SECTION 3.1.

MULTIPLE SPECIES

CHAPTER 3.1.1.

ANTHRAX

SUMMARY

Description and importance of the disease: *Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and some avian species can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped bacterium,* Bacillus anthracis*. The disease has world-wide distribution and is a zoonosis.*

The disease is mediated mainly by exotoxins. Peracute, acute, subacute and, rarely, chronic forms of the disease are reported. Ante-mortem clinical signs may be virtually absent in peracute and acute forms of the disease. Subacute disease may be accompanied by progressive fever, depression, inappetence, weakness, prostration and death. Acute, subacute, and chronic disease may show localised swelling and fever. In chronic disease, the only sign may be enlarged lymph glands.

Detection and identification of the agent: Bacillus anthracis *is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and colony morphology of* B. anthracis *is quite characteristic after overnight incubation on blood agar. The colony is relatively large, measuring approximately 0.3–0.5 cm in diameter. It is grey-white to white, non-haemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. The vegetative cells of* B. anthracis *are large, measuring 3–5 μm in length and approximately 1 μm in width. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential cell growth phase. The cells stain strongly Gram positive, and long chains are often seen* in vitro*, while paired or short chains are seen* in vivo*. Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with azure B or polychrome methylene blue* (*M'Fadyean's reaction*) *is fully diagnostic.*

Serological tests: *Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. The predominant procedure used is the enzyme-linked immunosorbent assay.*

Requirements for vaccines: *The most widely used livestock anthrax vaccine developed by Max Sterne in 1937 is a live, non-encapsulated, spore former held in suspension. In Russia and Eastern Europe, an equivalent type of vaccine is used* (*strain 55*)*. A list of producers is given in the World Health Organization anthrax guidelines.*

A. INTRODUCTION

Anthrax, an acute bacterial disease primarily of herbivores, is transmissible to humans. The aetiological agent, *Bacillus anthracis*, is a Gram-positive spore-forming rod-shaped bacterium. Anthrax is known by many names around the world including charbon, woolsorters' disease, ragpickers' disease, malignant carbuncle, malignant pustule and Siberian ulcer.

Animals become infected by ingesting spores, or possibly by being bitten by blood feeding insects such as stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*) that have fed on an infected

animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. To avoid environmental contamination with spores, post-mortem examinations of carcasses of animals suspected to have died of anthrax are discouraged and natural orifices such as mouth, nostril and anus should be closed (for example plugged with cotton wool or other suitable material soaked in an approved disinfectant) to prevent the formation of spores. There are regulations in most countries that prohibit post-mortem examination when anthrax is suspected. Recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent.

Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen with a dark semi-fluid pulp ('blackberry jam' consistency) and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death may be found.

Gram-positive rod-shaped *B. anthracis* is an obligate pathogen. Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus, B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

1. Zoonotic risk and biosafety requirements

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. *Bacillus anthracis* is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves and other protective clothing (including full personal protective equipment [PPE] depending on the situation) when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

The risk of inhaling infectious doses becomes significant in occupations involving the processing of animal by-products for manufacturing goods (industrial anthrax). These include the tanning, woollen, animal hair, carpet, bone processing, and other such industries, where the potential for aerosolisation of substantial numbers of spores increases the risk of exposure to infectious doses. It is important that industrial workers use appropriate personal protective clothing and equipment and follow standard operating procedures that minimise the risk of transmission. Efficient air extraction equipment should be positioned over picking, combing, carding and spinning machines. Air blowing machinery should never be used for cleaning equipment due to the risk of spore dispersal.

Clinical specimens and cultures of *B. anthracis* should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Vaccination of laboratory personnel is recommended.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of anthrax and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Demonstration of capsule	–	–	–	++	–	–
Demonstration of lack of motility	–	–	–	++	–	–
Gamma phage lysis	–	–	–	++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Penicillin susceptibility	–	–	–	++	–	–
PCR	–	–	–	++	+++/++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

Demonstration of encapsulated *B. anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of *B. anthracis* from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is often difficult, requiring demanding and labour-intensive procedures. However live spores may be recovered from the turbinate bones of dead livestock and wildlife for an extended period after death (M. Hugh-Jones, personal communication).

1.1. Culture and identification of *Bacillus anthracis*

1.1.1. Fresh specimens

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates. After overnight incubation at 37°C, *B. anthracis* colonies are grey-white to white, 0.3–0.5 cm in diameter, non-haemolytic, with a ground-glass surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a 'medusa head' or 'curled hair' appearance. Confirmation of *B. anthracis* should be accomplished by the demonstration of a capsulated, spore-forming, Gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 (Brown & Cherry, 1955) and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (World Health Organization [WHO], 2008).

Two tests for confirming the identity of *B. anthracis* are gamma phage lysis and penicillin susceptibility. The typical procedure for these tests is to plate a lawn of suspect *B. anthracis* on a blood or nutrient agar plate and place a 10–15 µl drop of the phage suspension on one side of the lawn and a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak into the agar before incubating the plate at 37°C. A control culture, e.g. the Sterne vaccine or the NCTC strain 10340, should be tested at the same time as the suspect culture to demonstrate the expected reaction for gamma phage lysis and penicillin susceptibility. If the suspect culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, because of lysis, and a clear zone will be seen around the penicillin disk indicating antibiotic susceptibility. Note that some field isolates of *B. anthracis* may be phage resistant or penicillin resistant. As the performance of the gamma phage lysis assay may be affected by the density of bacterial inoculum, Abshire *et al.* (2005) recommend streaking the suspect culture on the agar plate over several quadrants instead of using a lawn format and inoculating a drop of gamma phage on the

first and second quadrants on the plate. If antibiotic or phage resistant *B. anthracis* is suspected then polymerase chain reaction (PCR) diagnostic methods may be applied.

Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.

The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.

1.1.1.1. Stage one

i) Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.

ii) Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.

iii) Spread 100 µl of the culture from step ii on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.

iv) Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.

v) Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second 'wash' of 5 ml NB. Incubate at 37°C overnight.

vi) Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step iii.

1.1.1.2. Stage two

This is essentially the same procedure as Stage one, only uses the filtrate from step vi to harvest the phage from the plates.

vii) Prepare three Sterne strain lawns on BA, as in step iii. Incubate at 37°C for 30–60 minutes.

viii) Spread 100 µl phage from step vi. Incubate at 37°C overnight.

ix) To 9 ml of filtrate from step vi, add 1 ml of 10× concentrated NB.

x) Harvest the phage from step viii with 5 ml of the solution from step ix, followed by a second 5 ml wash with the rest of the solution from step vi.

xi) Add 10 ml of 1× NB.

xii) Incubate at 37°C overnight, filter and count.

1.1.1.3. Stage three

xiii) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step ii. Incubate on a rotary shaker at 37°C until just turbid.

xiv) Add the 20 ml of filtrate from step xii and continue incubation overnight.

xv) The resultant filtrate is checked for sterility and titrated in tenfold dilutions on lawns of the vaccine strain as in step vi to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque forming units per ml.

1.1.2. Capsule visualisation

Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. Thin smears may be prepared from blood from ear veins or other peripheral veins, exudate from orifices and, for horses and pigs, from oedematous fluid or superficial lymph nodes in the neck region. However if the animal has been dead more than 24 hours, the capsule may be difficult to detect. The bacteria should be looked for in smears of these specimens that have been dried, fixed and then stained with azure B (M'Fadyean's reaction). The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended (the chains are sometimes likened to a set of railway

carriages – so-called 'box-car' or 'jointed bamboo-rod' appearance). Gram stain does not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths but can be seen when the virulent bacterium is cultured for a few hours in a few millilitres of blood (defibrinated horse or sheep blood seems to work best). Alternatively, the capsule is produced when the virulent *B. anthracis* is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO_2 (20% is optimal, but a candle jar works well). The agar is prepared by weighing nutrient agar base powder required for a final volume of 100 ml but reconstituting the measured agar in only 90 ml of water. Autoclave and cool to 50°C in a water bath. Add 10 ml of a filter-sterilised (0.22–0.45 µm filter) 7% solution of sodium bicarbonate. Mix and pour into Petri dishes. The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualised by making thin smears on microscope slides, fixing, and staining with azure B or polychrome methylene blue (M'Fadyean's stain).

Polychrome methylene blue can be prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K_2CO_3 (to a final concentration of 1%) hastens the 'ripening' of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on *bona fide* samples. It has been found that stains that give positive reactions with cultures of *B. anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.

The method for capsule visualisation has been revised according to the description by Owen *et al.* (2013). The established simple polychrome methylene blue (PMB) staining method for blood or tissue smears from dead animals (M'Fadyean's reaction) is therefore replaced by azure B staining. It must be noted that the rarity of anthrax disease worldwide due to improvements in the control of the disease has led to quality controlled commercially produced PMB being difficult to obtain. In addition, reports of inaccurate results using alternative methylene blue-based stains has also become a concern. Hence, for laboratories requiring a reliable M'Fadyean stain for rapid detection, the recommended approach is to use commercially pure azure B (Owen *et al.,* 2013).

This microscopy method was validated by Aminu *et al.* (2020), for the detection of *B. anthracis* in field-collected blood smears. Four capsule-staining techniques were evaluated in an investigation of livestock mortalities suspected to be caused by anthrax. Field-prepared blood samples were tested by microscopy and the results indicated that the median sensitivity and specificity of microscopy using azure B were found to be comparable with those of the recommended standard, polychrome methylene blue (Aminu *et al.* 2020).

Azure B stain is prepared by constituting into a solution of 0.03 g azure B in 3 ml of 95% ethanol or methanol to which is then added 10 ml of 0.01% KOH (0.23% final azure B concentration). This can then be used immediately and throughout the tests. If stored in the dark at room temperature, the shelf life is at least 12 months. Smears to be stained should be fixed with ethanol or methanol (95–100%), not by heat, and the stain left for 5 minutes before washing off for optimum effect (Owen *et al.,* 2013).

In making smears for staining, only small drops of blood or tissue fluid are needed, and a thin, small smear is best. After fixing by dipping the smear in 95–100% alcohol for about 5 minutes and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop. After 5 minutes, the stain is washed with water, blotted, air-dried, and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.

1.1.3. Other specimens

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on non-selective agars. The following procedure is suggested:

i) The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 15–30 minutes. Turnbull *et al.* (2007) have demonstrated that heat activation of spores can be conducted at a temperature range of 60–70°C with holding times not exceeding 15–30 minutes for best recovery.

ii) Tenfold dilutions to 10^{-2} or 10^{-3} are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (Knisely, 1966; WHO, 2008). All plates are incubated at 37°C. For combined selection and differentiation, blood agar-based media can provide advantages. In addition, PLET agar contains the toxic thallium acetate. An alternative for this is the so-called TSPB agar, a blood agar that has high selectivity against Gram-negative bacteria due to the addition of trimethoprim, sulfamethoxazole and polymyxin B (Hudson *et al.,* 2007; Rosenblatt & Stewart, 1974). TSPB agar is prepared by dissolving 40 g/litre nutrient agar base (e.g. SIFIN). The mixture is autoclaved and uniformly cooled to 45°C before adding 50 ml/litre sterile sheep blood (5%), trimethoprim (13.1 mg/litre), sulfamethoxazole (20 mg/litre) and polymyxin B (30,000 IU/litre). After mixing thoroughly, the agar is dispensed into Petri dishes.

iii) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described above.

PLET medium (Knisely, 1966; WHO, 2008) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at o30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.

Reports of procedures for direct detection of *B. anthracis* in soils and other environmental specimens using the PCR are emerging. None of these has become routinely applicable at the present time.

Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. Examples of when this might occur are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals. Due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified. Adult mice or guinea-pigs are the animals of choice. If the samples involved are soils, the animals should be pretreated, the day before testing, with both tetanus and gas gangrene antiserum. The samples are prepared as described for culturing, including heat-shocking at 62.5°C for 15 minutes. Mice are injected subcutaneously with 0.05–0.1 ml; guinea-pigs are inoculated intramuscularly with up to 0.4 ml (0.2 ml in each thigh muscle). Any *B. anthracis* present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

1.2. Immunological detection and diagnosis

It needs to be borne in mind that *B. anthracis* is antigenically very closely related to *B. cereus,* which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

1.2.1. Ascoli test

Ascoli (1911) published a procedure for the detection of thermostable anthrax antigen in animal tissue being used for by-products. This uses antiserum raised in rabbits to produce a precipitin reaction. The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. This test appears to be used only in Eastern Europe.

To perform the Ascoli test, put approximately 2 g of sample in 5 ml of saline containing 1/100 final concentration of acetic acid and boil for 5 minutes. The resultant solution is cooled and filtered through filter paper. A few drops of rabbit antiserum (see preparation below) are placed in a small test tube. The filtrate from the previous step is gently layered over the top of the antiserum. A positive test is the formation of a visible precipitin band in under 15 minutes. Positive and negative control specimen suspensions should be included.

Antiserum is prepared in rabbits by the subcutaneous inoculation of Sterne anthrax vaccine on days 1 and 14. On days 28 and 35, the rabbits receive 0.5 ml of a mixture of several strains of virulent *B. anthracis* not exceeding 10^5 colony-forming units (CFU)/ml suspended in saline. Alternatively, the live virulent bacteria can be inactivated by prolonged suspension in 0.2% formalised saline, but the antigen mass needs to be increased to 10^8–10^9 CFU/ml. The suspension should be checked for inactivation of the *B. anthracis* before animal inoculation by culture of 0.1 ml into 100 ml of nutrient broth containing 0.1% histidine and, after incubation at 37°C for 7 days, subculture on to blood or nutrient agar. The dose regimen for the formalised suspension after initial vaccination on days 1 and 14 is increasing doses of 0.1, 0.5, 1, and 2 ml given intravenously at intervals of 4–5 days. Following either procedure, a test bleed at 10 days after the last injection should determine whether additional 2 ml doses should be administered to boost the precipitin titre.

1.2.2. Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (Ezzell & Abshire, 1996), it does not lend itself to routine diagnosis.

1.3. Confirmation of virulence with the polymerase chain reaction

Confirmation of virulence can be carried out using the PCR. The following instructions are taken from the WHO (2008). Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by suspension of a loop of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Examples of suitable primers (Beyer *et al.,* 1996; Hutson *et al.,* 1993) for confirming the presence of the pXO1 and pXO2 plasmids are given in the table below.

Target	Primer ID	Sequence (5' → 3')	Product size	Concentration
Protective antigen (PA)	PA 5 3048–3029	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp	1 mM
	PA 8 2452–2471	GAG-GTA-GAA-GGA-TAT-ACG-GT		
Capsule	1234 1411–1430	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bp	0.2 mM
	1301 2257–2238	TCC-CAC-TTA-CGT-AAT-CTG-AG		

PCR can be carried out in 50 µl volumes using the above primers, 200 µM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and 2.5 units of DNA polymerase, all in NH_4 buffer, followed by the addition of 5 µl of template DNA. A 2% agarose gel has been found to work best with these small fragments.

Alternatively, premixed, predispensed, dried beads available commercially can be used. These are stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions. The template can be added in a 2.5 µl volume.

The following PCR cycle can be used: 1 × 95°C for 5 minutes; 30 × 95°C for 0.5 minute followed by 55°C for 0.5 minute followed by 72°C for 0.5 minute; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that the primers given in the table above have proved successful for confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) specimens or environmental samples. They may be unsuitable, however, for direct detection of *B. anthracis* in such specimens or samples. A choice of alternatives can be found in Jackson *et al.* (1998) and Ramisse *et al.* (1996). For the rare possibility that an isolate may lack both pXO1 and pXO2, a chromosomal marker should also be run; primers for these are also described in Jackson *et al.* (1998) and Ramisse *et al.* (1996). Ågren *et al*. (2013) published a very comprehensive study on the *in-silico* and *in-vitro* evaluation of 35 PCR-based methods for 20 chromosomal markers of *B. anthracis*. The PL3 (target: part of pro-phage type 3) assay (Wielinga *et al*., 2011) was identified as one of the best performing assays in this study and could be used for routine diagnostics.

Real-time PCR assays have been developed for enhanced speed, sensitivity and specificity of detection of pXO1, pXO2 and chromosomal genes of *Bacillus anthracis* and other closely related *Bacillus* spp. (e.g. Hadjinicolaou *et al*., 2009; Hoffmaster *et al*., 2002; Irenge *et al*., 2010; Qi *et al*., 2001; Rao *et al*., 2010). Selection of a particular assay will be dependent on the fitness for purpose and source of starting material (e.g. isolates, clinical specimen, environmental sample), requirement to differentiate from other *Bacillus* spp. or vaccine strains, demonstration of genetic diversity or confirmation of isolate identity. It is important that the laboratory conducting real-time PCR evaluate the performance of the test for their purpose and complete a validation analysis to ensure that it has been optimised and standardised for its intended use (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

Molecular typing techniques for *B. anthracis* like canonical single-nucleotide polymorphisms typing (e.g. Van Ert *et al*., 2007) and variable number tandem repeat analysis (e.g. Keim *et al*., 2000) are appropriate for use in specialised laboratories. Molecular typing based on whole genome sequencing e.g. core genome multilocus sequence typing may be useful to elucidate the diversity of *B. anthracis* genotypes circulating, to determine connections between outbreak events and supports infection chain tracing (Abdel-Glil *et al*., 2021).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne (1937). He derived a rough variant of virulent *B. anthracis* from culture on serum agar in an elevated CO_2 atmosphere. This variant, named 34F2, was incapable of forming a capsule and was subsequently found to have lost the pXO2 plasmid, which codes for capsule formation. It has become the most widely used strain world-wide for animal anthrax vaccine production. In Central and Eastern Europe, an equivalent $pXO2^-$ derivative, Strain 55, is the active ingredient of the current livestock vaccine. A list of manufacturers of anthrax vaccine for use in animals is given in Annex 5 of WHO (2008).

The following information concerning preparation of the anthrax vaccine for use in animals is based on Misra (1991) and the WHO (1967). Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Anthrax vaccine production is based on the seed-lot system. A seed lot is a quantity of spores of uniform composition processed at one time and maintained for the purpose of vaccine preparation. Each seed lot is no more than three passages from the parent culture and must produce a vaccine that is efficacious and safe for use in animals. It is recommended that a large

seed lot be prepared from the parent strain and preserved by lyophilisation for future production lots. The parent culture can be purchased[1].

2.1.2. Quality criteria

The seed lot is acceptable for anthrax vaccine if a vaccine prepared from the seed lot or a suspension harvested from a culture derived from a seed lot meets the requirements for control of final bulk with respect to freedom from bacterial contamination, safety and efficacy (immunogenicity).

2.2. Method of manufacture

2.2.1. Procedure

i) Preparation of the master seed

Seed lots are cultured on solid media formulated to promote sporulation of the organism. The solid medium formula for casein digest agar (sporulation agar) given in Misra (1991) is: 50 g tryptic digest of casein; 10 g yeast extract; 0.1 g $CaCl_2.6H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.05 g $MgSO_4.7H_2O$; 0.03 g $MnSO_4.4H_2O$; 5.0 g K_2HPO_4; 1.0 g KH_2PO_4; 22 g agar; 1000 ml deionised or distilled water. The ingredients are dissolved in the water with the appropriate amount of heating; the solution is adjusted to pH 7.4, distributed into Roux bottles (120 ml per bottle) or other appropriate container, sterilised by autoclaving and cooled in the horizontal position. After the agar has solidified, excess liquid should be removed aseptically and the bottles left in an incubator (37°C) for at least 2 days to dry and to check the sterility.

Volumes of 2 ml of vaccine seed should be spread across the agar in Roux bottles, which should be incubated at 37°C until at least 80% sporulation is apparent by microscopic examination of aseptically extracted loopfuls (at least 72 hours). The growth is harvested with 10 ml per bottle of sterile deionised or distilled water and checked for purity. After washing three times in sterile deionised or distilled water with final suspension, also in sterile deionised or distilled water, sterilised lyophilisation stabiliser is added and the suspension is dispensed into lyophilisation vials and freeze-dried.

Attenuated vaccine strains can gradually lose their antigenicity over repeated subculturing conditions. Therefore, it is recommended that master seed lots be made in bulk and kept within three passages from the original seed culture. A large number of master seed stocks should be prepared.

ii) Preparation and testing of the working seed

Reconstitute a vial of seed stock and inoculate several slants (approximately 10 ml) of sporulation (casein digest) agar. Incubate at 37°C for 72 hours and store in a refrigerator. Test the slants for purity by culture on to nutrient agar plates and in nutrient broth (0.1 ml in 100 ml of nutrient broth). The latter should be subcultured on to nutrient agar after incubation at 37°C for 7 days and should be a pure culture of *B. anthracis*. A sample of the broth culture should also be checked for lack of motility.

Volumes of seed needed for a production run should be calculated on the basis of harvesting the spores from each slant with 10 ml of sterile deionised or distilled water and using this to inoculate five Roux bottles.

iii) Preparation of vaccine concentrate

Roux bottles with casein digest agar are prepared as for the master seed in Section C.2.2.1.i above. One Roux bottle can be expected to yield about 2000 doses of vaccine. Each Roux bottle is inoculated with 2 ml of working seed suspension and incubated at 37°C with porous plugs for several days until small loopfuls of culture from randomly selected bottles show at least 90% of the organisms to be in sporulated forms when examined in wet mounts by phase contrast (phase bright spores) or following staining for spores. The growth from each

1 UK Health Security Agency, Microbiology Services, Porton Down, Salisbury SP4 0JG, UK (www.culturecollections.org.uk/)

bottle is then harvested with 20 ml of physiological saline. Tests for contaminants should be carried out by subculture to nutrient agar plates and inoculation of 100 ml nutrient broth with 0.1 ml of harvested spores followed by subculture to nutrient agar after 7 days at 37°C and by tests for motility. Acceptable harvests (i.e. those showing no evidence of contaminants) are pooled.

iv) Glycerination

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool of vaccine concentrate. Saponin (0.1% final concentration) may also be added at this point if it is to be included as an adjuvant. Mix thoroughly (the inclusion of sterilised glass beads may be helpful). Carry out a purity test and hold for 3 weeks at ambient temperature to allow lysis of any vegetative bacteria, determine the viable spore count and store under refrigeration thereafter.

v) Determining titre and dilution for use

The number of culturable spores in the product is then calculated by spreading tenfold dilutions on nutrient agar plates. The suspension is diluted so that the final bulk contains the number of culturable spores desired. The diluent should contain the same proportions of saline, glycerol and (if being included) saponin as present in the vaccine concentrate. The vaccine should contain a minimum of $2–10 \times 10^6$ culturable spores per dose for cattle, buffaloes and horses, and not less than $1–5 \times 10^6$ culturable spores per dose for sheep, goats and pigs.

vi) Filling the containers

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in WHO (1965). Basically, the final bulk is distributed to containers in an aseptic manner in an area not used for production, and any contamination or alteration of the product must be avoided. The vaccine may be lyophilised after distribution into appropriate dosage containers. Containers are sealed as soon as possible with a material that is not detrimental to the product and that is capable of maintaining a hermetic seal for the life of the vaccine.

2.3. Requirements for substrates and media

Please refer to Misra (1991) for detailed information on substrates and media used for anthrax vaccine production.

2.3.1. In-process controls

i) Purity of the seed lot

Purity tests consist of microscopic examination of stained smears with culture and motility tests as described in Section C.2.2.

ii) Safety of the seed lot

Not less than 5×10^9 culturable spores should be injected subcutaneously into each of three healthy, 1–2-year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.

iii) Immunogenicity of the seed lot

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with 5×10^6 viable spores and observed for 21 days. At least 80% of the animals should survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses (LD_{50}) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.

2.3.2. Final product batch tests

i) Sterility and purity

The vaccine is a live culture of *B. anthracis* spores; sterility does not apply, but the batches must be tested for freedom from contamination (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Safety

Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.

iii) Batch potency

Efficacy or immunogenicity is tested on the final bulk as follows: at least ten healthy 300–500 g guinea-pigs are inoculated with a sheep dose of the vaccine. The guinea-pigs are observed for 21 days, and at least 80% of the animals must survive the observation period. Surviving immunised guinea-pigs and three non-vaccinated controls are challenged with an appropriate dose of virulent *B. anthracis*. A recommended challenge is 200 LD_{50} of the Pasteur II strain (17JB). If, by 10 days after challenge, all vaccinated guinea-pigs survive and control animals die, the final bulk is deemed to be satisfactory. If any vaccinated animals die during the post-challenge observation period from a cause other than anthrax, and death is not associated with the vaccine, the test may be repeated.

2.4. Requirements for authorisation

2.4.1. Safety requirements

i) Target and non-target animal safety

The vaccine has been shown to cause disease in some goats and llamas; this may be related to the saponin adjuvant. The vaccine is not recommended for use in pregnant animals, nor in animals destined for slaughter within 2–3 weeks of vaccination. Local regulations may specify other time periods in some countries or regions, but there is no scientific reason for regarding meat from clinically healthy animals as unfit for human handling or consumption after a holding period of 2 weeks following vaccination. Concurrent administration of antibiotics to vaccinated animals is contraindicated as the antibiotic will interfere with the vaccine. Antibiotics should not be given for several days before and after vaccination.

Accidental human inoculation is treated by expressing as much of the inoculum as possible from the injection site and washing the wound thoroughly with soap and water. Medical attention should be sought if infection develops.

ii) Reversion–to-virulence for attenuated/live vaccines

The 34F2 strain of *B. anthracis* is known to be stable and cannot produce capsule *in vitro*.

iii) Environmental consideration

Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated.

2.4.2. Efficacy requirements

i) For animal production

Not applicable.

ii) For control and eradication

The recommended dose for cattle and horses is a minimum of 2–10 × 10^6 culturable spores; for sheep, goats and pigs, it is 1–5 × 10^6 culturable spores. The vaccine should contain these

spores in an appropriate volume, e.g. 2×10^6/ml. Immunity should be good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.

Bacillus anthracis spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).

As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantage

There are no vaccines based on biotechnology available for anthrax.

3.2. Special requirements for biotechnological vaccines, if any

Not applicable.

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NB: There are WOAH Reference Laboratories for anthrax (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on anthrax diagnostic tests, reagents and vaccines

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.