CHAPTER 3.6.11.

GLANDERS AND MELIOIDOSIS

SUMMARY

***Description and importance of the disease**: Glanders is a contagious and fatal disease of horses, donkeys, and mules, caused by infection with the bacterium* Burkholderia mallei. *The pathogen causes nodules and ulcerations in the upper respiratory tract and lungs. The skin form is known as 'farcy'.*

Melioidosis is an infectious disease caused by Burkholderia pseudomallei. *Melioidosis is uncommon in humans and animals and sometimes resembles glanders in horses. This chapter focuses on the disease in horses.* Burkholderia mallei *has evolved from* B. pseudomallei *by reduction of genetic information and is phylogenetically considered as a clone, i.e. a pathovar of* B. pseudomallei.

Control of glanders and melioidosis requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of reactors. Stable hygiene and manure management are imperative. As B. mallei *and* B. pseudomallei *can be transmitted to humans, all infected or contaminated (or potentially infected or contaminated) material must be handled in a laboratory with appropriate biosafety and biosecurity controls following a biorisk analysis.*

***Detection of the agent:** Smears from fresh material containing* B. mallei *bacteria may reveal Gram-negative nonsporulating, nonencapsulated rods.* Burkholderia mallei *grows aerobically and prefers media that contain glycerol. Standard media for isolation of* B. pseudomallei *can be used and selective enrichment techniques have been developed. The presence of a capsule-like cover has been demonstrated by electron microscopy in both agents. Unlike the* Pseudomonas *species and the closely related bacterium* B. pseudomallei, B. mallei *is nonmotile. For identification, biochemical phenotyping can be used. Commercially available biochemical identification kits lack diagnostic sensitivity. MALDI-TOF spectra have been made available for both agents in the past years. Whole genome sequences have been published. Specific monoclonal antibodies and polymerase chain reaction (PCR), as well as real-time PCR assays are available.*

***Serological tests:** Complement fixation is an accurate and reliable serological method for diagnostic use in glanders in equids. A rose bengal plate agglutination test for glanders has been developed. An immunoblot test based on a crude formalin preparation of* B. mallei *antigens from isolates of different geographical regions is also a sensitive and specific assay. These tests may also be positive in horses with melioidosis. Enzyme-linked immunosorbent assays show promise for specific diagnosis in equids once their validation is complete.*

***Mallein test:** The mallein test is a hypersensitivity skin test against* B. mallei. *The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. Mallein, a water soluble protein fraction of the organism, is injected intradermo-palpebrally. In infected animals, the eyelid swells markedly within 1–2 days. This test may also be positive in horses with melioidosis.*

***Requirements for vaccines and diagnostic biologicals:** There are no vaccines. Mallein purified protein derivative is commercially available.*

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates. It is a zoonotic disease and has been known since ancient times. It is caused by the bacterium *Burkholderia mallei* (Yabuuchi *et al.*, 1992) and has been variously classified in the past as *Pseudomonas, Pfeifferella*, *Loefflerella, Malleomyces* or *Actinobacillus*. It is a serious contagious disease in equids and outbreaks may also occur in felids living in the wild or in zoological gardens. Susceptibility to glanders has been proven in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle, and pigs are resistant. Small ruminants may be infected if kept in close contact with glanderous horses (Wittig *et al.*, 2006). Glanders generally takes an acute form in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course and horses may survive for several years. Chronic and subclinical 'occult' cases are potential sources of infection due to the permanent or intermittent shedding of bacteria (Wittig *et al.*, 2006). Khan *et al.* (2013) reviewed the disease, its epidemiology, diagnosis and control.

In horses, inflammatory pustules and ulcers develop in the nasal conchae and nasal septae, which give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. The formation of reddish nodular abscesses with a central grey necrotic zone in the lungs is accompanied by progressive debility, febrile episodes, coughing and dyspnoea. Diarrhoea and polyuria can also occur. In the skin form ('farcy'), the lymphatics are enlarged and 0.5–2.5 cm sized nodular abscesses ('buds') develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyogranulomatous nodules are sometimes found in the liver and spleen. Discharges from the respiratory tract and skin are infective, and transmission between animals, which is facilitated by close contact, inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common. The incubation period can range from a few days to many months (Wittig *et al.*, 2006).

Glanders is transmissible to humans by direct contact with diseased animals or with infected or contaminated material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer *et al.*, 1997). However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies. A chronic form with abscessation can occur (Neubauer *et al.*, 1997).

Glanders has been eradicated from many countries by statutory testing, culling of infected animals, and import restrictions. It persists in numerous Asian, African and South American countries and can be considered a re-emerging disease. Glanders can be introduced into glanders-free areas by movement of equids (Neubauer *et al.*, 2005).

Burkholderia pseudomallei is the causative agent of melioidosis and originates from South-East Asia (areas within latitudes 20°N and 20°S) (Limmathurotsakul *et al.,* 2016). It is an important soil bacterium, denitrifying organic materials and is ubiquitous in those areas. Thus, *B. pseudomallei* is stable in the environment. It is also naturally resistant to various antibiotics and disinfectants (Currie, 2015; O'Connell *et al.,* 2009; Sprague & Neubauer, 2004). *Burkholderia pseudomallei* has an extremely broad host range including wildlife, farm animals and humans (Rush & Thomas, 2012). *Burkholderia pseudomallei* has regularly been isolated from abscesses in various organs (spleen, liver, lung, lymph nodes), from milk in cases with mastitis, or from faeces in diarrhoea cases. This chapter focuses on the disease in horses, which may closely resemble glanders, but more diverse presentations also occur. Melioidosis in horses has been reported as peracute cases with high fever, septicaemia, limb oedema, diarrhoea and death occurring within 24 hours, or acute cases with limb oedema, slight colic and intestinal hypermotility. More typically, melioidosis runs a subacute to chronic course from 3 weeks to 3 months with no loss of appetite. Further signs reported are emaciation, oedema and lymphangitis of the limbs, mild colic, diarrhoea, pneumonia, cough and nasal discharge. Skin involvement may initially resemble fungal eczema, later becoming papular without abscess formation. Acute meningoencephalitis and keratitis may be seen. If infection is acquired *per os*, intestinal signs may predominate. It is reported that the lungs are always affected and show signs of acute bronchopneumonia and numerous abscesses. Severe enteritis, multiple microabscessation in the kidneys, and necrotic foci in liver and spleen may be found at necropsy. Ulcers on the mucosa of the upper respiratory tract and cicatrical scar tissue on the septum nasi and epiglottis may be found. Ulcers and nodules in the skin and subcutis of the limbs may be confused with farcy. Conjunctivitis and keratitis have been reported, though rare. The incubation period for naturally occurring infection in animals is not known. Spread may be by infected animals or contaminated materials in the animals' environment. Infection is thought to occur by inoculation, ingestion or inhalation of environmental organisms. Inapparent infection may occur. (reviewed by Sprague & Neubauer, 2004). In humans the disease is frequently fatal despite timely institution of therapy (Currie, 2015; Kingsley *et al.,* 2016b). No approved antibiotic regimens for horses have been recommended.

Melioidosis can be considered an emerging disease and is present in many regions worldwide (Limmathurotsakul *et al.,* 2016). Control of melioidosis requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of reactors. In areas with a temperate climate, special thought has to be given to stable hygiene (disinfection, removal of faeces several times a day, reduction of the use of water for cleaning, disinfection of hooves and lower limbs, movement control) and soil hygiene. Suitable methods to prevent spread by treatment of manure, waste water or rodent control have not been investigated or reported (Dodin, 1992). Dodin (1992) stated that the enzootics in France declined and the infected soils were cleared only after all infected animals were removed. Consequently, Sprague & Neubauer (2004) proposed that equids with melioidosis should be culled immediately in non-endemic countries to prevent local establishment of environmental reservoirs that would pose an ongoing risk of infection for humans and animals.

When handling suspect or known infected animals or fomites, stringent precautions must be taken to prevent self-infection or transmission of the bacteria. Laboratory samples must be securely packaged, kept cool (not frozen) and shipped as outlined in Chapter 1.1.3 *Transport of biological materials*. All manipulations with potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of glanders 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
Detection of the agent						
PCR	–	–	–	+	–	–
Culture	–	–	–	+	–	–
Detection of immune response						
Complement fixation	++	++[(a)]	+++	+	+++	–
ELISA	+	+	++	+	++	–
Mallein skin test	+	+	+	+	+	–
Western blotting	+	+	++	+	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]Horse samples only – care needed with interpretation of test on donkey samples.

For melioidosis, culture and polymerase chain reaction (PCR) methods may be used to detect and identify the organism for confirmation of clinical cases, as described in the text below, but serological methods are not yet available for this infection.

1. Interpretation of tests for the diagnosis of glanders

Confirmation of a diagnosis of glanders should be based on the isolation and identification of *Burkholderia mallei* in a sample from an equid or a product derived from that equid; or the identification in such samples of antigen or genetic material specific to *B. mallei.* Supporting evidence may be provided by positive serological test results such as a titre of 1/5 in the complement fixation test (CFT), confirmed by a second test with equal or higher sensitivity and higher specificity, e.g. *B. mallei*-specific lipopolysaccharide (LPS)-western blot, I-enzyme-linked immunosorbent assay (ELISA) (indirect) (based on a recombinant protein from type VI secretion system) or C-ELISA (competitive ELISA) (based on *B. mallei*-specific monoclonal antibodies).

2. Detection of the agent

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections affecting the nasal mucous membranes, sinuses or the skin. Among these are strangles (*Streptococcus equi*), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), pseudotuberculosis (*Yersinia pseudotuberculosis*) and sporotrichosis (*Sporotrichium* spp.). Glanders should be excluded from suspected cases of epizootic lymphangitis (*Histoplasma farciminosum*), with which it has many clinical similarities. In horses and humans in particular, glanders should be distinguished from melioidosis.

2.1. Morphology of *Burkholderia mallei*

Burkholderia mallei organisms are fairly numerous in smears from fresh lesions, but scarce in older lesions. Smears should be stained with methylene blue or Gram stain. The Gram-negative rods have rounded ends, are 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size. The bacteria are generally located extracellularly and frequently stain irregularly and poorly when Gram stain is used. They do not have a readily visible capsule under the light microscope and do not form spores. The presence of a capsule-like cover has been verified by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the *Pseudomonas* group and its close relative *B. pseudomallei*, *B. mallei* has no flagellae and is therefore nonmotile (Sprague & Neubauer, 2004). Nonmotility is the most important phenotypic characteristic diagnostically and must be demonstrated when pure culture is available. The organisms are difficult to detect in tissue sections, where they may have a beaded appearance. In culture media, they vary in appearance depending on the age of the culture and type of medium. In older cultures, there is much pleomorphism. Branching filaments form on the surface of broth cultures (Neubauer *et al.*, 2005).

2.2. Cultural characteristics

It is preferable to attempt isolation from unopened, uncontaminated lesions. *Burkholderia mallei* is aerobic and facultative anaerobic only in the presence of nitrate, growing optimally at 37°C. It grows well, but slowly, on culture media, including sheep blood agar. Incubation of cultures for 72 hours is recommended; glycerol enrichment is particularly useful. The tiny greyish shiny colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; hence careful observation is needed not to overlook the bacteria after 72 hours of incubation. After a few days on glycerol agar, a confluent, smooth, moist and slightly viscous cream coloured growth can be observed. On continued incubation, the growth thickens and becomes dark brown and tough. *Burkholderia mallei* also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On nutrient agar, the growth is much less effusive, and growth is poor on gelatine. Various commercially available *Burkholderia* selective agars enable the growth of *B. mallei* (Glass *et al.*, 2009). Even in fresh samples obtained under sterile conditions *B. mallei* is often overgrown by other bacteria, which makes isolation extremely difficult (Wernery, 2009).

Confirmation of the identity of suspected isolates is by biochemical reactions or by PCR. Growth characteristics may alter *in vitro*, so fresh isolates should be used for identification reactions. The positive biochemical reactions include reduction of nitrates, utilisation of arginine by arginine dihydrolase, assimilation of glucose, N-acetyl glucosamine and gluconate. Strain to strain variation is observed in the assimilation reactions of arabinose, fructose, mannose, mannitol, adipic acid, malate, trisodium citrate, phenyl acetic acid and VP reaction, which needs an incubation time of 48 hours. Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures. Various biochemical characteristics can be used for the differentiation of *B. mallei* from *B. pseudomallei*, as *B. mallei* has lost many fermentative activities during phylogeny (Neubauer *et al.,* 1997). Commercially available laboratory biochemical identification systems can be used for confirmation that an organism belongs to the *Pseudomonas* group. In general, however, commercially available systems are not suitable for unambiguous identification of members of the steadily growing number of species within the genus *Burkholderia* (Glass & Popovic, 2005, Hemarajata *et al*., 2016; Inglis *et al*., 2005; Kingsley *et al*., 2016a; Lau *et al*., 2015; Zong *et al*., 2012). Lack of motility is therefore of special relevance. Cell matrix-assisted laser desorption/ionisation mass spectrometric typing can be used (Cunningham & Patel, 2013; Karger *et al*. 2012). A bacteriophage specific for *B. mallei* is available. Reference laboratories or specialised laboratories use *B. pseudomallei*-specific antibodies that are not commercially available and that have not been validated for use in equids (Sprague & Neubauer, 2004)

All prepared culture media should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are functioning correctly.

In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be useful, as well as pre-treatment with penicillin (1000 units/ml for 3 hours at 37°C). A semi-selective medium (Xie *et al.*, 1980) composed of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or tryptone agar (0.1%) has been developed. Heavily contaminated samples should also be streaked onto stiff blood agar (3% agar) which inhibits the growth of *Proteus* spp., and onto Sabouraud dextrose agar which inhibits the growth of many Gram-positive and Gram-negative bacteria in glanders samples. These samples should also be streaked onto blood agar and incubated for 24 hours anaerobically to inhibit the growth of obligate aerobes. Isolation of *B. mallei* from the anaerobic plates needs a further 24 hours' incubation at 37°C. PCR methods may also prove useful for testing contaminated samples.

No validated procedures for the isolation of *B. pseudomallei* from horse samples exist. Cultivation may be successful from samples of ulcers, lesions or excretions. Standard media, i.e. blood or MacConkey agar, are used for isolation of *B. pseudomallei* and selective enrichment techniques e.g. Ashdown agar, Galimand's broth or *B. pseudomallei* selective agar (BPSA) have been developed (Limmathurotsakul *et al*., 2012; Peacock *et al*., 2005; Prakash *et al*., 2014; Roesnita *et al*., 2012; Sprague & Neubauer, 2004; Trung *et al*., 2011). Colony morphology varies from smooth to rough depending on the medium and strain, and often becomes wrinkled after a few days of incubation. A metallic sheen over the area of confluent growth on Columbia agar is an important feature for the presumptive identification of *B. pseudomallei* (Dance *et al*., 1989).

Outside the body, *B. mallei* shows little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely (Neubauer *et al.*, 1997). Under favourable conditions, however, it can probably survive a few months. *Burkholderia mallei* can remain viable in tap water for at least 1 month. For disinfection, benzalkonium chloride (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective. Phenolic disinfectants are less effective (St. Georgiev, 2008).

Burkholderia pseudomallei can survive in water up to 16 years (Pumpuang *et al.,* 2011), in muddy water for up to 7 months and in soil in the laboratory for up to 30 months. Chlorine has only a bacteriostatic effect on the agent as bacteria were recovered from water containing up to 1000 p.p.m. free chlorine (review: Sprague & Neubauer, 2004).

National regulations and guidelines for handling and application of disinfectants should be observed.

2.3. Identification of *Burkholderia mallei* by polymerase chain reaction (PCR) and real-time PCR

Several PCR and real-time PCR assays for the identification of *B. mallei* and differentiation of *B. pseudomallei* have been developed (review Lowe *et al.*, 2014).

One conventional PCR and one real-time PCR assay were evaluated using samples from a natural outbreak of glanders in horses (Scholz *et al.*, 2006; Tomaso *et al.*, 2006). These two assays are therefore described in more detail, but inter-laboratory studies are needed to confirm the robustness of these assays. The guidelines and precautions outlined in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* should be observed.

2.3.1. DNA preparation

Single colonies are transferred from an agar plate to 200 µl deionised water. After heat inactivation (for example 99°C for 30 minutes), the DNA isolation can be performed using commercial DNA preparation kits for gram-negative bacteria (see Scholz *et al.,* 2006 and Tomaso *et al.,* 2006). Alternatively, heat-inactivated bacteria from pure cultures (eg 99°C, 10 minutes) can b used directly for PCR.

Tissue samples from horses (skin, lung, mucous membrane of the nasal conchae and septae, liver and spleen) that have been inactivated and preserved in formalin (48 hours, 10% v/v) are cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), incubated overnight in sterile saline at 4°C, and minced by freezing in liquid nitrogen, followed by grinding with a mortar and pestle. Total DNA is prepared from 50 mg tissue using a commercial extraction kit according to the manufacturer's instructions. DNA is eluted with 80 µl dH_2O or as appropriate for the kit used.

For *B. pseudomallei,* kits have been evaluated for the extraction of DNA from tissue samples (Obersteller *et al*., 2016).

2.3.2. PCR assay (Scholz *et al.,* 2006)

The assay may have to be adapted to the PCR instrument used with minor modifications to the cycle conditions and the concentration of the reagents used.

The oligonucleotides used by Scholz *et al.,* (2006) are based on the differences between the *fliP* sequences from *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-IS407-flip-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma- IS407-flip-r (5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. The PCR uses 50 µl ready-to-go master mix, 15 pmol of each primer, and 4 µl of template DNA. Thermal cycling conditions are 94°C for 30 seconds and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with nucleic acid stain. No template controls containing PCR-grade water instead of template and positive controls containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons of former runs or amplification failure. The lower detection limit of this assay is 10 fg or 2 genome equivalents.

2.3.3. Real-time PCR assay (Tomaso *et al.,* 2006)

The assay should be adapted to the real-time PCR instrument used, e.g. the cycling vials should be chosen according to the manufacturer's recommendations, the concentration of the oligonucleotides may have to be increased, or the labelling of the probes altered.

The oligonucleotides used by Tomaso *et al.* (2006) are based on differences in the *fliP* sequences of *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic probe is synthesised with 6-carboxy-fluorescein (FAM) at the 5'-end and black hole quencher 1 (BHQ1) at the 3'-end. Oligonucleotides used were Bma-flip-f (5'-CCC-ATT-GGC-CCT-ATC-GAA-G-3'), Bma-flip-r (5'-GCC-CGA-CGA-GCA-CCT-GAT-T-3') and probe Bma-flip (5'-6FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ1-3'). The 25 µl reaction mixture consists of 12.5 µl 2× master mix, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl template DNA. Thermal cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 cycles at 95°C for 25 seconds and 63°C for 1 minute. Possible contaminations with amplification products from former reactions are inactivated by an initial incubation step using uracil *N'*-glycosilase. The authors suggest including an internal inhibition control based on a bacteriophage lambda gene target (Lambda-F [5'-ATG-CCA-CGT-AAG-CGA-AAC-A-3] Lambda-R [5'-GCA-TAA-ACG-AAG-CAG-TCG-AGT-3'], Lam-YAK [5'-YAK-ACC-TTA-CCG-AAA-TCG-GTA-CGG-ATA-CCG-C-DB-3']), which can be titrated to provide reproducible cycle threshold values. However, depending on the sample material a PCR targeting a housekeeping gene may be used additionally or as an alternative. No template controls containing 4 µl of PCR-grade water instead and positive controls containing DNA of *B. mallei* have to be included in each run to detect amplicon contamination or amplification failure.

The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that was consistently detectable in three runs with eight measurements each is 60 fg DNA or four genome equivalents (95% probability). The intra-assay variability of the *fliP* PCR assay for 35 pg DNA/reaction is 0.68% (based on Ct values) and for 875 fg 1.34%, respectively. The inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA 2.76%, respectively.

To date, a positive result in real-time PCR confirms the diagnosis '*Burkholderia mallei*' for an isolate and the diagnosis 'glanders' in clinical cases. It has to be kept in mind, however, that future genetic evolution may well result in *B. mallei* clones that can no longer be detected by these standard PCRs.

The sensitivity of the PCR assays for clinical samples is unknown. A negative result therefore, is no proof of the absence of *B. mallei* in the sample and other diagnostic means must be applied for confirmation.

2.4. Other methods

Molecular typing techniques for *Burkholderia* isolates such as PCR-restriction fragment length polymorphism (Tanpiboonsak *et al.*, 2004), pulsed field gel electrophoresis (Chantratita *et al.*, 2006), ribotyping (Harvey & Minter, 2005), multilocus sequence typing (MLST) (Godoy *et al.*, 2003), or variable number tandem repeat analysis (Currie *et al.*, 2009) are only appropriate for use in specialised laboratories. Molecular typing and whole genome sequencing may be useful in the future (Gilling *et al.*, 2014; McRobb *et al.*, 2015; Price *et al.*, 2015).

3. Serological tests

3.1. Complement fixation test in horses, donkeys, and mules

The CFT is an accurate serological test that has been used for many years for diagnosing glanders. It will deliver positive results within 1 week post-infection and will also recognise sera from exacerbated chronic cases. Application of rigorous quality control in the formulation of CFT antigens, complement and haemolytic systems are crucial for the performance of this test as its specificity and sensitivity are critically dependent on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). However, the specificity of CFT has been questioned (Neubauer *et al.*, 2005). The CFT is valid for horses, mules and camels; if used in donkeys particular care is needed to avoid misdiagnosis.

3.1.1. Antigen preparation

i) The stock culture strain of *B.mallei* stored at –80°C is revived by plating onto sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.

ii) From this 48 hours culture, a loopful (0.5 mm diameter) is inoculated to 5 ml of brain–heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

iii) 1 ml from the above culture broth is further inoculated to 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle agitation.

iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.

v) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 1 hour, and clarified by centrifugation at 3000 rpm for 10 minutes.

vi) The clarified product is stored as concentrated antigen in brown glass bottles to shield from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

vii) Aliquots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline containing 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for the CFT is prepared when performing the test.

The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for up to 48 hours and washing them off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.

3.1.2. CFT procedure

i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatine (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine or other commercially provided CFT buffers.

ii) Diluted serum is inactivated for 30 minutes at 58–60°C. Serum of equidae other than horses should be inactivated at 63°C for 30 minutes. Camel serum is inactivated for 30 minutes at 56°C.

iii) Twofold dilutions of the sera are prepared using veronal buffer or alternative commercially available CFT buffers in 96-well round-bottom microtitre plates.

iv) Guinea-pig complement is diluted in the chosen buffer and 4 or 5 complement haemolytic units-50% (CH_{50}) are used.

v) Sera, complement and antigen are mixed in the plates and incubated for 1 hour at 37°C. An alternative procedure is overnight incubation at 4°C.

vi) A 2-3% suspension of sensitised washed sheep red blood cells is added.

vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 ***g***.

When using commercially available CFT-antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

i) Positive control: a control serum that gives a positive reaction;

ii) Negative control serum: a control serum that gives a negative reaction;

iii) Anti-complementary control (serum control): diluent + inactivated test serum + complement + haemolytic system;

iv) Antigen control: diluent + antigen + complement + haemolytic system;

v) Haemolytic system control: diluent + haemolytic system;

vi) Complement control: diluent + complement titration + antigen + haemolytic system.

3.1.3. Reading the results

The absence of anti-complementary activity must be checked for each serum; anti-complementary sera must be excluded from analyses. A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. False-positive results can occur, and animals can remain positive for months. Moreover, *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (Neubauer *et al.*, 1997). Healthy non-glanderous equids can show a false positive CFT reaction for a variable period of time following a mallein intradermal test.

3.2. Enzyme-linked immunosorbent assays

Both plate and membrane based ELISAs have been used for the serodiagnosis of glanders, but none of these procedures has been able to differentiate between *B. mallei* and *B. pseudomallei*. An avidin–biotin dot ELISA has been described, but has not yet been widely used or validated. The antigen used is a concentrated and purified heat-inactivated bacterial culture. A spot of this antigen is placed on a nitrocellulose dipstick. Using antigen-dotted, pre-blocked dipsticks, the test can be completed in approximately 1 hour. An I-ELISA was shown to be of limited value for the serological diagnosis of glanders (Sprague *et al.*, 2009). An I-ELISA based on recombinant *Burkholderia* intracellular motility A protein (rBimA) showed a promising sensitivity of 100% and a specificity of 98.88% (Kumar *et al.*, 2011). Pal *et al*. (2012) used also recombinant proteins to develop an ELISA.

A C-ELISA that makes use of an uncharacterised anti-LPS MAb has also been developed and found to be similar to the CFT in performance (Katz *et al.*, 2000). The C-ELISA was used again on a panel of horse sera originating mainly from Middle Eastern countries (Sprague *et al.*, 2009). A commercially available C-ELISA has recently been developed using anti-s *B. mallei* LPS MAb along with antigen prepared from a regional *B. mallei* isolate. This showed higher sensitivity than CFT in identifying field cases. The C-ELISA has been evaluated on donkey sera and reliable results obtained in an infection trial. Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components will offer the possibility to develop more specific ELISAs that will help to resolve questionable test results of quarantined imported horses (Neubauer *et al.*, 1997).

For melioidosis, no serological techniques have been validated for use in veterinary medicine nor are any commercially available.

None of these tests has been fully validated to date.

3.3. Immunoblot assays

Immunoblot assays were developed for the serodiagnosis of glanders, but further validation was impossible because of the lack of a positive serum control panel (Katz *et al.*, 1999). Recently, the development of an immunoblot using *B. mallei* LPS antigen was reinitiated. The aim was to obtain a more sensitive test than the CFT in order to retest false positive CFT sera in non-endemic areas (Elschner *et al.*, 2011). The developed assay is based on crude antigen preparations of the *B. mallei* strains Bogor, Zagreb and Mukteswar, which are also the basis of most CFT antigen formulations. The antigens are separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to nitrocellulose membranes. Anti-*B. mallei* LPS antibodies in a serum sample reacting to the antigen on the blot strip are visualised by animal species-specific (phosphatase) conjugate and the NBT-BCIP (Nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The immunoblot is scored positive if the banding pattern of the *B. mallei* LPS ladder within the 20–60 kDa region is clearly visible, suspicious if a weak colour reaction is detected and negative if no reaction is seen. 171 sera of glanderous horses and mules from Pakistan and Brazil and 305 sera of negative German horses were investigated and all glanders positive and negative animals were diagnosed correctly, however the test has not been fully validated to date. This test is not able to differentiate glanders from melioidosis infection and it has not yet been evaluated for use in donkeys because of the lack of a significant number of positive control sera.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

3.4. Other serological tests

The rose bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; and has been validated in Russia. In a study in Pakistan the RBT showed a sensitivity of 90% and a specificity of 100% (Naureen *et al.*, 2007). The antigen is a heat-inactivated bacterial suspension coloured with Rose Bengal, which is used in a plate agglutination test.

The accuracy of other agglutination and precipitin tests is unsatisfactory for control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

4. Tests for cellular immunity

4.1. The mallein test

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. See section C below for details of its preparation and availability. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. It depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional diagnostic methods.

The intradermo-palpebral test is the most sensitive, reliable and specific mallein test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced other methods. 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

No data are available for use of this PPD for equids with melioidosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available for glanders or melioidosis.

Mallein PPD is available commercially[1]. The following information outlines the requirements for the production of mallein PPD.

1. Seed management

Three strains of *Burkholderia mallei* are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged in guinea-pigs.

2. Production

Dorset-Henley medium, enriched by the addition of trace elements, is used for the production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of *B. mallei*, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch's steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40 % is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight during which the light brownish to greyish precipitate settles.

The supernatant is decanted and discarded. The precipitate is centrifuged for 15 minutes at 2500 ***g*** and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and has a pH of 6.7. This mallein concentrate is centrifuged again and the supernatant diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected regularly for any signs of contamination, and suspicious flasks are discarded. A typical growth of the *B. mallei* cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

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4. Batch control

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency.

Sterility testing is performed according to the European Pharmacopoeia guidelines.

The examination for safety is conducted on five to ten normal healthy horses by applying the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be no less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil adjuvant. Cattle can also be used instead of horses. The production batch is bio-assayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured with callipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

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NB: There are WOAH Reference Laboratories for glanders (please consult the WOAH web site: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and diagnostic biologicals for glanders

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.