CHAPTER 3.6.10.

GLANDERS AND MELIOIDOSIS

SUMMARY

Description and importance of the disease*: Glanders is a highly contagious and fatal disease of horses, donkeys, and mules caused by ~~infection with~~ the bacterium* Burkholderia mallei*. ~~The~~ It is an obligate mammalian pathogen that spreads by direct host-to-host transmission and causes nodules and ulcerations in the skin, lymph nodes, upper respiratory tract and lungs. The ~~skin~~ cutaneous form is known as 'farcy'. Glanders persists in parts of Asia, Africa, South America and is re-emerging in the Middle East. Melioidosis ~~is an infectious disease~~, caused by* B. pseudomallei*. ~~Melioidosis is uncommon~~, is an infectious disease with a wide host range. Unlike* B. mallei, B. pseudomallei *is an environmental pathogen present in ~~humans and animals~~ soil and ~~sometimes resembles glanders~~ water. Human and animal cases of melioidosis have been reported mainly in South-East Asia and Australia, but the disease is now emerging in Africa and the Americas. This chapter focuses on ~~the disease in horses.~~* B. mallei *and* B. pseudomallei infections *in equidae, which share clinical similarities due to their genetic proximity.* Burkholderia mallei *has evolved from* B. pseudomallei *by genomic reduction ~~of genetic information~~ and is ~~phylogenetically~~ considered ~~as~~ a phylogenetic clone, ~~i.e. a pathovar~~ of* B. pseudomallei*.*

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

Detection of the agent: *Smears from fresh ~~material~~ specimens containing* B. mallei *~~bacteria~~ or* B. pseudomallei *may reveal Gram-negative nonsporulating, nonencapsulated rods. Electron microscopy reveals a capsule-like structure in both bacteria.* Burkholderia mallei *grows aerobically and prefers ~~media that contain~~ glycerol~~. Standard~~ containing media, ~~for isolation of~~* Burkholderia pseudomallei *can ~~be used~~ grow on standard media 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 for identification. Commercially available biochemical identification kits lack diagnostic sensitivity. MALDI-TOF [1] spectra ~~have been made~~ are 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. Whole genome sequences have been published for both bacteria.*

Serological tests: *Complement fixation is an accurate and reliable serological method for ~~diagnostic use in~~ the diagnosis of glanders in equids and is the test recommended for individual animal freedom from infection prior to movement alongside the newly validated recombinant double-antigen enzyme-linked immunosorbent assays (ELISA). Other ELISAs are promising but require further validation*

1 MALDI-TOF: Matrix assisted laser desorption ionisation time of flight [mass spectrometry]

~~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~~ from different geographical regions is also a sensitive and specific assay. None of 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~~ can distinguish between infections caused by* B. mallei *and* B. pseudomallei. Furthermore, the occurrence of *B. pseudomallei* in tropical soils may influence the results and must be considered.

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

Requirements for vaccines and diagnostic biologicals: *~~There are~~ No vaccines are avaiblable. A mallein purified protein derivative is commercially available.*

A. INTRODUCTION

Glanders is a bacterial disease ~~of~~ that primarily affects perissodactyls or odd-toed ungulates, such as horses, donkeys and mules. It is a zoonotic disease and has been ~~known~~ recognised since ancient times. ~~It is caused by the bacterium.~~ The causative agent, *Burkholderia mallei* (Yabuuchi *et al.*, 1992) ~~and~~ has previously been ~~variously~~ classified in ~~the past~~ genera such as *Pseudomonas, Pfeifferella*, *Loefflerella, Malleomyces* or *Actinobacillus*. ~~It is a serious contagious disease in~~ Although equids ~~and outbreaks may also occur in~~ are the primary hosts, glanders can infect other species. ~~Outbreaks have been reported in wild felids living in the wild or in zoological gardens and~~ zoo animals. Susceptibility to glanders has been proven demonstrated in camels, small ruminants, bears, wolves ~~and dogs.~~ Carnivores (lions, tigers, leopards, cats, dogs, polar bears, wolves, walruses, jackals and hyenas) can be infected through the ingestion of contaminated meat. Camels, small ruminants, sheep and goats have been infected with glanders, which had been in close proximity to an infected horse. ~~Carnivores may become infected by~~ eating, often through ingestion of infected meat, but cattle, and pigs are resistant. Small ruminants may be infected if kept in or 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 infected animals. Cattle, pigs, rats and birds are potential sources of infection due to the permanent or intermittent shedding of bacteria relatively resistant (Wittig *et al.*, 2006).

~~Khan *et al.* (2013) reviewed the disease, its epidemiology, diagnosis and control.~~

In ~~horses~~ equids, inflammatory pustules and ulcers develop in the nasal conchae and ~~nasal septae, which give rise to~~ septa, producing a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. In the lungs, the formation of reddish nodular abscesses with a central grey necrotic zone ~~in the lungs~~ is accompanied by progressive ~~debility, febrile episodes, coughing~~ weakness, fever, cough and dyspnoea. Diarrhoea and polyuria can also occur. In the ~~skin~~ cutaneous form ('farcy'), the lymphatic~~s~~ vessels 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~~ Respiratory and ~~skin~~ cutaneous discharges are ~~infective,~~ infectious and transmission between animals~~, which is facilitated~~ occurs 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~~ through equipment such as harnesses. The incubation period can vary from days to months (Wittig *et al.*, 2006). In horses, the disease is often chronic and can persist for years. Chronic and subclinical 'occult' cases pose a risk due to intermittent or permanent bacterial shedding (Wittig *et al.*, 2006). In donkeys and mules, glanders typically presents acutely with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia), leading to death within days. Khan *et al.* (2013) reviewed the disease, its epidemiology, diagnosis and control. ~~The incubation period can range from a few days to many months (Wittig *et al.*, 2006).~~

Glanders is ~~transmissible~~ transmitted to humans ~~by~~ through direct contact with ~~diseased~~ sick animals or with infected or contaminated material. ~~In the untreated~~ Without treatment, acute ~~disease,~~ cases have a ~~the~~ mortality rate ~~can reach 95% within 3 weeks~~ of up to 90% (~~Neubauer *et al.*, 1997~~ Van Zandt *et al.*, 2013). However, ~~survival is possible if the infected person is treated~~ early and aggressively ~~by~~ treatment with multiple ~~systemic antibiotic therapies~~ antibiotics can improve survival. A chronic form with ~~abscessation can~~ abscess formation may occur (Neubauer *et al.*, 1997).

Glanders has been eradicated from many countries ~~by statutory~~ through compulsory testing, culling of infected animals, and import restrictions. However, it persists in ~~numerous Asian, African~~ parts of Asia, Africa and South ~~American~~ America. It is re-emerging in countries in the Middle East, India, Pakistan, Nepal, and ~~can be considered~~

~~a re-emerging disease~~ Brazil. Glanders can be introduced into ~~glanders~~ disease-free areas by movement of infected equids (Neubauer *et al.*, 2005).

Melioidosis is caused by *B. pseudomallei* ~~is the causative agent of melioidosis~~, a soil-dwelling bacterium historically endemic to South-East Asia (Cambodia, Indonesia, Laos, Malaysia, Myanmar, Singapore, Thailand, and ~~originates from~~ Vietnam) and northern Australia. Its range is expanding and now includes the Pacific, South ~~East~~ Asia ~~(areas within latitudes 20°N~~, Africa, and ~~20°S)~~ the Americas (Limmathurotsakul *et al.,* 2016). ~~It is an important soil bacterium, denitrifying organic materials and is ubiquitous in those areas. Thus, *B.* *Burkholderia pseudomallei* is stable in the environment. It is~~ and it also naturally resistant to various antibiotics and disinfectants (Currie, 2015; O'Connell *et al.,* 2009; Sprague & Neubauer, 2004). *Burkholderia pseudomallei* ~~has~~ is an ~~extremely broad~~ opportunistic pathogen with a wide host range, including ~~wildlife, farm animals and~~ humans ~~(Rush & Thomas, 2012). *Burkholderia pseudomallei* has regularly been isolated~~, mammals, birds and reptiles (Birnie *et al.*, 2022).

In humans, melioidosis can cause a wide range of signs, 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~~ localised infections to severe systemic disease. Despite timely and aggressive multi-antibiotic therapy, the disease ~~in horses, which may closely resemble glanders, but more diverse presentations also occur~~ is often fatal (Meumann *et al.*, 2024).

Melioidosis in horses ~~has~~ is rare ~~and often presents~~ but can present with signs similar to glanders. Occurrences have 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~~ mild colic and intestinal hypermotility. More typically, melioidosis ~~runs~~ has a subacute to chronic course ~~from~~ of 3 weeks to 3 months ~~with no~~ without loss of appetite. ~~Further signs~~ Other reported ~~are~~ signs include 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~~ occur. If the infection is acquired *per os*, intestinal signs may predominate. ~~It is reported that~~ The lungs are always ~~affected and show~~ reported to be involved with signs of acute bronchopneumonia and ~~numerous~~ multiple 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, although rare, have been reported~~, though rare~~. The incubation period for naturally occurring infection in animals is ~~not known. Spread~~ unknown (Sprague & Neubauer, 2004). Experimental infection of horses with *B. pseudomallei* (via oral or subcutaneous routes) failed to generate lesions in the nasal septum or conchae; however, typical pyogranulomas indistinguishable from those caused by glanders were observed in the lungs and other organs, and the pathogen was detected in the bladder (Wernery *et al.,* 2019a; 2019b). The disease may be spread by infected animals or by contaminated materials in ~~the animals'~~ their 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~~ are recommended for horses.

~~Melioidosis can be considered an emerging disease and is present~~ in many ~~regions worldwide (Limmathurotsakul *et al.*, 2016). Control~~ In regions where *B. pseudomallei* is not endemic (e.g. non-tropical countries), control of melioidosis requires testing of suspected clinical cases, screening of apparently normal equids, and elimination of reactors. ~~In areas with a temperate climate, special thought has to be given to~~ Emphasis should be placed on maintaining stable hygiene practices (disinfection, removal of faeces several times a day, ~~reduction of the~~ reduced 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 outbreak at the ~~enzootics~~ Jardin des Plantes in France ~~declined and~~ in the ~~infected soils were cleared only after all~~ 1970s highlighted the risks of importing 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,~~ which led to the contamination of ~~environmental reservoirs that would pose an ongoing risk of infection for~~ zoo animals, humans and ~~animals~~ nearby cavalry horses, facilitating the spread of the disease across the country. It took a decade to bring the epidemic under control. In addition, the infection can be imported from species other than equidae. When handling ~~suspect or known infected~~ animals ~~or fomites, stringent precautions must be taken~~ suspected or confirmed to ~~prevent self-infection or transmission of the bacteria. Laboratory samples must~~ be infected with *B. mallei* or *B. pseudomallei*, or when handling potentially contaminated materials, strict biosafety protocols are essential. This includes securely ~~packaged, kept~~ packaging specimens to prevent spillage, keeping specimens cool (not frozen) ~~and shipped as outlined~~ during transport, and following the transport guidelines for biological materials in Chapter 1.1.3 *Transport of biological materials*. All manipulations ~~with~~ of potentially infected/contaminated material must be performed at an appropriate biosafety and containment level as determined by a biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Outside its host, *B. mallei* has limited resistance to environmental factors such as desiccation, heat, light ~~or~~ and chemicals, making its survival beyond 2 weeks ~~is~~ unlikely (Neubauer *et al.*, 1997). However, it can persist for several months under favourable conditions, and can remain viable in tap water for at least 1 month. Effective disinfectants include benzalkonium chloride (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, 1% sodium hydroxide, 2% formalin, 1% potassium permanganate. Phenol and quarternaly

amonium compounds are ineffective and potassium permanganate. Phenolic disinfectants are less effective (St. ~~Georgiev, 2008~~). In contrast, *B. pseudomallei* is more resilient, surviving up to 16 years in water (Pumpuang *et al.,* 2011), 7 months in muddy water and up to 30 months in laboratory soil. Chlorine has only a bacteriostatic effect on *B. pseudomallei,* with bacteria recoverable even in water containing up to 1000 ~~p.p.m.~~ ppm free chlorine (review: Sprague & Neubauer, 2004).

B. DIAGNOSTIC TECHNIQUES

Test methods available for the diagnosis of glanders and their purpose are listed in Table 1. For melioidosis, *B. pseudomallei* can be specifically confirmed by culture and polymerase chain reaction (PCR). Although serological tests developed for glanders may cross-react and give an indication of potential melioidosis cases, these tests are not fully validated for melioidosis. This issue is particularly relevant in areas where *B. pseudomallei* is present in the soil, as environmental exposure may lead to positive test results.

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

Method	Purpose					
	Population freedom from infection[a]	**Individual animal freedom from infection prior to movement[b]**	**Contribute to eradication policies[c]**	**Confirmation of clinical cases[d]**	**Prevalence of infection – surveillance[e]**	**Immune status in individual animals or populations post-vaccination**
Detection of the agent						
Conventional PCR	☐☐☐	☐☐☐	☐☐☐	~~☑☐☐~~ ☑☑☐	☐☐☐	☐☐☐
Real-time PCR	☐☐☐	☐☐☐	☐☐☐	☑☑☑	☐☐☐	☐☐☐
Culture	☐☐☐	☐☐☐	☐☐☐	~~☑☐☐~~ ☑☑☐	☐☐☐	☐☐☐
Detection of immune response						
Complement fixation[f]	☑☑☐	☑☑☐[a]	☑☑☑	☑☑☐	☑☑☑	☐☐☐
ELISA[g,h]	~~☑☐☐~~ ☑☑☐	~~☑☐☐~~ ☑☑☐	~~☑☑☐~~ ☑☑☑	~~☑☐☐~~ ☑☑☐	~~☑☑☐~~ ☑☑☑	☐☐☐
Recombinant double-antigen ELISA	~~☑☑☑~~ ☑☑☐	~~☑☑☑~~ ☑☑☐	~~☑☑☑~~ ☑☑☐	~~☑☑☑~~ ☑☑☐	~~☑☑☑~~ ☑☑☐ [h]	☐☐☐
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~~ See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.

[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.

[c] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.

[d] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.

[e] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.

[f] Horse samples only – care needed with interpretation of test on donkey samples.

[g] Only validated ELISAs should be used, especially for mules and donkeys, which are not always included in validation studies.

[h] Special consideration given in tropical regions where *B. peudomallei* is present in soils.

~~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 and melioidosis

Confirmation of a diagnosis of glanders ~~should be based on the~~ or melioidosis requires isolation and identification of ~~*Burkholderia* *B. mallei* in a sample~~ or *B. pseudomallei*, respectively, from an equid sample or ~~a~~ derived product ~~derived from that equid; or the identification in such samples~~, or detection of ~~antigen or~~ their specific genetic material ~~specific to *B. mallei*. Supporting evidence may be provided by~~ in these samples. In animals showing clinical signs, a positive ~~serological test results such as a titre of 1/5 in the~~ complement fixation test (CFT) result, confirmed by a second test ~~with~~ of equal or ~~higher~~ greater 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~~ may indicate glanders or melioidosis and should prompt further investigation for direct detection of the agent. The serological tests to be used depend on the prevalence of the disease (Sprague *et al*., 2009).

2. Detection of the agent

Cases ~~for~~ requiring specific ~~glanders~~ investigation for glanders or melioidosis should be clinically differentiated ~~on clinical grounds~~ from other chronic infections ~~affecting~~ of the nasal mucous membranes, sinuses or the skin. ~~Among these are~~ These include 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 capsulatum 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* and *B. pseudomallei*

Burkholderia mallei ~~organisms are fairly numerous~~ is often found in smears from fresh lesions, but ~~scarce~~ less commonly in older lesions. Smears ~~should~~ can be stained with methylene blue or Gram's 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's 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~~ coat has been ~~verified~~ observed by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from ~~unfavourable~~ adverse 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~~ for diagnosis 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~~ their appearance ~~depending on~~ varies according to the age of the culture and the type of medium. ~~In~~ Older cultures~~, there is much pleomorphism~~ are highly pleomorphic. Branching filaments form on the surface of broth cultures (Neubauer *et al.*, 2005).

In contrast, *B. pseudomallei* is motile due to the presence of flagella and can be found in both acute and chronic lesions. These Gram-negative rods are 2–4 µm long and 0.4–0.8 µm wide, with rounded ends. Smears from lesions or cultures can be stained with Gram's stain or methylene blue. *Burkholderia pseudomallei* is primarily extracellular, but may be present within macrophages during infection. It has a polysaccharide capsule that aids in immune evasion and survival in the environment, although this capsule is not readily visible under the light microscope. In tissue sections, *B. pseudomallei* may appear granular or clumped. On solid media, the colony morphology of *B. pseudomallei* varies, with some strains forming smooth colonies and others developing a wrinkled appearance, depending on the strain and culture conditions.

2.2. Cultural characteristics

~~It is preferable to attempt isolation from unopened~~ Isolation of *B. mallei* is most effective when samples are taken from fresh, uncontaminated lesions~~.~~ to avoid bacterial overgrowth. Key organs include the lungs, liver, spleen and lymph nodes in the vicinity of the lesions. The nasal cavity, trachea and skin lesion (if present) should also be sampled.

Burkholderia mallei is an aerobic ~~and facultative anaerobic only~~ bacterium that can grow anaerobically in the presence of nitrat~~e, growing optimally at 37°C~~. It grows ~~well, but~~ best, albeit slowly, at 37°C. It can be grown on a variety of ~~on~~ culture media, including sheep blood agar. ~~Incubation of cultures for 72 hours~~

~~is~~ The recommend~~ed; glycerol~~ incubation period is 72 hours. Glycerol enrichment is particularly useful. The tiny greyish ~~shiny~~ shining colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; ~~hence~~ so careful observation is needed to not ~~to overlook~~ miss 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~~ is observed. On ~~continued~~ further 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~~ forming a slimy pellicle ~~forms~~. Growth on nutrient agar~~, the growth~~ is much less ~~effusive~~ profuse, and growth ~~is poor~~ on gelatine~~. Various~~ is poor. Several commercially available *Burkholderia* selective agars ~~enable~~ allow 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~~ making 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).~~

A novel selective medium has been described for the isolation of *B. mallei* from equine specimens (Kinoshita *et al.*, 2019). This agar was prepared by combining 5 g of proteose peptone no. 3, 10 g of tryptone pancreatic digest of casein, 0.25 g of magnesium sulfate, 1 g of sodium pyruvate, 5 g of sodium chloride, 40 ml of glycerol, 3 mg of crystal violet (3 ml from 0.1% w/v crystal violet in distilled water), 50 mg of cycloheximide (5 ml from 1% [w/v] cycloheximide in absolute ethanol), 15 g of agar, and distilled water (960 ml). The mixture is sterilised at 121°C for 15 minutes. After cooling to 45°C, the medium is supplemented with three antimicrobials: 16.7 mg of ticarcillin disodium salt (668 µl from 25 mg/ml stock solution), 197.8 mg of fosfomycin sodium (1520 µl from 130 mg/ml stock solution), 40,000 units of polymyxin B (400 µl from 100,000 units/ml stock solution). For heavily ~~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~~ and pre-treatment with penicillin (1000 units/ml for 3 hours at 37°C) have proven to be useful. A semi-selective medium has been developed (Xie *et al.*, 1980) ~~composed~~ consisting 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 on~~to~~ 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~~ plated on blood agar and incubated ~~for 24 hours~~ anaerobically for 24 hours 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.~~

Although there are no validated ~~procedures~~ methods for ~~the isolation of~~ isolating *B. pseudomallei* from ~~horse~~ equine samples ~~exist. Cultivation may be successful from samples of~~, cultivation from ulcers, lesions or ~~excretions. Standard media, i.e.~~ urine can be successful (Wernery *et al.*, 2019a). Initial isolation can be performed on blood agar or MacConkey agar~~, are used for isolation of *B. pseudomallei* and selective enrichment techniques e.g~~. Selective media such as Ashdown agar, Galimand's broth ~~or *B. pseudomallei* selective agar (~~and BPSA~~) have been developed (Limmathurotsakul *et al.*, 2012;~~ improve detection (see website Melioidosis - Home) (Peacock *et al.*, 2005~~; Prakash *et al.*, 2014; Roesnita *et al.*~~,

2012; Sprague & Neubauer, 2004; Trung *et al*., 2011). Colony morphology varies from). *Burkholderia pseudomallei* colonies may appear smooth to rough depending on the medium and strain, and often becomes or wrinkled after, often with a few days of incubation. A metallic sheen over the area of confluent growth on Ashdown or Columbia agar is an important feature.

All culture media must be quality controlled to ensure growth of the suspect organism from samples with low bacterial loads.

Biochemical tests such as the API Gallery are useful for the presumptive identification of *B. pseudomallei* (Dance *et al*., 1989) *B. mallei* and *B. pseudomallei*. The API 20NE system is used to identify non-enteric, oxidase-positive bacteria. *Burkholderia mallei* and *B. pseudomallei* share several similar and different biochemical characteristics. Typical positive results for *B. mallei* include nitrate reduction, oxidase activity and arginine dihydrolase activity. It also shows positive assimilation of glucose, N-acetylglucosamine and gluconate. However, *B. mallei* does not produce urease, which may help to distinguish it from *B. pseudomallei* (Neubauer *et al*., 1997). In contrast, *B. pseudomallei* also shows positive nitrate reduction, oxidase activity and glucose assimilation. It typically has positive or variable arginine dihydrolase activity. Unlike *B. mallei*, *B. pseudomallei* is urease positive, a key differentiator between the two bacteria. Although commercial biochemical systems can confirm membership of the *Pseudomonas* group, they are generally not reliable for accurate identification of the diverse *Burkholderia* genus (Glass & Popovic, 2005; Inglis *et al*., 2005; Lau *et al*., 2015; Zong *et al*., 2012). These results should be interpreted alongside other identification methods, such as MALDI-TOF MS[2] (Cunningham & Patel, 2013; Karger *et al*. 2012), which is a more advanced technique, although database inconsistencies can occasionally lead to misidentifications, or PCR, which remains the most reliable method for accurately distinguishing between the two species.

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.

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

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

PCR methods available for *B. mallei* and *B. pseudomallei* are listed in Tables 2 and 3.

Table 2. Conventional PCR methods available for *Burkholderia mallei* and *B. pseudomallei*

Pathogen/ target gene	Primer (5'–3')	Concentration of working solution	Cycling parameters[(a)]
Method 1: Scholz *et al.* (2006); GenBank Accession No.: NC_006350, NC_006351; amplicon size: 989bp			
Burkholderia mallei/fliP	Bma-IS407-fliP-f: 5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3' Bma-fliP-r: 5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3'	10 µM 10 µM	94°C/10 min, 35 × (94°C/30 sec, 65°C/30 sec, 72°C/60 sec); 72°C/7 min

[(a)]Conditions of used polymerases and mastermixes must be considered.

2 MALDI-TOF MS: Matrix assisted laser desorption ionisation time of flight mass spectrometry

Table 3. Real-time PCR methods available for *Burkholderia mallei* and *B. pseudomallei*

Pathogen/ target gene	Primer/probe (5'–3')	Concentration of working solution	Cycling parameters[a]
Method 1: Tomaso *et al.* (2006); GenBank Accession No.: NC_006350, NC_006351			
Burkholderia mallei/fliP	Bma-flip-f: CCC-ATT-GGC-CCT-ATC-GAA-G Bma-flip-r: GCC-CGA-CGA-GCA-CCT-GAT-T probe Bma-flip: FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ	10 µM 10 µM 10 µM	(50°C/2 min)[b]; 95°C/10 min; 45 × (95°C/25 sec, 63°C/60 sec)
Method 1: Thibault *et al.* (2004); GenBank Accession No.: AF074878			
Burkholderia pseudomallei/orf11	orf11F: ATC-GCC-AAA-TGC-CGG-GTT-TC orf11R: CAA-ATG-GCC-ATC-GTG-ATG-TTC orf11pro: FAM-TCG-GCG-AAC-GCG-ATT-TGA-TCG-TTC-C-BHQ	50 µM 50 µM 20 µM	(50°C/2 min)[b]; 95°C/10 min; 45 × (95°C/10 sec, 60°C/45 sec)

[a]Conditions of used polymerases and mastermixes must be considered. [b]Uracyl N-glycosylase pre-treatment in association with inclusion of uracil instead of thymine in the master mix helps to prevent contamination from previous amplifications.

Burkholderia pseudomallei and *B. mallei* are closely related bacteria with different environmental and disease profiles. *Burkholderia pseudomallei* has a highly flexible genome, influenced by frequent gene transfers and rearrangements. In contrast, *B. mallei* has evolved into a strict mammalian pathogen through genome reduction, resulting in a more uniform population, although it is still evolving.

Several PCR ~~and real-time PCR~~ assays have been developed for the specific detection and identification of *B. mallei* and ~~differentiation of~~ *B. pseudomallei* ~~have been developed~~ (reviewed by Lowe *et al.*, 2014). ~~One~~ A conventional PCR and ~~one~~ a real-time PCR assay ~~were~~ have been 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~~ presented in more detail~~, but~~ in this chapter, although real-time PCR is recommended because of its higher analytical sensitivity. Inter-laboratory studies are still needed to confirm ~~the~~ their robustness ~~of these assays~~. The guidelines and precautions outlined in Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases of terrestrial animals* should be ~~observed~~ followed.

2.3.1. DNA preparation

~~Single~~ From agar cultures, single colonies are transferred ~~from an agar plate~~ to 200 µl of deionised water. After heat inactivation (for example ~~99~~ 100°C for ~~30~~ 20 minutes), the ~~DNA isolation~~ supernatant obtained after centrifugation at 8000 ***g*** for 10 minutes 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 be~~ used directly for PCR or subjected to DNA extraction using commercial kits designed for Gram-negative bacteria.

Tissue samples from horses (e.g. skin, lung, mucous membrane of the nasal conchae and septae, liver and spleen) ~~that have been inactivated and preserved in formalin~~ can be processed for DNA extraction using commercially available kits. Formalin-fixed tissues (48 hours, 10% v/v) ~~are~~ should be 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), and incubated overnight in sterile saline at 4°C~~, and minced by freezing~~. Samples are then frozen in liquid nitrogen, ~~followed by grinding~~ crushed with a mortar and pestle~~.~~ and total DNA is ~~prepared from 50 mg tissue using a commercial extraction~~ extracted using kits 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 DNA extraction ~~of DNA~~ from ~~tissue samples~~ formalin-fixed, paraffin–embedded tissues for the detection of *B. pseudomallei* (Obersteller *et al.*, 2016).

2.3.2. PCR ~~assay (Scholz *et al.*, 2006)~~ assays for *B. mallei* detection

~~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.~~

Scholz *et al.* (2006) developed a conventional PCR method targeting differences in the *fliP* gene to specifically differentiate *B. mallei* from *B. pseudomallei* (Table 2). The PCR reaction contains *Taq* polymerase, primers and DNA template. Amplicons are stained with a nucleic acid dye before visualisation under UV light on agarose gels. No-template and *B. mallei* positive controls ensure reliability.

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

~~The assay should be~~ This method was then adapted ~~to the~~ for 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~~ by Tomaso *et al.* (2006, Table 2) and incorporates a 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')~~ that binds to the amplified fragment, increasing the sensitivity and specificity of the test compared with conventional PCR. The 25 µl reaction ~~mixture~~ volume 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~~ A pre-incubation step ~~using~~ with uracil ~~*N*'~~-glycosilase. ~~The authors suggest including an~~ N-glycosylase (50°C for 2 minutes) and the use of uracil instead of thymine in the master mix helps to prevent contamination from previous amplifications. An internal PCR 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~~ (endogenous or exogenous), along with no-template and *B. mallei* positive controls ~~containing DNA of *B. mallei* have to be included in each run to detect amplicon contamination or amplification failure~~, must be included to ensure reliability.

~~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 PCR result ~~in real-time PCR~~ confirms the diagnosis ~~'~~of *Burkholderia mallei*~~'~~ for an isolate and the diagnosis of 'glanders' in clinical ~~cases. It has to be kept in mind, however,~~ samples. The target of these two PCR methods is based on a mobile element in the *B. mallei fli*P gene, which distinguishes it from *B. pseudomallei*. It is important to note that future genetic evolution ~~may well result in *B. mallei* clones that can no longer be detected by these standard PCRs.~~ could lead to the loss of this element in *B. mallei* strains, potentially causing false-negative results with *fli*P-based PCR tests (Laroucau *et al.*, 2021). The use of multiple PCR systems is therefore recommended, in complement to serological methods, especially in regions where *B. mallei* strains are poorly characterised (Laroucau *et al.*, 2021).

The sensitivity of the PCR assays for clinical samples is unknown, so a negative result ~~therefore, is no proof of~~ does not rule out the ~~absence~~ presence of *B. mallei* ~~in the sample~~ and other diagnostic means must be ~~applied~~ used for confirmation.

2.3.3. PCR assay for *B. pseudomallei* detection

No PCR methods have been validated for the detection of *B. pseudomallei* in equine samples, but several PCR systems are available for the specific identification of this species. Among the available targets, the *orf11* PCR (Thibault *et al.,* 2004) (Table 2) is commonly used, for identification or as differential diagnosis with *B. mallei*.

~~2.4. Other methods~~

2.4. Molecular typing methods and whole genome sequencing

~~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),~~ Multi-locus sequence typing (MLST) (Godoy *et al.*, 2003) reveals considerable diversity among *B. pseudomallei* strains, with variation associated with geographical origin. In contrast, *B. mallei* strains are nearly identical worldwide and are characterised by a single sequence type (ST40). Multi-locus ~~or~~ variable number of tandem repeat analysis (MLVA) has been developed to differentiate *B. mallei* strains (Currie *et al.*, 2009; Singha *et al.*, 2021), ~~are only appropriate for use in~~ but it requires specialised ~~laboratories. Molecular typing~~ laboratory equipment and is mainly used to compare strains from the same outbreak.

More advanced methods such as whole genome sequencing (WGS) and molecular typing techniques such as core genome MLST (cgMLST) (Appelt *et al.*, 2022; Brangsch *et al.*, 2022) and single nucleotide polymorphism (SNP) typing ~~may be useful in the future (Gilling *et al.*, 2014; McRobb *et al.*, 2015; Price *et al.*, 2015~~ Girault *et al.*, 2018) offer new opportunities to study both *B. mallei* and *B. pseudomallei*. These methods provide the highest resolution and discriminatory power for differentiating isolates and conducting epidemiological studies.

3. Serological tests

Burkholderia mallei evolved from *B. pseudomallei*, which means that infections caused by the two species are currently indistinguishable by serological tests (Neubauer *et al.*, 1997; Wernery *et al.*, 2019b). As a result, serological methods cannot reliably differentiate between the two species, and definitive diagnosis can only be made by direct pathogen identification. WOAH Reference Laboratories can provide positive reference sera for serological testing.

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

The CFT is ~~an accurate~~ a reliable serological test ~~that has been used~~ for ~~many years for diagnosing~~ the diagnosis of glanders and establishing freedom from infection prior to movement. It ~~will deliver positive results~~ detects infections within 1 week ~~post~~ of infection and ~~will also recognise~~ can identify sera from exacerbated chronic cases. ~~Application of rigorous~~ Strict quality control ~~in the formulation~~ of CFT ~~antigens~~ reagents (antigen, complement and haemolytic ~~systems are crucial~~ system) is essential for the performance of this test ~~as its~~, with both specificity and sensitivity ~~are critically dependent~~ depending on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). However, the specificity of the CFT has been questioned (Neubauer *et al.*, 2005). The CFT ~~is valid~~ has been validated for horses, mules and camels~~; if used~~, but care must be taken when using the test 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~~ should be 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~~ A loop (0.5 mm diameter) of the 48-hour culture is then inoculated into 5 ml of brain–heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

iii) After 24 hours, 1 ml ~~from~~ of the above culture broth is further inoculated into 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle ~~agitation~~ shaking.

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 ~~.~~ and the filtrate is re-heated ~~again~~ by exposure to live steam for 1 hour ~~, and.~~ It is then clarified by centrifugation at 5000 ***g*** for 10 minutes.

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

vii) Aliquots of ~~antigen are prepared by diluting~~ the concentrated antigen are prepared by dilution 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.~~ and prepared at the time of testing. The resulting antigen consists mainly of lipopolysaccharides (LPS).

~~The resulting antigen consists primarily of lipopolysaccharides (LPS).~~ An alternative ~~procedure~~ method is to use young cultures ~~by growing the organism~~, in which *B. mallei* is grown on glycerol–agar slopes for up to 48 hours and ~~washing them off~~ washed with normal saline. A suspension of the culture is heated at 70°C 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~~ inoculation onto blood agar plates.

Commercial antigens are available (Khan *et al*., 2011).

3.1.2. CFT procedure

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

ii) The diluted serum is inactivated at 58–60°C for 30 minutes ~~at 58–60°C~~. Serum of equidae other than horses should be inactivated at 63°C for 30 minutes ~~.~~, while camel serum ~~is~~ should be inactivated at 56°C for 30 minutes ~~at 56°C~~.

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

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

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

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

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

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

~~*Recommended*~~ *Controls to verify test conditions:*

i) Positive control: a control serum ~~that~~ known to give~~s~~ a positive reaction;

ii) Negative control serum: a control serum ~~that~~ known to give~~s~~ a negative reaction;

iii) Anti-complementary control (serum control~~): diluent~~, for each serum to be tested): CFT buffer + inactivated test serum + complement + haemolytic system;

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

v) Haemolytic system control: ~~diluent~~ CFT buffer + haemolytic system;

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

3.1.3. Reading the results

~~The absence of~~ When all test controls (antigen, haemolytic system, complement, negative and positive controls) are valid and no anti-complement~~ary~~ activity ~~must be checked for each~~ is

detected in the test serum~~; anti-complementary sera must be excluded from analyses. A sample that produces~~, the degree of haemolysis of the test serum can be evaluated. A serum with 100% haemolysis at ~~the~~1/5 dilution is negative, 25–75% haemolysis is ~~suspicious~~ suspect, and no haemolysis (100% fixation) is positive. False-positives ~~results can~~ may occur~~,~~ and animals ~~can~~ may remain positive for months. ~~Moreover,~~ Animals infected with *B. mallei* and *B. pseudomallei* ~~and *B. mallei*~~ produce cross-~~react and cannot be differentiated~~reactive antibodies, making them indistinguishable by ~~serology~~ CFT (Neubauer *et al.,* 1997~~). Healthy non-glanderous equids~~; Wernery *et al*., 2019b). An intradermal mallein test can ~~show a false~~ produce CFT-positive ~~CFT reaction~~ results 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).~~

Several in-house and commercial enzyme-linked immunosorbent assays (ELISAs) based on crude or recombinant antigens have been developed for glanders, but systematic validation studies according to Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, are lacking. A recent comparative study of five ELISAs using crude suspension or recombinant antigens showed promising results (Elschner *et al*., 2019). A commercial recombinant double antigen-based ELISA was validated with 400 negative sera from Germany and 370 positive field samples from equidae collected in India and Pakistan (Elschner *et al*., 2021). This ELISA showed a significantly higher specificity (99.8%) than the CFT (97%) and similar sensitivities (CFT 96.5%, ELISA 98.1%).

The presence of *B. pseudomallei* in the environment may expose equids living in tropical regions where the bacterium is endemic, potentially leading to infection. However, reported cases of melioidosis remain rare. Exposure to the bacterium can induce a detectable serological response, particularly when sensitive assays such as the commercial recombinant double antigen-based ELISA are used. This assay detects *B. mallei* antibodies (Elschner *et al.,* 2021) and also cross-reacts with *B. pseudomallei* antibodies (Desoutter *et al.,* 2024; Gasqué *et al.,* 2024; Laroucau *et al.,* 2025). This validated ELISA is therefore recommended for: i) to confirm clinical cases (in combination with direct diagnostic methods to distinguish between glanders and melioidosis), alongside associated epidemiological investigations; and ii) in the context of international trade, to prevent exposed or infected animals from entering populations and regions that are free from glanders and melioidosis. In areas endemic for melioidosis, where exposure to *B. pseudomallei* may be frequent, apparently healthy equids with a positive ELISA result should undergo follow-up testing and movement restrictions may be warranted. Further studies are needed to clarify the infectious status and epidemiological role of such animals.

More recently, an in-house competitive ELISA for *B. mallei* infection has been developed based on a monoclonal antibody to *B. mallei* lipopolysaccharide (Wernery *et al*., 2021).

For melioidosis, ~~no~~ although serological ~~techniques~~ tests developed for glanders may cross-react if not specifically designed for glanders, none have been validated ~~for use in veterinary medicine nor are any commercially available~~ to date.

3.3. Immunoblot assays

Immunoblot assays ~~were~~ have been developed for ~~the serodiagnosis of~~ glanders~~, but further validation was impossible because of the lack of a positive serum control panel~~ (Elschner *et al.,* 2011; Katz *et al*.,

1999). ~~Recently, the development of an~~ This test was recently included in a comparative assays study with the CFT and a commercial recombinant double antigen-based ELISA using 400 glanders negative from Germany and 370 glanders positive field samples from horses, mules, and donkeys (Elschner *et al.*, 2021). The immunoblot ~~using *B. mallei* LPS antigen~~was ~~reinitiated. The aim was to obtain a~~ ~~was~~ is significantly more ~~sensitive test~~ specific (99.2%) than the CFT ~~in order to retest false positive CFT sera in non-endemic areas (Elschner *et al.*, 2011).~~ (97.0%). Considering the comparable sensitivities of CFT (96.5%) and the immunoblot (97.3%), the immunoblot is a suitable confirmatory test. However, the test is best suited to single samples, as it is time-consuming and requires experienced laboratories. The ~~developed assay~~ test is based on crude antigen preparations ~~of the~~ from *B. mallei* strains (Bogor, ~~Zagreb and~~ Mukteswar, ~~which are also the basis of most CFT antigen formulations. The antigens are~~ and Bahrain1) 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~~ using a species-specific (phosphatase) conjugate and the NBT-BCIP (nitro-blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The ~~immunoblot~~ test is ~~scored~~ considered positive if ~~the banding~~ a clear scale band pattern ~~of the *B. mallei* LPS ladder within~~ appears in the 20–60 kDa ~~region is clearly visible, suspicious~~ range, suspect if a weak colour reaction is ~~detected~~ observed 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~~ occurs. This test cannot 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~~.

United States	**Comment category:** Addition **Proposed amended text:** A chemiluminescent immunoblot assay was also developed and evaluated with near perfect agreement to the colorimetric assay method (Grause et al., 2024). **Rationale:** Recommend adding the above text and corresponding reference which provide information on the recent development and evaluation of an equivalent alternative immunoblot assay method for detecting glanders. This assay method may benefit some diagnostic laboratories, particularly those performing high-volume testing. The United States Department of Agriculture National Veterinary Services Laboratories (NVSL) tests approximately 12,000–15,000 samples annually for glanders as part of import testing, with some samples requiring confirmatory glanders testing. To improve turnaround time and result interpretation, there was a need for a western blot assay with faster time to results and greater ease of use. A chemiluminescent western blot assay was subsequently developed and evaluated using 224 of the 370 glanders-positive samples previously analyzed by Elschner et al. (2021), along with 100 negative samples. Near-perfect agreement was observed between the colorimetric and chemiluminescent immunoblot assay methods (Grause et al., 2024). **Supporting evidence**: Grause et al., 2024. Development and validation of a chemiluminescent western blot assay for glanders (Burkholderia mallei) serodetection - PubMed

~~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 and validated in Russia for the diagnosis of glanders in horses and other susceptible animals~~; and has been validated in Russia~~. The test is based on a heat-inactivated bacterial suspension stained with rose bengal, which is used in a plate agglutination test. 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.~~

However, the accuracy of ~~other agglutination and precipitin tests~~ this test is ~~unsatisfactory~~ not ideal for control programmes~~.~~ , as horses with chronic glanders ~~and those~~ or in ~~a debilitated condition~~ poor health may 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 (mallein PPD)~~, which~~ is ~~available~~ a commercially~~, is a~~ available solution ~~of water-soluble protein fractions of~~ prepared from a heat-treated *B. mallei* suspension (see section C below for details of its preparation and availability). Although the test is not ~~generally~~ widely recommended because of animal welfare concerns, ~~however~~ it ~~can~~ may be useful in remote ~~endemic~~ areas where sample transport or proper cooling of samples is not possible. ~~It depends on~~ The test detects hypersensitivity to mallein in infected horses ~~being hypersensitive to mallein~~. However, advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional diagnostic methods. In addition, transient seroconversion after mallein injection has been demonstrated in healthy uninfected horses, resulting in false positive CFT results.

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~~ replaced other methods. The test involves the intradermal injection of 0.1 ml of ~~concentrated~~ mallein PPD ~~is injected intradermally~~ into the lower eyelid ~~and the test~~. The reaction is read at 24 and 48 hours. A positive reaction is characterised by a marked oedematous swelling of the eyelid, ~~and there may be~~ possibly accompanied by a purulent discharge from the inner canthus or conjunctiva~~. This is usually accompanied by~~ and a rise in body temperature. ~~With~~ A negative ~~response, there is usually no~~ reaction typically shows minimal or ~~only a little~~ no swelling ~~of the lower lid~~.

No data are available ~~for~~ on the use of ~~this~~ mallein PPD ~~for~~ in equids with melioidosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines ~~are available for~~ against glanders or melioidosis.

Mallein PPD is available ~~commercially~~[3] from institutes such as the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey or the Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 (aprovizionare@pasteur.ro). The following information outlines the requirements for the production of mallein PPD.

1. Seed management

Mallein PPD is produced using three strains of *B. 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~~ To maintain virulence and antigenicity, the strains ~~may~~ can 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.

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.

3 ~~Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey; Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 *aprovizionare@pasteur.ro*~~

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United States	**Comment category:** Addition **Proposed reference:** Grause JF, Elschner MC, Ledesma NA, Murphy G. Development and validation of a chemiluminescent western blot assay for glanders (*Burkholderia mallei*) serodetection. J Vet Diagn Invest. 2024 Mar;36(2):283-286. PMC10929638. **Rationale:** Recommend adding the above reference which provides information on the recent development and evaluation of an equivalent alternative immunoblot assay method for detecting glanders. This assay method may benefit some diagnostic laboratories, particularly those performing high-volume testing. See corresponding chapter comment for further detail. **Supporting evidence**: n/a

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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.