CHAPTER 2.5.11.

GLANDERS

SUMMARY

Glanders is a contagious and fatal disease of horses, donkeys, and mules, and is caused by infection with the bacterium Burkholderia mallei (*the name recently changed from* Pseudomonas mallei *and was previously classified as* Pfeifferella*,* Loefflerella, Malleomyces *or* Actinobacillus)*. The disease causes nodules and ulcerations in the upper respiratory tract and lungs. A skin form also occurs, known as 'farcy'. Control of glanders requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of positive reactors. It is transmitted to humans and all infected/contaminated or potentially infected/contaminated material must be handled in a laboratory that meets the requirements for Containment Group 3 pathogens*.

Identification of the agent: *Smears from fresh material may reveal Gram-negative nonsporulating, nonencapsulated rods. The presence of a capsule-like cover has been demonstrated by electron microscopy. The bacteria grow aerobically and prefer media that contain glycerol. Unlike the* Pseudomonas *species and the closely related bacterium* Burkholderia pseudomallei*,* Burkholderia mallei *is nonmotile. Guinea-pigs are highly susceptible, and males can be used, if strictly necessary, to recover the organism from a heavily contaminated sample. Commercially available biochemical identification kits lack diagnostic sensitivity. Specific monoclonal antibodies and polymerase chain reaction* (*PCR*) *as well as real-time PCR assays are available. The latter have also been evaluated in recent outbreaks.*

Mallein and serological tests: *Complement fixation test and enzyme-linked immunosorbent assays are the most accurate and reliable serological tests for diagnostic use. The mallein test is a sensitive and specific clinical test for hypersensitivity against* Burkholderia mallei*. Mallein, a water soluble protein fraction of the organism, is injected subcutaneously, intradermo-palpebrally or given by eyedrop. In infected animals, the skin or the eyelid swells markedly within 1–2 days. A rose bengal plate agglutination test has recently been developed in Russia; it has been validated in Russia only.*

Requirements for vaccines and diagnostic biologicals: *There are no vaccines. Mallein purified protein derivative is currently available commercially from the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey.*

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates with zoonotic potential known since ancient times. It is caused by the bacterium *Burkholderia mallei* (the name recently changed from *Pseudomonas mallei* (Yabuuchi *et al.*, 1992) and has been classified in the past as *Pfeifferella*, *Loefflerella, Malleomyces* or *Actinobacillus*). Outbreaks of the disease may occur in felines living in the wild or in zoological gardens. Susceptibility to glanders has been proved in camels, bears, wolfs and dogs. Carnivores may become infected by eating infected meat, but cattle and pigs are resistant (Minett, 1959). Small ruminants may also be infected if kept in close contact to glanderous horses (Wittig *et al.*, 2006). Glanders in the acute form occurs most frequently in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia); death occurs within a few days. In horses, glanders generally takes a more chronic course and they may survive for several years. Chronic and subclinical 'occult' cases are dangerous sources of infection due to permanent or intermittent shedding of bacteria (Wittig *et al.*, 2006).

In horses, inflammatory nodules and ulcers develop in the nasal passages and 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 nodular abscesses 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 nodular abscesses ('buds') of 0.5–2.5 cm develop, which ulcerate and discharge yellow oily pus. Nodules are regularly 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, by 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 (Monlux, 1984; Wittig *et al.*, 2006).

Glanders is transmissible to humans by direct contact with diseased animals or infected/contaminated material. In the untreated acute disease. 95% mortality can occur 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 (Kahn & Ashford, 2001; Srinivasan *et al.,* 2001). A chronic form with abscessation also occurs (Neubauer *et al.*, 1997). When handling suspect or known infected animals or fomites, stringent precautions should be taken to prevent self-infection or transmission of the bacterium to other equids. Laboratory samples should be securely packaged, kept cool and shipped as outlined in Chapter 1.1.1 Collection and shipment of diagnostic specimens. All manipulations with potentially infected/contaminated material must be performed in a laboratory that meets the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.

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

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections of the nasal mucosae or sinuses, and from strangles (*Streptococcus equi* infection), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), pseudotuberculosis (*Yersinia pseudotuberculosis*) and sporotrichosis (*Sporotrichium* spp.). Glanders should be excluded positively from suspected cases of epizootic lymphangitis (caused by *Histoplasma farciminosum*), with which it has many clinical similarities. In humans in particular, glanders should be distinguished from melioidosis (*B. pseudomallei* infection), which is caused by an organism with close similarities to *B. mallei* (Minett, 1959).

a) Morphology of *Burkholderia mallei*

The organisms are fairly numerous in smears from fresh lesions, but in older lesions they are scanty (Wilson & Miles, 1964). They should be stained by methylene blue or Gram stain. The organisms are mainly extracellular, fairly straight Gram-negative rods with rounded ends, 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size. They often stain irregularly and do not have a readily visible capsule, under the light microscope, or form spores. The presence of a capsule-like cover has been established 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 *Burkholderia pseudomallei*, *Burkholderia mallei* has no flagellae and are therefore nonmotile (Krieg & Holt, 1984; Sprague & Neubauer, 2004). The organisms are difficult to demonstrate in tissue sections, where they may have a beaded appearance (Miller *et al.*, 1948). 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).

b) Cultural characteristics

It is preferable to attempt isolation from unopened uncontaminated lesions (Miller *et al.*, 1948). The organism is aerobic and facultatively anaerobic only in the presence of nitrate (Gilardi, 1985; Krieg & Holt, 1984), growing optimally at 37°C (Mahaderan *et al.,* 1987). It grows well, but slowly, on ordinary culture media, 72-hour incubation of cultures is recommended; glycerol enrichment is particularly useful. After a few days on glycerol agar, there is a confluent, slightly cream-coloured growth that is smooth, moist, and viscuous. With continued incubation, the growth thickens and becomes dark brown and tough. It also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On plain nutrient agar, the growth is much less luxuriant, and growth is poor on gelatin (Steele, 1980). In samples not obtained under sterile conditions *B. mallei* is regularly overgrown by other bacteria.

Alterations to characteristics may occur *in vitro*, so fresh isolates should be used for identification reactions. Litmus milk is slightly acidified by *B. mallei*, and coagulation may occur after long incubation. The organism reduces nitrates. Although some workers have claimed that glucose is the only carbohydrate that is fermented (slowly and inconstantly), other workers have shown that if an appropriate medium and indicator are used, glucose and other carbohydrates, such as arabinose, fructose, galactose and mannose, are

consistently fermented by *B. mallei* (Evans, 1966). Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures (Krieg & Holt, 1984). A commercial laboratory test kit (e.g. API [Analytical Profile Index] system: Analytab Products, BioMerieux or Biolog [Hayward, California]) can be used for easy confirmation that an organism belongs to the *Pseudomonas* group. In general, commercially available systems are not suited to unambiguously identifying members of the steadily growing number of species of the genus *Burkholderia* (Glass & Popovic, 2005). Lack of motility is then of special relevance. A bacteriophage specific for *B. mallei* is available (Woods *et al.,* 2002).

All culture media prepared 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 working 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 of use, as has pretreatment with penicillin (1000 units/ml for 3 hours at 37°C) (Minett, 1959). A selective medium has been developed (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%).

Outside the body, the organism has 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 (Steele, 1980). For disinfection, benzalkonium chloride or 'roccal' (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective against *B. mallei* (Mahaderan *et al.,* 1987). Phenolic disinfectants are less effective.

c) Laboratory animal inoculation

Guinea-pigs, hamsters and cats have been used for diagnosis when necessary. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into a male guinea-pig. As this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended (Neubauer *et al.*, 1997). Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of the lesions. Additional steps are used when the test material is heavily contaminated (Gould, 1950). The Strauss reaction is not specific for glanders, and other organisms can elicit it. Bacteriological examination of infected testes should confirm the specificity of the response obtained.

d) Confirmation by polymerase chain reaction and real-time PCR

In the past few years, several PCR and real-time PCR assays for the identification of *Burkholderia mallei* have been developed (Bauernfeind *et al.,* 1998; Lee *et al.*, 2005; Sprague *et al.*, 2002; Thibault *et al.*, 2004; Ulrich *et al.*, 2006; U'Ren *et al.*, 2005), but only a PCR and a real-time PCR assay were evaluated using samples from a recent outbreak of glanders in horses (Scholz *et al.*, 2006; Tomaso *et al.*, 2006). These two assays will be described in more detail here. However, the robustness of these assays will have to be demonstrated in the future by interlaboratory studies. The guidelines and precautions outlined in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, have to be taken into account.

- **DNA preparation**

Single colonies are transferred from an agar plate to 200 µl lysis buffer (5× buffer D [PCR Optimation Kit, Invitrogen, DeShelp, The Netherlands, 1/5 diluted in ultra-pure water]; 0.5% Tween 20 [ICI, American Limited, Merck, Hohenbrunn, Germany]; 2 mg/ml proteinase K [Roche Diagnostics, Mannheim, Germany]). After incubation at 56°C for 1 hour and inactivation for 10 minutes at 95°C, 2 and 4 µl of the cleared lysate are used as template in the PCR or the real-time PCR assay, respectively.

Tissue samples of horses (skin, liver, spleen, lung, and conchae) 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 over night in sterile saline at 4°C, and minced using liquid nitrogen, a mortar and a pestle. Total DNA is prepared from 50 mg tissue using the QIAamp Tissue Kit™ according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA is eluted with 80 µl dH_2O of which 4 µl are used as template.

- **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 chemicals used.

The oligonucleotides used in by Scholz *et al.* (2006) are designed based on differences of 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. PCR is done using 50 µl ready-to-go mastermix (Eppendorf, Hamburg, Germany) and 15 pmol of each primer. Thermal cycling conditions are 94°C for 30 seconds; 65°C for 30 seconds and 72°C for 60 seconds. This cycle is repeated for 35 times. A final elongation step is added (72°C for 7 minutes). Visualisation of the products is done by UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with ethidium bromide. No template controls containing PCR-grade water instead of template and positive controls containing DNA of *B. mallei* have to be included in each run to detect contamination by amplicons of former runs or amplification failure.

The lower detection limit is 10 fg or 2 genome equivalents.

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

The assay has to be adapted to the real-time PCR instrument used with minor modifications, e.g. the cycling vials have to be chosen according to the manufacturer's recommendations and the concentration of the oligonucleotides may have to be doubled or the labelling of the probes has to be changed. The authors used a MX3000P™ real-time PCR system (Stratagene, Amsterdam, The Netherlands) and 96-well plates (ThermoFast 96 ABGene™, Rapidozym, Berlin, Germany).

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The 25 µl reaction mixture consists of 12.5 µl 2× TaqMan™ Universal MasterMix (Applied Biosystems, Foster City, USA), 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl sample. Thermal cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 (50) 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 to include 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 give reproducible cycle threshold values. However, depending on the sample material a house keeping gene targeting PCR 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.

e) Other methods

The genome of the *Burkholderia mallei* type strain ATCC 23344^T was sequenced in 2004 (Nierman *et al.*, 2004). Several genomes of other isolates followed and revealed a wide genomic plasticity. Passages in different host species or culture media may provoke considerable sequence alterations (Romero *et al.*, 2006). The loss of the ability to produce LPS and/or capsule polysaccharide upon ongoing culture due to mutation is a well known fact and results in reduced or absent virulence and influences serologic tests (Neubauer *et al.*, 2005). Several molecular typing techniques have successfully been introduced. Simple molecular techniques like PCR-restriction fragment length polymorphism (Tanpiboonsak *et al.*, 2004) and pulsed field gel electrophoresis (Chantratita *et al.*, 2006) can be used for further discrimination of isolates. Ribotyping using restriction enzymes *Pst*I and *Eco*RI in combination with an *E. coli* 18-mer rDNA probe produced 17 distinct ribotypes within 25 *B. mallei* isolates (Harvey & Minter, 2005). These techniques are still the in-house tests of specialised laboratories as an extensive strain collection is necessary. Multilocus sequence typing (MLST) can be done with purified DNA so there is no need for excessive cultivation of the agent or the keeping of strain collections. Web-based analysis might even enhance diagnostics (Godoy *et*

al., 2003). No specific histopathology features can be described for lesions caused by *B. mallei.* For immunuhistochemical analysis, *B. mallei* specific hyperimmune sera of rabbits can be used.

2. Serological tests and the mallein test

a) Complement fixation test (a prescribed test for international trade)

Although not as sensitive as the mallein test, the CF test is an accurate serological test that has been used for glanders diagnosis for many years (Blood & Radostits, 1989). It is reported to be 90–95% accurate, serum being positive within 1 week of infection and remaining positive in the case of exacerbation of the chronic process (Steele, 1980). Recently, however, the specificity of CF testing has been questioned (Neubauer *et al.*, 2005).

- **Antigen preparation (Kelser & Reynolds, 1935)**

i) Flasks of beef infusion broth with 3% glycerol are inoculated with log-phase growth *B. mallei* and incubated at 37°C for 8–12 weeks.

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

iii) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 75 minutes on 3 consecutive days, and clarified by centrifugation.

iv) The clarified product is concentrated to one-tenth the original volume by evaporation on a steam or hot water bath.

v) Concentrated antigen is bottled in brown-glass bottles to protect from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

vi) Lots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline with 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and store at 4°C. The final working dilution is determined by a block titration. The final working dilution for CF test use is made at the time the CF test is performed.

The resulting antigen is primarily lipopolysaccharide. An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for 12 hours and washing 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.

- **Test procedure (NVSL, 2006)**

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

ii) Diluted serum is inactivated for 30 minutes at 56°C. The USDA complement fixation protocol calls for inactivation for 35 minutes (NVSL, 2006). (Serum of equidae other than horses should be inactivated at 63°C for 30 minutes.)

iii) Twofold dilutions of the sera are prepared in 96-well round-bottom microtitre plates.

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

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

vi) A 2% suspension of sensitised washed sheep red blood cells is added. The USDA protocol calls for confirmation of positive reactions in a tube test using 3% sheep red blood cells (NVSL, 2006).

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

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. Unfortunately, false-positive results can occur, and *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (Blood & Radostits, 1989; Neubauer *et al.*, 1997). Also healthy horses can have a false positive CF reaction for a variable period following a mallein intradermal test.

b) Enzyme-linked immunosorbent assays

Both plate and membrane (blot) enzyme-linked immunosorbent assays (ELISAs) have been reported for the serodiagnosis of glanders, but none of these procedures has been shown to differentiate serologically between *B. mallei* and *B. pseudomallei*. Blotting approaches have involved both dipstick dot-blot and

electrophoretically separated and transferred western blot methods (Katz *et al.,* 1999; Verma *et al.,* 1990). A competitive ELISA that uses an uncharacterised anti-lipopolysaccharide monoclonal antibody has also been developed and found to be similar to the CF test in performance (Katz *et al.,* 2000). Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components offers the potential for more specific ELISAs in the foreseeable future that will help resolve questionable test results of quarantined imported horses (Burtnick *et al.,* 2002; Feng *et al.,* 2006; Khrapova *et al.,* 1995; Neubauer *et al.*, 1997). At this time, none of these tests has been validated.

c) 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*. The test 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 methods of diagnosis to be employed (Allen, 1929).

- **The intradermo-palpebral test**

This is the most sensitive, reliable and specific test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced the ophthalmic and subcutaneous tests (Blood & Radostits, 1989): 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.

d) Other serological tests

The avidin–biotin dot ELISA has been described (Verma *et al.,* 1990), but has not yet been widely used or validated. The antigen is heat-inactivated bacterial culture that has been concentrated and purified. A dot of this antigen is placed on a nitrocellulose dipstick that is then used to test for antibody against *B. mallei* in equine serum. Using antigen-dotted, preblocked dipsticks, the test can be completed in approximately 1 hour. Serum or whole blood can be used for the test, and partial haemolysis does not impart any background colour to the antigen-coated area on the nitrocellulose. Recently, polysaccharide microarray technology has offered a new promising approach to improve sensitivity in serology (Parthasarathy *et al.,* 2006).

The rose bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; this test has been validated in Russia only. 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 tests and precipitin is unsatisfactory for use in control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.

Mallein PPD for use in performing the intradermo-palpebral and ophthalmic tests is produced commercially by the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey.

The ID-Lelystad has provided the following information on requirements for 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 through guinea-pigs.

2. Method of manufacture

Dorset–Henley medium, enriched by the addition of trace elements, is used for 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 and a light brownish to greyish precipitate settles.

The supernatant fluid is pipetted off 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 a pH of 6.7 will be obtained. This mallein concentrate has to be centrifuged thoroughly and the supernatant is 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 frequently for any signs of contamination, and suspect 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 from five to ten normal healthy horses by carrying out 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 not 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 or incomplete Freund's adjuvant. Cattle can also be used instead of horses. The production batch is bioassayed 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 by calipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

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NB: There are OIE Reference Laboratories for Glanders
(see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and diagnostic biologicals for glanders