirmation by the polymerase chain reaction (PCR) is becoming increasingly accepted on a stand-alone basis for many types of specimen and is increasingly available worldwide through commercial kits. Retrospective diagnosis by enzyme-linked immunosorbent assay (ELISA) in animals that have survived infection is possible, but specific antigen for this is expensive and the test is more a research tool than of practical day-to-day value in the field.

4. Anthrax in humans

Human incidence

Naturally acquired human anthrax infection generally results from contact with infected animals, or occupational exposure to infected or contaminated animal products. The incidence of the natural disease depends on the level of exposure to affected animals.

Reported animal:human case ratios in a country or region reflect the economic conditions, quality of surveillance, social traditions, dietary behaviour, etc. in that country or region.

In contrast to animals, age- or sex-related bias is generally not apparent in humans, though males generally have higher occupational risk rates in many countries.

Infectious dose in humans

The evidence is that humans are moderately resistant to anthrax but that outbreaks occur.

Infectious doses are difficult to assess but in individuals in good health and in the absence of lesions through which the organism can gain ready access, ID_{50}s are generally in the thousands or tens of thousands and anthrax is not considered a contagious disease.

Epidemiology and transmission

Anthrax in humans is traditionally classified in two ways: (i) based on how the occupation of the individual led to exposure differentiates between *non-industrial anthrax*, occurring in farmers, butchers, knackers/renderers, veterinarians, etc., and *industrial anthrax*, occurring in those employed in the processing of bones, hides, wool and other animal products; (ii) reflecting the route by which the disease was acquired. This distinguishes between *cutaneous anthrax*, acquired through a skin lesion, *ingestion (oral route) anthrax*, contracted following ingestion of contaminated food, primarily meat from an animal that died of the disease, and *inhalational anthrax*, from breathing in airborne anthrax spores.

Non-industrial anthrax, resulting from handling infected carcasses, usually manifests itself as the cutaneous form; it tends to be seasonal and parallels the seasonal incidence in the animals from which it is contracted. Cutaneous anthrax transmitted by insect bites and anthrax of the alimentary canal from eating infected meat are also non-industrial

forms of the disease. Industrial anthrax also usually takes the cutaneous form but has a far higher probability than non–industrial anthrax of taking the inhalational form as a result of exposure to spore-laden dust.

Deliberate release

B. anthracis has always been high on the list of potential agents with respect to biological warfare and bioterrorism. This reflects the ability of humans to generate massive exposures that vastly exceed the maximum exposure that can occur naturally. This means that there is no conflict between, on the one hand, the statements that humans are moderately resistant to anthrax infection and, on the other hand, the choice of anthrax spores by an aggressor. Important lessons have been learnt from the anthrax letter events in the USA in 2001.

Clinical manifestations

Cutaneous anthrax accounts for > 95% of human cases worldwide. The lesions (eschars) are generally found on exposed regions of the body almost invariably accompanied by marked oedema extending some distance from the lesion. The incubation period ranges from a few hours to 3 weeks, most often 2 to 6 days. Although antibiotic treatment will rapidly kill the infecting bacteria, the characteristic lesion will take several days to evolve and possibly weeks to fully resolve, presumably reflecting toxin-induced damage and repair. Clinicians need to be aware of the delay in resolution and not prolong treatment unnecessarily or resort prematurely to surgery.

Ingestion or oral route anthrax takes two forms – *oropharyngeal*, in which the lesion is localized in the buccal cavity or on the tongue, tonsils or posterior pharyngeal wall, and *gastrointestinal*, where the lesion may occur anywhere within the gastrointestinal tract, but mostly in the ileum and caecum. Sore throat, dysphagia and regional lymphadenopathy are the early clinical features associated with oropharyngeal anthrax, with development of extensive oedematous swelling of the neck and anterior chest wall. Tracheotomy may be necessary. Symptoms of gastrointestinal anthrax are initially nonspecific and include nausea, vomiting, anorexia, mild diarrhoea and fever. These may be mild but are occasionally severe, progressing to haematemesis, bloody diarrhoea and massive ascites. The incubation period is commonly 3–7 days.

In inhalational (pulmonary) anthrax, symptoms prior to the onset of the final hyperacute phase are also nonspecific, and suspicion of anthrax depends on knowledge of the patient's history. Symptoms include fever or chills, sweats, fatigue or malaise, non-productive cough, dyspnoea, changes in mental state including confusion, and nausea or vomiting. Chest X-rays reveal infiltrates, pleural effusion and mediastinal widening. Mediastinal lymphadenopathy is likely. The median incubation period is 4 days (range 4–6 days) but may be up to 10 or 11 days. Reflux of spores into the alimentary tract with development of lesions there may affect the time of onset of symptoms.

Meningitis (haemorrhagic leptomeningitis) is a serious clinical development which may follow any of the other three forms of anthrax. The clinical signs are neck pain with or without flexion, headache, changes in mental state, vomiting and high-grade fever. There is an intense inflammation of the meninges with accompanying oedema resulting in a markedly elevated cerebrospinal fluid (CSF) pressure and the appearance of blood in the CSF.

In any of the forms sepsis may develop after the lymphohaematogenous spread of *B. anthracis* from a primary lesion. The mild initial phase of nonspecific symptoms is followed by the sudden development of toxaemia and shock with dyspnoea, cyanosis, disorientation with coma and death, all occurring within a period of hours.

Differential diagnosis

The differential diagnosis of the anthrax eschar of cutaneous anthrax includes a wide range of infectious and non-infectious conditions: boil (early lesion), arachnid bites, ulcer (especially tropical); erysipelas, glanders, plague, syphilitic chancre, ulceroglandular tularaemia; clostridial infection; rickettsial diseases; orf, vaccinia and cowpox, rat bite fever, leishmaniasis, ecthyma gangrenosum or herpes. Generally these other diseases and conditions lack the characteristic oedema of anthrax. The absence of pus, the lack of pain and the patient's occupation may provide further diagnostic clues.

In the differential diagnosis of oropharyngeal anthrax, diphtheria and complicated tonsillitis, streptococcal pharyngitis, Vincent angina, Ludwig angina, parapharyngeal abscess and deep tissue infection of the neck should be considered.

The differential diagnosis in gastrointestinal

anthrax includes food poisoning (in the early stages of intestinal anthrax), acute abdomen due to other reasons, and haemorrhagic gastroenteritis due to other microorganisms, particularly necrotizing enteritis due to *Clostridium perfringens* and dysentery (amoebic or bacterial).

Inhalational anthrax may be confused with mycoplasmal pneumonia, legionnaires' disease, psittacosis, tularaemia, Q fever, viral pneumonia, histoplasmosis, coccidiomycosis, or malignancy.

Confirmation of diagnosis

With early cutaneous anthrax lesions, vesicular fluid in the untreated patient will yield *B. anthracis* on culture and reveal capsulated bacilli in appropriately stained smears. In older lesions, detection in smears or by culture requires lifting the edge of the eschar with tweezers and obtaining fluid from underneath. The fluid will probably be sterile if the patient has been treated with an antibiotic.

In the case of pulmonary or gastrointestinal forms, where the history has not led to suspicion of anthrax, confirmatory diagnosis will usually take place after the patient has died or, if correct treatment is initiated early enough, after recovery. Blood culture is useful and culture from sputum in suspected inhalational anthrax or from vomitus, faeces and ascites in suspected intestinal anthrax should be attempted. Also where appropriate, culture should be attempted from pulmonary effusions, CSF or other body fluids or tissues. Culture may not always be successful, but would be definitive when positive. In patients who have received prior antibiotic therapy, culture is likely to be unsuccessful and confirmation of diagnosis will be difficult without the tests only available in specialized laboratories. The conclusion from analyses of the October–November 2001 anthrax letter events in the USA was that nasal swabs were not useful as clinical samples.

Techniques available in specialized laboratories for retrospective confirmation of diagnosis are serology, immunohistochemistry, tests for anthrax-specific protective antigen and PCR. In the Russian Federation, Anthraxin^{T} is widely used for retrospective diagnosis.

Prognosis

Essentially any of the forms of anthrax is treatable if the diagnosis is made early enough and with the appropriate supportive therapy. In the non-cutaneous forms, the problem of making the correct early diagnosis is particularly difficult, so these are associated with particularly high mortality. Following recovery, resolution of small- to medium-size cutaneous lesions is generally complete with minimal scarring. With larger lesions, or lesions on mobile areas (e.g. eyelid), scarring and contractures may require surgical correction to return normal functioning and large cutaneous defects may require skin grafting. Some patients recovering from the 2001 anthrax letter events in the USA complained of long-term fatigue and problems with short-term memory. No organic basis for these complaints has been identified but they indicate that convalescence may require weeks to months depending on the severity of illness and patient-related factors.

5. Pathogenesis and pathology

Pathogenesis

Much of our understanding about the pathogenesis of anthrax dates from excellent pathological studies in the 1940s to 1960s. The histopathological and bacteriological events following uptake of spores percutaneously or by inhalation or following ingestion, and the differences apparent when relatively susceptible and relatively resistant host species are compared, were well described.

The focus of attention during the 1980s was largely on how the three toxin components, the Protective Antigen (PA), the Lethal Factor (LF) and the Oedema Factor (EF) interact with each other and with host cells, and the effects of these interactions. The relationship between these effects and death from anthrax has been the target of interest in the 1990s and to the present day, with the latest opinion being that death results from the toxin acting through a non-inflammatory mechanism that results in hypoxia. Much remains to be learnt about the details of how the toxin so acts, and also about how it produces the non-lethal manifestations of anthrax, such as the cutaneous lesion. The role of inflammatory cells in the disease process remains to be clarified.

Virulence factors

The two principal virulence factors of *B. anthracis* are the toxin complex and the polypeptide capsule. The function of the capsule in the pathogenesis of the disease is a relatively neglected area and most of the information available on it also dates to pre-1970.

In general terms, its role has long been viewed as protecting the bacterium from phagocytosis, but the details of how this occurs and how it influences the pathogenesis of the disease have not been elucidated. However, new focus is beginning to turn to this again now.

There is reason to believe that other less prominent virulence factors exist, and these are also receiving some attention, albeit usually motivated by a search for vaccine antigen candidates.

6. Bacteriology

The causative agent of anthrax is *Bacillus anthracis*, belonging to the genus *Bacillus*, the Gram-positive, aerobic, endosporeforming rods. In vivo, or under the right in vitro culture conditions of bicarbonate and/or serum and carbon dioxide atmosphere, it produces its polypeptide capsule, which is a reliable diagnostic feature. Capsulated bacilli, often square-ended ("box-car") in appearance and in chains of two to a few, in smears of blood or tissue fluids are diagnostic. In old or decomposed animal specimens, or processed products from animals that have died of anthrax, or in environmental samples, detection requires selective isolation techniques.

Confirmation of identity

Confirmation of identity and differentiation from near relatives is generally easy with both traditional and molecular techniques. PCR depends on the unique specificity of the toxin and capsule and their genes.

Molecular basis

Homologues of genes of phenotypic characters are shared with near relatives but many are not expressed by *B. anthracis* due to truncation of the *plcR* regulatory gene.

Spores and rapid detection

As assessed by heat resistance, sporulation of vegetative forms shed by the dying animal becomes detectable at about 8–10 hours but may not be complete until 48 hours, depending on environmental conditions. Following exposure to a germinant, germination of spores commences rapidly, one report stating that > 99% completion is achieved within 10 minutes at 30 °C. Another study showed 22 °C to be the optimum temperature for germination of *B. anthracis* spores, with 61% to 63% of spores germinating in 90 minutes, and the same study found no correlation between germination rate and the innate resistance of an animal to anthrax.

Numerous attempts were made in the 1960s, 1970s and 1980s to develop antigen-based rapid, species-specific spore detection systems, but invariably cross-reactivity with other common environmental *Bacillus* species proved insurmountable. Claims now exist that anthrax spore-specific epitopes are present on at least an immunodominant exosporium protein.

7. Treatment and prophylaxis

Choices of antibiotics (humans)

Anthrax is responsive to antibiotic therapy provided this is administered early in the course of the infection.

Penicillin has long been the antibiotic of choice but, where this is contraindicated, a wide range of alternative choices exist from among the broad-spectrum antibiotics. Ciprofloxacin and doxycycline have received high profiles as primary treatment alternatives in recent years. Doxycycline has the disadvantage of penetrating poorly to the central nervous system (CNS).

Concerns about penicillin resistance are probably overstated in recent literature. Reports of case-treatment failures due to penicillin resistance number two or three in all of history. However, since it has been demonstrated that penicillin resistance can be induced in at least some strains, the fundamental principle is that adequate doses should be administered when penicillin is being used for treatment.

In severe life-threatening cases, intravenous penicillin or another chosen primary antibiotic – for example, ciprofloxacin – may be combined with another antibiotic, preferably one which also has good penetration into the CNS. Clarithromycin, clindamycin, vancomycin or rifampicin are suggested as supplementary antibiotics for inhalational anthrax and streptomycin, or other aminoglycosides, for gastrointestinal anthrax; vancomycin or rifampicin are suggested for anthrax meningitis.

Ciprofloxacin and doxycycline are generally not considered suitable for children (< 8 to 10 years of age) and should only be used in this age group in emergency situations. Penicillin (in combination with rifampicin or vancomycin in life-threatening infections) is suitable for pregnant women and nursing mothers; as with children, ciprofloxacin or doxy-

cycline (again in combination with rifampicin or vancomycin) may be considered in emergency situations, possibly switching to amoxicillin when sensitivity tests indicate that this is appropriate.

Generally, immunocompromised persons can be given the same treatment as immunocompetent persons, but special consideration may be needed for patients with renal or hepatic insufficiency.

Duration of treatment in naturally occurring anthrax

The infecting *B. anthracis* is generally killed very rapidly by antibiotic therapy but the clinical effects of the toxin may continue for some time afterwards. The clinician should be aware of this in deciding the duration of treatment. Suggested durations are 3–7 days for uncomplicated cutaneous anthrax and, in the absence of clinical experience with short-course antibiotic therapy in systemic anthrax, 10–14 days in cases of systemic anthrax. (See also *Exposure during deliberate release* below.)

Supportive treatment and care

In severe systemic infection, symptomatic treatment in an intensive treatment unit in addition to antibiotic therapy may be life-saving.

Opinions differ on the value of corticosteroids.

Immunotherapy

Treatment of anthrax with hyperimmune serum, developed in various animal species, predates antibiotics by several decades. It was considered effective and is still an available treatment in China and the Russian Federation. Hyperimmune globulin from human vaccinees and monoclonal antibody-based approaches to passive protection are now being revisited. Novel concepts under consideration for late-stage therapy include, for example, humanized monoclonals targeting toxin component interactions.

Exposure during deliberate release

Where known or suspected inhalation of substantial numbers of anthrax spores has occurred, if a non-live vaccine is available, simultaneous administration of vaccine and antibiotics may be considered, with the antibiotic prophylaxis continued for about 8 weeks to allow for development of adequate vaccine-induced immunity. (This would not be applicable in the case of the live human vaccines in China and the Russian Federation.)

Treatment in animals

Penicillin, together with streptomycin if thought advisable, is the treatment of choice for animals showing clinical illness thought to be due to anthrax. A few countries, however, do not permit antibiotic treatment of livestock for anthrax, requiring slaughter with appropriate disposal instead.

Prophylaxis (vaccines)

Control of anthrax begins with control of the disease in livestock, and vaccination of livestock has long been the hub of control programmes. Livestock anthrax vaccines are available in most countries, particularly those that experience outbreaks or sporadic cases on an annual basis.

Vaccines for administration to humans are only produced in four countries and are nominally for use only in persons in at-risk occupations within their borders. Their availability is, consequently, very restricted at present.

Prophylaxis (antibiotic)

Prolonged antibiotic prophylaxis is only recommended for persons known to have been, or strongly suspected of having been, exposed to very substantial doses of aerosolized spores in a deliberate release scenario. Antibiotics generally should not be administered prophylactically for other situations and should only be used for treatment, not prophylaxis, unless there is a real danger of a very substantial exposure having taken place. This is unlikely to be the case in most natural exposure scenarios (as opposed to a human-made situation). Where sufficient fear of a substantial exposure in a natural situation exists (e.g. consumption of meat from a poorly cooked anthrax carcass), antibiotic prophylaxis may be considered but should only be of ± 10-day duration. In other suspected natural exposure situations, the relevant medical personnel should be notified and the individual(s) concerned should report to them immediately for treatment should a spot/pimple/boil-like lesion develop, especially on exposed areas, or flulike symptoms appear.

Where possible exposure is anticipated, but has not yet happened (e.g. preparing to dispose of carcasses in an outbreak), use of proper personal protec-

tion methods is the correct approach, not antibiotic prophylaxis.

8. Control

The basis of control and, in the case of livestock, eradication of anthrax is breaking the cycle of infection. If a potential infectious source is known to exist, this should be eliminated without delay. Recalling that natural anthrax is primarily a disease of herbivorous mammals, control largely centres around control in livestock.

In the event of a case or outbreak occurring in livestock, control measures consist of correct disposal of the carcass(es), decontamination of the site(s) and of items used to test and dispose of the carcass(es), and initiation of treatment and/or vaccination of other animals as appropriate.

The best disposal method is incineration.

Livestock vaccines are available in most countries. Vaccines for humans, in contrast, are not widely available.

Enzootic wildlife areas with hands-off management policies only exert anthrax control measures in emergency situations, or when endangered species are threatened. This has to be viewed slightly differently in the case of smaller or commercial game management areas. Intersectoral cooperation is important in areas where such wildlife areas abut with livestock areas, or where wildlife and livestock intermingle.

9. Anthrax surveillance

Effective *surveillance* is essential to prevention and control programmes for anthrax and encompasses mechanisms for disease detection, confirmation of diagnosis, reporting, collation of data and feedback of the data to the source.

Reporting requires mechanisms for easy communication of cases and also some incentives for reporting or disincentives for not reporting.

Control of anthrax among humans depends on the integration of veterinary and human health surveillance and control programmes. Routine cross-notification between the veterinary and human health surveillance systems should be part of any zoonotic disease prevention and control programme, and close collaboration between the two health sectors is particularly important during epidemiological and outbreak investigations.

The primary objectives of any anthrax surveillance system are to prevent or reduce livestock losses and to prevent human disease. The cardinal actions to achieve this are:

- education of both those who will be involved in the surveillance and all who own or handle livestock, meat, hides and other animal products;
- correct diagnosis;
- implementation of control measures;
- reporting.

These need to be supported by well-formulated case definitions (animal and human) and appropriate laboratory back-up.

1. The disease and its importance

1.1 The natural disease

Anthrax is primarily a disease of herbivores although few, if any, warm-blooded species are entirely immune to it. According to Sterne (1959), amphibians and reptiles are naturally resistant but warming of cold-blooded animals allows them to be experimentally infected. From the earliest historical records until the development of an effective veterinary vaccine midway through the present century (Sterne, 1937b; Sterne et al., 1939), and the subsequent advent of antibiotics, the disease was one of the main causes of uncontrolled mortality in cattle, sheep, goats, horses and pigs worldwide. Reports of the World Organisation for Animal Health (OIE)[1] show that the disease is still enzootic in most countries of Africa and Asia, a number of European countries and countries/areas of North and South America; it still occurs sporadically in many other countries.

1.2 Deliberate release – bioaggression

Bacillus anthracis has always been high on the list of potential agents with respect to biological warfare and bioterrorism (Klietmann & Ruoff, 2001; see also section 3.3.10) and as is well known, it has now been used in that context in the anthrax letter events in 2001 in the USA with severe consequences (Jernigan et al., 2001; Inglesby et al., 2002).[2] This, unfortunately, has attached an unjustified doomsday image to the naturally-occurring disease. It is important however to clearly distinguish the natural and deliberate release situations: the massive exposures that can be created in man-made deliberate release scenarios cannot be remotely emulated by nature. The natural disease is readily controllable in contrast to the biowarfare/bioterrorist act (see also section 4.2.3).

[1] OIE World Animal Health Information Database (WAHID) Interface: http://www.oie.int/wahid-prod/public.php?page=home.

[2] Please refer also to *Emerging Infectious Diseases*, 8(10), 2002.

2. Etiology and ecology

2.1 Spores and vegetative forms

Anthrax is a bacterial disease caused by the spore–forming *Bacillus anthracis*, a Gram-positive, rod-shaped bacterium (see chapter 6), the only obligate pathogen in the large genus *Bacillus*.

2.1.1 Cycle of infection

When conditions are not conducive to growth and multiplication of the vegetative forms of *B. anthracis*, they start to form spores. Sporulation requires the presence of free oxygen. In the natural situation, this means the vegetative cycles occur within the low oxygen environment of the infected host and, within the host, the organism is exclusively in the vegetative form. Once outside the host, sporulation commences upon exposure to the air and the spore forms are essentially the exclusive phase in the environment.

It is very largely through the uptake of spores from the environment that anthrax is contracted. The cycle of infection is illustrated in Fig. 1. Within the infected host the spores germinate to produce the vegetative forms which multiply, eventually killing the host (see chapter 5). A proportion of the bacilli released by the dying or dead animal into the environment (usually soil under the carcass) sporulate, ready to be taken up by another animal. This uptake by the next host may happen at any time, from less than one hour to many decades later.

2.1.2 Sporulation and germination in the environment

(See also section 6.5.)

2.1.2.1 Nature of the spore

The bacterial spore is a resting form of the organism. The essential parts of the vegetative cell (the vegetative cell genome precursors in a dehydrated state, small acid-soluble proteins (SASPs) that bind to and protect the DNA as well as acting as amino acid sources during germination, and specific organic acids which act as energy sources for germination) lie in a "core" surrounded by a thick protective cortex, spore coats and a proteinaceous exosporium. The inner layer of the cortex is the precursor of the vegetative cell wall and the receptor for germinants lies in the interface between the cortex and spore coats. The proenzyme of a germination-specific cortex-lytic enzyme (GSLE) is activated when the germinant attaches to the receptor. The active GSLE allows uptake of water by the cortex for initiation of germination. The cortex also plays a role in the resistance of the spore to heat. The spore coats, which represent approximately 50% of the volume of the spore, supply the first line of resistance to chemicals and physical disruption. The function of the loose-fitting exosporium is not known but may have a role in adhesion to surfaces.

Spores are markedly resistant to biological extremes of heat, cold, pH, desiccation, chemicals (and thus to disinfection), irradiation and other such adverse conditions. The organism can persist in the spore state for long periods of time awaiting the moment when conditions favour germination and multiplication. The ability of anthrax spores to persist in the soil and other environments for decades is legendary (Titball et al., 1991; Quinn & Turnbull, 1998; de Vos & Turnbull 2004; section 2.1.2.6).

2.1.2.2 Sporulation and survival

The rate and extent of sporulation by vegetative cells shed from infected animals is affected in a complex manner by the environmental conditions into which they fall. Temperature, humidity, water activity (a_w, available water within the microenvironment), pH, oxygen availability, sunlight and the presence of certain cations, particularly Mn++, are all influencing factors. Maintenance of the organism in the spore state and thus its persistence in the environment is also influenced by water activity, temperature, pH

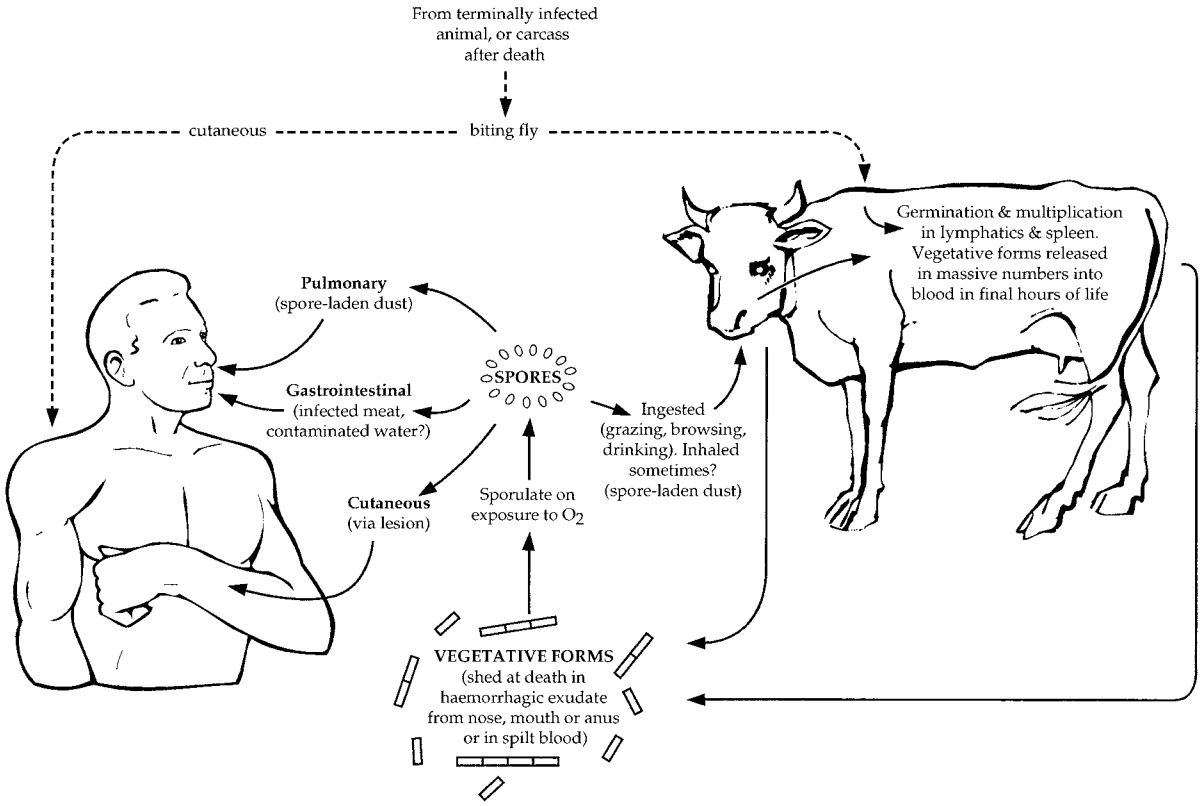


Fig. 1 Cycle of infection in anthrax. The spore is central to the cycle, although vegetative forms may also play a role in establishing infection when, for example, humans or carnivores eat meat from an animal that died of anthrax or when biting flies transmit the disease. The infectivity of vegetative forms is difficult to establish since it is close to impossible to prepare truly spore-free vegetative cell suspensions in the laboratory.

and the presence of nutrients and germinants (see section 2.1.2.4).

Although, in the laboratory, the vegetative forms of *B. anthracis* grow and multiply readily on or in normal laboratory nutrient agars or broths, the evidence is that, in natural circumstances, they are more "fragile" than the vegetative forms of other *Bacillus* species, dying more spontaneously in simple environments such as water or even milk, and being more dependent on sporulation for species survival (Turnbull et al., 1991; Bowen & Turnbull, 1992; Lindeque & Turnbull, 1994). See also sections 2.1.2.3, 2.1.2.4, and Annex 6.

2.1.2.3 Subsidiary cycles ("Sporulate or die")

Spores will germinate outside an animal if conditions permit. For bacterial spores in general, these conditions include temperatures between about 8 °C and 45 °C, pH between 5 and 9, a relative humidity greater than 96% and the presence of adequate nutrients (Sussman & Halvorson, 1966). The extent to which they may then germinate, multiply as vegetative bacilli and resporulate, setting up subsidiary cycles in the environment, remains a topic of debate (Titball et al., 1991). While it has been shown that environmental cycling can be induced experimentally (Minett & Dhanda, 1941; Titball et al., 1991; Anon., 2004; see also section 2.1.2.4), the level of nutrient required for this to become possible is probably not reached very frequently under natural conditions. If the spores germinate, the emergent vegetative cells might generally be expected to die spontaneously (section 2.1.2.2) or as a result of competition from soil microflora (Sterne, 1959), or both. Then, unless further cases occur, *B. anthracis* is eliminated within a period of years (Sterne, 1959; section 2.1.2.4).

The conserved nature of *B. anthracis* as a species (see section 2.3) also belies the concept of frequent environmental cycling. Overall, it seems that the fragile vegetative forms shed by the dead animal die rapidly in most environmental conditions and depend (i) on sporulation in a proportion of their population for their survival, and (ii) on their next animal host for multiplication. This can be described as "sporulate or die". For this reason, for all practical purposes, *B. anthracis* can be regarded as an obligate pathogen.

The rarity of environmental cycling is not universally accepted and Kaufmann (1990) believed that certain features of naturally-occurring anthrax are better explained by a pattern of *B. anthracis* multiplication in soil than by mere persistence of spores. He considered that the frequent association of outbreaks with rain ending a period of drought – "the onset of a distinct rainy season when animals disperse to graze the newly emergent vegetation" – is best explained by a concomitant burst of growth of *B. anthracis* in the soil. The conclusions of a recent study (Saile & Koehler, 2005) were that *B. anthracis* spores could germinate and establish populations of vegetative cells in the rhizosphere of grass plants, even supporting horizontal gene transfer. Certainly the fact that contamination levels at a carcass site can sometimes remain apparently undiminished for years after the death of the animal despite exposure of the site to wind, rain and sunlight (Turnbull et al., 1998) is difficult to explain in terms other than that localized multiplication has occurred. De Vos (1990) and Dragon et al. (1996) believed that the ecoepidemiological patterns of anthrax in, respectively, the Kruger National Park, South Africa, and bison in northern Canada were in line with the "incubator area" hypothesis of Van Ness (1971). (See section 2.1.2.4.)

2.1.2.4 Temperature, a_w, pH and calcium

There is a surprising shortage of reliable data on germination, multiplication and sporulation of *B. anthracis* under different conditions of temperature, water activity and pH. Howie (1949) noted that a nutrient broth culture of *B. anthracis* formed spores within 6 hours at 37 °C as assessed by heat resistance and that, in an ice-chest (0–4 °C) bacilli did not spore but died in 6 days. In a recent study (Reyes, Turnbull & LeBron, unpublished data, 2006), vegetative cell preparations of five *B. anthracis* and two *B. cereus* strains were transferred to sporulation agar slants that were held at selected temperatures in refrigerated water baths. The critical temperature for three of the *B. anthracis* and the two *B. cereus* strains was found to lie between 9 °C and 12 °C. At 12 °C they grew and sporulated (as determined by heat resistance), albeit requiring up to 2 weeks before spores were detectable, while at 9 °C, none grew or sporulated and numbers declined to unrecoverable. The other two *B. anthracis* strains declined and failed to sporulate at 12 °C.

According to the experience of Turnbull (personal communication, 2002), spores, again as identified by heat resistance, appear in 37 °C blood agar plate cultures within 6–8 hours.

The laboratory study of Davies (1960) demonstrates the dramatic extent to which temperature and relative humidity affect sporulation and how temperature affects germination. Basically, as assessed from stained smears:

- at 37 °C and 100% RH, sporulation was first apparent at 6 hours and was complete by 12 hours;
- at 37 °C and 90% RH, spores were first seen at 12 hours and sporulation was complete at 16 hours;
- at 37 °C and 80% RH, these time points were delayed to 16 and 24 hours;
- at 37 °C and RH below 50%, variable sporulation was seen at 34 hours;
- at 26 °C with RH 100% and 90%, sporulation was first seen at 24 hours and was complete at 28 hours; the corresponding time points were delayed with decreasing RH but at all RH values down to 20%, sporulation was complete by 60 hours.

In laboratory terms, germination is a much faster process than sporulation and, as assessed by loss of heat resistance, is apparently complete 2–10 minutes after exposure of the spores to germinants, such as alanine, tyrosine or adenosine (Sussman & Halvorson, 1966). In Davies's study (1960) of the influence of temperature (as judged by visualization of 10% vegetative forms):

- no germination occurred at 46 °C and 18 °C; while at
- 44 °C, germination was first seen at 12 hours and was fully present by 16 hours;
- 42 °C, germination was first seen at 3 hours and was fully present by 6 hours;
- 39 °C, germination was first seen at 2 hours and was fully present by 3 hours;

- 37 °C, germination was first seen at 2 hours and was fully present by 6 hours;
- 30 °C, germination was first seen at 4 hours and was fully present by 8 hours;
- 25 °C, germination was first seen at 10 hours and was fully present by 12 hours;
- 20 °C, early germination was not seen but was fully present at 16 hours.

Lindeque & Turnbull (1994) inoculated blood taken from wild animals just after death from anthrax into soil and water. In sandy soils, pH 7.5 to 8.2, sporulation (as detected by surviving 62.5 °C for 15 minutes) was first apparent at about 10 hours and was complete by 24 hours; in chalky karstveld soils (pH 7.9–8.1), sporulation only occurred reluctantly (total counts fell extensively and spore counts only equalled total counts at 96 hours) but was first seen at about 4–5 hours. The specific ambient temperatures are not given but appear to have ranged between about 20 °C and 30 °C over the course of 24 hours. In waterhole water similarly inoculated in the laboratory with blood collected just after death and held at 27–29.5 °C, sporulation had commenced by 15 to 24 hours; spores accounted for 100% of the total count at 68 hours. Although the pH of the water before addition of the blood was > 9, after addition of the blood it was effectively neutral.

Further comments are made in section 2.2.3 on the importance of temperature and relative humidity for the ecology of anthrax in relation to climate.

The belief of an association of high soil pH with "favourable sites" for anthrax persistence dates back to at least the statement of Higgins (1916) that "a suitable soil must be slightly alkaline". Minett & Dhanda (1941) found that multiplication in sterilized soil of natural pH 6.69 was enhanced by the addition of slaked lime ($CaCO_3$ 9.45%, CaO 68.32%). A water content of ≥ 10% (optimum 25%) of dry soil and a temperature ≥ 17 °C (optimal 30 °C) were necessary for growth to occur (see section 2.1.2.3 for discussion on multiplication in soil under natural conditions). Analogy with information on *B. cereus* (Sussman & Halvorson, 1966) suggests that the rate and yield of germination may be influenced by temperature in a manner that varies with pH and that spores will not germinate at a pH of < 5, a temperature of < 8 °C and relative humidity of < 96%. Titball & Manchee (1987) showed that germination of Vollum strain spores was minimal at 9 °C and that the optimum germination temperature in the presence of germinant L-alanine was 22 °C.

Van Ness & Stein (1956) and Van Ness (1971) cite the results of Minett & Danda and those from a thesis by Whitworth (1924) as well as their own analysis of outbreaks in relation to pH in support of their hypothesis that "anthrax occurs in livestock that live upon a soil with a pH higher than 6.0, and in an ambient temperature above 15.5 °C". On the basis of this, they mapped "the distribution of soils considered capable of supporting anthrax" in the USA (Van Ness & Stein, 1956). The "incubator area" hypotheses of Van Ness (Van Ness, 1971) have been cited frequently in the literature since the publication of his paper (it is sometimes forgotten that these were theories and were not demonstrated scientifically). These hypothesized "incubator areas" are depressions in calcareous or alkaline locations, collecting water and dead vegetation which, in turn, provide a medium suitable for germination and multiplication of anthrax spores.

The association of calcium and anthrax "favourable sites" seems to have been recognized in the Russian Federation over a century ago within the "chernozem concept", the process of soil calcification from an underlying calciferous parent material, studied by Dokuchaev as early as the late 19th century (Smith, personal communication, 2003). In a retrospective analysis of anthrax in the USA over the 100-year period 1900–2000 (Smith, personal communication, 2003) found that the occurrence of the disease in livestock at the county level is firmly linked to chernozem soils (defined as a calcium rich, neutral-to-alkaline soil suitable for prairies, grasslands and cereal grain cultivation). Livestock anthrax mortality rates in areas with such soils, analysed for the period 1945–1955, were > 21-fold greater than other areas (Smith et al., 2000). Smith (personal communication, 2003), in the first quantitative study on the possible link between soil calcium and pH, and anthrax ecology carried out in the Kruger National Park, South Africa, also noted that areas where soil calcium was greater than 150 milliequivalents per gram and pH was greater than 7.0 had a greater than 7 times higher anthrax death rate than areas lacking these parameters. Furthermore Smith et al. (2000) demonstrated that different genetic types of *B. anthracis* may be "restricted" in their geographical ranges by an adaptation (or lack of it) to differing ranges of soil calcium and pH.

Calcium is integral to the dehydration of vegetative cell genome precursors necessary for effective long-term storage in the spore, and Dragon & Rennie (1995) proposed that exogenous calcium in calcium-rich soils may act as a buffer to leaching of calcium from the spore core, thereby enhancing preservation of the spore.

It may be that there is a variable ability to survive within a population of spores in the environment, but that a moist alkaline (pH 9) and calcium-rich environment will favour spore survival for long periods. This seems to be supported by evidence from observations in wildlife parks and reserves. It is possible that shorter-term survival in agricultural environments – as evidenced by the general experience over decades that eradication of the disease from an affected area can be achieved with a vaccination programme of about three years' duration – may be at least partially explained in terms of germination and failure to survive and resporulate at the lower pH of most agricultural soils. (See also section 2.1.2.6.)

2.1.2.5 Physical movement of spores

Some differences of opinion are apparent in the literature as to the mobility of anthrax spores in the environment. Contaminated carcass sites in the generally dry, dusty soils of the Etosha National Park, Namibia, are noteworthy both for how discrete they remain and for how high levels of contamination can persist there for years despite seasonal winds (bacteriologically proven to move some of the spores) and rain, sometimes severe in nature (Lindeque & Turnbull, 1994; Turnbull et al., 1998b). It was proposed that strong attachment of spores to soil might be responsible for this, at least in part. Similarly, in the almost 40 years between the trials on Gruinard Island and predecontamination sampling, there had been negligible, if any, spread of contamination from the original detonation and testing sites (Manchee et al., 1990) although the highest levels of spores were recorded below the soil surface (Manchee et al., 1983). On the other hand, de Vos (1990) believed that epidemics of anthrax in the Kruger National Park arose from the concentration of spores in depressions as a result of run-off into the depressions following rain, reflecting the "incubator area" theory of Van Ness. The incubator and concentrator hypotheses have also been considered in relation to the persisting anthrax in the Great Slave Lake region of Canada (see section 2.2.5). As noted in section 2.1.2.4, however, the concentration/incubator theories have not been confirmed bacteriologically.

Historically, in the Netherlands and the United Kingdom (at least), the main enzootic livestock areas traditionally lay "downstream" from tanneries and the implication has been that watercourses have carried contaminated tannery effluent, depositing them in ditches and streams a few kilometres away. The occasional incidents or outbreaks that still occur in these areas are frequently associated with recent site disturbance (dredging or digging). Similarly, the large Australian outbreak in 1997 was believed to have been initiated by the movement of earth and the disturbance of old anthrax graves associated with the levelling of irrigation land on the index property. In support of the belief that this was an explosive point-source outbreak was the finding that all the isolates collected across the outbreak area were the same strain (Keim et al., 2000). Anthrax had not been recorded previously in the outbreak area since record-keeping began in 1914 (Seddon, 1953). One example was the outbreak in Washoe county, Nevada, in August 2000, one of the "persistent pockets" of anthrax in the USA, which followed ditch-clearing work (Hugh-Jones, 2000). Another good example is given in the report of Turnbull et al. (1996): two ponies died after grazing in a field which had been scarified and re-seeded and in which a bullock that had died of anthrax had been buried 50 years before. The first pony was not examined for cause of death but the second was confirmed as an anthrax case. Bacteriological mapping of the field pinpointed the burial site (the ponies had died in their stable). Although the circumstantial evidence for an association between soil disturbance and outbreaks is strong however, it is almost invariably anecdotal and unsupported by bacteriological evidence. The corollary is that farming operations frequently involve soil disturbance and that anthrax infection does not result.

The relevance of the infectious dose to the importance of physical movement of *B. anthracis* in transmitting the disease is addressed in chapter 3.

2.1.2.6 Persistence of anthrax spores

The ability of anthrax spores to remain viable for very long periods has become almost legendary but there is little well-documented information on this. Jacotot & Virat (1954) found anthrax spores prepared

by Pasteur in 1888 to still be viable 68 years later, and Wilson & Russell (1964) reported that anthrax spores had survived in dry soil for 60 years. In 1992 Bowen & Turnbull (Turnbull, personal communication, 2002) found *B. anthracis* in samples of the plaster and lagging of London's King's Cross railway station roof space and dated this to infected horse hair used to bind the plaster when the building was constructed a century before (only in 1908 was the Horse Hair Act passed in the United Kingdom, requiring the sterilization of horse hair used in buildings). The longest survival claim is probably that of de Vos (1990) who recovered anthrax spores from bones retrieved during archaeological excavations at a site in the Kruger National Park, South Africa, that were estimated by carbon-dating to be 200 ± 50 years old. The condition that appears most to favour long survival is dryness. However, other conditions that discourage spores from germinating may also play a role in persistence. Manchee et al. (1990) noted that data from annual sampling between 1946 and 1969 of the contaminated site on Gruinard Island (where an estimated 4 x 1014 spores were dispersed by explosive means in 1942 and 1943 during the Second World War) predicted a decay to undetectable by 2050. The island, off the west coast of Scotland, has a wet cool climate and a highly organic soil with pH of 4.2–4.7; the low pH is probably the main factor inhibiting germination (Titball et al., 1991). Where germination occurs, the temperature may not permit growth and resporulation and the emergent vegetative forms probably die.

In contrast, Turnbull (personal communication, 2002) reports not infrequently finding that *B. anthracis* and other *Bacillus* species stored on agar slopes had died, particularly when the lids of the bottles containing the slopes had become loose, allowing the agar to dry out.

Turnbull et al. (1992b) observed that environmental isolates of *B. anthracis* from sites with a history of anthrax spore contamination in the distant past quite frequently lacked pXO2 and, less frequently, both pXO1 and pXO2. They hypothesized that, under stressful environmental conditions such as within sewage or in the harsh semidesert circumstances of the Etosha National Park in Namibia, *B. anthracis* could spontaneously lose one or both virulence plasmids. When first cultured, the Kings Cross isolates were a mixture of capsulating and non-capsulating cells, possibly representing a population in the transition stage. However, the precise causes and events responsible for the loss of one or other of the plasmids and the time or times during the germination, outgrowth, multiplication and resporulation at which these events occur is not known.

2.2 Seasonality

2.2.1 Seasonality and incidence

Anthrax is a seasonal disease. Typically, an outbreak in an enzootic area follows a prolonged hot dry spell, which in turn was preceded by heavy rains or flooding, or with rain ending a period of drought. However, because conditions and circumstances that predispose to outbreaks vary from location to location, seasonality shows corresponding divergences from this pattern in different locations (Quinn & Turnbull, 1998). The patterns in Australia from 1914 to 1951 are described by Seddon (1953).

Much has been written and hypothesized about the effects of season, rainfall, temperature, soil, vegetation, host condition and population density on the epidemiology of anthrax, but little agreement exists on the roles played by these factors in the incidence of the disease, and this is a topic in need of further research. Nevertheless, the primary conditions affecting the seasonality of anthrax in any one place would appear to be temperature and rains (or drought) and the associated humidity. The paper of Minett (1952) is especially worth studying in any review of the relationship between climate and incidence of anthrax.

2.2.2 How climate acts

Climate probably acts by:

- affecting the animal either: (i) directly, by influencing the way in which it makes contact with the spores through, for example, grazing closer to the soil in dry periods when the grass is sparse, or enforced grazing at restricted sites when water becomes scarce; or (ii) indirectly through its effect on the general health of the animal and its level of resistance to infection; and/or
- affecting the ability of *B. anthracis* to germinate and/or sporulate.

Kaufmann and colleagues (Kaufmann, personal communication, 2004) attempted to verify the close-grazing concept in several outbreaks in southern USA and were unable to find a difference between

affected and control pastures in terms of density of animals per hectare, or of grass length, but it was acknowledged that the situation might be different in more arid climates.

The large outbreak in Australia in 1997 occurred when there was abundant irrigated pasture.

2.2.3 Climate and sporulation

Anthrax enzootic areas are generally found in warmer climates. However, there are a number of well-known areas where the disease occurs or has occurred near or above the Arctic Circle, e.g. in wood bison in the Wood Buffalo National Park, Alberta, and the MacKenzie Bison Sanctuary, Northwest Territories, Canada, and in caribou in the Taymyr Peninsula, northern Siberia. In summer, these areas approach 24-hour daylight and warm seasonal temperatures are adequate for sporulation.

The general association with warmer climates is probably attributable to the relationship between temperature and water activity, and rates of sporulation of bacilli shed from victims of the disease. The vegetative form appears to survive poorly outside the animal host, and the outcome of the race to sporulate or die is temperature-dependent (section 2.1.2.3). Sporulation may be incomplete, or not achieved at all below certain temperatures, and in temperate or cool climates, the disease can be expected to disappear with time (section 2.2.5) unless reintroduced through importation.

The importance for the ecology of anthrax of the effect of temperature and relative humidity on the rate of sporulation was noted by Minett (1950). Studying thin layers of anthrax blood under different atmospheric conditions, he concluded that humidity of < 60% inhibited sporulation, while above that level the speed of sporulation was temperature-dependent. He went on to use this to predict which regions of India had a relatively high risk of anthrax prevalence (Minett, 1951). Lindeque & Turnbull (1994) noted that in the Etosha National Park in Namibia the overall peak anthrax activity is in the rainy season, in contrast to most other enzootic localities, where it is in the dry season. This might be related to the fact that the relative humidity in the very dry semidesert Etosha is generally below 60%–70% during the dry months. The water activity (a_w) of the soil is likely to be as important as the atmospheric humidity and, although the two are related, the relationship varies from time point to time point.

2.2.4 Climate and germination of spores

The relationship between temperature and water activity and germination probably also influences the ecoepidemiology of anthrax, although this will be to a lesser extent than with sporulation (see section 2.1.2.3). Within the temperature, pH and relative humidity limits specified in section 2.1.2.4, germination may be induced but, if adequate nutrient is not present or other undefined conditions are not optimal, the emerging vegetative cells will die out.

2.2.5 Seasonality and stress

Some modification of the theory put forward in section 2.2.3 on the expectation that the disease will disappear in temperate or cool climates may be needed to explain the persisting anthrax in the bison community of the Great Slave Lake region of northern Canada. The hypothesis currently put forward by Canadian researchers is that persisting anthrax in this environment is attributable to either or both spore concentration through the action of rainwater in wallows visited by bulls every year and/or subclinical infection through chronic exposure. Outbreaks occur when the animals become immunocompromised from the stress that results when meteorological conditions lead to diminished food and water sources. They crowd around the remaining sources, with heat, high concentrations of insects and hormonal changes heralding the onset of the rutting season (Dragon et al., 1999; see also section 3.3.7). The invariable occurrence of natural outbreaks in hot dry weather, as opposed to feed-related winter outbreaks, supports the hypothesis that innate immunity is depressed under such climatic stress, thereby reducing the necessary minimal infective dose sufficient for some exposed grazing animals to become infected and diseased. Stress-inducing factors of these types are, in fact, thought to be important in the seasonality of anthrax in any anthrax-enzootic environment worldwide.

The stress theory is not entirely new. Hutyra et al. (1946) cited a study by Opperman showing that the oral infectious dose for sheep was reduced from 200 000 to 51 000 spores if the sheep were starved for a few days. They also noted that anthrax sometimes developed after journeys of 4–5 days in road or rail trucks, especially in cattle in summer, the animals presumably being infected prior to the start of the journey.

2.2.6 Seasonality and insects

Where insects may play a significant role in anthrax transmission, their own seasonal incidence would be expected to be reflected in the seasonality of anthrax. Davies (1983) noted that the peak incidence of anthrax cases in the large anthrax epidemic in Zimbabwe in 1978–1979 coincided with the peak period for tabanid flies, in contrast with *Stomoxys* species, which were equally prevalent throughout the year. Furthermore, the importance of flies in anthrax transmission can be expected to vary greatly in different regions and countries of the world, generally being of greater importance in hotter climates than in cooler ones. The role of flies in the transmission of anthrax is covered in detail in section 3.3.5.

2.2.7 Seasonality and human activities

In nonenzootic areas of the world, seasonal occurrence may reflect human activities rather than the direct effect of the local climate on the disease. This was especially apparent in Europe in the early part of the 20th century, when the dependence in winter on feed supplements imported from enzootic countries led to a relatively high incidence of anthrax in winter months (Minett, 1952).

2.3 Strains and ecoepidemiology

B. anthracis appears to be one of the most monomorphic species known, i.e. isolates from whatever type of source or geographical location are almost identical phenotypically and genotypically. Phenotypically, strain differences are only apparent in nonquantifiable or semiquantifiable characteristics, such as colonial morphology, flocculation in broth culture, cell size, multiplication rate, sporulation efficiency, LD_{50} in animal tests, etc. The genetic basis for these differences has not been established and at the molecular level genomic differences long proved difficult to detect. The biochemical, serological or phage-typing methods available in the case of other pathogens have proved of no value for identifying different strains of *B. anthracis*.

In the past five years important progress has been achieved in determining phylogenetic relationships among isolates worldwide, through multilocus variable number tandem repeat (VNTR) analysis (MLVA) (Keim et al., 2000; Keim & Smith, 2002). MLVA examines a number of DNA segments within the chromosome and plasmids of *B. anthracis* for the presence of strain-dependent patterns of repeated specific nucleotide sequences, and has enabled a broad separation of isolates into two major clonal groups, A and B, with four or more minor clusters in the A branch and two minor clusters in the B branch. The A branch is the most common worldwide, with the B1 branch found only in southern Africa and B2 scattered worldwide outside Africa (Keim & Smith, 2002). Why the A strains have a wide and common global distribution and the B strains are so restricted in numbers and distribution is unknown. Hugh-Jones (personal communication, 2003) suggests that this might be attributable to a hypothetical ability by A, but not B, strains to establish latent infection (see section 3.3.8).

Evidence for differences in growth and sporulation characteristics in different genotypes was found by Smith & Hugh-Jones (Keim & Smith, 2002), who have suggested that such differences represent adaptation to different environmental conditions. They illustrate this concept with two genotypes responsible for anthrax at different latitudes in Alberta, Canada. The more northerly strain was found to have higher (in vitro) growth and sporulation rates. Smith & Hugh-Jones suggest that this represents an adaptation for the colder climate, where carcasses cool faster and time after death for maximal growth and sporulation is limited.

VNTR analysis is a specialist technique currently restricted to a few laboratories with the necessary capabilities.[1] MLVA has played a major role in criminal investigations into the source of the anthrax letter events in the USA in the last quarter of 2001.

It seems reasonable to attribute the exceptional degree of species monomorphism to the fact that *B. anthracis* encounters opportunities to multiply less often than most other bacterial and pathogenic species. Given the truth of the statements in sections 2.1 and 2.2 that opportunities to multiply in the environment are rare, multiplication cycles depend almost exclusively on infections in animal hosts – and these, in turn, may only occur following considerable time intervals between sequential hosts. Furthermore, since multiplication occurs almost exclusively in the animal host, the vegetative form

[1] Enquiries about strain-typing may be addressed to Dr Paul Keim, Department of Biological Sciences, Northern Arizona University, P.O. Box 5640, Flagstaff, AZ 86011, USA.

of the organism is rarely exposed to the mutagens, phages or other environmental factors responsible for strain variation in most bacterial species.

With the *B. anthracis* genome sequence now revealed (Ivanova et al., 2003; Read et al., 2003), improved understanding may be expected in the foreseeable future of the relationship between strain and what are described above as nonquantifiable or semiquantifiable phenotypic characteristics.

2.4 Anomalies of history

The periodicity of anthrax over the centuries is a phenomenon receiving little attention in the literature. At the time of Pasteur's interest in anthrax, the incidence of the disease had surged to the point where some contemporary observers felt animal husbandry was doomed in France. The question arising is why was anthrax a greater problem in 19th century France than in the previous century, and could this be attributed to the global cooling that marked the 18th century, or was it a direct outcome of the industrial revolution with its increased trade in and processing of contaminated animal materials?

It is also difficult to attribute the decline of the disease in the USA from the 19th century to the present day to control programmes which, at best, were confined to ad hoc actions at the time of an epizootic. Once again the question arises, could global warming have played a role in the decline of the disease in the USA?

For a specific country such as Australia, the industrial revolution and development of agricultural practices were probably the major factors in introducing anthrax. In Australia, it is believed that the outbreaks occurred not from direct feeding of bone flour to livestock but as a result of cattle and sheep grazing land that had been treated with bone flour for horticultural purposes. Anthrax has not been recorded among the native fauna of Australia.

3. Anthrax in animals

3.1 Host range, susceptibility and infectious dose

Anthrax is primarily a disease of herbivores (chapter 1). However, reports of its occurrence in a wide range of omnivores, carnivores and other vertebrates are not entirely uncommon, although outbreaks affecting large numbers of carnivorous animals are very rare (see section 3.4.7).

It is the common perception that *B. anthracis* is not an invasive organism. This is reflected in the generally much higher experimental LD_{50}s by inhalational or oral routes as compared with parenteral challenges in susceptible laboratory species, such as the guinea-pig, mouse or rabbit (Druett et al., 1953; Watson & Keir, 1994). In fact, this had been recognized and studied in the early 1900s. Eurich & Hewlett (1930) cite and discuss seven publications between 1921 and 1928 reporting that animals were much more readily infected percutaneously than by other routes. The following quote from Eurich & Hewlett (1930) illustrates the knowledge of the time:

> "Sanarelli (1925) found that large numbers of anthrax bacilli or of spores may be given by mouth to laboratory animals without infecting, and that the blood of an infected animal may be injected per anum without harm. Virulent anthrax spores enclosed in small gelatin capsules may be swallowed by mice and guinea-pigs without harm, though virulent spores can be recovered from the faeces for a week (Holman, 1922)."

Aitoff (1922, also cited by Eurich & Hewlett, 1930) demonstrated that anthrax bacilli would not infect via the conjunctiva, even through conjunctival ulcers. Similarly in livestock, Sterne (1966) noted that "cattle, which are notoriously difficult to kill by parenteral injection, can regularly be killed by dosing with virulent spores made up in pellets of food. Whether the infection is by the buccal or pharyngeal route during rumination, or whether invasion occurs from the stomach, is not known". Experimental support for this is given by Schlingman et al. (1956).

According to Lincoln et al. (1967), each animal species has a characteristic pattern in the development of septicaemia, with death occurring when circulating bacilli reach a characteristic concentration which, in turn, is directly correlated to the toxin level in blood. An inverse relationship was noted between resistance to infection and susceptibility to the anthrax toxin complex. Rats, highly resistant to infection (parenteral LD_{50} =10^6 spores), had terminal bacteraemia in the range of 10^4 to $10^{5.9}$ bacilli/ml of blood. In contrast mice, very susceptible to infection (parenteral LD_{50} = 5 spores) had terminal bacteraemia in the range of 10^7 bacilli/ml of blood. In challenge with toxin, rats died within 2–20 hours of injection with 15–280 units of toxin/kg, whereas the lethal dose for a mouse was 1000 units of toxin/kg. Two decades later, when purified toxin components had become available, Ezzell et al.(1984) confirmed this earlier observation in Fisher rats, which succumbed to 40 µg PA combined with 8 µg LF, mice to 12.5 µg PA with 2.5 µg LF, and guinea-pigs to 250 µg PA with 50 µg LF. This translates approximately to the µg/kg body weight values given in Table 1.

TABLE 1

Susceptibility of laboratory-animal species to lethal toxin (PA in combination with LF)[a]

SPECIES	WEIGHT (ADULT, APPROX.)	µg/kg BODY WEIGHT	
		PA	**LF**
Mouse	55 g	225	45
Guinea-pig	325 g	250	50
Rat (Fisher)	1000 g	120	25

[a] See section 5.5.3.
Source: Ezzell et al., 1984.

Ranges of published LD_{50}s for anthrax by the parenteral route (Schlingman et al., 1956; Watson & Keir, 1994) are:

- < 10–50 for guinea-pigs
- < 10–151 for mice
- 100 for sheep
- 3000 for rhesus monkeys
- 5000 for rabbits
- 10^6 for rats
- 10^9 for pigs
- 5 x 10^{10} for dogs.

There was 100% mortality in nine species of wild Utah rodents injected intracutaneously with 80–100 spores of *B. anthracis* strain M 36, but in two species of kangaroo rats, doses of 20 000 or 40 000 spores killed fewer than half of the animals tested (Marchette et al., 1957).

Ranges of published LD_{50}s by the inhalational routes (Watson & Keir, 1994; Zaucha et al., 1998) are:

- 16 650–40 000 in guinea-pigs
- 14 500 in mice
- 4000–750 000 in monkeys
- 1.8 x 10^7 in dogs
- 2.7 x 10^7 in pigs
- 10^5 in rabbits.

Ingestion LD_{50}s in guinea-pigs and rabbits exceeded 10^8 spores (Druett et al., 1953).

Minimum infectious dose (MID) estimates are only rarely available, but:

- British biological warfare work in the early 1940s established that the aerosol MID for sheep was 35 000 spores (Fildes, 1943) and that the dose needed to ensure lethal infection by the oral route in sheep, horses and cattle was 5 x 10^8 spores (Carter & Pearson, 1999).
- The respiratory MID estimate of 35 000 spores for sheep is consistent with the respiratory LD_{50} of 200 000 cited by Lincoln et al. (1967).
- Schlingman et al. (1956) found that oral administration of 150 million spores proved fatal to most cattle.
- De Vos (1990) and de Vos & Scheepers (1996) recorded that 100–250 spores of a strain isolated from a kudu in the Kruger National Park consistently resulted in death from anthrax when administered parenterally in impala while the oral LD_{50} with the same strain in these animals was approximately 15 million spores.
- In a study on 50 pigs given doses of 10^7–10^{10} spores in feed containing grit (Redmond et al., 1997), the majority showed clinical illness with recovery, and just two died with confirmed anthrax 6 and 8 days respectively after ingestion of the spores; these were estimated to have received 1.6 x 10^7 and 7.8 x 10^7 spores respectively.
- In a letter dated 14 August 1971 from Keppie to Sterne, Keppie concludes: "The [parenteral] sheep LD_{100} dose appears to be approximately 75–225 spores". Data from the letter appear in Table 2.

The importance of knowing the MID by various exposure routes is apparent when attempting to reconstruct the epidemiology of certain outbreaks. However, the optimal method of computing an MID from experimental exposure data is subject to debate (Haas, 2002). Since it is thought that, in the natural situation, animals generally acquire anthrax by ingestion of spores, and that some sort of lesion is necessary for the establishment of infection (see section 3.3.1), LD_{50}s or MIDs, particularly parenterally determined, only provide a rudimentary guide to likely infection in the field. Extrapolating experimental findings to the natural situation should also be done cautiously, taking into consideration the many factors influencing infectivity, such as the strain of *B. anthracis*, the route of infection, the species, breed or strain and state of health of the animal concerned, the times and sites at which tests are done, etc. It is difficult to relate the very large oral doses that are apparently needed experimentally to infect species generally regarded as susceptible to anthrax to the levels of environmental contamination that these animals are likely to encounter naturally and that result in infection and outbreaks in the field. One possible explanation for, or contribu-

TABLE 2

Lethal doses of the Vollum strain in sheep

Spores/sheep[a]	55 000	5500	750	550	225	75	55
Dead at (hours)	36	48	60	60	60	60/108	Lived

[a] The specified dose was administered to two sheep subcutaneously (bleb under the skin).
Source: Keppie, personal communication, 1971.

tion to, the seasonality of anthrax (see section 2.2.5) may be that, for reasons of reduced innate immunity, the MID for at least one or two individuals in the community drops sufficiently in the high season for them to contract infection and then become foci of infection for others.

Innate or natural resistance is covered further in chapter 5.

3.1.1 Mixed infections

It was an early observation (Eurich & Hewlett, 1930, cite four publications between 1889 and 1927) that simultaneous injection of other organisms, such as staphylococci, *B. coli*, *B. typhosus* and cowpox vaccine, delayed or prevented death from anthrax in guinea-pigs, mice or rabbits. Fuerst apparently suggested that the mutual antagonism of anthrax bacilli and cowpox vaccine might be used to prevent losses in cattle incurred with the anthrax vaccine (then the Pasteur vaccine, which Eurich & Hewlett record as having mortality rates of 0.1% in cattle, 0.19% in horses and 0.62% in sheep).

3.2 Incidence of anthrax in animals[1]

Thanks to successful national programmes, there has been a progressive global reduction in livestock anthrax cases over the past three decades. This has had many beneficial aspects but has also created some problems. The latter follow from lack of experience of the disease on the part of younger veterinarians, who may fail to recognize or are slow to diagnose cases. The result of this is that single cases become multiple, sometimes with human exposure, whereas previously it would have been limited to the initial deaths. The other problem is public ignorance of the disease, with the consequent sale and slaughter of affected livestock. This is a particular problem with sheep, where help is requested only when several to many are dead. Because of the resulting environmental contamination, this turns a trivial problem into one that will have long-lasting repercussions. There is also the perennial problem of owners failing to vaccinate livestock in the years immediately following an outbreak while the environmental risk is still present. In Europe as in parts of North America, truly sporadic outbreaks follow from soil disturbance – ditch-clearing, laying water lines, bulldozing roads – where there was an unrealized risk from forgotten cattle graves of past cases (see sections 2.1.2.5, 3.3.2, 8.3.3).

As a result of successful prevention, the disease is absent or only sporadic in the middle and higher latitudes of Europe and the Russian Federation. But it is still common in some countries bordering the Mediterranean (Albania, Greece, southern Italy, Spain and Turkey). In Canada, apart from its continued incidence in and around the Wood Bison National Park in northern Alberta, it is sporadic in southern Alberta and Saskatchewan. Recent events have demonstrated that it has been a long-lasting problem in southern Manitoba because of the cost of vaccination. A change in the provincial law whereby ranchers can now buy vaccine on prescription has resulted in vaccination being affordable and thus routine and successful. In the USA, the disease is confined to a few persistent pockets in North and South Dakota, Minnesota, Nebraska, Nevada and Texas. Elsewhere erratic singular cases have occurred. In western Texas, however, a hyperenzootic situation has arisen in an area of white-tailed deer ranching (see section 3.4.7).

The true situation in Latin America is uncertain and awaits proper definition, largely because the disease is frequently ignored, especially in small ruminants, and underreported. There is a lack of diagnostic facilities. It is enzootic in El Salvador, Guatemala and Mexico, with decreasing incidence as one moves further south. It is absent in Belize and in all of the Caribbean, except Haiti. The situation in Colombia is obscured by civil unrest. The disease is well reported in Chile, but with excess human cases indicating control defects. It is enzootic in Argentina, Bolivia and Peru, and sporadic in western Uruguay and in parts of Brazil.

In South Africa it continues at a low sporadic incidence probably as a result of control being now largely dependent on the livestock owners. Outbreaks occur in wildlife of the Kruger National Park, as in the national parks in Botswana, Namibia, Uganda and the United Republic of Tanzania. The efficient control programmes of the past in Zambia and Zimbabwe are a matter of memory and the disease is now hyperendemic, with significant human losses each year. Epidemics occur in Chad and Ethiopia. The endemic situation in West Africa is made worse by civil wars.

[1] This section summarizes information from different sources. Past and updated official information on disease occurrence is available through the OIE World Animal Health Information Database (WAHID) Interface: http://www.oie.int/wahid-prod/public.php?page=home.

In the Middle East, Israel and the West Bank and Gaza Strip are possibly free. Elsewhere the disease is sporadic, and the Islamic Republic of Iran is attempting eradication. Various levels of endemicity occur in nearby Georgia, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan and northern Afghanistan and Pakistan.

Anthrax is a severe problem in southern and eastern India, with a significant human incidence because the disease is poorly controlled. Outbreaks in wildlife also occur. It is absent however from the western state because of the low soil pH.

The disease is a continuing problem in western China, but sporadic in the eastern provinces. Thailand, although essentially free, is afflicted by infected animals imported from Myanmar, some of which have reached Bangkok. The disease is enzootic in Cambodia, Viet Nam and a number of Indonesian islands. Malaysia is free. Sporadic outbreaks have occurred in China, Province of Taiwan, Japan, Philippines, and the Republic of Korea; the situation in the Democratic People's Republic of Korea is unknown. The disease occurs sporadically in limited areas of Australia, although large outbreaks have occurred at about 30-year intervals over the past 60 years; the disease there is associated with grazing animals. Anthrax has not been recorded in New Zealand for more than 60 years.

Unfortunately, the mere absence of reported outbreaks is no proof of absence of the disease. Insufficient examination of unexpected livestock deaths and reporting deficiencies are worldwide surveillance defects. The restrictions on the use of meat and bonemeals in ruminant feed as a result of bovine spongiform encephalopathy (BSE) cases in Europe has played a significant role in stopping the recycling of the pathogen.

3.3 Transmission, exacerbating factors and epidemiology in animals

3.3.1 Acquisition of the disease

Although anthrax has been recognized for centuries, little is known about the disease, and among the most basic questions frequently asked, but not yet answered, is how precisely do grazing and browsing animals acquire it? The epidemiology of anthrax centres around the dose necessary for infection to occur, and this section needs to be read in conjunction with section 3.1.

The sporulated forms shed by an animal dying or dead from anthrax generally provide the source of infection of other animals (Fig. 2). As noted in section 3.1, it has long been the traditionally held belief that ingestion of the spores while grazing is a frequent mode of uptake. Since *B. anthracis* is apparently noninvasive, it is believed that a lesion is necessary for the initiation of infection. In view of associations between higher incidence and dry hot conditions, theories have arisen that at such times the animal is forced to graze dry spiky grass close to the soil. The spiky grass and grit produce the orogastrointestinal lesions and, if the soil is contaminated with anthrax spores, these may enable infection to occur. The concept that spiky grass and grit produce lesions is, however, not totally consistent with some epidemiological observations that neither length of grass on pasture nor density of cattle grazing affected pastures correlated with the risk of infection (Fox et al., 1973, 1977).

Ingestion of soil may not be necessary for initiation of infection in grazing animals. In one early study of plants grown on contaminated soil, contamination was alleged to be readily demonstrable on a range of farm crops (Williams, 1932). For example, 92% of corn plants were reported to be culture-positive for *B. anthracis* from the stems to the tips of the leaves. It is possible to be sceptical about this report, and one would want the identities of the isolates to be reconfirmed but, if this pattern does occur on pasture, close grazing would not be essential for infection.

"Barn anthrax" was an earlier term for animal infections acquired from fodder. An incident at a Pennsylvania dairy farm during 1971 is illustrative of such an outbreak (Kaufmann, personal communication, 2004). Five dairy animals, three cows and two heifers, died over a 10-day period in August. The heifers were kept in a barn with a concrete floor, about a kilometre from the barn where the dairy cows were housed. The heifers were never allowed on pasture and subsisted solely on hay; the primary feed source of the cows was hay, although they did some grazing. Both hay and soil from the farm were culture-positive for *B. anthracis*.

It is difficult to believe that an animal grazing over dry dusty contaminated soil is not also inhaling spores, and inhalation may well be a mode of infection also. Pulmonary anthrax has been reported in cows (McCulloch, 1961; Bell & Laing, 1977). Acquisition of anthrax through fly-bites probably occurs also. The roles of aerosols and flies are discussed further in sections 3.3.4 and 3.3.5.

Reflecting the generally high infectious dose and noninvasive character of *B. anthracis* in the natural disease (section 3.1), direct animal-to-animal transmission is understood to occur to an insignificant extent, except in the case of carnivores feeding on other victims of the disease.

3.3.2 Transmission as a result of trade in animal products

In economic and public health terms, the importance of the disease lies in its ability to affect large numbers of livestock at one time and to be spread from these to others great distances away. Anthrax carcasses pose a hazard to humans and other animals both in the vicinity and at a distance through their meat, hides, hair, wool or bones. Hides, hair, wool and bones may be transported long distances for use in industries, feedstuffs or handicrafts. Livestock may acquire the disease continents away from the original infection source through contaminated feedstuffs, or from spores that have reached fields in industrial effluent.

In countries with advanced agriculture, feedstuffs with contaminated ingredients are traditionally the primary source of infection, especially for dairy cows. These ingredients can either be improperly-treated locally-produced meat and bonemeals salvaged from moribund stock or, more likely today, infected bones or contaminated meat and bonemeal imported from enzootic regions. Hugh-Jones & Hussaini (1975) noted an association between the incidence of anthrax in England and Wales from 1938 to 1972 with changes in patterns of importation of meat and bones, or of treatment of these. A decline in incidence in the period 1938–1946 was associated with cessation of imports of bones and meat meals during the Second World War. An increase of importation of these again in 1946–1957 was accompanied by an increase in anthrax, but towards the end of that period, some of the large feed compounders replaced untreated bonemeal with steamed bone flour, and there was a concurrent drop in the incidence of the disease. Finally reintroduction of meat and bonemeal between 1962 and 1964 was again associated with an increase of anthrax in cattle.

It was recognized as long ago as 1911 (Stockman, 1911) that crops such as oats and soya beans, which normally would not be expected to contain *B. anthracis* spores, could become cross–contaminated by bagging in sacks made of infective materials, such as shoddy wool or horsehair, or during shipping together with dry hides. Stockman wrote: "There are outbreaks in which the circumstantial evidence indicates that infection has been introduced with turnips which have been grown on land manured with crushed bones". Jackson (1930) stated that instances of cross-contamination of feedstuffs of plant origin had occurred as a result of the hulls of cargo ships and other containers not being cleaned out after transporting infective materials, or following the reuse of sacks previously used for movement of contaminated materials of animal origin. Such occurrences are unlikely today due to the BSE restrictions on the feeding of rendered meat and bonemeal to ruminants.

Tanneries have long been associated with transmission of anthrax. Stockman (1911) wrote: "It has been found that the disease is exceptionally prevalent on certain sewage farms which are known to receive tannery or knackery drainage". In 1916, Higgins similarly recorded: "One of the most commonly reported sources of infection is that through water and other wastes coming from tanneries". The anecdotal association of outbreaks of anthrax in livestock and disturbance of soil and tannery wastewater was referred to in section 2.1.2.5, and also features in Fig. 3. On occasion, an incident or outbreak can be related directly to disturbance of an anthrax carcass burial site (Turnbull et al., 1996), while at other times it takes the less focused form of disturbance of the environment downstream from the site of an industrial process involving animal products, such as a tannery.

3.3.3 Hay, grain, etc.

Williams (1932) recounts the problems encountered in controlling anthrax in Texas in the early 20th century, citing a study at the Louisiana Experiment Station demonstrating the potential for anthrax transmission by crops grown on contaminated soil (see also section 3.3.1). In the past, the former Soviet Union has insisted that hay shipped there from the United States be certified as "free of contamination", presumably reflecting some previous experience of cross-infection. It is unlikely, however, that hay or grain or other crops are significant contributors to the transmission of anthrax today.

3.3.4 Aerosols and wind

It seems reasonable to assume that spores are inhaled by animals grazing over the dry dusty site

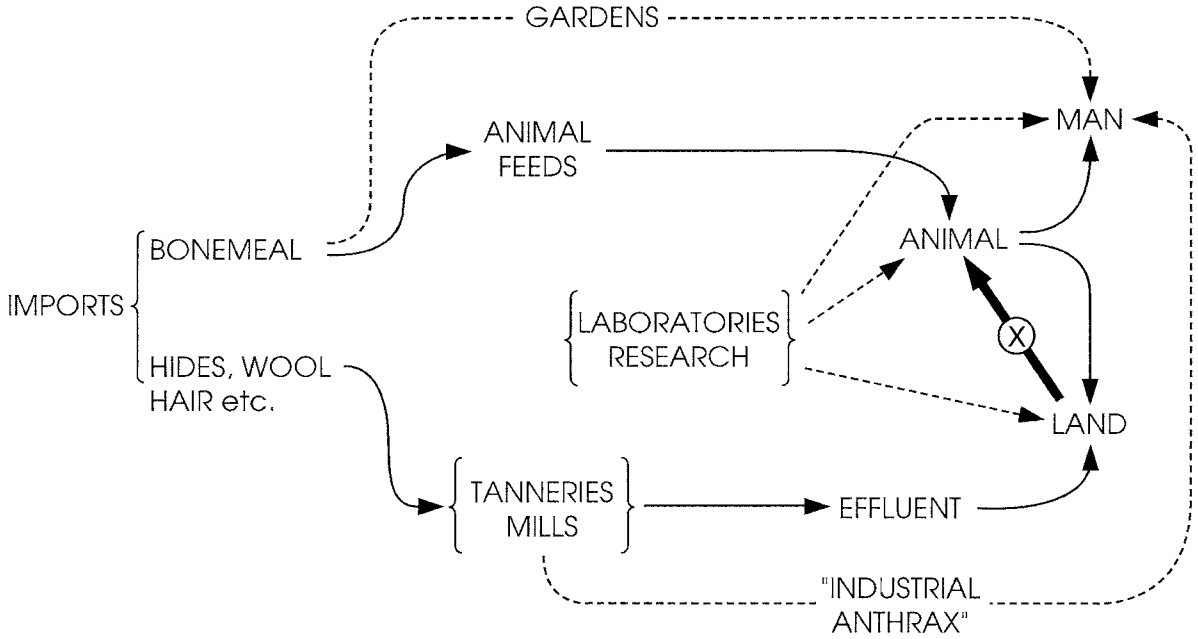


Fig. 3 Importation from endemic countries to other countries, and cycles within the importing country[a]

[a] X indicates the predominant route to animal infection.

where another animal died of anthrax at some point in the past (section 3.3.1). On the basis of the experimental findings (see section 5.2) that inhaled spores may remain in ungerminated form in the lungs for months after uptake, it is conceivable that a form of chronic carriage with onset of disease some time after uptake may result (see also section 3.3.8).

As far as long-distance transmission is concerned, basically, anywhere dust can travel, so can anthrax spores. However, the importance of this to transmission of the disease is debatable. Turnbull et al. (1998b) sampled air 6–18 m downwind of old anthrax sites with contamination levels of 2×10^4 to 1.6×10^6 cfu/gram in the semidesert Etosha National Park, Namibia. Sampling was carried out before and after disturbance to simulate animals moving across the site. The highest aerosol concentration found was approximately 2 spores per 100 litres of air. From published respiratory rates, this amounted to a time needed to inhale one spore of 5–7 minutes in sheep and goats, 0.3–0.7 minutes in large animals, and 2.5 minutes in a human. From this, it would seem that the dilution effect of wind spread is too great for wind to contribute greatly to the transmission of the natural disease.

In the event, albeit probably rare, that a case does occur as a result of spores moved by wind, it establishes a potential new focus of infection for other animals. This may have been the case in an instance recorded in Australia many years ago where a storm passing sequentially across an anthrax endemic area and then a clean area was followed by cases of the disease in seven herds in the "clean" area a few weeks later (Henry, 1936).

In contrast to the natural disease, the potential for aerosol spread in a deliberate release event was demonstrated in the accidental release at Sverdlovsk in the former Soviet Union in 1979 (Meselson et al., 1994). Sheep reportedly died of anthrax in a village approximately 60 km distant from the point of release at the research institute, with an estimated 2% death loss overall on affected premises (see also section 4.2.2.3).

3.3.5 *Insects*

Insects have been incriminated in the transmission of anthrax since at least the 19th century (Budd, 1863; Henning, 1893) and, at the turn of the 20th century, biting flies, particularly *Hippobosca* and *Tabanus* species, were considered important as transmitters of

the disease (Morris, 1918, 1919, 1920; Sen & Minett, 1944; Sterne, 1959). Davies (1983) believed that tabanids (horseflies) were responsible for the expansion of the huge epidemic in Zimbabwe in 1978–1979 by "hops" from community to community. At the time there were heavy rains which would have encouraged large hatches of tabanids. Rao & Mohiyudeen (1958) also explained the spread of anthrax in India in terms of biting flies. *Pangonia* spp., biting flies that target hippos, may have played an important role in periodic outbreaks in hippos (Bengis, personal communication, 2005). Non-biting blowflies (*Chrysoma albiceps* and *C. marginalis*) have been incriminated as the principal vector of anthrax in browsing wild herbivores in the Kruger National Park, South Africa (Braack & de Vos, 1990; de Vos, 1990; de Vos & Bryden, 1996). Blowflies feed on the body fluids of anthrax carcasses and then deposit highly contaminated faeces or vomit on the leaves of trees and bushes in the vicinity. Browsing herbivores may then contract anthrax when eating the contaminated leaves.

The ability of the biting stable flies (*Stomoxys calcitrans*) and non-biting houseflies (*Musca domestica*) or *Calliphoridae* (bluebottles, blowflies, etc.) to transmit anthrax was demonstrated experimentally by several workers in the early 1900s (reviewed by Sen & Minett, 1944). Transmission by *Tabanus rubidus* was readily effected in horses and carabao (Kraneveld & Djaenoedin, 1940) and when *M. domestica* and *Calliphora erythrocephala* that had fed on incisions in the flanks of goats that had died of anthrax were brought into contact with the cauterized skin of healthy goats (Sen & Minett, 1944). However, the disease was not induced by bringing infected *M. domestica* into contact with the eyes of healthy goats. Anthrax was also transmitted between infected and healthy guinea-pigs by *S. calcitrans* and by mosquito species *Aedes aegypti* and *A. taeniorhynchus* (Turell & Knudson, 1987). Schuberg & Boing (1913) are cited without reference by McKendrick (1980) as also having demonstrated the ability of mosquitoes to transmit anthrax.

Ticks collected from terminally ill animals have been found to carry *B. anthracis* (Stiles, 1944; Buriro, 1980; Akhmerov et al., 1982) but they probably do not play a significant role in the transmission of the disease as interhost transfer of adult ticks is rare.

Dose is again pertinent. Small insects may pick up too few *B. anthracis* from a sick or dead animal to definitely induce infection in another host (Greenberg, 1971, 1973), although this may be countered by the cumulative effect of several or numerous bites. During "epidemics", such as in Texas or Canada, affected stock have been noted to be "black" with biting flies. However, it is necessary to bear in mind the finding by Schlingman et al. (1956) that lethal infection could not be induced in cattle by parenterally administered doses of up to 6×10^8 spores of the Vollum strain. The Vollum strain is regarded as less virulent than many other strains but, if this is taken as the minimum lethal parenteral dose for cattle, and terminal blood counts in anthrax victims at death are in the order of 10^8 cfu/ml (see section 3.1), it would imply that 1–2 ml (cumulative) of blood needs to be injected by tabanid mouthparts. In the experimental studies reviewed and carried out by Sen & Minett, the transmission success rate was generally quite low. It can be seen, however, that this mode of transmission could be expected to select out more virulent strains requiring lower volumes of injected blood (Hugh-Jones, personal communication, 2003).

The time between the exposure of the insect vector and the exposure of the second host to that insect was of consequence. The transmission rates in Turell & Knudson's study were 17% for stable flies and 12% for mosquitoes, and all transmissions occurred in guinea-pigs exposed ≤ 4 hours after uptake of blood by the insect from the bacteraemic animal.

According to Kraneveld & Mansjoer (1939) the bacilli did not multiply in the gut of tabanids; some were excreted in vegetative state, some died and some were able to sporulate. De Vos (de Vos & Turnbull, 2004) states that blowflies may be lifelong carriers, although vegetative *B. anthracis* disappear from their digestive tracts within two weeks of feeding on a carcass.

The possible association between flies and the seasonality of anthrax is discussed in section 2.2.6.

3.3.6 Other modes of mechanical transmission

Cattle drives in the 19th and early 20th centuries have long been regarded as a major means of the early spread of anthrax in Canada and the USA. A comparison of the distribution of affected counties in the first half of the 20th century (Hugh-Jones, unpublished data) shows that, for the period up to 1932, there was a 1:3.99 chance of a county having livestock anthrax if it was within 10 km of a south-to-north cattle trail ($p < 0.0003$); for trails going east

to west (essentially all these were immigrant trails, such as the Oregon and California trails), the chance was 1:0.60 ($p < 0.4175$).

Livestock began arriving in large numbers in what became the anthrax-endemic regions of northern Canada as early as 1880 from herds in the northwestern American states and southern Alberta, and may have been the source of the disease (Dragon et al., 1999). Alternatively, it has been hypothesized that anthrax in northern Canada originated from Wainwright in southern Alberta, along with brucellosis and tuberculosis, when excess plains bison that had been in contact with local cattle were moved to the north in the mid-1920s. The reflux of *B. anthracis* bison strain GT5 down into central Alberta probably came from contact between southern cattle grazing in the north at Fort Vermilion during the summer and wandering bison bands out of the Wood Bison National Park (Hugh-Jones, personal communication, 2004).

Livestock movements along the Silk Road probably took anthrax eastwards into western China.

The spread of anthrax by carrion eaters was discussed by Kraneveld & Mansjoer (1941) and vultures and other scavengers have long been suspected of transmitting the disease various distances in southern Africa (Pienaar, 1967; de Vos, 1990; Lindeque & Turnbull, 1994; Lindeque et al., 1996a). Similarly gulls, ravens and possibly black bears were considered as possible vectors in the bison outbreaks in northern Canada (Dragon et al., 1996). Certainly anthrax spores have been found on/in the feathers, faeces or digestive tracts of these animals (Lindeque & Turnbull, 1994; Dragon et al., 1996; Lindeque et al., 1996a), but the numbers are generally low and below what is generally regarded as the "normal" infectious doses of other animals. However, as with aerosols and other modes of mechanical transmission, if and when an occasional case does occur, it may set up a new focus of infection for other susceptible animals in the vicinity.

3.3.7 Age and sex

In general, although reliable evidence is difficult to come by, the incidence of anthrax in wild animals seems to be slightly biased towards adulthood and males (Pienaar, 1961; Moynihan, 1963; Brunsdon, 1968; Ebedes, 1976; de Vos, 1990; Lindeque & Turnbull, 1994; de Vos & Bryden, 1998). However, this may be apparent rather than real, reflecting factors other than resistance to infection, such as differences in behaviour or feeding habits or, in the case of age, the relative ease with which carcasses of adults that have died of anthrax may be detected as compared with those of the young animal. Physiological factors may be involved also. Weinstein (1938) showed that administration of thyroxine, testosterone and pituitary, thymus and pineal gland extracts to mice increased their resistance to anthrax infection while insulin, oestrin, progesterone and adrenal cortex extract did not.

Of particular interest are the bison in an anthrax-enzootic area in northern Canada where the majority of carcasses found are sexually mature bulls. Special surveillance techniques during the 1993 outbreak established that bull-bias in mortality was real and not due to calf carcasses being more difficult to spot (Gates et al., 1995). Except for one outbreak in the summer of 2001, where an equal number of bull and dam carcasses were found, a bull-bias of > 80% has been observed in all known anthrax outbreaks in the Canadian bison herds (Dragon & Elkin, 2001). Explanations for this range from the rutting behaviour of the bulls causing exposure to spore-laden dust, to chronic infection becoming acute from stress-related factors during the rutting season. Bulls use the wallows for dominance displays against each other and generally die within 100 metres of their individual wallows. Bulls generally graze closer to the ground than cows and calves. The next bull adopting the wallow of one that has died and grazing around it becomes relatively exposed to the deposited spores (Hugh-Jones, personal communication, 2004). Also, females rarely wallow and calves, being suckled, do not graze much; they may also have maternal antibody.

Information found on age- and gender-related incidence in livestock is limited. Significantly disparate attack rates were noted between bulls, cows and calves in Louisiana and Texas during outbreaks in the 1970s, with males having the highest attack rates and the incidence in calves being nil (Fox et al., 1973, 1977). In Louisiana, the attack rates in steers and cows were similar, suggesting that a factor unique to intact males resulted in their increased susceptibility to infection. The explanation offered for the age-specific differential attack rate has been that calves, as they are suckling, would ingest less forage than adults on the same pasture (Fox et al., 1973).

No calves were infected in the outbreak in

Victoria, Australia, in 1997. This could have been due to movement of the weaned dairy calves to a raising shed away from the herd.

3.3.8 Prolonged incubation, carrier state, chronic and latent infections

A differentiation must be made between prolonged incubation, carrier state, and chronic and latent infection. The lengthy period between exposure and onset of clinical illness that was observed in experimental monkeys exposed to spore aerosols (see section 5.2) and in humans exposed in the Sverdlovsk accidental release incident (Meselson et al., 1994) are examples of prolonged incubation. Another example is the vaccine trial of Lindley (1955) described in the next paragraph. A carrier state, in the sense of animals harbouring the specific organisms of a disease without overt symptoms and being capable of transmitting the infection, has not been demonstrated in anthrax. A chronic infection results in antibody production and deterioration in health of the infected individual. Latent infection implies that spores are lodged in one or more lymph glands or other sites, successfully germinating and initiating infection when the innate immunity is depressed. However, in some of the instances cited below it is difficult to make the distinction between prolonged incubation, carriage, and chronic and latent infection, and the terminology of the original reports has been retained in the following paragraphs.

As an example of prolonged incubation, Lindley (1955) observed anthrax deaths in two goats, 47 days and 51 days post-challenge with the virulent Gwandu strain of *B. anthracis* administered as a spore suspension in 50% glycerin. Both animals had been housed in an indoor stable, and no infections occurred in unchallenged animals at the facility. The one that died 51 days post-challenge had been vaccinated. The unvaccinated animal, in particular, illustrates a potential long incubation period.

Chronic anthrax infections have been reported in swine (Hutyra et al., 1946). Only a few decades ago, meat inspectors in slaughterhouses periodically observed discrete anthrax lesions in the lymph nodes of the mesentery, pharynx or submandibular region of swine. The lymph nodes were generally enlarged, and the cut surface was brick red or studded with small greyish-yellow necrotic foci, sometimes diffusely necrotic and containing dry caseous material. Lymph nodes of this description were found nine months after the 1952 three-state epidemic in pig herds in Ohio, Indiana and Illinois affecting 490 herds and attributed to contaminated pig feed (Hugh-Jones, personal communication, 2004). Presumably, other resistant scavenger species such as dogs can also develop chronic lesions.

Among susceptible species, Weidlich (1934) reported the case of a cow producing apparently normal milk containing anthrax bacilli for some months. Provost & Trouette (1957) isolated *B. anthracis* from the abdominal lymph nodes of apparently healthy cattle in an endemic area of Chad. Culture collection strain ASC 65 was from the milk of one of four chronically infected cows in Brazil. The bacterium was isolated from these cows (apparently healthy at the time, although two unvaccinated animals subsequently died) during routine mastitis screening tests on milk samples in 1983[1] (see also Annex 6, section 3).

Evidence of carriage in susceptible herbivores has been reported. De Moulin & Soemanegara (1937) noted that spores administered subcutaneously in highly immunized animals were still present at the site of inoculation ≥ 7 weeks later in cattle, ≥ 2 months in horses, buffalo and sheep and ≥ 6 weeks in chickens. Dormant spores were found to still be circulating in the blood of black rats at 30 days by Walker et al. (1967). Ferguson (1981) reported a carrier state in pigs that had recovered from the disease.

A serological survey in the enzootic Etosha National Park, Namibia, showed that Park herbivores rarely have natural anti-anthrax antibodies, while most of the Park carnivores have them (Turnbull et al., 1992a). This was interpreted as indicating that infection in herbivores usually leads to death. In contrast, however, it was found that 87% of bison bulls in the Canadian Mackenzie Bison Sanctuary had specific anti-anthrax antibodies when tested eight months after an outbreak in 1993 (Turnbull et al., 2001b). The implication in this instance is that a significant proportion of the bison survived *B. anthracis* infection or were chronically infected. Passively transferred or partial immunity can delay onset of overt disease by many days, which again represents a form of latent infection (Turnbull, 1990b).

Latent infections may conceivably be the occasional explanation for the long-distance spread of

[1] Information from Dr P. Baptista, Department of Agriculture, South Rio Grande State, Brazil.

anthrax. However, the success of the application of the OIE 20-day incubation period for anthrax control in livestock over the past 50 years is testimony to the rarity of this, if it occurs at all.

Much has been made in recent years (in relation to formulating post-exposure prophylaxis in humans) of the finding that inhaled spores may lie dormant in the lungs of monkeys for weeks before being cleared by alveolar macrophages, showing no evidence of germination until within the macrophages (Barnes, 1947a; Henderson et al., 1956; Widdicombe et al., 1956; Ross, 1957; Friedlander et al., 1993; see section 5.2). The relevance to the natural disease and its spread in animals is uncertain.

3.3.9 Anomalies and unknowns of anthrax epidemiology

Despite anthrax being a disease that has been so well known for so long, many seemingly simple epidemiological questions remain unanswered. A variety of explanations have been developed on the factors that lead to outbreaks of anthrax. The explanations remain just speculation and vary with respect to different environments, different continents and whether domestic or wild animals are involved.

Examples of continuing unknowns follow.

3.3.9.1 How does an animal acquire anthrax?

How an animal contracts the disease remains in the realm of theory (see section 3.3.1).

3.3.9.2 Not always point-source?

Outbreaks of anthrax are generally considered to be of the point-source type; direct herbivore-to-herbivore transmission is thought not to occur (with the exception of transmission as a result of osteophagia) and certainly does not occur under laboratory conditions (i.e. in animal cages in research studies). However, it is difficult to explain periodic explosive epizootics, or unexpected spread to areas that were previously unaffected, in terms of point-source infection.

The history of outbreaks in Australia is that outbreaks start as a point-source death with 2, 3 or 4 further cases occurring in 5–7 days, and 7–8 deaths in a further 5–7 days. This pattern of spread has been attributed to the social cohort of dead animals licking and sniffing around the carcass, and this was the theory accepted in the large 1997 outbreak in Victoria State.

Fly-borne spread (section 3.3.5) is strongly suspected of being the means of spread in such events.

3.3.9.3 Why do outbreaks predominantly affect one species?

Large explosive outbreaks usually affect only one species in the affected area while the incidence in other, equally susceptible species in the vicinity remains sporadic. This has been known for a long time. Higgins (1916) wrote: "In certain outbreaks a single species of animal may show a more marked susceptibility than others which are apparently similarly exposed". The phenomenon may be explained in terms of the different ecological niches of the various species and that they are, in fact, not at equiprobable risk within the same setting. A simple example might be that if biting flies are responsible for the outbreak, they bite the primary species preferably or exclusively.

Susceptibility data based on experimental results do not correlate well with the occurrence of deaths under natural circumstances (Sterne, 1959). Cattle are quite resistant to experimental infection but are very prone to infection in nature. In contrast, sheep are quite susceptible to experimental infection but may not be so readily infected in nature, even on premises where affected cattle are grazing. Consistent with Sterne's observation, a large outbreak in California in 1968 resulted in 176 animal deaths (169 cattle, 4 mules and 3 horses), yet sheep in close proximity were not affected (Kaufmann, personal communication, 2004). Sterne also pointed out that goats, despite being even more susceptible than sheep to experimental infection, accounted for even fewer cases than sheep (see below).

Wise & Kennedy (1980) noted that New South Wales, Australia, could be divided into a northern zone in which bovine and ovine anthrax occurred with equal frequency, and a southern zone where bovine anthrax was about four times as common as ovine, with death rates in cattle 13 times greater than in sheep. In the 1997 outbreak in Victoria, some 202 cattle and 4 sheep died. The reasons for such area variations in the two species are unknown, particularly as they are interspersed, often on the same property, across all these areas. Although cattle and sheep do exhibit different grazing behaviours, it is hard to define the reason why one species is favoured over the other for infection in one area or property.

In the wild, anthrax does not affect all herbivo-

rous species equally, and there is an apparent preference by the disease for particular species in any one region. Zebras, for example, are the most commonly affected in the Etosha National Park in northern Namibia, with kudu only occasionally affected. Assessments of all recorded anthrax deaths (uncorrected for total populations) are in the order of 45% for zebra versus 0.8% for kudu (Lindeque & Turnbull, 1994). In the Kruger National Park, South Africa, the kudu is the principal host, accounting for > 50% of all recorded anthrax cases with zebra falling into a relatively small group of "other affected species" (de Vos, 1990). The ecological and behavioural factors responsible for these differences tend to depend on circumstantial evidence, but appear to revolve around blowflies in the Kruger National Park which, after feeding on anthrax carcasses, fly to nearby shrubbery and deposit infected blood on the leaves, which then become the source of infection for the browsing kudus. The analagous situation with goats and sheep is referred to above; goats are allegedly infected less often than sheep under equiprobable risk situations, despite being more susceptible to experimental inoculation. Goats, like kudus, are browsers; sheep, like zebras, are grazers.

Few disagree that grazing, browsing and flies are the main variables in the differing equations; flies are the vectors of spores from dead carcasses to foliage in the vicinity. Other aspects relating to the manner in which plants are eaten are also likely to affect acquisition of anthrax. Bovines pull plants out of the ground when grazing, thereby ingesting a lot of soil. In contrast, sheep and horses bite plants off at ground level, taking in very little soil (Hugh-Jones, personal communication, 2004).

Strain differences come to mind as an obvious explanation in relation to these anomalies. In Texas, goats and white-tailed deer frequent the same areas but, if horseflies are transmitters of the disease, they fail to infect the goats (Hugh-Jones, personal communication, 2004). Is this strain-related? This has yet to be proven (see section 2.3).

3.3.10 Transmission resulting from deliberate release – bioaggression

The realities of transmission of anthrax as a result of deliberate release were made very clear by the anthrax letter events in the USA between September and December 2001, although those were targeted at humans rather than animals. The potential damage that could be inflicted from targeting livestock has, however, been recognized for at least a century. The 1899 Hague Convention, adopting the unratified Brussels Declaration of 1874, prohibited "the spreading … of diseases on enemy territory". Although the illegality of biological warfare was spelt out in the 1902 German General Staff handbook on land war, Germany ran an anti-animal sabotage programme from 1915 to 1918 largely targeted at neutral countries (Argentina, Norway, Romania, Spain and the United States) to disrupt their shipments, primarily of horses destined for military use, to the enemy belligerents. Glanders and anthrax, the agents involved, were delivered by crude inoculation with a needle dipped into the cultures. No data exist on the level of damage inflicted by this programme, although it was considered a "success" by the German army (Wheelis, 1999).

It seems that after the First World War, thinking about biological warfare (BW) became focused on antihuman agents, and veterinary sabotage was no longer considered relevant in the German programme (Wheelis, 1999; Geissler, 1999). Possibly owing to a misunderstanding in intelligence coding, perception that Germany had an antilivestock biological warfare programme led to a British development and production capability retaliation-in-kind programme (Carter & Pearson, 1999). Between September 1942 and April 1943, 5 million cattle-cakes were prepared and stockpiled ready for targeting Germany's wartime agriculture section. Encapsulated within the centre of each cake were 5×10^8 anthrax spores (established MID for cattle – see section 3.1) of the Vollum strain. The cakes were never used and were destroyed shortly after the war.

The possibility that the epidemic of anthrax in Zimbabwe (then Southern Rhodesia) in 1979–1980 was the result of deliberate release has been raised. Epidemiological details of a large proportion of the > 10 000 human cases were published (Davies, 1982, 1983) and "Contact with a dead bovine carcass is always given as the manner in which anthrax is acquired. … Confirmation of anthrax as the cause of cattle deaths is available from all areas in which (human) anthrax is thought to have occurred" (Davies 1982); "two thirds of the country has been affected by bovine anthrax in a very short time" (Turner, 1980). Although further data on the livestock cases were not published, it was believed that tabanids (horseflies) spread the disease among animals

(Davies, 1983; see section 3.3.5), and the breakdown in veterinary services resulting from the insurgency situation occurring at the time prevented the setting up of vaccination campaigns (Davies, 1982) and of education programmes aimed at stopping people from butchering the carcasses. However, some years later, accusations arose in the public press and minor publications that this had been a "deliberate use of anthrax as an agent of BW, directed at African-owned cattle" (Nass, 1992) and that "the use of anthrax spoor to kill off cattle of tribesmen assisting the guerillas … was carried out" (Martin, 1993). In defence of the official stance that this was a natural outbreak resulting from the breakdown of the veterinary services (commercial farms, where vaccination was still possible, remained virtually free of infection – Davies, 1983), it was pointed out by government officials that other diseases, such as rabies and tick-borne infections, escalated several 100-fold at the same time.

The accidental release of anthrax at Sverdlovsk in the former Soviet Union in 1979 (see also sections 3.3.4, 4.2.1.1, 4.2.1.3, 4.2.2.3) originated from a microbiology laboratory and caused the death of a significant number of livestock up to approximately 60 km from the point of release (Meselson et al., 1994).

3.4 Clinical manifestations; incubation periods

The incubation period in susceptible laboratory animals ranges from about 36 to 72 hours. In susceptible livestock, it may be a little longer. Schlingman et al. (1956) found an incubation period of 3–7 days following oral challenge. The normal incubation period in naturally-infected cattle is stated to be 1–14 days or more (Kaufmann, personal communication, 2004). For trade purposes, the OIE incubation period for anthrax is 20 days (Annex 4). The requirement that animals for export will not have been in contact with an infected animal or premises in the previous 20 days appears to have been effective in controlling the spread of anthrax through international trade and also seems to work in controlling dissemination of infection through domestic trade (Turner, personal communication, 2004).

In an experimental study on pigs, the majority of 45 pigs challenged orally with high doses of the Ames strain of *B. anthracis* showed nonspecific clinical signs of illness (fever, anorexia, lethargy, dullness, shivering, constipation, loose faeces, blood in the faeces and ataxia) between 1 and 8 days after challenge; rectal temperatures increased at 24 hours, peaking at 48 hours and returning to normal by 7 days. Two of the animals died suddenly on days 6 and 8 (Redmond et al., 1997).

A dose-response curve in rhesus monkeys challenged with 10^5 to 5 x 10^{10} spores showed that death occurred in progressively shorter times from 50 to 20 hours (Klein et al., 1962).

The first signs of an anthrax outbreak are one or more sudden deaths in affected livestock, although farmers may reflect retrospectively that the animals had shown signs such as having been off their food or having produced less milk than usual. During the systemic phase, the animals become distressed, appear to have difficulty breathing and cease eating and drinking. Swellings in the submandibular fossa may be apparent; temperatures may remain normal for most of the period or may rise. The animal can remain responsive to treatment well into this period, but if treatment fails it lapses into coma followed by death from shock. In highly susceptible species, the period between onset of visible symptoms and death may be just a few hours; the course of these events is more protracted in more resistant species or in immunized animals when the protective effect of the vaccine is wearing off. In studies on immunized laboratory animals, this has been shown to be associated with reduced terminal bacteraemia and reduced circulating toxin (Turnbull, 1990b).

The history is of major importance to immediate suspicion of anthrax. In particular, if deaths occur unexpectedly in areas known in the past to have experienced anthrax, whether recently or not, then the diagnosis of anthrax should be regarded as a possibility. This is even more the case if the deaths follow recent disturbance of the land such as digging, ploughing, dredging of watercourses and so on (see sections 2.1.2.5, 3.3.2). Anthrax should always be considered in the event of sudden deaths in zoos or other captive facilities where meat from knackeries or the equivalent are regularly fed to animals.

Cutaneous lesions, analogous to the eschars of human cutaneous anthrax (section 4.4), are rarely reported in animals. Maculopapular swellings with vesicle formation and oedema were reported in intracutaneous infections in wild Utah rodents (Marchette et al., 1957) and carbuncular lesions are referred to in sections 3.4.1, 3.4.5 and 3.4.6.

3.4.1 Bovines

In the bovine, anthrax often occurs as an acute febrile disease without obvious localization, although subacute disease with throat swellings may be encountered in tropical countries, possibly through buccal lesions from chewing infected bones (Sterne, 1959). A steep rise in temperature is a typical sign, and irritability may be followed by dullness. If no treatment is given, death usually occurs in 2–3 days with the animal showing cramplike symptoms and shivering. The urine may be blood-stained and blood may exude from the rectum and other natural openings. Often advanced signs are minimal, and the apparently healthy animal may fall in an apoplectic seizure and die within a few minutes to a few hours. Earlier clinical opinions that recovery is not an infrequent event (Sterne, 1959, 1966) are supported by more recent serological evidence (Turnbull et al., 1992a). According to Sterne (1959), oedematous or carbuncular lesions are associated with immunized animals when their resistance is waning.

Oedematous swellings along the neck, flanks or lumbar region have been described in cattle with experimental anthrax infections (Jackson et al., 1957).

The situation regarding milk is discussed in Annex 6, section 3. A single report of *B. anthracis*-associated bovine abortion was seen (Gibbons & Hussaini, 1974).

3.4.2 Sheep and goats

Anthrax usually takes the form of a peracute apoplectic infection. This may be modified in animals that have been immunized.

3.4.3 Horses

Horses may show acute symptoms and die in 2–3 days. Intestinal lesions may result in colic and diarrhoea. Sterne (1959) associated large oedemas on the breast, abdomen, neck and shoulders with cases transmitted by biting flies. It is to be assumed he observed this in southern Africa. Recovery sometimes occurs.

3.4.4 Pigs

Pigs are regarded as more resistant to anthrax than cattle, sheep, goats and horses, but herd outbreaks with significant mortality can occur. Their greater resistance is reflected in the greater evidence of local signs, such as swellings of the throat and pharyngeal and cervical lymph glands. As reviewed by Redmond et al. (1997), outbreaks in pigs were recorded with particular frequency in the 1950s and may have reflected postwar husbandry practices of feeding food waste which inadvertently included meat and bones from anthrax carcasses. At that time also, it was accepted that there were two principal manifestations of porcine anthrax: the pharyngeal and intestinal forms.

The pharyngeal form, not frequently reported now, was related to scavenging or purposeful feeding of carcasses to swine and characterized by an ulcerative stomatitis, laryngitis, and markedly oedematous swelling of the throat that could interfere mechanically with respiration, feeding and drinking. There is reference in Nieberle & Cohrs (1967) to demonstration of invasion via the epithelium overlying the tonsils. One theory is that it was associated with lesions from bone chips in food waste, but Kaufmann (personal communication, 2004) recalls that major outbreaks in the United States in the early 1950s were due to contaminated bonemeal which was finely ground and did not contain jagged chips.

The intestinal form, thought to be associated with contaminated mineral supplements, is less obvious than the pharyngeal form, lacking the obvious swellings and associated effects. Because the animals frequently recover, its occurrence may often have gone undiagnosed. Anorexia, vomiting, diarrhoea (sometimes bloody) or constipation have been reported. In an experimental study on 50 pigs (Redmond et al., 1997), anorexia, lethargy, dullness, shivering, constipation or loose faeces, blood in the faeces and ataxia were the symptoms variously noted in 33 animals showing symptoms. In 45 of the animals, rectal temperatures indicating pyrexia rose in 24–48 hours after ingestion of anthrax spores, decreasing to a minimum at about 8 days. Marked seroconversion occurred in all but 4 of the animals, indicating that infection with recovery was the common outcome (only 2 pigs died). The induced infection took exclusively the intestinal form.

It is possible that the two manifestations of the disease in pigs relates to whether vegetative cells are being consumed in addition to spores. Infection resulting from consumption of meat and bones from infected carcasses, which would contain both vegetative cells and spores, may be associated with the pharyngeal form of gastrointestinal anthrax. The

intestinal form is associated with consumption of spore-contaminated mineral supplements which would lack the vegetative forms (see also section 4.2.2.4).

The number of circulating *B. anthracis* in the blood of pigs at death is very low compared with most other species. This has practical consequences for diagnosis (see section 3.5.1).

3.4.5 Dogs and cats

Dogs are considered very resistant to anthrax, but reports in dogs that have scavenged anthrax carcasses are not exceptionally rare. In the United Kingdom, within living memory, retired foxhounds scavenging when dead cows were collected from the hunt area acquired the disease (Hugh-Jones, personal communication, 2004). Villagers in endemic countries of at least sub-Saharan Africa recognize anthrax in their cattle and, although they will eat meat from the carcass (sections 4.1 and 9.7), they will not eat the enlarged spleen; instead, they will frequently feed these to dogs and cats. Several dogs and cats were reported to have died of presumed anthrax during an epidemic involving some 500 cattle and 50 humans in the Zambezi plains area of Western Province, Zambia, in 1990–1991. Sera from 3 of 3 dogs that had eaten meat from suspected livestock anthrax victims had measurable antibody titres (Muyoyeta, Bbalo & Turnbull, unpublished data, 1994).

Severe inflammation and oedematous swelling of the throat, stomach, intestine and of the lips, jowls, tongue and gums may be seen. Carbuncular lesions of the jowls occur. Infection may generally not be lethal. A few reports of anthrax in domestic cats allegedly exist also (Eurich & Hewlett, 1930; Whitford, 1979).

3.4.6 Birds

Sterne (1959) referred to "epizootics amongst birds", but without reference or details. References found to cases of anthrax in avian species are largely confined to birds in captivity or domesticated state. While there is some logic to reports of the disease in eagles in captivity following inadvertent feeding with an anthrax carcass (Keymer, 1972; Turnbull, 1990), more surprising are the citations found to the disease in not overtly carnivorous birds including ducks, a hen, geese, artificially-infected poultry and pigeons, and even a crested crane. Again without reference or detail, Sterne (1959) stated that domestic ostriches readily contract anthrax. A recent case (June 2005) in an ostrich in the Etosha National Park, Namibia (Lindeque, Versfeld & Turnbull, personal communication, 2005) reflects the report of cases in 5 wild ostriches in the 1970s in the Park by Ebedes (1976). Other early references exist of the disease in ostriches (Viljoen et al., 1928; Ebedes, 1976; Hugh-Jones & de Vos, 2002). Pasteur (cited by Klemm & Klemm, 1959) attributed resistance to anthrax in birds to their body temperature being higher than that of a mammal, and Higgins (1916) stated that anthrax may be contracted by birds "providing … their normal body temperature is lowered". Pasteur's experiments with hens, attributing resistance against anthrax in birds to their high body temperature, are described in detail by Vallery-Radot (1923).

According to Sterne (1959), the apoplectic type of death is usual, although less acute infection, with carbuncular lesions on the comb or extremities, also occurs. As reviewed by Snoeyenbos (1965), the lesions of anthrax in the ostrich, duck and eagle are similar, with haemorrhagic enteritis and oedematous swellings, particularly in the neck. Haemorrhages may be present on the surface of many organs, such as the heart. Excessive fluid is commonly present in the thoracic and abdominal cavities. The spleen, liver and kidneys are swollen and congested.

Reference to a vulture belonging to a travelling menagerie that became very sick during an outbreak of anthrax affecting many of the animals is made in section 3.4.7. De Vos (personal communication, 2006) recalls confirming death from anthrax in one vulture by blood smear and subsequent culture from the blood. It was, however, the only bird found dead out of a very large number feeding on carcasses during a large outbreak of the disease in the Kruger Park, South Africa.

3.4.7 Other animals

Anthrax is well known as a disease of wild mammals. The largest number of reports comes from national parks in southern and central Africa, where a wide variety of species are affected. It has also been reported for many years in white-tailed deer in the southern USA (Marburger & Thomas, 1965; Yu et al., 2002) and has been observed to be enzootic in free-ranging bison in northern Canada for over four decades (Gainer & Saunders, 1989; Gates et al., 1995; Dragon & Elkin, 2001). In these areas, occasional sec-

ondary cases occur in other species (including carnivores) making contact with the primary species. Although the disease is enzootic in other parts of the world, there seems to be very little information on its occurrence in wildlife in these parts, presumably as a result of lack of resources to investigate causes of death in the wild.

Carnivores are, in general, significantly more resistant than herbivores (section 3.1) but can die from infection. In enzootic areas, resistance in wild carnivores is enhanced by the humoral immunity acquired naturally as a result of frequent exposure to scavenging carcasses of anthrax victims (Turnbull et al., 1992a). The antibody titres in the carnivores reflect (i) the level of anthrax activity in an area, and (ii) the habits of those carnivores. It seems likely that the carnivores depend on the antibodies to protect them from lethal infection. The increasing incidence of canine distemper in lions in African parks may be behind increased numbers of observed cases of anthrax in lions in recent years; a side-effect of the distemper is to damage the immune system.

Cheetah appear to represent an unusual group among carnivores in relation to anthrax. It has recently been observed in Namibia that cheetah suffer a high mortality from anthrax (Lindeque et al., 1998). Cheetah do not normally scavenge and may not, therefore, get the same chance as other carnivores to build up acquired immunity. Their unique genetic constitution may also play a role in susceptibility to infection through its effect on their immune system.

Among other animals, anthrax has been recorded in camels (Eurich & Hewlett 1930; Punskii & Zheglova, 1958; Musa et al., 1993), carabao (Steele, 1954), mink (Greener, 1947; Sterne, 1959; Hugh-Jones & Husseini, 1975), badgers, ferrets and racoons (Somers, 1911; Greener, 1947) and foxes (Eurich & Hewlett, 1930; Greener, 1947). A 1945 handbook (Anon., 1945) states: "In the domestic animals, sheep (excepting Algerian sheep which are immune), cattle, camels and horses are susceptible in the order named". Whitford (1979) tells us that Gayot (1952) showed that, in fact, Algerian sheep were as susceptible as other sheep. Cases of the disease in moose have been seen in both Canada and the USA (Dragon & Elkin, 2001; Kaufmann, personal communication, 2004).

The list of species that have experienced anthrax in captivity (zoos, capture and release facilities, wildlife orphanages, etc.) is wide-ranging (Hugh-Jones & de Vos, 2002). Mostly these have been carnivores fed on abattoir or knackery offal or, inadvertently, on portions of carcasses from animals that died of anthrax (Lyon, 1973; Hugh-Jones & Husseini, 1975; Orr et al., 1978; Turnbull, 1990b; Tubbesing, 1997; Hugh-Jones & de Vos, 2002). A particularly interesting very early report (Somers, 1911) describes an outbreak occurring in Bolton in the United Kingdom in a travelling menagerie that resulted in the deaths of two racoons, a coypu, an English badger, two pumas, a leopard, an ant-eater, two polar bears and a lioness. Two lions and a vulture became very sick but recovered. The animals had been fed raw meat from several sources and the particular source was not traced. A very recent report is that of Grigoryan (2002) in which a silver puma, a serval and a black lynx died of anthrax in an Armenian zoo after being fed contaminated meat purchased at a local market. Reference was made in section 3.1 to susceptibility tests carried out on 12 species of wild Utah rodents injected intracutaneously with spores of *B. anthracis* strain M 36 (Marchette et al., 1957).

Sudden death in apparently healthy animals with a very short period of disorientation before falling is the normal clinical picture up to death. Death may be accompanied by bloody discharges from natural orifices (Fig. 2) but it is not a constant. Rapid bloating of the carcass, incomplete *rigor mortis* and the absence of clotting of the blood are other common characteristics. The bloody discharges were not seen in Texas deer dying in various epidemics and only rarely in Canadian bison dying of anthrax (Gates et al., 1995; Dragon & Elkin, 2001).

Clinical information for carnivores is supplied by Lyon (1973) and Tubbesing (1997). Anorexia, lethargy, dyspnoea, serous nasal discharge, unilateral facial and/or submaxillary swelling with convulsions and death are variously noted by Lyon in the 13 carnivorous animals with anthrax in the Chester Zoo, United Kingdom, in 1971. Tubbesing records the death from anthrax of a leopard showing no clinical signs prior to death, a second leopard which became lethargic and anorexic, a caracal noted to be listless, anorexic and having signs of facial oedema, a lion showing signs of lethargy and partial anorexia and a lioness with lethargy, anorexia and facial swelling. All the animals that developed clinical signs died within 24 hours of the onset of symptoms and all were found at death to have facial oedema, most pronounced in the submandibular area.

The recent report (Leendertz et al., 2004) of an outbreak of anthrax in chimpanzees in the Taï National Park, Côte d'Ivoire, is apparently the first record of naturally acquired anthrax in non-human primates. In experimentally induced inhalational anthrax in rhesus monkeys, irritability, weakness and anorexia 1–4 days before death were observed, the animals dying 3–8 days after exposure to the infecting spores (Friedlander et al., 1993).

It seems uncertain whether cold-blooded animals can contract anthrax. Greenfield (1880) stated that "frogs have been found capable of infection with the anthrax poison". Snails, especially Arion fuscus, were claimed to be carriers by Karlinski (cited by Hutyra et al., 1946). This may represent passive carriage resulting from environmental contamination rather than true infection. As noted in section 1.1, Sterne (1959) stated that amphibians and reptiles are naturally resistant but warming of cold-blooded animals allows them to be experimentally infected; unfortunately he did not cite the relevant publication(s).

The large epizootic of anthrax in hippopotami in Zambia in 1988–1989 (Turnbull et al., 1991) was accompanied by a higher-than-usual mortality in crocodiles. However, these were not examined bacteriologically and the excess deaths were attributed by crocodile experts to gout from overeating (Turnbull, personal discussions, 1989). In the Chester Zoo outbreak in the United Kingdom (Lyon, 1973) affecting 13 carnivorous mammals, a western diamond-backed rattlesnake also died, but again was not examined to establish whether anthrax was the cause of death.

It is worth noting that in a number of endemic areas, such as in western Texas, various susceptible exotic species are now raised in semidomesticated environments for hunting. For example, in 2001 a large enzootic of anthrax occurred among domesticated white-tailed deer and exotic ungulate species in central Texas. Presumably further examples exist in Africa and probably other regions of the world where anthrax is endemic. These animals cannot be mass-vaccinated, and thus represent susceptible populations and foci of persisting disease in these regions (Yu et al., 2002).

3.5 Diagnosis

3.5.1 Blood smear rationale

In most species, at death from anthrax (the pig being a notable exception) the blood is usually teeming with the capsulated anthrax bacilli, provided the animal has not been treated. Numbers may also be lower in immunized animals that succumb to the disease (Turnbull, 1990b). Published figures for terminal *B. anthracis* blood counts are (approximate cfu/ml) mice 10^7, guinea-pigs 10^8–10^9, rats 10^4–10^7, sheep and goats 10^8, rhesus monkeys 10^4–10^9, chimpanzees 10^9, zebras 10^6–10^8, elephants 10^6–10^8, springbok 10^8, blue wildebeest 10^8, cheetah 10^8 (Lincoln et al., 1967; Turnbull, 1990b; Friedlander et al., 1993; Lindeque & Turnbull, 1994; Fritz et al., 1995; Lindeque et al., 1996a). In the occasional case, however, the terminal bacteraemia is unexpectedly low (Friedlander et al., 1993; Lindeque & Turnbull, 1994); this was actually recognized almost a century ago (Stockman, 1911).

Numbers of *B. anthracis* in the blood at death in pigs are invariably very low. This was recognized from very early on in the history of bacteriology (Stockman, 1911) and has been stated or confirmed frequently (Hudson, 1953; Sterne, 1959; Shaw et al., 1963; Edginton, 1990; Redmond et al., 1996a, 1997).

Apart from the cheetah (section 3.4.7), little relevant information relating to carnivores was found. Tubbesing (1997) was unable to visualize the capsulated bacilli in any of the blood smears from the two leopards or the two lions dead from anthrax on his farm (see section 3.4.7).

These figures are relevant to understanding the value of the blood smear for diagnosis. Lincoln et al. (1967) found that it became very difficult to observe *B. anthracis* in blood when numbers were below about 5×10^4 per ml.

3.5.2 Smear and culture

The blood characteristically clots poorly or not at all upon death from anthrax and is dark (sometimes described as "tarry") and partially haemolysed. In a carcass that remains intact and unscavenged, it is usually easy to obtain the necessary drops of blood for smear and/or culture by means of a syringe from an appropriately accessible vein for about 24 hours after death. Alternatively, a dry swab inserted into a small incision in a region well supplied with blood vessels (the ear is traditionally recommended) can be used to make a smear and for culture (Annex 1, section 7.5). After fixing and staining as described in Annex 1, section.9.3, the capsulated bacilli may be looked for in the smears under a microscope. The haemorrhagic oronasal or anal exudate will also yield *B. anthracis* on culture, but selective isolation

from other environmental contaminating bacteria may be necessary.

B. anthracis does not compete well with putrefactive bacteria and, with increasing age of the carcass, the capsulated bacilli become more difficult to visualize. Smears, as a diagnostic procedure, become unreliable about 24 hours after death, although capsular material may still be observed some time after the bacilli themselves can no longer be seen. Blood in transit at ambient temperatures for over 72 hours may not reveal organisms on examination or culture.

Where blood from a fresh carcass fails to reveal the presence of *B. anthracis*, but anthrax is still a possible or likely diagnosis, the capsulated bacilli will probably be visible in other body-fluid smears made from fine needle aspirates of affected areas, such as submandibular swellings, regional lymph nodes, mesenteric fluid, etc., or cultured from such specimens. This would be appropriate in pigs, for example, in which terminal bacteraemia is invariably low compared with most other species (see section 3.4.4).

Milk is not generally regarded as being useful for diagnostic purposes. There appear to be very few reports of isolation of *B. anthracis* from the milk of affected animals. Rare exceptions are instanced in Annex 6, section 3.

Regulations in most countries prohibit the opening and postmortem examination of animals where anthrax is the suspected cause of death. Contamination of the environment by spilled body fluids with subsequent spore formation is thereby avoided. In suspect cases, if negative results are obtained with the procedures described above, deeper examination of the cause of death may be necessary, "paying due regard to the possibility of anthrax existing and taking all reasonable precautions against infective material being disseminated" (Stockman, 1911). This could start with scalpel incision to obtain a smear or culture from a lymph node near the surface, progressing to more internal organs such as mesenteric lymph nodes or spleen.

If anthrax was not suspected and the carcass has been opened inadvertently, the dark unclotted blood and markedly enlarged haemorrhagic spleen will probably be immediately apparent. The mesentery may be thickened and oedematous and peritoneal fluid may be excessive. Petechial haemorrhages may be visible on many of the organs and the intestinal mucosa may be dark red and oedematous with areas of necrosis. The subcutaneous and intramuscular tissues may be oedematous. As indicated above, smears and cultures should be made from the mesenteric fluid and lymph nodes. Where anthrax has been diagnosed after a carcass has been opened, special attention should be paid to decontamination of the site at which the postmortem examination was carried out and of the tools and materials that were used (see Annex 3, sections 6 & 7).

When a carcass is old or putrefied, *B. anthracis* can often be cultured from residual skin or blood-stained material for some days after death, but this becomes progressively less easy the longer the time between death and examination. If the carcass has been opened and residual lymph node or spleen samples are available, these should be taken for culture. Diagnosis becomes increasingly dependent with time on isolation of spores from surface swabs of the remaining skeleton, particularly in the nostrils and eye sockets, or from soil or other environmental samples around or under the carcass that had been contaminated by the oronasal or anal exudates or spillages of body fluids.

3.5.3 Diagnosis in animals that have been treated before death

It may not be possible to find the bacilli in smears or to isolate *B. anthracis* from animals that were treated before death (see Annex 1, section 8.2); treatment can sterilize the blood and tissues but, if sufficient toxin has been formed, the animal may still die. Tests based on antigens, if available, may be the only practical approach (section 3.5.4). If it is sufficiently important to confirm the cause of death as anthrax, injection of spleen or lymph node extracts into mice or guinea-pigs may result in infection of the test animals by any residual viable *B. anthracis* (see Annex 1, section 12). Otherwise diagnosis may have to be made on the basis of the history and associated circumstances and findings (such as a positive feed sample that had been fed to the animal that died).

Immunohistochemical studies of fixed tissue or smears of aspirates have been shown to be a sensitive and specific method of diagnosis (Fritz et al., 1995; Guarner et al., 2003).

3.5.4 Diagnosis based on tests for antigens

The thermostable antigen precipitin test devised by Ascoli (1911) (see Annex 1, section 11.1) is still used

in several countries of Europe and the Far East for detecting residual antigens in tissue in which it is no longer possible to demonstrate *B. anthracis* microscopically or by culture. However, it should be borne in mind that it is not a highly specific test; the antigens being detected are shared by other *Bacillus* species. The test relies on the fact that, if *Bacillus* antigens are present in the tissues, this probably represents *B. anthracis* infection since infections with other *Bacillus* species are rare. Care has to be taken if the tissue being examined has been grossly contaminated with environmental materials (soil, sand, etc.) which frequently harbour large numbers of other *Bacillus* species.

A simple, rapid and highly sensitive and specific chromatographic device, a more reliable and more sensitive alternative to the Ascoli test and utilizing a monoclonal capture antibody detecting the anthrax–specific protective antigen, has now been designed and shown to be useful for rapid on-site diagnosis in the field (Burans et al., 1996; Tubbesing, 1997; Muller et al., 2004) and not to give false reactions in recently vaccinated animals (Muller et al., 2004). Unfortunately, this has not become commercially available. These types of device are covered in section 6.2.

3.5.5 Molecular diagnosis

Genetically-based confirmation by PCR is becoming increasingly accepted on a stand-alone basis for many types of specimen and is increasingly available worldwide through commercial kits. It is still advisable to attempt to confirm bacteriologically (i) a positive to avoid the chance of a false positive, and (ii) a negative in case the PCR test was insufficiently sensitive.

3.5.6 Retrospective diagnosis (seroconversion)

Historically, there has been little need for serological support for the diagnosis of anthrax in animals. Either the animal had anthrax, recognized from the recent history of the herd or site, and was treated accordingly, or it died. Most of the interest in developing serological testing has been for research on humoral responses in humans, and – to a lesser extent – animals, to vaccines and for epidemiological studies involving naturally acquired seroconversion in humans, livestock and wild mammals.

Currently accepted as the best serological procedure is the ELISA in microtitre plates coated with the Protective Antigen (PA) and Lethal Factor (LF) components of the anthrax toxin. The toxin antigens appear to be truly specific for *B. anthracis*. PA and LF are available commercially[1] but are costly. This tends to mean that anthrax serology is currently confined to a few specialist laboratories. Examples of the successful field application of anthrax serology in animals are given elsewhere (Turnbull et al., 1992a; Redmond et al., 1996a; 1997; Turnbull et al., 2001b, 2004b).

3.5.7 Differential diagnosis

For differential diagnosis, other causes of sudden death should be considered. Among these are African horse sickness, botulism, blackleg (*Clostridium chauvoei*), peracute babesiosis, chemical poisoning (heavy metals, other poisons), ingestion of toxic plants, snake bite, lightning strike or metabolic disorders such as lactic acidosis, magnesium deficiency, bloat. An outbreak of Rift Valley Fever in Kenya in 1997 was initially thought to be anthrax (WHO, 1997a). The differential diagnosis list will inevitably vary by species and geographical area, and the above list is not exhaustive.

[1] From www.listlabs.com.

4. Anthrax in humans

4.1 Human incidence

The major sources of naturally acquired human anthrax infection are direct or indirect contact with infected animals, or occupational exposure to infected or contaminated animal products. The incidence of the natural disease in humans is dependent on the level of exposure to affected animals and, for any one country, national incidence data for non-industrial cases reflect the national livestock situation. Historical analysis of epidemiological data globally reveals the following approximate ratios: in northern Europe and countries with similar epidemiological situations there has been one human cutaneous case per 10 livestock carcasses butchered; in Africa, India, and the southern Russian Federation, there can be some 10 human cutaneous and enteric cases per single carcass owing to rural malnutrition in the former and poor veterinary supervision in the latter (see section 3.2). This has produced significant numbers of human cases each year in Chad, Ethiopia, India, Zambia and Zimbabwe. While enteric anthrax is frequently lethal, subclinical cases, which provide subsequent immunity, are believed to occur with some frequency also (see section 4.4.1). These would contribute indirectly to persistence of the disease by engendering the concept among indigenous populations that the risk of contracting lethal disease from consuming meat from animals having succumbed to sudden death is not very high.

Some caution should be exercised in making projections of potential human cases based on fixed human:animal ratios. Economic conditions, surveillance data quality and dietary habits are examples of variables that may dramatically alter the situation from area to area. For example in the United States and north-western Europe, cutaneous anthrax associated with animal anthrax has been rare since the first half of the 20th century, with most cutaneous cases being associated with processing of imported goat hair, hides and other animal products. Despite the rarity of the human disease since then, many thousands of animal cases have occurred. Similarly, in Haiti human cutaneous anthrax is quite common, but reports of animal anthrax are essentially non-existent despite a well-documented problem with *B. anthracis*-contaminated goatskin products. The value of hides and cultural demands for caretakers in at least some regions of Africa to preserve as much as possible from dead animals to present later to the owner exacerbate the problem of persisting contaminated animal parts.

Unlike cutaneous anthrax, ingestion anthrax is notably rare in Haiti, presumably because of the local practice of cooking all meat well before consumption. In other countries such as Thailand, ingestion anthrax is associated with consumption of undercooked meats and in sub-Saharan Africa, the value of the meat from an animal that has died unexpectedly outweighs the perceived risks of illness that might result from eating it (section 9.7). Evidently intestinal anthrax was quite a common disease on the Korean peninsula prior to about 1940 and was still seen in the 1990s (Oh et al., 1996).

Industrial anthrax incidence data can be inferred from the volume and weight of potentially affected materials handled or imported, taking into account the quality of prevention, such as vaccination of personnel and forced ventilation of the workplace. These relationships are essentially all that can be used for many countries where human anthrax is infrequently, erratically or incompletely reported. In addition, certain countries suppress anthrax reporting at the local or national levels.

Human case rates for anthrax are highest in Africa and central and southern Asia. Where the disease is infrequent or rare in livestock, it is rarely seen in humans. However with low sporadic incidence comes forgetfulness of the risk and, when a case in livestock does occur, it may result in a surge in the number of cases and people exposed.

In contrast to reports of anthrax in animals (see section 3.3.7), age- or sex-related bias is generally not apparent in human anthrax (Heyworth et al., 1975; Martin, 1975; Davies, 1985; Kobuch et al., 1990; Kaufmann & Dannenberg, 2002) and differences in incidence have been readily explained in terms of likely exposure of the different groups to the organism (Davies, 1985; Kobuch et al., 1990). The lack of obvious age- or sex-related differences was also noted in the records of 112 anthrax cases occurring in 7 villages bordering the Tarangire National Park in the United Republic of Tanzania between 1986 and 1999 (Turnbull, personal communication, 2002). There is, however, a bias towards higher occupational risk of exposure to anthrax in men in many countries.

4.2 Susceptibility: data for risk assessments

4.2.1 Evidence that humans are moderately resistant

4.2.1.1 Circumstantial and historical evidence

Circumstantial evidence indicates that humans are moderately resistant to anthrax. Historical human:animal case ratios in handlers of anthrax carcasses suggest a relative resistance on the part of humans. Before vaccines and antibiotics became available, and at a time when understanding of industrial hygiene was relatively basic, workers in at–risk industrial occupations processing animal products were exposed to significant numbers of anthrax spores on a daily basis. In the United Kingdom, although case rates in terms of total numbers with apparently equal exposure are not known, it seems likely that the average of 40 cases of industrial anthrax a year during the 50-year period 1900–1949 (1979 cases total) (Anon., 1959) represents a very low infection:exposure ratio. In four mills in the USA, in which unvaccinated workforces, varying in size from 148 to 655, were "chronically exposed to anthrax", annual case rates were only 0.6% to 1.4% (Brachman et al., 1962). In one mill, workers were found to be inhaling 600–1300 anthrax spores over an 8-hour shift without ill-effect (Dahlgren, 1960), and in two goat-hair mills, *B. anthracis* was recovered from the nose and pharynx of 14 of 101 healthy workers. It needs to be remembered that the vast majority of the cases that did occur in these at-risk occupations were cutaneous infections (section 4.4.1).

More recently, the analysis by Meselson et al. (1994) of the accidental release of anthrax spores from a microbiology facility in the former Soviet Union (the Sverdlovsk incident in 1979) showed that the large majority of human cases were mapped within a narrow zone approximately 4 km long downwind from the source, while cases in cattle and sheep occurred in a zone up to 50 km in the same downwind direction. In this event, the value of exposure:infection ratio estimates as an indicator of relative resistance to infection is limited by the fact that the effectiveness of the public health measures in preventing development of human cases is not known. While tabulated clinical cases numbered 77, some 59 000 persons were considered eligible by the public health authorities for "prophylactic immunization against anthrax". The attack rate, in actual fact, may have been higher than 77 (Gumbel, 1991) (see also section 4.2.2.3).

4.2.1.2 Wildlife workers

Despite extensive exposure to anthrax carcasses, cases among workers in wildlife reserves are exceedingly rare (Quinn & Turnbull, 1998; de Vos and Turnbull, 2004). In three decades of specimen collection from hundreds of anthrax carcasses by unvaccinated researchers, rangers and wardens with minimal other personal protective equipment in the Etosha National Park, Namibia, there have been just two anecdotal, unconfirmed cases of cutaneous anthrax. Serology failed to detect evidence of past infection in seven rangers/researchers deemed to have had a high potential exposure to anthrax (Turnbull et al., 1992a). In the recent outbreak in the Malilangwe Trust, Zimbabwe (Clegg et al., 2006), two individuals dealing with carcass disposal developed cutaneous lesions on their hands. One did not seek treatment but recovered without complications and developed measurable antibodies; the other was given antibiotic treatment and did not measurably seroconvert (Turnbull, Clegg & Wenham, unpublished data).

Anthrax was first confirmed in northern Canada in 1952 when two employees of Wood Buffalo National Park were treated for the cutaneous form of the disease after handling a dead bison (Gates et al., 1995). In 1962, during the first recorded large-scale epizootic in bison, the biologist who discovered the first carcasses developed cutaneous anthrax after performing several postmortem examinations without any protective gear (Pyper & Willoughby,

1964). Later during the same outbreak, a backhoe operator helping to bury carcasses developed inhalation anthrax. He had repeatedly crawled under his machine to clean the blades of contaminated soil, hair and offal. The individual was a heavy smoker, which may have contributed to the development of disease. Prompt medical treatment resulted in his survival.

In the Kruger National Park, South Africa, "during three major epidemics in wildlife, many necropsies were performed, and large teams of workmen were used to track down, sometimes cut up and burn anthrax carcasses, but none contracted the disease" (de Vos & Turnbull, 2004). Other wildlife parks in central and southern Africa similarly report a negligible incidence in wildlife workers, despite the fact that levels of personal care probably drop somewhat during animal epidemics, which result in large numbers of carcasses and associated stress from pressure of work. Serological evidence of infection was detected in 12 of 24 persons in a village on the Luangwa River in Zambia who were believed to have eaten meat from hippos that had died of anthrax during the epizootics of 1987 and 1988, but there were no records of clinical cases (Turnbull et al., 1991).

4.2.1.3 Outbreaks in humans

Although the evidence indicates that humans are relatively resistant to anthrax, outbreaks and epidemics do occur in humans; sometimes these are sizeable. Among the most notable was the epidemic in Zimbabwe which began in 1979 and was still smouldering in 1984–1985. More than 10 000 persons were affected, albeit with a low (1%–2%) case-fatality rate (Turner, 1980; Davies, 1982; Kobuch et al., 1990; Pugh & Davies, 1990). In the epidemic in the Gambia reported by Heyworth et al. (1975), 448 cases of human cutaneous anthrax were diagnosed with just 12 known deaths. In 2000, apparently "hundreds" were affected in the Afar region of Ethiopia, many with oral and gastroenteric infections. Consistent among these cases was the skinning and butchering of sick and dead animals, handling contaminated meat and eating raw or inadequately cooked meat.

Mortality rates will have been reduced in these outbreaks by the availability of penicillin but occasionally case-fatality rates are substantial, such as in the Sverdlovsk incident in the former Soviet Union, with 66 known deaths in 1979 (Abramova et al., 1993; Meselson et al., 1994), and possibly many more (Gumbel, 1991). In the 2001 bioterrorist anthrax letter events in the USA, exposure rates are not known but it seems likely that the 22 clinical cases (11 inhalational [5 deaths] and 11 cutaneous) represented a small proportion of the numbers of persons exposed. The widespread prophylactic use of antibiotics is likely to have played a significant role in the final case rate also (Jernigan et al., 2001; Inglesby et al., 2002).

4.2.2 Infectious dose

4.2.2.1 General

In general terms, human infectious doses have not been established. Clearly data were generated from human experimentation in units 731 and Ei 1644 in Japan during the Second World War, but the published subcutaneous and oral MID_{50} doses of 10 and 50 mg (Harris, 1999), respectively, are hard to interpret without further information. On the basis that 1 mg dry weight of spores contains a little over 10^9 cfu, the subcutaneous dose at least is so much higher than what would be expected from the historical and epidemiological evidence that only a small number of spores are needed to initiate cutaneous anthrax when infecting through a breach in the skin.

The issue of inhaled dose, greatly highlighted by the anthrax letter events in the USA, remains unresolved. Haas (2002) compares the curves developed by Druett et al. (1953) and Glassman (1966). Druett et al.'s data suggest an RID_2 of approximately 2300 spores in contrast to 9 spores on the probit slope published by Glassman. (RID_2 is the dose at which 2% of the population exposed to that dose via the respiratory route would develop a clinical infection, but not necessarily die.)

As a generalization, the severity of the resulting infection undoubtedly depends on several factors such as route of infection, nutritional and other states of health on the part of the infected person, and possibly also on the relative virulence of the infecting strain. For the purpose of risk assessment, dependency on information from animal tests is unavoidable. Some of the published data on infectious and lethal doses in animals are given in section 3.1.

The issue of the human MID for anthrax by various routes of exposure is an important consideration in remediation of contaminated sites, i.e. how safe are sites in which it may be impossible or unfeasible to eliminate low levels of contamination? Cutaneous

and inhalational exposure are of primary concern, but gastrointestinal exposure may also be of concern, depending on the circumstances. Risk assessments following the 2001 anthrax letter events in the USA resulted in the conclusion that there is no safe level of contamination. It has long been anecdotal wisdom that it only requires a single spore reaching the correct site to initiate anthrax infection and, as noted in section 4.2.2.2, much depends on the probability of that spore reaching that site. Certainly the probability may be dependent in part (i.e. the chance of infection for a particular dose may be dependent in part) on the individual's underlying disease(s) and immune status.

4.2.2.2 Cutaneous infections

A century's experience of the natural disease in humans suggests that it probably does not take many spores to initiate a cutaneous infection once the necessary access to subepidermal tissues is achieved, despite the implication otherwise in the experimental data from Japanese Unit 731 (section 4.2.2.1). It is generally accepted that *B. anthracis* is non-invasive and that the spores must gain access to subepidermal tissue through a cut or abrasion before infection can occur, although this has been challenged: in a series of laboratory-acquired cutaneous anthrax cases, preceding trauma at the site of the lesion was noted by only 5 of the 25 patients (Ellingson, 1946).

The risk of infection reflects the chance of the spores gaining access and, in general, this is a low-probability event. This risk is greatly reduced in at–risk occupations by appropriate clothing and gloves, dressing of wounds, and other hygienic practices.

4.2.2.3 Inhalational infections

Based on two large studies in monkeys, the estimated human LD_2 ranges from 9 to 2300 inhaled spores, depending on whether exponential dose-response or a log-probit regression analysis is used (Druett et al., 1953; Glassman, 1966; Haas, 2002) (LD_2 is the dose at which 2% of the target population receiving that dose by the specified route would die). In the Sverdlovsk accidental release incident, sheep reportedly died of anthrax in a village 54 km distant from the point of release at the research institute (Meselson et al., 1994). If the respiratory MID for sheep is 35 000 spores (see section 3.1) and the minute respiratory volume for sheep is 8 litres, a cloud passing the affected premises would have contained more than 2187 spores/litre of air, assuming that the cloud originated as a single puff and transited the premises in 2 minutes. The concentration could have been lower with a more prolonged release but, either way, this suggests that the inhaled dose for humans near the research institute may have been greater than Meselson et al.'s originally proposed 9 spores. As already covered in section 4.2.1.1, the account of Gumbel (1991) suggests that there may have been a higher death rate than that reported by Meselson et al. (1994). Many of those who succumbed had predisposing respiratory illness.

Recorded inhalation LD_{50}s in non-human primates range from 2500 to 760 000 spores (Meselson et al., 1994; Watson & Keir, 1994). The United States Department of Defense based its strategies on an estimate that the LD_{50} for humans is 8000 to 10 000 spores (Meselson et al., 1994). This may date from work during the Second World War, or shortly thereafter, when it was seen that "something in excess of 10 000 inhaled spores were needed to kill most species" (Carter & Balmer, 1999). However the only direct, but semiquantitative, data on inhalation infectious doses in humans come from the studies in goat-hair processing mills referred to in section 4.2.1.1. It is well established that, at sizes above 5 µm, particles face increasing difficulty in reaching the alveoli of the lungs (Druett et al., 1953). The likelihood of inhaled spores penetrating far enough to induce inhalation anthrax therefore depends greatly on the size of the particles to which they are attached.

The overall conclusion from the available evidence is that, deliberate release scenarios apart, the risk of pulmonary anthrax outside industrial situations is very low. For example, addressing concerns over hazards in the vicinity of anthrax carcass sites, Turnbull et al. (1998b) analysed air samples 3–9 m downwind from disturbed dry dusty anthrax-carcass sites with soil contamination levels of 10^4–10^6 anthrax spores per gram in Namibia, and found that, even at the highest concentrations found in the air, it would require about 2.5 minutes for an average human undergoing moderate activity to inhale 1 cfu of spores. Clearly the dilution factor rapidly reduces the risk of infection.

In the anthrax letter events in the USA in late 2001, in which 11 persons contracted inhalational anthrax (Jernigan et al., 2001; Anon., 2002a), the exposure

doses were not determined. It is possible, although unproven, that exposures were < 10 000 spores but that these individuals represented only a small proportion of persons actually exposed reflecting, again, the "low probability event" nature of anthrax infection and the historically low infection rate:exposure ratio discussed in sections 4.1, 4.2.2.1 and 4.2.2.2. In this case, however, evidence of subclinical infection, such as might be anticipated in at least a proportion of a large number of persons exposed to sublethal doses, surprisingly was not found by serological surveys (Quinn et al., 2002).

These events also underscored the problem of overdependence on experimental animal LD_{50} data in risk assessments for human exposures to "weaponized" preparations of *B. anthracis*. Bioaggression centres around how many casualties can be inflicted on the opposing force, which requires focusing on LD_{50} estimates. In contrast, the focus in public health, which emphasizes the prevention of any avoidable illness, is the MID by alternative routes of exposure. Age, underlying disease status, immunological deficiencies, etc. all need to be taken into account. Sizeable doses may be necessary to deliver the necessary number of respiratory LD_{50}s[1] to young healthy soldiers in an act of aggression, but the minimum respiratory infectious dose might be considerably less for the elderly or immunocompromised, and those with a poor lung-clearance efficiency. Those that died in the Sverdlovsk incident were mostly over 45 years of age, smokers with emphysema or suffering from welder's lung, also with emphysema – conditions having a serious impact on lung-clearance capacity for inhaled particles (Hugh-Jones, personal communication, 2003).

4.2.2.4 Oral route infections

The oral MID_{50} dose determined from human experimentation in units 731 and Ei 1644 in Japan during the Second World War is 50 mg, equivalent to approximately 10^{11} spores (section 4.2.2.1). No other information on infectious doses in humans by the oral route was found, but what is true for the skin is probably largely true for the oropharyngeal and gastrointestinal epithelium, namely that the chance of infection is likely to be enhanced by, if not dependent on, the existence of a lesion in the epithelium through which spores can gain entry and establish an infection. Uptake of bacilli and/or spores through the tonsillar epithelium and M cells overlying Peyer's patches in the small intestine may be possible routes of entry also.

Reports of oral LD_{50}s from tests in animals, even for species regarded as highly susceptible to infection, range from 10^6 to 10^8 cfu of spores with MIDs of approximately 1.5 to 5 x 10^8 (see section 3.1). Illness with recovery and seroconversion may occur with non-lethal doses in both humans and animals (see section 4.4.1). The extent to which the outcome reflects activities (colonization, germination, toxin production, etc.) within the gut, as opposed to the point at which very large numbers of spores simply overcome natural intestinal barriers, is unknown at present.

Virtually nothing is known about the relative infectivity of vegetative cells as compared to spores and the possible importance of this to oral infectious dose. (It is not always appreciated that it is almost impossible to prepare spore-free vegetative cell preparations with which to study the infectivity of vegetative cells experimentally.) In pigs, however, the pharyngeal form of ingestion anthrax generally results from consumption of meat and bones from infected carcasses, which would contain both vegetative cells and spores, while the intestinal form is associated with consumption of spore-contaminated mineral supplements (section 3.4.4). By analogy, in humans the oropharyngeal form has been reported in persons eating undercooked meat (presumably containing vegetative cells) from water buffalo (Sirisanthana et al., 1984). Intestinal manifestations presumably result from the presence of spores which survive the gastric juices.

4.2.2.5 Treatability

The fact that anthrax is readily treated if diagnosed at a sufficiently early stage of infection also needs to be taken into account when assessing risks. Awareness of the likelihood of exposure having taken place is clearly an important part of the equation. Of the 11 patients diagnosed as having inhalational anthrax following the deliberate-release anthrax letter events of late 2001 in the USA, 6 were successfully treated (Anon., 2002a) proving that even this most dangerous form of the disease is susceptible to timely intervention. Antibiotic combinations were

[1] 55 000 spores appears to be the number currently quoted for healthy young adults (Hugh-Jones, personal communication, 2003).

thought to provide a therapeutic advantage over single antibiotics (see section 7.3.1.6).

In evaluating the prognosis, the pathogenic role of the anthrax toxin complex needs to be kept in mind (see section 5.5.3) In cutaneous anthrax, progressive evolution of the lesions continues for about 24 hours after bacteriological cure is achieved (Ellingson et al., 1946); similar progression of disease is probable in the other clinical forms of the disease. As long ago as the 1950s (Keppie et al., 1955) it was noted that, in guinea-pigs, once bacteraemia had reached ± 3 x 10^6 chains/ml (approximately 8 hours before death), termination of the bacteraemia and removal of the infection by streptomycin treatment delayed but did not prevent death. Similarly, in both monkeys and rats challenged with sterile anthrax toxin, antiserum is effective in preventing death if given early but not late in the post-challenge period (Lincoln et al., 1967).

4.2.3 Bioaggression versus natural disease

B. anthracis has always been high on the list of potential agents with respect to biological warfare and bioterrorism (section 1.1; Klietmann & Ruoff, 2001), and unfortunately has now been used in that context in the 2001 anthrax letter events in the USA with severe consequences (sections 4.2.1.3, 4.2.2.3). Regrettably, this has attached an unjustified doomsday image to the naturally-occurring disease in regions of the world where it is no longer common and the name "anthrax" frequently engenders unnecessary fear, for example in relation to occasional cases in animals, or to contaminated burial or industrial (e.g. tannery) sites.

It is important to separate the two situations, i.e. the natural and the deliberately-induced disease. Nature cannot remotely reproduce the overwhelmingly massive exposures that can be created in human-made deliberate release scenarios. The natural disease is always readily controllable. The "worst case" natural contamination in the environment is found at the carcass sites of animals that have died of anthrax. Contamination levels at such sites rarely exceed 1 million anthrax spores per gram of soil, and mostly are less than 1000 per gram (Lindeque & Turnbull, 1994; Dragon et al., 2005). Levels in other types of inadvertently contaminated environments (soils at tannery sites, horsehair plaster, etc.) rarely exceed a few units or tens of spores per gram (Turnbull, 1996). Natural environmental exposure to infectious doses in the normal course of human endeavour is generally, therefore, an unlikely event (see section 4.2.1.2). This contrasts to scenarios with exposure to many millions of doses which can only be created artificially.

The public health implications of deliberately-induced anthrax outbreaks and their use as a biological weapon are largely beyond the scope of this publication; the reader is referred to WHO (2004). (See also section 4.3.3.)

4.3 Epidemiology and transmission: the forms of anthrax

4.3.1 Naturally acquired anthrax

Anthrax in humans is classically divided in two ways. The first type of classification, which reflects how the occupation of the individual led to exposure, differentiates between *non-industrial anthrax*, occurring in farmers, butchers, knackers/renderers, veterinarians and so on, and *industrial anthrax*, occurring in those employed in the processing of bones, hides, wool and other animal products. The second type of classification, reflecting the route by which the disease was acquired, distinguishes between *cutaneous anthrax*, commonly held to require a prior skin lesion as a prerequisite for infection (section 4.2.2.2), *ingestion or oral route (enteric) anthrax* contracted following ingestion of contaminated food, primarily meat from an animal that died of the disease, or conceivably from ingestion of contaminated water (section 4.2.2.4) and *inhalational (pulmonary) anthrax* from breathing in airborne anthrax spores (section 4.2.2.3).

Non-industrial anthrax, resulting from handling infected carcasses, usually manifests itself as the cutaneous form; it tends to be seasonal and parallels the seasonal incidence in the animals from which it is contracted. Cutaneous anthrax transmitted by insect bites, and anthrax of the alimentary canal from eating infected meat, are also non-industrial forms of the disease. Industrial anthrax also usually takes the cutaneous form but has a far higher probability than non-industrial anthrax of taking the inhalational form as a result of exposure to spore-laden dust.

Occasional cases of laboratory-acquired anthrax have occurred (Pike et al., 1965; Collins, 1988), but this was principally before appropriate laboratory-containment procedures and facilities were put in place.

As with animals (section 3.3.5), mechanical transmission by biting insects is believed to be at least an occasional mechanism by which anthrax is contracted by humans in some countries (Rao & Mohiyudeen, 1958; Davies, 1983).

4.3.2 Chances of person-to-person transmission

Humans almost invariably contract anthrax directly or indirectly from infected animals, and the epidemiology of human incidents and outbreaks reflects that of the animals from which the humans contract the disease. The disease is generally regarded as being non-contagious and, as with animals (section 3.3.1), records of person-to-person transmission exist but such instances are very rare (Lalitha et al., 1988; Quinn & Turnbull, 1998).

Also rare are examples of humans acting as intermediate fomites. Eurich & Hewlett (1930) record a schoolboy contracting anthrax after playing football with "factory hands still clothed in their overalls" and a woman who became infected through washing her husband's overall (the precise occupations of the factory hands and husband are not specified). Punskii & Zheglova (1958) describe an outbreak of human anthrax in Turkmenistan in 1956 that resulted in 37 cutaneous cases. The source was meat from a camel that had died suddenly. In two children, who had no contact with the camel meat, the infection was regarded as having resulted from sleeping with their parents who had cutaneous lesions following exposure to the raw meat. In the outbreak described by Heyworth et al. (1975), the spread was effected by a shared toilet article. Pugh & Davies (1990) recorded a case of anthrax in an untrained orderly who had handled the soiled dressing of a patient's cutaneous lesion. The source of the infection in the case of infant cutaneous anthrax during the 2001 anthrax letter events was considered to have been the spore-contaminated workplace of the infant's mother.

Of the rare reports of person-to-person transmission, all were cases of cutaneous anthrax. It has not been reported for inhalational or ingestion anthrax. However, it tends to be forgotten that the total all-time number of recorded cases of inhalational anthrax in world history is probably less than a thousand. This compares with, probably, hundreds of thousands of cutaneous cases in the same time frame. Because inhalational anthrax is an occupational disease of a few specific at-risk occupations, it is likely that the number of recorded cases is very close to the number of actual cases. The number of all-time documented cases of ingestion anthrax is also probably less than a thousand, but because of the nature of the acquisition of ingestion anthrax, namely consumption of meat from infected animals in endemic areas, it is probable that the all-time number of actual cases greatly exceeds the number of recorded cases. However, it is still likely to lie only in the high thousands.

Statistically, therefore, the chances of person-to-person transmission occurring from the inhalational or ingestion forms of anthrax are far less than the chances in the case of cutaneous anthrax (which is, itself, very low). Pathologically, before death of the infected person, the chance of another person being exposed to infective material from the first person is far greater in cutaneous anthrax than in the other forms because the cutaneous lesion is exposed, whereas the developing infection is contained within the body in the other forms until the very terminal stages of illness. After the death of the infected person, the chances of infection by a second person handling the cadaver become equal whichever form of anthrax the first person developed.

4.3.3 Bioaggression: mass disruption

Envisaged scenarios in planning for potential acts of bioaggression utilizing *B. anthracis* have invariably taken the form of attacks with aerosolized spores. Carus (2001), reviewing 269 traceable instances in the past century of allegations that terrorist, criminal or covert state operators used, acquired, threatened to use, or thought of using biological agents, found that, of 191 that could be substantiated, over half were anthrax threats. The majority of these involved the implied use of aerosolized spores. Updates of Carus's figures by Dolnik & Pate (2001) and Turnbull & Abhayaratne (2002) show a huge surge in similar anthrax hoaxes in 2001 in association with the anthrax letter events, and yet more in 2002. It was always appreciated, however, that the release of large numbers of aerosolized spores would lead to cutaneous exposure, and probably ingestion of substantial numbers of spores as well as inhalation. It is technically feasible to deliberately release enormous concentrations of spores (see section 4.2.3); the potential for long-term environmental contamination with re-aerosolization of substantial amounts is a real possibility.

The anthrax letter incident demonstrated that even a small number of casualties can cause a significant anxiety level in a community. Despite the implementation of on-site clinics for employees at contaminated facilities, one major Washington, D.C., emergency department had 801 patients presenting for evaluation of possible exposure to anthrax in just the first two weeks following initial newspaper accounts about the incident (Sporn et al., 2002). The media coverage of the incident blanketed the airways, raising general awareness of the problem and the powdered nature of the material. Thousands of requests to examine powders from a myriad of sources overwhelmed the laboratory system. Even innocuous situations, such as finding spilt sugar on tables, led to calls to emergency management agencies. The reaction illustrates the fact that a terrorist does not have to kill large numbers of people to induce panic. In this regard, anthrax spores have been aptly referred to as "weapons of mass disruption". The importance of effective risk communication cannot be overestimated in response to a terrorist incident. In addition to the immediate response requirements for treatment, postexposure prophylaxis, and environmental risk assessment following the use of *B. anthracis* in a terrorist attack, there is a great demand from the public, resulting from the terror effect, for support and information. Clear, early and effective risk communication can assist in mediating this public concern and fear, thereby reducing the overload on the health and other emergency services.

Although surface contamination may be limited at points remote from the primary release area of "weaponized" agent powder, in the vicinity of the release, particularly within buildings, the potential for re-aerosolization of the undispersed primary preparation presents a major health risk. Larger particles that settle out near the point of primary dispersal present a definite hazard. In the anthrax letter events, resuspension of anthrax spores following the attacks of 2001 was shown in several settings (Dull et al., 2002; Weis et al., 2002) and the secondary aerosolization by normal office activities was documented in one office that was contaminated when a letter containing powdered agent was opened (Weis et al., 2002).

4.4 Clinical disease in humans

4.4.1 Historical information

Historically, cutaneous anthrax has always accounted for > 95% of human cases globally. All three forms (cutaneous, alimentary tract and inhalational) are potentially fatal if untreated, but the cutaneous form is more often self–limiting. Data from pre–antibiotic and vaccine days indicate that 10%–40% of untreated cutaneous cases may be expected to result in death with some geographical and temporal variations. In German tannery workers during the period 1910–1921, the case-fatality rate was 15.8% (Smyth, 1924); data from British at-risk industries indicated that 10%–25 % of untreated cases were expected to result in death (Anon., 1918, 1959); in the USA from 1919 to 1924, the mortality was approximately 31% with yearly variance from 13% to 39% (Smyth et al., 1926). With treatment, < 1% are fatal. However, in the days when the disease was seen more commonly, cutaneous cases were occasionally so mild as "not to merit treatment" (Gold, 1967). Certainly the clinical significance of cutaneous anthrax when untreated should not be downplayed, and any suspect case should be treated. In general, however, the rare fatalities seen today are caused by obstruction of the airways by the accompanying extensive oedema when the lesion is on the face or neck, and sequelae of secondary cellulitis or meningitis.

Overt inhalational and alimentary tract cases are more often fatal, largely because they go unrecognized until it is too late for effective treatment. Of 75 notifications of inhalational anthrax in England and Wales during the period 1900–1959, 74 (98.7%) were fatal (Anon., 1959). Similarly in the USA, of the 18 recorded cases of naturally acquired inhalational anthrax in the 20th century, 16 (88.9%) were fatal (Brachman & Kaufmann, 1998). However, serological and epidemiological evidence suggests that undiagnosed low-grade or subclinical versions of both these forms of the disease (and also the cutaneous form) with recovery can also occur, and may not be infrequent, among exposed groups (Brachman et al., 1960; Norman et al., 1960; Heyworth et al., 1975; Sirisanthana et al., 1988; Turnbull et al., 1991, 1992a; de Lalla et al., 1992; Oh et al., 1996; Van den Bosch, 1996). Eurich & Hewlett (1930) state definitely that "instances of recovery from pulmonary anthrax have been recorded". Latent infections are also discussed in section 3.3.8.

Development of meningitis is a dangerous possibility in all three forms of anthrax.

4.4.2 Cutaneous anthrax

Anthrax eschars are generally seen on exposed unprotected regions of the body, mostly on the face, neck, hands and wrists. Generally cutaneous lesions are single, but sometimes two or more lesions are present. For example, with infection resulting from skinning an infected dead animal, multiple lesions may be seen on hands, wrists or arms.

The incubation period ranges from as little as 9 hours to 3 weeks, mostly 2 to 6 or 7 days. In the October–November 2001 anthrax letter cases in the USA, the mean/median incubation period for the 11 cutaneous cases was 5–6 days, with a range of 1–12 days (Inglesby et al., 2002; Jernigan et al., 2002; Phillips et al., 2003). Shlyakhov (Anon., 1996) noted an average incubation period of 2–3 days, range 9 hours to 10 days, in an analysis of 1215 cases of cutaneous anthrax in the former Soviet Socialist Republic of Moldavia between 1946 and 1950. He cites a 1929 publication by a Russian author on 30 persons who developed cutaneous anthrax following vaccination against rabies with a syringe previously used for dispensing anthrax spores; the incubation periods were 1 day (1 patient), 3 days (2 patients), 4 days (2 patients), 5 days (2 patients), 6 days (10 patients), 7 days (5 patients), 9 days (1 patient) and 12 days (1 patient). Salmon (who gave his name to *Salmonella*) also recorded a case in which an anthrax pustule developed within 12 hours of contact with a new horse brush (Salmon, 1896).

The general scenario is as follows:

Day 0 Entry of the infecting *B. anthracis* (usually as spores) through a skin lesion (cut, abrasion, etc.) or (possibly as vegetative forms or vegetative forms and spores) by means of a fly-bite.

Days 2–3 A small pimple or papule appears (see Fig. 4).

Days 3–4 A ring of vesicles develops around the papule. Vesicular fluid may be exuded. Unless the patient was treated, capsulated *B. anthracis* can be identified in appropriately stained smears of this fluid, and the bacterium can be isolated by culture (Annex 1, section 10). Marked oedema starts to develop. Unless there is secondary infection, there is no pus and pathognomonically the lesion itself is not painful, although painful lymphadenitis may occur in the regional lymph nodes and a feeling of pressure may result from the oedema. The lesion is usually 1–3 cm in diameter and remains round and regular. Occasionally a lesion may be larger and irregularly shaped (Fig. 5).

Days 5–7 The original papule ulcerates to form the characteristic eschar. Topical swabs will not pick up *B. anthracis*. Detection in smears or by culture requires lifting the edge of the eschar with tweezers (this gives no pain unless there is secondary infection) and obtaining fluid from underneath. The fluid will probably be sterile if the patient has been treated with an antibiotic. Oedema extends some distance from the lesion. Systemic symptoms are low-grade fever, malaise and headache. If the cutaneous reaction is more severe, especially if located on the face, neck or chest, clinical symptoms may be more severe with more extensive oedema extending from the lesion, toxaemia, a change in mental status, high fever, hypotension, regional lymphadenomegaly and the patient unable to eat or drink. Tracheotomy is a life-saving procedure in patients having a cutaneous lesion on the face or neck with an extensive oedema leading to compression on the trachea. This clinical manifestation is very dangerous (Doganay et al., 1987; Doganay, 1990).

Day 10 (approx.) The eschar begins to resolve; resolution takes several weeks and is not hastened by treatment. Clinicians unaware of this suffer from concern that the treatment has been ineffective (see section 7.3.1.5). A small proportion of untreated cases develop sepsis or meningitis with hyperacute symptoms.

Time to resolution will depend on the size, location and local severity of the lesion. The initial crust separates several weeks after onset, with subsequent healing by granulation. Sometimes the separation of the crust is delayed and the lesion may become secondarily infected. In this situation, the crust should be excised surgically. Lesions characterized by "malignant oedema" (this is a historical misnomer resulting from the "cauliflower" appearance of the lesion; there is, in fact, no malignant process involved) can be expected to take months to heal. Very large lesions may require skin grafts, and lesions in locations such as the eyelid may require surgical intervention due to scarring.

4.4.2.1 Differential diagnosis

A history of exposure to contaminated animal materials, occupational exposure and living in an endemic area is important when considering a diagnosis of anthrax. A painless, pruritic papule, surrounding vesicles and oedema, usually on an exposed part of the body, is suspicious. Clinical diagnosis is confirmed by the demonstration of Gram-positive encapsulated bacilli from the lesion and/or positive culture for *B. anthracis* from the lesion and/or positive specialist tests as described in section 4.4.2.2.

The differential diagnosis of the anthrax eschar includes a wide range of infectious and non-infectious conditions: boil (early lesion), arachnid bites, ulcer (especially tropical); erysipelas, glanders, plague, syphilitic chancre, ulceroglandular tularaemia; clostridial infection; rickettsial diseases; *Rhizomucor* infections, orf, vaccinia and cowpox (Lewis-Jones et al., 1993), rat-bite fever, leishmaniasis, ecthyma gangrenosum or herpes. Generally these other diseases and conditions lack the characteristic oedema of anthrax. The absence of pus, the lack of pain, and the patient's occupation may provide further diagnostic clues. The outbreak of Rift Valley Fever, referred to in section 3.5.7 and initially thought to be anthrax in livestock, also affected numerous humans.

In differential diagnosis of the severe forms, orbital cellulitis, dacrocystitis and deep tissue infection of the neck should be considered in the case of severe anthrax lesions involving the face, neck and anterior chest wall. Necrotizing soft tissue infections, particularly group A streptococcal infections and gas gangrene, and severe cellulitis due to staphylococci, should also be considered in the differential diagnosis of severe forms of cutaneous anthrax. Gas and abscess formation are not observed in patients with cutaneous anthrax. Abcess formation is only seen when the lesion is infected with other bacteria such as streptococci or staphylococci.

4.4.2.2 Immunological and other tests

The rapid hand-held, on-site, immunochromatographic detection and diagnostic devices that have been developed in recent years are discussed in section 6.2.

In the case of retrospective diagnosis of anthrax, serology can be supportive (sections 3.5.6, 4.4.1). Purified protective antigen and lethal factor (see section 5.5.3) are available commercially from List Laboratories).[1]

Turnbull et al. (1992a) detected seroconversion in 17 of 38 (44.7%) patients with bacteriologically-confirmed cutaneous anthrax, and a further 5 patients in whom bacteriological confirmation was not successful. The percentage of seroconverters (71%) was much higher in the 14 persons for whom paired sera were examined than in the remaining 24 persons (29%). Similarly, seroconversion was only found in 5 of 21 (24%) individuals from whom blood was obtained ≤ 7 days after the first appearance of lesions as compared with 15 (83%) of the 18 persons bled ≥ 8 days after the appearance of lesions. The failure to seroconvert by the 21 individuals with bacteriologically confirmed anthrax and 19 other persons clinically diagnosed as anthrax cases was interpreted as indicating that treatment early in the course of the infection prevented elaboration of sufficient antigen to induce a detectable antibody response. Studies in non-human primates showed that early antibiotic treatment after a known challenge with *B. anthracis* spores abrogated a detectable antibody response (Friedlander et al., 1993). Negative results should therefore be interpreted with caution and in the light of the full patient history.

Quinn et al. (2004) analysed sera from 16 individuals with confirmed or suspected cutaneous or inhalation anthrax resulting from the 2001 anthrax letter releases in the USA, and one laboratory worker with laboratory-acquired cutaneous anthrax also associated with that event. In 6 patients surviving inhalation anthrax, anti-PA (anti-protective antigen) IgG was detected 11–22 days after the onset of symptoms (15–28 days after likely exposure). Anti-PA IgG was also detectable in the serum from 10 of the 11 patients with bioterrorism-associated cutaneous anthrax and the one patient with laboratory-acquired infection. In these cutaneous cases anti-PA IgG was detectable at 12 days after the onset of symptoms (24 days after estimated exposure). One cutaneous anthrax patient was seronegative at day 18 after the onset of symptoms but had detectable anti-PA IgG at day 34. In one cutaneous anthrax patient, anti-PA IgG was not detectable at 4, 5, 47 and 253 days after the onset of symptoms. Anti-PA IgG was detectable 8–16 months post-symptoms in all 6 survivors of inhalation anthrax and in 7 of 11 persons suffering from the cutaneous form of the disease. There was a positive correlation between

[1] www.listlabs.com.

serum toxin neutralizing activity and anti-PA IgG levels.

Immunohistochemistry, noted by Fritz et al. (1995) in experimental inhalation anthrax in rhesus monkeys to have diagnostic value, proved to be an invaluable aid to confirmation of diagnosis in the anthrax letter events in the USA in 2001, with the particular advantage over other diagnostic tests of being able to detect anthrax-specific antigens in tissues regardless of treatment (Shieh et al., 2003). In one patient, definitive diagnosis retrospectively depended on this technique being applied to a skin biopsy taken as long as 9 days after treatment had begun. At present, however, the method is confined to specialist laboratories with access to appropriate specific antibodies.

In the Russian Federation, a skin test utilizing Anthraxin™,[1] first licensed in the former Soviet Union in 1962, has become widely used for retrospective diagnosis of human and animal anthrax and for vaccine evaluation (Shylakhov et al., 1997). This is a commercially produced heat–stable protein–polysaccharide–nucleic acid complex without capsular or toxigenic material, derived from oedematous fluid of animals injected with the vaccine STI-1 or the Zenkowsky strains of *B. anthracis*. It is sterilized by autoclaving. The test, which is still used in the Russian Federation (Cherkasskiy, personal communication, 2003), involves intradermal injection of 0.1 ml of Anthraxin™. A positive test is defined as erythema of ≥ 8 mm with induration persisting for 48 hours (Shlyakhov et al., 1997). This delayed-type hypersensitivity is seen as reflecting anthrax cell mediated immunity and was reportedly able to diagnose anthrax retrospectively some 31 years after primary infection in up to 72% of cases (Shlyakhov et al., 1997). It was used with success in a retrospective investigation of a series of cases occurring in a spinning mill in Switzerland where synthetic fibres were combined with goat hair from Pakistan (Pfisterer, 1990). The diagnostic reliability of Anthraxin™, like Ascoli test antigen (Annex 1, section 11.1), depends on the nature of anthrax rather than on the specificity of the antigens involved.

With the same provisos as given in section 3.5.5, the PCR (section 6.3.2; Annex 1, section 10.7.4) has now become accepted as a sensitive method for detecting anthrax-specific DNA in clinical samples (Ellerbrok et al., 2002; Shieh et al., 2003).

4.4.2.3 Precautions

Surgical tools should be sterilized without delay after use, and dressings should be incinerated (Annex 1, sections 7.8 & 7.9). The wearing of surgical gloves by medical staff and orderlies is recommended, but risks to these staff are not high. Direct human–to–human transmission is exceedingly rare (see section 4.3.2); standard contact precautions for management of patients are recommended with any form of anthrax (Ashford et al., 2000). For the most part, this involves wearing disposable gloves and gown or laboratory coat while taking specimens or dressing lesions, changing gloves after the relevant action and before touching anything else and properly disposing of the gloves, thorough handwashing at the end of procedures, ensuring decontamination and disinfection of gown/laboratory coat, bedding and other items that may have become contaminated from the patient's infected site and ensuring that other appropriate procedures for cleaning and disinfection are in place (Garner, 1997; see also Annex 1, sections 7.8 & 7.9). Vaccination of medical staff and orderlies is not necessary.

4.4.3 Ingestion (oral route/enteric) anthrax

4.4.3.1 Signs and symptoms

There are two clinical manifestations of anthrax that may result from ingestion of *B. anthracis* in contaminated food or drink – oropharyngeal anthrax and gastrointestinal anthrax. The oropharyngeal form is the less commonly seen.

The suspicion of alimentary canal anthrax depends largely on awareness and alertness on the part of the physician as to the patient's history and to the likelihood that he/she has consumed contaminated food or drink. Response to treatment can be good (Van den Bosch, 1996) and there is evidence that mild undiagnosed cases with recovery occur (Ndybahinduka et al., 1984; Turnbull et al., 1992a; Oh et al., 1996; Centers for Disease Control and Prevention, 2000; see also sections 4.2.2.4 and 4.4.1).

The incubation period is commonly 3–7 days. The spectrum of disease ranges from asymptomatic to severe, terminating in sepsis, septic shock and death. In developing countries, mild cases with gastroenteritis attract little attention and the patients

[1] Antiplague Research Institute, Sovetskaya St., 13/15, Stavropol 355106, Russian Federation; fax: +7 8652 260312).

with severe infections, leading to death within 2–3 days, may never reach a medical facility. In endemic areas, all physicians should be aware of gastrointestinal anthrax.

Oropharyngeal anthrax

This appears to be a relatively infrequent manifestation in regions where ingestion anthrax is not uncommon (Sirisanthana & Brown, 2002). The lesion is generally localized in the oral cavity, especially on the buccal mucosa, tongue, tonsils or posterior pharynx wall. In some cases, lesions may be present at two or more sites along the gastrointestinal tract. The oral lesion is generally 2–3 cm in diameter and covered with a grey pseudomembrane surrounded by extensive oedema. When the lesion is localized on tonsils, the affected tonsil is also intensely oedematous and covered with a grey or white pseudomembrane. Tonsil lesions may be ulcerated. Pharyngeal culturing is important in the diagnosis. Microscopic examination of a Gram-stained smear from the lesion reveals polymorphonuclear leukocytes and Gram-positive bacilli, and the culture may be positive for *B.anthracis* (Doganay et al., 1986; Sirisanthana et al., 1984; Navacharoen et al., 1985; Onerci & Ergin, 1993; Beatty et al., 2003).

The main clinical features are sore throat, dysphagia and painful regional lymphadenopathy in the involved side of the neck. The illness may progress rapidly, and oedema around the lymph node may result in extensive swelling of the neck and anterior chest wall. Bacteraemia may develop and blood culture may be positive for *B.anthracis*. The overt infection leads to toxaemia, acute respiratory distress and alteration in mental state. The patient develops acute respiratory distress syndrome and may require respiratory support. This clinical picture is followed by shock, coma and death. The lesion and extensive oedema may lead to airway obstruction. In this situation, tracheotomy is frequently required (Sirisanthana et al., 1984, 1988; Doganay et al., 1986; Inglesby et al., 2002). Even with treatment, mortality can be high (Doganay et al., 1986).

Gastrointestinal anthrax

The gastrointestinal anthrax lesion may occur anywhere within the gastrointestinal tract – the oesophagus, stomach, duodenum, jejunum, terminal ileum or caecum, but mostly in the ileum and caecum. The character of the lesion is generally ulcerative, usually multiple and superficial, surrounded by oedema. These lesions may bleed, haemorrhage may be massive and fatal, in some cases with stomach infection. Intestinal lesions may also lead to haemorrhage, obstruction, perforation or any combination of these. Some cases are complicated with massive ascites and this leads to shock and death. Pathological examination of intestinal anthrax shows mucosal ulceration with oedema, and enlarged and haemorrhagic regional lymph nodes. Necrosis is sometimes present. The infection may also be disseminated, and sepsis with pulmonary or meningeal involvement may result.

The symptoms of gastrointestinal anthrax are initially nonspecific and include nausea, vomiting, anorexia, fainting spells, asthenia, mild diarrhoea and fever, and headache. In these instances, patients will probably not seek medical treatment and, if they do, intestinal anthrax may not be considered in differential diagnosis. In some cases, approximately 24 hours later the symptoms may become severe and include acute diarrhoea, nausea, vomiting, abdominal pain. With progression of the illness, abdominal pain, haematemesis, bloody diarrhoea, massive ascites and signs of suggestive acute abdomen (rapid increase in abdominal girth and paroxysms of abdominal pain) appear. Kanafani et al. (2003), reporting on > 100 cases over a 14-year period in Lebanon, record that laparotomy at this later stage revealed yellowish and thick ascetic fluid, hypertrophied mesenteric lymph nodes, mostly in the ileocaecal region, and substantial oedema involving one segment of the small bowel, caecum or ascending colon. Toxaemia, sepsis and shock may develop, followed by death. The time from onset of symptoms to death has most frequently varied from 2 to 5 days (Dutz et al., 1970; Nalin et al., 1977; Bhat et al., 1985; Sirisanthana & Brown, 2002; Kanafani et al., 2003). The incubation period is typically 1–6 days (Beatty et al., 2003), although it may be as long as 10 days (Kanafani et al., 2003).

There is evidence that not all untreated cases end in toxaemia, sepsis and death and that, after the initial symptoms, recovery occurs (see sections 4.4.1, 4.4.3.1).

4.4.3.2 Confirmation of diagnosis

Diagnosis of oropharyngeal anthrax is covered in section 4.4.3.1. For the gastrointestinal form, see section 4.4.4.2.

4.4.3.3 Differential diagnosis

In the differential diagnosis of oropharyngeal anthrax, diphtheria and complicated tonsillitis, streptococcal pharyngitis, Vincent angina, Ludwig angina, parapharyngeal abscess, and deep-tissue infection of the neck should be considered.

The differential diagnosis in gastrointestinal anthrax includes food poisoning (in the early stages of intestinal anthrax), acute abdomen owing to other reasons, and haemorrhagic gastroenteritis caused by other microorganisms, particularly necrotizing enteritis caused by *Clostridium perfringens* and dysentery (amoebic or bacterial).

4.4.4 Inhalational (pulmonary, mediastinal, respiratory) anthrax

4.4.4.1 Symptoms and course of the disease

The term "inhalational anthrax" has largely replaced the older names for this form of the disease, the most common of which was "pulmonary anthrax", reflecting the fact that active infection occurs in the lymph nodes, rather than the lung itself, and that bronchopneumonia does not occur. The inhaled spores are carried by macrophages from the lungs, where there is no overt infection, to the lymphatic system where the infection progresses. Germination and initial multiplication begin within the macrophages while in transit to the lymph nodes (Hanna & Ireland, 1999). The vegetative cells kill the macrophages and are released into the bloodstream where they continue to multiply and lead to fatal septicaemia (see also section 5.2).

Symptoms prior to the onset of the final hyperacute phase are nonspecific, and suspicion of anthrax depends on the knowledge of the patient's history. Analysis of 10 of the 11 inhalational cases associated with the anthrax letter events of 2001 in the USA (Jernigan et al., 2001; Inglesby et al., 2002) revealed a median incubation period of 4 days (range 4–6 days) and a variety of symptoms at initial presentation including fever or chills (n=10), sweats (n=7), fatigue or malaise (n=10), minimal or nonproductive cough (n=9), dyspnoea (n=8), changes in mental state including confusion (n=5) and nausea or vomiting (n=9). All patients had abnormal chest X-rays with infiltrates (n=7), pleural effusion (n=8) and mediastinal widening (n=7). Mediastinal lymphadenopathy was present in seven cases. In the previously best–documented set of five case reports of inhalational anthrax, Plotkin et al. (1960) also recorded headache, muscle aches and development of a cough in four patients and mild pain in the chest in one. Jernigan et al. (2001) drew attention to profound sweating as a prominent feature in their patients not emphasized in previous reports.

In contrast to the median incubation period of 4 days found in the anthrax letter inhalation cases, Brookmeyer et al. (2001) estimated it to have been 11 days in the Sverdlovsk outbreak. One consideration that should be kept in mind is the possibility that reflux of spores from the respiratory tract into the alimentary tract may occur with the development of lesions there, and that this may affect time of onset of symptoms. However, while high exposure may lead to swallowing as well as inhaling spores, it is the alternative view that enteric manifestations result from toxic action being carried to the gastrointestinal tract via the bloodstream rather than from concurrent ingestion anthrax.

The mild initial phase of nonspecific symptoms is followed by the sudden development of dyspnoea, cyanosis, disorientation with coma, and death. In Plotkin et al.'s cases, treatment was unsuccessful in four of the patients, and death occurred within 24 hours of onset of the hyperacute phase.

The typical clinical course of this form of the disease is consistent with the lesion development within the mediastinal lymph nodes before the development of bacteraemia. The assault on the lung appears to be two-pronged. In the initial phase, the blockade of the lymphatic vessels develops, in association with symptoms such as a sensation of tightness of the chest. Lymphatic stasis is associated with oedema, which may be apparent above the thoracic inlet, and pleural effusion. Histological sections of the lung may reveal bacilli within the lymphatic vessels. In the acute phase, damage associated with septicaemia occurs. This is manifested morphologically by the changes described by Dalldorf et al. (1971). Occasional patients do not develop the mediastinitis which usually typifies this form of the disease. Mediastinal widening has been found to be a relatively frequent manifestation of other diseases, leading to the recommendation for computerized axial tomography (CAT) scans to demonstrate lymph node involvement.

Recent findings using computerized tomography (CT) scans combined with autopsy observations have enhanced clinical interpretation of early inhalational anthrax evolution (Galvin et al., 2001). The earliest

detectable specific finding pointing to inhalational anthrax is mediastinal widening on posteroanterior (PA) chest X-rays. However, mediastinal widening is not a rare finding in a series of patients presenting at a hospital emergency department. Imaging in inhalational anthrax patients using a non-contrast spiral CT will reveal hyperdense lymph nodes in the mediastinum associated with oedema of mediastinal fat. The hyperdensity of the lymph nodes represents haemorrhage and necrosis, following spore germination and vegetative growth with exotoxin elaboration. Lymphatic stasis resulting from the damaged lymph nodes leads to dilatation of pulmonary lymphatics which originate in the pleura and drain towards the hilum, following interlobular septa in association with blood vessels. The stasis manifests as an early onset pleural effusion and peripheral infiltrates, representing thickened bronchovascular bundles, detectable on chest X-ray. These findings mark fully developed initial stage illness. Ultimately, the bacteria escape from the damaged lymph nodes and invade the blood stream via the thoracic duct. Once the bacteraemia and associated toxaemia reach a critical level, the severe symptoms characteristic of the acute phase illness are manifest. During the acute phase illness, damage of the lung tissue becomes apparent on X-ray. This damage results from the action of anthrax toxin on the endothelium of the lung's capillary bed (Dalldorf et al., 1971). Primary damage of the lung is not normally a feature of the initial phase illness and primary pulmonary infection is an uncommon presentation (see also section 5.2).

The X-ray picture of the lung appears to be a very sensitive diagnostic aid with multiple abnormalities, including mediastinal widening, paratracheal fullness, pleural effusions, parenchymal infiltrates and mediastinal lymphadenopathy (Jernigan et al., 2001).

As stated in section 4.4.1, the number of recorded cases of inhalational anthrax in history is lower than might be perceived from the high profile given to this manifestation, and it has long been suspected, with some supportive evidence, that undiagnosed low-grade inhalational infections with recovery may occur in at-risk occupations.

4.4.4.2 Confirmation of diagnosis (inhalational and ingestion anthrax)

Clinical diagnosis is dependent on a high index of suspicion resulting from knowledge of the patient's history (see sections 4.4.3.1 and 4.4.4.1). Early symptoms are nonspecific and flulike, with mild upper respiratory tract signs in inhalational anthrax or resembling mild food poisoning in intestinal anthrax. Where the history has not led to suspicion of anthrax, confirmatory diagnosis of pulmonary or gastrointestinal anthrax will usually take place after the patient has died or, if correct treatment is initiated early enough, after recovery.

Albrink & Goodlow (1959) demonstrated in monkeys with inhalational anthrax that detectable bacteraemia occurred before the fulminant clinical phase set in. However, treatment resulted in negative cultures after just one or two doses of antibiotics. In the 2001 anthrax letter cases in the USA, blood culture was positive in all patients who had not received prior antibiotic therapy (Jernigan et al., 2001). Sputum for staining and culture was not consistently collected; however, positive isolation from sputum would supply a definitive diagnosis. In general, visualization of the Gram-positive, capsulating bacilli and verification by culture should be attempted with pulmonary effusions, CSF, or other body fluids or tissues of suspected anthrax cases. Similarly, isolation from vomitus, faeces and ascites in intestinal anthrax may not always be successful, especially if the patient has been given antibiotics, but would be definitive when positive. The conclusion from analyses of the October-November 2001 events in the USA was that nasal swabs were not useful as clinical samples (Inglesby et al., 2002). See also Annex 1.

Immunohistochemical staining of pulmonary effusions or of bronchial biopsies (if available) proved valuable in the diagnosis of treated inhalational anthrax patients in the anthrax letter events in the USA in 2001 (Guarner et al., 2003; Shieh et al., 2003). As stated in relation to cutaneous anthrax, however, the method is currently confined to specialized laboratories with access to appropriate specific antibodies. Also as with cutaneous anthrax, direct PCR on clinical specimens from suspected inhalational anthrax cases is also now regarded as an acceptable diagnostic procedure (Ellerbrok et al., 2002; Shieh et al., 2003).

Specialized laboratories may be able to demonstrate anthrax toxin in fluid specimens (serum or oedematous fluid), or show these to be positive by PCR. This would permit an earlier diagnosis than culture and should still be positive in advanced cases where treatment has rendered culture negative. In

the case of patients who survive, antitoxin antibodies may be demonstrable in convalescent sera (section 4.4.2.2). The rather older Anthraxin^{T} hypersensitivity test referred to in section 4.4.2.2 may also be applicable.

Belated treatment can sterilize the blood and tissue fluids while still not preventing toxin-induced death (section 4.2.2.5). Among the 11 cases of inhalational anthrax resulting from biological terrorism in 2001, 4 (36%) died despite antibiotic therapy. Post mortem, if the sterilizing effect of treatment has not occurred, the capsulated *B. anthracis* will usually be visible in capsule-stained smears of these fluids, and should be easily isolated from them by bacteriological culture. Again, tests for toxin or PCR on these fluids would still be positive when treatment has rendered them smear- or culture-negative.

Guidelines for confirmation of diagnosis of oropharyngeal anthrax are given in section 4.4.3.1. With regard to gastrointestinal infection, confirmation of diagnosis by culture may not be possible before death in the absence of a known prior event raising the suspicion of anthrax, at least in the index case(s). In those who survive, retrospective diagnosis by serology may be supportive. Where it is considered that anthrax is the cause of gastrointestinal symptoms, examination of ascetic fluid by smear, culture and/or toxin tests and/or PCR may again be used to confirm the diagnosis. Where anthrax has not been suspected prior to death and post mortem, characteristic signs of intestinal anthrax are dark haemolysed unclotting blood, enlarged haemorrhagic spleen, petechial haemorrhages throughout the organs, and a dark oedematous intestinal tract, ulcerated or with areas of necrosis. With inhalational anthrax, the haemolysed unclotting blood, enlarged haemorrhagic spleen and petechial haemorrhages throughout the other organs are again seen and the mediastinal lymph nodes are always affected with haemorrhagic necrotizing lymphadenitis.

Differentiation between inhalational and gastrointestinal anthrax at autopsy may be difficult, and the decision as to how the disease was contracted may have to be based, at least in part, on the patient's history. The problem arises when varying sized (petechial to ecchymotic) haemorrhages occur in the gastrointestinal tract wall secondary to generalized spread of the infection (septicaemia/bacteraemia). Such haemorrhages occur in a number of diseases secondary to septicaemia/bacteraemia and are not indicative of primary infection via the gastrointestinal tract, reflecting instead irregularly distributed damage to the vascular bed caused by microorganisms and/or their toxins circulating in the bloodstream. Staging the lesions found at various locations within the body is an important means of determining the primary route of initial infection. The most advanced lesions will be located in the area of initial infection. For example, in gastrointestinal anthrax, the presence of a thickened, oedematous zone within the intestinal or stomach wall with an ulcerative lesion on the mucosal surface is typically present. The haemorrhages due to bacteraemia/septicaemia, in contrast, will not have marked thickening of the gut wall associated with them. Also, mesenteric adenopathy and ascites are usually present in gastrointestinal anthrax. Although, with gastrointestinal anthrax, the lungs may show damage similar to that found in patients dying of inhalational anthrax, the mediastinal lymph nodes, if affected at all, will have relatively less advanced pathological changes than those in the mesenteric lymph nodes. Microscopic study of the lesions by an experienced pathologist may be needed to determine their relative stage of progression.

4.4.4.3 Differential diagnosis (inhalational anthrax)

Alternative diagnoses to be considered are mycoplasmal pneumonia, legionnaires' disease, psittacosis, tularaemia, Q fever, viral pneumonia, histoplasmosis, coccidiomycosis, malignancy.

4.4.4.4 Retrospective diagnosis

Quinn et al. (2004) showed that seroconversion can be expected in persons who recover from inhalational anthrax (see section 4.4.2.2).

4.4.5 Anthrax meningitis

Meningitis due to anthrax is a serious clinical development which may follow any of the other three forms of anthrax. Anthrax meningitis is a haemorrhagic leptomeningitis with symptoms of neck pain with or without flexion, headache, changes in mental state, vomiting and high-grade fever. There is an intense inflammation of the meninges with accompanying oedema (referred to in Russian descriptions as "cardinal's cap" – Hugh-Jones, personal communication, 2004). A markedly elevated CSF pressure and the appearance of blood in the CSF are followed rapidly by disorientation, loss of consciousness and

death (Levy et al., 1981; Koshi et al., 1981; Lalitha et al., 1990, 1996; George et al., 1994; Kanungo et al., 2002; Jernigan et al., 2001). Neurological signs that have been noted include cranial nerve palsies, myoclonus, fasciculations, decerebrate posturing and papilloedema (Lanska, 2002; Sejvar et al., 2005). A striking characteristic of anthrax meningitis is its haemorrhagic component; few other central nervous system (CNS) infections are associated with this finding (Sejvar et al., 2005). The prognosis is extremely poor; only a very few instances of survival as a result of early recognition of the problem and prompt treatment are on record (Khanne et al., 1989; Lalitha et al., 1996; see also section 7.3.1.9). Post mortem, evidence of meningeal involvement was noted in over half the individuals who died following the 1979 Sverdlovsk accidental release incident (Abramova et al., 1993) and was observed in the one patient that had the appropriate postmortem evaluation in the anthrax letter cases in the USA (Jernigan et al., 2002).

On occasion meningeal signs are the first manifestation of disease. This is apparently the result of early translocation of the spores to the meninges. Kaufmann (personal communication, 2004) recalls a patient who had florid haemorrhagic meningitis and trivial mediastinal involvement. His death stemmed from the meningitis.

Differential diagnosis should take into account acute meningitis of other bacterial etiologies and other cerebral afflictions, such as cerebral malaria or subarachnoid haemorrhage (Kanungo et al., 2002). The definitive diagnosis is obtained by visualization of the capsulated bacilli in the CSF and/or by culture.

4.4.6 Anthrax sepsis

Sepsis develops after the lymphohaematogenous spread of *B. anthracis* from a primary lesion (cutaneous, gastrointestinal or pulmonary). Clinical features are high fever, toxaemia and shock, with death following in a short time. Evidence confirming that there is no such thing as asymptomatic anthrax sepsis, or that there is only symptomatic sepsis in anthrax has not been found.

In the differential diagnosis, sepsis due to other bacteria should be considered. Definitive diagnosis is made by the isolation of *B. anthracis* from the primary lesion and from blood cultures or by detection of the toxin or DNA (deoxyribonucleic acid) of *B. anthracis* in these specimens.

4.4.7 Long-term effects

Following the anthrax letter incidents of 2001, some of the patients complained of long-term problems such as undue fatigue upon minimal physical activity and problems with short-term memory. Reissman et al. (2004) carried out an assessment of these long-term effects in 15 patients approximately one year after infection. Many of the individuals continued to report fatigue and joint complaints, and cognitive impairment and psychological distress. No organic basis for these complaints was found, and the authors noted that similar long-term medically unexplained health problems and poor life-adjustment have been observed in persons surviving Lyme and legionnaires' diseases, possibly representing post-traumatic stress disorder. Convalescence, therefore, may require weeks to months depending upon severity of illness and patient-related factors.

In recovery from cutaneous anthrax, resolution of small- to medium-size cutaneous lesions is generally complete with minimal scarring. With larger lesions, or lesions on mobile areas (e.g. eyelid), scarring and contractures may require surgical correction to return normal functioning and large cutaneous defects may require skin grafting.

4.4.8 Second and subsequent attacks

Records of individuals contracting anthrax a second time exist, but are rare. Brachman (1954) refers to reports of two cases of anthrax occurring in England in 1912 in patients who had had cutaneous anthrax previously, and two further cases in the USA recorded in 1920. Hodgson (1941) records the case of a veterinarian contracting cutaneous anthrax on three occasions and in another individual whose second infection occurred only six weeks after the first. Heyworth et al. (1975) record the case of a girl treated for cutaneous anthrax in both 1971 and 1972. Tsurkan et al. (1980) note a case in which an eschar was located on the back of the right hand initially and then, several weeks later, a second eschar on the lower third of the right leg. The course of the secondary infection was less severe. One United Kingdom report (Anon., 1982) lists an "M 50y slaughterman with malignant pustule on the hand after handling pigs, had cutaneous anthrax 25 years ago". Shylakhov (1996) records observing 3 cases of second infection after 8, 15 and 20 years respectively, in three persons. In one of them, the eschar was located at the same site on the arm as 15 years previously. Martin

(1975) states that "reinfection of the skin was seen not infrequently at Rassa (Ethiopia), and the second lesion was usually noted to be less severe than the initial one". A physician in Turkey reports seeing two cutaneous anthrax infections in an individual approximately one year apart (Doganay, personal communication, 2004).

It is unclear whether Christie (1969) saw second infections himself, but the impression is gained that he did from his statement: "These can occur although sometimes the lesion is very small and there are no vesicles, no eschar, and no oedema. Anthrax bacilli can be isolated from scrapings of the lesion, but unless anthrax is suspected, it is unlikely that such examination of a small pimple will be made, and the sore will probably heal undiagnosed".

Christie implies, therefore, that second attacks may occur more frequently than realized, often going unrecognized.

Kaufmann (personal communication, 2005) notes that, at the time of the anthrax letter events in the USA in 2001, some infectious disease experts considered that patients recovering from inhalational anthrax should be maintained on antimicrobial therapy for 60 days, or longer, to prevent reinfection from spores which remained lodged within their lungs (see also section 5.2).

5. Pathogenesis and pathology

5.1 Events after entry through a lesion

The common perception is that *B. anthracis* is not invasive (section 3.1) and that, except when taken up by the pulmonary route, it needs a lesion through which to enter the body (section 3.3.1). As stated in section 4.2.2.2, this perception was queried by Ellingson et al. (1946) (see also section 5.3).

Our knowledge of the microscopic events following entry into a lesion comes mainly from studies conducted some years ago in parenterally-infected laboratory animals, with a general picture of events from initial entry to death resulting from the accumulated observations on a number of different species.

Cromartie et al. (1947a) and Bloom et al. (1947) observed that virulent spores injected into the skin of susceptible animals (mice, guinea-pigs or rabbits) germinated and gave rise in about 2–4 hours to a small oedematous area containing capsulated bacilli. The lesion increased in size and became defined by a zone of altered capillaries through dilatation, congestion and swelling of the endothelial cells and diapedesis of neutrophils. Macrophages and fibrin deposits appeared and the efferent lymphatics became dilated. With continued multiplication of the bacilli in the lesion came fragmentation of the connective tissue as the oedema spread extensively from the injection site. Some necrosis and haemorrhage occurred. The bacilli were visible in foci surrounded by large zones of oedema apparently free of the organism, and proliferation in the initial lesion continued to the point of death. Phagocytosis was apparently minimal. According to Sterne (1966), particularly if infection has been with a small number of spores, the primary lesion may be insignificant compared with that in the draining lymph node. The bacilli migrate from the primary lesion by means of the lymph vessels to the local lymph nodes where multiplication occurs, releasing a continuous stream of organisms into the efferent lymph vessels and thence to the spleen and other lymphoid tissues where multiplication continues (Widdicombe et al., 1956).

Some differences are apparent between species: in guinea-pigs, the terminal bacteraemia seems to be caused by actual multiplication in the blood during the last few hours of life, while in cattle, the blood may be free of organisms as late as 30 minutes before death with the same massive terminal bacteraemia (Keppie et al., 1955; Sterne, 1959, 1966). Published doubling times during the final 10–14 hours of bacteraemia are approximately 45 minutes in mice, 53 minutes in guinea-pigs, 95 minutes in sheep and 115 minutes in rats (Keppie et al., 1955; Trnka et al., 1958, cited by Lincoln et al., 1961; Lincoln et al., 1961). In rhesus monkeys, the minimum observable level of about 5000 bacilli per ml of blood was observed by Klein et al. (1962) and Lincoln et al. (1964) 11.5 to 12 hours before death, with the septicaemia progressing to its terminal level (see section 3.5.1). The organisms were first detected a few hours earlier (17 hours before death) in lymph from the thoracic lymph duct (Klein et al., 1962).

In monkeys succumbing to infection following subcutaneous challenge, the local lymph nodes became haemorrhagic and oedematous with necrosis of lymphatic elements and of blood-vessel walls, and phagocytosis of the bacilli was apparent here. The spleen was depopulated of lymphoid elements and contained extensive necrotic cellular debris and masses of bacilli (Gleiser, 1967). In resistant animals (rats, dogs, pigs) studied by Bloom et al. (1947), *B. anthracis* germinated and proliferated as in the susceptible species for up to about 4 hours, after which they lost their capsules and proliferation ceased, with the bacterial cells dying, disintegrating and disappearing by about 70 hours. By 26 hours, the lesions had become infiltrated with neutrophils and macrophages, the zone becoming necrotic by 72 hours. At 10 days, resolution commenced with the

subepithelial appearance of dense fibrous tissue and epithelial regeneration. Bloom et al. (1947) evidently believed that natural resistance was attributable to an anthracidal substance – a basic polypeptide containing a large amount of lysine – produced by leucocytes.

Splenic enlargement (the classic large soft spleen) is characteristic of septicaemia. The animal world is subdivided into those species with and those without sinusoidal spleens. The extreme enlargement that occurs in cattle and sheep with septicaemic anthrax relates to their having a distensible sinusoidal spleen. Septicaemia in human (or non-human primate) anthrax results in a large soft spleen but not so markedly enlarged as in cattle.

5.2 Events after entry by inhalation

Species differences were noted by Gleiser (1967) and Gleiser et al. (1968) in aerosol infection studies comparing pigs and dogs, as representative of more resistant hosts, with sheep and rhesus monkeys representing more susceptible hosts. Responses in the lungs to the presence of the spores were seen in the lungs of the pigs and dogs, but not in the sheep and monkeys. Discrete, intensely haemorrhagic and cellular lesions surrounded by dense masses of fibrin in the more resistant animals were interpreted as representing an ability to "wall off" the invading organisms into local foci of infection and were seen to explain the lack of systemic infection. In earlier studies in rhesus monkeys (Young et al., 1946; Henderson et al., 1956), it had been observed that the inhaled spores apparently did not germinate in the lungs but were ingested by motile macrophages which carried them through the undamaged epithelium to the lymphatics (see also section 3.3.8).

Germination began on the way to or on arrival at the tracheobronchial lymph nodes, and the vegetative cells freed from the phagocytes then proliferated (Ross, 1957). The infection spread through the efferent lymph duct into the bloodstream. Some of the bacteria reached the peribronchial lymph nodes within 15 minutes of inhaling the spores (Young et al., 1946). Clearance from the lung was apparently inefficient, however; Henderson et al. (1956) found that, in rhesus monkeys exposed to aerosolized spores but protected from contracting anthrax by combined penicillin and vaccine prophylaxis, 15%–20%, 2%, and 0.5%–1% of the spores remained in the lungs at 42, 50 and 75 days respectively after exposure, and even after 100 days trace levels of spores were present. This was confirmed by Friedlander et al. (1993) in a similar study in which the last anthrax death occurred on day 58 in a group of rhesus monkeys exposed to aerosolized anthrax spores and maintained on doxycycline for 30 days after exposure.

In the terminal stages in monkeys infected by the respiratory route, the intrathoracic lymph nodes and spleen showed changes similar to those seen in the local lymph nodes of animals infected subcutaneously (see section 5.1; Gleiser, 1967).

5.3 Events after entry by ingestion

In cattle and sheep, the intestinal lesions are focal to diffuse haemorrhagic necrotic enteritis of the small intestine (Nieberle & Cohrs, 1967). The tendency for localized lesions to develop in Peyer's patches suggests a possible role of the M cell in the uptake of the anthrax bacillus (Hutyra et al., 1946; Jensen, 1974). In one study of experimental infection in cattle, the primary lesion in 18 of 19 steers was an oedematous and haemorrhagic area in the small intestine, 4–6 inches (10–15 cm) long and located about 16 feet (ca. 5 m)from the pylorus. Haemorrhage and oedema clearly demarcated the lesion from contiguous intestine (Jackson et al., 1957). In swine, pharyngeal anthrax results from penetration of the bacilli through the superficial epithelium overlying the tonsils or from the tonsilar fossae (Nieberle & Cohrs, 1967). Records of anthrax pathology in domestic animals are notably devoid of lesion descriptions for pre-existent lesions in the pathogenesis of the disease, again raising the question of the invariable need for pre-existent lesions in the pathogenesis of the disease (see section 5.1).

5.4 The role of phagocytosis

5.4.1 Macrophages

From the histopathological observations outlined in sections 5.1 to 5.3, it would appear that phagocytosis plays a more important role in the dissemination of *B. anthracis* after entry into the body by aerosol, as compared with cutaneous (and, presumably, alimentary tract) exposure. The possible involvement of macrophages in the uptake of *B. anthracis* in the intestine was alluded to in section 5.3.

It seems clear that spores lodged in the alveoli following inhalation are phagocytosed by, and germinate within, alveolar macrophages as they move

to the lymph nodes (Ross, 1957; Guidi-Rontani et al., 1999; Dixon et al., 2000). As reviewed by Guidi-Rontani & Mock (2002), phagocytosis is mediated by receptors for the Fc portion of IgG. The ability of the emergent vegetative cells to multiply has been demonstrated in cultured macrophages (Dixon et al., 2000) but seemingly not all agree that germinated spores multiply within macrophages (Moayeri & Leppla, 2004). Guidi-Rontani & Mock (2002) postulate that *B. anthracis* spores may possess a unique system for detecting specific germinants within the macrophage, although putative germinants remain to be identified. As reviewed by Moayeri & Leppla (2004), lethal and oedema toxins (section 5.5.3), expressed at the spore stage and by newly germinated spores, play an early role in survival of the germinated cell within the macrophage and apparently contribute to its death.

In addition to the role of macrophages in facilitating spore germination and possibly outgrowth, various laboratories have documented a defensive role for macrophages in protecting the host from infection. Guidi-Rontani et al. (2001) showed that, although spores could germinate in macrophages, growth of the germinated spore did not occur in their in vitro system. Welkos et al. (2002) and Cote et al. (2004) observed an apparent protective sporicidal activity by macrophages in vitro and in vivo. Piris-Giminez et al. (2004) described the bacterial activity of a macrophage phospholipase against *B. anthracis*. The roles that spore dose, mode of infection and other factors play in the relative extent to which macrophages abet and facilitate the infection of the host versus protecting the host from infection require further study. The in vitro macrophage systems used to date are of necessity artificial; studies that track infection in real time in vivo are needed.

The involvement of phagocytosis in anthrax acquired through the epithelium is less clear at present. The role of the capsule of *B. anthracis* (see section 5.5.1) has long been regarded as being to protect the bacterium from phagocytosis (Sterne, 1959) and the observation of little evidence of phagocytosis by Bloom et al. (1947) suggests that the vegetative cells themselves make their way to the lymph nodes via the lymphatics in these cases. Researchers in the first half of the 20th century (reviewed by Sterne, 1959) believed that the capsule inhibits phagocytosis by neutralizing anthracidal substances present in normal serum and leucocytes. Whether or not the capsule is also essential to protecting the emerging cells when phagocytosed spores germinate within macrophages is discussed in section 5.5.1.

Complement C5-derived chemoattractants may be important in the recruitment of macrophages (Welkos et al., 1989).

Vegetative cells survive within the host as extracellular bacilli avoiding phagocytosis by means of the protective capsule (see section 5.5.1).

That macrophages may have a central role in the lethality of anthrax was demonstrated in experiments showing that laboratory mice were rendered insensitive to anthrax lethal toxin when their native macrophage population had been depleted by silica injection. The mice could be made sensitive again by injection of a toxin-sensitive macrophage cell line (Hanna et al., 1993). In contrast, Cote et al. (2004) showed that depletion of macrophages by other means increased the susceptibility of mice to infection. Supplementing the macrophage population in mice by treating them with additional exogenous macrophages enhanced their resistance to infection (Cote et al., 2004, 2006). Animals harbouring lethal toxin-resistant macrophages are, however, also susceptible to the toxin (Leppla, personal communication, 2004). The point at which these toxin-related observations manifest themselves in the actual infection has yet to be elucidated (see also section 5.5.3).

The role of macrophages in the pathogenesis of anthrax is now the subject of sophisticated research (Chai et al., 2004; Bergman et al., 2005).

5.4.2 Neutrophils (polymorphonuclear leukocytes)

Histopathology indicates that anthrax bacilli elicit a neutrophil response, and that both the lethal and oedema toxins enhance migration of neutrophils (Wade et al., 1985). Perversely, however, one role of the oedema toxin component of anthrax toxin (section 5.5.3) is to prevent mobilization and activation of neutrophils and thereby to suppress their phagocytosis of the bacteria (Leppla et al., 1985; O'Brien et al., 1985; Leppla, 1991). A recent report (Zenewicz et al., 2005) states that neutrophils are essential for early control of vegetative bacterial growth, although others (Cote & Welkos, 2005) considered that they played a relatively minor role in the early host response to anthrax spores.

5.5 Virulence factors

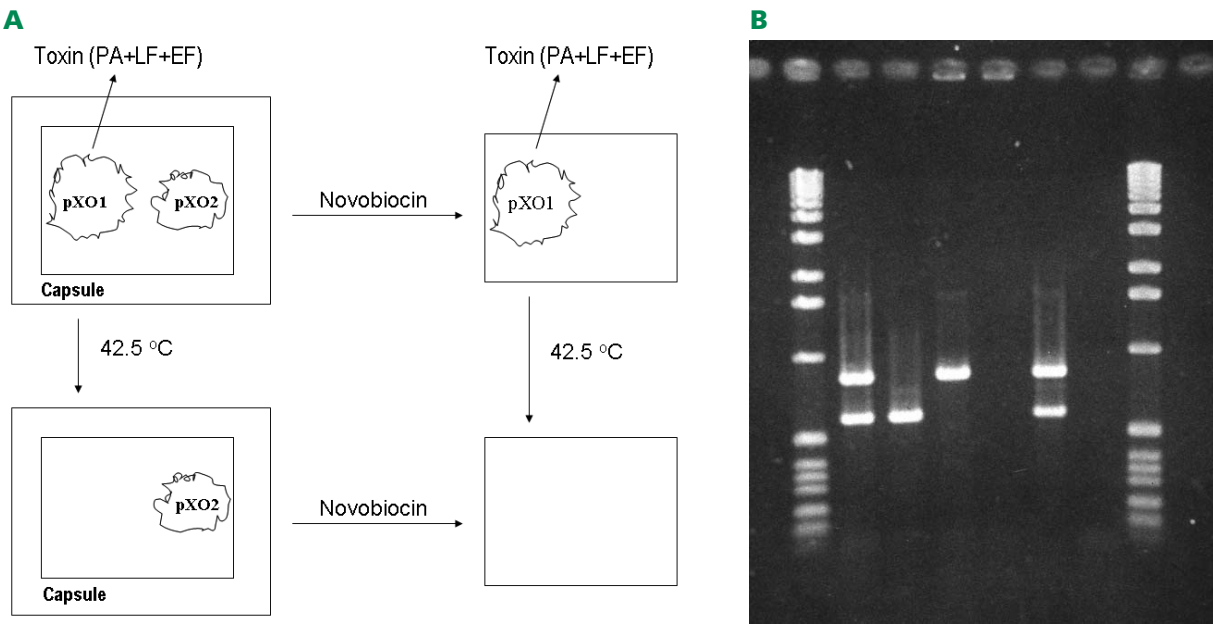
In the first half of the 20th century, it was held that death from anthrax was due to circulatory failure from capillary blockage, hypoxia and depletion of nutrients by the exceedingly large numbers of bacilli. This mechanism was suggested by the light-microscopic appearance of dilated capillaries stuffed with bacilli. With the electron microscope, however, the bacilli were seen to be merely trapped within fibrin and leukocyte thrombi and subsequently it was demonstrated that death was attributable to a toxin (Smith & Keppie, 1954; Keppie et al., 1955; see section 7.1.1). Opinion has now circled back to favouring hypoxia as the ultimate cause of death, albeit with toxin-induced vascular collapse leading to this (Moayeri et al., 2003; see also 5.5.3).

The toxin complex and the capsule are the two known virulence factors of *B. anthracis*. Both are plasmid-mediated. The genes for the toxin components and virulence gene regulator *atxA* are located on a large (182 kb) plasmid designated pXO1 and the genes for capsule synthesis and degradation and their regulators *acpA* and *acpB* are located on a smaller (95 kb) plasmid, pXO2 (Koehler, 2002; Drysdale et al., 2004; Candela et al., 2005; Fig. 6A). The *atxA* gene, originally named for its control of the anthrax toxin genes, is also the master regulator of the capsule biosynthetic gene operon via a positive effect on transcription of *acpA* and *acpB* (Drysdale et al., 2004). Thus there is communication between the two plasmids and some interdependence between the two virulence factors. Both toxin and capsule expression are modulated by CO_2 or bicarbonate ion and, in strains cured of pXO1, increasing the CO_2 level will increase expression of *acpA* and *acpB* and thereby alleviate the need for *atxA* (Koehler, personal communication, 2004).

Fig. 6 Plasmids and PCR for virulence factor genes of *B. anthracis*

A. Diagrammatic representation of plasmids of *B. anthracis* and the results of their selective curing. Top left: fully virulent cell producing both toxin complex and capsule. Top right: curing of pXO2 results in the Sterne-type vaccine strain. Bottom left: curing of pXO1 results in the Pasteur-type vaccine strain. The bacterium can be cured of both plasmids (bottom right).

B. PCR is useful for confirming the virulence of an isolate by amplification of targeted virulence gene sequences such as the protective antigen gene (*pagA*) and one or more of the genes in capsule biosynthesis operon (*capBCAD*). Lanes 2 and 6 show the presence of both capsule and toxin (PA component) genes, representing fully virulent *B. anthracis*. Lane 3 shows the presence of a gene for one of the toxin components (PA) but no capsule gene; the strain would be similar to the Sterne vaccine strain. Lane 4 shows the presence of a capsule gene but no toxin gene; the strain would be similar to the Pasteur vaccine strain. The DNA in lane 5 lacks both capsule and toxin genes. Lane 7 is a water control. (Lanes 1 and 8 are molecular weight markers.)



Thus capsule production in pXO1-/2+ derivatives is better under 20% CO_2 than under 5% CO_2, for example (Green et al., 1985).

Loss of either plasmid, pXO1 or pXO2, results in considerable reduction in (though not complete loss of) virulence, and this has been the basis of anthrax vaccines since the end of the 19th century (Fig. 6B), i.e. well before the molecular basis of *B. anthracis* virulence was understood (Turnbull, 1991; see section 8.6.2). There has been one suggestion that virulence differences and loss of virulence with age or storage can be linked to plasmid copy number (Coker et al., 2003).

5.5.1 The polypeptide capsule

The role of the poly-γ-D-glutamic acid capsule has long been regarded as being to protect the bacterium from phagocytosis (see section 5.4.1; Sterne, 1959). Early researchers believed that it inhibits phagocytosis by neutralizing anthracidal substances in serum and leucocytes. Ezzell & Abshire (1996) showed that the capsule appeared early during the process of germination of spores, which would suggest it has a protective role for emerging cells when phagocytosed spores germinate within macrophages. However Guidi-Rontani et al. (2001), using fluorescence imaging analysis to follow germination of *B. anthracis* spores and study survival of the germinated entities within primary mouse macrophages, concluded that the capsule played no role in this process. The observation of Meynell & Lawn (1965) was that, in vitro in the dividing vegetative cells, capsulation occurred at the end of exponential growth and that the cells divided equatorially with the capsules partitioned among the progeny.

Elucidation of the chemical nature of the capsule dates back to 1933 when Tomcsik & Szongott obtained a nitrogenous, polysaccharide-free, capsular material from *B. anthracis*. This was identified by Ivanovics & Bruckner (1937a, 1937b) as poly-D-glutamic acid, and how the glutamic acid residues were linked became the subject of a substantial number of studies (reviewed by Zwartouw & Smith, 1956a, and Fouet & Mesnage, 2002), with the final conclusion by Zwartouw & Smith (1956a) that they were linked by their γ-carboxyl groups. They also established that the antiphagocytic activity of the capsule was due to the multivalent negative charge of the polyglutamate ion.

The synthesis of the polypeptide through transamination between α-ketoglutaric acid and L-aspartic acid was worked out by Herbst (1944) and Housewright & Thorne (1950). The size of the polyglutamaic chains varies with the growth conditions, being 20–50 kDa when produced in vitro but in the order of 215 kDa in vivo (reviewed by Fouet & Mesnage, 2002). The biochemical structure of the capsule formed by *B. anthracis* is indentical to that formed by *B. licheniformis* with the same L-glutamic acid precursor (Thorne, 1956; Leonard & Housewright, 1963).

Ivanovics (1939) injected isolated capsular material intravenously into mice and rabbits and found that it was rapidly excreted by the kidneys without being broken down. He considered that this explained why the capsular material was harmless in itself and also why it was poorly antigenic. Weak immunogenicity of the surrounding capsule presumably aids *B. anthracis* in evading host immune response to its presence; at the same time, the capsule prevents antibodies to deeper surface antigens reaching those antigens. Tomcsik & Ivanovics (1938) did, however, succeed in developing antibodies in rabbits and recorded that they resulted in "certain immunity" in the rabbits and passively immunized mice. In more recent studies, opsonic IgG antibodies to the poly-γ-D-glutamic acid conjugated to proteins have been produced, and the suggestion made that the conjugates might be contributory as vaccine additives (Schneerson et al., 2003; Wang et al., 2004). A more definitive claim that the capsule could induce protective antibodies has been made by Chabot et al. (2004, 2005). Wang & Lucas (2004) immunized various strains of inbred mice and concluded that the antibody response patterns defined the capsule as a thymus-independent type 2 antigen.

The capsule does not form under normal aerobic culture and, in in vitro cultures, requires an atmosphere of elevated CO_2 together with the presence in the medium of serum and/or bicarbonate, or both (sections 6.1, 6.3.1.6; Annex 1, section 3.7). Meynell & Meynell (1964) showed that sera from different species had marked differences in capsule-promoting activity and that the contribution of the sera was not nutritional but rather that it absorbed a dialysable inhibitor of capsule production present in the medium. The inhibitor was thought to be a fatty acid interfering with the assimilation of CO_2. It could also be absorbed by 0.2% (w/v) activated charcoal which was as efficient as serum in promoting capsulation. They showed that there was a relationship between pH and the threshold concentration of HCO_3- at

which the capsule would be formed under any one atmospheric CO_2 concentration. They also noted that glucose suppressed capsulation. Later (Meynell & Meynell, 1966), using mutants with different nutritional requirements for capsulation, they concluded that, while HCO_3- made capsular synthesis possible, it was not required for the formation of the capsule itself. They also concluded that the inhibitory fatty acid(s) act by interfering with uptake or utilization of HCO_3-. Finally they showed that capsule formation was not inhibited by tetracycline and therefore was not synthesized like a protein.

Most of the research of recent years on the pathogenesis of anthrax has focused on the structure and function of the toxin, and the detailed role of the capsule is a topic still to be revisited properly with today's molecular tools. Welkos (1991) showed that, in inbred strains of mice, cap+/tox- strains do possess a degree of virulence but, in the absence of toxin, the pathogenesis of these strains was unexplained. That there is strain variation in virulence indicates that there may be more to this than simple elaboration of the capsule, and the possibility that the phenomenon may be related to some other pXO2 gene(s), possibly together with chromosomal gene(s), has not been ruled out (Welkos, personal communication, 2004).

5.5.2 Polysaccharide

As reviewed by Smith & Zwartouw (1956), the production of polysaccharide material by *B. anthracis* was noted by several authors in the 1940s and 1950s. At least some of this was cell-wall associated. It precipitated with antipneumococcus type XIV serum and was present in both virulent and avirulent strains, and completely inactive in tests for aggressive activity. The conclusion was that it plays no important role in the virulence of *B. anthracis*.

5.5.3 The anthrax toxin complex

The toxin complex, which consists of three synergistically acting proteins, Protective Antigen (PA, 83kDa), Lethal Factor (LF, 90 kDa) and Oedema Factor (EF, 89 kDa), is produced during the log phase of growth of *B. anthracis*. LF in combination with PA (lethal toxin) and EF in combination with PA (oedema toxin) are now regarded as responsible for the characteristic signs and symptoms of anthrax. A considerable amount is known now about all three toxin components; they have been sequenced and the crystal structures of all three have been worked out with the roles of the domains within each molecule broadly elucidated (Petosa et al., 1997; Pannifer et al., 2001; Drum et al., 2002; Lacy & Collier, 2002).

According to the currently accepted model (Fig. 7), PA binds to receptors on the host's cells and is activated by a host-cell surface furin-like protease which cleaves off a 20 kDa (PA_{20}) piece leaving exposed an EF/LF receptor-binding site. This "activated" PA_{63} fragment combines with six other PA_{63} fragments to form ring-shaped heptamers that bind EF and LF competitively and forms channels through which the complex is internalized by endocytosis (Lacy & Collier, 2002). Following acidification of the endosome, the LF and EF are released into the host-cell cytosol. The protein tumour endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) have been shown to function as cellular receptors, although it is not clear which is relevant in vivo (Bradley et al., 2001; Scobie et al., 2003; Moayeri & Leppla, 2004). According to Ezzell et al. (1992, 2005), the cleavage of PA_{83} into PA_{63} and PA_{20} occurs in the blood through the action of a serum protease, and the binding of EF and LF occurs here rather than at the cell surface just prior to endocytosis.

The literature now generally treats the combinations of PA with LF, and of PA with EF, as two separate toxins, referred to respectively as lethal and oedema toxins. They fall into the A-B class of toxins (binary toxins) with the enzymatically active "A" and binding "B" domains as discrete structures (Lacy & Collier, 2002).

EF is an adenylate cyclase which, by catalysing the abnormal production of cyclic-AMP (adenosine monophosphate) (cAMP), produces the altered water and ion movements that lead to the characteristic oedema of anthrax. High intracellular cAMP concentrations are cytostatic but not lethal to host cells. EF has been shown to impair neutrophil function and incapacitates phagocytes and cytokine pathways (reviewed by Moayeri & Leppla, 2004) and its role in anthrax infection may be to prevent activation of the inflammatory process. Recent presentations (Firoved et al., 2005; Quesnel-Hellmann et al., 2005) reported that oedema toxin (EF + PA) disrupted cytokine networks during infection, and that purified oedema toxin administered to BALB/cJ mice intravenously resulted in circulatory and cardiac dysfuntions, tissue damage and focal haemorrhaging, accumulation of several cytokines in the sera and death.

LF is a highly specific zinc-dependent protease that cleaves the amino termini of six mitogen-activated protein kinase kinases (MAPKK), and thereby disrupts pathways in eukaryotic cells concerned with regulating activity of molecules through phosphorylation cascades (Duesbury et al., 1998; Vitale et al., 1998, 2000). The affected signalling pathways are involved in cell growth and maturation as well as cellular stress responses; the manner in which the disruption of these pathways leads to the known effects of lethal toxin has yet to be fully elucidated but may act by impairing the ability of endothelial cells to initiate an immune response by destabilizing cytokine IL-8 mRNA (Batty et al., 2005) or through other modes of inhibition of cytokine gene expression (Tonello et al., 2005).

Advancing knowledge of the structure of the toxin components and the stages of interaction of these with the host cell, from binding of PA to internalization and catalytic actions of EF and LF in the cytosol, are reviewed by Lacy & Collier (2002).

On the basis of mouse and tissue culture models, the primary cell type affected in anthrax pathogenesis has for some years been regarded as the macrophage (Friedlander, 1986; Hanna et al., 1993; see also section 5.4.1). Low levels of lethal toxin cleave MAPKK-3. High levels of lethal toxin lead to lysis of the macrophage by an as yet unknown mechanism (Pannifer et al., 2001). The relationship between macrophage cell death and death of the infected host is under investigation, although it seems clear that macrophage lysis is not required for lethal toxin lethality (Moayeri et al., 2003). It is emerging that the effect of lethal toxin on other cell types may also be integral to the pathogenesis of anthrax. For example, lethal toxin impairs host B and T cell immune responses by its action on dendritic cells (Moayeri & Leppla, 2004). The action of lethal toxin is further discussed in section 5.5.4, and the current wisdom is that death is the result of the toxin acting through a non-inflammatory mechanism that involves hypoxic injury but not macrophage sensitivity to toxin (Moayeri et al., 2003).

The inverse relationship between susceptibility to infection and susceptibility to the toxin was discussed in section 3.1.

5.5.4 Toxin and terminal haemorrhage

The characteristic terminal haemorrhage to the exterior from the orifices of the animal at death – an essential part of the organism's cycle of infection (Figs 1&2) – is caused by the action of the toxin on the endothelial cell-lining of the blood vessels, histologically visible as necrosis, which results in their breakdown and bleeding. Anthrax toxin acts directly on the intact membranes of endothelial cells, making them permeable to plasma and causing intravascular thrombosis (Beall & Dalldorf, 1966; Dalldorf, 1967; Dalldorf et al., 1971). This is manifested in petechiae in the visceral organs and haemorrhages leading to bleeding from the orifices. Kirby (2004) found that lethal toxin (PA+LF), but not oedema toxin (PA+EF), induces apoptosis in cultured human endothelial cells. He hypothesized that lethal toxin action on endothelial cells, primarily through inhibition of the extracellular signal-regulated kinase (ERK) pathway, contributed to vascular pathology and haemorrhage during systemic anthrax. The disruption of key functions of the endothelium by lethal toxin has been studied further more recently (Warfel et al., 2005).

The degree of endothelial damage appears related to the number of bacilli. In early infection of the lymph nodes, only oedema is present. As the number of bacilli rises, concomitant toxaemia increases to a critical level, at which the toxin causes endothelial cell injury with resulting thrombosis and haemorrhage.

5.5.5 S-layer

In common with many, but not all, bacteria, *B. anthracis* forms the proteinaceous paracrystalline sheath over the peptidoglycan cell surface known as an S-layer. Very few bacteria share with *B. anthracis* both an S-layer and a capsule (Fouet & Mesnage, 2002) and this may be pertinent to the pathogenicity of *B. anthracis*. S-layers have been shown in other bacterial species to be virulence factors, and this may be the case with *B. anthracis* (Fouet et al., 1996; Fouet & Mesnage, 2002).

The S-layer of *B. anthracis* comprises two components, a 94-kDa protein termed Sap (surface array protein) and a 94-kDa protein known as EA1 (extractable antigen 1) (Ezzell & Abshire, 1988; Fouet & Mesnage, 2002). Sap is cell-associated but also released into the surrounding medium in cell or culture suspensions; EA1 is cell-associated and only released if the cells are washed with sufficient vigour (Fouet & Mesnage, 2002; Williams & Turnbough, 2004).

The presence of an S-layer is not required for capsulation of *B. anthracis* bacilli, but the presence of all three is necessary for maximal resistance to complement pathway-mediated defences (Fouet & Mesnage, 2002). This may be related to protection of the emerging germinating cell which, at early stages, is not wholly surrounded by capsule (Ezzell & Abshire, 1996).

5.5.6 Exosporium

In common with spores of all *Bacillus* species, the spores of *B. anthracis* comprise a core surrounded by peptidoglycan cortex, in turn surrounded by the multiple-layered proteinaceous spore coats. These apparently form the outermost layer for some *Bacillus* species but, in the case of the *B. cereus* group, there is an additional loose-fitting, two-layer exosporium comprising about 2% of the mass of the spore. The inner layer has a hexagonal crystal structure, while the outer layer consists of hairlike filaments – the so-called "hirsute nap". About 50% of the exosporium is protein; the precise number and identities of the component proteins are not yet known, although several have been described.

The function of the exosporium is not known, but it may be the basis of the hydrophobicity of spores and, thereby, play a role in adhesion both in the environment and in vivo. It is speculated that it is involved in the interaction between spores and uptake by, or germination within, macrophages, although it is also stated that spores devoid of the exosporium are as infectious as those that have it. (For relevant references, see Steichen et al., 2003; Liu et al., 2004; Redmond et al., 2004).

5.5.7 Other potential virulence factors

A number of general and specific observations suggest that there is more to the virulence of *B. anthracis* than simply its ability to elaborate the toxin and the capsule. The following are examples of these:

- While the toxin and capsule components, the genes encoding these and the plasmids carrying these genes are seemingly identical in different *B. anthracis* strains, the strains themselves possess different LD_{50}s.
- Strains cured of one or other of the plasmids, and therefore able to produce toxin or capsule, but not both, retain a level of virulence (Welkos, 1991; see section 5.5.1).
- There is substantial evidence that some tox+/cap+ strains can overwhelm immunity induced by toxin-based vaccines more readily than other strains.
- There is some evidence that *B. anthracis* can elaborate a thiol-activated cytolysin, anthrolysin O (Shannon et al., 2003; Mosser et al., 2005; Thomason et al., 2005). Analogous cytolysins in other pathogens are established virulence factors.
- While *B. subtilis* spores injected into the footpad of a mouse are cleared rapidly, those of a *B. anthracis* strain cured of both pXO1 and pXO2 persist, implying chromosomal involvement in the ability of *B. anthracis* to persist (Pezard et al., 1991).
- Evidence exists of chromosomal loci with a role in the anthrax infectious process (Stepanov, Mikshis & Bolotnikova, 1996; Stepanov et al., 1996, 1999).
- The regulator *atxA*, known to be the anthrax toxin activator and responsible for up-regulation of the capsule genes, has been shown to be involved in the regulation of numerous other genes on pXO1, pXO2 and the chromosome. These genes may have direct or indirect roles in the pathogenesis of *B. anthracis* (Bourgogne et al., 2003).
- Strain comparisons of germination within macrophage cultures suggested a requirement for multiple germinant receptor genes located on both the chromosome and pXO1 for host-specific germinants and initiation of the infectious cycle (Hanna, 2001).
- Compared to *B. subtilis*, *B. anthracis* harbours a large number of genes predicted to be important for amino acid and peptide utilization, and which may provide an advantage during its life-cycle within an animal host. Moreover, several chromosomally encoded proteins have been noted which may contribute to pathogenicity, including haemolysins, phospholipases and iron uptake entities (Read et al., 2003).
- Germination operon *gerX* is located on plasmid pXO1 and is unique in being the only germination operon known to be located on a virulence plasmid (Guidi-Rontani & Mock, 2002). The *gerX*-encoded proteins may be virulence factors, contributing to the pathogenesis of *B. anthracis*.
- A recent claim has been made (Heninger et al., 2005) that overwhelming murine septicaemia can be induced independent of toxin function.

6. Bacteriology

6.1 Description

B. anthracis, the causative agent of anthrax, is a Gram-positive, aerobic or facultatively anaerobic, endospore-forming, rod-shaped bacterium approximately 4 µm by 1 µm, although under the microscope it frequently appears in chains of cells. In blood smears, smears of tissues or lesion fluid from diagnostic specimens, these chains are two to a few cells in length (see Fig. 8A); in smears made from in vitro cultures, they can appear as endless strings of cells – responsible for the characteristic tackiness of the colonies (see Fig. 8B) and for the flocculating nature of broth cultures. Also characteristic is the square-ended ("box-car" shaped) appearance traditionally associated with *B. anthracis* vegetative cells, although this may not always be very clear. In the presence of oxygen, and towards the end of the exponential phase of growth, one ellipsoidal spore (approximately 2 µm by 1 µm in size) is formed in each cell; this does not swell the sporangium and is generally situated centrally, sometimes subterminally (see Fig. 8C).

In the absence of oxygen and under a high partial pressure of CO_2 in the presence of bicarbonate (HCO_3-), the vegetative cell secretes its polypeptide capsule (Fig. 8D; see section 5.5.1) and it is one of the two established in vivo virulence factors of *B. anthracis*. The capsule is also a primary diagnostic aid (see section 4.4.2, 4.4.4.2, Annex 1, sections 8–10 and Fig. 8A).

6.2 Detection and isolation

In appropriate blood or other body fluid samples, or tissue specimens collected within a few hours of death from animals (see section 3.5) or humans with anthrax, *B. anthracis* is readily visualized in capsule-stained smears and readily isolated in pure culture. The same applies to smears of fluid from cutaneous lesions of humans prior to treatment (see section 4.4.2). Procedures are given in Annex 1, sections 8–10.

In old or decomposed animal specimens, or processed products from animals that have died of anthrax, or in environmental samples, detection is likely to involve a search for relatively few *B. anthracis* within a background flora of other bacteria, many of which will probably be other *Bacillus* species, in particular the closely related *B. cereus*. In this case, selective techniques are necessary. Procedures for the isolation of *B. anthracis* from such specimens are given in Annex 1, sections 8 & 10.

Such is the nature of the properties of *B. anthracis* that few agents which differentially select between *B. anthracis* and other *Bacillus* species do so in favour of *B. anthracis*, and those that do only do so unconvincingly. Of the selective media that have been proposed, the most successful historically is polymyxin-lysozyme-EDTA-thallous acetate (PLET – Fig. 8E and Fig. 14) agar (Knisely, 1966), although care should be taken to prepare this correctly (Annex 2). Success has also been noted with the trimethoprim and sulfamethoxazole blood agar formulation (Annex 2).[1] As yet no selective enrichment broth system has been devised for *B. anthracis*, although significant attempts have been made (Bowen, 1999) and, pending development of such a system, the sensitivity of in vitro detection of *B. anthracis* by conventional means in environmental samples or specimens from old or decomposed animals, or from processed animal products, is in the order of 10 cfu/g or /ml depending on the sample processing protocol employed (Manchee et al., 1981; Dragon & Rennie, 2001). Justification today for the use of laboratory animals to isolate *B. anthracis* is exceedingly unusual and confined to "last resort" circumstances.

PCR detection systems (Annex 1, section 10.7.4) have been developed for *B. anthracis* (Beyer et al., 1996; Patra et al., 1996a; Sjöstedt et al., 1996, 1997; Jackson et al., 1998), but it will probably be some time before

[1] http://www.ourfood.com/Anthrax.html.

they become totally stand-alone and generally available for use in the non-specialist laboratory. A positive by this method is close to being accepted on a stand-alone basis now for clinical specimens and simple environmental samples such as tap water and air samples. In case the reason for a negative by PCR is the presence of inhibitors, negatives may need bacteriological support. Clinical specimens and more microbiologically "complex" samples, such as faeces, muddy water or soil, will generally need a DNA extraction stage and will also need bacteriological support to confirm positives or negatives.

Rapid immunoassays for detection or confirmation of identity of *B. anthracis* are available commercially.[1] The absolute specificities of these are, as yet, unproven and positives using these generally need to be confirmed bacteriologically or by PCR, or both. The updated immunochromatographic device described by Burans et al. (1996) and used by Tubbesing (1997) and Muller et al. (2004) utilizes a monoclonal capture antibody to the anthrax protective antigen (PA) bound to a nitrocellulose membrane, and a second monoclonal antibody specific for a different epitope of PA bound to colloidal gold particles which become visible when they accumulate at the capture sites. The assay can detect as little as 25 ng/ml of PA and is performed in a few minutes without the need for special reagents. It therefore lends itself to direct diagnosis of cases of anthrax by detection of PA in the blood or body fluids, or to retrospective analysis of extracts from the types of sample of animal origin for which the Ascoli test was designed (Annex 1, section 11.1). As such, it has excellent specificity and sensitivity, but it has not become commercially available.

6.3 Identification and confirmation

6.3.1 Practical differentiation

With rare exceptions, it is generally easy to identify *B. anthracis* and to distinguish it from other *Bacillus* species, including other members of the *B. cereus* group (*B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*). For all practical purposes, an isolate is *B. anthracis* if it:

- has the characteristic colonial morphology (Parry et al., 1983) on nutrient or blood agar (matt appearance, fairly flat, similar to *B. cereus* but generally rather smaller, more tacky, white or grey-white on blood agar, and often (but by no means always) having curly tailing at the edges (Fig. 8B)
- is non-haemolytic or (very rarely) only very weakly haemolytic on sheep or horse blood agar;
- is non-motile;
- is sensitive to the diagnostic 'gamma' phage and penicillin (Fig. 8F);
- is able to produce the capsule in blood or when cultured on bicarbonate-serum agar under CO_2 (Fig. 8D; Annex 1, section 10.8.2).

Biochemical tests or commercial identification kits can contribute virtually nothing to this for identifying *B. anthracis*, although they may usefully supply the identity of an isolate initially, but mistakenly, thought possibly to be *B. anthracis*. Despite this, conventional biochemical and physiological identification characters for *B. anthracis* are generally included in microbiological textbooks, although the evidence is that this rarely represents first-hand experience on the part of the author(s). In addition, there is an increasing tendency in textbooks for *B. anthracis* to be listed simply as a member of "the *B. cereus* group" stating that, apart from being non-haemolytic, non-motile and pathogenic, it exhibits the same physiological and biochemical identification characteristics as *B. cereus*. This leads to these texts being of limited practical value, and possibly even misleading, for the microbiologist encountering a suspicious isolate. For the record, however, conventional physiological and biochemical characteristics for *B. anthracis* are given in Table 3 and sections 6.3.1.1 to 6.3.1.14. The profiles given in the majority of textbooks are largely derived from the related milestone monographs of Smith et al. (1952) and Gordon et al. (1973), although it should not be forgotten that these had been preceded by some excellent texts dating back to the previous century (Slater & Spitter, 1898; Whitby, 1928; St John-Brooks, 1930; Knight & Proom, 1950).These characteristics have been rechecked (Turnbull, unpublished data, 2005).

B. anthracis databases have been developed for the commercial API[2] and Biolog[3] systems (Baillie et al., 1995; Cogne et al., 1996). The API 50CHB system for the identification of *Bacillus* species is based on acidification of 49 carbohydrates (and is designed to be – and should be – used in conjunction with miscellaneous tests in the API 20E strip). The Biolog system

[1] Examples can be found on www.osborn-scientific.com, www.responsebio.com and www.tetracore.com, and from New Horizons Diagnostics,Columbia, MD 21045, USA; e-mail: NHDiag@aol.com.
[2] BioMérieux SA, 69280 Marcy-l'Etoile, France.
[3] Hayward, California 94545, USA.

TABLE 3

Primary physiological and biochemical characteristics of *B. anthracis* (all incubations at 35 °C)

CHARACTERISTIC	REACTION	COMMENT
Spores and cells		Spores central/subterminal, not swelling sporangium (see section 6.3.1.1).
Haemolysis on sheep/horse blood agar	–	See section 6.3.1.2.
Egg yolk reaction	+	Look under the colony (see section 6.3.1.3).
Motile	–	See section 6.3.1.4.
Susceptible to "gamma" phage	+	Phage-resistant isolates have been encountered on rare occasions (see section 6.3.1.5).
Susceptible to penicillin	+	Penicillin-resistant isolates have been encountered on rare occasions (see section 7.1.2).
Capsule production	+	Naturally occurring Cap- isolates are occasionally encountered (see section 6.3.1.6).
Oxidase	+	
Ammonium salt glucose	+	Negative only accepted after incubation for 14 days.
Ammonium salt arabinose	–	- " -
Ammonium salt xylose	–	- " -
Ammonium salt mannitol	–	- " -
Ammonium salt salicin	–	- " -
Catalase production	+	
Indole	–	Negative only accepted after incubation for 14 days.
Voges-Proskauer (VP)	+	Negative only accepted after incubation for 14 days. Also see section 6.3.1.7.
Citrate utilization	–	Negative only accepted after incubation for 14 days. Also see section 6.3.1.8.
Growth in 5% NaCl	v	Negative only accepted after incubation for 14 days. Also see section 6.3.1.9.
Gelatin hydrolysis	+	
Nitrate reduction	+	See section 6.3.1.10.
Casein hydrolysis	+	See section 6.3.1.11.
Starch hydrolysis	+	See section 6.3.1.12.
Urease	–	Negative only accepted after incubation for 14 days.
Phenylalanine deamination	v	Negative only accepted after incubation for 14 days. Also see section 6.3.1.13.
Propionate utilization	–	Negative only accepted after incubation for 14 days. Also see section 6.3.1.14.

v = variable.

is based on "carbon source metabolic fingerprints" in 96-well trays. These supply useful characterization data for comparing *B. anthracis* with other *Bacillus* species but a read-out giving the identity of an isolate as *B. anthracis* must be confirmed with either the simple tests listed in the first paragraph of this section and/or the PCR (section 6.3.2 and Annex 1, section 10.7.4)

6.3.1.1 Spore and cell morphology

In blood or tissue smears from infected humans or animals, the bacteria are seen in short chains of two to a few cells, frequently square-ended (box-car) or "vertebrate" in shape (not apparent in Fig. 8A). In contrast, when grown in laboratory media, long chains are often apparent under the microscope, although with quite wide-ranging strain-to-strain and media-to-media differences. Cell size, or chain width, and

partial loss of Gram-positivity at 24–48 hours on nutrient or sporulation agar also vary from strain to strain. The long chains (Fig. 8C) are the reason for the characteristic tackiness of *B. anthracis* colonies and for the stringy growth frequently encountered with *B. anthracis* in broth cultures. Turnbull (unpublished data) found that chaining could be selected out by passage through semisoft agar; the resulting cells in the passaged culture were present singly or in short chains or small clumps, and the colonies no longer had the tackiness of typical *B. anthracis* colonies.

Strain-to-strain differences can be expected in the degree of sporulation on sporulation agar at any given time period, and in the size and dimensions (length and breadth) of the ellipsoidal spores, which do not swell the sporangia (Fig. 8C). The spores usually appear within 48 hours and are often apparent after 24 hours, but several (5 or 6) days should be allowed before concluding that spores are not formed if they are not seen at 48 hours. Lipid globules are apparently not so readily visible in the protoplast of the vegetative cells as with at least some *B. cereus*.

6.3.1.2 Haemolysis

Fellows (1996) showed that *B. anthracis* was haemolytic on blood agar made with sheep red cells that had been washed with buffered saline containing calcium and magnesium, and Popov et al. (2004) refer to the haemolytic activity and haemolytic proteins of *B. anthracis*. Similarly, the anthrolysin O of Shannon et al. (2003), Mosser et al. (2005) and Thomason et al. (2005) is presumably haemolytic. Reports are also occasionally encountered of haemolysis in blood or on agar containing blood of certain species, including human. It is unclear whether, when seen, this is equivalent in appearance to the strong haemolysis of *B. cereus*. (See also section 6.4.1.)

6.3.1.3 Lecithinase

B. anthracis either produces lecithinase to a lesser extent than its close relatives, *B. cereus* and *B. thuringiensis*, or produces a lecithinase with a lower activity (McGaughey & Chu, 1948; Zwartouw & Smith, 1956b). In the lecithinase test on egg yolk agar, the zone of opalescence or "halo" almost always seen around colonies or areas of growth of *B. cereus* and *B. thuringiensis* is only sometimes visible around *B. anthracis*, probably only becoming apparent at 48 hours (35–37 °C) and usually in a narrow band when present. Opalescence should be looked for under the colony/area of growth by scraping away some of the colony material. It can be seen here after 24 hours incubation at 35–37 °C. While *B. anthracis* grows well on conventional egg yolk agar, it grows less well than *B. cereus* on Kendall's BC egg yolk-mannitol agar (Parry et al., 1983); the growth of *B. anthracis* on this medium (24–48 hours) is greyish as compared to the deep purple of *B. cereus* and, in contrast to *B. cereus*, a zone of opalescence does not form around the growth of *B. anthracis*. Once again, colony material must be scraped away to see the underlying LV (lecithovitellin) reaction.

6.3.1.4 Motility

As reviewed by Sterne & Proom (1957), a number of claims exist in the early literature of motile forms of *B. anthracis*. Sterne & Proom considered that most, if not all, of these claims could be explained in terms of contaminants or misidentification. Logan & Berkeley (1984) and Logan et al. (1985) found that, while 144 of their 149 strains of *B. cereus* were motile, none of 37 strains of *B. anthracis* they examined were motile. Although examination for motility is always listed as one of the primary identification tests for *B. anthracis*, it is doubtful that the test is often done on new isolates or checked more than rudimentarily and occasionally on culture collection cultures. Certainly, the first obvious appearance for diagnostic test purposes is lack of motility, but in view of the existence of genes associated with motility (see section 6.4.1), and even one recent report (Liang & Yu, 1999) that includes electron micrographs showing flagella, perhaps the "absoluteness" of non-motility in *B. anthracis* should be revisited.

6.3.1.5 Diagnostic phage

An anthrax phage was described as early as 1931 (Cowles, 1931). McCloy (1951) described a phage, designated phage W, isolated from strain W bacillus which she believed to be an atypical *B. cereus* or unusual *B. anthracis* strain. The "gamma" phage was first recorded by Brown & Cherry (1955) as a "new variant isolated from the original strain W bacteriophage". This phage lysed all of 41 *B. anthracis* strains, but none of 89 *B. cereus* strains or 134 strains representing 8 other *Bacillus* species. The subsequent work of Buck et al. (1963) in the early 1960s with phages isolated from 25 lysogenic *B. anthracis* strains, and the work 30 years later by Redmond et al. (1996b)

with almost identical results using a set of 25 phage isolates from different cultures of *B. anthracis*, have indicated that either there exists a family of closely related phages, of which the gamma phage is one, or that selective pressures have resulted in variants of one phage with slightly different properties.

In so far as the phages are spontaneously produced and plaques appear on culture lawns in an unpredictable manner, they are considered temperate. However, it has long been noted (McCloy, 1951, 1958; Buck et al., 1963) that, in being able to lyse their own original host and in that they can be grown in their homologous host, this gamma family "provides a striking exception to the rule of immunity" (McCloy, 1958). Buck et al. (1963) termed this phenomenon "dismunity" and seemed to attribute it to the formation of mutant forms of the phages.

Just to what extent the diagnostic phages used in various laboratories around the world and often referred to as "gamma phages" are unadulterated descendants of McCloy's gamma phage is now uncertain. For this reason, the term "diagnostic phage" is preferred.

Some care needs to be taken in defining precisely what is meant by phage sensitivity. The titre of the phage suspension is of consequence, and interpretation of what is seen in the zone of effect is somewhat subjective. For the purposes of Table 3, the titre of the phage suspension is ≥ 10^9 pfu/ml and any zone of effect, which may be graded from ± to 4+, represents sensitivity. (Note: this is different from the manner in which antibiotic sensitivity is read, where colonies growing in a zone of clearing result in an interpretation of "resistant".) For phage sensitivity tests, a total absence of effect is regarded as true resistance. On this basis, in formal studies on susceptibility/resistance, Buck et al. (1963) found that 7 of 264 (2.7%) *B. anthracis* isolates were phage-resistant, and 3 of 64 (3.1%) non-anthrax *Bacillus* species were lysed by anthrax phage. Similarly, Redmond et al. (1996b) found that 1 of 87 (1.2%) strains of *B. anthracis* were phage-resistant, and 2 of 47 (4.2%) non-anthrax *Bacillus* species were lysed by anthrax phage. In further tests (unpublished), Turnbull and colleagues found no positives among 14 *B. cereus*, 10 *B. megaterium*, 6 *B. pumilus*, 5 *B. subtilis*, 5 *B. circulans*, 4 *B. mycoides*, 4 *B. firmus*, 4 *B. sphaericus*, 3 *B. licheniformis*, 3 *B. thuringiensis*, 2 *B. amyloliquefaciens*, 1 *B. lentus*, and 2 *Brevibacillus brevis* from collections at the Food Safety and Microbiology Laboratory, Health Protection Agency, Colindale, London, United Kingdom, and the Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, United Kingdom. This is also supported by the unpublished results of tests on many hundreds of isolates from routine environmental samples performed over a period of years by Turnbull and colleagues, in which only three phage-resistant *B. anthracis* isolates were encountered (from a tannery dump site in the United Kingdom, from a human case of anthrax in Zimbabwe, and from three animals in the Etosha National Park, Namibia). Likewise, the finding of a phage-sensitive non-anthrax *Bacillus* species was a very rare event.

Subsequent examination of the three "phage-resistant" *B. anthracis* isolates retained in a culture collection indicated that this resistance was not so clear-cut, suggesting it had represented some type of lysogenic immunity at the time of isolation which has, since then, become "dismunity" (see above, this section) (Turnbull, unpublished observations).

It should be noted that these phages do not lyse capsulated *B. anthracis* cultures (McCloy, 1951; Meynell, 1963; Turnbull, unpublished data) and only attack vegetative cells during the multiplication phase. As discussed in section 2.1.2.3, the vegetative form of *B. anthracis* rarely exists or multiplies outside the animal host, and therefore is rarely exposed to exogenous phage. This raises the following question: what is the evolutionary role of the anthrax phage? One possibility is that lysis is a laboratory-induced side-effect and is not representative of the true and yet-to-be elucidated role of this virus, or group of viruses, in the life-cycle of *B. anthracis*.

6.3.1.6 Capsule

Spontaneous loss of ability by virulent *B. anthracis* to produce the capsule has been recognized since at least Sterne's observations (1937a, 1937b) and it was out of these observations that Sterne's livestock vaccine was developed (sections 5.5, 8.6.2). Chu (1952) attached numerical values to this, finding that laboratory stock cultures put out noncapsulated variants to extents ranging from 0.14% to 32.4%. This appears to result from spontaneous loss of pXO2, although the triggering event and point in the life-cycle at which this occurs has yet to be elucidated. The not infrequent presence of pXO1+/2- (and occasionally pXO1-/2-) forms of *B. anthracis* in environmental sites and samples with histories of potential anthrax con-

tamination was noted by Turnbull et al. (1992b) and Turnbull (1996), and was attributed to natural curing of one or both plasmids by unidentified environmental stresses.

6.3.1.7 Voges-Proskauer reaction

Although definitely VP-positive, some difficulty may be experienced in this test due to occasional reluctance by *B. anthracis* to grow in glucose-phosphate broth. In the event of a negative test, the broth should be checked to determine whether growth took place, or whether the culture died out. Positivity manifests itself as a gentle rust colour as compared with the much deeper red of an *Enterobacter cloacae* control.

Logan (personal communication, 2005) found the VP test to be inconsistent within the genus *Bacillus* using the commercial API 20E strip, no matter how well controlled the variables were, but all *B. anthracis* strains were positive as compared with 93% of 149 *B. cereus* strains being positive (Logan & Berkeley, 1981; Logan & Berkeley, 1984; Logan et al., 1985).

6.3.1.8 Citrate utilization

This is given as "variable" (as compared with "positive" for *B. cereus*) by Gordon et al. (1973), Parry et al. (1983), Turnbull et al. (1990a) and Turnbull & Kramer (1991), as well as various editions of Bergey's *Manual of systematic bacteriology*. Elsewhere (Turnbull & Kramer, 1995), *B. anthracis* and *B. cereus* are combined under "*B. cereus* group" and citrate utilization is again inscribed "variable". In fact, using the procedure of Parry et al. (1983), in which slants are stabbed and streaked with a needle dipped into a saline suspension of culture with turbidity equivalent to MacFarland standard 0.5, *B. anthracis* appears to be unable to survive and is negative in this test.

Using the commercial API 20E system, Logan et al. (1985) and Logan & Berkeley (1984) found all 37 of their strains of *B. anthracis* to be negative as compared with 89% of their 149 *B. cereus* strains being positive for this character.

6.3.1.9 Growth in the presence of 5% and 7% salt

Textbooks generally link *B. anthracis* together with *B. cereus* in the "*B. cereus* group" as growing in the presence of 7% salt. In fact, *B. anthracis* generally seems to be reluctant to grow in the presence of 5% salt. One or two strains may show a little growth in the presence of 5% salt, but this is much reduced compared to the 0% control. Others will not show visible growth. Whether this growth is seen or not, the cultures in 5% (and 7%) salt tend to become nonviable within a week.

6.3.1.10 Nitrate

Kiel et al. (2000) discuss the fact that *B. anthracis* maintains a strong nitrate reductase activity and, unlike other facultative anaerobes, the presence of oxygen does not prevent the utilization of nitrate. They relate this to the initiation of sporulation through nitration of the *SpoOA* gene product when the vegetative cells shed by the infected animal encounter the oxygen of the air.

Logan et al. (1985) and Logan & Berkeley (1984) found all 37 of their strains of *B. anthracis* to be nitrate-positive when tested using the API 20E systems compared with only 81% of their 149 *B. cereus* strains.

6.3.1.11 Casein

Strain differences can be expected in the size and nature of the haloes which, after 48 hours at 35 °C, range from narrow (1–2 mm) opaque zones around the growth to totally translucent zones of about 4 mm surrounded by opaque zones of approximately 2 mm width.

6.3.1.12 Starch hydrolysis

As with casein hydrolysis, strain differences can be expected in the extent and visibility of the hydrolysis. Plates should be left 48 to 96 hours before reading. Rapid colony spreading occurs without alterations of these readings beyond this point. Flooding the plate with a 1:5 dilution of Lugol's iodine solution is generally necessary to show up the hydrolysis.

6.3.1.13 Phenylalanine deamination

Gordon et al. (1973) and other texts, including various editions of Bergey's *Manual of systematic bacteriology*, specify that *B. anthracis* (and *B. cereus*) fails to deaminate phenylalanine. In tests done by Turnbull (unpublished) using Method 2 in Cowan & Steel's identification manual (Cowan, 1974), greening of the agar surface was found with 6 of 12 *B. anthracis* cultures and browning in a further 5. These were interpreted as positive tests.

6.3.1.14 Propionate utilization

The results of this test can vary with inoculum size. Following the procedure of Parry et al. (1983)

described in section 6.3.1.8, *B. anthracis* is negative in this test. Positives may result in an inconsistent manner when larger inocula are used. In fact, in an international reproducibility trial of standardized characterization tests for *Bacillus*, Logan & Berkeley (1981) judged this test to be inherently unreliable.

6.3.2 PCR

PCR is becoming more widely available as a means of confirming the presence of the virulence factor (capsule and toxin) genes, and hence that an isolate is, or is not, virulent *B. anthracis*. For routine purposes, primers to one of the toxin genes (usually the Protective Antigen gene) and to one of the enzymes mediating capsule formation are adequate (Annex 1, section 10.7.4). In laboratories not equipped for PCR tests, if doubt remains at the end of the procedures given in Annex 1, sections 10.7.1 to 10.7.3 as to the definitive identity of a suspect *B. anthracis* isolate, inoculation into a mouse or guinea-pig may be the only way remaining to determine conclusively if it is virulent *B. anthracis* (Annex 1, section 12). However this should be a last resort procedure and confined to situations where a definitive identification is essential.

6.4 Molecular composition

6.4.1 The genome

The *B. anthracis* chromosome is a circular DNA molecule of 5 227 293 base pairs encoding 5508 predicted protein-coding sequences (Read et al., 2003).

Elucidation of the genome sequences of *B. anthracis*, *B. cereus* and *B. subtilis* has revealed that the majority of *B. anthracis* genes are homologues of genes found in *B. cereus* and other closely-related species. These include genes encoding such testable phenotypes as haemolysins, enterotoxins, phospholipases, beta-lactamases and other enzymes, and even genes associated with motility (Read et al., 2003). Lack of expression by *B. anthracis* of many phenotypes appears to lie primarily in a truncation of the *PlcR* positive regulator gene, which has been shown to increase production of many of these enzymes. In the case of motility, there are also deletions of genes in the flagellum operon. Low expression of lecithinase and haemolytic activity, or expression under specific inducing conditions, are explained in terms of alternative regulatory mechanisms (Klichko et al., 2003; Read et al., 2003).

The *AtxA* transcriptional regulator of the anthrax toxin genes located on the pXO1 plasmid (see section 5.5) appears to be a master regulator of genes on both the plasmids and chromosome of *B. anthracis* (Bourgogne et al., 2003). There is some evidence that *PlcR* and *AtxA* may be incompatible (Mignot et al., 2001, 2003).

While homologues of the plasmid-encoded toxin and capsule genes and their associated regulatory loci were not found on the *B. cereus* ATCC14579 (type strain) genome, the presence in other *B. cereus* strains of genes with pXO1 and pXO2 sequence identities supports evidence of mobility on the part of the anthrax plasmid genes (Read et al., 2003). Thus, comparative genome analysis was supportive of the hypothesis that *B. anthracis* evolved from a *B. cereus* ancestor. Other than the acquisition of the pXO1 and pXO2 plasmids, the major phenotypic differences between *B. anthracis* and its relatives appear, therefore, to be the result of altered gene expression rather than absence or presence of specific chromosomal genes (Read et al., 2003).

6.4.2 Strains

See section 2.3.

6.5 Spores

The position of the spore as the centre of the cycle of infection of *B. anthracis* and the environmental factors influencing sporulation, germination and survival of the emergent cells are discussed in section 2.1.2. From the public health and veterinary public health standpoints, the rate of sporulation of vegetative forms released by the animal that has died of anthrax is probably of more relevance than the rate of germination.

6.5.1 Sporulation

Reports on conventional studies of sporulation rates of *B. anthracis* were not found, but there is little reason to believe that the rate varies greatly from other *Bacillus* species. In a culture with synchronized growth, the seven stages of sporulation commence at the top of the exponential phase of growth and are complete 7–8 hours later (Doi, 1989; Nicholson & Setlow, 1990). Resistance to UV (ultraviolet) locks in at about 4–6 hours (stages 4 and 5) and resistance to heat and chemicals at around 5–7 hours (stages 5 and 6) after onset of the sporulation process. These are under optimal conditions; under suboptimal

conditions it can take much longer (Davies, 1960). In a field study (Lindeque & Turnbull, 1994), the earliest time at which spores were detected in the soil at a site in the Etosha National Park (Namibia) where a springbok had just died was 8 hours; by 24 hours, spores accounted for 75% of the total count, and complete sporulation was achieved between 32 and 48 hours. The findings in soils and waterhole water inoculated in the laboratory with blood collected just after death are given in section 2.1.2.4, and other information on sporulation can be found in sections 2.1.2.2 and 2.1.2.4.

6.5.2 Germination

The process of germination is dependent upon the action of a germinant on a trigger site within the spore. Common germinants include L-alanine and ribosides, and sometimes germination can be induced by combinations of chemicals that are not germinants on their own (McCann et al., 1996). "Heat-shocking" the spores, i.e. exposing them to 60 °C–70 °C for several minutes (10–30), predisposes them to germination and results in more synchronous germination within a spore population. Heat-shock temperature-time combinations in the literature vary from 60 °C for 90 minutes to boiling for one minute. Turnbull et al. (2006) found evidence of damage to *B. anthracis* spores at 80 °C for 10 minutes, and almost complete killing at 90 °C for 10 minutes.

Germination is a rapid process. Fernelius (1960) found that, following germination of *B. anthracis* spores by alanine, tyrosine and adenosine and, as measured by loss of heat resistance, > 99% germination had occurred in 8 minutes at 30 °C and in 16 minutes at 15 °C. Based on loss of refractility and resulting optical density change, Titball & Manchee (1987) recorded germination as a rather slower process, with 61% to 63% of spores germinating in 90 minutes under optimal conditions, which consisted of low spore concentrations at 22 °C. This optimal temperature is some 10 °C lower than that recorded for other *Bacillus* species; the authors considered that it might represent selection for the best conditions for establishing cutaneous infection. Studying germination of *B. anthracis* spores in sera from 11 species, they found that serum alanine ranged from 0.01 to 0.35 mmol/l, and serum tyrosine from 0.02 to 0.08 mmol/l. Germination rates varied in the different species. They concluded, therefore, that there was no evidence of a relationship between germination ability and the innate resistance of an animal to anthrax, as had been proposed by Hachisuka (1969).

Lincoln et al. (1967) similarly failed to correlate spore germination within phagocytes with susceptibility of an animal to anthrax.

6.6 Other surface antigens: anthrax-specific epitopes and detection methodology

Reliable detection of *B. anthracis* in the environment has traditionally depended on selective culture and identification of the emergent bacilli. This is a slow process (two days or more) and there has long been a desire, particularly in relation to bioaggressive scenarios, for more instant methods of detecting anthrax spores. Numerous attempts were made in the 1960s, 1970s and 1980s to develop antigen-based rapid, species-specific spore-detection systems, but invariably cross-reactivity with other common environmental *Bacillus* species proved insurmountable. Hope that monoclonal antibodies to anthrax spore-specific epitopes might provide reliable bases for rapid detection tests arose in the late 1980s (Phillips et al., 1988). Progress on finding and validating monoclonal antibodies that had the necessary specificities, sensitivities, affinities, etc., for application in detection systems appears to have been slow, with the next published report being in 1998 (Park et al., 1998), and the most recent being that of Mangold et al. (2005) recording the development of anthrax-specific monoclonal antibodies to spore and vegetative cell antigens. Rapid detection being a major issue for defence concerns, it is certain that unpublicized systems are in use.

Claims now exist that anthrax spore-specific epitopes are present on at least one immunodominant exosporium protein (see section 5.5.6).

The target epitopes of some anthrax-specific monoclonal antibodies proposed or used for detection of anthrax spores are unidentified, but suspected of residing in the vegetative cell S-layer extractable antigen EA1 (section 5.5.5) retained on the spores. For general and veterinary public health detection purposes, antibody-based systems utilizing such monoclonals are likely to be satisfactory. In bioaggression scenarios, the possibility that the determined aggressor may have effectively cleaned his/her spores may have to be borne in mind.

6.7 Transport of clinical and environmental samples

Movement of infectious or contaminated materials from the site of origin to a diagnostic or reference laboratory, both within a country and internationally, presents a risk of spread of diseases if the materials inadvertently escape into the environment during transit.

The international regulations for the transport of infectious substances by any mode of transport are based upon the recommendations made by the Committee of Experts on the Transport of Dangerous Goods (UNCETDG), a committee of the United Nations Economic and Social Council. The recommendations are presented in the form of Model Regulations. The United Nations Model Regulations (UNMR) are reflected in international law through international model agreements.[1]

WHO has developed a document to help understand the current regulatory framework and support compliance with current international regulations for the transport of infectious substances and patient specimens by all modes of transport, *Guidance on regulations for the transport of infectious substances 2007–2008*, applicable as of 1 January 2007 (WHO, 2007). This document is regularly updated to reflect modifications made to relevant sections of the UNMR.

The reader is encouraged to refer to this WHO document for details on transport of clinical and environmental samples.

[1] http://www.unece.org/trans/danger/danger.htm.

7. Treatment and prophylaxis

7.1 Overview

7.1.1 Background

In a review of treatment in the early days of antibiotics half a century ago, Herman Gold (1955) wrote: "penicillin and the broad spectrum antibiotics … have simplified the management of anthrax so that one can safely say that, in man, this disease has lost its serious connotations". Since that time, it has become well established that prompt and timely antibiotic therapy usually results in dramatic recovery of the individual or animal infected with anthrax. Any fresh isolate of *B. anthracis* from naturally acquired cases of anthrax in animals or humans can be assumed to have a very high probability of being sensitive to penicillin and, being cheap and readily available almost everywhere, this remains the basis of treatment schedules in animals and in humans in developing countries.

The organism is also sensitive to numerous other broad-spectrum antibiotics. Should the use of penicillin be contraindicated, a wide range of alternative choices exist from among the aminoglycosides, macrolides, quinolones and tetracyclines. Chloramphenicol has been stated to be a satisfactory alternative and shows in vitro sensitivity but, in early experimental studies, Lincoln et al. (1964) found that it was not effective in mice or monkeys. Vancomycin, clindamycin, clarithromycin, rifampin, levofloxacin, gatifloxacin, moxifloxacin, ofloxacin, imipenem, cefazolin and linezolid have, in papers published after the anthrax letter events, been named as meriting consideration on the basis of in vitro sensitivities (Editorial Note, 2001a; Inglesby et al., 2002; Sejvar et al., 2005). Published MICs (minimum inhibitory concentrations) are available for some of these (Table 4).

As discussed in section 5.5, the basis of death in anthrax is the toxin. This was first demonstrated conclusively by Smith & Keppie (1954) when they showed that, if bacteraemia in experimentally infected animals was cleared with streptomycin after a certain critical stage of the infection, the animals still died. The importance of early administration of antibiotics still needs to be stressed; administered too late, they may clear the infection but still fail to save the patient or animal.

7.1.2 β-lactamases and "penicillin resistance"

The ability of *B. anthracis* to elaborate penicillinase was recognized over half a century ago (Barnes, 1947b; Turnbull et al., 2004a). Inducible β-lactamase production in a number of strains was demonstrated by Lightfoot et al. (1990) following exposure to a subinhibitory level of flucloxacillin. Induction of β-lactamase was again noted in relation to the anthrax letter events in the USA, leading to published statements that penicillins are not recommended for treatment of anthrax (Anon., 2001a, 2001c; Editorial Note, 2001a), "particularly if the number of organisms present is high, as appears typical with inhalational disease" (Bell et al., 2002). A second reason given for concerns about the use of penicillin was the poor penetration of β-lactams into macrophages, the site where *B. anthracis* spores germinate (Bell et al., 2002).

In reality, reports of naturally-occurring resistance to penicillin among fresh patient or animal isolates are exceedingly rare and, again reviewed by Turnbull et al. (2004a), appear to number just 5 cases, not all of which were well substantiated with further studies. The proportion (11.5%) of penicillin-resistant isolates of *B. anthracis* recorded by Cavallo et al. (2002) in their series is surprisingly high, but the culture histories are not given, apart from the information that 67 (70%) were isolated from environmental sources. It is possible that some of the isolates were related to each other and that this may account for this high percentage. The report highlights the problem discussed by Turnbull et al. (2004a) of defining precisely what is meant by "strain" when it comes

TABLE 4

Comparison of reports on MICs for *B. anthracis*

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a] S (≤)	BREAKPOINTS[a] R (≥)	INTERPRETATION[b] S	INTERPRETATION[b] I	INTERPRETATION[b] R
ABT 492	Frean et al. (2003)	Agar dilution	28	0.016–0.063	≤0.06 /0.03	0.063	na	na			
Amikacin	Doganay & Aydin (1991)	Agar dilution	22	0.03–0.06	0.03	0.06	16	64			
		Disc diffusion	22						100		
Ampicillin	Doganay & Aydin (1991)	Agar dilution	22	0.03–0.125	0.03	0.03	0.25	0.5			
		Disc diffusion	22						100		
	Odendaal et al. (1991)	Disc diffusion	44						100		
Ampicillin + sulbactam	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.03	0.015	0.015	8	32			
		Disc diffusion	22						100		
Amoxicillin	Cavallo et al. (2002)	Agar dilution	96	0.125–16	0.125	4	0.25	0.5	88.5		11.5
	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.03	0.015	0.015					
	Jones et al. (2003)	Microdilution	12	≤0.06	≤0.06	≤0.06			100		
Amoxicillin + clavulanic acid	Jones et al. (2003)	Microdilution	12	≤0.06/0.03	≤0.06/0.03	≤0.06/0.03	4	8	100		
	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.015	0.015	0.015			100		
		Disc diffusion	22						100		
	Lightfoot et al. (1990)	Agar dilution	70	0.03–64	0.125	0.06			99		1
	Turnbull et al. (2004a)	Etest	45	0.016–0.5	0.032	0.047			100		
		Agar dilution	10	0.015–0.06	0.03	0.06			100		
Azithromycin	Jones et al. (2003)	Microdilution	12	1–4	2	4	2	8	50	50	
	Sumerkan et al. (1996)	Agar dilution	34	0.5–4	1	4					
	Turnbull et al. (2004a)	Etest	73	1–12	3	6			26	64	10
Aztreonam	Cavallo et al. (2002)	Agar dilution	96	1–>128	128	1–>128	4	32			100
	Doganay & Aydin (1991)	Agar dilution	22	>128	>128	>128					
		Disc diffusion	22								100
Cefaclor	Coker et al. (2002)	Etest	25	0.125–0.75	0.38	1.65	8	32	100		
Cefamandole	Odendaal et al. (1991)	Disc diffusion	44						68	32	
Cefazolin	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.03	0.015	0.015	8	32			
Cefdinir	Frean et al. (2003)	Agar dilution	28	1–16	2	8	1	4			
Cefditoren	Frean et al. (2003)	Agar dilution	28	4–8	8	8	na	na			
Cefoperazone	Doganay & Aydin (1991)	Agar dilution	22	0.5–4	2	4	16	64			
		Disc diffusion	22						100		

TABLE 4 CONTINUED

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a]		INTERPRETATION[b]		
							S (≤)	R (≥)	S	I	R
Cefotaxime	Doganay & Aydin (1991)	Agar dilution	22	8–32	32	32	8	64			
		Disc diffusion	22						5	13	82
	Odendaal et al. (1991)	Disc diffusion	44							100	
	Turnbull et al. (2004a)	Etest	76	3–>32	>32	>32			1		99
		Agar dilution	10	16–64	32	32				100	
Cefoxitin	Cavallo et al. (2002)	Agar dilution	96	1–64	8	32	8	32	74	15.3	10.7
	Odendaal et al. (1991)	Disc diffusion	44						93	7	
Ceftazidime	Doganay & Aydin (1991)	Agar dilution	22	128–256	128	128	8	32			
		Disc diffusion	22						5	0	95
Ceftizoxime	Doganay & Aydin (1991)	Agar dilution	22	16–64	32	32	8	64			
		Disc diffusion	22						5	13	82
Ceftriaxone	Cavallo et al. (2002)	Agar dilution	96	4–64	32	32	8	64		100	
	Doganay & Aydin (1991)	Agar dilution	22	16–32	16	32					
		Disc diffusion	22						9	50	41
	Jones et al. (2003)	Microdilution	12	4–16	4	8					
	Mohammed et al. (2002)	Microdilution	65	4–32	16	32			22	78	
Cefuroxime	Coker et al. (2002)	Etest	25	6–48	21.33	32	4	32	4	76	20
	Doganay & Aydin (1991)	Agar dilution	22	16–64	64	64					
		Disc diffusion	22						5	9	86
Cephalexin	Coker et al. (2002)	Etest	25	0.38–2	1.5	1.5	na	na	100		
Cephalothin	Cavallo et al. (2002)	Agar dilution	96	0.125–32	0.5	16	8	32	83.2	12.2	4.6
Cephalothin	Doganay & Aydin (1991)	Disc diffusion	22						100		
Cephradine	Doganay & Aydin (1991)	Disc diffusion	22						100		
Cethromycin (ABT 773)	Frean et al. (2003)	Agar dilution	28	0.016–0.063	0.031	0.063	na	na			
Chloramphenicol	Cavallo et al. (2002)	Agar dilution	96	1–4	2	2	8	32	100		
	Doganay & Aydin (1991)	Agar dilution	22	1–2	2	2					
		Disc diffusion	22						100		
	Mohammed et al. (2002)	Microdilution	65	2–8	4	4			100		
	Odendaal et al. (1991)	Disc diffusion	44						100		

TABLE 4 CONTINUED

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a] S (≤)	BREAKPOINTS[a] R (≥)	INTERPRETATION[b] S	INTERPRETATION[b] I	INTERPRETATION[b] R
Ciprofloxacin	Cavallo et al. (2002)	Agar dilution	96	0.03–0.5	0.06	0.25	0.5	na	100		
	Coker et al. (2002)	Etest	25	0.032–0.38	0.094	0.094			100		
	Doganay & Aydin (1991)	Agar dilution	22	0.03–0.06	0.03	0.06			100		
		Disc diffusion	22						100		
	Esel et al. (2003)	Agar dilution	40	<0.008–0.12	0.03	0.06			100		
	Jones et al. (2003)	Microdilution	12	0.03–0.25	0.03	0.12			100		
	Frean et al. (2003)	Agar dilution	28	0.016–0.125	0.031	0.031			100		
	Lightfoot et al. (1990)	Agar dilution	70	0.03–0.06	0.06	0.06			100		
	Mohammed et al. (2002)	Microdilution	65	0.03–0.12	0.06	0.06			100		
	Turnbull et al. (2004a)	Etest	76	0.032–0.094	0.064	0.094			100		
		Agar dilution	10	0.06	0.06	0.06			100		
Clarithromycin	Frean et al. (2003)	Agar dilution	28	0.063	0.125	0.125	2	8			
	Sumerkan B et al. (1996)	Agar dilution	34	0.03–0.25	0.06	0.12					
Clindamycin	Cavallo et al. (2002)	Agar dilution	96	0.125–1	0.125	0.25	0.5	4	100		
	Doganay & Aydin (1991)	Agar dilution	22	0.5–1	1	1					
		Disc diffusion	22						95	5	
	Mohammed et al. (2002)	Microdilution	65	≤0.5–1	≤0.5	1			94	6	
	Odendaal et al. (1991)	Disc diffusion	44						93	7	
	Frean et al. (2003)	Agar dilution	28	0.063–0.125	0.125	0.125					
Cotrimoxazole	Cavallo et al. (2002)	Agar dilution	96	<4/76	<4/76	<4/76	2/38	4/76		9	91
Doxycycline	Coker et al. (2002)	Etest	25	0.094–0.38	0.23	0.34	4	16	100		
	Cavallo et al. (2002)	Agar dilution	96	0.125–0.25	0.125	0.25			100		
	Esel et al. (2003)	Agar dilution	40	≤0.016–0.03	≤0.016	0.03					
	Frean et al. (2003)	Agar dilution	28	0.031–0.125	0.063	0.063					
	Jones et al. (2003)	Microdilution	12	≤0.008–0.015	0.015	0.015			100		
Erythromycin	Cavallo et al. (2002)	Agar dilution	96	0.5–4	1	1	0.5	8	95.4	4.6	
	Doganay & Aydin (1991)	Disc diffusion	22						100		
	Frean et al. (2003)	Agar dilution	28	0.5–8	1	2					
	Jones et al. (2003)	Microdilution	12	0.5–2	0.5	1			100		
	Lightfoot et al. (1990)	Agar dilution	70	0.25–1	0.5	1			na	na	0
	Mohammed et al. (2002)	Microdilution	65	0.5–1	1	1			3	97	
	Odendaal et al. (1991)	Disc diffusion	44						100		
	Sumerkan et al. (1996)	Agar dilution	34	0.25–1	0.5	1			100		
	Turnbull et al. (2004a)	Etest	69	0.5–4	1	1.5			15	85	
		Agar dilution	10	0.5–2	1	2			10	90	
Fusidic acid	Odendaal et al. (1991)	Disc diffusion	44						16	64	

TABLE 4 CONTINUED

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a] S (≤)	BREAKPOINTS[a] R (≥)	INTERPRETATION[b] S	INTERPRETATION[b] I	INTERPRETATION[b] R
Gatifloxacin	Cavallo et al. (2002)	Agar dilution	96	0.125–0.125	0.125	0.125	2	8	100		
	Esel et al. (2003)	Agar dilution	40	0.016–0.06	0.03	0.06					
Gentamicin	Cavallo et al. (2002)	Agar dilution	96	0.125–0.5	0.25	0.5	4	16	100		
	Doganay and Aydin (1991)	Agar dilution	22	0.03–0.25	0.06	0.125			100		
		Disc diffusion	22						100		
	Lightfoot et al. (1990)	Agar dilution	70	0.06–0.5	0.125	0.25			100		
	Odendaal et al. (1991)	Disc diffusion	44						97.8	2.2	
	Turnbull et al. (2004a)	Etest	75	0.064–0.5	0.25	0.38			100		
		Agar dilution	10	0.25–0.5	0.25	0.5			100		
Levofloxacin	Cavallo et al. (2002)	Agar dilution	96	0.03–1	0.125	0.25	2	8	100		
	Esel et al. (2003)	Agar dilution	40	0.016–0.12	0.06	0.12					
Imipenem	Cavallo et al. (2002)	Agar dilution	96	0.125–2	0.125	0.125	4	16	100		
Methicillin	Odendaal et al. (1991)	Disc diffusion	44						100		
Mezlocillin	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.06	0.06	0.06	na	na			
		Disc diffusion	22						100		
Nalidixic acid	Cavallo et al. (2002)	Agar dilution	96	0.125–32	4	8	8	16	94.8	4.2	1
Netilmicin	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.125	0.06	0.125	8	32			
		Disc diffusion	22						100		
	Odendaal et al. (1991)	Disc diffusion	44						100		
Novobiocin	Odendaal et al. (1991)	Disc diffusion	44						86.5	13.5	
Ofloxacin	Cavallo et al. (2002)	Agar dilution	96	0.06–2	0.25	0.25	1	8	99	1	
	Doganay & Aydin (1991)	Agar dilution	22	0.03–0.06	0.06	0.06					
		Disc diffusion	22						100		
Olamufloxacin (HSR 903)	Frean et al. (2003)	Agar dilution	28	0.016–0.03	0.031	0.031	na	na			
Pefloxacin	Cavallo et al. (2002)	Agar dilution	96	0.03–1	0.125	0.5	1	4	100		

TABLE 4 CONTINUED

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a]		INTERPRETATION[b]		
							S (≤)	R (≥)	S	I	R
Penicillin	Cavallo et al. (2002)	Agar dilution	96	0.125–16	0.125	8	0.12	0.25	88.5		11.5
	Coker et al. (2002)	Etest	25	<0.016–0.5	0.042	0.236			88		12
	Doganay & Aydin (1991)	Agar dilution	22	0.015–0.03	0.015	0.015			100		
		Disc diffusion	22						100		
	Jones et al. (2003)	Microdilution	12	≤0.06	≤0.06	≤0.06			100		
	Esel et al. (2003)	Agar dilution	40	0.016–0.03	0.016	0.016			100		
	Lightfoot et al. (1990)	Agar dilution	70	0.015–64	0.06	0.125			99		1
	Mohammed et al. (2002)	Microdilution	65	≤0.06–128	≤0.06	≤0.06			97		3
	Odendaal et al. (1991)	Disc diffusion	44						84	16	
	Turnbull et al. (2004a)	Etest	74	<0.016–>32	<0.016	0.023			99		3
		Agar dilution	8	0.015–0.5	0.015	0.015					
Piperacillin	Cavallo et al. (2002)	Agar dilution	96	0.25–32	1	1	8	16	99	1	
	Doganay & Aydin (1991)	Agar dilution	22	0.125–0.5	0.25	0.5					
		Disc diffusion	22						100		
Rifampin	Cavallo et al. (2002)	Agar dilution	96	0.125–0.5	0.125	0.125	1	4	100		
	Mohammed et al. (2002)	Microdilution	65	≤0.25–0.5	≤0.25	0.5			100		
	Sumerkan et al. (1996)	Agar dilution	34	0.06–0.25	0.25	0.25					
Streptomycin	Cavallo et al. (2002)	Agar dilution	96	0.5–2	1	1	8	16	100		
	Doganay & Aydin (1991)	Agar dilution	22	1–4	2	4					
	Odendaal et al. (1991)	Disc diffusion	44						100		
Sulphamethoxazole	Odendaal et al. (1991)	Disc diffusion	44								100
Sulphatriad	Odendaal et al. (1991)	Disc diffusion	44						2.25	2.25	95
Teicoplanin	Cavallo et al. (2002)	Agar dilution	96	0.125–0.5	0.25	0.5	8	32	100		
Temafloxacin	Frean et al. (2003)	Agar dilution	28	0.031–0.125	0.063	0.125	na	na			
Tetracycline	Doganay & Aydin (1991)	Disc diffusion	22				1	na	100		
	Lightfoot et al. (1990)	Agar dilution	70	0.6–1	0.125	0.125			100		
	Mohammed et al. (2002)	Microdilution	65	0.03–0.06	0.03	0.06			100		
	Odendaal et al. (1991)	Disc diffusion	44						100		
	Turnbull et al. (2004a)	Etest	71	0.016–0.094	0.023	0.032			100		
		Agar dilution	10	0.015–0.06	0.015	0.03			100		
Tobramycin	Coker et al. (2002)	Etest	25	0.25–1.5	0.75	0.97	4	16	100		
	Doganay & Aydin (1991)	Agar dilution	22	0.25–1	0.25	1					
		Disc diffusion	22						100		
Tosufloxacin	Frean et al. (2003)	Agar dilution	28	<0.003–0.008	<0.003	<0.008	na	na			

TABLE 4 CONTINUED

ANTIBIOTIC	STUDY	TEST METHOD	NO. OF STRAINS	RANGE	50%	90%	BREAKPOINTS[a] S (≤)	BREAKPOINTS[a] R (≥)	INTERPRETATION[b] S	INTERPRETATION[b] I	INTERPRETATION[b] R
Trimethoprim	Odendaal et al. (1991)	Disc diffusion	44								100
Trimethoprim + sulfamethoxazole	Doganay & Aydin (1991)	Agar dilution	22	1.6/8–3.2/16	3.2/16	3.2/16	2/38	4/76			
		Disc diffusion	22						100		
Vancomycin	Cavallo et al. (2002)	Agar dilution	96	0.25–2	1	1	4	32	100		
	Doganay & Aydin (1991)	Agar dilution	22	0.25–1	1	1			95	5	0
		Disc diffusion	22						95	5	0
	Mohammed et al. (2002)	Microdilution	65	0.5–2	2	2			100		
	Turnbull et al. (2004a)	Etest	74	0.75–5	2	3			99	1	
		Agar dilution	10	1–4	4	4			100		

[a] NCCLS MIC interpretive standards for Gram-positive and/or aerobic bacteria except ciprofloxacin, penicillin and tetracycline for which the newly available NCCLS breakpoints for *B. anthracis* are given.

[b] Percentages.

na = information not available.

to *B. anthracis*, and how one knows for certain that two cultures are unrelated. This becomes relevant when concluding that a particular proportion of a set of *B. anthracis* cultures are resistant to penicillin. It is also not known if the dependence on culture collection strains for these types of analysis adversely affects the proportions of resistant strains recorded. There was some discrepancy between the results of Turnbull et al. (2004a) and those of Coker et al. (2002) on the same strains; Turnbull et al. did not observe penicillin resistance in two of the three strains constituting Coker et al.'s 12% resistant strains, all culture collection strains.

It is important that penicillin should be judged by its performance in the field, and faith in it should not be lost on the basis of surmise and remote laboratory experiments. It has withstood the test of time as the first choice of treatment in those parts of the world where anthrax is or has been common, and being cheap and readily available almost everywhere, it needs to remain the recommended antibiotic in both animals and humans, at least in developing countries (Tasyaran & Erol, 2002). Probably the fundamental principle is that adequate doses should be administered when penicillin is being used for treatment (see also section 7.3.1.4). It is reasonable to add a second drug in cases showing signs of systemic involvement (Tasyaran & Erol, 2002). This is treated more extensively in section 7.3.1.6.

With the recent completion of sequencing of the *B. anthracis* genome, it has become apparent that the gene for penicillinase is an example of a significant number of genes shared with close relative *B. cereus* which, though present, have become inactivated in recent evolutionary history and are not normally expressed (see section 6.4.1). The genes for the second β-lactamase, a cephalosporinase, are expressed by virtually all *B. anthracis* strains, inactivating second- and third-generation cephalosporins. Chen et al. (2003) noted that functional β-lactamases were encoded but that gene expression was usually not sufficient to confer resistance to β-lactam agents. The underlying genetic basis of β-lactamase expression in *B. anthracis* is being elucidated further (Chen et al., 2004).

7.1.3 Supportive care

In pulmonary or gastrointestinal anthrax or cutaneous disease with systemic signs, symptomatic treatment in an intensive care unit in addition to antibiotic therapy may save the patient's life. The availability of supportive care, including mechanical ventilatory support, probably enhanced the survival rate in the 2001 anthrax letter cases (see also section 7.3.1.9). As covered in section 7.3.1.1, the value of corticosteroids is debatable. The possible value of immune therapy is discussed in sections 7.3.4 and 7.3.4.1.

7.1.4 Vaccines

Anthrax in its natural state being primarily a disease of herbivorous animals (section 1.1), its control in both animals and humans depends to a very great extent on its prevention in livestock (principally cattle, sheep, goats and horses), good hygienic practices when an animal dies of anthrax and antibiotic treatment when a case occurs, at least in a human. Livestock anthrax vaccines are available in almost all countries that experience outbreaks or sporadic cases on an annual basis. These are comprised of live spores of attenuated strains (Annex 5).

Analogous live spore vaccines are produced for human use in China and in the Russian Federation. Cell-free vaccines containing anthrax protective antigen are produced and licensed for human use in the United Kingdom and the USA. Fundamentally, these vaccines are targeted at persons in occupations with a high risk of exposure to the disease. In former years, these were workers in factories that processed hides, bones, wool, hair and other materials of animal origin imported from endemic regions of the world, and vaccination was associated with a major reduction of anthrax in such individuals (Turnbull, 2000). There has been a notable shift in recent years to the administration of these vaccines to personnel in occupations related to defence. Their availability has become correspondingly more restricted.

7.2 Response to outbreaks in animals

7.2.1 General principles and approaches

7.2.1.1 Early treatment for animals

Following the first incident of anthrax in a herd, the remaining animals should be moved immediately from the field or area where the index case died, and regularly checked at least three times a day for two weeks for signs of illness (rapid breathing, elevated body temperature), or of submandibular or other oedema. Any animal showing these signs should be separated from the herd and given immediate treatment. Where such close observation and individual animal handling is difficult or not possible or likely

to be stressful, the whole herd should be treated with long-acting antibiotic that provides at least 72-hours cover.

Clinical experience has frequently demonstrated that animals, especially cattle, will respond favourably to treatment even though apparently in the terminal stages of anthrax. Even if they go on to die (death in anthrax results from the effect of the toxin – section 7.1.1), the infecting load of *B. anthracis* will have been greatly reduced, if not entirely eliminated, thereby significantly reducing the chance of subsequent transmission from the carcass to other animals.

In certain countries, antibiotic treatment is not permitted and slaughter policies are in place (section 7.2.2.4).

7.2.1.2 Vaccination for animals

In endemic areas, or if there is concern that the outbreak may spread, the herd should be vaccinated (section 8.7). Further anthrax deaths can be expected to cease within 8 to 14 days of vaccination.

Decontamination of the site(s) where the index case or other case(s) died should be carried out (see section 8.3 and Annex 3). Subject to local regulations giving different instructions, herd quarantine can be lifted 21 days after the last death (see Annex 4).

Where animals are scheduled to be moved for local or international livestock and meat trade purposes, it is important to check whether there are local advisories in place specifying a withholding period following vaccination before which animals may be moved to other premises, or sent to slaughter. OIE standards are given in Annex 4.

More information on animal vaccines is given in section 8.6.2 and in Annex 5, section 2.

7.2.1.3 Treatment and vaccination in animals

Where the response to an outbreak consists of treatment first, followed by vaccination, it should be remembered that, in those animals that are treated, a suitable period of time should be allowed between the end of treatment and the start of vaccination, otherwise the treatment will prevent the live vaccine taking effect. The precise time lapse will depend on the antibiotic used but, in the absence of specific studies on the minimum withdrawal times before the vaccine can be administered, 8–12 days would appear logical for long-acting antibiotics. It should also not be forgotten that if the livestock are consuming feedstuffs containing antibiotics, or are being individually treated (e.g. intramammary antibiotics for mastitis), this is likely to render the vaccine ineffective (Lee et al., 1961; Webster, 1973).

Long intervals between outbreaks in a given area despite a lack of vaccination programmes are a notable feature in animal anthrax epidemiology (Fox et al., 1973, 1977). As implied in section 7.2.1.1, treatment alone can halt an outbreak and further cases may not occur on cessation of treatment.

7.2.2 Specific procedures

7.2.2.1 Antimicrobial therapy in animals

The recommended procedure for treating animals showing clinical illness in which anthrax is thought to be the likely or possible cause is immediate intravenous administration of sodium benzylpenicillin as directed by the manufacturer's instructions (usually in the range 12 000–22 000 units per kg of body weight) followed 6–8 hours later by intramuscular injection of long-acting benethamine penicillin (manufacturers' instructions usually recommend a dose within the range of 6000–12 000 units per kg of body weight) or other appropriate preparation such as Clamoxyl® (15 mg/kg), a long–acting preparation of amoxycillin. If long-acting preparations are unavailable, procaine penicillin (the dose recommended by manufacturers is usually 6000–12 000 units/kg) can be used for intramuscular injection, but should be administered again after 24 and 48 hours.

Streptomycin acts synergically with penicillin (Lincoln et al., 1964) and penicillin/streptomycin mixtures are available commercially. Recommended doses of streptomycin to be administered together with penicillin intramuscularly are 5–10 mg per kg body weight in large animals and 25–100 mg per kg body weight in small animals.

Lincoln et al. (1964) noted in studies with rhesus monkeys that, for penicillin, the route of administration was important, with intramuscular injection being more effective than intravenous.

Veterinary experience in the United Kingdom is that, in contrast to advice frequently found in textbooks, treatment with tetracyclines may not be fully effective (Taylor, personal communication, 1997).

Attention should be paid to manufacturers' recommendations regarding precautions and limitations of use of their antibiotics, including aspects relating to withdrawal periods after use in food animals.

Cost and availability are likely to be major considerations in the choice of treatment. For example, combined penicillin and streptomycin treatment can be expected to cost twice as much as penicillin alone.

7.2.2.2 Supportive therapy for animals

Symptomatic treatment may also be useful, and a range of possible agents is available. Supportive therapy with an agent such as flunixin (an analgesic with anti-inflammatory, antipyretic and anti-endotoxic properties) may be advantageous although it will add significantly to the cost of the therapy. As with steroids (section 7.3.1.10), it is, with current understanding of the pathogenesis of anthrax, difficult to see a scientific basis for efficacy with this type of therapeutic agent.

7.2.2.3 Hyperimmune serum therapy for animals

Hyperimmune serum has been used in the past for treating anthrax cases (Sterne, 1959) and it was generally considered that homologous sera (e.g. serum prepared in cattle for bovine use, etc.) were more effective than heterologous sera, although in rabbits equine antisera were twice as effective as bovine antisera (Spears, 1955). In Spears's experiments, substantially increased time to death was a notable feature in animals that were not fully protected, indicating that serum therapy could only be regarded as supportive rather than a stand-alone action. This appeared to be understood in 1935 (Gochenour et al., 1935) when it was found that antiserum in combination with spore vaccine was more effective over time than antiserum alone.

Serum treatment of livestock is still practised in the Russian Federation at a rate of approximately 5 cases a year (Cherkasskiy, personal communication, 2002). The antiserum is produced in horses hyperimmunized with vaccine strain 55-VNIIVVM (see Annex 5, section 4.1). As far as could be ascertained, antiserum for this purpose is not produced or routinely used elsewhere for therapy against anthrax in animals.

The protective effect of immune serum administered therapeutically was demonstrated in monkeys (Henderson et al., 1956; Lincoln et al., 1964). These were challenged by the inhalation route and, in Henderson et al.'s experiments, once the effects of the immune serum wore off at about 20 days, the animals began to die of anthrax from continued uptake into the lymphatics of spores that still remained in the lungs. The possibility of relapse after the effect of this therapy has worn off was thereby illustrated. Lincoln et al. (1964), anticipating this outcome, administered the forerunner to the United States and United Kingdom human vaccines on day 8 and had no deaths. Lincoln et al. also found that intramuscular and intravenous administration of antiserum were equally effective.

7.2.2.4 Countries prohibiting treatment in livestock

It should be noted that treatment of animals is forbidden in some countries. Veterinary requirements in these countries are that, in a herd that has experienced a case of anthrax, other animals showing signs of illness must be killed without spilling of blood or exsanguinations, and the unopened carcass must be disposed of appropriately (section 8.3.2). It is understood that certain countries require the slaughter of the entire herd following a case of anthrax; this draconian approach is unnecessary and wasteful. Anthrax is not a chronic infection; if treated, animals will certainly recover. Killing sick animals instead of treating them is costly and alienates owners, even if compensated, and may leave them unwilling to report illness in their animals in the future. This is counterproductive.

7.2.2.5 Therapy in wildlife

While the use of antibiotics for controlling anthrax in wildlife is, generally speaking, unlikely to be feasible, it has reportedly been done with success in an outbreak in the United Republic of Tanzania where a single treatment of roan, sable antelope and kudu, 50 animals in total, by direct darting, appeared to arrest an outbreak (Jiwa, personal communication, 1995). In September 2002, a number of lions in the Etosha National Park (Namibia) were observed with severely swollen heads. Anthrax was confirmed in two of these but a third was captured and treated with a single dose of 15 ml (2250 mg) of procain benzylpenicillin (long acting) and lived (Jago, personal communication, 2006). The second lion that died was the mother of two cubs and was lactating heavily. The cubs were apparently healthy.

7.3 Treatment of humans

7.3.1 Developing countries, naturally acquired anthrax

7.3.1.1 Mild uncomplicated cases

Penicillin G is still the drug of choice in the therapy of naturally-occurring anthrax in most parts of the world. In mild uncomplicated cases of cutaneous anthrax, the treatment usually recommended is intramuscular procaine penicillin, 500 to 600 mg (800 000 to 1 million units) every 12–24 hours for 3–7 days (procaine penicillin is frequently marketed in vials containing 800 000 units in developing countries). Intravenous antibiotic therapy is not recommended in mild cases. If the patient rejects intramuscular injection, penicillin V (500 mg taken orally every 6 hours) or amoxicillin (500 mg orally every 8 hours for 3–7 days) are acceptable alternatives. Cutaneous lesions usually become sterile within the first 24 hours of such regimens and the accompanying oedema usually subsides within 24 to 48 hours but, although early treatment will limit the size of the lesion, it will not alter the evolutionary stages it must go through (Gold, 1955; Kobuch et al., 1990).

7.3.1.2 Severe or potentially life-threatening cases

In patients exhibiting signs of systemic involvement, such as with inhalational or gastrointestinal anthrax, meningoencephalitis, sepsis, or cutaneous anthrax with extensive oedema, antibiotics should be given intravenously. Penicillin G is generally the first choice, 2400 mg (4 million units) every 4–6 hours by infusion (total daily dose should be 20–24 million units) until the patient's symptoms resolve with the temperature returning to normal. At this point consideration may be given to switching to the intramuscular procaine penicillin regimen described in section 7.3.1.1. Also in severe cases, penicillin or another chosen antibiotic, e.g., ciprofloxacin, may be combined with another appropriate agent, preferably one that penetrates well to the central nervous system (CNS) (see also section 7.3.1.7). Penicillin G may be combined with clindamycin or clarithromycin in treating inhalational anthrax, or with an aminoglycoside (streptomycin is suggested) in gastrointestinal anthrax. The synergistic action of streptomycin when combined with penicillin has already been noted in relation to treatment of animals (section 7.2.2.1). In the USA, the most up-to-date recommendation for anthrax meningoencephalitis (Sejvar et al., 2005) is a fluoroquinolone (levofloxacin has also been shown to be effective in an animal model – Deziel et al., 2005) with two additional agents that penetrate well to the CSF (see section 7.3.1.9).

Use of more than one antibiotic is discussed further in section 7.3.1.6. Suggested doses are given in Table 5. As a general principle, in life-threatening infection with *B. anthracis*, low doses of antibiotics should not be used.

General measures for treatment of shock may be life-saving since death is caused, at least in part, by toxin-induced shock. Intubation, tracheotomy or ventilatory support may be required in the event of respiratory problems, for example in cases where compression on the neck from oedema is leading to danger of tracheal obstruction; once oedema is extensive it can be difficult to find the trachea at operation. Vasomotor support may be called for when there is haemodynamic instability. If administration of the appropriate volume of intravenous fluid fails to raise the systolic blood pressure above 90 mm Hg, a vasoactive drug such as dopamine or dobutamine can be given. Primary haematological, renal or liver function disorders are not generally seen.

7.3.1.3 Therapy in children

Penicillin is again the antibiotic of choice for treatment of anthrax in children. Generally the approach taken at least in Africa has been to give half-adult doses to children of less than 10 years of age (Martin, 1975; Turnbull, personal observations).

In mild uncomplicated paediatric cases of cutaneous anthrax, penicillin V should be given by the oral route at a dosage of 25–50 mg/kg/day in 3–4 doses, or amoxicillin according to the following formulae:

- weight > 20 kg, 500 mg orally every 8 hours for 3–7 days;
- weight < 20 kg, 40 mg/kg orally every 8 hours or intramuscular procaine penicillin (25 000 to 50 000 units/kg/day divided into 1–2 doses) for 3–7 days.

In severe or life-threatening cases, penicillin G should be administered intravenously in a dose of 300 000 to 400 000 units/kg/day for systemic anthrax and for cutaneous anthrax if (i) there are signs of systemic toxicity, (ii) the lesion(s) is/are located on the head and neck, and (iii) extensive oedema is present. As with adults (section 7.3.1.2), consideration should be given to combining the penicillin with rifampicin or vancomycin (Sejvar et al., 2005). Penicillin combined

TABLE 5

Suggested antibiotic administration regimens for anthrax

ANTIBIOTIC	DOSAGE FOR ADULTS	DOSAGE FOR CHILDREN
Penicillin V	500 mg orally, 4 times/day	25–50 mg/kg/day orally in 4 divided doses
Penicillin G, procaine	0.6–1.2 mU IM every 12–24 h	25 000–50 000 U/kg/day IM
Penicillin G, sodium or potassium	4 mU intravenously every 4–6 h	300 000–400 000 U/kg/day in divided doses every 4–6 hours
Ampicillin	1–2 g intravenously every 4–6 h	50–200 mg/kg/day intravenously divided every 4–6 h
Amoxicillin	500 mg orally every 8 h	Weight > 20 kg: 500 mg orally every 8 h Weight < 20 kg: 40 mg/kg orally in 3 doses every 8 h
Ciprofloxacin[a]	500 mg orally every 12 h 400 mg intravenously every 12 h	Not generally recommended for children. In emergency, 10–15 mg/kg twice daily, not to exceed 1 g/day
Clarithromycin[b]	500 mg PO or IV every 12 h	Not suggested
Clindamycin[b]	150–300 mg orally every 6 h 600–900 mg IV every 6–8 h	(oral) 8–25 mg/kg/day every 6–8 h (IV) 15–40 mg/kg/day in 3–4 divided doses
Doxycycline	100 mg every 12 h	Not generally recommended for < 8 years. In emergency, ≤ 8 years, 2.2 mg/kg twice daily > 8 years and weight > 45 kg: 100 mg twice daily > 8 years and weight < 45 kg: 2.2 mg/kg twice daily
Erythromycin	500 mg orally every 6 h	30–50 mg/kg/day in 4 divided doses
Rifampicin[c]	0.6–1.2 g daily IV or PO in 2–4 divided doses	10–20 mg/kg/day every 12–24 h
Streptomycin[d]	1 g/day IM	20–40 mg/kg/day IM
Tetracycline	500 mg orally every 6 h	Not approved for children
Vancomycin[c]	1 g IV every 12 h	40 mg/kg/day in 2 or 4 divided doses every 6–12 h

PO = peroral; IV = intravenous; IM = intramuscular.

[a] For therapy in life-threatening cases in patients allergic to penicillin, a combination of a fluoroquinolone with two additional agents which penetrate well to the CSF is recommended.

[b] The combination of penicillin G with clindamycin or clarithromycin is suggested for therapy of inhalation anthrax.

[c] The combination of penicillin G with rifampicin or vancomycin is suggested for therapy of anthrax meningoencephalitis.

[d] The combination of penicillin G with streptomycin or other aminoglycosides is suggested for therapy of gastrointestinal anthrax.

with streptomycin may be given in gastrointestinal anthrax, and penicillin combined with clindamycin or clarithromycin may be given in inhalation anthrax. Suggested doses are given in Table 5. As with severe cases in adults, once the patient's temperature returns to normal, consideration may be given to switching to the regimen described for mild cases.

According to Sejvar et al. (2005), in view of the recognized potential of ciprofloxacin to cause arthropathy in children, the United States Working Group on Civilian Biodefense has recommended the use of ciprofloxacin in anthrax-related emergencies in children owing to the severity of the disease.

See section 7.3.1.7 for a discussion of alternatives to penicillin and section 7.3.2.1 for a discussion of postexposure prophylaxis.

7.3.1.4 Adequate doses of penicillin

The possibility that subinhibitory concentrations of penicillins may induce β-lactamase and the importance of using adequate doses of penicillin when this is chosen for treatment is covered in section 7.1.2. This needs to be appreciated in developing country situations, where there may be a temptation to economize.

7.3.1.5 Duration of treatment

The appropriate duration of treatment is a subject for debate. *B. anthracis* cannot be isolated from cutaneous lesions 24–48 hours after commencement of antibiotic therapy (Ellingson et al., 1946) and Kobuch et al. (1990) could see no advantage to continuing treatment of cutaneous anthrax beyond 4 days. A report from Ethiopia (Martin, 1975) records that 100 patients with cutaneous anthrax were treated with a single intramuscular dose of procaine penicillin, 600 000 units, and 99 of these were sent home with the invitation to return if complications occurred. Only 5 returned on account of further developments. Continuation of treatment for 7–14 days or longer has become standard, but frequently it is not appreciated that the lesions, or other toxin-related systemic damage, will continue to progress through their cycles of development and resolution regardless of the elimination of the infecting *B. anthracis*. Excessive antibiotic treatment may be wasteful and counterproductive, possibly giving rise to adverse side-effects. Supportive therapy becomes more important after the first few days. For guidance, it is suggested that the duration of antibiotic therapy in uncomplicated cutaneous anthrax be 3–7 days but, in the absence of clinical experience with short-course antibiotic therapy in systemic anthrax, therapy in cases of systemic anthrax should be continued for 10–14 days.

The same reasoning applies to treatment of alimentary tract cases; after the first 24 to 72 hours of antibiotic treatment, which can be expected to have eliminated viable anthrax bacilli, patient survival becomes dependent on supportive therapy while systemic damage caused by the toxin is overcome.

The topic of postexposure prophylaxis following substantial aerosol exposure in deliberate release events is covered in section 7.3.2.1.

7.3.1.6 Use of more than one antibiotic

The concept of using two antibiotics is not a new one. Forty years ago, Lincoln et al. (1964), following animal studies showing that penicillin together with streptomycin was more effective than either antibiotic alone, recommended that streptomycin should be administered together with penicillin in the treatment of septicaemic anthrax.

Recently, in the aftermath of the anthrax letter events in the USA in 2001, it was felt that the use of more than one antibiotic may have been life-saving in the 6 of 11 individuals who survived inhalational anthrax infection (Inglesby et al., 2002; Jernigan, 2001) and, while acknowledging that controlled studies to support a multidrug approach are not available, multidrug regimens were recommended for cases with signs of systemic involvement. As stated in section 7.1.2, it is certainly reasonable to add a second drug in cases showing signs of systemic involvement, utilizing two antibiotics to which *B. anthracis* is typically sensitive initially, with at least one of these able to penetrate well to the CNS. It may be appropriate to revert to one drug when progression of symptoms ceases, temperature returns to normal and the sensitivity profiles of any isolate have been established. Suggested antibiotic combinations for severe or life-threatening anthrax infections are given in section 7.3.1.2.

7.3.1.7 Alternatives to penicillin

In the event of allergy to penicillin, effective alternatives that are likely to be available in developing countries are tetracyclines and erythromycin (see also section 7.1.1). If ciprofloxacin or doxycycline are available, these are now accepted as best alternatives, although it needs to be remembered that doxycycline penetrates poorly to the CNS (see section 7.3.1.6). For uncomplicated cases, suggested doses are given in Table 5.

In severe, potentially life-threatening cases, ciprofloxacin or doxycycline (also see section 7.3.2.1) should be given intravenously, preferably together with another antibiotic having good CNS penetrability (see section 7.3.1.2) until the patient's clinical condition permits switching to oral therapy. For adults, the recommended dose of intravenous ciprofloxacin is 400 mg every 12 hours and the recommended intravenous dose for doxycycline is 100 mg every 12 hours. The intravenous form of doxycycline is not available in some developing countries.

Ciprofloxacin and doxycycline are normally considered not ideal for children owing to their potential side-effects. Doxycycline is generally not recommended for use in children < 8 years old, owing to staining of teeth and the inhibition of bone growth associated with tetracyclines, and ciprofloxacin has been shown to cause cartilage toxicity in immature animals. However, as discussed in section 7.3.1.3, ciprofloxacin is recommended in anthrax-related emergencies in children owing to the severity of the disease.

The topic of prolonged postexposure prophylaxis for children following substantial aerosol exposure in a deliberate release event is covered in section 7.3.2.1.

7.3.1.8 Pregnancy

There are few reports of anthrax during pregnancy both in humans and animals. Kadanali et al. (2003) found only four human cases reported in the literature; three of these occurred before the availability of antibiotics and all three patients died. The fourth patient, in the Islamic Republic of Iran, went into labour within 48 hours after the appearance of the lesion; she died shortly after admission to hospital from massive oedema of head and neck, which interfered with her breathing. The neonate had no evidence of congenital infection and lived.

All the reports and Kadanali et al.'s own two (cutaneous anthrax) cases were in the last trimester of pregnancy. Kadanali et al. considered it advisable to use penicillin instead of more fetotoxic drugs, such as ciprofloxacin. Treatment was successful in both mothers, though both delivered preterm babies; neither of these showed any evidence of congenital infection. The authors concluded that anthrax during pregnancy could be successfully managed with penicillin, but preterm delivery could be a complication.

Sejvar et al. (2005) support the use of penicillin G (in combination with rifampicin or vancomycin) to treat naturally-occurring anthrax meningitis in pregnant women in endemic countries. As with children (section 7.3.1.3), while recognizing that ciprofloxacin has the potential to cause arthropathy in children, owing to the severity of anthrax meningitis, they state that ciprofloxacin should still be considered for pregnant women under such circumstances.

Published recommendations from the United States Centers for Disease Control and Prevention (CDC) following the anthrax letter events were generally careful to specify that the recommendations were for postexposure prophylaxis for prevention of inhalational anthrax after exposure to intentionally released anthrax spores, and specified the same regimen of ciprofloxacin or doxycycline for pregnant women under such circumstances as for non-pregnant ones (Anon., 2001c). For cutaneous anthrax, Carucci et al. (2002) similarly recommend the same doses of ciprofloxacin or doxycycline for pregnant and non-pregnant women, adding that ciprofloxacin is favoured over doxycycline. Carucci et al. do not state that this is related to bioterrorism, but their recommendation that duration of treatment should be 60 days indicates that it was.

Further discussion of prolonged postexposure prophylaxis for pregnant women and nursing mothers following substantial aerosol exposure in a deliberate release event is given in section 7.3.2.1.

7.3.1.9 Treatment of anthrax meningoencephalitis

Anthrax meningoencephalitis is a life-threatening infection, and mortality is very high. Currently, the first-choice treatment is penicillin G or a fluoroquinolone combined with rifampicin. These have the dual benefits of effective activity against *B. anthracis* and rapid penetration into the CSF. The intravenous form of rifampicin is not yet available in some developing countries. Another choice would be penicillin G or a fluoroquinolone in combination with vancomycin, but vancomycin is costly. For patients allergic to penicillin, a combination of a fluoroquinolone with two additional agents that penetrate well to the CSF, such as a β-lactam (penicillin, ampicillin or meropenem), rifampicin or vancomycin is recommended (Sejvar et al., 2005). A regime of vancomycin, 1 g intravenously every 12 hours, combined with rifampicin (600–1200 mg/day) for 10–14 days is another alternative.

Lessons from the few recorded instances of survival in cases of anthrax meningoencephalitis suggest that the doses of penicillin G should be 20–24 million units/day divided for intravenous administration every 2–4 hours, and a daily dose of rifampicin, 600–1200 mg/day (intravenous administration is suggested but it may also be given via an enteral tube).

The suggestion given in the third edition of these guidelines (1998) that penicillin may be replaced by chloramphenicol for patients who are hypersensitive to penicillin has now been withdrawn. Chloramphenicol has been stated to be a satisfactory alternative but, in early experimental studies, Lincoln et al. (1964) found that chloramphenicol was not effective in mice or monkeys. It is bacteriostatic rather than bactericidal against *B. anthracis*, and some strains may be resistant to it (Athamna et al., 2004). Furthermore, chloramphenicol has the potential for serious side-effects on bone marrow. In vivo data regarding its effectiveness in the treatment

of severe anthrax infections is lacking, and there is a wide availability of more effective alternatives (Sejvar et al., 2005).

Doxycycline should not be used if meningitis is suspected because of its lack of adequate central nervous system penetration (Bell et al., 2002; Sejvar et al., 2005).

It should be stressed that the therapy failure rate is very high in cases of anthrax meningoencephalitis; in 47 cases covered by four reports (Levy et al., 1981; George et al., 1994; Lalitha et al., 1996; Kanungo et al., 2002), there were only two survivors. Delayed suspicion of the true cause of illness, with initial diagnoses ranging from cerebral malaria to subarachnoid haemorrhage, was considered by Kanungo et al. to be responsible in part for the high treatment failure rate.

Supportive therapy is very important in anthrax meningoencephalitis; respiratory support and anti-oedema therapy for the brain may be required. Essential supportive therapy includes the early institution of assisted respiration, fluid and electrolyte supplement and anticerebral oedema measures, such as 100 ml of 20% mannitol intravenously every 8 hours and hydrocortisone, 100 mg every 6 hours. Dexamethasone is generally suggested.

Useful references concerning anthrax meningoencephalitis are given in section 4.4.5.

7.3.1.10 Immuno- or otherwise compromised individuals

Although written with the deliberate release event in mind, recommended therapy for the immunocompromised individual was the same as that for immunocompetent persons in Anon. (2001c) and Carucci et al. (2002). Special consideration may be needed for patients with renal or hepatic insufficiency.

7.3.1.11 Corticosteroids

The swelling seen in an anthrax infection is caused by the action of oedema toxin, and inflammation is fairly minimal. As discussed in section 5.5.3, the EF component of the toxin is anti-inflammatory in nature. Theoretically, therefore, steroids should be of little value. In practice, some reports indicate that these have been administered with evidence of benefit, but others (Kobuch et al., 1990) have concluded that they were ineffective, discontinuing their use. In experimental studies on therapy in rhesus monkeys, Lincoln et al. (1964) concluded that hydrocortisone did not have a statistically significant effect on survival. In section 7.3.1.9, hydrocortisone is suggested as part of supportive treatment in the event of the very dangerous meningoencephalitic complication. In such situations, it is reasonable to give corticosteroids the benefit of the doubt on the basis that any help they may offer is welcome.

7.3.1.12 Surgery in gastrointestinal anthrax

It should be pointed out that some value a surgical approach to management of advanced intestinal anthrax (Binkley et al., 2002; Kanafani et al., 2003). Kanafani et al. propose that the management of cases of gastrointestinal anthrax should consist of:

- initiation of intensive intravenous antibiotic therapy as soon as diagnosis is made;
- in patients not improving with medical therapy, wide resection into seemingly healthy tissue with primary anastomosis;
- continuous drainage of the ascites, as fluid will continue to accumulate for several days after surgery;
- aggressive replacement of protein and electrolyte losses.

7.3.2 Treatment in high-economy (developed) countries

7.3.2.1 Reaction to bioterrorism

Fear resulting from increasing numbers of anthrax hoaxes, especially in the USA, and then the 2001 anthrax letter events, also in the USA, have led to recommended treatment schedules that would frequently not be possible in developing countries.

Postexposure prophylaxis

Normal adults. Fundamentally, these schedules involve prolonged treatment (duration up to 60 days) with ciprofloxacin or doxycycline for "postexposure prophylaxis" where exposure to aerosolized anthrax spores is known to have, or suspected of having occurred (Bell et al., 2002). This is based on the understanding of the possible persistence of inhaled spores in the lungs resulting from demonstrations that anthrax spores may remain lodged for many weeks in the lungs of monkeys exposed by the aerosol route and kept on antibiotics, and that the animals succumb to the disease once the antibiotic treatment is stopped (Henderson et al., 1956; Friedlander et al., 1993). It became accepted wisdom that, in cases where known or suspected inhalation

of anthrax spores had taken place, especially if this was likely to have been substantial, simultaneous administration of vaccine and antibiotic treatment should be considered, with the treatment continued for about 6 weeks to allow for development of adequate vaccine-induced immunity.

At the time of the 2001 anthrax letter events in the USA, the situation was complicated by lack of availability of vaccine, but as this became available, potentially exposed persons were offered extended antibiotic treatment with or without three doses of anthrax vaccine through an investigational new drug (IND) protocol (Shepard et al., 2002). In theory, it remains logical to consider simultaneous administration of a non-live vaccine and antimicrobial therapy where there is strong evidence that inhalation of substantial numbers of anthrax spores has taken place, with the antibiotic treatment being continued for about 6 weeks to allow for development of adequate vaccine-induced immunity. In practice, these vaccines are only available on a restricted basis in the United Kingdom and the United States (see section 7.1.4 and Annex 5, section 3).

The approach would have to be somewhat different in the case of the live human vaccines in China and the Russian Federation (Pomerantsev et al., 1996; Stepanov, Mikshis & Bolotnikova, 1996). The situation here is analogous to the livestock vaccine (see section 7.2.1.3).

In the USA, in response to biopreparedness initiatives, ciprofloxacin and doxycycline were approved by the Food and Drug Administration (FDA) in 2000 and 2001, respectively, for use in antimicrobial prophylaxis against anthrax (Shepard et al., 2002).

Children, nursing mothers, pregnancy. This subsection should be read in conjunction with sections 7.3.1.3, 7.3.1.7, 7.3.1.8 and 7.3.1.10.

Initially following the anthrax letter events of 2001 in the USA, the recommendation made (Anon., 2001c) was that, for infants and children (age not clearly defined), amoxicillin may be used for the 60-day prophylaxis proposed in the event of inhalation of spores following a deliberate release incident, when the incriminated *B. anthracis* was determined to be susceptible to penicillin, but that initial treatment of infants and children with inhalational or systemic anthrax should consist of intravenous ciprofloxacin or doxycycline plus one or two additional antimicrobial agents. Subsequently, because of safety concerns in relation to ciprofloxacin and doxycycline (see section 7.3.1.7), amoxicillin (in three daily doses) was to be offered to children and nursing mothers for postexposure prophylaxis, although this was not an FDA-approved protocol (Bell et al., 2002; Shepard et al., 2002).

After the anthrax letter events, the Committee on Obstetric Practice of the American College of Obstetricians and Gynecologists (ACOG) issued an opinion paper on the management of (asymptomatic) pregnant or lactating women who had been exposed to anthrax spores (ACOG, 2002). Confining treatment to those for whom a high risk of exposure had been confirmed, prophylaxis would be 500 mg of ciprofloxacin every 12 hours for 60 days. Women taking ciprofloxacin when they discover they are pregnant should continue the course for 60 days, but once the bacteria are confirmed as penicillin-sensitive, the patient should be switched to amoxicillin, 500 mg orally every 8 hours for 60 days. Doxycycline should be used in the event of allergy to penicillin and ciprofloxacin, although penicillin desensitization should be considered.

Adherence and adverse events with prolonged therapy

Adherence rates and records of adverse events in prolonged antibiotic therapy have now been analysed (Jefferds et al., 2002; Shepard et al., 2002; Williams et al., 2002).

More than 2000 workers in the Washington D.C. Processing and Distribution Center of the United States Postal Service were advised to complete 60 days of postexposure prophylaxis following the anthrax letter events. Of 245 workers surveyed, 98 (40%) reported full adherence, 45 (18%) discontinued and never restarted, and 102 (42%) were classified as intermediate (Jefferds et al., 2002). Adverse effects and concern over possible long-term adverse effects were cited as major influencing factors for discontinuing prophylaxis. It proved impossible, however, to distinguish the effects of stress from adverse effects of the antibiotics in many cases. Adherence was heavily dependent on frequent visits by public health staff.

In a survey of 100 individuals from a similar group of 1122 potentially exposed persons in Connecticut offered long-term antibiotic prophylaxis, 94 acquired antibiotics but only 68 actually started taking them; of these 21 discontinued early (Williams et al., 2002).

As a result of the literature on treatment associated with the anthrax letter events, 85 individuals who came into contact with infected carcasses during the large wildlife outbreak in the Malilangwe Trust area (Zimbabwe) in August to November 2004, were put on what at the outset was to be a 60-day course of doxycycline. This was discontinued after 2 weeks in all but those who were exposed on a daily basis to infected carcasses; they continued to take it for approximately 35 days. A few of these individuals suffered side-effects which included moderate to severe sunburn depending on fairness of skin, and three persons suffered blackening and lifting of nails exposed to the sun, as well as loss of appetite; administration of the antibiotic was discontinued in these persons (Clegg et al., 2006b).

On a broader basis, Shepard et al. (2002) recorded the overall adherence as "poor" (44%), ranging from 21% of persons regarded as potentially exposed in the New York Morgan postal facility to 64% in the Washington Brentwood facility. A total of approximately 10 000 persons across the eastern USA were offered > 60 days of postexposure antimicrobial prophylaxis. The rate of serious adverse effects was low, with no deaths, although mild adverse events that did not fulfil criteria as serious were common. Some of these were attributed to above-average symptom awareness and anxiety.

None of these papers made recommendations for the future, nor was simultaneous vaccination discussed.

7.3.2.2 Treatment for naturally acquired anthrax in high-economy countries

The issue of treatment of occasional cases of naturally acquired anthrax that still occur periodically in developed countries has not been raised in the vast amount of recent literature resulting from the bioterrorist threats and events of recent years. Unless there is reason to believe that the infection represents possible exposure to substantial numbers of inhaled spores, it is suggested that simple regimens of limited duration be adhered to, but possibly utilizing two antibiotics to which *B. anthracis* is typically sensitive initially, reverting to one when progression of oedema ceases in the case of uncomplicated cutaneous anthrax, or when the temperature has returned to normal in systemic cases, and the sensitivity profiles of any isolate have been established (sections 7.3.1.5, 7.3.1.6).

7.3.2.3 Antibiotic prophylaxis in natural anthrax scenarios

Prolonged antibiotic prophylaxis is only a recommendation for persons known to have been, or strongly suspected of having been, exposed to very substantial doses of aerosolized spores in a deliberate release scenario. Antibiotics should not be administered in that way for other situations. Antibiotics should only be used for treatment, not prophylaxis, unless there is a real danger of a very substantial exposure having taken place. This is almost certainly not the case in any natural exposure scenario (as opposed to a human-made situation). Where sufficient fear of a substantial exposure in a natural situation exists (e.g. consumption of meat from a poorly cooked anthrax carcass), antibiotic prophylaxis may be considered but should only be of ± 10 day duration. In other suspected natural exposure situations, the relevant medical personnel should be notified, and the individual(s) concerned should report to them immediately for treatment should a spot/pimple/boil-like lesion develop, especially on exposed areas, or flulike symptoms appear.

Where possible exposure is anticipated, but has not yet happened (e.g. preparing to dispose of carcasses in an outbreak), use of proper personal protection methods (Annex 1, section 7.1.2) is the correct approach, not antibiotic prophylaxis. Again, however, the persons concerned should consult their medical practitioner without delay should any unusual lesion or flulike illness develop within 3 or 4 weeks of the exposure.

7.3.3 Vaccination for humans

See sections 7.1.4, 8.6.3, 8.6.4.1 and Annex 5, section 3.

7.3.4 Immunotherapy (hyperimmune serum therapy)

The use of serum therapy for treatment of anthrax predates by several decades the realization that death was caused by toxin action. In 1930, Eurich & Hewlett wrote that "as regards malignant pustule, serum treatment is now the method of choice, taking the place of excision".

A couple of anecdotes concerning Professor Eurich that appeared in the *Sunday Telegraph* (United Kingdom) in June 1992 may be of interest. The first states: "Professor F.W. Eurich, a German immigrant, settled in Bradford and devoted much of his life to seeking a cure for anthrax, or woolsorter's disease,

so prevalent in the city at the turn of the century. … He liked to recall how at Heidelberg he had watched one student challenge another to a duel at dinner with the accusation: you have been staring at my sausage". In another, a letter, Mrs E.G. Croisdale, Hereford, United Kingdom, wrote: "My late father, J.W. Hillas, was one of Professor Eurich's patients to be cured of anthrax in the early years of this century. … My father owed his life to Professor Eurich … thus I owe my life to this great man".

At the time Eurich was working, antisera were developed in asses, sheep and oxen (horses are not named) and standardized by a protection test in rats. Doses and routes varied from 30–40 ml daily by the subcutaneous route, or occasionally intravenously, to single intravenous doses of 100 ml. Efficacies were widely acclaimed. Gold (1955) refers to treatment (apparently in the 1930s) of 21 cases with "an optimum dose [of anti-anthrax serum] that varied from 250 to > 1000 ml given intravenously. This therapy, although effective, resulted in prolonged morbidity from the severe horse sensitivity reactions that ensued". According to Lincoln et al. (1964), "the cures effected by antiserum alone in the 1920s and 1930s appear to be more numerous than those following use of modern antibiotics in the 1940s and 1950s". In the former Soviet Union, recommendations on treatment of any type of anthrax infection put primary emphasis on use of antiserum (Shlyakov, 1957, cited by Klein et al., 1962). Klein et al. (1962) appreciated that the role of antiserum was to neutralize the toxin. Lincoln et al. (1964) were demonstrating the greater efficacy of a combination of antibiotics, antiserum and active immunization over any one of these approaches alone in the treatment of late-stage inhalational anthrax in rhesus monkeys.

Despite the history, the use of anti-anthrax serum for treatment of human anthrax appears to have been abandoned by about 1950 in most western countries. However, purified immunoglobulin fractions from hyperimmune horse serum are still prepared for human treatment in China (Dong, 1990; Dong, personal communication, 2002) and the Russian Federation (Anon., 1996; Cherkasskiy, personal communication, 2002) (see Annex 5, section 4). According to Cherkasskiy (personal communication, 2002), this treatment is administered to approximately 2–3 persons each year in the Russian Federation.

The topic was revisited scientifically by Little et al. (1997), who found in protection tests in guinea-pigs that only antibodies to the PA component of the anthrax toxin (section 5.5.3) provided passive protection against live spore challenge. Antibodies to the lethal and oedema factor components did not. One monoclonal antibody to PA of several monoclonal antibodies tested provided a delay in time to death.

Following the anthrax letter events in the USA in 2001, a call was made for a biological defence initiative based on developing, producing and stockpiling specific antibody reagents, for use in protection against biological warfare threats including anthrax (Casadevall, 2002). Certainly one response to this has been the development of clinical-grade hyperimmunoglobulin from donor persons immunized with the United States anthrax vaccine.[1] At the research level, reports have appeared on toxin-neutralizing Fabs from human donors vaccinated against anthrax (Wild et al., 2003), human monoclonal anti-PA antibodies (Maynard et al., 2002; Sawada-Hirai et al., 2004; Cui et al., 2005; Mohamed et al., 2005; Peterson et al., 2006) and murine anti-LF monoclonal antibodies (Lim et al., 2005). The protective effects in all but the tests of Mohamed et al. (2005) and Peterson et al. (2006) were based on antitoxic activities. In live spore challenge tests, Mohamed et al. (2005) and Peterson et al. (2006) obtained good protection in rabbits when they were combined with low doses of ciprofloxacin, and also in mice and guinea-pigs. Mohamed et al. (2005) found their antibody still protected 50% of animals when administered 36 hours after challenge, In Peterson et al.'s case, the antibody had to be administered within 12 hours of challenge to be highly effective. Kozel et al. (2004) targeted their monoclonal antibodies to capsular antigen, obtaining a high degree of protection in mice against virulent *B. anthracis* infection in a pulmonary model.

The value of these types of antibodies for amelioration of infection once symptoms have commenced has yet to be demonstrated. Nevertheless, they remain important for potential situations in which the infecting agent has been engineered to be multiresistant to antibiotics.

7.3.4.1 Novel immune and other therapies

Antibiotics are only effective against anthrax if administered early enough in the course of the infection (section 7.1.1). After a certain point, enough toxin has been formed to cause death of the host even if

[1] www.cangene.co/products-hyperimmunes.htm.

the antibiotic treatment has killed all the *B. anthracis*. Among other aftermaths of the 2001 anthrax letter events in the USA have been some intense efforts to develop therapies that will continue to be effective after antibiotics can offer no hope. Among the approaches being taken are therapies targeting:

- one or more of the points of interaction between the toxin components and the host cell, such as the host-cell receptors, or the receptors on PA63 for LF and EF (see 5.5.3 and Fig. 7);
- the toxin-induced events within the host cell;
- other virulence factor entities.

Probably among the most advanced in their development are humanized monoclonal antibodies targeting toxin-component interactions. For example, the toxin-neutralizing antibodies mentioned by Maynard et al. (2002), referred to in 7.3.4, operated by competing with the host-cell receptor for PA binding. Similarly, the Fab fragments developed by Wild et al. (2003) functioned by binding in a manner that inhibited the interaction of LF with PA. EluSys Therapeutics[1] reports on the Web the development of a heteropolymer system using two monoclonal antibodies chemically joined together; one binds to anthrax toxin and the other to red blood cells which then carry the bound toxin to the liver for destruction. Human Genome Sciences, Inc.[2] also has a product well into its development stage based on human or humanized neutralizing anti-PA monoclonal antibodies. A non-immunologically based approach is illustrated in the report of Sarac et al. (2004) that furin inhibitor, hexa-D-arginine, might be an effective therapeutic agent by preventing cleavage of PA to its active form.

An example of therapy targeting toxin-induced events within the host cell is the observation by Shen et al. (2004) that adefovir diphosphate, a drug approved to treat chronic hepatitis B virus infection, inhibits the adenylyl cyclase activity of EF and thereby EF-induced cAMP accumulation (see section 5.5.3).

Illustrating other virulence factor entities are the reports of Gold et al. (2004) suggesting that interferon may have a benefit as an immunoadjuvant therapy and of Kozel et al. (2004) on their monoclonal antibodies to capsular antigen (section 7.3.4). An intriguing suggestion for novel therapy was the proposed use of gamma phage lysin by Schuch et al. (2002) that lysed *B. anthracis* cells from without in in vitro tests, and protected mice against infection with a phage-sensitive *B. cereus* used as a simulant for *B. anthracis*. Success in this case for real anthrax treatment would depend on the lysin's ability to penetrate the *B. anthracis* capsule in genuine anthrax infections. Direct phage therapy, an idea that has been considered from time to time in the past for anthrax, has to overcome the fact that the phage does not attack capsulated *B. anthracis* cells (section 6.3.1.5).[3]

7.3.5 Recurrence after treatment; second infections

Recurrence of disease on termination of treatment is very rare, but convalescent cases should remain under observation for at least a week after treatment has been discontinued.

Equally rare are reports of second infections, although Martin (1975) states: "Reinfection of the skin was seen not infrequently at Rassa (Ethiopia), and the second lesion was usually noted to be less severe than the intial one. This accords with the observations of Sinderson (1933) and Hodgson (1941)". Hodgson's case was a veterinarian who was infected on three occasions. Shlyakhov (1996) records observing three cases of a second cutaneous anthrax infection occurring respectively 8, 15 and 20 years after the first attack. Two of the individuals were veterinarians and, in one of them, the carbuncle was located in the same place on the arm as 15 years previously.

[1] Pine Brook , New Jersey, USA.
[2] Maryland, USA.
[3] Developments in this field may be followed by consulting the USDA site: http://www.fda.gov/cber/summaries.htm.

8. Control

8.1 Introduction

This section should be read in conjunction with Annexes 3, 5 & 6.

Control measures are aimed at breaking the cycle of infection depicted in Fig. 9. Each of the following actions must be rigorously implemented:

- Cut off infection source.
- Dispose of anthrax carcasses correctly (point X).
- Correctly disinfect, decontaminate and dispose of contaminated materials (point Y).
- Vaccinate exposed susceptible animals (point Z1) and, where possible, humans in at-risk occupations (point Z2).

In addition, in some outbreak circumstances in relevant countries, it may be appropriate to consider fly-control measures (point A).

8.2 Discontinuation of infection source

In outbreaks having a defined infection source, clearly discontinuing this source is an essential first step to breaking the cycle of infection (see point Y in Fig. 9). If the infection can be traced to feeding, for example, the feed source should be immediately withdrawn from the index farm and from all others that received it and destroyed. Moving other animals away from the affected area is an important early action. If flies are suspected of being important vectors, fly control should be considered. Detailed practical actions are given in Annex 6.

8.3 Disposal of anthrax (animal) carcasses

See point X in Fig. 9. Please refer also to the OIE Code for general guidance for the disposal of dead animals.[1]

8.3.1 Principles involved

Because sporulation of *B. anthracis* requires oxygen and therefore does not occur inside a closed carcass, regulations in most countries forbid postmortem examination of animals when anthrax is suspected. Most, if not all the vegetative *B. anthracis* cells in the carcass are killed in a few days by putrefactive processes. Nevertheless, with the characteristic (though not invariable) terminal serosanguinous exudates from the nose, mouth and anus, contamination of the environment around an anthrax carcass can still be expected. The precise length of time after which no viable *B. anthracis* remain within a carcass is unpredictable but depends greatly on climatic conditions, particularly temperature.

8.3.2 Alternatives

8.3.2.1 Livestock

In most countries, the preferred method of disposal of an anthrax carcass is incineration. Controlled heat treatment or "rendering" has been proposed in at least one country of the European Union, but no records were found of this having been done, or of relevant legislative documentation. Where neither of these approaches is possible, for example owing to lack of fuel, burial is the remaining less satisfactory alternative. As discussed in section 3.2, history has many examples of new outbreaks following disturbance of old burial sites.

Consideration might be given to treating anthrax carcasses with 10% formalin, leaving them in situ for some days before disposal while natural putrefaction processes within the carcass kill the vegetative anthrax organisms. The formalin would have the action of killing anthrax organisms shed by the dead animal, preserving the skin so that it retains the anaerobic environment within the putrefying carcass. It may also deter scavengers that would otherwise open up the carcass and thereby increase the contamination, and flies that might spread the

[1] *General guidance for the disposal of dead animals*. In: Terrestrial Animal Health Code, Appendix 3.6.6. Paris, World Organisation for Animal Health (OIE), 2007 (http://www.oie.int/eng/normes/mcode/en_chapitre_3.6.6.htm).

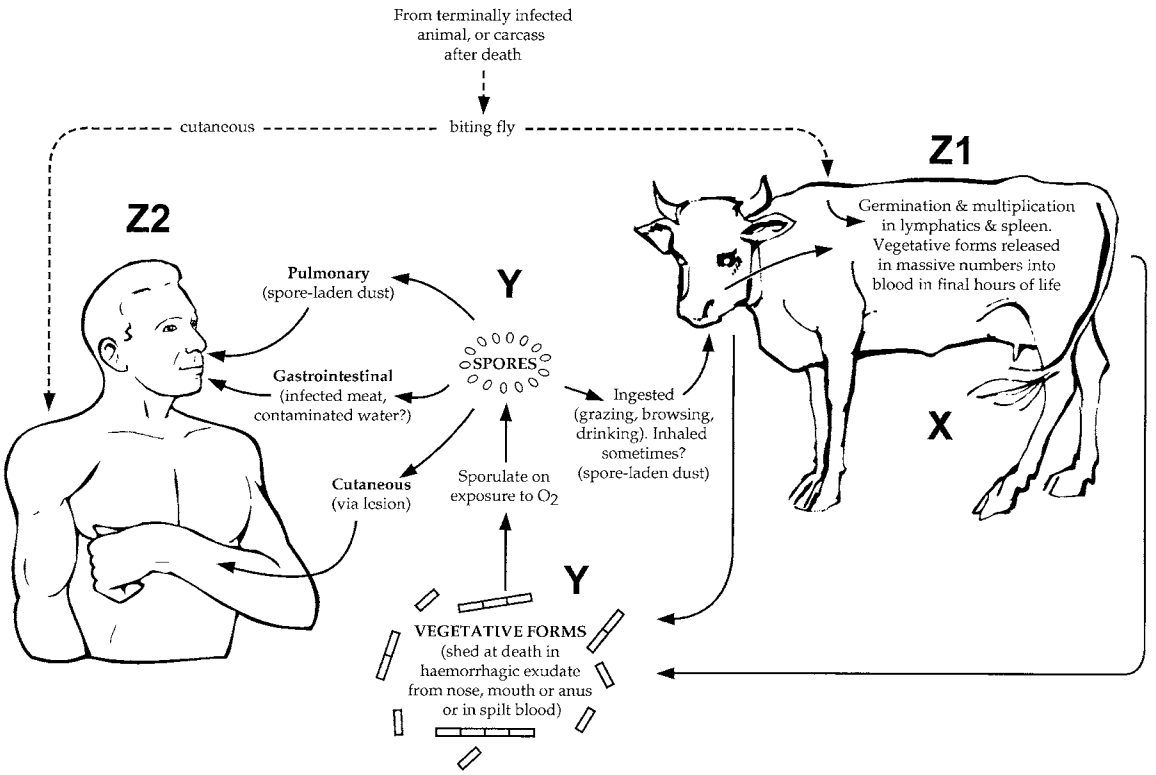


Fig. 9 Breaking the cycle of infection[a]

[a] See text and Fig.1.

disease. However, it was the experience in the major outbreak in wildlife in Zimbabwe (Clegg et al., 2006a, 2006b), that formalin treatment of carcasses per se did not deter scavengers or flies (Clegg & Wenham, personal communication, 2005).

The procedure adopted in the large livestock outbreak in Victoria (Australia) in 1997 was to burn carcasses at the death site or as near as possible to that site. External spore contamination was minimized by spraying with 5% formaldehyde. Where incineration could not be done at the death site and transportation to another site for incineration was necessary, this was done by loading the formalin-sprayed carcass onto double-thickness plastic on a low-loading trailer, and wrapping it in the plastic (Turner, personal communication, 2003).

In some developing country situations where burial, incineration or rendering is not feasible, the last resort may be to leave the carcass unmoved in situ and ensure that it is inaccessible to other animals, particularly scavengers, or people. This is achieved by covering with tarpaulins, branches of trees, corrugated iron or other available materials. Hazard signs should be posted around sites in this case. This again allows the putrefactive process to take effect, although residual environmental contamination may still remain, and either the site should be scorched after putrefaction is complete or it should be treated with 10% formalin. Alternatively, it should be made inaccessible to other animals indefinitely by fencing, capping with concrete or other impervious material, covering with brushwood, or growing impenetrable undergrowth.

Hugh-Jones (personal communication, 2005 reports that in Argentina, carcasses are first covered with lime (see section 8.3.3), then a tarpaulin, and left for 9 months before the bones are collected. This is said to stop scavenging and is cheaper than burying.

8.3.2.2 Large outbreaks in wildlife

Suggested practical actions in the event of an anthrax outbreak in wildlife are given in Annex 6, section 5.

In national parks from which livestock are excluded and which have "hands-off" management policies for all but emergency situations, control actions may be regarded as interference with natural processes. This is discussed in sections 8.9 and 8.10. Frequently, carcasses of animals that have died of anthrax on a sporadic basis will only be seen some time after death, if they are seen at all, and they will have been opened up by scavengers. It is likely in enzootic areas that for every one seen by park officials, many are not seen. Consequently, it is impractical to attempt to rigidly enforce "burn or bury" action plans. It can also be counterproductive (i) environmentally, through depletion of scarce wood for burning or from pollution if fuel such as diesel fuel is used, and (ii) by taking underresourced human time from more valuable activities.

Where action is seen to be necessary, all carcasses are again best burnt but, if this is impractical, they should be buried. In the case of burial (and possibly burning also) consideration may be given to spraying the carcasses and surrounding ground with 10% formalin to minimize the number of spores which may survive and resurface, causing cases again at some point in the future (section 3.2).

8.3.3 Burial

Periodic reports of viable anthrax spores at burial sites of animals that died many years previously, and incidents and outbreaks in animals associated with such sites, have testified to the unreliability of burial procedures for long-term control of the disease (section 3.2). Disturbance of such sites, for example by ploughing or laying drainage, presumably brings the spores to the surface. Even without site disturbance, spores can work their way up to the soil surface. In either case, this may result in new livestock cases as shown by Turnbull et al. (1996). Further disadvantages of burial sites are that scavengers may dig down to reach the carcass, and in dry dusty areas, the digging process can spread the contaminated soil extensively. In Wood Buffalo National Park, Canada, where burials of bison that had died of anthrax were carried out in the 1960s, the raised burial mounds became excavation sites for foxes and wolves building their dens and nesting sites for ants (Dragon, personal communication, 1997). In Africa as an example, it is hard to stop dogs from digging up buried anthrax (cattle) carcasses.

The origins of the common recommendation to bury with lime appear to be lost to history. In theory heat would be generated on contact with body fluids, and that would thereby be expected to hasten decomposition of the carcass. It may have been applied originally to deny corpses (human or animal) to relatives/owners after death where that was officially needed (Hugh-Jones, personal communication, 2004). It is now uncertain just what lime does to buried carcasses, whether it accelerates their disintegration or actually preserves them. Lindner & Böhm (1985) believed that at least as far as lime treatment of tannery effluent is concerned, this probably followed the use of lime as a cheap chemical for disinfecting sewage sludge. However, they showed that at 20 kg/m^3, lime failed to inactivate anthrax spores in sewage sludge. In raising the pH of the soil, addition of lime when burying anthrax carcasses may actually be counterproductive to minimizing long-term spore contamination (section 2.1.2.4).

In summary, burial should be discouraged in favour of incineration.

8.3.4 Incineration

Guidelines on incineration procedures are given in Annex 3, section 7. Ideally, the soil surrounding and under the carcass, particularly around the nasal and anal regions, should be decontaminated and then incinerated with the carcass.

Incineration must be carried out with appropriate care to ensure complete burning from beneath. Usually this involves raising the carcass off the ground before the process is started. Mobile commercial incinerators designed to ensure this are available (see Figs 10A–D). It must be appreciated that spores that have soaked into the soil may survive the incineration process, although isolation of *B. anthracis* from incineration sites is rare. The down-directed blow-torch shown in Figs 10E& F illustrates an alternative incineration procedure that ensures severe scorching of the soil to several centimetres of depth (see also Annex 3, section 7).

Comments are occasionally encountered opposing incineration on the basis that anthrax spores may survive the fire and become aerosolized in the updraft. In general, circumstantial evidence does not

support the contention that incineration of anthrax carcasses results in the transmission of anthrax in this manner, and the rapid dilution effect on any spores that may become airborne in viable state reduce the chances of these causing an infection to next to nil. The generally high infectious doses for anthrax by routes other than through a lesion even in the more susceptible species supports this contention (see sections 3.1, 3.3.4, 4.2.1). Nevertheless, suspicion that airborne transmission from carcass incineration occurs has arisen. Henton & Briers (1998) described an outbreak of anthrax in the summer of 1995–1996 in the Kimberley district of South Africa affecting goats, cattle, sheep, horses, roan antelope, gemsbok, laldu and springbok. The outbreak developed by spreading from the initial focus in a south-westerly direction over a distance of 270 km (40–50 km wide). They considered the distances involved too great for the spread to be accounted for in terms of insect transmission, and believed it to result from windborne spores emanating from incineration sites. In support of this possibility, they demonstrated that *B. anthracis* could be isolated in low numbers from cotton wool held in the smoke above three carcasses and from the face mask of one of the veterinary officers assisting in the incinerations.

Blenkharn & Oakland (1989) were able to isolate Gram-positive bacteria, predominantly *Bacillus* species, from the base of the exhaust stack of a hospital waste incinerator with design-specified operating temperatures of 800 °C and 1000 °C in the primary and secondary chambers respectively, thereby demonstrating that there is no room for complacency. However, numbers were very low, averaging 56 cfu per cubic metre (range 0–400 cfu per cubic metre, i.e. < 1 cfu per litre) and would be subject to rapid further dilution on leaving the chimney. A badly constructed pyre producing smoke with little or no flame might result in a higher survival rate of organisms collected by the updraft.

An additional consideration is that anthrax organisms in an unopened carcass are in the vegetative form and are readily susceptible to heat and other adverse conditions. The spores will be confined to where the blood has been shed through the body orifices and will mostly be in the soil beneath these points. Relatively few spore forms, therefore, will enter the fire and updraft; vegetative forms will almost certainly not survive. If concern persists, consideration might be given to pretreating the carcass and associated contaminated soil with 10% formalin a few hours before incineration to minimize the number of viable spores present (section 8.3.2.1; Annex 6, section 1).

8.3.5 Rendering

Rendering is essentially a cooking process that results in sterilization of raw materials of animal origin such that parts of carcasses may be utilized safely for subsequent commercial purposes.

There are a number of variations of the rendering process, broadly divided into batch processes and continuous processes. In general, the raw materials are finely chopped and then passed into a steam–heated chamber and subjected to temperatures ranging from 100 °C to 150 °C for 10–60 minutes (this does not include the time taken to bring the material to the peak temperature or the subsequent cooling period time).

The rendering procedure involves correct performance at each of three stages: collection, transport and treatment of the carcass (Riedinger et al., 1975; Riedinger, 1980; Strauch, 1991). These should be supervised by veterinary authorities. The carcass should be bagged and the bag, collection machinery, materials and tools, and the carcass site itself appropriately decontaminated and disinfected. The rendering plant must be properly divided into "dirty" and "clean" areas, which must not be connected via a common drain to avoid possible cross-contamination by back-flow. The dirty side must be suitably equipped for disinfection of the transport vehicles and other equipment involved. Wastewater from the dirty side must be collected and treated by heat or chemicals (preferably heat) to destroy the spores. Before the heat treatment, carcasses should be broken down into pieces not larger than 10 cm^3. In the case of anthrax carcasses, this should be done with very careful attention to hygiene during the process, with the necessary disinfection and decontamination of the rendering premises, tools, clothing, waste run-off, etc.

Controlled heat treatment is then carried out with temperature, pressure and time of sterilization recorded.

As well as careful hygiene control on the dirty side of the rendering plant, the level of hygiene being maintained on the clean side should also be monitored at least twice yearly by the veterinary authorities.

8.4 Human cases: infection control in management

The risk of human–to–human transmission is not a serious one given that sensible precautions are taken (section 4.3.2). For example, cutaneous anthrax lesions should be dressed for the first 24–48 hours after treatment; disposable gloves should be worn, or gloves that can be sterilized, while applying the dressing and during subsequent disposal of specimens or sterilization of materials and equipment (see Annex 3, sections 3 & 4).

Prophylactic antibiotics and vaccination are not necessary for health workers or family contacts, although these individuals should know that their medical practitioner should be consulted if suspicious sores or illness develop that might have arisen from their contact.

In fatal cases, postmortem examinations should be discouraged; cremation is preferable to burial where local custom permits. It is advisable for the body to be placed in an impervious body bag for transport from the place of death, and preferably the body should not be extracted from the bag. Where only burial is permitted, the bagged body should be placed in a hermetically sealed coffin and buried without reopening. Useful guidelines are available elsewhere (Healing et al., 1995; Young & Healing, 1995). Bedding and contaminated materials should be bagged and incinerated, autoclaved or fumigated as appropriate. Whether room fumigation is necessary will depend on the perceived level of contamination in the room where the patient died (see Annex 3, sections 3.1–3.3).

8.5 Fumigation, disinfection, decontamination

See point Y in Fig. 9.

8.5.1 Principles

In addition to helping break the cycle of anthrax infection locally, disinfection, decontamination and correct disposal of infected/contaminated material are of considerable importance in preventing long-distance and international transmission of anthrax. In non-endemic countries, risks arise largely from animal products – wool, hair, hides, bone, etc. – imported from endemic regions. Regulations regarding importation of untreated animal products vary from country to country, but several importing countries take the view that the financial costs that would be incurred from legislating for the sterilization of such imports would be disproportionate to the benefits.

In many countries, some or all of the following requirements are in place to limit the risks of importation or dissemination of products contaminated with anthrax spores:

- Products must be accompanied by a certificate signed by a veterinary official in the country of export certifying that they derive from anthrax-free sources (see Annex 4).
- Products regarded as having a high chance of containing anthrax spores are subject to some form of monitoring or control.
- Finished or raw materials from certain countries may be subject to import restrictions, such as treatment before export.
- Finished or raw materials from certain countries may have to undergo a general sporicidal treatment after arrival and before processing or distribution.

In the case of hides and skins (where a sterilization procedure that does not damage the materials has never been devised), the exporting countries nowadays often require the initial processing stages to be carried out before export for financial benefit. This has contributed to a reduction in anthrax-contaminated hides and skins now reaching non-endemic countries as compared with a few years ago, although on the other hand, exporting countries have experienced increased incidence of the disease downstream from the preprocessing tanneries through lack of adequate control programmes (Hugh-Jones, personal communication, 2004).

Practical approaches to disinfection and decontamination of animal products are given in Annex 3, section 6. However, it is stressed that long-term global control depends almost entirely on the application of appropriate measures to prevent the disease among livestock in enzootic exporting countries. Ideally, national policies should ensure that materials known to be contaminated with anthrax spores are appropriately disposed of and are not included in any industrial process.

8.6 Prophylaxis

8.6.1 The nature of protection in anthrax

Four decades of research have resulted in the perception that protection against anthrax in the sus-

ceptible host depends almost entirely on the host's immune response to a single antigen – the protective antigen (PA) component of the anthrax toxin (Ivins & Welkos, 1988; Turnbull et al., 1988; Turnbull, 1991; Friedlander et al., 2002; see section 5.5.3), and the design of putative novel vaccines has been based on this. Immune responses to the other two toxin components, the lethal and oedema factors, may contribute to or enhance protection. One group has proposed that at least the lethal factor component of the anthrax toxin has an important role to play (Price et al., 2001). In contrast, another group (Cohen et al., 2000) obtained protection without the development of measurable anti-PA antibodies and suggested that other, spore-associated antigens may contribute in a significant manner to protective immunity. As yet, no other such antigens have been identified, although alternative possible candidate vaccine antigens continue to be sought, such as S-layer proteins (Fouet et al., 1999; Mesnage et al., 1999; see section 5.5.5) and the collagen-like surface glycoprotein BclA, a major antigen of the exosporium (Sylvestre et al., 2003; Steichen et al., 2003). The possibility that the capsule may be capable of enhancing protective efficacy has also been mooted (section 5.5.1).

Although it has long been believed that the effectiveness of both animal and human vaccines depends on the induction of anti-PA antibodies, it has also been recognized for some years that measurable anti-PA antibodies in the blood of an individual are not, in themselves, a guarantee of protected status although, the theory continues, they must be there for the individual to be protected (Ivins & Welkos, 1988; Turnbull et al., 1988; Ivins et al., 1990a, 1990b; Turnbull et al., 1990b). Certainly the immune response is complex; the recently-acquired knowledge that the macrophage is central to lethality in anthrax (see section 5.4.1) and evidence that Th1 and Th2 responses may be involved in protective efficacy (McBride et al., 1998) – possibly to different extents in different species – indicate that cellular immunity has a role in protection, although this is undefined at present. The enhanced efficacy of future vaccines over existing ones may depend on improved stimulation of the cellular immune response in parallel with the humoral response to PA (see section 8.6.4).

The mechanism whereby an immune process based on antitoxin immunity protects against a disease characterized by rapid in vivo growth of the bacilli is not known yet. Neutralization of the effect of the toxin on macrophages may be a major factor. It is also possible that some action of the toxin that results in the release of nutrients needed for multiplication is inhibited. PA appears to be expressed very early in the germination of spores and is rapidly released. Antibodies to PA may have a role in retarding germination or enhancing spore clearance by phagocytic cells (Welkos et al., 2002; Cote et al., 2005).

8.6.2 Animal vaccines

Gochenour et al. (1935) summarize the situation at the end of the first third of the 19th century:

> "Undoubtedly, Pasteur's ability to protect animals against anthrax by vaccination was heralded at the time as a sure means of preventing that dreaded disease of livestock. The vaccines subsequently prepared by Pasteur did much to control the disease. Experience has shown, however, that the Pasteur vaccine had definite limitations. … Accordingly, numerous investigators undertook the task of developing anthrax biologics … as a result of [which] a number of products have been developed for the control of anthrax. For the immunization of animals against anthrax, the veterinarian has, therefore, a number of biologics at his command, namely, anti-anthrax serum, anti-anthrax serum and anthrax-spore vaccine used simultaneously, anthrax-spore vaccine (single injection), anthrax-spore vaccine (intradermic), anthrax-spore vaccines (2, 3, or 4 injection), anthrax-spore vaccine in saponin solution, anthrax aggressin, and two kinds of killed-organism anthrax bacterins, one being a whole-culture anthrax bacterin, and the other a washed-culture bacterin."

Most anthrax vaccines for animals in use around the world today utilize the toxigenic, non-capsulating (pXO1+/pXO2–) *B. anthracis* strain $34F_2$ derived from a virulent bovine isolate in the 1930s (Sterne, 1937a, 1937b; Alper, 1996). The Pasteur-type vaccines (pXO1-/2+), which carried a ≥ 3% mortality risk, have now been abandoned in almost all countries. The animal vaccines that use strain $34F_2$ are essentially as originally formulated (Sterne, 1939) with approximately 10^7 spores per ml suspended in 0.5% saponin in 50% glycerine-saline (Annex 5, Table 18).

Animal vaccines against anthrax should be prepared in accordance with the *Requirements for anthrax spore vaccine (live – for veterinary use), Requirements for biological substances No. 13* (WHO, 1967), the *Manual for the production of anthrax and blackleg vaccines* (FAO, 1991) and the *Manual of diagnostic tests and vaccines for terrestrial animals* (OIE, 2008).

The WHO *Requirements for anthrax spore vaccine (live – for veterinary use)* points out the considerable differences in quality that may exist between anthrax vaccines. Glycerine and saponin are important to vaccine performance. Vaccine strains should be maintained carefully since unencapsulated *B. anthracis* variants may lose their immunogenic powers on subculture (Sterne et al., 1939; Sterne, 1959). Since, in theory, the vaccine is easy to prepare, many countries undertake their own manufacture (Annex 5, section 2.13). However, it is important that careful quality control be exercised to avoid resurgence of anthrax because inadequate vaccines are being used.

The protective effect of a single dose of strain $34F_2$ vaccine is said to last about one year (Sterne, 1939), and annual boosters are recommended for livestock in endemic areas. The duration of the protection has never been systematically studied in livestock or laboratory animals, and this is an area of research that needs attention. A small study set up to examine the effect of maternal antibody on the response of calves to vaccination, described in Annex 5, section 2.11.3, showed that after two doses 4–5 weeks apart, and with dose 1 administered at 5–9 weeks of age, titres had fallen to just measurable by 5–6 months after dose 2. Some information has also emerged from studies on vaccinated wildlife. In a study on antibody levels to PA in vaccinated zebra in the Etosha National Park in Namibia (Lindeque et al., 1996b) it was evident that two initial doses approximately 8 weeks apart were necessary for the development of dependably measurable antibody titres, and the decline in titre one year after the second booster indicated that the next booster should be administered no later than that. A study in vaccinated cheetah utilizing passive protection in mice (Turnbull et al., 2004b) showed that more than one initial dose was necessary to ensure substantial protective immunity, and that lasting immunity might not set in until after a subsequent booster. Although the manner in which the vaccinations were given in that study did not permit the recommendation of a precise schedule, it was felt that the logical schedule, at least in that species, would be two initial doses two months or more apart, followed by annual boosters.

Strain $34F_2$ and its analogs in China and the Russian Federation lack the genes for capsule formation but still produce the toxin (i.e. they are pXO1+/2-). They thus possess "reduced" virulence, rather than being totally avirulent (Welkos et al., 1986; section 5.5.7) and occasional losses occur. Overdosing with the live spore vaccines based on these strains is hazardous. Certain species such as goats (Sterne, 1939) appear to be especially susceptible to adverse reactions, and the vaccines need to be used with extra caution in these (see Annex 5, section 2.5).

The extent to which vaccination should be applied to control an outbreak will be determined by the nature of the outbreak. Sporadic confined outbreaks will only require one herd vaccination, whereas annual vaccination may be required for extensive periods following widespread outbreaks (see also section 8.7).

Other limitations of the live spore vaccines in use today are (i) limited duration of effect (animals in enzootic areas should be immunized annually as discussed above); (ii) they should be administered parenterally; and (iii) efficacy and unwanted side-effects may be greatly influenced by small faults in production or administration. Items (i) and (ii) become important in developing countries where appropriate equipment and manpower are limiting factors; item (iii) has increasingly become relevant as "local" production has tended to replace manufacture by a few major companies or centres.

8.6.3 Human vaccines

In China and in the Russian Federation, live spore vaccines are prepared and licensed for human use (Annex 5, section 3). The Russian vaccine, dating from the 1930s and 1940s (Shlyakhov & Rubinstein, 1994), uses strain STI-1, analogous in its derivation to Sterne's $34F_2$ (STI was derived from strain "Krasnaya Niva", isolated from a horse that had died of anthrax (Cherkasskiy, personal communication, 2002). It was licensed for administration by scarification in 1953 and by subcutaneous injection in 1959. Good target populations for clinical trials existed in the southern European and middle Asian Soviet republics, where a 75%–84.2% rate of effectiveness was apparently recorded (Demicheli et al., 1998). The available information is that the Russian vaccine may be procured outside the Russian Federation. China however only produces enough to meet its national needs, and the Chinese vaccine is not available outside the country.

Trials were conducted by Russian workers in the 1950s and 1960s on the possibility of carrying out

vaccination by the inhalational route. Large inhaled doses (20–50 million spores or more) of live vaccine strain spores were found to be needed to induce substantial protective immunity in various animal species by the inhalational route (Anon., 1967; Lebendinskii, 1971; Ogarov & Gapochko, 1975). At this level of dose, generalized infection by the vaccine strain occurred. Lebendinskii (1971) reviewed a number of papers published between 1958 and 1967 describing the testing of aerosolized "dust" anthrax vaccines on some 4000 humans using doses of 15–300 million STI strain spores. A further 260 persons were vaccinated with highly dispersed liquid aerosols of STI strain spores. Although the only pathophysiological changes noted were a transient moderate leucocytosis with slight shift to the left, as monitored by the Anthraxin^{T} test (section 4.4.2.2), the percentage of positive skin tests, which peaked at 3 months, depended directly on the dose of vaccine inhaled.

In the United States and the United Kingdom, nonliving human vaccines developed in the 1950s and 1960s, and licensed in 1972 and 1979 respectively, are produced (Turnbull, 2000; Friedlander et al., 2002). However, their availability has recently been largely restricted to their respective national needs, for the most part defence-related. However, they remain nominally available for persons in other at-risk occupations, primarily industrial workers concerned with processing of animal products from endemic regions. For further information, the relevant organization given in Table 19 should be contacted directly. The United Kingdom vaccine is an alum-precipitated cell-free culture filtrate of strain $34F_2$, while the United States vaccine is an aluminium hydroxide-adsorbed cell-free culture filtrate of a non-capsulating, non-proteolytic derivative of bovine isolate V770. No evidence has been found of anthrax vaccines for humans produced or licensed in other countries.

There are no human efficacy data for the current United States and United Kingdom vaccines and the extent and duration of the protection afforded in humans by vaccination with these is uncertain. In the case of the United Kingdom vaccine, the original administration schedule was 2 doses of 1 ml at days 1 and 10 with an annual booster. This was based on protection tests in monkeys challenged with 10–15 LD_{50} of spores by the inhalation route 7 days (group 1), 1 year (group 2) and 2 years (group 3) after dose 2. All animals in groups 1 (10 animals) and 2 (10 animals) survived; 1 of 7 in group 3 died. All controls died (Darlow et al., 1956). Three-dose schedules were adopted a few years later and subsequent assessment of the efficacy of the United Kingdom vaccine depended on observations in the 1960s of decreasing incidence in the wool and hide industries where vaccination programmes were in place, while infection rates outside these trades remained steady (Darlow & Pride, 1969), and of a fourfold decline in the number of reported cases (including both cutaneous and inhalational forms of the disease) between the early 1960s and the late 1970s, in parallel with the introduction of the vaccine (CDSC, 1981). There is also no case on record of anthrax in a human vaccinated with the United Kingdom vaccine.

With the United States vaccine, now commonly referred to as AVA (anthrax vaccine adsorbed), a study of mill workers when the vaccine was first being introduced recorded a protection rate of 93% (Brachman et al., 1962). In its early days of use in mill workers processing animal products from anthrax-endemic regions of the world, four cases were recorded in partially-vaccinated persons and one in a fully-vaccinated individual (Brachman et al., 1962). This vaccine has also evolved somewhat since then, and more recent evaluations in rhesus monkeys indicated that it is able to confer a high degree of protection against aerosolized spore challenge (Ivins et al., 1996, 1998).

The United States and United Kingdom vaccines have been associated with complaints of reactogenicity, although this rarely exceeds mild erythema, soreness and swelling at the site of injection lasting 2–3 days (Turnbull, 2000). The topic is being studied in depth for the United States vaccine (Marano et al., 2005). No adverse effect "in more than 30 years" has been claimed for the Russian STI vaccine (Shlyakhov & Rubenstein, 1994).

In certain circumstances, including following known or suspected exposure as a result of a deliberate release event, postexposure vaccination at the same time as administration of antibiotics may be appropriate (see section 7.3.2.1).

Vaccination of humans who are not occupationally exposed to anthrax is inappropriate. For the general public, control of the disease should be done through control in livestock.

8.6.4 Prospective new vaccines

8.6.4.1 Human vaccines

The basic prerequisite for protection against anthrax is an adequate humoral immunity to the protective antigen (PA) combined with an appropriate cellular immune response (section 8.6.1). This should be taken into account in the design of new vaccines. While cellular immune stimulation can be achieved by means of an effective adjuvant, the surest way of ensuring this combined humoral and cellular response is to immunize with live vaccines. The current animal vaccine (section 8.6.2) is a live vaccine, and its greater efficacy over the nonliving human vaccines (section 8.6.3) in animal protection studies is attributed to its greater stimulation of cellular immune responses in the recipients (Ivins & Welkos, 1988; Turnbull et al., 1988; Turnbull, 1991).

When first introduced for administration to workers in at-risk occupations, the United States human anthrax vaccine of the time was shown to be clearly associated with protection against the disease (Brachman et al., 1962). That paper contains the only existing reference to anthrax in a fully-vaccinated person (section 8.6.3); since then, there have been no other such cases on record. However, protection tests in laboratory animals have resulted in a confusing view of the efficacy that can be expected from the currently licensed human nonliving vaccines for a given challenge situation, and of the duration of the protection they afford. Being essentially simple culture filtrates, the vaccines are somewhat undefined, lack consistency in production and involve a cumbersome dosing schedule. They are occasionally associated with complaints of side-effects (Turnbull, 2000). The case for the development of new vaccines rests on these issues.

The aims for prospective new vaccines are that they should be:

- well-defined with well-characterized ingredients and mode of action;
- effective against challenge with any anthrax strain;
- safe, giving no dangerous side-effects in any species;
- long-lasting;
- easy to administer rapidly and by non-skilled persons;
- requiring just one dose, or very few doses;
- orally administrable, especially for livestock and at-risk wildlife species;
- cheap;
- environmentally acceptable.

The next-generation human vaccines are likely to be parenterally administered nonliving vaccines consisting of purified recombinant PA with adjuvant. Two such vaccines are in clinical trials, and progress towards licensure for one or both of these can be tracked on the Web through standard search engines. At present, only certain aluminium salts are approved for use as adjuvants in human vaccines; these induce Th2 responses predominantly. It is thought that this may be advantageous for promoting protective immunity in primates and that it may also account for the apparently better performance of the current licensed Al^{3+}-adjuvanted vaccines in protecting monkeys as compared with guinea-pigs and mice in which, it is theorized, stimulation of Th1 responses may be more advantageous (see also section 8.6.1).

For subsequent-generation human vaccines, pressure is likely to arise for the vaccine to be based on more than just PA, and to include at least one or both of the other toxin components, LF and EF, and possibly other antigens such as S-layer proteins or even the capsule (Fouet et al., 1999; Friedlander et al., 2002). In this case a mutant form of the PA that can no longer combine with LF and EF (Singh et al., 1989) and which is, therefore, non-toxigenic while still being fully immunogenic, is likely to be involved. It may be that this will take the form of a DNA vaccine (Price et al., 2001) rather than a protein vaccine, with the DNA encoding the desired vaccine antigens, whole or mutated as desired. At this stage it is difficult to predict how many doses would be needed for effectiveness, whether a non-parenteral route of administration may be possible, or the likely duration of the conferred protection.

8.6.4.2 Veterinary vaccines

It is probably due in part to the known effectiveness of the Sterne strain and analogous vaccines, and in part to the relative unimportance in veterinary spheres of anthrax as a day-to-day disease, that there has been little interest shown in developing new livestock vaccines. The capability to do so certainly exists. Vaccines producing stronger initial immunity, and immunity of longer duration with lower potential risks of casualties, would certainly

be desirable. Particularly useful would be a vaccine that is unaffected by antibiotics, permitting simultaneous treatment and vaccination during outbreaks.

8.6.5 Studies on orally administered live spore vaccines

As indicated in section 8.6.4.1, the ideal vaccine would be an oral formulation that:

- meets the requirements of manufacturers for a readily-standardized production with batch-to-batch reproducibility;
- meets the requirements of regulatory bodies for readily-demonstrated pharmacological and safety data;
- meets the needs of recipients for a single-dose administration resulting in rapid development of immunity and lasting efficacy with no undesirable effects;
- could also be a combination vaccine covering multiple-disease entities;
- could be administered with equal effectiveness to humans, domestic animals or wildlife as needed;
- could be administered in an easy and non-invasive manner;
- would be cheap.

Current anthrax vaccines are administered by parenteral injection. Claims of immunization with a level of success by oral delivery of anthrax vaccines appear to be confined to the reference by Ebedes (1976) to an oral vaccine that protected guinea-pigs from repeated challenge with virulent anthrax spores, the reports of Rengel (1993) and Rengel & Boehnel (1994, 1995) on the results of feeding Sterne vaccine strain spores to guinea-pigs, unpublished claims in Texas of reduced disease in white-tailed deer given Sterne-strain spores mixed in with their feed (Hugh-Jones, personal communication, 2004), and partial protection in challenged mice that had been immunized with a *Salmonella typhimurium* construct expressing the *B. anthracis* protective antigen (PA) gene (Coulson et al., 1994).

The evidence for *Bacillus* species being capable of germinating, colonizing and/or multiplying in the intestinal tract is tenuous. Granum et al. (1993) express the opinion that the diarrhoeal syndrome of *B. cereus* may result from in situ production of the enterotoxin within the intestine rather than from ingestion of preformed toxin, basing this on the ability of the bacterium to grow anaerobically. The same group (Andersson et al., 1998) considered their demonstration of hydrophobic attachment of the spores of certain strains of *B. cereus* to monolayers of Caco-2 human epithelial cells to indicate that such strains might be capable of epithelial adhesion and colonization within the intestine. Similarities between *B. cereus* diarrhoeal-type food poisoning and food poisoning by *Clostridium perfringens*, which is known to colonize the intestine, is another argument, by analogy, for *Bacillus* species being able to colonize, germinate and multiply within the intestine. It is well documented that LD_{100}s and LD_{50}s for fully virulent *B. anthracis* by the oral route are enormously high, even in species regarded as highly susceptible to infection (see section 3.1). Most reports indicate figures of 10^8 or higher, although de Vos (1990) gives an oral LD_{50} of about 1.5×10^6 spores for impala. This compares with parenteral route LD_{50}s in the order of tens or hundreds for the same species where documented. Illness and recovery following oral challenge clearly can occur with non-lethal doses (Schlingman et al., 1956; Jackson et al., 1957; Redmond et al., 1996a, 1997) followed by seroconversion (Jackson et al., 1957; Redmond et al., 1996a, 1997). To what extent these oral-dose data reflect some, if limited, colonization or toxin production within the gut, as opposed to simply reflecting the points at which a proportion of the very large numbers present overcome the natural intestinal barriers (Walker & Owen, 1990), is impossible to say.

The bottom line is that it is not known whether vaccine strains such as the Sterne strain or recombinants, such as *B. subtilis* WB600 (pPA101), are capable of producing PA/rPA in situ and, if so, to an extent where immunity can result. In a study to address this question (Turnbull et al., 2001a), guinea-pigs were fed by stomach gavage on days 1, 21 and 42 with 1 ml volumes of spore suspensions of *B. subtilis* WB600 pPA 101-1, *B. anthracis* Sterne animal vaccine and *B. globigii* (control group), all pre-adjusted to 10^8 cfu per ml. Faecal samples were collected from each individual animal before each feeding session for IgA anti-spore or anti-PA antibody analysis and, after each feeding session, all faeces were collected to day 6 for determination of numbers of excreted spores. Following test bleeding, the guinea-pigs were challenged intramuscularly with 10^3 cfu of *B. anthracis* Vollum strain spores on day 64. In all three groups, the administered organisms were recovered exclusively in the spore form in the faeces and became

undetectable by day 6. There was no evidence of multiplication within the gastrointestinal tracts of the animals. Increasingly rapid declines in faecal counts, reflecting accumulating immunity, were not readily apparent with successive feeding sessions, and IgG and IgA anti-spore or anti-PA antibodies were not detected in faecal and/or serum samples. All the guinea-pigs succumbed to the challenge with no significant differences in time to death between the livestock vaccine (*B. anthracis*) group, the recombinant *B. subtilis* producing PA group and *B. globigii* controls. The indications, therefore, were that the vaccine strains failed to colonize, produce PA and thereby induce immunity.

Similarly, Hugh-Jones and colleagues (personal communication, 2005) carried out a field trial in 2004 with 15 goats, feeding them 60 times the prescribed parenteral dose of Sterne vaccine together with crushed pecan nuts. Just one of the animals developed a low anti-PA titre.

8.7 Decisions on treatment and/or vaccination of livestock

While vaccination of livestock is the fundamental control measure in enzootic areas with seasonal recurrence of the disease, when an incident occurs unexpectedly in a non-endemic area, antibiotic treatment of exposed animals may be preferable to vaccination – or at least more immediately practical – as the primary control measure. Vaccination may be added as an adjunct if prolonged incubation periods are of concern and there is reason to fear that an incident is going to develop into an outbreak. In fact, in temperate climates such as the United States, where years may elapse between anthrax outbreaks in a given locality, anecdotal evidence suggests that significant decreased livestock losses may be associated with a control programme of first administering a long-acting antibiotic followed by vaccination after 7 to 10 days.

The decision on whether to vaccinate should be made soon after infection with anthrax has been confirmed. With livestock, when infection has been confirmed in the first case, immediate vaccination of all animals thought to have an equal chance of exposure can be expected to result in no further cases. On the other hand, where multiple cases have already occurred, further cases may occur over the next week or two despite vaccination, presumably in animals already incubating the disease. In this case, antibiotic treatment followed by vaccination after 7–10 days is the best control option (Annex 5, section 2.2).

Only animals thought to have an equal chance of exposure need be vaccinated if separation from other animals can be assured. If separation cannot be assured and other animals will have access to the infected group and/or infected site(s), or if multiple cases have occurred, neighbouring herds and flocks should be vaccinated. In an outbreak situation ring vaccination may need to be applied to a distance of 1 km beyond an infected property. If biting flies are considered responsible for the spread of the outbreak, the ring should cover an area exceeding the distance normally travelled by the flies.

All animal vaccines are live vaccines, and their use requires a withholding period prior to slaughter for human consumption. This period may be stipulated on the label of the vaccine or in the accompanying leaflet, and varies from 3 to 6 weeks. Withholding periods from slaughter of up to 6 weeks are also required by some countries for the purposes of trade in meat products. There is no withholding period for milk destined for human consumption following vaccination of milking animals with Sterne $34F_2$ strain vaccine. There are no known reports of illness in humans following the consumption of animal products from animals immunized with that vaccine.

Anthrax vaccines, being living organisms, are restricted to veterinary use in some countries, and in some jurisdictions they can only be used with the approval of the official veterinary service. It is important that local requirements be met.

8.8 Duration of veterinary vaccination programmes

Despite the well–known longevity of anthrax spores, decline in spore numbers, as evidenced by reduced outbreaks, does occur through decay and/or dispersal at contaminated sites (see also sections 2.1.2.4, 2.1.2.6). It was the experience of Max Sterne, and others since, that pastures associated with anthrax continued to give rise to cases for up to three years after the index cases. Therefore, if there has been an outbreak on a farm, the stock should be revaccinated annually for at least three years to prevent further cases.

8.9 Intersectoral issues: the question of eradication

As with any zoonosis, intersectoral cooperation is essential for effective control of anthrax. Public health officials should be notified by the veterinary authorities in the event of outbreaks in livestock so that they become alert to the possibility of associated human cases. Likewise, medical authorities should notify veterinary health officials when a human case is encountered (see section 9.1.2).

Cooperation between livestock officials and wildlife managers in enzootic zones is more complex. While the target of the former is eradication from the region, the disease is regarded in large game-management areas where it is not rate-limiting in any of the species as an integral part of natural population-control mechanisms. In these, action, usually vaccination, is often seen as being necessary only when endangered species are at risk, and is usually targeted specifically at that species (section 8.3.2.2). Beyond this, control actions are regarded as constituting unwarranted interference with natural processes. In this instance, cooperation between livestock and wildlife management needs to take the form of joint efforts to minimize mingling of susceptible livestock and wildlife species. Fencing is one approach, although this interferes with the normal migrations of several of the susceptible wildlife species, and therefore should only be done advisedly. Annual vaccination of livestock that are likely to be in regular direct or indirect contact with susceptible wildlife species is another approach.

Eradication from livestock areas not in contact with enzootic wildlife is feasible (section 8.8), given sufficient time together with unremitting efficient application of the control procedures discussed in this chapter.

8.10 Control in wildlife

While large national wildlife parks may adopt the "hands-off" management policies outlined in sections 8.3.2.2 and 8.9, this may be inappropriate for commercial or smaller parks or sustainable resource development management areas that cannot sustain the financial losses resulting from the disease. Suggested approaches to anthrax control for these are given in Annex 6, section 5.

9. Anthrax surveillance

9.1 Introduction

9.1.1 Understanding the concept of surveillance

Surveillance is the collection, collation and analysis of health data that enables the prompt dissemination of the information to those who need to know, in order that appropriate action may be taken. Effective surveillance is essential to any prevention and control programme and encompasses the mechanisms for disease detection, confirmation of diagnosis, reporting, collation of data and reporting back of the data to the source. Detection of rare diseases requires well-trained veterinary or human health-care providers. Confirmation of reports of suspicious cases requires appropriate laboratory support. Reporting requires mechanisms for easy communication of cases, and also some incentives for reporting or disincentives for not reporting. Many countries rely on disincentives for not reporting diseases under surveillance (reportable diseases). Regulations exist for reporting certain diseases (usually of high epidemic potential or with high morbidity or mortality rates) in most countries of the world.

Because of its epidemic potential, associated high morbidity and mortality rates, wide-ranging occurrence, and history of threatened use – and now actual use in the 2001 anthrax letter events – as a biological terrorist weapon, anthrax in humans or animals should be included on all national notifiable-disease lists.

Surveillance systems vary widely in objectives and methodology. The surveillance scheme outlined below may be adapted to accommodate the local veterinary and public health systems within a country. It should be noted that efforts to improve certain attributes of surveillance, such as specificity, may detract from other attributes such as timeliness.

9.1.2 Intersectoral cooperation

As with most zoonotic diseases where animals serve as the primary sources of human infection and epidemics (as opposed to zoonoses, where both humans and animals may be infected from common environmental sources), control of anthrax among humans depends on the integration of veterinary and human health surveillance and control programmes. Routine cross-notification between the veterinary and human health surveillance systems should be part of any zoonotic disease prevention and control programme, and close collaboration between the two sectors is particularly important during epidemiological and outbreak investigations.

High-risk areas for human disease may be identified pre-emptively by review of veterinary surveillance information by veterinary or public health authorities. Because herbivorous livestock and wild animals are more susceptible to *B. anthracis* than humans, and because in general soil sources do not represent a threat to humans directly, the potential threat to human populations is through contaminated animal products. The exceptions to this rule are biological warfare or terrorism, or laboratory settings where the spores of *B. anthracis* may be manipulated and concentrated in an unnatural way to represent a direct threat to human health through respiratory, gastrointestinal or cutaneous exposure – see also section 4.2.3. However, many countries rely on detection of human disease as the alert of anthrax epizootic activity. In these cases, humans may be considered sentinels (see also section 4.1). Ideally, this should not be the case, and enzootic and epizootic surveillance and reporting should lead to the implementation of anthrax control strategies that prevent human cases altogether. Realistically however, in many countries the support and infrastructure for human health surveillance programmes is greater than for veterinary equivalents. In such cases, when detection depends on the

appearance of a human case, the control of anthrax among livestock is delayed and the losses suffered by farmers will be greater than if a functional veterinary surveillance programme is in place. This is because only a fraction of the livestock cases are ever identified and the disease goes unreported and uncontrolled.

9.2 Objectives

The design of a surveillance system for anthrax depends in part on its objectives. The primary objectives of any anthrax surveillance system are to prevent or reduce livestock losses and to prevent human disease. To achieve these objectives, the surveillance system should emphasize education of the producers and front-line veterinarians in the detection, confirmation and reporting of cases. This detection, confirmation and reporting should be followed by a strong response from the veterinary health system to control the disease. Prevention of cases among livestock depends on knowledge of enzootic regions and on vaccination of livestock in those affected areas. Gaining knowledge of risk areas is a secondary objective of surveillance for the disease.

Identification of the characteristics of the disease in the affected populations, and evaluation of prevention and control activities by monitoring the incidence of the disease in both animal and human populations, should be among other objectives chosen by the national surveillance programme.

9.3 Surveillance reporting outline

9.3.1 Local level

Animal and human health-care professionals, with their supporting laboratories, form the front line of any zoonotic disease surveillance system. Surveillance begins with the first point of contact by a veterinarian or clinician in the event of a suspicious case. In an optimal situation, concern over a suspicious case should result in either contact with local health authorities (veterinary or human) or the initiation of a case-reporting form.

Depending on the objectives of the surveillance system, a case form should be completed on every suspected or confirmed case. Depending on veterinary or public health resources, this case-report form may be completed by the local health-care professionals attending the suspicious case, or preferably by health authorities at the local level (municipal or county).

Figs 11&12 are examples of human and veterinary case-report forms. Minimum data elements for human case-reporting are:

- case classification (e.g. suspected or confirmed);
- clinical form of the case (cutaneous, gastrointestinal, inhalational, meningeal);
- identifying information, and a unique identification code.

Depending on the objectives of the surveillance system, other pertinent data might include age, sex, geographical information, occupation, date of presentation, exposure history, nationality, date of clinical onset, treatment and outcome.

The local level (health-care centre, private clinic and physician, and other health personnel) is the first point of official contact with the infected patient and the point at which surveillance data should first be collected. Suspected rather than confirmed cases may be reported from this level to higher levels. The tasks at this level are diagnosis and case management, including treatment and health education plus, resources permitting, case and outbreak investigation. The laboratory criteria for the confirmation of any suspicious case may be included on the same case-report form, or the form may be simplified to only include whether the case was confirmed or not. Local health authorities may be asked to assist in diagnosis and case management.

Local health authorities are responsible for reporting to the intermediate level (i.e. state) or to the central level (i.e. federal), depending on the government structure. Also, local health authorities will be expected to mount the early epidemiological investigation targeted at:

- identifying and controlling the source of infection (including additional case-finding, case-confirmation, identification of source of infection and tracing sources of infection from infected animals or animal products);
- reducing population morbidity and mortality through initiation of control measures depending on the authority, epidemiological situation and guidelines.

Control measures for anthrax in livestock are given in chapter 8 and Annex 4. Depending on the epidemiological details of any human outbreak, control measures for humans are based on removal of the source of infection from human contact or separation

Fig. 11 Sample anthrax case-report form (human)

Suspect ..... **Confirmed** ..... **Case ID:** .....

Patient information **Anthrax form:** ..... **Cutaneous,** ..... **Inhalational,** ..... **Gastrointestinal**

1. Patient name: First ..... Last .....
2. Age ..... Gender: ☐ Male ☐ Female
3. Residence location:

   Address .....

   .....
4. Occupation
5. Patient status 1. ☐ Outpatient 2. ☐ Inpatient 3. ☐ Admitted and discharged 4. ☐ Died
6. Date of illness onset ..... / ..... / .....
7. Date first received antibiotics ..... / ..... / .....
8. Date admitted ..... / ..... / .....
9. Date died ..... / ..... / .....
10. Date of autopsy ..... / ..... / .....

Brief history of present illness (include dates)

Name and route of medication	Date started
1.	..... / ..... / .....
2.	..... / ..... / .....

1. Contact with animals or animal products (e.g. livestock, hair, hides, meat, or other potential animal sources)

   ☐ Yes ☐ No ☐ Unknown

If YES, animal or product	Type of contact and location	Source confirmed anthrax positive?
1.	.....	..... (Y, N, U)
2.	.....	..... (Y, N, U)
3.	.....	..... (Y, N, U)

Laboratory confirmation

Specimen	Date collected	Test method	Laboratory	Result

Reporting information

1. Date reported ..... / ..... / .....
2. Person reporting case
   - Name
   - Title and professional status
   - Address
   - Phone # Other contact method
3. Patient's medical professional
   - Name
   - Title and professional status
   - Address
   - Phone # Other contact method
4. Other medical examiner
   - Name
   - Professional status
   - Contact information

Fig. 12 Sample anthrax case-report form (animal)

Suspect ..... **Confirmed** ..... **Case ID:** .....

1. Species ..... 2. No. affected .....

3. Animal IDs (if different from case IDs) .....

4. Age(s) ..... 5. Gender(s) ☐ Males ☐ Females

6. Location ..... Address .....

7. Date of 1st case .....

8. Brief history of incident/outbreak (with dates)

(continue on back of sheet if more space needed)

9. Information to identify possible sources

Has anthrax occurred in this herd before?	☐ Yes ☐ No ☐ In the last year ☐ In the last 10 years ☐ > 10 years ago
Has anthrax occurred at this site before?	☐ Yes ☐ No ☐ In the last year ☐ In the last 10 years ☐ > 10 years ago
Was the animal/herd in contact with, or located near another herd/animal with anthrax	☐ Yes ☐ No ☐ Details
Is there any reason to suspect the feed?	☐ Yes ☐ No ☐ Details
Has there been any unusual occurrence or activity on or near the premises, such as soil disturbance?	☐ Yes ☐ No ☐ Details

10. Vaccination history in this herd or at this site

11. Control measures instituted

12. Laboratory confirmation

Specimen	Date collected	Test method	Laboratory	Result

13. Reporting information

Date reported

Person reporting the incident/outbreak (name):

Professional status

Address

Phone/fax/other

of human populations from the infectious source(s); however, these control measures may vary widely in detail and will depend on the particular exposure situation.

9.3.2 Intermediate level

Depending on the political infrastructure, data from the report forms may be managed at an intermediate level such as a state health or veterinary department. Here the data may be collated and analysed for feedback to the local (municipal or county) level. Usually, the intermediate level collates and analyses data from local levels. In addition, personnel at this intermediate level may serve as support to the local level. The tasks of the intermediate level are:

- aspects of case-confirmation and case-management that cannot be undertaken at the local level;
- analysis of data from local levels;
- epidemiological investigations, tracing sources of infection from infected animals or animal products;
- initiation and monitoring of control measures.

9.3.3 Central level

Reporting of cases should also be transmitted to the central level. Depending on national resources, the central level will analyse and report the data back to the intermediate and local levels. In addition, the central level may formulate national policies and allocate resources based on the surveillance information. The central level may also provide technical support (e.g. laboratory or epidemiological) to the intermediate and local levels as appropriate, and it is responsible for reporting summary surveillance information to international authorities such as WHO and OIE.

9.4 Human surveillance

9.4.1 Case definition

Details of the clinical presentation of anthrax in humans can be found in chapter 4.

The suggested case definitions below may need to be adapted to local needs and laboratory capabilities available for confirmation of diagnosis (WHO, 1997b).

9.4.1.1 Clinical criteria

Anthrax is an illness with acute onset characterized by several clinical forms as described in detail in chapter 4, especially when supported by a suggestive case history. These could be summarized in a concise case definition for surveillance purposes as follows.

Localized form

Cutaneous. Skin lesion evolving over 2–6 days from a papular through a vesicular stage, to a depressed black eschar invariably accompanied by oedema that may be mild to extensive.

Systemic forms

Gastrointestinal. Abdominal distress characterized by nausea, vomiting, anorexia and followed by fever.

Pulmonary (inhalation). Brief prodrome resembling acute viral respiratory illness, followed by rapid onset of hypoxia, dyspnoea and high temperature, with X-ray evidence of mediastinal widening.

Meningeal. Acute onset of high fever possibly with convulsions and loss of consciousness, meningeal signs and symptoms.

9.4.1.2 Laboratory criteria

Several laboratory assays may be considered as supportive diagnostic criteria. The availability or reliability of these assays may vary from location to location, and therefore the definition of confirmed and suspected may be modified according to local laboratory capacity. However, it is thought that every country should be able to establish the necessary capabilities within the guidelines given in Annex 1 and meet the following laboratory criteria for diagnosis of anthrax:

- isolation and confirmation of *B. anthracis* from a clinical specimen collected from an affected tissue or site; or
- other supportive laboratory tests, including:
  - evidence in fresh tissue or blood samples with traditional M'Fadyean (polychrome methylene blue) stained smears;
  - evidence of *B. anthracis* DNA by PCR from specimens collected from an affected tissue or site;
  - demonstration of *B. anthracis* in a clinical specimen by immunohistochemical staining; or
  - other laboratory tests (e.g. serology).

9.4.2 Case classification

9.4.2.1 Confirmed case

A confirmed case of anthrax in a human can be defined as a clinically compatible case of cutaneous, inhalational or gastrointestinal illness that is laboratory-confirmed by:

- isolation of *B. anthracis* from an affected tissue or site; or
- other laboratory evidence of *B. anthracis* infection based on at least two supportive laboratory tests.

Note: it may not be possible to demonstrate *B. anthracis* in clinical specimens if the patient has been treated with antimicrobial agents.

9.4.2.2 Suspected case

A suspected case of anthrax in a human may be defined as:

- a clinically-compatible case of illness without isolation of *B. anthracis* and no alternative diagnosis, but with laboratory evidence of *B. anthracis* by one supportive laboratory test; or
- a clinically-compatible case of anthrax epidemiologically linked to a confirmed environmental exposure (infected animal product, contaminated fomite, or other source).

9.4.3 Special considerations for human surveillance

A single case of anthrax in a human population should result in a report and investigation.

Because the disease is rare in many countries, continuing education programmes for health-care providers should include information on recognition and confirmation of this disease.

Certain at-risk populations may warrant active surveillance (the implementation of case-finding activities among a subpopulation) on a regular basis. One example of such a need was found among hair and wool industry workers in Europe and the United States in the 20th century. Because of an especially high risk, these workers were more intensively observed for anthrax than the general population by the occupational health systems and authorities serving these groups.

9.5 Veterinary surveillance

9.5.1 Case definitions

9.5.1.1 Clinical description

Please refer also to the OIE Code for general guidance for animal health surveillance.[1]

Details of the clinical presentation of anthrax in animals can be found in chapter 3. In general in non-immunized cattle, sheep or goats, anthrax is usually a peracute disease characterized by septicaemia and sudden death, frequently (but not universally) with bleeding from orifices and subcutaneous haemorrhages. Other reported symptoms in cattle, horses, sheep and some wild herbivores consist of fever, dyspnoea, agitation and convulsions followed by death. In pigs, carnivores and primates, the main symptoms are local oedema and swelling of the face and neck. Failure of the blood to clot, absence of *rigor mortis* and the presence of splenomegaly are the most important necropsy findings (but not necessarily pathognomonic). (See also sections 3.4 and 3.5.)

9.5.1.2 Laboratory criteria for diagnosis

Internationally recognized standard diagnostic techniques and their interpretation for diagnosis of anthrax in animals are described in section 3.5 and Annex 1, as well as in the *Manual of standards for diagnostic tests and vaccine* (OIE, 2008).

9.5.2 Case classification

9.5.2.1 Suspected

A suspected case is a case that is compatible with the clinical description. In enzootic areas all sudden deaths should be regarded as suspected anthrax cases.

9.5.2.2 Confirmed

A confirmed case is a suspected case that is laboratory-confirmed. A diagnosis based on clinical signs may be difficult, especially when the disease occurs in a new area, and a confirmatory laboratory examination should be carried out as described in section 3.5 and Annex 1.

[1] *General guidance for animal health surveillance.* In: Terrestrial Animal Health Code, Appendix 3.8.1. Paris, World Organisation for Animal Health (OIE), 2007 (http://www.oie.int/eng/normes/mcode/en_chapitre_3.8.1.htm).

9.5.3 Special considerations for veterinary surveillance

- In countries or areas free of anthrax, the disease is treated as an exotic or foreign disease, and measures to be adopted are based on primary prevention, mainly the control of imported animals and animal products (see Annex 4).
- In enzootic countries or areas, whether or not animal vaccination is carried out, all suspected sudden deaths in animals should be investigated as possible cases of anthrax. Flocks or herds in direct or indirect contact with positive human cases should be investigated, as the human infection will have derived from animal cases and any infected herds or flocks should be identified and vaccinated. The primary prevention measures mentioned above should also be in place. In enzootic countries or areas where animal vaccination has been discontinued, particular emphasis should be placed on continued surveillance.
- Because the numbers of animals may be very large on individual affected properties, the unit of reporting may in some cases be the property or farm rather than the animal.
- Whenever possible, global positioning data should be included in case-reporting to ensure that affected areas are properly located and recorded.

In general, the occurrence and reporting of anthrax may cause economic harm to affected animal producers. For that reason, it is important that national programmes for anthrax control include no penalties for reporting and compensation for additional costs to the farmer. Anything that discourages reporting should be avoided, and everything that encourages reporting should be embraced. Suggested approaches to ensuring this are:

- Compensation to the owner should be available for any carcass proven (i) to be a case of anthrax, and (ii) to have been incinerated or disposed of by another officially approved method. Ideally, disposal of the carcass should be carried out by the official veterinary service.
- The initial vaccination of the herd or flock should be carried out by the official veterinary service.
- Quarantine should be kept as non-onerous as possible; it need not be longer than 21 days after vaccination of the affected herd has been carried out (see section 7.2.1.2); or, if cases continue to occur after vaccination, 20 days after the last case, according to the OIE-designated period (Annex 4).
- Animal health officers should be properly equipped with specimen-collection materials, equipment and materials necessary for disinfection, clean-up and disposal, and with antibiotics and vaccine plus syringes and needles. They should also be appropriately trained (e.g. not to administer vaccine and antibiotics simultaneously – see section 7.2.1.3) and, furthermore, should have the necessary transport and suitable office support for reporting cases.
- The laboratory technician should be supplied with the appropriate equipment, materials and training for confirmation of diagnosis.
- Appropriate educational literature for all persons involved, from the farmer/owner to ministry levels, and information packets for the media should be produced.
- Every effort should be made to get local understanding and popular support for the policy of carcass destruction and herd vaccination.
- The reporting system should be based more on incentives than penalties, although penalties for failure to report may be necessary. The reporting should not incur cost (e.g. forms, postage, etc.) to those required to make reports.
- Treatment and vaccination of the remainder of the herd/flock and the services of the animal health officers in assisting with cleaning and disinfection should be free of charge.
- Affected herds/flocks should be revaccinated annually for three years (sections 8.8, 8.9).

9.6 Communication of disease data

Maps offer an efficient tool for communicating data on the prevalence of diseases or the location of at-risk populations. In order to facilitate disease control and surveillance, WHO has developed the HealthMapper. This software is specifically designed for use by public health administrators working at national and district levels. The HealthMapper simplifies the collection, storage, updating, retrieval and analysis of public health data. Core to the HealthMapper is the geographical database. This database contains the standardized data of individual countries with regard to various features such as administrative boundaries, population by administrative level, roads, rivers, forests and elevation. Such data enable the linkage of disease data such as cases per district with background data such as population size

or environmental factors. Furthermore, standardized data facilitate the assessment of trends over time and the comparison of disease prevalence across geographical areas. The geographical database is developed and updated in collaboration with national ministries of each partner country. The completeness of these data varies from country to country. The HealthMapper geographical files may be used in conjunction with ESRI Inc. products.

In addition to the HealthMapper, WHO has launched the Global Health Atlas. This is more suitable for comparisons across countries than within countries. The Global Health Atlas is an electronic platform that builds on the HealthMapper and brings together standardized databases on infectious diseases and on core health statistics. The Global Health Atlas contains data on demography, environmental factors, socioeconomic conditions and health indicators.[1]

9.7 Templates for control programmes from the Model Country Project

In the third edition of these guidelines (Turnbull et al., 1998a), Appendix 7 was devoted to the outcome of, and lessons from, the Model Country Programme formulated by the WHO anthrax working group (Editor's note 1996b). While it was felt that it was not necessary to include the entire appendix in this edition, it was considered that the templates drawn up to assist national authorities to define their problems in achieving the ideal in regard to surveillance, reporting and control could still be useful. The templates were designed to enable a simple comparison between "textbook" approaches to control and what actually occurs in reality in any one country. This, in turn, will make it easier for that country's national authorities to develop an action plan to reduce the discrepancy between the ideal and the reality. These templates (modified) are reproduced in Tables 6 –11. Guideline users may compare the situation in their country ("actual") to the "ideal" and decide what action they should take to bring their control programmes nearer the ideal.

TABLE 6

Surveillance	
Ideal	All unexplained livestock deaths or suspected cases must be investigated with laboratory support.
Actual	To what extent is "all" not achieved? How can this be improved?
Constraints (on achieving the ideal)	To what extent do the following prevent the ideal being achieved and how can their influence be reduced: • distances involved; • remoteness of affected premises; • lack of transport; • delays in specimen delivery to laboratory; • laboratory materials unavailable.
Importance of surveillance	Confusion as to cause of death is avoided. Demonstrates that control measures are working.

Note. Every unexpected death in livestock should automatically result in a blood or tissue smear and sample for examination by veterinary authorities (see Annex 1, sections 8.1 & 9, Table 14) at the earliest opportunity. A person of appropriate standing in each community might be recruited to implement this; he/she should be given adequate instruction, including on safety issues.

Since it is a frequent experience that smears may fail to reveal anthrax bacilli, especially if the carcass was not fresh at the time of making the smear (i.e. if the smear is not made within, for example, 48 hours after death), laboratory culture should be regarded as an essential back-up procedure for diagnosis. This should be made possible by provision of the appropriate equipment, materials and instruction at the district veterinary laboratory level at least.

[1] HealthMapper: http://www.who.int/health_mapping/tools/healthmapper/en/index.html; Global Health Atlas: http://www.who.int/health_mapping/tools/globalatlas/en/index.html; World health statistics: http://www.who.int/statistics.

TABLE 7

Reporting	
Ideal	Reporting and information must be efficient, involving: • keeping good records; • mandatory reporting of deaths.
Actual	Reporting does occur, but to what extent does its efficiency need to improve? How can this be improved? (e.g. would better cooperation between the local established social hierarchy and the veterinary public health officials be the way to improve this?)
Constraints (on achieving the ideal)	To what extent do the following prevent achieving the ideal and how can their influence be reduced: • the size of the areas being monitored; • the remoteness of some of the villages; • the inaccessibility of many of the communities; • shortage of basic materials at the veterinary assistant level.
Importance of reporting	The success of control efforts cannot be monitored without good reporting.

Note. A concerted effort should be made to ensure that hospitals and human clinics inform veterinary officials when they see a case of anthrax in a person.

TABLE 8

Disposal	
Ideal	After confirmation as being a case of anthrax, a carcass should not be opened and should be burnt (or, the less preferred option, buried) in situ, or in an incinerator or rendered (see section 8.2).
Actual	Are carcasses butchered and sold for human consumption? Is the value of the meat and hides seen as outweighing risks of serious illness? Is burning not feasible owing to shortage of fuel? Is burial a possible alternative?
Constraints (on achieving the ideal)	To what extent do the following prevent achieving the ideal and how can their influence be reduced: • attitudes of stock owners; • traditions; • lack of understanding of why only some of those handling an anthrax carcass or eating the meat acquire disease.
Importance of disposal	Avoidance of environmental contamination. Avoidance of spread of anthrax.

Note. Options on the best official approach to dealing with anthrax carcasses in developing country situations range from leaving it unopened for several days, possibly first wetting with 10% formalin (section 8.3.2.1; Annex 6, section 1), to allow putrefaction to kill the anthrax bacilli within the carcass, to a mandatory incineration policy, with many other approaches in between. Research is needed to determine practical and effective procedures appropriate to the local circumstances.

TABLE 9

Disinfection	
Ideal	Disinfectants should be held in reasonable quantities at field stations. Veterinary assistants and stock owners should be trained in their use.
Actual	To what extent is this possible?
Constraints (on achieving the ideal)	To what extent do the following prevent achieving the ideal and how can their influence be reduced: • lack of availability of suitable disinfectants; • cost; • hazards posed by the appropriate disinfectants; • the type of premises where anthrax carcasses are handled.
Importance of disinfection	Disinfection is a valuable control measure. It needs to be included in the control programme wherever possible.

Note. Provision, supply and storage of appropriate types of disinfectants are clearly a problem for developing countries, mainly because these are imported at a very high cost. Research is needed on the design of disinfection procedures applicable to the economic and other conditions prevailing in such countries.

TABLE 10

Vaccination	
Ideal	A vaccine meeting acceptable standards should be available. Field officers should have the necessary storage facilities to maintain full vaccine viability. A contingency stock should be readily available. Vaccination for the exposed animals in the first outbreak should be provided as a control measure by the official veterinary service.
Actual	Is vaccination coverage as it should be or very variable? Is a ready supply of the vaccine rapidly available? Are there storage facilities for the vaccine at field level? Are vaccine campaigns of adequate duration? Is vaccination supplied and applied by the official veterinary service?
Constraints (on achieving the ideal)	To what extent do the following prevent achieving the ideal and how can their influence be reduced: • perceived cost to the stock owner; • perception of failure from the cycle of "free" vaccination → retroactive immunization → perceived failure → loss of confidence in vaccination on the part of the stock owner.
Importance of vaccination	Vaccination and safe disposal of carcasses are the hub of anthrax control in endemic areas.

Note. For maximum success, vaccination as a control measure should be applied *together with* other control measures and continued for a full specified period (frequently three years is the period specified in a region with a history of regular or periodic outbreaks). Attention needs to be paid to problems that may arise if antibiotics are being administered to the animals for any reason (see section 7.2.1.3, Annex 6, section 1.4). It makes sense in many endemic regions to use combined vaccines, such as BQ/Anthrax vaccine.

Vaccines are generally available (see Annex 5). Problems largely relate to the attitudes of farmers (Dietvorst, 1996a), cost, and logistics of carrying out and evaluating the effectiveness of vaccination campaigns. Examples of the sort of resistance on the part of farmers to vaccination, and the rationale for this resistance, are well covered by Dietvorst (1996a). As a first example, vaccination initiated in response to an outbreak has led to the situation in which the vaccine has been administered to animals already infected and which have died shortly after. This results in, at best, loss of faith in the vaccine and, at worst, a belief that the vaccine killed the animal(s). A second example is a belief that animals must be rested for two weeks after vaccination; frequently farmers feel they are unable to cease work for this period. The earliest identification of anthrax infection and application of annual vaccination of susceptible animals before the anthrax season in endemic areas will overcome most of these negative attitudes.

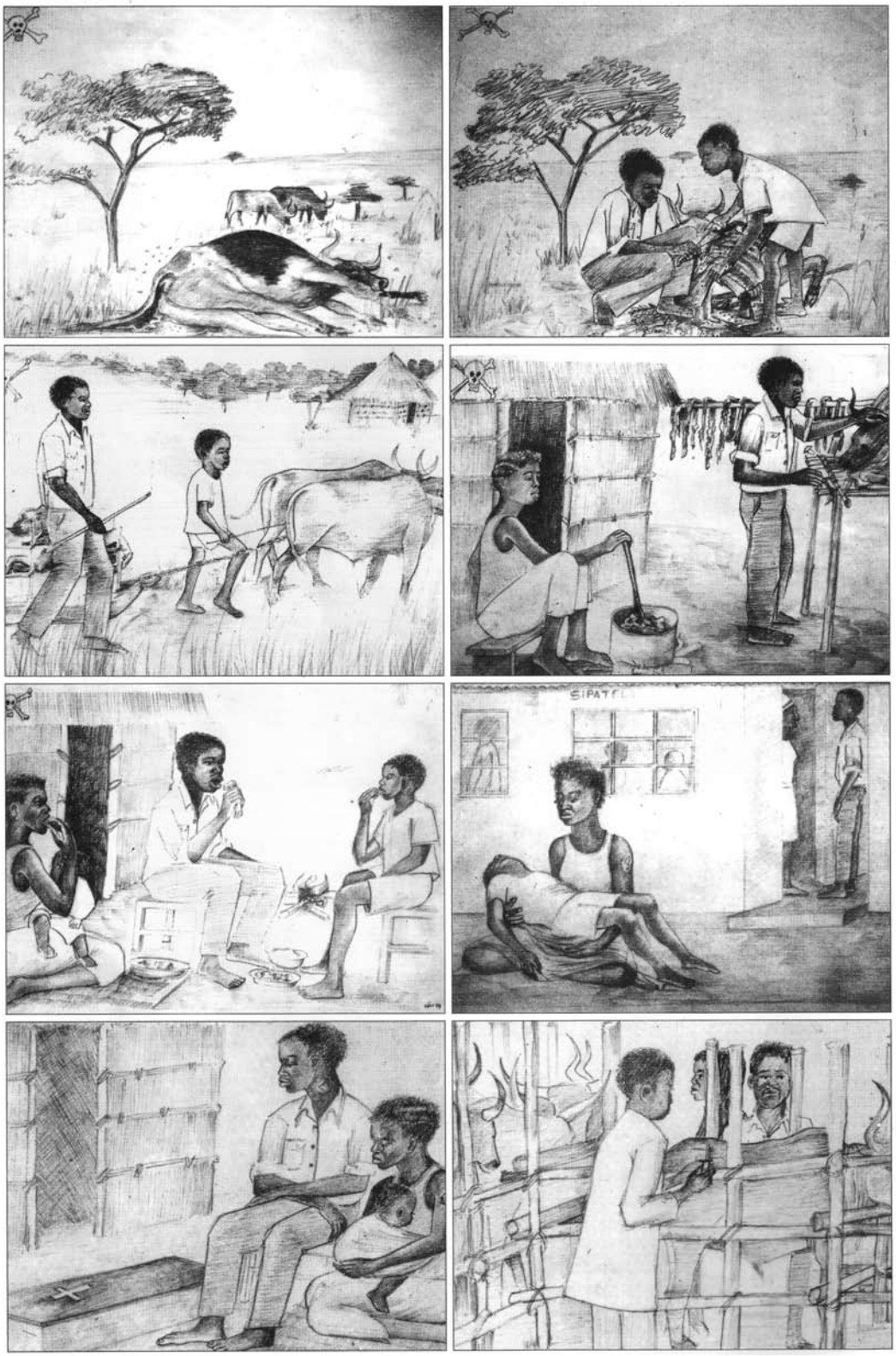
TABLE 11

Education	
Ideal	Educational material on anthrax should be supplied to field or other appropriate stations for distribution in the affected community.
Actual	Have booklets, posters, radio programmes, videos, etc. been prepared? Have they reached the relevant communities (see Fig. 13)? Is there a need for continuation, expansion (more issues covered) and extension (more affected people reached)?
Constraints (on achieving the ideal)	To what extent do the following prevent achieving the ideal and how can their influence be reduced: • distances involved and inaccessibility of some communities; • printing, paper or production costs; • dissemination/distribution costs.
Importance of education	The community gains a better understanding of the rationale and the limitations of control actions.

Note. Inherent in all the control measures is the underlying need for educational programmes and materials. Of foremost importance is education of the farmer/owner to recognize and report suspected anthrax and take proper action over the disposal of the carcass. Appropriate education should also be available for instruction of veterinary, medical and other officials in confirmation of diagnosis and correct action thereafter. Finally, the community itself should understand the rationale as well as the limitations of control actions. The clear need for information broadsheets, manuals, videos and films for disseminating information at courses, seminars and village meetings has been addressed in an exemplary manner in Zambia (Dietvorst 1996a), where posters, booklets and a radio play (Dietvorst, 1996b) have been developed (Fig. 13).

Fig. 13 Educational material (opposite)

The pictures are from a book, and also featured on a poster, conveying the story of the problems the fictional Liseli family suffered following the death of their cows from anthrax. The message to farmers/owners of livestock is that they should not handle and butcher the carcasses of animals that have died unexpectedly or eat meat from such carcasses, but rather that they should call a veterinary official to supervise correct disposal of the carcass. (Source: Dietvorst, 1996b.)


SIPATI

Annexes

ANNEX 1

Laboratory procedures for diagnosis of anthrax, and isolation and identification of *Bacillus anthracis*[1]

1. Anthrax and the microbiology laboratory; operational safety

With some country-to-country variation in safety level definitions and requirements, recommendations for the manipulation of the causative agent of anthrax, *Bacillus anthracis*, generally are that BSL (biosafety level) 2 practices, containment equipment and facilities are appropriate for diagnostic tests, but BSL3 standards should be used when the work involves:

- producing quantities of the organism;
- activities with high potential for aerosol production.

and possibly also:

- activities with antibiotic-resistant strains.

In the case of Member States with limited resources and unable to operate at BSL3, it is pertinent to remember that *B. anthracis* is not highly infectious, and that humans are moderately resistant (see section 4.2.1). For diagnostic test purposes, therefore, good laboratory practice (Table 12) at all times is the important factor in carrying out the necessary tests safely. Large numbers of the organism should not be generated in uncontained laboratory situations, and manipulation of liquid cultures or suspensions should be kept to a minimum. It may be appropriate to distinguish between fully virulent strains and those lacking virulence factors, such as vaccine strains.

Only a few confirmatory tests require liquid suspensions, e.g. preparing smears, testing for capsule production in blood and suspension of the organism for PCR (polymerase chain reaction). Small volumes (< 2.5 ml) are needed for these tests.

Work for further tests, such as bacterial counts, sterility tests, etc. involving liquid cultures should be done in biological safety cabinets in laboratories meeting as nearly as is possible the criteria for basic BSL3, or at least BSL2, laboratories. These criteria are readily obtained on the Internet.

2. Principal features of good laboratory practice

1. Use and storage of personal protective equipment (PPE):
   - Individuals should wear gowns or laboratory coats with elastic cuffing, and disposable gloves.
   - Gowns/coats should be hung in a specified place near the entry to the laboratory and should be cleaned or disposed of at suitable intervals or when defective.
   - PPE should be removed on leaving the work area and should not be worn outside the laboratory.
2. The laboratory door should be kept closed.
3. Appropriate disinfectant (usually hypochlorite solution, 10 000 ppm – see Annex 3, section 4.1) should be prepared freshly on a regular basis:
   - this should be available, ready for immediate use in the event of a spillage;
   - benches should be wiped down with the disinfectant after work is completed.
4. Appropriate containers (generally screw-capped, non-breakable) should be used for specimens, cultures, etc., and proper carriers or secondary containers should be used for moving cultures around the laboratory.
5. Eating, chewing, drinking, taking medication, smoking, applying cosmetics and mouth pipetting are strictly prohibited in the laboratory.
6. Storage of contaminated materials should be done safely:

[1] Significant use has been made in this annex of the operating procedures drawn up for the *Manual for laboratory diagnosis of anthrax* (WHO, 2003) recently produced by the WHO Regional Office for South-East Asia.

TABLE 12

Criteria, equipment and materials for laboratory diagnosis of anthrax

LABORATORY DIAGNOSIS	CAPABILITY LEVEL	CONDITION	EQUIPMENT AND MATERIALS NEEDED
Suspect	Peripheral Intermediate Central/reference	The smear shows Gram-positive, square-ended rods in pairs or short chains, occasionally singly, in association with a suggestive clinical history.	Specimen transportation kits Sterile swabs Microscope Microscope slides and cover slips Immersion oil and lens paper Ethanol (or methanol) ≥ 95% Stains: polychrome methylene blue stain (quality controlled by reference laboratory for capsule staining); Gram stain Test tubes/screw-capped bottles Gloves, high quality particle-filtering face masks, gowns, Bunsen burner or spirit lamp Disinfectant spray "gun" Disinfectant: sodium hypochlorite (bleach) Autoclave (and spore disks/strips)[a]
Presumptive	Intermediate Central/reference	Smear stained with polychrome methylene blue shows dark blue square-ended rods in pairs or short chains, occasionally singly, surrounded by pink capsule. Primary culture has typical characteristics. Other helpful tests or antigen-detection devices based on protective antigen are becoming available.	As above, plus biological safety cabinet preferably with fumigation capability Incubator Centrifuge Water bath CO_2 incubator/candle jar Equipment for culture: loops, Petri dishes, pipettes and tips, screw-capped bottles or tubes, flasks, etc. *Media and reagents* culture media (blood agar, nutrient agar, heart infusion agar, brain-heart infusion broth, etc.) PLET agar (and/or other selective agar) ingredients other stains – spore stain gamma phage (quality-controlled by reference laboratory for efficacy) penicillin discs defibrinated horse blood (blood from other species also suitable) horse serum (serum from other species also suitable) sodium bicarbonate Sterne vaccine strain of *B. anthracis* for controlling phage and penicillin tests or, if possible, a wild-type isolate for controlling phage, penicillin and capsule tests *Further disinfectants* formalin (38%–40% formaldehyde solution) or paraformaldehyde with neutralizer ammonium carbonate/bicarbonate Antigen detection devices if available
Confirmed	Intermediate, if suitably equipped Central/reference	Confirmatory tests show culture is *B. anthracis*. PCR confirms presence of toxin and capsule genes.	As above, plus Hazard-level 3 laboratory[b] with biological class 3 safety cabinet equipment and materials for PCR media and materials for antimicrobial susceptibility testing room fumigation capability when necessary.

[a] The destructive function of the autoclave should not be assumed but should be checked with a spore disc or strip (available commercially).

[b] The ideal is a BSL (biosafety level) 3 facility containing a class 3 cabinet. In some laboratories, a class 2 cabinet with respirator in a BSL 3 facility or a class 3 cabinet in a BSL 2 facility, are the less ideal alternatives.

- "Tools" (pipettes, tips, loops, spreaders, etc.) should be housed safely after use, awaiting autoclaving (e.g. in strong autoclavable bags) or fumigation, or they should be fully immersed in jars of disinfectant (10% bleach or formalin).
- Contaminated items and materials awaiting reuse or disposal should be housed in strong leak-proof containers, preferably within autoclavable bags. Spillage within these should be avoided.

7. Infectious disposable waste (see 6 above) should be autoclaved, preferably followed by incineration also. Reusable articles should be autoclaved, fumigated or otherwise sterilized before cleaning.
8. Procedures should be performed so as to minimize production of potentially contaminated aerosols or dusts.
9. Hands should be thoroughly washed with soap and water before leaving the facility, using disposable towels or an air drier after washing. (Ideally a hand-washing basin should be sited by the laboratory door, adjacent to the hooks for laboratory gowns/coats.)
10. An accident and incident emergency plan should be in place.
11. Laboratory workers should receive initial and regular revision training on the above.

3. General bacteriology of anthrax

The basic bacteriology of *B. anthracis* and its identification characteristics have been described in chapter 6. Further practical information is given here. *B. anthracis* is a non-fastidious, facultatively anaerobic bacterium organism which grows readily on simple laboratory media. The optimum temperature for growth is 35–37 °C.

3.1 Nutrient agar

After overnight incubation at 35–37 °C colonies are large, 2–3 mm in diameter, irregular, raised, dull, opaque and greyish-white with "frosted glass" (ground glass) appearance. Occasionally the colonies may have fringed edges or put out curled protrusions (tailing). This is the so-called "Medusa head appearance" but is not encountered as frequently as textbooks often suggest, and varies from batch-to-batch of media. The colony is notably tacky in consistency.

3.2 Blood agar

After overnight incubation at 35–37 °C on horse or sheep blood agar (BA), colonies of freshly isolated *B. anthracis* are white, or grey-white and non-haemolytic, 2–4 mm in diameter, again with a slightly moist, matt appearance. Fringed edges or tailing is sometimes seen as with nutrient agar (Fig. 8B).

3.3 Spores

Spores develop at the end of the log phase of multiplication. For diagnostic purposes, they can generally be visualized in smears of standard laboratory agar plate cultures (e.g. blood agar or nutrient agar) after 20–24 hours of incubation at 35 °C to 37 °C. The spores are central/subterminal, ellipsoidal and do not swell the vegetative cell (Fig. 8C). Strain-to-strain differences occur (section 6.3.1.1). In Gram-stained preparations, the developing spores appear as unstained areas within the cell. With malachite green/safranin (or malachite green/basic fuchsin) staining, the spores are stained green and the vegetative forms are pink (Fig. 8C). In the Ziehl-Neelsen staining, spores are pink and the vegetative forms are blue. When fully mature (dormant), the spores can also be seen as refractile egg-shaped bodies under phase contrast. (See also sections 6.3.1.1 and this annex, sections 9.3.4 & 9.3.5.)

3.4 Capsules

Capsules are not formed during normal aerobic in vitro culture. They can be induced either by growth in bicarbonate agar containing serum under a 5%–20% carbon dioxide atmosphere, or in defibrinated blood or in serum (defibrinated horse blood seems to work best) (see also sections 6.3.1.6 and this annex, section 10.7.2).

3.5 Broth cultures

Growth is frequently very floccular, especially in static cultures, due to the tendency of *B. anthracis* to form long chains in vitro (section 6.3.1.1). Being non-motile, the strands settle as a deposit which comes up as silky strands on shaking the broth gently.

3.6 Selective agars – PLET and TSPBA

Selective media are needed for the isolation of *B. anthracis* from clinical materials or environmental samples heavily contaminated with other bacteria. Bowen (1999) concluded that the best selective sys-

tem was the polymyxin, lysozyme, EDTA and thallous acetate (PLET) agar of Knisely (1966) (Fig. 14). PLET agar was also chosen as the most selective and sensitive (3–5 spores per gram of soil) detection medium for all the work leading up to and following decontamination of Gruinard Island (Manchee et al., 1981, 1983). Dragon & Rennie (2001) recorded that, compared with blood agar, as few as 33% of the viable anthrax spores present in a sample germinated and outgrew, but Bowen (1999) and Samaan & Turnbull (unpublished results) found that losses on PLET were normally nil. The single exception was strain LSU 62, a 1962 bovine isolate from Poland, which, uniquely, did not grow on PLET (Turnbull et al., 2004a). Whether this is a laboratory adaptation phenomenon is not known.

While it would seem that PLET agar, prepared well (Annex 2), is an excellent selective isolation medium for *B. anthracis*, it does generally require at least 36 hours of incubation to read. Its other disadvantage lies in the ingredient, thallous acetate, which is highly toxic and environmentally unfriendly in terms of disposal.

After incubation at 37 °C for 36–48 hours, the colonies of *B. anthracis* are 2–3 mm, roughly circular, creamy-white with ground-glass texture. Colonies are usually smaller in size on this medium compared to those on nutrient or blood agar, and lack the tackiness. Tailing edges are not seen.

Trimethoprim-sulfamethoxazole agar medium[1] has been recommended by some. Polymyxin at the same concentration as used in PLET may give extra selective advantage. This medium is referred to here as TSPBA; instructions for its formulation are given in Annex 2. *B. anthracis* colonies are recognizable earlier on TSPBA than PLET and at the same rate as conventional blood agar; being a blood agar, it retains the value of showing up haemolysis in the case of any haemolytic species that break through.

B. anthracis growth and colony morphology on TSPBA are indistinguishable from those on BA (sections 6.3.1, and this annex, section 3.2; Fig. 8B).

3.7 Bicarbonate agar

Colonies of fully virulent isolates are mucoid in nature on this medium when incubated overnight under CO_2 due to capsule formation. Vaccine strains lacking the capsule genes, such as the Sterne strain, are rough (see this annex, section 10.7.2.2; Fig. 8D). The formula for bicarbonate agar is given in Annex 2.

4. Special features

4.1 Susceptibility to penicillin G

Fresh isolates of *B. anthracis* from cases of anthrax are almost always susceptible to penicillin (see section 7.1.2). In its simplest form, this involves spreading a portion of a nutrient or blood agar plate with the culture under test, and placing a 10U penicillin disc at some point within the area of spread. The zone of susceptibility will be visible after overnight incubation at 35–37 °C (Fig. 8F; see also this annex, section 10.7.1).

4.2 Susceptibility to the diagnostic ("gamma") bacteriophage

The diagnostic ("gamma") phage has the ability to lyse *B. anthracis* grown aerobically on blood or other nutrient agar and rarely lyses any other *Bacillus* species. Phage-resistant isolates are encountered, but this is rare (see section 6.3.1.5). There are a variety of ways this test can be done, but the simplest is to pick the suspect colony to a segment of a blood agar plate with a 1 µl inoculating loop as shown in Fig. 8F and place a 15 µl drop of phage suspension in the centre of the area over which the loop has been spread. The plate is incubated overnight at 35–37 °C (see also this annex, section 10.8.1).

4.3 Animal pathogenicity test

Definitive identity of a suspect *B. anthracis* isolate used to be done by inoculating the organism into a mouse or a guinea-pig and confirming the cause of death by smear or isolation. However, for ethical reasons animal inoculations are only done now under exceptional circumstances (see also this annex, section 12).

5. Case definition based on laboratory findings

For the purposes of investigation and control activities, the diagnostic definitions in Table 12 are proposed for anthrax. This is based on the model outlined in the *Manual for laboratory diagnosis of anthrax* (WHO, 2003) in which laboratories in any country can be broadly subdivided on the basis of their resources and capabilities into: (i) peripheral (district), able to receive clinical samples and carry out basic procedures by which to establish initial

[1] As found on http://www.ourfood.com/Anthrax.html.

"suspect" diagnosis of anthrax so that immediate control measures can be instituted; (ii) intermediate (regional/provincial), which may be equipped to biosafety level 2; and (iii) central (reference) able to reconfirm identifications and perform further tests.

6. Sample processing and containment

6.1 Type of specimen

The approach taken will depend on the type of specimen being examined which, for the purposes of examination, will fall broadly into: (i) fresh specimens from untreated animals or humans; (ii) specimens from treated animals or humans; (iii) specimens from old and decomposed animal carcasses or from animal products; or (iv) environmental specimens, including those from suspected deliberate release events.

6.2 Clinical specimens and materials

Specimens from lesions or from freshly dead humans or animals may be handled at hazard levels lower than level 3 (see this annex, section 2) with the following safety precautions:

- use of adequate protective clothing (gloves, gowns with tight wrists and ties at the back). If the samples are not being processed in a safety cabinet, protective eye-shields and good-quality face masks may be advisable to protect the operator from other (non-anthrax) infectious agents that might be present;
- availability of high-quality, properly positioned facilities for hand-washing;
- careful dressing of skin abrasions.

Old dried-up specimens, such as old hides, that are liable to give off dust during processing, should be handled in a biosafety cabinet, preferably class 3.

6.3 Environmental and suspect deliberate release samples

Environmental samples from sites suspected of having been contaminated naturally (e.g. carcass sites) are best handled in a biological safety cabinet. Samples under suspicion of being artificially contaminated *must* be handled in a biosafety cabinet, again, preferably a class 3 cabinet. Suspect powders *should strictly* be processed in a well-constructed class 3 safety cabinet. A deliberately contaminated environmental sample is potentially very dangerous, and the processing of suspect environmental samples should be restricted to a proper hazard-level 3 laboratory with the correct facilities, most likely in the central/reference laboratory.

7. Specimen collection

7.1 Equipment and materials for specimen collection

7.1.1 All-purpose kit

In the case of human specimens (Table 13), these must be collected by the attendant medical professionals.

The following list is for guidance in relation to specimen collection:

- leak-proof specimen containers, wide-mouth in the case of environmental samples;
- secondary containers for "double-bagging";
- secure carrying containers (e.g. good-quality cool box, metal box, plastic mailing pots, etc.);
- sterile swabs, forceps, scissors, syringes (1 ml) and needles (approx. 19 gauge), spatulas or spoons;
- sterile water and/or saline;
- microscope slides and slide carriers;
- culture plates and inoculating loops (if appropriate to make primary culture at the site);
- "sharps" disposal containers;
- labels and markers or pens;
- adhesive tape;
- autoclavable discard bags for disposables;
- autoclavable discard bags for tools, clothing, boots, etc.;
- tock hypochlorite solution and water to make up working solution (5000–10 000 ppm) and hand-washing facilities (e.g. large water container and basin);
- paper towels.

7.1.2 Personal protective equipment

7.1.2.1 Specimen collection from a human or animal situated indoors

- Laboratory coat, gown or overall, as appropriate to the situation, should be worn. Sleeves should be long with elastic cuff.
- Double disposable gloves and (if appropriate, e.g. large animal dead on floor) overshoes or sterilizable boots should be used. The outer gloves should be changed as necessary to avoid spreading contamination. Skin should not be exposed between the gloves and the sleeves.

TABLE 13

Guidelines on appropriate specimens from humans suspected of being infected with *B. anthracis*

CLINICAL PICTURE	SPECIMEN[a]	QUANTITY	CONTAINER	OTHER ACTION
Cutaneous anthrax	Vesicular fluid	3	Sterile swabs	Make a smear with one of the swabs for M'Fadyean (capsule) test. Use other for culture. Test with antigen detection device if available.
Inhalational/ pulmonary anthrax	Blood	10 ml	Blood collection tubes (anticoagulant not needed)	Make a smear with a drop of the blood for M'Fadyean (capsule) test as well as culture. Test with antigen detection device if available.
	CSF	0.5 ml	Sterile screw-capped container	Make a smear for M'Fadyean (capsule) test as well as culture.
	Nasal swab	2	Sterile swabs	Culture.
Gastrointestinal anthrax[b]	Blood	10 ml	Blood culture bottles	Make a smear with a drop of the blood for M'Fadyean (capsule) test as well as culture. Test with antigen detection device if available.
	Ascitic fluid	2 ml	Sterile screw-capped container	Make a smear with a drop of the fluid for M'Fadyean (capsule) test as well as culture. Test with antigen detection device if available.
	Peritoneal fluid	2 ml	Sterile screw-capped container	Make a smear with a drop of the fluid for M'Fadyean (capsule) test as well as culture. Test with antigen detection device if available.
Anthrax meningitis	CSF	0.5 ml	Sterile screw-capped container	Make a smear for M'Fadyean (capsule) test as well as culture.
	Blood	10 ml	Blood culture bottles	Make a smear with a drop of the blood for M'Fadyean (capsule) test as well as culture. Test with antigen detection device if available.

[a] Where possible collect specimen before administration of antibiotics to the patient.
[b] It may be appropriate to investigate suspected oropharyngeal anthrax in the same way as cutaneous anthrax.

- Existing cuts or abrasions should be dressed before putting on personal protective equipment (PPE).

7.1.2.2 Specimen collection from animals in the field

See Table 14 for guidance on appropriate specimens to collect.

Preferably a veterinarian or microbiologist trained in handling disease-causing agents should do the sample collection. This may not always be possible, or only possible with a substantial delay, and farmers/ owners/managers may have to collect the samples. The following advice aims at covering either situation:

- You will need an apron or coverall if you anticipate extensive handling of the carcass.
- You will also need disposable covers for your hands and feet (see below) and strong bleach solution (10 000 ppm).
- Dress cuts or abrasions on exposed areas, especially hands and arms.
- The professional approach is to wear apron or coverall, disposable gloves and overboots, or boots that can be disinfected. It may be appropriate to wear two pairs of disposable gloves (double gloving); the outer gloves can then be changed as and when needed without exposing the hands. Minimal alternatives are strong plastic bags as overboots and, for the hands, evert a plastic bag, insert the hand that will touch the carcass into the everted bag and grasp tissue to be sampled; insert swab, or cut off sample with other hand; reverse bag over sample or swab and seal and label the bag. In the case of cutting off a piece of tissue, insert the cutting implement into another plastic bag for transport to where it can be disinfected (strong bleach for 1 hour) or sterilized (boiled for 30 min or pressure cooked for 15–20 min).

TABLE 14

Guidelines on appropriate specimens from animals suspected of having died from anthrax

CIRCUMSTANCE	SPECIMEN	CONTAINER	OTHER ACTION
Fresh carcass	Blood from vein (0.1 ml) or, if opened (e.g. by scavengers), blood and fluid from body cavity or piece of highly vascularized tissue (usually ear clipping).	Small vial, or leave in syringe.	Use for smear and culture.[a] The smear can be prepared on the spot. Test with antigen detection device if available.
Putrefied carcass	Piece of highly vascularized tissue and swabs of vascularized regions (nostrils, eye socket, any bloody material). Bloody soil from under head or tail.	Swab tubes. For soils, sealable specimen container.	Culture animal specimens on BA (preferably with polymyxin) and selective agar. Culture soil on selective agar.
Very old carcass, hides, bones, soil around/ under carcass, etc.	Swabs of nostrils, eye sockets. Soil from where body fluids believed to have fallen.	Swab tubes. For soils, sealable specimen container.	Culture on selective agar.

[a] Smear and culture should be done within hours of collecting blood. Vegetative cells disintegrate in blood held for much more than a day. If a delay in reaching the laboratory is expected, the smear should be made on a slide immediately after collection and the blood should be collected on a dry swab. This will encourage sporulation of the *B. anthracis* on the swab, which is then reliable for culture for long periods.

- After specimen collection, discard disposable items into disposal bags for subsequent sterilization or incineration. Similarly, non-disposable items should be put into discard containers for subsequent sterilization or disinfection. Care should be taken to ensure that sharp objects are in a container they cannot pierce easily. The containers themselves should be sterilized, incinerated or disinfected.
- Wash hands thoroughly with soap and water.

7.1.2.3 Environmental samples

- Disposable or reusable apron or coverall (as appropriate to the potential hazard of the sample) should be worn.
- Where the possibility exists of aerosolizing and inhaling dust, a respirator is advisable, preferably a quality assurance tested full-face respirator. (*Caution: the operator should be trained by a qualified person in correct wearing and use of the respirator.*)
- For samples related to known or suspected deliberate release, a quality assurance tested full-face respirator should be regarded as mandatory. (*Caution: the operator should be fitted and trained by a qualified person in correct wearing and use of the respirator.*)
- Double disposable gloves and overshoes or sterilizable boots should be worn. The outer gloves should be changed as necessary to avoid spreading contamination.
- Existing cuts or abrasions should be dressed before putting on PPE.

7.2 Safety procedures for specimen collection

7.2.1 During specimen collection

- Before specimen collection, put on the chosen clothing, including double gloving. Ensure disinfectant, disposal bags and hand-washing equipment are ready.
- Existing cuts or abrasions should be dressed before putting on PPE.
- After specimen collection, rinse or wipe down gloved hands with 10% hypochlorite solution and discard outer gloves.
- Discard used PPE into disposal bags, separating autoclavable and non-autoclavable items. Inner gloves should be discarded last. Sharps should be placed in a sharps container.
- Wash hands.

7.2.2 Using disinfectants, fumigants, etc.

- Prepare hypochlorite solutions (10 000 ppm) freshly every day. Preferably handle sodium hypochlorite wearing gloves and eye protection. Avoid spilling it on clothes. Remember it corrodes ferrous metals.
- Use formalin in well-ventilated areas, wearing gloves and face shield while handling it. If handling it in an enclosed space with little ventilation, or if large volumes are involved, a full-face

chemical respirator should be worn (*the operator should be fitted and trained by a qualified person in correct wearing and use of the respirator*). It is injurious to skin and mucous membranes.

7.3 Labelling

The following information should be recorded:

- a reference code or number marked in indelible ink on the container;

and, either on the container or on a sample documentation sheet:

- the date and time of sampling;
- the location of the sampling point;
- the type of sample;
- the reason for sampling;
- the identity of the person taking the sample.

7.4 Collection of human specimens for anthrax diagnosis

To a great extent, the specimen that can be collected, or that will yield *B. anthracis*, will depend on the condition of the patient and stage of the disease. For example, it may not be possible to isolate *B. anthracis* from the vesicular fluid of a cutaneous lesion if the patient has been treated, and vesicular fluid may no longer be available if a cutaneous lesion is older than 3–4 days. As another example, it will not be possible to isolate *B. anthracis* from blood until the very last few hours of life. With this proviso, Table 13 provides guidelines.

7.5 Collection of animal specimens for anthrax diagnosis

Legislation in most countries forbids postmortem examination of animals that have died of anthrax. Animals that have died suddenly and unexpectedly should not be necropsied unless anthrax has been ruled out as the cause of death (see section 3.5.2).

7.6 Collection of environmental samples for examination for B. anthracis

- Exposed surfaces are swabbed with moistened swabs, which are "double-bagged" (see also this annex, section 7.7) and sent to the laboratory.
- Water is collected by means of a syringe without needle and double-bagged.
- Food samples are collected with sterile spoons or other suitable sterile collecting devices into small sterile containers and double-bagged.
- Soil samples are collected with sterile spoons or other suitable sterilized tools into sterile, sealable containers (e.g. specimen cups with screw-on lids) and double-bagged.
- The cautions outlined in this annex, sections 6.3 and 7.1.2.3, pertain to dust samples, or suspect powders. For most purposes, swabs or sterile gauze "wipes", premoistened with sterile water, are best. Dry swabs may be used if there is special reason not to use wet ones, such as not damaging evidence, but they will only collect small amounts of sample. The swabs are transferred to an appropriate container and double-bagged. It may also be possible, depending on the circumstances, to transfer dust to a sterile container with a sterile spatula; this should obviously be done carefully so as not to create aerosols. If vacuum collectors, purpose-designed to collect these types of sample into Hepa filter collectors, are available, then these should be the method of choice.

7.7 Containment for transport ("double-bagging")

- The specimens should be collected into sterile containers as indicated in Tables 13 &14, using aseptic techniques.
- The containers should be wiped down with hypochlorite (10 000 ppm) and, with outer gloves changed first, put into an outer, secondary container (double-bagged). If the secondary container is a plastic bag, then this should be of good quality. It should, in turn, be sealed and, for transport, be put into a good-quality cool box or a strong plastic or metal container with a lid that can be made secure.
- The secondary and outer containers should bear the relevant hazard labels.

Generally, specimens should be stored at 2–8 °C. Preferably they should be transported in cool boxes, especially in hot weather and when the time interval between collection and delivery to the laboratory is likely to be more than 1–2 hours.

The use of dry swabs for samples which cannot be examined immediately is covered in this annex, section 8.1 and Table 14.

For shipping of samples by mail or courier, the appropriate procedures with relevant paperwork must be followed.

7.8 Disinfection, decontamination and discard

Basically all specimens and used disposables should be autoclaved when finished with. Whether in the laboratory or in the field, these should have been collected into autoclavable bags or other suitable containers which are then autoclaved at 121 °C for ≥ 1 hour, preferably followed by incineration.

Contaminated autoclavable non-disposable items should also be deposited in autoclavable containers and ultimately autoclaved.

Microscope slides, coverslips and other sharp items should be placed in autoclavable sharps containers and autoclaved, preferably followed by incineration.

There may be circumstances where it is appropriate to immerse items in hypochlorite solution (10 000 ppm) initially and then to autoclave and incinerate them later.

Disinfect or fumigate non-autoclavable materials (see Annex 3, section 3.3).

Laboratory clothing should be autoclaved before being sent to the laundry. Non-disposable boots should be washed down into an autoclavable basin or bucket, and the washings autoclaved. The boots then should be disinfected by immersion in hypochlorite (10 000 ppm available chlorine) or 10% formalin (see Annex 3, section 6.5) and allowing them to dry for about 30 min before reuse.

7.9 Fumigation/UV

Equipment that cannot be autoclaved, boiled or immersed in disinfectant solutions should be fumigated (see Annex 3, section 3.3). Where fumigation is not readily achieved and a safety cabinet fitted with a UV light is being used, this should be utilized applying the same principles of arranging the items to be sterilized in such a way as to ensure the UV light reaches into and around them to maximum extent (see Annex 3, section 3.3.1). UV should not be relied on alone for decontamination, but should be used in conjunction with wiping the items to be decontaminated with towelling moistened with hypochlorite or possibly formalin, paying attention to the cautions given in Annex 3, section 1.1.

Ideally, cabinets and rooms should be fumigated when suspected of being contaminated. Where this is not possible, they should be given a very thorough floor-to-ceiling wipe-down with hypochlorite solution (10 000 ppm).

8. Types and conditions of specimens versus ease of diagnosis

8.1 Fresh specimens from untreated animals or humans

Few difficulties should be encountered in: (i) identifying *B. anthracis* in M'Fadyean capsule-stained smears of blood, lymph or oedematous fluid from untreated animals shortly before or within one or two days after death from anthrax; or (ii) isolating *B. anthracis* from these types of specimen. Similarly, the bacteria should be readily visible in, or isolated from, vesicular fluid before treatment in humans or, so long as no treatment was given, from body fluids near to death or post mortem.

It should be noted, however, as indicated in the footnote to Table 14, that smears and culture should be done within hours of collecting blood. Vegetative cells disintegrate in blood held for much more than a day. If a delay in reaching the laboratory is expected, the smear should be made on a slide immediately after collection, and the blood should be collected on a dry swab. This will encourage sporulation of the *B. anthracis* on the swab, which is then reliable for culture for long periods

8.2 Specimens from treated animals and humans

Treatment of an animal suffering from anthrax may sterilize the blood and tissues even though the animal may go on to die from the effect of the toxin. Similarly, cutaneous lesions in humans will be quickly sterilized by treatment but will continue to pass through their stages of evolution and resolution (section 7.3.1.5). (See also sections 3.5.3 and 4.4.4.2.)

Residual forms of the capsulated bacilli may be visible in fluid smears from such animals or persons. Isolation attempts may be unsuccessful. Confirmation of diagnosis may be possible with a sensitive antigen-detection device for the toxin. It is hoped this will become more widely available in the future. In cases of extreme need to confirm diagnosis, a last-resort approach may be use of mice or guinea-pigs to isolate *B. anthracis* (this annex, section 12).

8.3 Specimens from old or decomposed animal specimens, or from animal products or environmental specimens

The problem likely to be encountered with this group of specimens is that detection will frequently involve a search for relatively few *B. anthracis* among many

other *Bacillus* species, particularly *B. cereus* (section 6.2). The selective approach covered in this annex, section 10.4 is necessary.

Environmental samples may vary greatly in their composition (different kinds of soil, water and wastewater, biowaste, food and feedstuff), in their content of toxic materials (e.g. organic or inorganic residuals used in tanneries), and the competing bacterial flora. Extensive experience in Germany (Böhm & Beyer, personal communication, 2005) has shown that environmental samples may contain substances which inhibit germination and growth of *B. anthracis*, and therefore appear negative on culture. Such conditions may result in false negative results if a sample-specific positive control is not included. Therefore in general all sample materials are best divided into two parts before processing, one being the true sample and the other used as the spiked positive control. In order to determine the limit of detection of the culture procedure, further portions of the sample may be spiked with tenfold dilutions of spores. In sufficiently contaminated materials, the effect of the inhibitor may be eliminated and the contaminating *B. anthracis* detected by suitable dilution of the initial sample suspension.

9. Microscopy for anthrax

9.1 Equipment and materials

The following equipment and materials will be needed:

- binocular microscope with good oil immersion lens
- microscope slides and cover slips
- pen/pencil/diamond pen/label as appropriate to label slides
- sharps container
- wash bottles and water, preferably deionized or distilled
- alcohol, 95%–100%
- immersion oil
- stain tray with slide holder
- inoculating loops
- Bunsen burner or spirit lamp
- Pasteur pipettes, preferably plastic disposable
- paper towel or other absorbent paper
- plasticine
- lens tissue
- autoclavable discard bag
- stock hypochlorite and working bottle for 10 000 ppm solution, preferably a spray bottle
- stains:
  - Gram stain
  - polychrome methylene blue (M'Fadyean).

9.2 Safety measures

- Wash the stain off with water into a tray containing hypochlorite solution (10 000 ppm). Leave overnight before discarding, or autoclave.
- Discard the used slides into the sharps container, which is autoclaved.
- Incinerate/autoclave or otherwise decontaminate other used items of equipment.
- Avoid contaminating the microscope, e.g. by changing outer gloves at appropriate times.
- Decontaminate the safety cabinet after use (see this annex, section 7.9 and Annex 3, section 3.3)

9.3 Preparation and staining of smears

9.3.1 Clinical (human or animal material)

- Make two thin smears of clinical/animal material by rolling over the swabs or spreading a small drop on a microscope slide, using a coverslip to do the spreading. The smear should be approximately 1.5 cm square and should not run to the edges or either end of the slide. The thinner the smear, the better (avoid thick smears).
- Air-dry.
- Fix by dipping in 95%–100% alcohol for one minute and redry.
- Stain one smear with Gram stain (this annex, section 9.3.3) and the other with polychrome methylene blue stain for the demonstration of capsule (M'Fadyean stain) (this annex, section 9.3.6).

9.3.2 Smears from cultures

- Transfer some growth from the primary isolation plate to about 0.5 ml of saline and emulsify to give a slightly cloudy suspension. If the culture is already in suspension (e.g a broth culture), transfer one or more loopfuls to about 0.5 ml saline, again to produce a slightly cloudy suspension.
- Using a 1 µl loop, transfer a drop of the suspension to a microscope slide and spread the drop well.
- Allow to dry and fix with heat or dipping in 95%–100% ethanol for one minute.
- Allow the ethanol to evaporate off and stain as required.

9.3.3 Gram stain

- Follow the standard method, washing off into hypochlorite solution (10 000 ppm) at each stage.
- Observe the typical morphology of the bacillus: in clinical material *B. anthracis* are Gram-positive thick, long, straight bacilli with square or truncated ends with parallel sides found usually single, in pairs or chains of 3 or 4 bacilli. The chain of bacilli with truncated and swollen ends gives a characteristic "bamboo stick" appearance. A further description is given in section 6.3.1.1.
- Remember this is not a suitable stain for demonstration of capsule.

9.3.4 Modified Ziehl-Neelsen stain for spores

- Air-dry and heat or alcohol-fix the smear.
- Cover the smear with carbol fuchsin.
- Heat for 3–5 minutes; do not allow the stain to boil.
- Wash off stain with water using wash bottle (into hypochlorite solution).
- Decolourize with alcohol until all traces of red are removed.
- Wash off stain with water using wash bottle (into hypochlorite solution).
- Counterstain with methylene blue for 1–2 minutes.
- Wash again (into hypochlorite solution) and allow to dry.
- Observe under oil immersion.

Spores will be stained red and vegetative forms blue.

9.3.5 Malachite green stain for spores

- Dry the films and heat or alcohol fix.

Either:

- place the slide over a beaker of boiling water, resting it on the rim with the bacterial smear uppermost;
- cover with 5% aqueous solution of malachite green;
- stain for 5 minutes, adding more stain solution if the stain covering the smear starts to dry;

or

- place the slide in a moist chamber (a petri dish with moistened filter paper will do);
- cover the film with 5% aqueous solution of malachite green;
- leave to act for 60 minutes.

Then, following either procedure:

- wash off stain with water using wash bottle (into hypochlorite solution);
- counterstain with 0.5% safranin or 0.05% basic or carbol fuchsin for 30 seconds;
- wash again (into hypochlorite solution) and allow to dry.

Spores appear green and the vegetative bacilli red (Fig. 8C)

9.3.6 Polychrome methylene blue stain for capsule (M'Fadyean reaction)

This is the ideal method for demonstration of the capsule:

- Put a large drop of polychrome methylene blue on the smear to cover it completely.
- Leave for 30–60 seconds.
- Wash off stain with water using wash bottle (into hypochlorite solution, 10 000 ppm) and allow to dry.
- When dry, examine under the 10x lens. The anthrax bacilli can be seen as tiny short threads. Switch to oil immersion and look for the capsule, which is seen clearly as pink amorphous material surrounding the blue-black bacilli (Fig. 8A).

A positive and negative control should be included with every test. The positive control will require a wild-type isolate, which should be securely stored, or conceivably the Pasteur ($pXO1^-/2^+$) if this can be acquired. The Sterne vaccine strain is a good negative control.

9.3.7 India ink method for capsule visualization

This is not a true staining method but highlights the capsule as a transparent halo around the bacillus. It is satisfactory with good capsule preparations, such as blood from a freshly dead animal or smears of bacilli from mucoid colonies on bicarbonate agar grown under CO_2 (see this annex, section 10.7.2.2). It may be less sensitive when smaller numbers of anthrax bacilli are present, or when the bacilli are dead and disintegrating as may be the case in specimens from old carcasses, or from animals/humans that were treated before the specimens were collected.

Procedure

Premix a loopful of the blood or other tissue fluid with a small drop of India ink on a clean slide such that a thin layer results when a cover slip is placed on top and pressed down lightly. If the India ink is too dark, dilute appropriately with water. One laboratory supplier of India ink for this purpose is Becton Dickinson Microbiology Systems, Maryland, USA (ref. 261194).

As before, the bacteria can be found by scanning under low power (10x objective) and then examined under oil immersion (100x) for the presence of the capsule.

As with polychrome methylene blue staining, a positive and negative control should be included with every test.

9.3.8 Fluorescent antibody staining for capsule

Mention should be made of the fluorescein-labelled anticapsule system developed by Ezzell & Abshire (1996). This now forms the basis of the capsule visualization tests used by the United States Laboratory Response Network.

10. Bacteriological confirmation

10.1 Equipment and materials

The following equipment and materials will be needed:

- binocular microscope with good oil immersion lens
- microscope slides and cover slips
- pen/pencil/diamond pen/label as appropriate to label slides
- sharps container
- wash bottles and water, preferably deionized or distilled
- alcohol, 95%–100%
- immersion oil
- stain tray with slide holder
- inoculating loops
- Bunsen burner or spirit lamp
- Pasteur pipettes, preferably plastic disposable
- paper towel or other absorbent paper
- plasticine (modelling clay)
- lens tissue
- autoclavable discard bags
- stock hypochlorite and working bottle for 10 000 ppm solution, preferably a spray bottle
- stains:
  - Gram stain
  - polychrome methylene blue (M'Fadyean) (quality controlled by reference laboratory for capsule staining), or other capsule stain
  - malachite green stain, or other spore stain
- culture media (blood agar, nutrient agar, heart infusion agar, brain-heart infusion broth, etc.)
- PLET agar and/or TSPBA ingredients (Annex 2)
- gamma phage (quality controlled by reference laboratory for efficacy)
- penicillin discs
- defribinated horse blood (blood from other species may also be used)
- horse serum (serum from other species may also be used)
- sodium bicarbonate
- incubator (with CO_2 facility ideally)
- water bath
- candle jar
- PCR equipment and reagents if appropriate.

10.2 Safety measures

- Place petri dishes (or other culture containers) in a purpose-designed carrier or secondary container, such as a sandwich box, for movement around the laboratory. The carrier or container should be labelled with the agent, the operator's ID and date.
- Discard the plates/tubes into autoclave bags. Autoclave, preferably followed by incineration.
- Discard used slides and other sharp items into the sharps container which is autoclaved and then, preferably, incinerated also.
- Incinerate/autoclave other used disposable items of equipment.
- Autoclave recyclable item.
- Fumigate or otherwise decontaminate non-disposable items of equipment which cannot be autoclaved.
- Decontaminate the safety cabinet after use (see this annex, section 7.9 and Annex 3, section 3.3).

10.3 Fresh human/animal materials

10.3.1 Smears

- Prepare two smears.
- Gram-stain one (this annex, section 9.3.3), stain the other with polychrome methylene blue (this annex, section 9.3.6) or other appropriate capsule stain.

Gram-positive bacilli in short chains, square-ended and, in the polychrome methylene blue-stained smears exhibiting capsules (Fig. 8A), are definitive. Culture is necessary for further characterization.

10.3.2 Culture

- Inoculate on blood agar (BA).
- Incubate plates at 37 °C for 18–24 hours.
- Read the plates for colony characters.

After overnight incubation, *B. anthracis* colonies are white with frosted glass appearance and non-haemolytic. They may exhibit some or extensive tailing and are exceptionally tenaceous when teased with an inoculating loop. These can now be tested and checked for penicillin and phage-sensitivity, capsule production confirmed and, where facilities are available, checked by PCR for the presence of toxin and capsule genes.

10.4 Old animal specimens, animal products, environmental samples

Caution: if the sample is associated with suspected deliberate release, the testing should be done by appropriately equipped and trained personnel at the relevant reference laboratory.

Old animal specimens, animal products and environmental samples differ from fresh specimens in that large numbers of other environmental bacteria, especially other *Bacillus* species, will be present and will outgrow and mask any *B. anthracis* that may be present, especially if it is present in low numbers. In this case, a selective medium is needed (see this annex, section 3.6 and Annex 2). The procedures given below are summarized in Fig. 15.

It should be noted that opinions differ as to the merit of centrifuging the suspended samples with a view to concentrating any anthrax spores present. Frequently this simply results in the concentration of the competing organisms. Depending on the type of sample, dilution may be more effective in revealing the presence of *B. anthracis* than concentration.

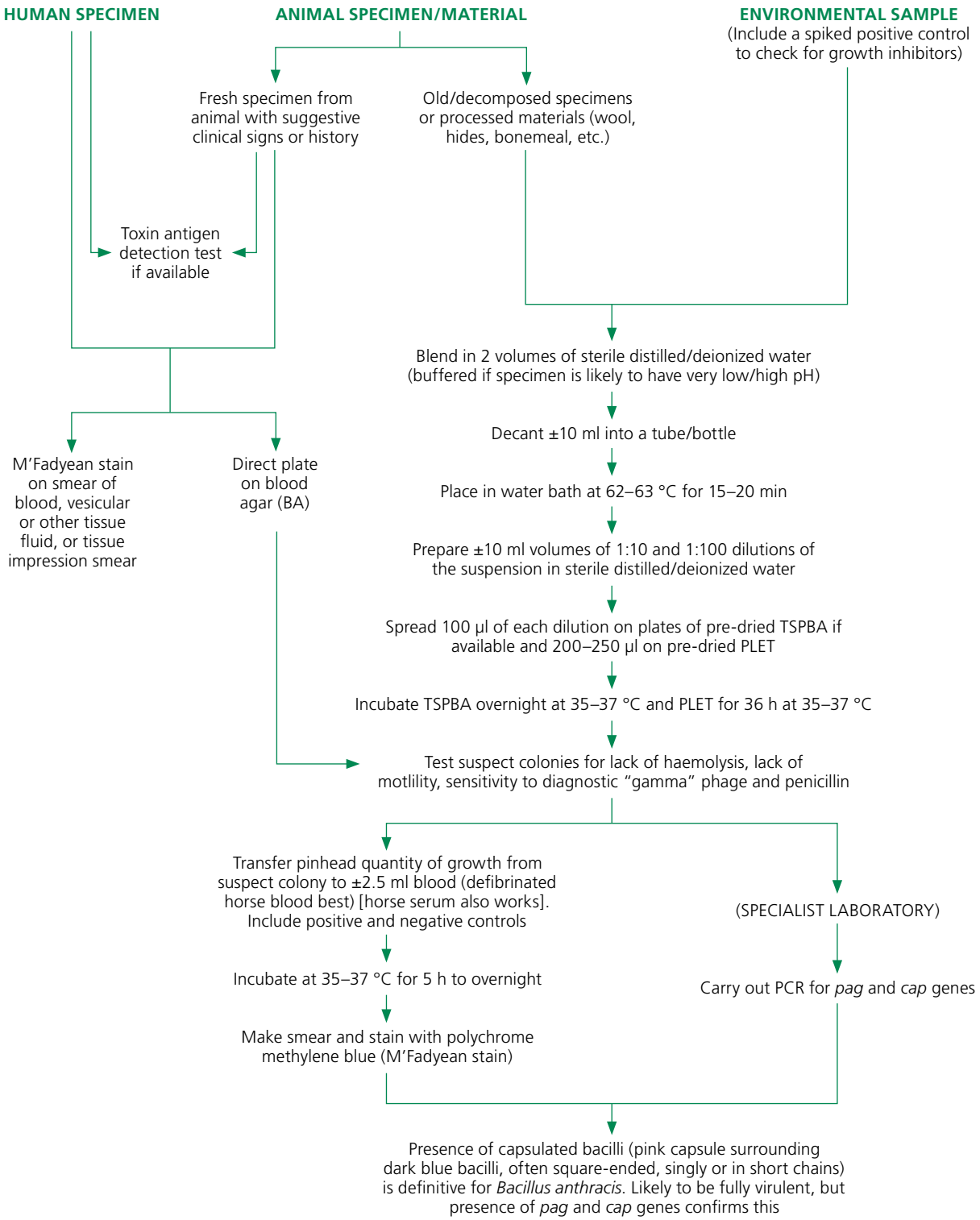
As pointed out in this annex, section 8.3, inhibitory substances may be present in some environmental samples which could prevent germination and growth of *B. anthracis*, and lead to false-negative results on culture. Appropriate controls are therefore needed. For example, a portion of the sample may be spiked with approximately 500–1000 cfu of spores of the Sterne $34F_2$ vaccine strain of *B. anthracis*.

Fundamental to the search for *B. anthracis* in environmental samples is a heating phase. This serves the dual purpose of killing non-sporing organisms that are present and heat-activating (heat-shocking) the *B. anthracis* spores, rendering them more predisposed to germination. Time/temperature combinations for *B. anthracis* spores found in many publications over the past 60 years range from 60 °C for ≤ 90 min to 80 °C for ≤ 30 min, but appear to have been invariably chosen arbitrarily. A recent study by Turnbull et al. (2006), aimed at determining the limits of flexibility that may be assumed in choosing a time/temperature combination for heat-treating *B. anthracis* spores, indicated that temperatures are best kept to ≤ 70 °C with no obvious reason for holding times > 15–30 minutes. Turnbull (Turnbull, 1996; Turnbull et al., 1998a) has normally opted for the conservative combination of 62–63 °C for 15–20 minutes, the time depending on the size of container or volume of liquid that has to be brought up to temperature, and the caution based on fear of losing spores in routine samples with low levels of contamination.

10.4.1 Examination of soil, material collected on a swab or filter, etc.

- Depending on type and quantity of sample and how collected, make an appropriate w/v suspension of the sample in sterile deionized water (SDW), e.g. 1 g of soil in 10 ml SDW, 10 g soil in 100 ml SDW, 1 ml SDW suspension from a swab sample, a membrane filter suspended in 5–10 ml SDW, etc.
- The same is done in parallel for the artificially-contaminated control.
- Both samples are shaken at 4 °C for several hours. If the samples contain a lot of insoluble matter (e.g. soil samples), they may then be filtered through a plug of gauze and the filtrate processed.
- Make 1:10, 1:100 and possibly 1:1000 dilutions by transferring 1 ml volumes to 9 ml SDW.
- Place in a 62–65 °C water bath for 15–20 minutes to kill all vegetative forms, and heat-shock any spores present.
- Spread 100–200 µl volumes of undiluted and diluted suspensions on 3 plates of predried blood agar or TSPBA plates and 250 µl volumes on 3 predried PLET agar plates.
- Incubate blood agar and TSPBA overnight and PLET agar for 36–48 hours at 37 °C. The suspected colonies are further isolated and identified (see this annex, section 10.3.2).

Fig. 15 Flow diagram of suggested procedures for isolation and identification of *B. anthracis* and confirmation of identity



10.4.2 Muddy/polluted water

If the water sample is highly particulate, treat as a suspension of soil (Fig. 15).

- Place approximately 10 ml of the suspended sample in a 62–65 °C water bath for 15–20 minutes to kill all vegetative forms and heat-shock any spores present.
- Spread 100 µl volumes on 3 plates of blood agar or TSPBA plates and 250 µl volumes on 3 PLET agar plates.
- Incubate blood agar and TSPBA overnight and PLET agar for 36–48 hours at 37 °C. The suspected colonies are further isolated and identified (see this annex, section 10.3.2).
- Include a spiked control as mentioned in this annex, section 10.4.1 if considered advisable.

10.4.3 Drinking-water or apparently clear water

Procedures that have been laid down formally in water quality and public health regulations for enteric pathogens (Anon., 2002b) have not been tested for *B. anthracis*. However, there is no reason to believe that the membrane filtration methods detailed in Anon. (2002b, parts 3, 8–10) would not be applicable to a search for *B. anthracis* in water. The suggested procedure based on this would be:

- Pass a known volume up to 100 ml of the sample through a 0.45 µm membrane filter (typically 47 mm in a sterile filter unit).
- Remove the filter unit funnel and transfer the filter to 10 ml of sterile distilled/deionized water and agitate firmly to resuspend organisms trapped by the filter.
- Place the suspension in a water bath at 62–65 °C for 20 minutes.
- Spread 100–200 µl volumes on 3 plates of predried blood agar or TSPBA and 250 µl volumes on 3 predried PLET agar plates. Incubate blood agar overnight and PLET agar for 36–48 hours at 37 °C.
- Look for typical *B. anthracis* colonies.

Alternatively:

- Bring two volumes of up to 100 ml of the sample to 62–65 °C and hold them at that temperature for 15–20 minutes.
- Pass each 100 ml through a 0.45 µm membrane filter (typically 47 mm in a sterile filter unit); remove the filter unit funnels and transfer one filter to a blood agar or TSPBA plate and the other to a PLET agar plate.
- Incubate the blood agar or TSPBA overnight and PLET agar for 36–48 hours at 37 °C.
- Look for typical *B. anthracis* colonies growing on the filters.

10.5 Examination of food

Food should be treated as environmental samples (this annex, section 10.4.1).

10.6 Examination of dusts and powders associated with suspected deliberate release

In a class 3 safety cabinet (see this annex, section 6.3), a sample of the dust or powder should be transferred by means of a dry swab (dry so as not to damage the evidence if follow-up is necessary) to approximately 0.5 ml of buffered saline. This should then be subcultured before and after heat treatment (62–65 °C for 20 minutes) on/in blood agar and other solid and/or broth media as considered appropriate to the circumstances.

In the event of a powder in an envelope or equivalent, the swab should be inserted through a corner of the envelope without opening the envelope wide at its top to minimize unwanted release of the sample.

10.7 Confirmatory tests

10.7.1 Phage and penicillin sensitivity

These tests can be done together in the simple manner illustrated in Fig. 8F. Up to six tests can be done on a BA plate. A control strain should be included in each batch of tests (the Sterne $34F_2$ vaccine strain, or equivalent, would be suitable).

The colony to be tested is spread over a segment of the plate, right down to the centre. A penicillin disk (2 or 10 U) is placed at the centre of the plate. A 10–15 µl drop of phage suspension is placed in the middle of the spread and allowed to dry in. The plate is incubated at 37 °C.

Phage and penicillin susceptibility can be read from about 6 hours to overnight. Haemolysis can also be checked.

Comments on phage titres and interpretation of zones in section 6.3.1.5 should be noted.

10.7.2 Induction of capsule formation

10.7.2.1 In blood

- Transfer a pinhead quantity of growth from a suspect colony to 2.5 ml defibrinated sheep or horse blood in a sterile test tube or small bottle.

- Incubate 5–18 hours at 35–37 °C.
- Transfer a drop with a 1 µl loop immersed to the bottom of the unshaken bottle or tube to a microscope slide and make a thin smear.
- Stain and examine as described in 9.3.6 above.
- A positive control strain should be included. This could be the Pasteur strain or equivalent if available, but may have to be a virulent wild-type isolate maintained for this purpose.

10.7.2.2 On bicarbonate agar plates

- Plate the suspect colony onto bicarbonate/serum agar (Annex 2).
- Incubate overnight at 35–37 °C under a 10%–20% CO_2 atmosphere (or in a candle jar).
- Capsulating *B. anthracis* appears as mucoid colonies (Fig. 8D). Make smears, stain and examine as described in 9.3.6 above. Although the capsule stains well when produced by this method, it does not appear so well circumscribed as when produced in vivo or in blood as described in 10.7.2.1 above.

10.7.3 Motility

B. anthracis is non-motile. Any of the established tests for motility can be used to check this with an isolate.

10.7.4 PCR

10.7.4.1 Introduction

The section on PCR in the third edition of these guidelines (Turnbull et al., 1998a) was the subject of some criticism. It is known that a number of defence-related laboratories in several countries have designed primers and PCR systems that have a high degree of reliability, but these are generally unavailable to the wider community.

The following protocols are kindly supplied by W. Beyer.[1] The methods were also provided in written form to the participants of the Anthrax Wetlab Workshop.[2]

10.7.4.2 Primers and protocols of choice

Of the primer systems described in the literature, only primers for the protective antigen (*pag*) and the lethal factor gene (*lef*) have not yet been shown to cause nonspecific results. Many primers described for the *capB* and *capC* genes and currently published primers targeting the chromosome of *B. anthracis* were shown to produce false positive results with the indigenous soil flora (Ramisse et al., 1999; Beyer et al., 1999; Ellerbrok et al., 2002).

10.7.4.3 DNA preparation

When PCR is simply being used to confirm suspicious colonies, short boiling of resuspended vegetative bacteria in PCR buffer is sufficient to extract DNA. If it is necessary to ensure that there are no viable spores in the DNA preparation, autoclaving of the culture material will also provide DNA suitable for the PCR protocols described here.

To prepare DNA from a non-selective enrichment culture or germinated spore suspensions, a DNA preparation kit is recommended. Depending on the target sequence of the PCR, the DNA preparation should either enrich for plasmid DNA or genomic DNA. In the former, the procedure should be able to isolate large, low copy number plasmids.

For the isolation of PCR compatible DNA from environmental samples, the procedure should also be able to remove polymerase inhibitors. The DNA preparation kits NucleoSpin® Plasmid (Macherey Nagel) and DNeasy Plant (Quiagen) can be used successfully for environmental samples. The DNeasyPlant-Kit of Quiagen is used according to the manufacturer's recommendations with the following modifications:

- The pellet from 1 ml of the non-selective enrichment broth or germinated spore suspension is used as starting material, resuspended in 400 µl buffer AP1 in the kit.
- After the second washing of the spin column with buffer AW, an additional washing with 500 µl pure ethanol is done. Be sure no residual ethanol is left after the subsequent centrifugation.
- The DNA is eluted in 1 x 50 µl of buffer AE, prewarmed to 70 °C.

10.7.4.4 Controls to be included in the diagnostic PCR

The following controls should be included in order to verify diagnostic findings:

- DNA prepared from an aliquot, or aliquots, of the original sample material spiked with known concentration(s) of a control strain of *B. anthracis*.

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[1] Institute for Environmental and Animal Health, University of Hohenheim, Stuttgart, Germany (initially published in *Tierärztliche Umschau*, 58:653–62).

[2] Global Health Security Action Group (GHSAG) Laboratory Network, 2004, HPA, Porton Down, United Kingdom.

This/these positive control(s) will provide information about any inhibition of germination or growth of *B. anthracis* during the preculture of the sample materials. Additionally it will provide an indication as to the sensitivity of the diagnostic procedure.

- Addition of 1 pg to 1 ng of purified genomic DNA of *B. anthracis* to the DNA prepared from the original sample material. This will reveal any inhibition effect that may be occurring on the PCR by polymerase inhibitors. Furthermore, it indicates the sensitivity of the PCR with the particular sample material being tested.
- Purified DNA (1 pg and 1 ng) of a pure culture of *B. anthracis*. This reaction serves as a positive control for the PCR. In a block cycler PCR, the amplicon should be visible for 1 ng of input DNA after the first round of amplifications, whereas 1 pg of DNA would be detectable after the nested PCR step only. In real-time PCR, 1 pg DNA or less should give a clear signal.
- DNA derived from the "negative in-process" control, e.g. *E. coli* cells. This control is handled and prepared simultaneously with both the original and the spiked sample materials, and serves as a negative control for contamination occurring throughout the entire process of culture, DNA isolation, and PCR.
- PCR premix without DNA. This reaction serves as a negative control for contamination during the preparation of the PCR premixes only.

10.7.4.5 Protocol for PCR in a block cycler instrument to detect the pag *or the* cap *genes*

Prepare a premix consisting of a volume of 50 µl per reaction containing 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 1 µmol/l of each primer, and 2.5 U polymerase.

Add 1 µg of T4 gene32 protein (Roche) to the standard premix during the first PCR. This single-strand binding protein enhances the efficacy of PCRs by a factor of 100 if the reaction is influenced by polymerase inhibitors (Beyer et al., 1995).

The use of a "hot start" protocol is advisable. Running a second PCR (nested PCR) is necessary: (i) if there is only a weak positive result with the first PCR run, e.g. due to inhibition of growth in the spiked control culture; or (ii) as a control reaction to verify the specificity of a positive result after the first PCR run. The nested PCR step may be omitted if the first PCR yields a negative result for the sample and the inhibition control, and the corresponding positive controls are positive.

The PCR conditions for a thermal block cycler running under block control are:

- 94 °C (83 °C for detection of *pag*) – 4 min
- 25 cycles (1st PCR); 30 cycles (nested PCR):
  - 94 °C (83 °C for detection of *pag*) – 1 min
  - 55 °C – 1.5 min
  - 73 °C – 1.5 min
- 72 °C – 9 min
- hold at 8 °C.

Primers used in the diagnostic PCR are shown in Table 15. Additional primer systems are provided in Table 16.

10.7.4.6 Protocols for real-time PCR in a LightCycler instrument

The LightCycler (LC) instrument (Roche) is designed for high-speed thermal cycling using air instead of thermal blocks. A capillary sample tube system ensures efficient heat transfer to the PCR samples. As a result, the time needed for each PCR cycle, including measurement of the sample fluorescence, is minimized to approximately 15–20 seconds. A 30–40 cycle PCR run can be completed within 20–30 minutes. The formation of amplification products can be monitored in real time.

The hybridization probe format is used for DNA detection and quantification. Two specially designed, sequence-specific oligonucleotides labelled with fluorescent dyes are applied for this detection method. This allows highly specific detection of the amplification product. Oligo 1 carries a fluorescein label at its 3' end, whereas oligo 2 carries another label (LC Red 640) at its 5' end. The sequences of the two oligonucleotides are selected so that they hybridize to the amplified DNA fragment in a head-to-tail arrangement. When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the LightCycler's LED (light emitting diode) filtered light source, and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the LC Red 640 attached to the second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength. This energy transfer, referred to as FRET

TABLE 15

Primers for nested PCR to detect the *pag*-gene

PRIMERA	SEQUENCE (5`–3`)	REFERENCE	BINDING SITE	GENE	ANNEALING
PA5	tcctaacactaacgaagtcg	Beyer et al. (1995)	2452–2471[a]	*pag*	55 °C
PA8	gaggtagaaggatatacggt	Beyer et al. (1995)	3048–3029[a]	*pag*	55° C
PA6	accaatatcaaagaacgacgc	Beyer et al. (1995)	2631–2651[a]	*pag*	55 °C
PA7	atcaccagaggcaagacaccc	Beyer et al. (1995)	2841–2821[a]	*pag*	55 °C

[a] Primer pairs are: PA5/PA8 for the first round of amplifications and PA6/PA7 for the nested PCR.

TABLE 16

Additional published primer systems

PRIMER	SEQUENCE (5`–3`)	REFERENCE	BINDING SITE	GENE	ANNEALING
MO1[g]	gctgatcttgactatgtgggtg	Makino et al. (1993)	2452–2473[a]	*capA*	65 °C
MO2[g]	ggcttcctgtctaggactcgg	Makino et al. (1993)	2739–2719[a]	*capA*	65 °C
BACA1FI[h]	acaactggtacatctgcgcg	Reif et al. (1994)	470–489[a]	*capB*	55 °C
BACA6RI[h]	gatgagggatcattcgctgc	Reif et al. (1994)	1073–1092[a]	*capB*	55 °C
CAP6[i]	tactgacgaggagcaaccga	Beyer et al. (1995)	506–525[a]	*capB*	55 °C
CAP103[i]	ggctcagtgtaactcctaat	Beyer et al. (1995)	1541–1522[a]	*capB*	55 °C
CAP9[k]	atgtatggcagttcaacccg	Beyer et al. (1995)	617–636[a]	*capB*	55 °C
CAP102[k]	acccactccatatacaatcc	Beyer et al. (1995)	1394–1375[a]	*capB*	55 °C
BA17[b]	gaaatagttattgcgattgg	Sjöstedt et al. (1995)	1230–1249[a]	*capB, capC*	54 °C
BA20[b]	ggtgctactgcttctgtacg	Sjöstedt et al. (1995)	210–2083[a]	*capC, capA*	62 °C
BA57[b]	actcgtttttaatcagcccg	Sjöstedt et al. (1997)	1603–1622[a]	*capC*	52 °C
BA58[b]	tggtaacccttgtctttgaat	Sjöstedt et al. (1997)	1867–1847[a]	*capC*	52 °C
57[c]	actcgtttttaatcagcccg	Ramisse et al. (1996)	1603–1622[a]	*capC*	57 °C
58[c]	ggtaacccttgtctttgaat	Ramisse et al. (1996)	186–1847[a]	*capC*	57 °C
67[c]	cagaatcaagttcccagggg	Ramisse et al. (1996)	1925–1944[a]	*pag*	64 °C
68[c]	tcggataagctgccacaagg	Ramisse et al. (1996)	2652–2671[a]	*pag*	46 °C
25[c]	ggtttagtaccagaacatgc	Ramisse et al. (1996)	1459–1478[a]	*cya*	59 °C
26[c]	cggcttcaagacccc	Ramisse et al. (1996)	1990–2004[a]	*cya*	59 °C
3[c]	cttttgcatattatatcgagc	Ramisse et al. (1996)	1238–1258[a]	*lef*	54 °C
4[c]	gaatcacgaatatcaatttgtagc	Ramisse et al. (1996)	1599–1622[a]	*lef*	54 °C
Cvi[d]	cactcgtttttaatcagccc	Beyer et al. (1999)	1602–1621[a]	*capC*	55 °C
Cri[d]	cctggaacaataactccaatacc	Beyer et al. (1999)	1808–1830[a]	*capC*	55 °C
PA-S[e]	cggatcaagtatatgggaatatagcaa	Ellerbrok et al. (2002)	3245–3271	*pag*	60 °C
PA-R[e]	ccggtttagtcgtttctaatggat	Ellerbrok et al. (2002)	3448–3425	*pag*	60 °C
Cap-S[f]	acgtatggtgtttcaagattcatg	Ellerbrok et al. (2002)	1673–1696	*capC*	60 °C
Cap-R[f]	attttcgtctcattctacctcacc	Ellerbrok et al. (2002)	1993–1940	*capC*	60 °C

[a] Positions derived from Makino et al. (1989) and Welkos et al. (1988).
[b] BA17/BA20 and BA57/BA58 were used as primer pairs in different trials.
[c] Primer pairs 3/4, 25/26, 57/58, and 67/68 were used in multiplexing.
[d–k] Given primers were used as pairs.

(fluorescence resonance energy transfer) is highly dependent on the spacing between the two dye molecules. Only if the molecules are in close proximity (a distance between 1 and 5 nucleotides) is the energy transferred at high efficiency. The intensity of the light emitted by the LightCycler Red 640 is filtered and measured by the LightCycler instrument's optics. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process.

(a) Detection of the pag *gene*

The premix (20 µl) consists of:

- 4 mM $MgCl_2$
- 0.5 µM of each primer (Ellerbrok et al., 2002):
  - BAPA-S: 5'-CGGATCAAGTATATGGGAATATAGCAA-3'
  - BAPA-R: 5-CCGGTTTAGTCGTTTCTAATGGAT-3'
- 0.2 µM of probe
  - BAPA-FL: 5'-TGCGGTAACACTTCACTCCAGTT CGA-X
- 0.2 µM of probe BAPA-LCRed 640 5'-CCTGTATCCACCCTCACTCTTCCATTTTC-P
- 1/10 vol. of FastStart mastermix (Roche)
- 5 µl DNA.

(b) Detection of the capC *gene*

The premix (20 µl) consists of:

- 4 mM $MgCl_2$
- 0.5 µM of each primer:
  - *CapS*: 5'-ACGTATGGTGTTTCAAGATTCATG-3' (Ellerbrok et al., 2002)
  - *CapA***: 5-GATTGCAAATGTTGCACCACTTA-3'
- 0.2 µM of probe
  - *CapC*-FL+: 5'-TATTGTTATCCTGTTATGCCATT TGAGATTTTT-X
- 0.2 µM of probe
  - *CapC*-LC Red640: 5'-AATTCCGTGGTATTGGAG TTATTGTTCC-P
- 1/10 vol. of FastStart mastermix
- 5 µl DNA.

(c) Experimental protocol

1. Pre-incubation step: 95 °C for 10 min, slope at 20 °C/sec.
2. Amplification (45 cycles): 95 °C for 10 sec; 55 °C for 20 sec; 72 °C for 30 sec, slope 20 °C/sec; one single signal acquisition at the end of annealing.
3. Denaturation: 95 °C for 0 sec, slope 20 °C/sec; 40 °C for 30 sec, slope 20 °C/sec; 80 °C for 0 sec, slope 0.1 °C/sec with continuous acquisition of the signal.
4. Cooling to 40 °C for 30 sec, slope 20 °C/sec.

10.7.4.7 Real-time PCR protocol for the specific detection of B. anthracis *chromosome*

(a) Sequence alignment and PCR design

The sequences of the B-type sasp gene of *B. cereus* (Gene bank No. M16813, gi: 143507) and of *B. anthracis*, (NCBI Ref. Seq. NC003995, Gene bank: AAAC01000001), contributed by TIGR, were aligned by the appropriate tool of the NTI8 software package.[1]

The PCR reaction mixture of 20 µl consists of:

- 4 mM $MgCl_2$
- 0.5 µM of each primer
  - ANT-F: 5'-GCTAGTTATGGTACAGAGTTTGCG AC-3'
  - ANT-Amt: 5'-CCATAACTGACATTTGTGCTTT GAAT-3'
- 0.2 µM of probe
  - ANT-FL: 5'-CAAGCAAACGCACAATCAGAAG CTAA G-X
- 0.2 µM of probe
  - ANT-LC: Red640: 5'-GCGCAAGCTTCTGGTGC TAGC-P
- 1/10 vol. of FastStart mastermix
- 5 µl DNA.

(b) Experimental protocol

1. Pre-incubation step: 95 °C for 10 min, slope at 20 v°C/sec.
2. Amplification (45 cycles): 95 °C for 10 sec; 57 °C for 20 sec; 72 °C for 30 sec, slope 20 °C/sec; one single signal acquisition at the end of annealing.
3. Denaturation: 95 v°C for 0 sec, slope 20 °C/sec; 40 °C for 30 sec, slope 20 °C/sec; 80 °C for 0 sec, slope 0.1 °C/sec with continuous acquisition of the signal.
4. Cooling to 40 °C for 30 sec, slope 20 °C/sec.

[1] Informax, Inc. Bethesda, United States. Primers and probes synthesized by TIB MolBiol, Berlin, Germany.

10.7.4.8 Commercial kit

The LightCycler *Bacillus anthracis* Detection Kit for the detection of capsule (*capB*) and PA (*pagA*) genes is available commercially from Roche Applied Sciences for the detection of both virulence plasmids of *B. anthracis*. Kits for a range of sample types, both clinical and environmental, are now available, for instance, from Idaho Technology.[1]

11. Antigen detection tests

11.1 Ascoli precipitin test (thermostable antigen test)

The purpose of this very old test dating from 1911 (Ascoli, 1911) is to supply rapid retrospective evidence of anthrax infection in an animal. It was designed to detect *B. anthracis* antigens in the tissues of animals being utilized in animal by-products, and thereby to reveal when these products contained ingredients originating from animals that had died of anthrax. Over the years, it has been one of the most valuable tools for controlling anthrax in most European countries and it remains in use, particularly in eastern Europe. Regular or occasional use of the test was indicated on returns of a survey by OIE in 2002 from Croatia, Germany, the Philippines, the Republic of Moldova and The former Yugoslav Republic of Macedonia. There may be other countries that use the test but did not reply.

It needs to be borne in mind that this test is not rigorously specific for *B. anthracis*. The thermostable antigens involved are common to other *Bacillus* species so the test depends on the fact that the only *Bacillus* likely to have proliferated within and throughout an animal depositing extensive precipitating antigens in the tissues is *B. anthracis*.

The test is not suitable for detection of *B. anthracis* in environmental specimens; numerous other *Bacillus* species can be expected to occur in these. It is hoped that immunochromatographic, on-site tests (6.2) will become widely available as the replacements of the future.

11.1.1 Procedure

- Chop or slice the specimen into fine pieces or strips.
- Boil approximately 2 g of the specimen for 5 minutes in 5 ml saline containing 1:100 (final concentration) acetic acid. Alternatively, soak in saline containing 0.5% phenol for 24–48 hours in a refrigerator.
- After cooling, filter through filter paper until completely clear.
- Insert a few drops of antiserum (11.1.2 below) in the bottom of a small test-tube and carefully add some of the filtrate down the side of the tube to form a layer of antigen above the antiserum. (As an alternative to using a test-tube, and more economical on the antisera, capillary tubes can be used as in the Lancefield test for streptococcal grouping.)
- Include appropriate positive and negative specimen controls.

11.1.2 Antiserum for the Ascoli test

Commercially prepared serum is available from:

- Bioveta plc, Komenského 212, 683 23 Ivanovice na Hané, Czech Republic; fax +42 507 932 84
- The National Institute of Animal Health, 3–1 Kannondai 3-chome, Tsukuba-shi, Ibaraki-pref, 305–0856 Japan; tel: +81 298 38 7713; fax: +81 298 38 7880
- DD "Vet Zavod Zemun", Batajnicki drum 4, Belgrade, Serbia
- National Anthrax Reference Laboratory, Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Institut für Bakterielle Infektionen und Zoonosen, Nationales Referenzlaboratorium für Milzbrand, Naumburger Straße 96a, D-07743 Jena, Germany; tel. +49 3641 804-0; fax +49 3641 804-228; web site: http://www.fli.bund.de/66+M52087573ab0.html
- C-C Pro: Gesellschaft für Herstellung und Vertrieb von Produkten für Cellculturen GmbH, Am Bahnhof 1, D-99986 Oberdorla; tel. +49 (700) 22 77 63 66; fax. +49 (700) 22 77 63 29; web site: http://www.c-c-pro.com/

It should be recalled that the following procedure was designed decades before the current era of fear of bioterrorism and associated strict control in many developed countries on access to virulent *B. anthracis*.

On days 1 and 14, rabbits are inoculated subcutaneously with animal anthrax vaccine (Sterne strain $34F_2$) (Annex 5, section 2). On days 28 and 35, further subcutaneous injections of 0.05 ml of a suspension in physiological saline of a mixture of several strains of virulent *B. anthracis* are administered. The viable count of this suspension should not exceed 100 000

[1] www.idahotech.com/reagents/it123dnakit.html.

colony-forming units/ml. After a further 10 days, a test-bleed will reveal the activity of the antiserum; if not adequate, further injections of the virulent *B. anthracis* suspension should be administered at 7–10 day intervals.

If considerations of safety prevent the use of live virulent *B. anthracis*, the mixture of several strains of *B. anthracis* can be suspended to a final count of 10^8–10^9/ml in physiological saline containing 0.2% formalin. This is held until sterile (at least 2 weeks). After the vaccine strain inoculations on days 1 and 14 as before, increasing doses of 0.1, 0.5, 1 and 2 ml of the killed suspension should be administered intravenously at approximately 4–5 day intervals. A test bleed should be done 10 days after the last injection. Further 2 ml doses can be administered if the titre is not adequate initially.

11.3 Other antigen detection tests

The rapid, hand-held, on-site, immunochromatographic detection devices that have been developed in recent years are discussed in section 6.2.

12. Isolation in animals

On account of increasing concern to eliminate the use of laboratory animals wherever possible, and of the increasing reliability and sophistication of alternative in vitro methods, the use of animals for isolation or confirmation of identity of *B. anthracis* can and should generally be avoided. It should be noted that EC Directive 86/609/EEC relating to protection of animals used for experimental and other scientific purposes pertains to members of the European Union. Several other countries have strict laws which make ad hoc use of an animal for isolation or confirmation of identification of *B. anthracis* virtually impossible nowadays.

There still are occasions, however, such as those where potential legal disputes may be involved, when confirmation of the presence or the virulence of *B. anthracis* is necessary. In the absence of a selective enrichment system (see section 6.2), inoculation of mice or guinea-pigs, essentially as done more than a century ago by Pasteur, is still the most sensitive isolation method. Pending the development of equally sensitive conventional immunological or DNA-based techniques, animal tests may offer the only chance of (i) confirmation of diagnosis in certain situations such as in the case of individuals or animals that were treated before specimens were taken, or (ii) detection of the organism when present in very low numbers in environmental samples, or in environmental samples containing sporostatic chemicals.

Confirmation of identity or of virulence can be done by injecting light suspensions (approximately 10 000 colony-forming units/ml) into mice (0.05–0.1 ml subcutaneously) or guinea-pigs (0.1–0.2 ml intramuscularly). Virulent *B. anthracis* will kill the animals after about 42–48 hours; M'Fadyean-stained blood smears examined at death will reveal large numbers of the capsulated bacilli which can also be isolated and confirmed bacteriologically.

In the rare situation in which it is necessary to use animals to isolate *B. anthracis* from soil or other environmental samples, the animals should be inoculated the day before with subcutaneous doses of mixed gas-gangrene antisera (extremely difficult to obtain, however) and antitetanus serum. Heated (62 °C to 65 °C for 15–20 min) soil extracts, as prepared for plating on selective or non-selective agar (see 10.4 above; Fig. 15), are then injected (0.05–0.1 ml subcutaneously in a mouse or up to 0.4 ml intramuscularly in a guinea-pig – 0.2 ml in each thigh muscle). M'Fadyean-stained blood smears from any animals that die are examined for the presence of the typical capsulated *B. anthracis* which can also be isolated and confirmed bacteriologically.

13. Retrospective confirmation; serology and delayed type hypersensitivity testing

Effective serological enzyme immunoassays (EIA) for confirmation of the diagnosis of anthrax have been designed and have proved to be useful diagnostic, epidemiological and research aids (Turnbull et al., 1992a; Quinn et al., 2004) (see section 4.4.2.2). The usual provisos for any serological confirmatory test apply, namely that: (i) two or more serum samples taken 2–4 weeks apart will give greater diagnostic reliability; (ii) if only one serum sample is collected, it will be of greater diagnostic value if collected more than a week after onset of symptoms; and (iii) negative or weak results be interpreted in the light of treatment the patient or animal may have received early on in the course of the infection. The last is particularly important in anthrax since antibiotic therapy rapidly kills infecting *B. anthracis* and, if carried out early enough in an infected individual, may prevent the elaboration of sufficient antigen to induce a detectable immune response (section 4.4.2.2).

Available immunoassays for anthrax are mostly based on antibodies to the toxin antigens, primarily the protective antigen component of the toxin (see section 5.5.3). Although not difficult to perform (any standardized EIA methodology may be used), they are, at present, confined to a few specialist laboratories capable of preparing the necessary purified toxin antigens or with the resources to purchase them.[1]

The Anthraxin^{T} delayed type hypersensitivity test is described in section 4.4.2.2 and involves intradermal injection of 0.1 ml of Anthraxin^{T}. A positive test is defined as erythema of ≥ 8 mm with induration persisting for 48 hours.

An immunochromatographic assay for detecting anti-PA antibody, analagous to the assay for detecting PA (section 6.2), might be valuable for retrospective confirmation of diagnosis.

The value of immunohistochemical staining of tissues for retrospective confirmation in the anthrax letter events in the USA is discussed in section 4.4.4.2.

[1] www.listlabs.com.

ANNEX 2

Media, diagnostic phage and stains

1. Media

1.1 Polymyxin-lysozyme-EDTA-thallous acetate agar (1 litre)

Polymyxin-lysozyme-EDTA-thallous acetate (PLET) (Knisely, 1966) is the best selective agar currently available for isolation of *B. anthracis* from old, decomposed or processed animal specimens, or environmental or food samples contaminated with other organisms including other *Bacillus* species. But its performance is heavily dependent on it being made well, with particular attention to the heart infusion agar being used and to adequate cooling of the molten agar before addition of lysozyme and polymyxin.

Difco heart infusion agar (or Difco heart infusion broth with agar base)[1] is made up according to the manufacturer's instructions with added EDTA (0.3 g/l) and thallous acetate (0.04 g/l – see below). Heart infusion agars of other manufacturers or other nutrient bases, such as brain-heart infusion, can be made to work, but the optimal concentrations need to be determined; the concentrations recommended for normal use may not be optimal for use in PLET. A starting point for determination of optimal concentration is 25 g/l of dehydrated broth plus agar at the manufacturer's recommended concentration.

After autoclaving, the agar is cooled to 50 °C and polymyxin (30 000 units/l) and lysozyme (300 000 units/l – see below) added. It is important that the agar is left at 50 °C for long enough to ensure that this temperature has been reached throughout the medium before the polymyxin and lysozyme are added. After swirling to ensure even suspension of the ingredients, the agar is poured into petri dishes.

1.1.1 Thallous acetate, polymyxin and lysozyme

(*Caution: thallous acetate is poisonous and should be handled with care; avoid skin contact or inhalation of the powder while weighing out. Store solutions in a well-labelled tube marked "Poison".*) Make a stock solution of thallous acetate based on the ratio of 0.1 g of thallous acetate dissolved in 2.5 ml deionized water. Addition of 1 ml of this to 1 litre of agar base gives a final concentration of 0.04 g/l.

Different lots of polymyxin and lysozyme will have different numbers of units/mg (U/mg). Adjust the following examples according to the activity of the lot being used. Aliquot and store in the -20 °C. Stability seems to be indefinite at -20 °C and freezing and thawing does not seem to harm them. However, it is better to aliquot and store in 0.25 or 0.5 ml volumes.

If, for example, the potency of the polymyxin as received from the supplier is 6000 U/mg, a convenient target concentration for a stock solution is 60 000 U in 0.5 ml (if the supplier does not indicate the potency, assume it is 6000 U/mg):

- this = 10 mg in 0.5 ml (sterile deionized water – SDW)
- weigh out, say, 200 mg into 10 ml SDW
- aliquot 0.5 ml or 0.25 ml volumes and put in -20 °C freezer.

When making PLET, thaw out and add 0.25 ml per litre to give a final concentration of 30 000 U/litre.

Similarly for lysozyme, if, for example, the lot as received from the supplier is 46 000 U/mg:

- 46 000 U ≡ 0.001 g
- 600 000 U ≡ [0.001 ÷ 46 000] x 600 000 = 0.6 ÷ 46 = 0.013 g
- weigh 20 x 0.013 g into 20 x 0.5 ml SDW = 0.261 g into 10 ml SDW
- again aliquot 0.5 ml or 0.25 ml volumes and put in -20 °C.

When making PLET, thaw out and add 0.25 ml per litre to give final concentration of 300 000 U/litre.

[1] Difco Laboratories, Detroit, Michigan 48232-7058, USA.

1.2 TSPBA (120 ml)

Formula

Mueller-Hinton agar for 120 ml supplemented with:

- peptone from beef 0.54 g
- yeast extract 0.54 g
- NaCl 0.6 g.

Weigh out the supplemented Mueller-Hinton agar base for 120 ml, but only make it up to 110 ml with distilled/deionized water. Dissolve thoroughly on a hot plate with magnetic stirrer. Autoclave and cool to 50 °C. Also warm up 6 ml blood (5%) and 2 ml each of the trimethoprim and sulphamethoxazole solutions, prepared as instructed below, to 50 °C. Add all these to the molten agar base plus 25 µl of polymyxin as made for PLET (above), swirl to mix without frothing and pour plates.

1.2.1 Preparation of trimethoprim and sulphamethoxazole solutions

The final concentrations of the trimethoprim in TSPBA should be 13–26 µg/ml, and the sulphamethoxazole 20–40 µg/ml with the polymyxin at the same concentration as for PLET.

Use trimethoprim lactate (the most soluble form of this drug). Dissolve 0.15 g trimethoprim lactate in 100 ml sterile distilled/deionized water (= 1.5 mg/ml). It takes about 5 min for this to dissolve at 30–37 °C with magnetic stirring. Store as 2 ml aliquots at -20 °C. The final 1:60 dilution results in 25 µg/ml of trimethoprim in the agar.

Add 0.12 g sulphamethoxazole to 50 ml sterile distilled/deionized water, and add 300 µl of 10% NaOH. Bring the temperature to 80 °C on a stirring hot plate with a magnetic stirrer. The sulfamethoxazole dissolves in about 1 min (= 2.4 mg/ml). Store as 2 ml aliquots at room temperature. The final 1:60 dilution will result in 40 µg/ml sulphamethoxazole in the agar.

When *B. cereus* is present, the *B. anthracis* is readily apparent as compared to normal blood agar where the *B. anthracis* are totally overgrown. While PLET is more selective, TSPBA plates can be read after overnight incubation as compared with 36–48 hours with PLET.

1.3 Sporulation agar

There are numerous alternative formulas for sporulation agar but the following, which are also probably the simplest, work well for *B. anthracis*.

Formula 1

— peptone	15.0 g
— yeast extract	3.0 g
— NaCl	6.0 g
— dextrose	1.6 g
— agar	12.0 g
— manganous sulphate	0.1 g
— deionized/distilled water	1000 ml

Sterilize at 121 °C for 20 minutes, cool to 50 °C, and dispense into the appropriate containers (bottles, petri dishes, tubes for slants, etc.) to set.

Formula 2

— nutrient agar	23 g
— $MnSO_4.H_2O$	0.025 g
— KH_2PO_4	0.25 g
— deionized/distilled water	1000 ml

Sterilize at 121 °C for 20 minutes, cool to 50 °C, and dispense into the appropriate containers (bottles, petri dishes, tubes for slants, etc.) to set.

1.4 Capsule agar

Weigh out the number of grams necessary to make 100 ml of nutrient agar as determined from the manufacturer's instructions and dissolve in 83 ml deionized or distilled water. Autoclave and cool to 50 °C in a water bath. Prewarm 10 ml of a filter-sterilized aqueous solution of 7% sodium bicarbonate and 7 ml of horse serum (filter-sterilized if necessary) to 50 °C and add these to the molten agar. Mix well and pour into petri dishes. The horse serum seems helpful but may not be essential. Serum from other species may be used but horse serum seems to be best.

The source of the nutrient agar may be important. If capsule formation is not obtained (see Annex 1, section 10.7.2.2), agar from another source should be tried. It is also best not to dry the plates before use, although excess liquid of condensation should be removed. Incubation under 10% to 15% CO_2 is recommended, although capsule may be apparent with a CO_2 level of 5%.

2. Diagnostic "gamma" phage

The background to the diagnostic phage is given in section 6.3.1.5. Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories. The phage may be propagated as follows:

Stage 1

1. Prepare a culture (approximately 10 ml) of the Sterne vaccine $34F_2$ strain of *B. anthracis* in nutrient broth until cloudy (± 10^8 cfu/ml). (Any nutrient broth will do.)
2. With a spreader, spread 75–100 µl of the culture on 3 predried blood or nutrient agar plates and allow to dry in. Keep the broth culture in the refrigerator; it will be needed in step 11 below and may be needed in step 8.
3. With a spreader, spread 50–100 µl of the phage suspension to be amplified over the same plates. Incubate at 37 °C overnight.
4. The growth on the plates should look "ropey" from the extensive lysis that occurred during growth. Harvest the phage-lysed growth on the agar plates in 5 ml of nutrient broth (i.e. suspend the growth with the help of a spreader and, with a pipette, transfer from the first plate to the second, and then to the third plate, and then to a tube) followed by a second "wash" of 5 ml nutrient broth. Make the final volume up to 10 ml with nutrient broth. Incubate at 37 °C, in a shaking incubator if possible (if not, shake regularly during the incubation), for 4–5 hours. (Note: there will be enough unlysed growth in and at the edge of the lysis zone to support more infection and lysis during the broth culture stage. So there is no need to add more bacteria at this point.)
5. Centrifuge at 2500 rpm for 30 minutes (preferably in a refrigerated centrifuge). Filter (0.45 or 0.2 µm). Label this "Stage 1" filtrate.

Stage 2

This is essentially the same procedure as Stage 1, only using the filtrate from step 5 to harvest the phage from the plates, i.e.:

6. Prepare Sterne strain lawns on 3 blood or nutrient agar, as in step 2.
7. Spread 100 µl filtrate from the Stage 1 filtrate of step 5 on each plate. Incubate at 37 °C overnight.
8. Harvest the phage-lysed growth on the plates from step 7 with 5 ml of the Stage 1 filtrate from step 5), followed by a second 5 ml wash with the rest of the Stage 1 filtrate. Again, there should be some growth in and at the edge of the lysis zone and enough to support more infection and lysis during the broth culture stage. So there should be no need to add more bacteria at this point. In the unlikely event of complete lysis – i.e. totally clear plates – add 0.5 ml of the nutrient broth culture from step 2.
   The result will be approximately 7 ml of harvested material. Make this up to 9 ml with nutrient broth.
9. Add 1 ml of 10x concentrated nutrient broth.
10. Incubate, on a shaker if possible (if not possible, shake at frequent intervals during incubation), at 37 °C for 4–6 hours. Centrifuge and filter (0.22 µm).
11. Check the resulting filtrate for sterility and titrate by spreading tenfold dilutions of the phage preparation on Sterne strain lawns prepared as in step 2 to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque-forming units per ml. Keep refrigerated at a temperature of between 2 °C and 8 °C. Freezing is probably unwise although the phage will survive it.
    Theoretically, any phage-sensitive strain of *B. anthracis* may be used for propagation and titration but, generally speaking, it is preferable for safety reasons to use the Sterne ($34F_2$) avirulent vaccine strain. The strain chosen should not be heavily infected with its own lysogenic phage.

3. Staining theory

Polychrome methylene blue is a complex mixture of methylene blue and substantial amounts of other homologs, primarily azure A and azure B, which are produced by oxidation ("ripening") that takes place in methylene blue solution upon standing. Natural ripening takes a year or more to complete but can be hastened by addition of 1% K_2CO_3 to Loeffler's alkaline methylene blue.

Two alternative formulae (others probably exist) for Loeffler's alkaline methylene blue are given in Table 17.

After addition of K_2CO_3 to 1% final concentration, distribute the stain in bottles to half full. Shake at intervals to aerate the contents. Test periodically for

TABLE 17

Alternative formulae for Loeffler's alkaline methylene blue

AGENT	FORMULA A	FORMULA B
Methylene blue	0.3 g	1.5 g
95% ethanol	30 ml	100 ml
Dissolve the methylene blue in the ethanol.		
KOH	100 ml of a 0.01% solution	3.3 ml of a 1% solution
Distilled/deionized water	–	330 ml

capsule staining ability. Quality control is not complete, however, until the stain has been validated on specimens from anthrax cases in the field.

Polychrome methylene blue is an ingredient of Wright's and Leishmann's stains. Both these are methanol solutions of a water-insoluble precipitate formed when eosin is added to a methylene blue solution. In Wright's stain, the methylene blue solution contains $NaHCO_3$ and is steamed before the eosin is added; in Leishmann's stain, the methylene blue solution is held for 12 hours at 65 °C followed by standing for 10 days. Giemsa stain is a deliberately formulated cocktail of the eosinates of methylene blue with prepurified azure A and azure B.

Romanowski in 1891 was the first to combine eosin with methylene blue so Wright's, Leishmann's and Giemsa stains are referred to as Romanowski stains. In theory these stains, in having the ingredients of polychrome methylene blue, should be usable for M'Fadyean reaction staining; in practice, reports suggest that Giemsa stains give variable results, possibly reflecting the variable presence of certain active but undefined impurities present in true polychrome methylene blue.

It is proposed that the best stain to use for normal diagnostic purposes in sudden-death cases is true polychrome methylene blue – i.e. the non-eosinated ripened Loeffler's methylene blue. However, in the extensive experience of Sterne (1959), confirmed by Lindeque in the Etosha National Park, Namibia (Turnbull, 1998a), the capsule loses its affinity for methylene blue during putrefaction and may no longer be visible with M'Fadyean's stain. Lindeque (personal communication, 1995) found that the Giemsa-type stains show up the characteristic square-ended shape of the anthrax bacillus better than M'Fadyean's stain, and this becomes advantageous in partially decomposed carcasses as encountered frequently in the wildlife situation.

Commercially produced M'Fadyean's stain is becoming difficult to obtain. In the experience of Lindeque & Turnbull, a particularly reliable stain is the blue stain of the CAM's Quick-stain kit.[1]

[1] CA Milsch [Pty] Ltd, PO Box 943, Krugersdorp, 1740, South Africa.

ANNEX 3

Disinfection, decontamination, fumigation, incineration

1. Introduction

This annex is concerned with control of anthrax through targeting the reservoirs of *B. anthracis*, taking into account the chemical and physical decontamination procedures to which it is susceptible, and detailing the practical details of these procedures.

1.1 Cautions

Bacterial spores are designed by nature to survive in the face of adverse conditions (i.e. levels of heat, radiation, desiccation, acidity, alkalinity and other chemical and physical conditions) that would rapidly kill other forms of life. It follows, therefore, that chemicals and procedures which can kill spores are necessarily highly lethal to less hardy cells, including those in human, animal and plant tissues.

The most widely used sporicides are chlorine (as in hypochlorite solutions or "bleach") and formaldehyde, with some use being made of hydrogen peroxide and other oxidizing agents, or glutaraldehyde. At the concentrations necessary to be effective as sporicides, these are potentially hazardous to human health if handled incorrectly.

Precautions, therefore, should be taken not to get these on skin or into the eyes or, especially with the aldehydes, not to inhale them. In the case of fumigation, the work should only be carried out by trained professionals with appropriate protective clothing and breathing apparatus.

Attention is drawn to the importance of handling the concentrated liquid disinfectants referred to with caution, using gloves and aprons or overalls and goggles or eye shields to prevent contact with skin or eyes. Clean water should be at hand for immediate washing or showering in the event of an accident while handling concentrated disinfectants. All containers of disinfectants should be properly and accurately labelled as to their contents. Peroxides may be explosive under certain circumstances.

Appropriate (chemical) respirators should be worn by personnel disinfecting or fumigating closed spaces (rooms, stables, etc.) and when opening up such places to ventilate them at the end of the disinfection or fumigation procedure. Respirators should be fitted and tested by qualified personnel, and users of respirators should be trained in their correct use by qualified personnel.

Irradiation by gamma ray or particle bombardment should only be done by properly trained persons in properly monitored facilities. In the case of UV irradiation, care should be taken to protect the eyes and not to expose eyes or skin to direct UV light sources.

Further cautions are given as appropriate in the sections that follow.

1.2 Choice of disinfectants, fumigants or procedures

Probably because *B. anthracis* is essentially an obligate pathogen and depends for the continued existence of the species on the survival of the spores in the environment between infections of successive hosts, it appears to produce particularly tenaceous spores. The results are that (i) only a few fairly formidable chemicals and procedures are capable of reliably killing anthrax spores, and (ii) information on the sporicidal activities of disinfectants, fumigants, and disinfection and fumigation procedures based on other *Bacillus* species should be viewed with caution.

If heat treatment or incineration of the contaminated material is possible, this should be done in preference to chemical decontamination and disinfection. For certain materials or animal by-products, irradiation with gamma rays or particle bombardment may be appropriate (see 6.6 below). As noted in Annex 1, section 7.9, UV irradiation should not be relied on alone for decontamination, but should be used in conjunction with wiping down items to be decontaminated with hypochlorite or possibly formalin.

1.2.1 Disinfectants

Lists of approved disinfectants published periodically in some countries may be misleading when selecting the sporicidal disinfectant to use for *B. anthracis*, and procedures that are both practical and effective have yet to be worked out for numerous situations.

The principal disinfecting agents for destruction of anthrax spores are formaldehyde, glutaraldehyde (at pH 8.0–8.5), hydrogen peroxide and peracetic acid (Dietz & Böhm, 1980; Böhm, 1990). Chlorine dioxide was the alternative chosen in the USA for decontamination of rooms following the anthrax letter events of 2001. Hypochlorites are sporicidal but are rapidly neutralized by organic matter and, therefore, while good for items like laboratory surfaces (not wooden ones) or glassware, or for water treatment, are unsuitable for disinfecting most environmental sites or materials. Hydrogen peroxide and peracetic acid are *not* appropriate if blood is present. The agents should have been tested for their sporicidal activity according to the recommendations below, and validity test results of two independent laboratories should be included on the manufacturer's product information sheet.

For environmental protection, and human and animal health hazard reasons, alternatives to formaldehyde as the recommended general purpose disinfectant have been sought (see 1.2.2 below) The information in this annex should be updated in the future when and if satisfactory alternatives have been identified.

1.2.2 Fumigants

The theoretical options for sporicidal fumigants are formaldehyde, ethylene oxide, methyl bromide, hydrogen peroxide vapour and chlorine dioxide. Formaldehyde and ethylene oxide have been labelled carcinogenic in some countries, and methyl bromide is scheduled to be eliminated for most uses under the Montreal Protocol on Substances That Deplete the Ozone Layer. Hydrogen peroxide, while being the most acceptable in environmental terms (see 3.3 below), requires elaborate apparatus and procedures and has other hazard factors, especially danger of explosion, that need to be borne in mind.

2. Efficacy tests for sporicidal disinfectants

Useful information on the sporicidal efficacies of disinfectant solutions may be obtained from the Kelsey-Sykes capacity test (Kelsey & Sykes, 1969), which is now published as a British Standard, BS 6905:1987 (Estimation of the Concentration of Disinfectants Used in "Dirty" Conditions in Hospitals by the Modified Kelsey-Sykes Test). However, it is officially concerned with the bactericidal, rather than the sporicidal, efficacy of a product. A United States Association of Official Analytical Chemists method (AOAC Official Method 966.04: Sporicidal Activity of Disinfectants) uses surgical silk sutures and porcelain "penicylinders". At present there is no European standard method for sporicidal efficacy testing. A procedure based on the methods accepted and used in Germany (German Society of Hygiene and Microbiology, 1972; German Veterinary Medical Society, 1976), also aimed at testing sporicidal efficacy on surfaces rather than in liquid suspensions, was detailed in an earlier version of this publication (Turnbull et al., 1998a).

2.1 Titration of available chlorine in hypochlorite solutions

Hypochlorite is a strong oxidizing agent and will oxidize iodide ions to form elemental iodine. The iodine so formed may be titrated with standard sodium thiosulphate using starch solution as an indicator. The starch solution can be made by making a paste of 0.1 g of soluble starch with a little water and transferring the paste to 100 ml of boiling water. Boil for one minute. Allow the solution to cool and add 2–3 g of potassium iodide. The solution should be kept in a stoppered bottle.

1. The chlorine solution to be tested should be diluted to an estimated 10 000 ppm.
2. Fill a clean 50 ml burette with 0.1M sodium thiosulphate solution.
3. Accurately pipette 5 ml of the solution being tested into a clean flask and acidify with 5 ml of glacial acetic acid. Then add approximately 0.2 to 0.3 g of potassium iodide to the solution which now becomes orange in colour.
4. Titrate the mixture by adding the sodium thiosulphate from the burette until the colour is pale yellow.
5. Add 5 drops of the starch solution and continue the titration until the blue colour of the starch is just detectable (it will look slightly pink now).
6. Note the burette reading and then continue to add the sodium thiosulphate dropwise. The burette reading immediately preceding the observation

of a colourless solution is the end point. Note the volume of sodium thiosulphate added from the burette and calculate the available chlorine in the test solution from the expression:

1 ml 0.1M sodium thiosulphate ≡ 0.00355 g chlorine.

Correcting for the original dilution of the concentrated sample and converting to a percentage:

Available chlorine (%w/v) = Titre x 0.00355 x 10 x 20 = Titre x 0.71.

3. Rooms, laboratories, animal houses, vehicles, etc.

3.1 Fumigation of rooms

(*Caution: this should only be done by trained professionals using PPE that includes a full-face respirator, fitted with a chemical filter and pretested for effectiveness. A formaldehyde dosimeter should be available also.*)

Note: formaldehyde is a gas which is soluble in water. The solution of formaldehyde in water is named "formalin". Fully saturated (100%) formalin has a concentration of approximately 37% formaldehyde. For simplicity, concentrations of formalin are used where possible below. So, for example, 10% formalin would be a 3.7% formaldehyde solution.

Rooms where surfaces cannot be cleared before decontamination and disinfection, such as laboratories, can be fumigated by boiling off (for rooms up to 25–30 m^3) 4 litres of 10% formalin in an electric kettle (fitted with a timing or other device to cut off the electricity when the fluid level has reached the element) and leaving overnight (or no less than 4 hours from the point in time when the boiling process has been completed) before venting.

Alternatively, paraformaldehyde can be vaporized in a pan on an electric element on the basis of 12 g per m^3 with simultaneous evaporation of 4 litres of water to supply the necessary humidity. For formaldehyde fumigation, room temperature should be > 15 °C.

(*Caution: vaporization of formalin or paraformaldehyde should not be done with gas or other naked flame heaters; formaldehyde is flammable. Avoid skin contact with formaldehyde solution or inhalation of formaldehyde vapour.*)

Neutralization of formaldehyde can be carried out by vaporizing 15.5 g of ammonium bicarbonate per m^3 or 13 g of ammonium carbonate per m^3 in a second pan on an electrically heated element. An electric fan will assist in circulating the ammonia, but it may still be 24–48 hours before the room can be entered without a respirator.

(*Note: ammonium carbonate and bicarbonate are hygroscopic. If they have become damp, greater weights than those given above should be vaporized to compensate.*)

The presence of absorbent material in the room (paper, cardboard, fabric, etc.) reduces the rate of clearance and, indeed, can reduce the effectiveness of the fumigation process. Where there is extensive absorbent material present, the exposure time and possibly the starting concentration of the formalin or paraformaldehyde should be raised to compensate.

Before fumigation commences, all windows, doors and other vents to the outside should be sealed with heavy adhesive tape. Hazard warning notices should be posted on the door(s) and, if appropriate, windows. To ensure complete access of the fumigant, items of equipment should be held above bench or floor surfaces by racks or by tilting to allow the fumigant to penetrate underneath. Proper chemical respirators should be on hand and at least one nitrocellulose disk or filter paper which has, beforehand, been dipped in a spore suspension and dried should be placed at some point in the room distant from the kettle. Preferably the spore preparation should be an accepted standard, such as *B. subtilis* var *globigii* (NCTC 10073) or *B. cereus* (ATCC 12826), but failing the availability of these, the spores of the Sterne vaccine strain ($34F_2$) of *B. anthracis* would do.

At the end of the fumigation, the spore disc(s) should be retrieved into a sterile petri dish and the windows or vents to the outside air should be opened up. (*Caution: a chemical respirator should be used for this. Respirators should be fitted and tested by qualified personnel and users of respirators should be trained in their correct use by qualified personnel.*)

A fan, or fans, assists the extraction. Doors into the room should be kept closed and other personnel prevented from passing near or through them until venting is complete. If a formaldehyde meter is available, venting should not be considered complete until levels of less than 2 ppm have been reached. In the absence of a meter, the odour of formaldehyde should have become almost undetectable before entry into the room without a respirator is allowed.

The effectiveness of the fumigation procedure

is checked by placing the spore disc(s) on plates of nutrient agar. In the case of formaldehyde fumigation, the nutrient agar should contain 0.1% histidine final concentration and be added as a filter-sterilized solution after the agar has been autoclaved and cooled to 50 °C. After overnight incubation at 37 °C, if fumigation was properly effective, the discs should show no bacterial growth.

Formaldehyde is becoming regarded as unacceptable in some places, at least for fumigation of large spaces, in particular in the USA on the basis of potential carcinogenicity. Following a peer-review process involving representatives of industry, academia and government, chlorine dioxide gas was identified by the United States Environmental Protection Agency as the best option for fumigation of buildings contaminated by the deliberate release events of October–November 2001 in the USA.

This requires the mixture of two solid precursor chemicals, sodium chlorite (NaClO) and sodium chlorate ($NaClO_3$), to produce chlorine dioxide (ClO_2) gas. The gas is an unstable compound and potentially explosive in air concentrations > 10 % v/v. It should therefore only be used as a fumigant by qualified personnel using the appropriate personal protective equipment, and generating equipment and a method based on liquid starting chemicals which cannot generate explosive concentrations (> 10% v/v).

Of the other effective sporicidal fumigants (vaporized hydrogen peroxide, methyl bromide, ethylene oxide), probably only vaporized hydrogen peroxide would be appropriate for attempts at room fumigation. It is by far the most ecologically acceptable, with the degradation products being oxygen and water. However it can be anticipated that more than one fumigation session may be needed before spore-strip tests pass completely. The process again requires the appropriate generating and personal protective equipment and should only be carried out by professionally qualified personnel.

3.2 Disinfection in rooms, animal houses, vehicles, etc.

Where fumigation is not an option, or following fumigation of a facility, such as an animal room, containing extensive soiled matter, disinfection should be carried out in a three-step process aimed at (i) preliminary disinfection, (ii) cleaning, and (iii) final disinfection. (*Caution: protective clothing, including eye cover and, at least with formalin, a combination chemical and biological respirator should be worn. Respirators should be fitted and tested by qualified personnel and users of respirators should be trained in their correct use by qualified personnel. Skin and eye contact with the disinfectants listed below or inhalation of their vapours should be avoided.*)

Stage 1: preliminary disinfection

One of the following disinfectants may be used in amounts of 1–1.5 litres per square metre for an exposure time of 2 hours:

- hypochlorite solution containing 10 000 ppm active chlorine (note: chlorine is rapidly neutralized by organic matter; if this is present, it should be washed down first with water and collected into suitable containers for autoclaving or aldehyde disinfection);
- 10% formalin (temperature should be ≥ 15 °C);
- 3% hydrogen peroxide solution.

Stage 2: cleaning

Where practical, cleaning of all surfaces should be done by straightforward washing and scrubbing using ample hot water or mild hypochlorite solution (5000 ppm active chlorine). The operator should wear protective clothing, face and hands included. Cleaning should be continued until the original colours and surfaces are restored and the wastewater is free of dirt particles. At the end of the process, residual water should be removed and disinfected and the surfaces dried.

Stage 3: final disinfection

For final disinfection, one of the following disinfectants should be applied at a rate of 0.4 litres per square metre for an exposure time of at least 2 hours:

- hypochlorite solution (10 000 ppm available chlorine)
- 10% formalin (temperature should be ≥ 15 °C)
- 3% hydrogen peroxide solution.

After the final disinfection, closed spaces such as rooms or animal houses should be well ventilated before recommissioning.

The effectiveness of the disinfection procedure cannot be assumed, and attempts should be made to confirm that it has been adequate by means of swabs and culture.

In the case of surfaces within a room, it may be considered appropriate to finish the disinfection process by fumigating the room itself as described in section 3.1 above.

3.3 Fumigation of safety cabinets; fumigation chambers

(*Caution: a full-face respirator fitted with a chemical filter and a formaldehyde dosimeter should be on hand for this procedure. Respirators should be fitted and tested by qualified personnel and users of respirators should be trained in their correct use by qualified personnel.*)

3.3.1 Formaldehyde fumigation

Prior to fumigation, items to be fumigated within the cabinet should be raised or angled in such a way as to ensure as near to all-round exposure as possible. Equipment can be placed on wire racks, boxes of tips placed at an angle, pipettors stood up in racks and so on.

Biosafety cabinets (volume 1–3 m^3) may be fumigated by boiling to dryness 25–50 ml of 40% formalin prepared by adding 1 part of undiluted formalin to 1.5 parts of water. Alternatively, paraformaldehyde may be vaporized in a pan on an electric element on the basis of 12 g per m^3 with simultaneous evaporation of 25–50 ml of water to supply the necessary humidity. The temperature should be ambient (> 15 °C) and exposure time at least 4 hours (often overnight is convenient). The cabinet can then be vented to the exterior (preferably directly to the exterior of the building) or neutralization of formaldehyde can first be carried out by vaporizing 15.5 g of ammonium bicarbonate per m^3 or 13 g of ammonium carbonate per m^3 in a second pan on an electrically-heated element. To ensure good mixing of the ammonia and formaldehyde, the cabinet blower, or a fan, should be switched on for about 10 seconds when between one third and two thirds of the ammonium bicarbonate/carbonate has vaporized. Allow 1 hour after the ammonium bicarbonate/carbonate has been fully vaporized for neutralization to be completed. If only venting to the room is possible, extraction fans from the room to the exterior should be switched on and at least 2 hours allowed before work is carried out in the room.

(*Caution: vaporization of formalin or paraformaldehyde should* ***not*** *be done with gas or other naked flame heaters: formaldehyde is flammable. Skin contact with formaldehyde solution or inhalation of formaldehyde vapour should be avoided.*)

Note: ammonium carbonate and bicarbonate are hygroscopic. If they have become damp, greater weights than those given above should be vaporized to compensate.

3.3.2 Other fumigants and procedures

Other oxidizing agent fumigants – hydrogen peroxide, ethylene oxide, chlorine dioxide, methyl bromide, etc. – are also effective. Hydrogen peroxide is especially appealing in that its degradation products are oxygen and water. However, the equipment needed for hydrogen peroxide fumigation is, at present, cumbersome, elaborate and expensive and is not universally available. It would not lend itself to routine fumigation of safety cabinets after every use. It is also reported to be readily neutralized by organic matter, including paper and cardboard (Rupert, personal communication, 2004). Ethylene oxide and methyl bromide are acutely toxic at concentrations of > 50 ppm and may cause skin burns and blistering; ethylene oxide is also explosive under alkaline conditions or if exposed to certain other chemicals. Although highly effective, ethylene oxide, methyl bromide and chlorine dioxide are really only to be recommended where the correct equipment and expertise in its use are available. Chlorine dioxide may cause discoloration.

Fumigation chambers should be properly constructed, airtight with a system of venting to the outside away from places of human or animal movement at the end of the fumigation procedure. The relative humidity within the chamber should be > 90% during the fumigation procedure (> 70% is adequate for ClO_2 –Rupert, personal communication, 2004).

Where fumigation is not possible or feasible, reliance for decontamination will probably depend on thorough hypochlorite wipe-down, possibly with UV support (Annex 1, section 7.9).

4. Chemical decontamination of materials contaminated with *B. anthracis*

4.1 Chlorine solutions

Commercially-prepared hypochlorite as supplied to laboratories, hospitals, etc. frequently takes the form of stock solutions having approximately 10% available chlorine (100 000 ppm). Thus, what is familiarly referred to in laboratories as "10% hypochlorite solutions" is a 1:10 dilution of the stock solution containing 10 000 ppm available chlorine. (Note: "bleach" as

sold in stores and supermarkets is frequently less concentrated, usually with 3%–5% available chlorine. This needs to be taken into account when making up daily working solutions.) If solid precursors of hypochlorous acid is available, stock solutions containing 100 000 ppm available chlorine should be prepared and the required dilutions made from this.

Unless a stabilizer such as 0.1% sodium carbonate is included, chlorine solutions are not highly stable and stock solutions should be titrated periodically to ensure that the correct level of available chlorine is present (see 2.1 above). Since stability is affected by concentration (and also by temperature and pH), subsequent dilutions should be made only as needed and these solutions should be changed frequently (preferably each day, but at least weekly). It should be remembered that chlorine solutions corrode metals and perish rubber, and that chlorine is rapidly neutralized by organic materials, including wood (as in wooden benches), soil, or specimens of blood or tissues.

Simple chlorine solutions are slow to kill spores (Jones & Turnbull, 1996). The sporicidal rate can be increased by using 50% methanol or ethanol to make the dilutions of the stock solution. However, the stability of these mixtures has not been established and, if used, these solutions should be made up fresh each day.

Simple garden-type spray bottles can be used for delivering hypochlorite solutions to surfaces prior to wiping down, although the chlorine will corrode the spring mechanism quite quickly and these spray bottles will need to be acquired and used on a semidisposable basis.

4.2 Rapid turnover items

Pipettes, disposable loops, microscope slides, sampling spoons, etc. may be immersed overnight in hypochlorite solutions with 10 000 ppm available chlorine. Small plastic items (loops, spoons, etc.) should then be transferred to an autoclave bin or bag for autoclaving, or to a bag for incineration. Glass items should be transferred to a sharps container for autoclaving and/or incineration. It is recommended that long plastic pipettes (1, 5, 10, 25 ml, etc.) are also discarded into sharps containers since they readily perforate autoclave bags.

4.3 Benches

Benches should be wiped down after use with hypochlorite solutions containing 10 000 ppm available chlorine. Because of their neutralizing effect on chlorine, wooden benches should be replaced by more suitable materials or covered with plastic or laminated sheeting, or with a proprietary covering designed for the purpose, such as Benchcote™.[1]

4.4 Spills and splashes on surfaces

Some thought should be given to the nature of the material spilled. For example, freshly growing *B. anthracis* cultures will have few, if any, spores and these will be incompletely dormant and more susceptible to disinfection procedures than, at the opposite extreme, purposely prepared spore suspensions.

In general, spills and splashes of cultures, or of materials known to be, or suspected of being contaminated with *B. anthracis* on floor, bench or apparatus should be covered with towelling and the towelling saturated with a hypochlorite solution containing 10 000 ppm available chlorine. The towelling should be left in place for at least 30 min before being transferred to an autoclave bin or bag and autoclaved, or to a bag for incineration. Vertical surfaces should be washed or wiped down thoroughly with cloths soaked in this solution. (*Caution: the operator should wear gloves and safety spectacles or goggles while doing this.*) In the event of substantial spills or splashes of spore suspensions, fumigation would be advisable after the initial hypochlorite decontamination. This would apply to the safety cabinet if the accident occurred within the cabinet, or the room if the accident occurred outside the cabinet.

Solutions of 10% formalin, 4% glutaraldehyde, 3% hydrogen peroxide or 1% peracetic acid are possible alternatives to hypochlorite, but the choice must be weighed against the greater personal protection needed when using these.

4.5 Biosafety cabinets

Decontamination of cabinets has been covered in Annex 1, section 7.9 and in section 3.3 above.

[1] Whatman International Ltd, Maidstone, United Kingdom.

5. Personal exposure

5.1 Spills and splashes on clothing

Rear-fastening laboratory gowns (surgical type) are the best type of overclothes to wear in laboratories working with *B. anthracis*, and disposable versions are available. In their absence, a plastic apron should be worn over the laboratory coat. Contaminated gowns/aprons/coats should be removed immediately and placed in autoclave bins or bags and autoclaved. Personal clothing that may still be contaminated – shoes, socks/stockings/upper garments if sleeves or collars are contaminated – should be removed as soon as possible and, if possible, autoclaved. Alternatively, they may be fumigated in a cabinet or fumigation chamber (section 3.3.2 above). Ideally, there should be an emergency shower and emergency clothing in the exit area that will allow the individual to put the contaminated clothes into an autoclave bag or bin, shower and dress to leave the area.

5.2 Spills and splashes on skin or in eyes

In case of contact (biological or chemical agents) with eyes, the eyes must be flushed out with copious quantities of water immediately for at least one full minute, preferably with running water. Ideally an eye-wash station should be included in the laboratory design. Avoid rubbing the eyes. The appropriate medical officer should be informed and the affected person kept under observation for at least a week.

In case of skin contact, the gross contamination should be washed off with water into a bowl and the washings subsequently neutralized by adding bleach and autoclaving. The skin should then receive a thorough soap and water wash (at least 2 minutes). The value of washing the skin itself with bleach is debatable since the contact time is too short to be effective. Bleach certainly should not be used on broken skin as it is likely to do more harm than good. Where the skin is broken (including needle-stick punctures), bleeding should be encouraged and the injury washed with copious amounts of water. The appropriate medical officer should be informed and the affected person kept under observation for at least a week.

5.3 Contamination in the mouth

At the outset, laboratory workers should be reminded that mouth pipetting in a microbiology laboratory is *unacceptable*. For contamination of the mouth with known or possible anthrax organisms, the mouth contents should be immediately spat out followed by thorough mouth washes with water. The appropriate physician should again be informed and the affected person be kept under observation for a week.

5.4 Suspected inhalation

In the event of suspected inhalational exposure, exhalation should be performed as hard as possible. Others present in the laboratory should be informed and, if necessary advised to evacuate the laboratory. The appropriate supervisor, safety officer and medical officer must be notified immediately and decisions on actions made without delay.

5.5 Exposure through sharps accidents

For sharps punctures (e.g. broken glass), gloves should be peeled off immediately and the wound encouraged to bleed under running tap water for 2–5 minutes followed by a thorough soap and water wash (washing wounds with disinfectant is not recommended). See also section 5.2 above.

6. Decontamination of animal products, environmental materials, etc.

6.1 Manure, dung, bedding, feed, etc.

Where possible, anthrax-contaminated materials to be disposed of, such as bedding, feedstuffs, manure, etc., should be incinerated or autoclaved (121 °C core temperature for 60 minutes). Immersion in 10% formalin for > 12 hours is an alternative, but full penetration of the fluids must be ensured and natural degradation of the fumigant to the point at which the material can be handled in some way will be slow (at least several weeks). Probably a way of neutralizing and degassing the fumigant should be worked out in advance of taking this route. (*Caution: avoid skin contact with formaldehyde solutions or inhalation of formaldehyde vapour. See cautions in section 1.1 above.*)

Slurry from livestock suffering outbreaks of anthrax may be also disinfected with formaldehyde by adding undiluted formalin with thorough stirring until a final concentration of 10% formalin is reached. The mixture should be left a minimum of four days with stirring for at least one hour each day before being further processed (Williams et al., 1992). (*Caution: avoid skin contact with formaldehyde solution or inhalation of formaldehyde vapour. See cautions in section 1.1 above.*)

Formalin degrades naturally to formic acid and thence to carbon dioxide and water (Goring & Hamaker, 1972). It is photooxidized by sunlight to carbon dioxide, and it reacts with nitrogen compounds in the air or soil to form formic acid which, in turn, degrades to carbon dioxide. Its half-life in air is generally less than one hour (WHO, 1991). Certain bacteria and yeast are also able to bring about this degradation by means of dehydrogenases. An alkaline pH neutralizes the formic acid and thereby increases the rate of degradation by pulling the equilibrium in that direction. Decomposition is most rapid at pH 7–8 and with added nitrogen (e.g. in ammonium carbonate). Buffering or addition of lime, to counteract the lowered pH as formic acid is produced, will aid degradation. The treated slurry can be spread on uncultivated land and ploughed in or otherwise buried.

6.2 Sewage sludge

Sewage sludge containing effluents from tanneries that process hides from enzootic areas may contain anthrax spores. Dewatered sewage sludge up to a dry-matter content of 8% should be disinfected by bringing to 10 % formalin and retaining for 10 hours or 3% peracetic acid for 30 minutes. The disinfection process is not affected by polyelectrolytes and may be enhanced by lime added for dewatering the sludge (Lindner et al., 1987). Formalin degrades fairly rapidly naturally (6.1 above); degradation in sewage sludge specifically is covered by Dickerson et al. (1954). (*Caution: avoid skin contact with formaldehyde or peracetic acid solutions or inhalation of their vapours. See cautions in section 1.1 above.*)

6.3 Water

It is difficult to give general advice on treatment of water. The approach chosen depends on what type of body of water is to be treated, the likely extent of the anthrax spore contamination, what volumes are involved and where the water is to go, and what it may be used for after treatment. However, the choices are much the same as with other materials covered within this annex.

Autoclaving is the surest way of killing spores but is only applicable to fairly small volumes of water. Boiling for 20–30 minutes is a generally effective option. As reviewed by Rice et al. (2004), many experimenters have concluded that boiling inactivates *B. anthracis* spores with the outside time in the order of 12 minutes. Rice et al. found that there was a critical difference in the effectiveness of boiling when there was a lid on the container in which the water was boiled.

Treatment by bringing to a concentration of 10% formalin and retaining for at least 10 hours is feasible for volumes up to about 100 000 litres, as may result from industrial wastes, but holding tanks must be available and methods of neutralization and discharge without danger to the environment must be established. The necessary safety cautions will apply (*see cautions in section 1.1 above*). Cost is a major factor in this approach also.

The merits of chlorination are debatable; the levels of chlorine necessary to ensure effective killing of spores may be hard to attain in large volumes and, if the body of water is on open ground, it is likely to contain organic matter which rapidly neutralizes the chlorine.

Filtration, as for water treatment, is probably effective as far as the emerging water is concerned, but leaves unsolved the problem of contaminated filter beds.

In general, each situation should be considered on an individual basis and the best solution worked out for the particular circumstances that exist.

6.4 Soil

If possible, soil at the site of an anthrax carcass should be removed up to a depth of 20 cm and incinerated or heat-treated (121 °C throughout for 60 minutes). If this is not possible, it should be disinfected with 10% formalin at 50 litres per m^2. Where it is necessary to decontaminate soil to greater depths, such as burial sites of anthrax carcasses, 10% formalin should be injected below the soil surface at a rate of 30 ml for every 10 cm of depth at 0.5 m horizontal intervals across the contaminated area. (*Caution: avoid skin contact with formaldehyde solution or inhalation of the vapour. See cautions in section 1.1 above.*)

It is sometimes not possible to achieve sufficient penetration of even small clods of soil by formaldehyde or other sporicide solutions to result in complete kill of anthrax spores (Turnbull et al., 1996), especially in the case of water–saturated or heavy soils. Decontamination failure may result when attempting chemical disinfection, and the effectiveness of any such attempt should be checked by subsequent culture.

The decision on the best approach to making a contaminated site safe depends substantially on

what the site is to be used for in the future. Where it is not feasible to incinerate or chemically decontaminate the soil or to remove it to an incinerator, the alternative is to close or seal off the site. Covering with concrete or tarmac for, say, a car park, is an alternative used in industrialized countries; planting with thorny bushes surrounded by a secure fence can be an aesthetic approach.

Further guidelines are supplied elsewhere (Turnbull, 1996).

6.5 Other materials – clothing, tools, etc.

Where possible, contaminated materials should be incinerated or autoclaved at 121 °C for 60 minutes. In the case of nondisposable items such as clothing, boots, tools, etc., excess dirt should be scraped off into incineration or autoclave bags and the items themselves should be soaked overnight (at least 8 hours) in 10% formalin. (*Caution: avoid skin contact with formaldehyde or glutaraldehyde solutions or inhalation of their vapours.*) Bleach is a possible alternative if discoloration or corrosion is not of consequence, and there is little organic material left on the items after scraping.

Decontamination and disposal procedures following collection and examination of clinical specimens are covered in Annex 1, sections 7.7 & 7.8.

6.6 Wool, hair or bristles

Disinfection stations exist in a number of countries which import wool, hair or bristles from endemic regions, and the names and addresses of these may be obtained from the relevant veterinary authorities that control imports and exports of animal products. The requirements of an importing country will be specified as part of the approval process for getting a permit. One established disinfecting protocol is the Duckering process. This involves five stages, each of 10 minutes duration at 40.5 °C: (i) immersion in 0.25%–0.3% soda liquor; (ii) immersion in soap liquor; (iii) two immersions in 2% formaldehyde solution (5% formalin); (iv) rinsing in water; and (v) the wool or hair is finally dried in hot air and baled.

In countries where irradiation facilities are available, the preferred approach is to test samples of wool and hair from a consignment and, if positive for *B. anthracis*, to sterilize the consignment by irradiation. The dose needed to guarantee freedom from viable spores in a contaminated lot is very high; the D_{100} in spore suspensions of 10^8 to 10^{10} per ml have been found to exceed 40 kGy (4 MRad) (Bowen et al., 1996). Calculations of exposure times need to take into account the size and density of the bales being irradiated.

6.7 Hides and skins

No hazard need be expected in situations where hides come from properly supervised slaughtering. Dry hides of uncertain origin within enzootic countries should, on the other hand, be regarded as being of high risk in terms of anthrax. Where possible these should be decontaminated by fumigation (formaldehyde or ethylene oxide) or by irradiation prior to processing. It is considered by some tannery experts, however, that no preprocessing disinfection protocol has been devised for hides and skins that does not damage them (Anon., 1959). However the dehairing stage, which involves sodium sulphide liming with a mixture of sodium sulphide and calcium hydroxide, exposes the skins to a significant level of sodium hydroxide at high pH which probably kills any *B. anthracis* spores present (Robertson, 1948; Lindner & Böhm, 1985).

Control processes in tanneries should therefore be primarily targeted at stages before dehairing, particularly dust control and treatment of effluent from initial washing and rehydrating stages. In tanneries processing raw hides from anthrax-endemic areas, these effluents should be treated by bringing to 10% formalin and holding for 10 hours, with adequate time being allowed for natural degradation of the formaldehyde before discharging to sewerage. Peracetic acid (3%) for > 30 minutes is an alternative but more expensive treatment.

Precautions should be taken to avoid cross-contamination of hides and skins pre- and post-treatment through appropriate controls on movement of personnel, equipment and the hides themselves.

6.8 Bone, bonemeal, hoof and horn

Feed ingredients of bone, hoof and horn origin imported from endemic countries are still the cause of incidents among livestock in nonendemic importing countries. Similarly, bonemeal in fertilizers is periodically suspected of being the source of anthrax infection in humans and animals. However, in many developed countries anthrax is becoming very infrequent and there has been a significant decline in the occurrence of anthrax from feed contamination following the BSE (bovine spongiform encephalopathy)

restrictions on the feeding of animal or ruminant materials to ruminants.

It is considered in most importing countries that mandatory requirements for sterilization of products of animal origin for international commerce would raise the costs of these products disproportionately to the human and animal health risks involved. Consequently few such countries have statutory requirements of this nature. However, some manufacturers consider it standard good practice to sterilize such products before placing them on the market, and this is certainly to be encouraged, particularly if they are to be used as fertilizers on land on which animals will subsequently graze. Similarly it should be a reasonable policy aim in any country to collect and process separately those raw bone, hoof and horn products obtained from regular and supervised slaughtering and those obtained from sources of uncertain origin, which present a higher risk in terms of anthrax.

Long-term control will be dependent on improved and effective control measures in the exporting countries. In the interim, control should depend on close adherence to the Terrestial Animal Health Code (Annex 4).

7. Guidelines on incineration of carcasses

The underlying physical principle that should be addressed in designing an efficient incineration procedure is that material underneath a flame can remain cool so that contaminated materials (ground, soil, carcass remains, etc.) that remain below the flames during incineration will remain contaminated. A number of approaches may be taken to ensure that incineration is fully effective, and the one of choice depends on available resources and other circumstances. Portable incinerators with gas-fired jets at base level and 0.25 m above base level, or flame guns which direct the flames downwards, are available in some countries (Fig. 10). These provide a good way of ensuring complete and effective incineration.

The following suggestions are offered to cover the different circumstances that may be encountered. It should be pointed out that all the procedures described below take many hours for a large domestic animal, such as a cow.

7.1 In-place incineration

7.1.1 Pit method[1]

For a large animal, a pit about 0.5 m deep and exceeding the length and breadth of the carcass by about 0.25 m on each side should be dug. A trench approximately 0.25 m wide by 0.25 m deep should be dug along the length of the centre of the pit extending beyond the ends by about 0.75 m; this serves the purpose of allowing air for the fire under the carcass. The bottom of the pit and the trench should be covered with straw which is then soaked in kerosene.

Above the kerosene-soaked straw, place a few pieces of heavy timber (or other type of beams which will hold the carcass well above the bottom of the pit) across the pit and then scatter thin pieces of wood over beams and straw. Then add larger pieces of wood and if available, coal, until the pit is filled to ground level. Saturate all the fuel with kerosene.

The carcass can then be drawn on to the pyre, preferably propped up so that it is lying on its back. Further kerosene should be poured over the carcass. The fire is started at either end of the longitudinal trench. Once the incineration is well under way (probably after about the first hour), the pyre should be covered with corrugated iron or other metal sheeting in such a way as to reduce heat loss without cutting off ventilation.

The approximate quantities of fuel that will be needed for a large domestic animal are 20 kg of straw, 10 litres of kerosene, and either 2 tonnes of wood or 0.5 tonnes of wood and 0.5 tonnes of coal. Note: it will be necessary to decontaminate the ground where the carcass lay and also the ground, equipment, etc. contaminated during the movement of the carcass (see section 6.4 above).

7.1.2 Pyre[2]

A pyre may be built up around the carcass so that it is incinerated precisely in the position in which it was found. Despite its reduced effectiveness compared to pit and raised incinerations which allow air to circulate underneath the carcass, a pyre may be the only cremation method available in remote areas where machinery necessary to position the carcass is unavailable or with large animals such as bison, elephant, giraffe and hippopotamus. After the initial burn, the ash and remains must be turned over

[1] Based on MAFF, 1992.
[2] Kindly supplied by D.C. Dragon and J.S. Nishi.

and burnt a second time. During carcass incineration trials in northern Canada during summer 2001, researchers regularly observed unburnt hair, hide, rumen, stomach lining and flesh beneath the ash after a single pyre incineration of bison carcasses. It was necessary to lift these remains from beneath the ashes and reburn them.

For a bovine-sized carcass, approximately 200 litres of a kerosene-based fuel and either 1 tonne of wood, or 300 kg of wood and 600 kg of coal, are needed. The wood should be in the form of 2 m length logs. The wood and coal should be piled around and on top of the carcass, and the pyre dosed with fuel and lit. When the primary incineration is complete, the ashes are allowed to cool. Personnel in appropriate protective gear (coverall, gloves, boots and respirator) should turn over the ashes and repile unburnt carcass remains with wooden poles. The poles are left with the remains and the pile reburned with further fuel. Consideration may be given to damping down or disinfecting the unburnt remains with 10% formalin prior to turning over the ashes, to minimize the chances of aerosolizing surviving spores (see Annex 6, section 1).

In the Canadian experience following outbreaks of anthrax in bison, the combination of in-place incineration and formalin treatment had an unforeseen benefit (Dragon et al., 2001). The combination of ash from the fire and formaldehyde-fixed organic matter changed the nutrient profile of the soil, and the carcass sites were recolonized by herbaceous, leafy plants such as yarrow, wild mint and lamb's quarter rather than the grass and sedge that grew previously. None of these plants are feed items for bison, and their size and density discouraged bison from wallowing at the carcass sites. Thus, the bison were kept spatially separated from any viable infectious anthrax spores remaining at the old carcass sites.

7.1.3 Raised carcass method[1]

This method may be appropriate when labour is scarce or the ground is unsuitable for the construction of a pit.

Place straw over a 2 m by 1.5 m area. Place two wooden beams (approximately 2 m lengths of small tree trunks, railway sleepers, etc.) over the straw parallel to each other, about 1.25 m apart and aligned with the direction of the prevailing wind. Soak the straw with kerosene and cover with thin and thick pieces of wood and coal if available. Place further stout cross-pieces of wood or other material across the two main beams to support the carcass. The fuel (wood or coal) is banked up on either side of the carcass (but not at the ends, where the air should be allowed to enter under the carcass), and the solid fuel and carcass are further doused with kerosene.

The fire can then be started and as before, when well under way, it should be covered with metal sheeting to retain heat but without inhibiting ventilation. Further fuel should be added if and when necessary.

Rather more fuel may be required than with the pit method. For a large domestic animal, an estimate is 0.75 tonnes coal + 0.5 tonnes wood or, if coal is unavailable, approximately 3 tonnes of wood, plus 20 kg straw and 20 litres of kerosene.

As with other approaches, it will be necessary to decontaminate the site where the carcass lay before incineration, and the ground and equipment contaminated while moving it from there to the cremation bed.

7.1.4 Gelled fuel

Anderson, Nevada Department of Agriculture, Animal Disease Laboratory (personal communication, 2000), described a gelled fuel terra torch system that was used to cremate bovine carcasses in a United States anthrax outbreak. SureFire – a powdered gelling agent designed to thicken fuels used in prescribed burning – was mixed into a recirculating tank containing a fuel mixture of diesel and regular gasoline. The thickened fuel was applied with a terra torch system and gave a hotter and more lasting fire than with fuel alone. The technique appeared to be an efficient method for the disposal of animal mortalities during emergencies. For example, an adult cow was reduced to ashes in about an hour using the powder in a 70/30 mixture of diesel and leaded gasoline. However, subsequent application of a similar formulation for gelled fuel in northern Canada failed to achieve adequate incineration of bison carcasses during an anthrax outbreak in the Slave River Lowlands (Nishi, Resources, Wildlife and Economic Development, Northwest Territories, personal communication, 2000). Possibly the difference was due to cooler temperatures and higher humidity in northern Canada compared to the affected United States region, or to a lower fat content in wild bison

[1] Based on MAFF, 1992.

compared to ranched cattle. With its advantages of ease of use, speed of disposal and minimal fuel requirements, the use of gelled fuel in anthrax carcass disposal remains promising and merits future study, either alone or in combination with wood or coal.

7.3 Commercial incinerators

7.3.1 Down-directed blow torches

An example of the use of down-directed blow torches for incineration of a carcass is shown in Fig. 10.

7.3.2 Portable incinerator

An example of incineration of a bovine anthrax carcass in a portable incinerator is shown in Fig. 10.

7.3.3 Centralized incinerators

Largely since the advent of the focus on BSE, commercial incinerators capable of taking whole bovine carcasses have now become available. It seems feasible for an anthrax carcass to be well-bagged in the same manner as if it were being taken to a rendering facility, and to be taken for incineration at one of these types of incinerator. The Australian procedure of spraying the carcass with formalin and loading it onto double-thickness plastic on a low-loading trailer and wrapping the carcass in the plastic (section 8.3.2.1) might be an approach worth considering. While these approaches would appear to be perfectly practicable, under the current legislation in certain countries, there may be problems obtaining local or national movement orders permitting the transport of the carcass.

As with the other approaches to carcass incineration, it will be necessary to decontaminate the site where the carcass lay before removal, and any equipment contaminated when bagging it.

8. Autoclave function

Frequent reference is made in this publication to sterilization by autoclaving. Autoclave function should be confirmed by inclusion of a spore strip, especially for "destruction runs" (i.e. where items are being sterilized prior to be disposed of), and even more particularly if the autoclaved items are not going to be incinerated. As with fumigation (section 3.1 above), the spore strip may be "home-made" using a filter paper which has, beforehand, been dipped in a spore suspension and dried. The spore preparation ideally should be an accepted standard, and preferably a thermophile, such as *Bacillus stearothermophilus* ATCC 7953, but failing the availability of this, the spores of the Sterne vaccine strain ($34F_2$) of *B. anthracis* would do. After autoclaving has been completed, the disc should be retrieved aseptically and placed on a plate of nutrient agar together with a control unautoclaved disc, and the plate incubated 1–3 days (if *B. stearothermophilus* is being used, the incubation temperature should be 55–60 °C).

Convenient commercial spore strips are readily available from hospital supply houses. Examples are the BTSure Biological Indicator[1] and Chemiclave® spore strips.[2] Again, an unautoclaved control should be included when incubating a spore strip used to check an autoclave cycle.

[1] Barnstead/Thermolyne, PO Box 797, Dubuque, Iowa 52004–0797, USA.
[2] Raven Biological Laboratories, Inc., Omaha Nebraska, USA; www.ravenlabs.com.

ANNEX 4

Terrestrial Animal Health Code[1]

Chapter 2.2.1 Anthrax

Article 2.2.1.1

There is no evidence that anthrax is transmitted by animals before the onset of clinical and pathological signs. Early detection of *outbreaks*, quarantine of affected premises, destruction of diseased animals and fomites, and implementation of appropriate sanitary procedures at *abattoirs* and dairy factories will ensure the safety of products of animal origin intended for human consumption.

For the purposes of the *Terrestrial Code, the incubation period* for anthrax shall be 20 days.

Anthrax should be notifiable in the whole country.

Standards for diagnostic tests and vaccines are described in the Terrestrial Manual.

Article 2.2.1.2

Veterinary authorities of importing countries should require:

for ruminants, equines and pigs

the presentation of an *international veterinary certificate* attesting that the animals:

1. showed no clinical sign of anthrax on the day of shipment;
2. were kept for the 20 days prior to shipment in an establishment where no case of anthrax was officially declared during that period; or
3. were vaccinated, not less than 20 days and not more than 6 months prior to shipment.

Article 2.2.1.3

Veterinary authorities of importing countries should require:

for products of animal origin (from ruminants, equines and pigs) intended for agricultural or industrial use

the presentation of an *international veterinary certificate* attesting that the products:

1. originate from animals not showing clinical signs of anthrax; or
2. have been processed to ensure the destruction of both bacillary and spore forms of *Bacillus anthracis* in conformity with one of the procedures referred to in Appendix X.X.X. (under study).

Article 2.2.1.4

Veterinary authorities of importing countries should require:

for fresh meat and meat products destined for human consumption

the presentation of an *international veterinary certificate* attesting that the products originate from animals which:

1. have shown no sign of anthrax during antemortem and postmortem inspections;
2. come from *establishments* which are not placed under quarantine on account of anthrax control and in which:
   a. there has been no case of anthrax during the 20 days prior to slaughter;
   b. no vaccination against anthrax has been carried out during the 42 days prior to slaughter.

[1] Terrestrial Animal Health Code. Paris, World Organisation for Animal Health (OIE), 2007 (http://www.oie.int/eng/normes/mcode/en_chapitre_2.2.1.htm#rubrique_anthrax).

Article 2.2.1.5

Veterinary authorities of importing countries should require:

for hides, skins and hair (from ruminants, equines and pigs)

the presentation of an *international veterinary certificate* attesting that the products originate from animals which:

1. have shown no sign of anthrax during antemortem and postmortem inspections;
2. come from *establishments* which are not placed under quarantine on account of anthrax control.

Article 2.2.1.6

Veterinary authorities of importing countries should require:

for wool

the presentation of an *international veterinary certificate* attesting that the products:

1. originate from animals showing no clinical signs of anthrax at the time of shearing;
2. originate from *establishments* where no case of anthrax has been reported since the previous shearing of all animals.

Article 2.2.1.7

Veterinary authorities of importing countries should require:

for milk and milk products intended for human consumption

the presentation of an *international veterinary certificate* attesting that the products:

1. originate from animals showing no clinical signs of anthrax at the time of milking; or
2. were processed using a heat treatment at least equivalent to pasteurization (under study).

ANNEX 5

Vaccines and therapeutic sera[1]

1. General

1.1 Vaccines

The history and theory of anthrax vaccines is covered in section 8.6. This annex covers the more practical details on vaccines and lists the names, addresses, telephone and fax numbers and other relevant data that could be traced on available anthrax vaccines.[2]

1.2 Therapeutic sera

The history and background to therapeutic sera for anthrax are given in sections 7.2.2.3 (for animals) and 7.3.4 (for humans).

2. Veterinary vaccines

Most veterinary vaccines are manufactured broadly in accordance with the *Requirements for anthrax spore vaccine (live – for veterinary use), Requirements for biological substances No. 13* (WHO, 1967), the *Manual for the production of anthrax and blackleg vaccines* (FAO, 1991) and the *Manual of diagnostic tests and vaccines for terrestrial animals* (OIE, 2008), or as updated in the *European Pharmacopoeia* or other appropriate pharmacopoeias. The active ingredients of these vaccines are the spores of the $34F_2$ "Sterne" strain suspended in glycerol with saponin added as an adjuvant, essentially as first formulated by Sterne (1939). The veterinary vaccines in China and the Russian Federation are similar in formulation but utilize other toxigenic, non-capsulating strains analogous to the Sterne strain. In Italy (Fasanella, personal communication, 2003), the Pasteur strain (non-toxigenic, capsulating) is still manufactured for vaccination of goats and horses. A different formulation, "Carbosap", is prepared for administration to cattle and sheep; this uses a toxigenic, capsulating strain with reduced virulence for most species. The basis of the reduced virulence is not known. In both vaccines the spores are suspended in 1% saponin. In this context, Sterne's comment (Sterne, 1939) that "it is still necessary to issue separate vaccines for goats and horses" should be noted.

For further information, enquiries should be addressed to the manufacturers direct.

The following subsections aim to address various considerations and questions that may arise regarding the use of veterinary anthrax vaccines and to highlight cautions applicable to them.

2.1 Storage

The vaccines should be stored in a refrigerator but not frozen (repeated freeze-thawing will result in reduced inocula).

2.2 Antibiotics

Since the active ingredient of the livestock vaccines is live (attenuated) *B. anthracis*, antibiotic treatment may be expected to interfere with vaccine performance. This was demonstrated in one small study in which three guinea-pigs receiving 100 000 U penicillin G intramuscularly in one leg at the same time as the Sterne strain vaccine in the other leg were not protected against challenge 3.5 weeks later with virulent *B. anthracis*. Three guinea-pigs receiving the vaccine but no penicillin were protected (Webster, 1973).

Animals being vaccinated should not receive antibiotics for several (7–10) days before or after vaccination. The vaccine may be rendered ineffective, for example, in cattle on antibiotics for growth promotion or receiving antimastitis therapy. If there are

[1] The lists provided in this annex are supplied for the benefit of users of these guidelines, but do not represent endorsement of the products by the World Health Organization (WHO). While every attempt has been made to ensure that the data are correct, WHO does not guarantee the accuracy of the information supplied in these lists. For confirmation of the data and for further information about the products, the reader should contact the relevant manufacturer direct.

[2] WHO invites any manufacturer not listed to notify Dr Ottorino Cosivi (at cosivio@who.int). Manufacturers are requested to inform WHO of any information that is not correct. Any corrections will be published in subsequent supplements or revisions of these guidelines.

concerns that antibiotics may have interfered with vaccine efficacy, the animals may be revaccinated after a period of two weeks.

2.3 Movement to other premises or for slaughter or trade

Where animals are scheduled to be moved for local or international livestock and meat trade purposes, it is important to check whether there are advisories in place specifying a withholding period before which animals may be moved to other premises, or sent to slaughter following vaccination (see sections 7.2.1.2 & 8.7). Basically, the vaccines are not recommended for use in animals destined for slaughter for human consumption within 6 weeks of vaccination (OIE, 2008). Local regulations, or the label on the vaccine being used, may specify longer periods, which may vary from 2–6 weeks. The specifications which pertain for the particular situation which exists and in that particular location should be established before any vaccination campaign is initiated.

2.4 Equines

Sterne, while stating in one paper (Sterne, 1939) that his vaccine had been entirely satisfactory during large-scale use on horses, in another paper the same year (Sterne et al., 1939) said that it was necessary to issue separate vaccines for goats and horses. He did not elaborate why this was the case, however. Later he stated (Sterne, 1946) that the same vaccine was now used for all animals and that, while cattle and sheep reacted very mildly, horses reacted more vigorously. He added, however, that no farmer had complained about the reactions. In 1959 he wrote (Sterne, 1959) that horses were slow to develop effective immunity following vaccination, taking a month or more as compared to less than a week in bovines. Lindeque et al. (1996) found that two initial doses approximately 8 weeks apart were necessary for development of dependably measurable antibody titres in zebra (section 8.6.2).

2.5 Goats (and llamas)

Goats are known to be prone to severe reactions to the vaccines. One possible approach to vaccination of goats is an initial schedule of two inoculations one month apart, with the first dose being one quarter of the standard recommended dose ("pre-inoculation dose"), and the second dose being the standard recommended dose. A single annual booster may be administered thereafter. One manufacturer recommends that injection of the vaccine in goats should be done in the tail-fold region (compared with the neck region in most species).

Llamas are frequently cited together with goats as being prone to severe reactions to the livestock vaccine, and this is again stated in section 8.6.2. However, this seems to be based on a single reference (Cartwright et al., 1987). In this, three 3-month-old calves in a herd of 20 llamas became ill three days after subcutaneous inoculation of the Sterne vaccine in the neck. Severe localized oedema developed at the inoculation site. One of the calves responded to penicillin, one died, and one was euthanized due to being moribund. None of the 17 older animals developed a local reaction. The facts may not support the grouping of llamas together with goats as especially prone to severe reactions to the vaccine.

2.6 Injection

Injection should be made through an area of clean dry skin.

2.7 Pregnant and lactating animals

The Sterne strain $34F_2$ livestock vaccine has been in use for well over half a century and is frequently administered in response to outbreaks. As outbreaks generally occur in summer or hotter seasons, pregnant animals are frequently among those vaccinated. There are apparently no records of adverse events related to the pregnancies, and the vaccine appears to be safe in pregnant animals (Berrier & Hugh-Jones, personal communication, 2006). A dose level of 10 spores of *B. anthracis* strain 193 with a mouse LD_{50} of about 100 spores was used as the "vaccine" in an unspecified number of cows. Excretion of *B. anthracis* was demonstrated between 1 and 9 days after vaccination with "100% recovery from all the cows tested". The relevance of this report is doubtful since the legitimacy of referring to inoculation with strain 193 as "vaccination" is questionable. A more relevant study resulted in no evidence that dairy cattle would shed the Sterne strain in milk following immunization. In this, no isolations of *B. anthracis* were made in milk samples collected from each of 49 vaccinated cows twice daily for 10 days post-vaccination (Tanner et al., 1978).

As mentioned in section 7.2.1.3, the vaccine may be rendered ineffective by antibiotics being used to treat mastitis. It may be necessary to wait until the

antibiotic level has fallen before vaccinating. In an outbreak situation, the animals should be carefully monitored (e.g. twice-daily temperature checks) during this waiting period.

2.8 Discard of vaccine and equipment

Being a live spore suspension, leftover vaccine vials, used syringes, needles, gloves, coveralls and other contaminated items should be disinfected, autoclaved or incinerated after completion of the operation (see Annex 3, section 6.5). It should be remembered that the vaccine is also potentially infectious to humans, so contaminated items should be handled with care (see section 2.10 below).

2.9 Milk from vaccinated animals

See section 8.7.

2.10 Accidental operator inoculation

Self-injection by the operator can give rise to infection but few, if any, serious infections from such events are on record. The experience of Ellard (personal communication, 2004) during one of the vaccination campaigns which followed the outbreaks in cattle in western Australia in 1994 (Forshaw et al., 1996) is probably one shared by many veterinarians over the years:

> "Vaccination of livestock was routinely undertaken using disposable vaccination guns with both the gun and any residual vaccine incinerated at the end of each day. I should mention that, when using this type of gun, it was not uncommon for the operator to self-inject if the animal struggles at an inopportune moment. This happened to me on at least two occasions without any adverse reaction to the vaccine. On each occasion the incident was reported and monitored, but no treatment was required."

In one anthrax outbreak in the United States in 1974, at least 12 people were accidentally inoculated with the Sterne strain vaccine. Clinical follow-up was available on seven persons who received small but unmeasurable amounts of the vaccine (probably 0.1 ml or less) at the time of the needle-stick accident. None of the seven developed a cutaneous lesion at the inoculation site. One developed febrile illness, with cervical and right axillary lymphadenopathy and possible aseptic meningitis, several days after he had punctured his right hand with a needle. However, blood or lymph node cultures were not obtained, and the cause of his illness was not determined (Fox et al., 1977).

Three clinical cases associated with "capsule negative" *B. anthracis* were noted elsewhere. None of these had any association with the Sterne or other vaccine strain; one isolate was from blisters and oedema on the hand and forearms of an immunocompromised individual who had handled 15th century leather in Poland, the second from the faeces of an individual in China with suspected intestinal anthrax, and the third from the blood of a child diagnosed as having endocarditis in Saudi Arabia, though not established as the cause of the condition (Editor's note, 1996a).

In the event of accidental self-inoculation by the operator, gentle pressure should be applied to the wound to squeeze out any inoculums, followed by thorough washing with soap and water. If saponin is included in the vaccine, there may be a painful local reaction at the site of inoculation. Medical advice should be sought if infection sets in.

2.11 Vaccine failures in livestock

Questions arise from time to time regarding cases of anthrax that occur in herds which have been vaccinated, or about continuing cases after vaccination to control outbreaks. Kaufmann et al. (1973, cited by Salmon & Ferrier, 1992) investigated an outbreak involving more than 4000 cattle, and found that 0.1% died (1.4 % of all deaths) 8–14 days after vaccination, and another 0.1% more than 15 days after vaccination. In the outbreak of 1987 described by Salmon & Ferrier, 5 of 10 deaths occurred 3, 5, 11, 68 and 126 days after vaccination and, in another case, 37 days after revaccination. Deaths continued after vaccination in the 1994 outbreak in cattle in western Australia. In Africa, where livestock owners sometimes do not understand the difference between vaccination and treatment, the knowledge that some of their animals may still die after vaccination may lead to distrust of vaccination and resistance to it being done (see section 9.7, Table 10).

Usually it is not possible to identify the specific reasons for these vaccine failures, but the following points may be helpful.

2.11.1 Varying responses and doses

Tests in guinea-pigs show that:

- The antibody response in different individuals may vary. Variable antibody titres are a feature in animals receiving a particular dose of a live spore vaccine.

- Receiving the correct dose is important. Very marked differences in titres are seen in groups receiving 1 million and 10 million spores in a dose.
- Protection tends to be less than 100% in animals if (i) they have only received a single dose, and (ii) the doses were less than 10^7 spores.

Extrapolating this to livestock, circumstances that lead to animals not receiving the correct dose are probably important. Examples of such circumstances in mass vaccination campaigns might be:

- The vaccine spores in the reservoir of the automatic syringe settle, so some animals get a reduced dose.
- The reservoir containing the vaccine runs out and some animals, although injected, actually receive no vaccine.
- The needle gets blocked, or the needles blunted, resulting in a reduced dose or no vaccine being administered.
- Some of the animals are fairly wild and move violently before delivery of the vaccine is complete.
- The animals were too young at vaccination, per se or because the vaccine effect was neutralized by maternal antibody.

Enzyme immunoassay studies on cattle vaccinated in response to the 1994 anthrax outbreak in western Australia (Forshaw et al. 1996) revealed a great variability in titres among animals with similar vaccination records; a few even exhibited low or negative titres despite multiple boosters (Ellard, Ellis & Turnbull, unpublished results). The reasons for this variability were not identified. It was not possible to establish a correlation, or lack of it, between low titre and succumbing to anthrax as the outbreak was brought under control. However, the observation underscores the need for care to ensure all animals receive the correct dose in a vaccination campaign, and also that all other conditions are favourable to optimal vaccine performance at the time of the campaign.

2.11.2 Vaccine potency

Another possible reason that should be considered in the event of vaccine failures is that the potency of the vaccine itself has fallen for reasons beyond the control of the person or team carrying out the vaccination.

2.11.3 Interference by maternal antibody

Questions arising following the vaccine campaign in response to the 1994 anthrax outbreak in western Australia (Forshaw et al., 1996) led to a study aimed at determining whether maternal antibody interfered with the response to anthrax vaccine in calves (Ellard, Ellis & Turnbull, unpublished results). Titres to the anthrax protective antigen in 13 calves from vaccinated dams were compared with those in 12 calves from unvaccinated dams. The calves received dose 1 of the live spore $34F_2$ vaccine at 5–9 weeks of age, and a second dose 4–5 weeks later (apart from two animals in the vaccinated dam group in which the interval between doses was 9 weeks).

Eight of the vaccinated dams had detectable antibody but only two had substantial titres. The calves of these two had measurable titres at birth, one substantial; the calves of 5 of the other 6 also appeared to be positive but with very low titres, and one calf from a negative dam also had a low titre. In the non-vaccinated group, one dam and her calf at birth and one other calf had evidence of antibody, again at low titre.

Detectable antibody was only present in 13 of the 25 sera at the time of vaccine dose 2 and, analysed by the unpaired Student's t-test, there was no significant difference between the two groups ($P > 0.05$). However, 4 and 9 weeks after dose 2, the trend was towards significantly higher titres in the calves from unvaccinated dams ($P = 0.034$ and 0.002 respectively) and when peak titres were compared, again the means were significantly higher in the calves from unvaccinated dams than in those from vaccinated mothers ($P = 0.006$). In both groups, however, the titres were not lasting and mostly had fallen close to baseline by 5–6 months after dose 2, with no significance between the two groups from 2 to 3 months after dose 2.

The results indicate that maternal antibody does interfere to some extent with the vaccine response in the calf, and this should perhaps be taken into account when planning vaccination schedules in premises experiencing anthrax. In the calves from vaccinated dams, 5 that received dose 1 at 9 weeks of age did not develop significantly different peak titres from 3 that received dose 1 at 5 weeks. The overall inference is that protection should be left to maternal antibody in calves from vaccinated cows and vaccination of the calves should not commence until at least 3 months of age.

Another consideration is illustrated in the finding that the anthrax attack rate in beef calves < 6 months of age was significantly lower than in older beef cattle during epizootics (Fox et al., 1977; see also section 3.3.7). This lower risk may be related to young beef calves subsisting mainly on their dam's milk and thus ingesting less contaminated pasture soil and grass. On most affected premises, the cows in these epizootics had not been previously vaccinated, thus eliminating maternal antibody as a factor. This lower risk of disease offsets to some degree the dampening effect of maternal antibody on vaccine response. This again supports the concept that haste to vaccinate calves after birth is not necessary.

It should be added however that the protective effect of maternal antibody against the natural disease has not been studied. In summary, the vaccination status of young calves on infected properties prior to their first vaccination should be considered highly variable and strict paddock management should be considered part of any anthrax control strategy. Cows and calves should be grazed on well-covered pasture with low incidence of disease history wherever possible.

It may be noted that manufacturers in at least Chile, Italy, Romania and Turkey (Table 18) make special recommendations in relation to vaccination of juvenile animals.

2.12 Vaccination of wild animals

Vaccines are not specifically produced for use in wild animals, but some of the vaccines listed in Table 18 are used by regional wildlife veterinarians and staff for vaccinating wild animals (see sections 8.6.2 & 8.7). India and Myanmar include elephants in their schedules (Table 18), albeit referring to domesticated representatives of the species. Although the prescribed method of administration of the vaccine in livestock is, with rare exceptions, the subcutaneous route (Table 18), frequently wildlife vaccination is done using darts, thereby administering the vaccine intramuscularly. Seemingly this is both effective and not dangerous for the animals (de Vos, 1990; de Vos & Scheepers, 1996; Turnbull et al., 2004b).

2.13 Manufacturers of live spore (veterinary) vaccines

Table 18 is based on equivalent tables in previous editions of these guidelines and updated by means of a questionnaire sent to the chief veterinary officers of Member States by the World Organisation for Animal Health (OIE) in October 2002. It should be noted that:

- A number of manufacturers listed in the 1998 guidelines have ceased production and have been removed from the list.
- Replies were not received from a number of countries, so no update was possible (this has been indicated where appropriate).
- One country indicated that it imports its vaccine from Madagascar, but there was no return from Madagascar itself to include in the list.

3. Human vaccines

The background to available and forthcoming human anthrax vaccines is given in section 8.6.3, where it is also pointed out that, of the four licensed vaccines that are produced globally, only the Russian one is nominally available outside national borders. With the others, availability is essentially restricted to their respective national needs. Even if cross-border availability became more possible, it should be remembered that the vaccines are only licensed for human use in the countries of origin. Table 19 is therefore included for completeness of information, but should be regarded as somewhat academic in terms of practical value.

4. Therapeutic sera/immunoglobulins

4.1 For animals

Antiserum (developed in horses) for serum therapy in animals is produced by, or available from:

- Bioplant, Orlov District, Orlov Oblast, 302501, Russian Federation. Tel:/fax: +7 (0) 862 41 37 08.

4.2 For humans

Purified IgG-F(ab)2 antibodies (developed in horses) for human therapy is produced by:

- Lanzhou Institute of Biological Products, 178 Yangchang Road, Lanzhou, China. Tel: +86 931 8340311 8621; fax: +86 931 834 3199.

A heterogeneous anti-anthrax immunoglobulin consisting of gamma and beta globulin fractions of hyperimmune horse serum is produced by:

- The Research Institute of Microbiology, 610024 Kirov, Oktyabrskiy Prospect, 119, Russian Federation. Tel: +7 (0) 8330 38 15 27.

TABLE 18

Manufacturers of livestock anthrax vaccine

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Argentina	Instituto Rosenbuch San José 1469 – (1136) Buenos Aires Tel./Fax: +54 11 4304 6922	Strain: R Strain: Sterne	Cattle: 2 ml s.c. Cattle: 1 ml s.c.
	San Jorge – Bago 9 de Abril 1251 – (1842) Monte Grande Pcia. Buenos Aires Tel. +54 11 4296 2990	Strain: Sterne	Cattle: 2 ml s.c. Sheep: 1 ml s.c.
	Bayer R Gutiérrez 3652 – (1605) Munro Buenos Aires Tel./Fax: +54 11 4762 7000	Strain: Sterne	Cattle: 2 ml s.c.
	Sanidad Ganadera Perú 1645/55 Buenos Aires Tel./Fax: +54 11 4307 9983	Strain: Sterne Strain: Chaco	Cattle: 2 ml s.c. Sheep: 1 ml s.c. Cattle: 2 ml s.c.
	Merial Int. Tomkinson 2054 – (1642) San Isidro Buenos Aires Tel./Fax: +54 11 4732 6700	Strain: Sterne	Cattle: 2 ml s.c. Sheep: 1 ml s.c.
	Agreed Valle Grande 3318 – (1636) Olivos Buenos Aires Tel./Fax: +54 11 4512 1554/5	Strain: Sterne	Cattle: 2 ml s.c.
	Immunovet Ruta 36 y Calle 78 – (1901) Olmos-La Plata Buenos Aires Tel./Fax: +54 221 4962 392	Strain: Sterne	Cattle: 2 ml s.c. Sheep: 1 ml s.c.
	Biogenesis Ruta Panamericana Km.38.2 – (1619) Garín Buenos Aires Tel.: +54 3327 448 300	Strain: Sterne	Cattle: 2 ml s.c. Sheep: 1 ml s.c.
Bangladesh (no update in 2002)	Animal Husbandry Laboratory Mohakhali Dhaka Animal Husbandry Laboratory Cossilla		
Botswana	Botswana Vaccine Institute Broadhurst Industrial Site Lejara Road Private Bag 0031 Gaborone Tel.: +267 3912711 Fax: +267 3956798	Sterne/saponin	Cattle, sheep and goats: 10×10^6 s.c. (1ml) Annual revaccination.

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Brazil	IRFA – Química e Biotecnologia Industrial Ltda. Estrada do Lami, 6133 Bairro Belém Novo 91780-120 Porto Alegre – RS Tel.: +55 51 258 1333/1241	Sterne $34F_2$	Cattle: 2 ml s.c. Sheep, goats, pigs, horses: 1 ml s.c.
	Laboratório Hertape SA Rod. MG 050 2001 CEP 35675-000 Juatuba – MG Tel.: +55 31 3535 8668	Sterne $34F_2$	Sheep, goats, pigs, cattle: 0.5 ml s.c.
	Vallée SA Av. Hum 1500 – Distrito Industrial CEP 39404-003 Montes Claro – MG Tel.: +55 38 3229 7000	Sterne $34F_2$	Cattle: 2 ml s.c. Sheep, goats, pigs: 1 ml s.c.
	Labovet Produtos Veterinários Ltda Av. Banco do Nordeste Galpão 22-A CEP 44052-510 Feira de Santana – BA Tel.: +55 75 622 3922/5946	Sterne $34F_2$	Cattle: 1 ml s.c. Sheep, goats, donkeys, pigs: 0.5 ml s.c.
	Laboratório Vencofarma do Brasil Ltda Trav. Dalva de Oliveira, 237 CEP 86030-380 Londrina – PR Tel.: +55 43 339 1350	Sterne $34F_2$	Cattle: 1 ml s.c. Sheep, goats, donkeys, pigs: 0.5 ml s.c.
	Laboratório Agromédica Ltda Rua José Maria de Lacerda, 1957 CEP 32210-120 Contagem – MG Tel.: +55 31 3333 0755/5223	Sterne $34F_2$	Cattle, horses: 1 ml s.c. Sheep, goats, pigs: 0.5 ml s.c.
	Leivas Leite SA – Ind. Químicas e Biológicas Rua Benjamin Constant 1637 CEP 96010-020 Pelotas – RS Tel.: +55 53 225 3666	Sterne $34F_2$	Cattle, horses.: 2 ml s.c. Sheep, pigs: 1 ml s.c.
Cameroon	Lanavet BP 503 Garoua Tel.: +237 227 1305 Fax: + 237 227 152	Sterne $34F_2$ in saponin Freeze-dried	Cattle – 10^7 spores in 1 ml; 0.5 ml for sheep and goats
Chile	Veterquímica Ltda Camino a Melipilla 5641 (Adm. Gral.) Camino a Lonquén 10387 (Ventas Maipú) Cerrillos, Santiago Metropolitana, 81 Cerrillos Tel.: +56 2 557 1222 Fax: +56 2 557 0774 E-mail: veter\@terra.cl	Sterne 1 ml suspension contains 10 000 000 *B. anthracis* spores	Cattle: 1 ml s.c. Sheep: 0.5 ml s.c. Generally, only one vaccination a year is necessary, preferably in spring. In high-risk zones, two vaccinations are recommended (in spring and autumn). First vaccination at 6 months.

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
China	National Control Institute of Veterinary Bioproducts & Pharmaceuticals Ministry of Agriculture 30 Baishiqiao Road Beijing 100081 Tel.: +86 256 8844 ext. 261 Fax: +86 831 6545	1. Strain: $34F_2$ Adjuvant: 20% aluminium hydroxide 2. "PA vaccine" Adjuvant: oil-in-water emulsion	1. Small animals: 1 ml Sheep, pigs: 2 ml Cattle, horses: 2 ml (all intracutaneous) 2. All animals 2 ml intracutaneously
Colombia (no update for any in 2002)	Laboratorios ERMA Av. El Dorado No. 90-31 Apartado Aereo 98835 Santafé de Bogotá Tel./Fax: +57 1 295 0900/410 2410 E-mail: Laberma@ col.1.telecom.com.co	Sterne, 10^7 spores/ml Adjuvant: saponin 50 ml flasks	Cattle and horses: 2 ml s.c. Sheep and goats: 1 ml s.c. Annual revaccination
	Laboratorios Probiol Ltda Diagonal 183 N 41-71 Apartado aereo 8001 Bogotá Tel.: +57 1 671 1023 Fax: +57 1 671 1066	Strain: $34F_2$ Adjuvant: (contact manufacturer)	Sheep, goats, pigs, cattle, horses: 2 ml
	LAVERLAM Carrera 5 No. 47-165 Salomnia Industrial Apartado aéreo 9985 Cali. Valle del Cauca Tel./Fax: +57 2 447 4411/447 4409	Sterne, 10^7 spores/ml Adjuvant: aluminium hydroxide 50 ml flasks	Cattle: 2 ml s.c. Other species: 1 ml s.c.
	Laboratorios V.M. Autopista Norte kilómetro 19 Urbanización Industrial El Pilar Apartado aéreo 15453 Santafé de Bogotá Tel./Fax: +57 1 676 0901/676 0823	Sterne, 10^7 spores/ml Adjuvant: saponin 10, 20, 50 and 100 ml flasks	Cattle and horses: 2 ml s.c. Sheep and goats: 1 ml s.c.
	VECOL Av. El Dorado No. 82–93 Apartado aéreo 7476 Santafé de Bogotá Tel./Fax: +57 1 263 3100/263 8331 E-mail: Vecol@insat.net.co	Sterne, 7–10 x 10^6 spores per ml in buffered glycerine 50 ml flasks and box of 10 x 10ml flasks	Cattle and horses: 2 ml s.c. Sheep, goats and pigs: 1 ml s.c.
Croatia	Veterina Ltd. Svetonedeljska 2 Kalinovica 10436 Rakov Potok Tel.: + 385 1 33 88 888 E-mail: veterina-info@pliva.hr	Strain: $34F_2$ Adjuvant: aluminium hydroxide gel Name: Antrax vaccine Bottles with 10 ml of vaccine	Horses, cattle, sheep, pigs: 0.5 ml s.c. Goats: 0.2 ml s.c. Annual revaccination. In anthrax-infested areas twice a year at 6 months intervals
Czech Republic (no update in 2002)	Bioveta plc Komenského 212 683 23 Ivanovice na Hané Tel.: +42 507 933 21-4 Fax: +42 507 932 84 (or 94)	Strain: $34F_2$ Adjuvant: saponin Name of product: "Antraxen inj.ad us. vet."	Sheep, goats, pigs, cattle, horses: 1 ml s.c.

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Ethiopia	National Veterinary Institute PO Box 19 Debre Zeit Tel.:+251 133 8411 Fax:+251 133 9300	Sterne $34F_2$ in saponin Freeze-dried	Cattle: 10^7 spores in 1 ml; 0.5 ml for sheep and goats
Ghana	Central Veterinary Laboratory Pong-Tamale PO Box 97 Tamale Tel./Fax: +233 71 22720	Anthrax spore vaccine Sterne $34F_2$ Adjuvant: saponin 15–20 million spores per dose	Cattle, sheep, goats, pigs: 1 ml s.c. Annual revaccination
Hungary (no update in 2002)	Phylaxia-Sanofi Veterinary Biologicals Co. Ltd PO Box 68 1475 Budapest Tel.: +36 1 262 9505 Fax: +36 1 260 3889	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats: 0.5 ml Cattle: 1 ml
India (no update in 2002)	Institute of Veterinary Preventive Medicine Ranipet – 632 402 North Arcot Ambedkar District Tamil Nadu Tel.: +91 4172 22633	Strain: $34F_2$ Adjuvant: none	Sheep, goats: 1 ml injected s.c. in tail-fold Cattle, pigs, horses, camels: 1 ml s.c. Elephants: 1 ml s.c. with second dose of 3 ml after 1 month
Indonesia (no update in 2002)	Pusat Veterinaria Farma Jalan Jenderal A. Yani 68-70 Kotak Pos WO.3 Surabaya 60231 Tel.: +62 31 816123 Fax: +62 31 814126	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats, pigs: 0.5 ml s.c. Cattle, horses: 1 ml s.c.
Italy	Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata Via Manfredonia 20 I-71100 Foggia Tel: +39 881 786 111/786 300 Fax: +39 881 786 362	Strain: Pasteur type 1 Adjuvant: saponin Strain: Carbosap Adjuvant: saponin	Goats, horses: 0.125 ml s.c. Cattle (≥ 6 months): 0.25 ml s.c. Cattle (< 6 months), sheep: 0.125 ml s.c.
Japan	Chemo-Sero-Therapeutic Research Institute 6-1 Okubo 1-chome Kumamoto-shi Kumamoto-pref. 860-8568 Tel.: +81 96 344 1211 Fax: +81 96 345 1345	Strain: $34F_2$ Adjuvant: none	Cattle and horses only: 0.2 ml s.c.
Kenya (no update in 2002)	Cooper Kenya Ltd Wellcome Centre Kaptagat Road (off Waiyaki Road) PO Box 40596 Nairobi Tel.: +254 580612 Fax: +254 632123	Strain: $34F_2$	Sheep, pigs: 0.5 ml s.c. Cattle, horses: 1 ml s.c. (It is recommended that goats should not be vaccinated without first consulting veterinary surgeon)

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Mali	Laboratoire Central Vétérinaire (LCV) BP 2295 Bamako Tel.: +223 22 3344 Fax: +223 22 9809	Sterne $34F_2$ in saponin	10^7 spores in 1 ml for cattle, sheep and goats
Morocco	Km 2 Route de Casablanca BP 4569 Rabat Tel.: +212 69 0454 Fax: +212 69 1689 E-mail: biopharma_ma@yahoo.fr	Sterne $34F_2$ in saponin	10^7 spores per 1 ml for cattle; 0.5 ml for sheep, goats and calves
Mozambique	National Veterinary Research Institute (INIVE) PO Box 1922 Maputo Tel.: +258 1 475 170/1 Fax: +258 1 475 172 E-mail: inive@cfmnet.co.mz; inivei@teledata.mz	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats: 0.5 ml s.c. Cattle: 1 ml s.c.
Myanmar	Livestock Breeding and Veterinary Dept. Research and Biologics Section Mukteswar	Strain: Sterne $34F_2$ in glycerol saline	Elephants, horses, mules, cattle and buffaloes: 1 ml s.c.
Nepal	Biological Products Division Directorate of Animal Health Veterinary Complex Tripureswar Kathmandu Tel.: +977 1 4252 348	Strain: Sterne in glycerine	All species: 1 ml s.c.
Netherlands	Central Institute for Animal Disease Control – Lelystad PO Box 2004 8200 AB Lelystad Tel.: +31 320 238 800 Fax: +31 320 238 668 Email: e.m.kamp@id.dlo.nl; f.g.vanzijderveld\@id.dlo.nl	Strain: $34F_2$ Adjuvant: saponin	Small animals: 0.5 ml s.c. Sheep, goats, pigs, young foals and calves: 0.5 ml s.c. Adult cows, horses: 1 ml s.c.
Niger	Laboratoire Central de l'Elevage BP 485 Niamey Tel.: +227 73 20 09/73 80 05 Email : radiscon@intnet.ne	Sterne $34F_2$ Adjuvant: saponin Lyophilized vaccine	Cattle, horses: 1 ml s.c. Small ruminants: 0.5 ml s.c.
Pakistan (no update in 2002)	Vaccine Production Laboratories Brewery Road Quetta	Details not obtained	
Philippines	Laboratory Services Division Bureau of Animal Industry Visayas Avenue, Diliman Quezon City	Strain: $34F_2$ Adjuvant: saponin	Pigs, sheep, goats: 0.5 ml s.c. Cattle, carabao, horses: 0.5 ml s.c.

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Republic of Korea (no update in 2002)	Choong Ang Animal Disease Laboratory Seo TaeJeon PO Box 312 408-1 Sa Jung Dong Choong-Ku TaeJeon Tel.: +82 42 581 2991 Fax: +82 42 581 5856	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats, pigs: 1.0 ml Cattle, horses: 2.0 ml
Romania (no update in 2002)	Institutul National de Medicina Veterinara "Pasteur" 77826 Sos. Giulesti 333 Sector 6 R-7000 Bucharest Tel.: +40 1 220 6486 Fax: +40 1 220 6915	Strain: 1190 R –Stamatin Adjuvant: saponin	Cattle (> 2 months): 0.5 ml s.c. Sheep, pigs (> 2 months): 0.2 ml s.c. Horses (> 6 months): 0.2 ml s.c. Goats (> 2 months): 0.1 ml s.c.
Russian Federation	Pokrov Biological Plant 601121, pos. Volginskiy Vladimirskaya oblast Tel.: +7 09243 6-7110	Strain: 55-VNIIVVM	Four versions to meet different needs Doses vary with different versions of the vaccine
South Africa	Onderstepoort Biological Products Private Bag X07 0110 Onderstepoort Tel.: +27 12 529 9111 Fax: +27 12 546 0216 E-mail: baty@obpvaccines.co.za	Sterne $34F_2$, 1 x 10^6 spores/ml Adjuvant: saponin	All species: 1 ml s.c.
Spain	Calier Barcelonés, 26 – Pol. Ind. Pla del Ramassa Apdo. 150 08520-Les Franqueses del Vallés Barcelona Tel.: +34 93 849 51 33 Fax: +34 93 840 13 98	Strain: Sterne Adjuvant: saponin	Ruminants: 20 x $10^{6.5}$ (1 ml) s.c.
	Ovejero Ctra. León – Vilecha 30 Apdo. Correos 321 24080 León Tel.: +34 902 235 700 Fax: +34 987 23 47 52	Strain: Sterne	Cattle: 1 ml Sheep: 0.5 ml 10 x 10^6 (1 ml) s.c.
	Iven Luis I, 56 – 58 – Pol. Ind. de Vallecas 28031 Madrid Tel.: +34 91 380 17 21 Fax: +34 91 380 00 61	Strain: Sterne Adjuvant: saponin	Cattle, horses, sheep, goats: 20 x $10^{6.5}$ (1 ml) s.c.
	Syva Avda. Párroco Pablo Díez, 49–57 24010 León Tel.: +34 987 80 08 00 Fax: +34 987 80 24 52	Strain: Sterne Adjuvant: saponin	Cattle, horses, sheep, goats, pigs: 10 x 10^6 (1 ml) s.c.
Syrian Arab Republic	Animal Health Directorate Tel.: +963 11 542 2500 Fax: +963 11 542 4761	Strain: $34F_2$ in buffered glycerin	Sheep, goats: 0.1 ml i.d Cattle, horses: 0.2 ml i.d

TABLE 18 CONTINUED

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
Turkey	Central Veterinary Research and Control Institute 06020 Ankara Tel.: +90 312 322 48 64/325 52 41 Fax: +90 312 321 17 55/325 52 41 E-mail: ehh.o@tr.net	Strain: $34F_2$, 10^7 spores/ml Adjuvant: saponin Vaccine composition: 50% glycerol (v/v), 50% physiological saline (v/v), 0.05–0.1% saponin	Horses, cattle, camels, buffaloes: 1 ml s.c. (into side of the neck or behind the shoulder) Sheep, goats, pigs: 0.5 ml s.c. (for sheep, into inside of a back leg; for goats, under the tail; for pigs, behind the ear) Colts, calves (2–6 months old): 0.5 ml s.c. Lambs, kids (2–6 months old): 0.5 ml s.c.
United Kingdom	Biotechnology Department Veterinary Laboratories Agency New Haw Surrey KT15 3NB Tel.: +44 1932 357641 Fax: +44 1932 357701	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats, pigs: 0.5 ml s.c. Cattle: 1 ml s.c.
United States of America	Colorado Serum Co. 4950 York Street Denver, CO 80216-0428 Tel.: +1 303 295 7527 Fax: +1 303 295 1923	Strain: $34F_2$ Adjuvant: saponin	All domestic farm animals: 1 ml s.c. Booster recommended 2–3 weeks after first dose in heavily contaminated areas
Uruguay (no update in 2002)	Interifa SA, Rhône Mérieux José Ma. Penco 3427 11700 Montevideo Tel.: +598 29 70 91 Fax: +598 23 78 52	Strain: $34F_2$ Adjuvant: saponin	Sheep, goats, pigs: 1 ml s.c. Cattle, horses: 2 ml s.c.
	Laboratorio Prondil S.A Barros Arana 5402 CP 12200 PO Box 15147 Dto.5 Montevideo Tel. : +598 25 13 32 54 Fax: +598 25 13 32 52 E-mail: pron@netgate.com.uy	Anthrax only Strain : $34F_2$ Adjuvant: saponin "Blanthrax": combination anthrax and blackquarter (pending registration) "Supervax": combination anthrax, blackquarter, botulism C, D (South African strains)	Sheep, goats, pigs: 0.5 ml s.c. Cattle, sheep, goats only: 2 ml s.c. Cattle, sheep, goats only: 2 ml s.c.
Viet Nam	National Veterinary Enterprise Phung Town Hoai Duc District Ha Tay Tel: +84 34 861 337 Fax: +84 34 861 779	Strain: $34F_2$ Adjuvant: saponin	Goats, pigs: 0.5 ml s.c. Cattle and horses: 30×10^6 s.c. (1 ml)
Zambia	Central Veterinary Research Institute PO Box 33980, Lusaka Tel.: +260 1 216031 Fax: +260 1 2334444/236283 E-mail: cvri@zamnet.zm	Sterne $34F_2$, 10^6 spores/ml Adjuvant: saponin	Cattle: 1 ml Sheep, goats, pigs: 0.5 ml

s.c. = subcutaneous; i.d. = intradermal.

TABLE 19

Manufacturers of human anthrax vaccine

COUNTRY	MANUFACTURER	DESCRIPTION	DOSE
China	Lanzhou Institute of Biological Products 178 Yanchang Road Lanzhou, Gansu 730046 Tel.: +86 931 834 0311-8621 Fax: +86 931 834 3199	Live spore suspension of strain A16R in 50% glycerol and distilled water No other active ingredient/adjuvant	Single dose (approx. 2 x 10^8 spores) by scarification of 50 µl in two spots on the skin with single booster after 6 or 12 months and annual boosters thereafter
Russian Federation	Research Institute of Microbiology 610024 Kirov Oktyabrskiy Prospect 119 Tel.: +7 8330 38 15 27 Fax: +7 8332 62 95 98	Lyophilized live spore suspension of strain STI-1 (Sanitary Technical Institute), Nikolaiv Ginsberg 1940 No other active ingredient/adjuvant	Initial 2 doses 20–30 days apart and single annual booster doses. Administered by scarification of 0.05 ml into the skin (approx. 5 x 10^8 spores), or, in urgent situations, injection s.c. of 0.5 ml (approx. 5 x 10^7 spores)
United Kingdom	Health Protection Agency Porton Down Salisbury Wiltshire SP4 0JG England Tel: +44 1980 612 100 Fax: +44 1980 611 096	Alum precipitated filtrate of strain $34F_2$ culture	3 primary doses of 0.5 ml i.m. at 3-week intervals with booster at 32 weeks and thereafter annually
United States of America	BioPort Corp 3500 N. Martin Luther King Jr Blvd, Lansing MI 48906 Tel.: +1 517 327 1500 Fax: +1 517 327 1501	Aluminium hydroxide-adsorbed filtrate of strain V770 culture	3 s.c. doses, 0.5 ml each, given 2 weeks apart followed by 3 additional s.c. injections, 0.5 ml each, at 6, 12, and 18 months. Subsequent booster injections of 0.5 ml annually

s.c. = subcutaneous; i.m. = intramuscular.

ANNEX 6

Contingency plan for the prevention and control of anthrax

1. Action in the event of an outbreak of anthrax in livestock

The control programme, instituted by the Department of Natural Resources and Environment, Victoria, Australia, in response to an outbreak of anthrax in cattle affecting some 83 farms during the first half of 1997, was subsequently established as an Australian standard and serves well as a global model for anthrax control. The following summary, based on the report by Turner (unpublished, 1997), Chief Veterinary Officer, Victoria, is included here by kind permission.

On each affected farm, the following measures should be applied:

1. Quarantine should be imposed on the flocks and herds in which anthrax has occurred. The quarantine should comprise limiting: (i) contact between infected and non-exposed herds/flocks; and (ii) the access of susceptible animals to infected sites until at least 14 days, and preferably 20 days (OIE incubation period), after they have been vaccinated.
2. The carcasses of infected animals should (see section 8.3) either be burnt at the site of death and the ashes buried deeply, or wrapped in double-thickness plastic, to prevent spilling of body fluids, and removed to a more suitable site (e.g. a quarry) to be burnt and the ashes buried. (Consideration may be given to removing the carcasses to suitable commercial incinerators or rendering plants.)
3. The site where the animal died should, where possible, be thoroughly scorched with a down-directed flame and/or disinfected with 10% formalin after disposal of the carcass. (*Caution: formalin should be handled with the appropriate cautions as indicated in Annex 3 and more specifically in its section 1.1.*)
4. On moving the herd out of the contaminated area/field/pasture, Hugh-Jones (personal communication, 2002) recommends that all animals be treated with a suitable long-acting antibiotic to abort covert incubating infections. If they are to be returned to the contaminated area, they should be vaccinated after the antibiotic has cleared (see section 7.2.1.3).
5. Affected properties should be quarantined for at least 20 days after the last case or the accepted period after vaccination (see Annex 5, section 2.3), whichever is later.
6. Any movement of susceptible livestock, or of risk items (hides, skins, carcasses, etc.) that have left the property in the 20 days before the first anthrax case, should be traced and appropriate action taken where necessary.
7. As far as possible, vehicles should remain on made roads in infected and vaccinated farms; where vehicles have to enter the pasture fields, the vehicle should be disinfected before exiting the property by washing down with water and detergent to remove mud, followed by a disinfectant wash. This should take place in a corner of the property where animals will not come in contact with the washings. Every attempt should be made to decontaminate the washings along the lines suggested in Annex 3, sections 6.2 & 6.4. Formalin 10% may be the best option. (*Caution: formalin should be handled with the appropriate cautions as indicated in Annex 3 and more specifically in its section 1.1.*)
8. People entering infected properties should wear protective clothing and footwear which can be disinfected or bagged for incineration before leaving the property.
9. All animals within a buffer zone, a few to several kilometres (1–3 properties) wide, around the infected property (-ies) should also be vaccinated and quarantined with the same post-vaccination holding conditions described under (5) above (see also section 8.7).

10. Milk from herds may not need to be destroyed (see section 3 below).

2. Other actions in the event of a case, or cases, of anthrax in livestock

The appearance of anthrax in animals from a previously uninfected premise may be dealt with in the following ways:

1. Identify, isolate and remove apparently healthy animals, and monitor these carefully for signs of illness (see (5) below and section 7.2.1).
2. Decontaminate soil, bedding, unused feed, manure, etc., or disinfect premises that may have become contaminated by exudations from the dying or dead animals (see Annex 3, sections 3.1, 3.2 & 6.1).
3. Avoid any unnecessary ante- and postmortem operation in animals on the premises (see section 3.5.2).
4. Destroy carcasses and their parts by burning, removal for rendering or, as a last resort, deep burial after disinfection, preferably with 10% formalin (see section 8.3 and Annex 3, section 7). If carcass disposal has to be delayed, the carcass and surrounding ground should be disinfected with 10% formalin and the carcass covered with strong plastic to prevent access of scavengers. The apparent failure of formalin to seriously deter scavengers and flies has been referred to in section 8.3.2.1.
5. As outlined in sections 8.6.2 & 8.7, and in Annex 5, section 2.2, vaccinate or monitor and treat other members of the herd or flock if there is reason to believe that they continue to be exposed to the source of the incident. It should be remembered that antibiotics and vaccine should not be administered simultaneously (see section 8.7 and Annex 5, section 2.2).
6. Control scavengers and minimize possible vectors such as flies, rodents and birds.
7. Carry out an epidemiological investigation to detect the source of infection (history of site, feed, disturbance of the environment, etc.) to identify the source of the incident (see section 9.3).
8. Take proper measures to avoid the contamination of water and soil and to prevent the spread of the infection to other farms and environments. Disturbed soil thought to be related to the incident should be fenced off and, ideally, not used for grazing again, at least until it has become well overgrown with vegetation, preferably of the type that inhibits access by susceptible animals (see section 8.3.2.1).
9. Alert public health authorities (see section 9.1.2).

3. Guidance on actions relating to milk from herds/flocks in which anthrax has occurred

Action to be taken on milk from a herd or flock experiencing cases of anthrax infection occasionally presents a dilemma for health authorities. Wasteful destruction of large quantities of milk, and consequent financial losses, may be avoided by considering the following points. OIE now recommends that, in the first place, only milk from healthy animals should be regarded as acceptable but, in the case of herds or flocks experiencing anthrax, milk should be pasteurized before being processed into products for human consumption.

It is accepted that milk from healthy animals in anthrax outbreaks does not pose a risk to humans for the following reasons:

1. Animals with anthrax do not usually discharge the infecting organism in their milk before death, although there have been occasional exceptions (see (2) below; sections 3.3.8 & 3.5.2; Annex 5, section 2.7). Secretion of milk would normally be expected to cease with onset of bacteraemia and illness. The organism would normally only gain entry to the milk-secreting glands through breakdown of blood-vessel walls at terminal stages of the disease, long after secretion of milk had stopped (M'Fadyean, 1909). Milk from other healthy animals in the herd/flock poses no risk of carrying anthrax organisms.
2. There are rare exceptions where the vegetative forms are excreted in milk (Weidlich, 1935). *B. anthracis* strain ASC 65 (from Baptista, Department of Agriculture, South Rio Grande State, Brazil) was chronically secreted in the milk from four cows and was isolated during routine mastitis-screening tests on milk samples in 1983. Two cows subsequently died. The two that survived had been vaccinated. In such an event it should be borne in mind that:

    - The organisms are in vegetative form.

- The organisms are unable to sporulate (Bowen & Turnbull, 1992) as a result of having minimal aeration and, where commercial processing follows, of rapid cooling to refrigeration temperatures.
- The pH rapidly falls on standing, increasing the killing effect on any vegetative cells present.
- The vegetative forms die quite rapidly in the milk (Bowen & Turnbull, 1992) and will be killed immediately by pasteurization (72–73 °C for 16 seconds, or HTST (high temperature short time) at 80–82 °C for 19–20 seconds).
- Any anthrax organisms reaching the bulked milk will have been considerably diluted; the infectivity of anthrax organisms for humans by the oral route is very low and vegetative forms will be killed by the gastric juices.

3. There appear to be no instances on record of human anthrax cases resulting from handling or consumption of milk from herds/flocks experiencing anthrax and processed dairy products have never been associated with human anthrax. Action on the part of farmers/owners and public health authorities aimed at reducing the minimal risk to zero risk should consist of:

   - milking only healthy animals; animals showing signs of illness should be set aside for appropriate treatment and the milk from those animals, and utensils used in its collection, should be sterilized;
   - ensuring hygienic practices are in place that prevent the environment of the premises from becoming contaminated with anthrax spores and, further, prevent milk becoming contaminated from the environment;
   - ensuring that all milk is rapidly cooled to 4 °C or less within 4 hours of milking and is held at this temperature until processed at a licensed dairy plant;
   - pasteurizing of all milk before processing for human or animal consumption.

If the veterinary inspector is satisfied that these requirements have been met, milk from healthy animals in herds/flocks in which cases of anthrax have occurred need not be excluded from processing, and bulked milk containing such milk need not be condemned.

4. Deliberate release into animal populations

Bioaggression scenarios involving the use of anthrax and targeted at animal populations are conceivable, and could take the form of airborne infection or deliberate infection of animal feeds or water with virulent spores. Reference is readily found on the Internet to declassified Second World War records of British retaliatory preparedness to respond to an anticipated biological attack from Germany, and in which several million cattle-cakes injected with anthrax spores were made ready for aerial drops over grazing areas in Germany. The cakes were never used and were destroyed by incineration after the war.

The response required to such an event would still involve the control principles outlined in sections 1 & 2 above. Infected and exposed animals would be defined by determining the source of infection and the nature of the release, and thereby the likely exposed population. Priorities could then be assigned to ensure that vaccination is carried out first in those herds and flocks believed to have highest exposure.

It is essential that all the other control procedures described in section 1 above are applied, along with vaccination, if further occurrence of disease is to be minimized in animals and humans. It will be important to safely dispose of carcasses to reduce environmental contamination as a further source of infection. It would be an essential extra task to develop appropriate press releases and to establish an education programme for the public about the disease and the control measures being applied, to prevent local panic.

5. Outbreaks in wildlife

5.1 Proaction plans and control actions

When it comes to wildlife, circumstances are likely to be very varied, and it is difficult to cover all eventualities within this section. Sporadic cases in large game-management areas (GMA) are likely to be seen as being of consequence only if livestock are at risk. Even with bigger outbreaks, in those large wildlife national parks from which livestock are excluded and which have “hands off” management policies for all but emergency situations, control actions may be regarded as interference with natural processes. This is discussed in sections 8.9 and 8.10. Each GMA that encounters anthrax, or knows it is at risk of

encountering anthrax, should have an action plan in place in line with its management policies and particular needs (Clegg et al. 2006a). For smaller commercial or sustainable GMAs, a proaction plan is advisable with prevention as the primary aim. The essence of this is to:

- ensure that risk factors and warning signs are recognized, for example, awareness of cases of anthrax in livestock in areas around the GMA;
- have surveillance in place which ensures that unexpected deaths are observed and diagnosed promptly and correctly;
- have good links with veterinary and public health services and good relations with surrounding communities;
- have an action plan in place;
- undertake relevant training of rangers and scouts on the nature of the disease, and prepare action plans and possibly educational material for surrounding communities;
- prevent the development of overdense populations of susceptible species in any part of the GMA;
- keep strategic stocks of vaccine and other items needed to implement the action plan as soon as an outbreak occurs;
- prepare information sheets for staff and tourists in case of an outbreak;
- make appropriate budgetary provision for the eventuality of an outbreak and the actions to be taken, e.g. for the extra personnel, vehicles, machinery, fuel, insecticides, disinfectants, protective clothing, veterinary and medical services, vaccine, information sheets, etc., that will be involved.

Where action is seen to be necessary, the following suggestions are offered:

- Identify if possible the source of the outbreak and isolate it, for example, by veld burning, or vulture decoys with uncontaminated meat.
- Liaise with veterinary and public health services and inform local communities, tourists, etc., by issuing information sheets.
- Take measures to prevent the infection being transmitted from the dead animals to live ones, e.g. covering carcasses to prevent access by scavengers, fly control, ring vaccination, etc. The problem is likely to be that it will not be possible to dispose of carcasses promptly or easily while the outbreak is active. In this event, the aim should be to keep carcasses unopened and intact for as long as possible to minimize the development of contamination; the quickest and most effective way may be to cover carcasses with tarpaulins or thick plastic, possibly wetting the carcass (and surrounding soil) with 10% formalin first. (*Caution: formalin should be handled with the appropriate cautions as indicated in Annex 3 and more specifically in its section 1.1.*) As mentioned in section 8.3.2.1 and section 1 above, this will help keep the skin intact and kill anthrax organisms. The apparent failure of formalin per se to seriously deter scavengers and flies has been referred to in section 8.3.2.1. Opened carcasses may also be treated with 10% formalin and covered until proper disposal actions can be implemented.
- If possible, capture and vaccinate at least a core of endangered or otherwise precious species, possibly restricting them to a fenced-off enclosure or other confined area. For maximum protection, it may be advisable to revaccinate after about 4 weeks.
- Take actions to encourage animals to move away from the area, e.g. close off or empty artificial waterholes.
- Set up monitoring to detect cases that might occur in the animals that have moved away but which might have been already infected.
- Before access to the affected area is reopened, all carcasses are best burnt but, if this is impractical, they should be buried, preferably disinfecting them first with 10% formalin (section 8.3 and section 2 above). (*Caution: formalin should be handled with the appropriate cautions as indicated in Annex 3 and more specifically in its section 1.1.*)
- Consider the possibility of disinfecting artificial water holes where this is feasible. Heavy chlorination (final concentration at least 5000 ppm) is probably the only practical approach available, at least for small holes, but its limitations should be appreciated (Annex 3, section 1.2.1). Whether it is necessary or feasible to treat large bodies of water, especially when extensive amounts of organic matter are present, and how to do so if it is deemed necessary, are topics needing research. Control may depend more on the water holes not playing a major role in maintenance of the outbreak than on the effectiveness of disinfection attempts.

It is difficult to advise on the value and cost-effectiveness of these suggested actions. History shows

that an explosive outbreak in a particular location is generally not followed by another one at the same location for several to many years. However, logic suggests that future outbreaks, even many years later, are less likely if measures are taken to minimize the residual environmental contamination resulting from an outbreak occurring today.

5.2 Models for answering frequently asked questions

Question. In an outbreak situation, should we put staff involved in burning or burying carcasses onto long-term antibiotic prophylaxis?

Answer. No. Prolonged antibiotic prophylaxis has only been a recommendation for persons known to have been, or strongly suspected of having been, exposed to very substantial doses of aerosolized spores in a deliberate release scenario. Antibiotics should not be administered in that way for other situations; they cause side-effects and there is the risk of producing resistant strains of other (unrelated) organisms which then do not respond if a person subsequently suffers another infection. Antibiotics should only be used for treatment, not prophylaxis, unless there is a real danger (see also section 7.3.2.3). There have been many epizootics of anthrax in African wildlife, but reports of cases in the humans dealing with these are exceedingly few in number (section 4.2.1.2), giving plenty of circumstantial evidence that the category of danger for staff is not very high. The appropriate approach is:

- Educate staff about the disease, including about not butchering and eating meat from the carcasses, and leaving the disposal of these to veterinary or other trained personnel.
- When disposing of carcasses, sensible protective equipment should be worn (coveralls, boots, gloves). If a lot of dust is created at a site where the dust is likely to be contaminated, a good-quality properly-fitted dust mask should be used that ensures breathing through it and not around it (see also Annex 1, section 7.1.2).
- Make sure that if any person develops a spot/pimple/boil-like lesion, especially on exposed areas, or flulike illness, he/she reports to the doctor, who can then give them penicillin or other chosen antibiotic(s) for 3–7 days (spots, etc.) or 10–14 days (flulike illness) (see section 4.4).

To avoid creating dust, objects should be dampened down, preferably with 10% formalin if they are likely to be contaminated. Clothing should be sterilized or, if disposable, burned after use. Everyone involved should be advised to be careful with formalin: it is effective for killing spores, and for the same reason it can damage human tissues.

Question. What are the environmental impact considerations when it comes to using formalin?

Answer. As first applied in a 10% solution, it will kill any living thing – microbe, plant, animal. However, it degrades readily through natural processes (see Annex 3, section 6.1).

Question. Can we expect naturally acquired immunity resulting from the outbreak, or vaccine-induced immunity to prevent a repeat outbreak?

Answer. The immunity from first-time vaccination may not be very long-lasting, It is better after anamnestic (the immune system's memory) responses following subsequent boosters. The pattern of history is that another large outbreak in the near future is unlikely, but whether it is the result of acquired immunity or not is not known. Tests have not confirmed or refuted it. Seasonal sporadic cases may be expected every year.

Question. Should the chlorine level be raised in the local water supply?

Answer. No. Filters and other water purification systems may be usefully checked, but chlorine needs to be at a very high concentration (approximately 5000 to 10 000 ppm) to be effectively sporicidal. If there is serious reason to fear the water, boiling for 20–30 minutes is probably the only option available (Annex 3, section 6.3).

Question. Can fish be carriers of anthrax? A number of animals dying from anthrax have done so in the dam. People catch fish from this for consumption. Is there a possibility that they could be infected through eating these fish? Some of the fish will have fed on the carcasses.

Answer. The following is a working model to build on:

1. If the fish was caught within a few hours of eating anthrax meat, and was opened up and filleted before eating, and was eaten ***uncooked***,

the risk would be a little less than for a person handling and eating the meat itself. There is a chance, probably in the order of 1:20 to 1:50, of contracting cutaneous anthrax from handling the opened fish, and probably around a 1:100 to 1:1000 chance of ingestion anthrax.

2. As in (1) above, but the fish ***is cooked*** before eating. There would still be the 1:20 to 1:50 chance of cutaneous anthrax from handling the fish before cooking, but a greatly reduced risk of ingestion anthrax, e.g. 1:1000 to 1:10 000 chance, or lower, depending on how the fish was cooked. The hotter the temperature and the longer the cooking period, the lower the risk.
3. As in (2) above, but the fish was not opened up before cooking. The chance of cutaneous anthrax is reduced to almost nil and the chance of ingestion anthrax to 1:1000 to 1:10 000, or lower, depending on the extent of the cooking.
4. As in (1) above, but the fish was caught 24 hours after eating the meat. Risks of cutaneous and ingestion anthrax would be greatly reduced to, for example, 1:100 to 1:500 (cutaneous) and 1:1000 to 10 000 (ingestion). The risks would decline fairly rapidly with time after that as the ingested anthrax spores are expelled from the fish.
5. As in (2) above, but the fish was caught 24 hours or more after eating the meat. The risks are getting very small to non-existent.

In summary, it depends on: (i) the precise habits (how the persons handle, treat and cook the fish before eating); (ii) the period of time between the fish eating the anthrax-infected meat and being caught; and (iii) the particular fish – some species will and some will not eat animal meat.

Anecdotal evidence indicates that humans are moderately resistant to infection. However, there can be no guarantee that one or two persons may not be unlucky and contract infection. The message is for them to know that they must report any sickness or developing spots or pimples to a medical practitioner for administration of penicillin or another chosen antibiotic in that event.

6. Precautions for exposed personnel

Persons who must handle animals known to be, or suspected of being, infected with anthrax or carcasses from such animals, or parts of such carcasses, should take the following precautions:

- avoid all blood-spilling operations (slaughtering included) on infected or suspect animals/carcasses;
- use protective clothing such as strong gloves, boots, coveralls, etc., as appropriate, to avoid direct contact with infected/contaminated materials. Cuts, abrasions or other lesions should be properly dressed before putting on the protective clothing. The equipment used must be adequately disinfected or appropriately destroyed (see Annex 1, sections 7.8 & 7.9);
- avoid any contact with other persons (family included) or animals, without first changing clothing, washing hands, and taking appropriate disinfection measures (see Annex 1, section 7);
- report to a physician any suspect symptoms appearing after contact with infected animals or materials;
- where there is a risk of aerosolization of spores, consider further precautions, such as damping down the material, possibly with 10% formalin. (*Caution: formalin should be handled with the appropriate cautions as indicated in Annex 3 and more specifically in its section 1.1.*)

As noted in section 8.6.3 and Annex 5, section 3, anthrax vaccines for humans are mostly unavailable outside certain countries and circumstances. If available, they should be considered for persons likely to have repeated exposure to animals infected with anthrax or animal products from such animals, or to *B. anthracis* itself. However, even when available, such vaccines require several doses over an extended time period to be effective, and the best approach is to use proper personal protection methods (Annex 1, section 7.1.2). Antibiotic prophylaxis is generally not to be recommended (section 7.3.2.3).

ANNEX 7

Sampling plans for environmental testing of potentially contaminated sites

1. Problems of sampling and interpretation of results

This annex provides some guidelines for those faced with testing sites that have histories suggestive of anthrax-spore contamination prior to development, for example, former tanneries, wool and hair or bone processing plants, slaughterhouses and laboratories, or fields where animals are alleged to have died of anthrax and been buried. In addition to requests for guidance on the examination procedures to be used, the questions generally asked are which samples need to be taken, how many, what quantities, how they should be collected and sent, and what precautions should be observed. In addition, those submitting samples frequently suffer considerable anxiety when faced with a positive result, and require guidance on the significance of a positive and the action that may need to be taken.

2. Laboratory examination of environmental samples

2.1 Limitations of examination procedures

Suggested procedures for examination of environmental samples are given in Annex 1, section 10.4.1. The limitations of these procedures should be recognized. It can be seen from these that, of the originally suspended sample, < 1% of sample is actually cultured. The procedure therefore relies heavily on the initial preparation resulting in a uniform suspension of any anthrax spores present. It is in fact probable that this is rarely achieved. Furthermore, the measured-out sample in the first step is normally a relatively small subsample of what was initially collected, and detection of any *B. anthracis* present depends on it being included in that subsample.

The ideal sample is a granular material which can be readily homogenized into even suspensions and which has very low numbers of other bacteria present; the sensitivity of the test for such a sample is about 5 anthrax spores per gram. This can be increased by increasing the number of plates spread, although that also increases the cost of the test. Soil samples usually contain numerous soil bacteria and, on plates spread with the undiluted suspensions, these tend to overgrow any anthrax bacteria that may be present. With these, the *B. anthracis* is most frequently found in the 10- and 100-fold dilutions, which means that, for most soils, the limits of sensitivity are more normally around 50 spores/g.

2.2 Quantitation

In the event of reporting a positive, clients are frequently anxious to have a quantitative assessment of the level of contamination in the sample. Attempts to accommodate this have frequently only led to confusion. Soil being a heterogeneous and complex system, clear dilution patterns rarely result on the plates onto which the undiluted and 10-fold and 100-fold dilutions are subcultured. A positive sample normally presents itself by the appearance of one to a few colonies on the 1:10 and 1:100 plates with no numerical dilution pattern. An added complication is that, on occasion, numbers increase with increasing dilution of suspension, presumably due to some growth inhibitor in the sample which is being diluted out. It is therefore advisable to divide samples broadly into:

- trace levels: < 5 colony-forming units on any of the subculture plates from the undiluted, 10-fold or 100-fold dilutions of a suspension of the sample in 1–2 volumes of sterile deionized water;
- moderate levels: ≥ 5 colony-forming units on the 100-fold and/or 10-fold dilution subculture plates (expressed as < 100 spores/g);
- higher levels: a clear dilution pattern is obtained making it possible to roughly quantitate the number of spores/g of sample.

It is rare to find colonies in sufficient numbers to make it possible to quantitate the level of contamination in soil samples and, generally, a statement of presence or absence of *B. anthracis* is all that can be supplied.

2.3 Reducing the limitations and increasing the sensitivity

At present, the only ways to increase the sensitivity of the present method are to examine more subsamples and to spread more than just one non-selective and one selective plate for each sample or subsample. Theoretically, there is no limit to the number of plates that may be spread and, if desired, the entire sample could be cultured this way. However this becomes expensive in terms of man-hours, media and plasticware without providing a great deal more enlightenment regarding the health risks to be associated with the site from which the sample is taken. Attempts to develop enrichment-broth systems for *B. anthracis* have invariably failed; the chemicals and antibiotics that might be considered for use as the active ingredients in such a system virtually always favour the competitors (mostly other *Bacillus* species) and inhibit the *B. anthracis*. Even the ingredients of selective PLET agar, which work quite effectively in solid media, fail to perform the same way in liquid suspensions.

Methods under development involving the polymerase chain reaction (PCR – Annex 1, section 10.7.4) may increase sensitivity in the future

2.4 Sensitivity of detection versus hazard

In conclusion, it is important to consider what, beyond academic satisfaction, is to be gained by more sensitive detection procedures than those that currently exist. On certain specific occasions, it may be important to find one spore if it is there but, more normally, examination of environmental samples is about whether a site represents a hazard to man or animals. While it is likely that sites having low levels of contamination with anthrax spores are missed in examinations as currently carried out, this defect is not reflected in unexplained cases or outbreaks of anthrax, suggesting that current sensitivity levels are satisfactory for normal purposes. When more sensitive detection procedures are developed and positive sites that would previously have been missed are identified, it will be important to keep in mind that the risk hazards at these sites have not increased.

3. Sampling plans

3.1 Distribution of bacteria

Environmental samples are generally heterogeneous systems; organisms of interest in such systems are neither evenly distributed nor even randomly distributed. In soil, microbial distribution is influenced by many factors such as depth, roots, animals and water. Sampling procedures are inevitably compromises between economy and accuracy.

3.2 Broadly statistical sampling

The problems related to achieving a 95% statistical chance of locating an area of contamination within a reasonable budget, or generating an impractical number of samples to examine, have been reviewed by Turnbull (1996). Cited guidance indicated that achieving the statistical goal would require: 66 sample points using a 10 m grid for a 0.5 hectare area; 140 sample points using a 20 m grid for 5 hectares; and 200 sample points using a 30 m grid for 16 hectares. Wilson & Stevens (1981) report that the compromise between statistical desirability and financial acceptability generally accepted for assessing chemical contamination in disused gaswork sites were spacings of 20–50 m, with a 25 m grid being fairly common. Based substantially on this, the British Standards Institute (1988) suggests that the minimum number of sampling points should be 15 for 0.5 hectares, 25 for 1 hectare and 85 for 5 hectares. The error in assuming that these samples are representative may be large but, in the case of anthrax, negative samples collected on this basis serve to offer assurance that gross contamination is not present.

It may be possible to further reduce the numbers of samples that need to be taken by:

- thoroughly investigating the site history to determine where the likely "hot spots" are, and confining the sampling to these. If it is considered that areas of greatest contamination can be pinpointed from the history of the site, it may be acceptable to sample from just that site and to conclude that, if *B. anthracis* is not found here, then the rest of the site can be assumed to have below detectable levels of the organism;

- considering the intended uses of the site and assessing whether contamination would be important (e.g. if the contaminated area will be covered by a hard surface, such as a car park, contamination of the underlying soil may be deemed irrelevant);
- designing the use of the land around its potential contamination (e.g. siting hard surfaces where contamination is suspected in the ground plan).

Another approach to reducing costs and making the sampling operation practical is to reduce the number of tests by pooling portions of samples drawn separately into composite samples from which a practical number of subsamples may be taken for testing. In the event of a positive in any of the composites, the individual samples making up that composite would have to re-examined separately.

The concept of "trigger concentrations" was also reviewed by Turnbull (1996). The idea here is to assist in determining the significance of contamination depending on the intended use of the site. The principle is that, if the samples from the site show values below the trigger concentrations, it is reasonable to regard the site as non-hazardous and to proceed with the development accordingly. The trigger concentrations would depend on the intended use of the site, being lowest for grazing land for example, where susceptible livestock will be directly exposed to the contamination, and highest for planned hard surfaces. Contamination above trigger concentrations for a particular planned use would indicate the need for remedial action before the development could proceed. Alternatively, a different use might be considered.

3.3 Depth of sampling

Decision on depth of sampling depends greatly on the type of site. At former industrial sites such as tanneries, wool mills, etc., contamination would have been largely of a surface nature and would only have penetrated to any depth through drains. Where site plans are available and drain paths can be identified, samples from appropriate depths would be worth taking. Otherwise initial sampling probably need only be from the top 0.25 m; a decision might be taken to test the sediment in drains that are found at a later stage. At carcass burial sites, clearly it is important to take samples up to about 2 m below surface.

4. Collection and shipment of samples

Collection of environmental samples is covered in Annex 1, section 7.1.2.3. Sensible but not extreme precautions are appropriate for sample collection. Ideally disposable coveralls and gloves should be worn and incinerated after use. Cuts and abrasions should be properly dressed before coveralls, gloves and boots are put on. Boots should be washed down with 10% formalin or strong hypochlorite after use, and the disinfectant itself should be left overnight before being discarded. In exceedingly dusty conditions, or where dust is being collected, for example around the inside of a disused tannery or bone-processing plant, dust masks are important.

5. Considerations of risk

Questions are frequently raised about the risks to health of (i) the workforce involved in the redevelopment of potentially contaminated sites, and (ii) humans or animals utilizing the sites after development. The actual risk to human health from contaminated soil and other environmental materials is very low. Experience shows that the levels of contamination in contaminated land sites rarely exceed a few spores per gram and, by taking appropriate precautions such as wearing coveralls and gloves, dressing wounds and taking other sensible hygienic precautions, such risks of infection as do exist are reduced essentially to nil. In addition, action plans should include the requirement to report any lesions or illness occurring within two weeks of carrying out the work to the relevant medical adviser. In the unlikely event that anthrax has been contracted as a result of the work, it is readily treatable with antibiotics (chapter 7).

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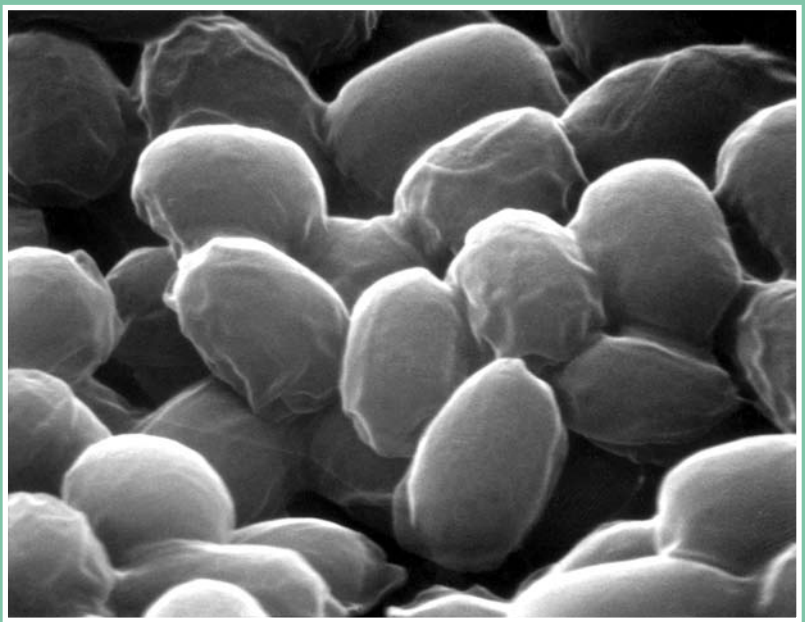
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