

HAEMORRHAGIC SEPTICAEMIA

SUMMARY

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterised by an acute, highly fatal septicaemia with high morbidity and mortality. It is caused by certain serotypes of Pasteurella multocida that are geographically restricted to some areas of Asia, Africa, the Middle East and southern Europe.

The diagnosis of HS depends on the isolation of the causative organism, P. multocida, generally from the blood or bone marrow of a dead animal, by cultural and biological methods, and the identification of the organism by biochemical, serological and molecular methods.

Isolation and identification of the agent: *Pure cultures of P. multocida can be obtained by streaking materials on to artificial media and the subsequent identification on the basis of the morphological, cultural, and biochemical characteristics of P. multocida.*

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination for 'capsular' typing using sheep red blood cells coated with bacterial extracts, 'somatic' typing by agar gel immunodiffusion tests using heat-treated cell extracts, or agglutination using acid-treated cells. Confirmation of the isolates can be made using molecular techniques.

Serology: *Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.*

Requirements for vaccines: *Effective vaccines against haemorrhagic septicaemia are formalin-killed bacterins, or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity.*

Seed cultures for the production of vaccines should contain capsulated organisms. Vaccines are standardised as to their bacterial density on the basis of turbidity tests and dry bacterial weight. Potency tests are carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Bain *et al.*, 1982; Carter & De Alwis, 1989; De Alwis, 1992; Mustafa *et al.*, 1978; Singh *et al.*, 1996). The disease has been recorded in wild mammals in several Asian and European countries (Carigan *et al.*, 1991; Rosen, 1981). In many Asian countries disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures). The disease is caused by *Pasteurella multocida*, a Gram-negative coccobacillus residing mostly as a commensal in the nasopharynx of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), corresponding to 6:B and 6:E (Namioka-carter system), are mainly responsible for the disease. In wild ruminants, serotype B:2,5 is predominantly present while serotype B:3,4 also has been reported from fallow deer (Aalbæk *et al.*, 2009). The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (Kumar *et al.*, 1996). The geographical distribution of HS includes some areas of Asia, Africa, the Middle East and southern Europe. It has never been confirmed in Mexico, Central or South America.

Clinically, HS caused by B:2 or E:2 strains is typified by fever, respiratory distress with nasal discharge, and frothing from the mouth, leading eventually to recumbency and death. Infection with serotypes A:1 and A:3 predominantly involves pneumonia and death. Septicaemia is the main characteristic feature in all forms of the disease. The incubation period varies from 3 to 5 days. In peracute cases, sudden death without clinical signs

may be observed (Carter & De Alwis, 1989; De Alwis, 1992). Water buffaloes are generally more susceptible to HS than cattle and show more severe forms of disease with profound clinical signs. Severe subcutaneous oedema of the mandible, neck and brisket is a distinctive feature of the disease. In endemic areas mortality is largely confined to older calves and young adults.

At *post mortem* most animals succumbing to HS typically show marked swelling of the neck caused by severe blood-tinged oedema. There are also abundant petechial haemorrhages in many tissues and organs, particularly in serosal membranes. The thoracic, pericardial and abdominal cavities may contain serosanguinolent fluid. The lungs are notably congested and oedematous, and foam is generally present in the nasal cavity, trachea and bronchi. Microscopically, there is interstitial pneumonia and pulmonary oedema as well as focal infiltrates of neutrophils and macrophages in many tissues. All these lesions are similar to those observed in severe sepsis and septic shock.

Massive epizootics may occur in endemic as well as non-endemic areas (Carter & De Alwis, 1989; De Alwis, 1992). HS has been identified as a secondary complication in cattle and water buffaloes following outbreaks of foot and mouth disease (FMD). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (Carter & De Alwis, 1989; De Alwis, 1992).

The diagnosis of the disease is based on the clinical signs, gross lesions, morbidity and mortality patterns. Confirmation requires the isolation and characterisation of the pathogen using conventional and molecular techniques. There are no confirmed reports of human infections with *P. multocida* B2 and E2; however, other serotypes do cause human infections and precautions should be taken to avoid exposure. The organism should be handled in biosafety level 2 laboratories.

B. DIAGNOSTIC TECHNIQUES

1. Isolation and identification of the agent

1.1. Cultural and biochemical methods

True septicaemia in HS occurs at the terminal stage of the disease, therefore blood samples should be taken from sick animals immediately before death. Animals in the early stages of the disease may not contain *P. multocida* in blood. The bacteria are also not consistently present in the nasal secretions or body fluids of sick animals.

A blood sample or swab collected from the heart is satisfactory only if taken immediately after death. If the animal has been dead for a long time, bone marrow from a long bone can be used for bacterial isolation. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage. If the blood samples are not transported to the laboratory within a few hours, they can be frozen. However repeated freezing and thawing may destroy the organism and is not advisable.

Blood smears from affected animals are stained with Gram, Leishman's or methylene blue stains. The organisms appear as Gram-negative, bipolar-staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopic examinations alone.

Blood samples, or swabs eluted into 2–3 ml sterile physiological saline, are cultured. Alternatively, the surface of a long bone is disinfected with alcohol and split open. The marrow is extracted aseptically and cultured. Direct culture is usually satisfactory only if the material is fresh and free from contaminants or post-mortem invaders that would otherwise overgrow any *Pasteurella* present.

For biological examinations, a small volume (0.2 ml) of eluted blood swabs or a portion of bone marrow in saline is inoculated subcutaneously or intramuscularly into mice. The mouse usually serves as a biological 'screen' for extraneous organisms. If viable *P. multocida* is present, the mice die 24–36 hours following inoculation, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood of the mice, even when the original samples come from relatively old carcasses. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests.

A suitable bacteriological medium for *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 g), sucrose (3 g), yeast extract (5 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (3 g), and distilled water to

1 litre. The pH is adjusted to 7.3–7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for 15 minutes. After cooling to 45–50°C, 5% calf blood (antibody-free *P. multocida*) is added (Wijewardana *et al.*, 1986). Conventional blood agar may also be used.

Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours' incubation at 37°C. Colonies grown on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. *Pasteurella multocida* does not grow on MacConkey agar. Gram-stained blood or tissue smears show Gram-negative, short, ovoid, bipolar-staining coccobacilli. A degree of pleomorphism will be noted, particularly in old cultures, with longer rods of varying length. The bipolar staining will be more evident with methylene blue or Leishman's stain.

HS organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulphide or urease, and fail to use citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

One property of HS-causing strains of *P. multocida* is the ability to produce the enzyme hyaluronidase (Carter & Chengappa, 1980). Having identified the genus and species by cultural characteristics and biochemical tests, hyaluronidase production may then be used as a specific test for HS-causing pasteurellae. It should be noted that B serotypes other than B:2 (or 6:B), and type E, are hyaluronidase negative.

A hyaluronic-acid-producing culture is streaked across the centre of a dextrose starch agar plate. The *Pasteurella* culture to be tested for hyaluronidase production is streaked at right angles. The plates are incubated at 37°C for 18 hours. Originally, hyaluronic-acid-producing *Streptococcus equi* was used, but a convenient culture for this purpose is a capsulated mucoid *P. multocida* type A culture. At the point of intersection, the mucoid growth of the hyaluronic acid producer will diminish into a thin line of growth, indicating the production of hyaluronidase by the test culture. Use of freshly prepared plates and a humidified incubator will facilitate hyaluronic acid production and, thereby, the interpretation of the test.

1.2. Serotyping methods

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (Namioka & Murata, 1961a), an indirect haemagglutination (IHA) test for capsular typing (Carter, 1955), an agglutination test using hydrochloric-acid-treated cells for somatic typing (Namioka & Murata, 1961b), the agar gel immunodiffusion (AGID) test (Anon, 1981; Heddeleston *et al.*, 1972; Wijewardana *et al.*, 1982), and the counter immunoelectrophoresis test (CIEP) (Carter & Chengappa, 1981).

Hyperimmune antisera for most of these tests are prepared against specific reference strains in rabbits. Cultures in CSY broth (6–8-hours old) are seeded on to CSY blood agar medium. After overnight incubation (18–20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of MacFarland's tube No. 4. Rabbits are inoculated intravenously at 3–4-day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. The rabbits are inoculated subcutaneously or intramuscularly 1 week after the last injection with 0.5 ml of a similar, but live, suspension. The animals are bled 10 days later. The serum is stored at –20°C, but small quantities for regular use are stored at 4°C with the addition of 1/10,000 merthiolate.

1.2.1. Rapid slide agglutination test (capsular typing)

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

1.2.2. Indirect haemagglutination test (capsular typing)

This was originally performed using antigen-sensitised human type 'O' red blood cells (RBCs) (Carter, 1955), but more recently sheep RBCs have been used (Sawada *et al.*, 1985; Wijewardana *et al.*, 1986). The antigen is prepared as follows:

A 6–8-hour broth culture of a reference strain is seeded on to CSY blood agar plates and incubated overnight at 37°C. The growth is harvested in 3 ml physiological saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3000 *g* for 15 minutes at 4°C, and the clear supernatant fluid is stored at –20°C. If a refrigerated centrifuge is not available, centrifugation at 1500 *g* for 30 minutes gives a supernatant fluid. This is used

as the antigen extract. A similar procedure is followed for preparing an antigen extract from an unknown strain that is to be typed.

Sheep blood is collected aseptically into an anticoagulant and centrifuged at 500 *g* for 10 minutes. The packed RBCs are washed three times in sterile physiological saline. The antigen extract from an unknown strain prepared by the method described above is used to sensitise the RBCs or absorbed on to the RBCs. This is done by adding 15 volumes of the antigen extract to the RBCs and incubating the mixture for 1 hour at 37°C with frequent shaking. The sensitised RBCs are recovered by centrifugation, washed three times in sterile physiological saline, and made up to a final 1% suspension in physiological saline. The type-specific hyperimmune antiserum (three volumes) is absorbed by the addition of packed RBCs (one volume) for 30 minutes at room temperature, and then centrifuged at 500 *g* for 10 minutes to pellet the RBCs. The absorbed antiserum is then inactivated by heating at 56°C for 30 minutes.

The test itself can be carried out in tubes or plates, and is performed in two rows. The test described below is for standard microtitre plates.

- i) The capsular extract of the unknown strain is prepared as described above and used to sensitise the sheep RBCs. The known type-specific hyperimmune sera raised in rabbits against types A, B, D and E are diluted as follows:
- ii) Using four separate rows of wells, the first wells are filled with 0.72 ml saline followed by 0.4 ml in the next six wells or more.
- iii) The type-specific hyperimmune sera are each separately diluted in each row by adding 0.08 ml of the serum to the first well and mixing with a pipette. From this well 0.4 ml is transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.
- iv) All the wells are each filled with 0.4 ml of antigen-adsorbed/sensitised RBCs, shaken slightly and left at room temperature. By the addition of the sensitised blood, the serum dilutions in the wells are doubled, i.e. 1/20 in well one, 1/40 in the second, and so on. A positive, negative and saline control are included in each test run.
- v) The first reading is taken after 2 hours and a final reading after 18 hours. A course agglutination of the RBCs along the sides of the concave wells is taken as a positive reading, and the formation of a button at the centre of the wells as negative. An arbitrary score of 1–4 is given depending on the size of the agglutination. An unknown strain is identified with the hyperimmune serum that has agglutination. In the absence of agglutination with all sera, the strain is considered to be untypeable.

While IHA can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as a quantitative test against these strains.

1.2.3. Agar gel immunodiffusion tests

AGID tests are used for what is described as ‘capsular’ as well as ‘somatic’ typing, depending on the antigens and antisera used. The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells.

- i) *Capsular typing*: The gel medium is 1.0% Noble agar, or equivalent product, in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1/10,000 (Anon, 1981; Wijewardena *et al.*, 1982). Antigens and antisera are the same as for capsular typing by the IHA method (Carter, 1955). The standard antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with standard homologous antigen.
- ii) *Somatic typing*: The gel medium consists of special Noble agar, or equivalent product, at a concentration of 0.9% in 0.85% sodium chloride solution.
- iii) For antigen preparation, the growth from each plate is harvested in 1 ml of 8.5% sodium chloride containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells are sedimented by centrifugation, and the supernatant fluid is used as antigen.

- iv) Antisera against 16 somatic types (Heddleston *et al.*, 1972) are prepared in chickens. Oil-emulsified bacterin¹ (1 ml) is injected subcutaneously into the mid-portion of the neck of 12–16-week-old male birds. A further injection is made 3 weeks later of 1 ml intramuscularly into the breast, 0.5 ml on each side of the sternum. The birds are bled 1 week later, and the serum is separated and preserved with 0.01% thiomersal and 0.06% phenol. Sera are tested against all somatic types and sera that cross-react are discarded. Some antisera preparations against B:2 may cross react with the somatic type 5.
- v) The test antigen is placed in the centre well and antisera against the different serotypes are placed in the peripheral wells. All haemorrhagic septicaemia serotypes (Asian and African) will react with type 2 antiserum. Cross-reactions may occur with type 5.

1.2.4. Counter immunoelectrophoresis

CIEP offers a rapid method for the identification of capsular types B and E cultures.

i) *Preparation of capsular substance*

Capsular substance is prepared in the same manner as described for the IHA test.

ii) *Preparation of hyperimmune antisera*

Antisera are prepared in rabbits as for the IHA test.

iii) *Medium for CIEP*

The medium for the CIEP consists of agarose (2.0 g), barbitone sodium (2.06 g), diethyl barbituric acid (0.37 g), distilled water (180 ml), and 1/1000 merthiolate (20 ml).

iv) *Veronal acetate buffer (barbitone buffer)*

The barbitone buffer consists of barbitone sodium (29.24 g), anhydrous sodium acetate (11.70 g), 0.1 N hydrochloric acid (180 ml), and distilled water to 3 litres. The pH should be 8.8.

v) *Preparation of slides*

The electrophoresis plates are prepared by precoating glass slides (57 mm × 70 mm) with 12 ml volumes of the medium. Seven wells, 4 mm in diameter and 7 mm apart, are cut in a row. A parallel set of wells is cut 6 mm (centre to centre) away from the other set of wells.

vi) *Test procedure*

The well on the side of the cathode is loaded with a 20 µl volume of capsular antigen, while an equal volume of type-specific antiserum is loaded on to the well on the side of the anode. Controls included in the test are 0.85% sodium chloride solution against positive antiserum, and capsular extract against negative rabbit serum as well as positive and negative control samples. The electrophoresis tank is filled with barbitone buffer, pH 8.8. The antigen and antiserum are electrophoresed for 30 minutes at 150 V (25 V/cm). The plates are then examined for precipitation lines.

vii) *Interpretation of the results*

The presence of a distinct line between the antigen and antiserum wells is considered to be a positive result.

1.2.5. Agglutination tests (somatic antigen)

The somatic 'O' antigen is prepared by a method similar to that described previously for the IHA test (Namioka, 1978; Namioka & Murata, 1961b). A 6–8-hour test culture is seeded on to CSY blood agar and incubated overnight. The growth is harvested in 2–3 ml of physiological saline containing 0.3% formalin per plate, and centrifuged at 3000 *g* for 15 minutes at 4°C (or 1200–

¹ The bacterial antigens in broth are covered by a light mineral oil (adjuvant) and then emulsified (stabilised) with an emulsifying agent, in this case lanolin or lanoline (wool fat). This has to be done as the watery phase with the bacteria (broth) will not mix with the oily phase (adjuvant). The proportion of oil to emulsifying agent will vary with different batches of lanolin and will have to be adjusted accordingly. The higher the percentage of lanoline, the higher the stability of the emulsion.

1500 g for 30–45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown's opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively.

Finally, a suspension of the residual cells, equivalent to Brown's opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the tests carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

1.2.6. Serotype designation

Broadly, two typing systems are adopted. One is 'capsular' typing by Carter's IHA test (Carter, 1955) or by AGID tests (Anon, 1981; Wijewardena *et al.*, 1982). The other is 'somatic' typing by the method of Namioka & Murata (Namioka, 1978; Namioka & Murata, 1961b; 1961c), and by the method of Heddleston *et al.* (1972). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination. Two systems commonly in use are the Namioka–Carter and the Carter–Heddleston systems. In the former system, Asian and African HS serotypes are designated 6:B and 6:E, respectively, while in the latter system they are designated B.2 and E.2, respectively.

1.2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is particularly necessary for *P. multocida* for which resistance to commonly used antimicrobial agents has been reviewed by Kehrenberg *et al.* (2001). AST methods are described in Chapter 2.1.1 *Laboratory methodologies for bacterial antimicrobial susceptibility testing*. The agar disk diffusion method has been used to test common fast-growing bacterial pathogens and is recognised to work well with *P. multocida* (Bauer *et al.*, 1966). Reliable results can be obtained with disk diffusion tests that use standardised methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behaviour of strains among clinically susceptible and resistant categorisations. Selection of the most appropriate antimicrobial agents to test is a decision best made by each laboratory in accordance with the needs of veterinary practitioners and the drugs available for veterinary use in the country. The following agents have proven their clinical efficacy: penicillin, amoxicillin (or ampicillin), cephalothin, ceftiofur, cefquinome, streptomycin, gentamicin, spectinomycin, florfenicol, tetracycline, sulfonamides, trimethoprim/sulfamethoxazole, erythromycin, tilmicosin, enrofloxacin (or other fluoroquinolones) and norfloxacin.

1.3. Nucleic acid recognition methods

1.3.1. *Pasteurella multocida*-specific PCR assay

PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (Mifflin & Blackall, 2001; Townsend *et al.*, 1998a). The rapidity and high specificity of two of the *P. multocida*-specific assays (Mifflin & Blackall, 2001; Townsend *et al.*, 1998a) provide optimal efficiency without the need for additional hybridisation. The *P. multocida*-specific PCRs (Mifflin & Blackall, 2001; Townsend *et al.*, 1998a) identify all subspecies of *P. multocida*. The Mifflin & Blackall PCR (Mifflin & Blackall, 2001) was described as giving a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2, while the Townsend *et al.* PCR (1998a) gave a false positive with *P. canis* biovar 2 (it has not been tested against *P. avium* biovar 2). Recently, both *P. avium* biovar 2 and *P. canis* biovar 2 have been re-named as *P. multocida* (Christensen *et al.*, 2004) – meaning that both the Townsend *et al.* (1998a) and the Mifflin & Blackall (Mifflin & Blackall, 2001) PCR assays are now regarded as being specific for *P. multocida*. Some difficulties remain as it is now known that sucrose-negative *P. multocida*-like organisms from large cat bite wounds form two groups. While both are positive in the Mifflin & Blackall *P. multocida*-specific PCR (2001) only one group has been confirmed as true *P. multocida* by other genotypic methods (Christensen *et al.*, 2005). The Townsend *et al.* (1998a) PCR is described in the following paragraph.

A fraction of an isolated colony of the suspect organism is transferred directly into the PCR mixture. Alternatively, template DNA can be obtained from 2 µl of either a mixed or pure broth culture. All currently used methods for the preparation of template DNA produce reproducible results with the KMT1 primers (Townsend *et al.*, 1998a), and allow detection of ≤10 organisms per reaction. The sensitivity and specificity of the *P. multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. *Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if the specimen is from an old carcass or from tonsil or nasal swabs. In such cases, the swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.

Primer sequences (Townsend *et al.*, 1998a):

P. multocida-specific PCR: KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 0.5 u *Taq* DNA polymerase. Thermocycler parameters are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination.

1.3.2. *Pasteurella multocida* multiplex capsular PCR typing system

Identification of the genes involved in the biosynthesis of the *P. multocida* A:1 (Chung *et al.*, 1998) and B:2 (Boyce *et al.*, 2000) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (Boyce *et al.*, 2000). Moreover, with the use of serogroup specific multiplex PCR, conflicting results as regards to typing of some strains could be confirmed (Townsend *et al.*, 2001). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (Townsend *et al.*, 2001). The *P. multocida*-specific primers are included as an internal control for species identification. In the multiplex capsular PCR typing system, the amplicon band giving the typing result may be unclear. In such cases, removal of the *P. multocida*-specific primers (KMT1T7, KMT1SP6) from the mixture can improve the result.

Primer sequences (Townsend *et al.*, 2001):

Multiplex capsular PCR:	CAPA-FWD	5'-TGC-CAA-AAT-CGC-AGT-CAG-3'
	CAPA-REV	5'-TTG-CCA-TCA-TTG-TCA-GTG-3'
	CAPB-FWD	5'-CAT-TTA-TCC-AAG-CTC-CAC-C-3'
	CAPB-REV	5'-GCC-CGA-GAG-TTT-CAA-TCC-3'
	CAPD-FWD	5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'
	CAPD-REV	5'-CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG-3'
	CAPE-FWD	5'-TCC-GCA-GAA-AAT-TAT-TGA-CTC-3'
	CAPE-REV	5'-GCT-TGC-TGC-TTG-ATT-TTG-TC-3'
	CAPF-FWD	5'-AAT-CGG-AGA-ACG-CAG-AAA-TCA-G-3'
	CAPF-REV	5'-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'
	KMT1T7	5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
	KMT1SP6	5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

Size of resulting fragments:

Serogroup A	CAPA-FWD/CAPA-REV	1044 bp
Serogroup B	CAPB-FWD/CAPB-REV	760 bp
Serogroup D	CAPD-FWD/CAPD-REV	657 bp
Serogroup E	CAPE-FWD/CAPE/REV	511 bp
Serogroup F	CAPF-FWD/CAPF-REV	851 bp

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 1 u *Taq* DNA polymerase. In the original publication (Townsend *et al.*, 2001) it is suggested to use a standard cycling programme as per *P. multocida*-specific PCR assay. However, the

cycling programme should be optimised to and validated for the model of thermocycler in use. Agarose gel electrophoresis is as described above.

1.3.3. HS-causing type-B-specific PCR assay

Presumptive identification of HS-causing type-B-specific *P. multocida* is also possible by PCR amplification (Townsend *et al.*, 1998a). Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620 bp fragment with the KTSP61 and KTT72 primers.

Primer sequences (Townsend *et al.*, 1998a):

HS-causing type-B-specific PCR	KTT72	5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
	KTSP61	5'-ATC-CGC-TAA-CAC-ACT-CTC-3'

Conditions for HS-causing type-B-specific PCR are as described for *P. multocida*-specific PCR. The usefulness of these primers has been reported for identification of serogroup B strains.

HS-causing type-B-specific PCR primers can also be used in a multiplex PCR with the *P. multocida*-specific primers, dramatically decreasing the time required for *P. multocida* detection and presumptive identification of the HS-serotype. Multiplex PCR conditions are as described above except that 3.2 pmol of each of the four primers and 1 u *Taq* DNA polymerase are used. The use of the multiplex *P.-multocida*-specific/HS-causing type-B-specific PCR on suspect organisms can confirm the identity and provide a presumptive serotype within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.

1.3.4. *Pasteurella multocida* type A specific PCR

Primers useful for typing of serogroup A strains with several somatic types have been reported to be useful in specific identification of isolates (Gautam *et al.*, 2004).

Primers:

RGPMA5:	5'-AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA-3'
RGPMA6:	5'-ATT-TGG-CGC-CAT-ATC-ACA-GTC-3'

PCR conditions:

Template DNA (50 ng) is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each dNTPs, 1.5 mM MgCl₂, 20 pmol of each primer and 1 unit *Taq* DNA polymerase. Standard amplification conditions are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 6 minutes. Amplified products are separated by agarose gel electrophoresis (1.5% agarose gel) in 0.5 × TBE buffer at 5 v/cm for 2 hours.

The PCR amplification yields a product of 564 bp.

The test can be applied on direct culture, boiled cell lysate and infected tissues.

1.3.5. Genotypic differentiation of isolates

Once presumptive (or definitive) identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis with the enzyme *HhaI* has proved useful for characterisation of type B HS-serotypes. Among 71 *P. multocida* capsule serogroup B isolates, 20 DNA fingerprint profiles were observed. With HS-causing serogroup strains, 13 unique profile among 54 isolates resembling the profile of the somatic serotype 2 reference strain have been reported (Wilson *et al.*, 1992). In contrast, while a single *HhaI* profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following *HpaII* digestion. *HpaII* appear to generate finer subdivisions than those achieved with the use of *HhaI* (Wilson *et al.*, 1995). Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E *P. multocida* isolates (Townsend *et al.*, 1997a). Genetic diversity of HS-causing *Pasteurella multocida* strains of animal or bird origin could be obtained by sequence analysis of the 16S rRNA gene. A study in the United Kingdom using 79 field isolates recovered from various species revealed nineteen 16S rRNA types that clustered into two distinct phylogenetic lineages (Davies, 2004). On the other hand, sequence analyses of Indian isolates of *P. multocida* serogroup B from different animal species did not reveal considerable variation (Dey *et al.*,

2007). Multilocus sequence typing (MLST), a sequence-based typing system based on seven housekeeping genes has been used to identify strain diversity of bovine isolates of *P. multocida* (Davies *et al.*, 2004). However, these techniques are largely used for research purposes and require specialised equipment. Moreover these profiles are not unique to country of origin or host species.

PCR fingerprinting is feasible for any laboratory with PCR capability, with several methods previously used for *P. multocida* differentiation. Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), respectively, have been shown to be useful for epidemiological studies of *P. multocida* isolated from rabbits (Chaslus-Dancla *et al.*, 1996). Repetitive sequence PCR analysis of *P. multocida* has provided useful for discrimination of avian and swine isolates, although all HS-causing strains analysed demonstrated similar profiles (Townsend *et al.*, 1997b; 1998b). However, molecular variability among HS-causing strains of *P. multocida* belonging to serogroup B has been found recently. Using repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and single primer PCR, genotypic differentiation among five *P. multocida* serogroup B isolates have been reported (Biswas *et al.*, 2004). RAPD and AP-PCR analysis of HS-causing *P. multocida* isolates have not been previously described.

2. Serological tests

Serological tests for detecting antibodies are not normally used for diagnosis. The IHA test can be used for this purpose, following a method broadly similar to that described for capsular typing above. High titres detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. Deaths occur very suddenly and no carcasses are available for examination when notification is made. In such situations, high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.

A live HS vaccine prepared using an avirulent *P. multocida* strain B:3,4 (Fallow deer strain) has been used for control of the disease in cattle and water buffaloes over 6 months of age in Myanmar since 1989. It is administered by intranasal aerosol application (Carter *et al.*, 1991; Myint *et al.*, 2005). The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A local isolate of *P. multocida* representing the prevalent serotype is used. A well-capsulated, stable culture that produces large colonies of approximately 2 mm in diameter on CSY blood agar must be maintained. Seed cultures should be stored as semisolid nutrient agar stab cultures at room temperature, or as lyophilised cultures.

A calf is infected with the culture, and, within 2–3 hours of its death, blood is collected aseptically from the heart and stored at –20°C in 1 ml aliquots. A fresh aliquot is used for each new batch of vaccine. It is permissible to subculture this aliquot once or twice, provided the colony size does not diminish. A blood aliquot is thawed, plated on to CSY blood agar, and the growth is tested for agglutinability by the appropriate antiserum on a slide. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Seed lots must be shown to be:

- i) *Pure*: Free from adventitious agents.
- ii) *Safe*: Produce no adverse reaction in the target species when given as recommended.
- iii) *Efficacious*: Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.2.2.4 below.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g dry weight per litre of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinised physiological saline, by which means suspensions of any density can be achieved. This is laborious as each flask must be harvested separately and tested for purity. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports *P. multocida*.

There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (Thomas, 1968). The more finely dispersed the air, the better is the bacterial growth. Vessels of 20–40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours, about 25% of the working volume is harvested and replaced hourly. The harvests of continuous cultures are collected in relatively small volumes into separate vessels, but, after several days, the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15–18 hours, when the growth can be terminated by the addition of formalin to a final concentration of 0.5%. This procedure, where a large inoculum is employed and the growth is terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g/litre.

Dense cultures are also obtained using fermenters, where heat sterilisation of the tanks and culture can be carried out *in situ*, with automatic temperature, pH and aeration control devices. Liquid sterilisation systems by filtration, for heat-labile components, can also be built into the fermenter. A 100 litre batch fermenter will yield a minimum of 66,000 doses (each of 3 ml) of OAV, and even more doses if the density is high enough for dilution to a reference standard equivalent to 1.5 g/litre, dry weight/volume.

OAV is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanolin as emulsifying agent. The mineral oil and lanolin are first sterilised and, on cooling to 40°C, 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification is continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

APV is prepared by first adjusting the turbidity of the suspension to the reference standard as above, and diluting it with an equal volume of 0.5% formalinised physiological saline. The pH is adjusted to 6.5, and a hot 20% solution of potash alum is added to give a final concentration of 1% alum. After overnight storage with continuous agitation, the vaccine is bottled for use.

2.2.2. Requirements for substrates and media

A suitable sterilised medium for the aerated culture method is casein hydrolysate (2 g), sucrose (6 g), yeast extract (6 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (8.6 g), anhydrous potassium dihydrogen orthophosphate (1.36 g), and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter-sterilised or autoclaved for 10 minutes at 107°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

2.2.3. In-process controls

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects.

Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for *P. multocida*.

iii) Batch potency

Potency tests can be carried out by any of the following methods:

- a) Vaccination of cattle followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is not very feasible as cattle take a long time to develop adequate immunity after OAV;
- b) Vaccination of rabbits followed by direct challenge or passive mouse protection test using the rabbit sera; or
- c) Potency tests in mice, the most feasible method of the three.

Each of 50 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into ten groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range 10^{-1} – 10^{-10} ; 50 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose (LD_{50}) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

See chapter 1.1.8.

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

See chapter 1.1.8.

iii) Environmental consideration

See chapter 1.1.8.

2.3.2. Efficacy requirements

i) For animal production

A single dose of vaccine administered to young calves 4–6 months of age will protect susceptible animals for 3–4 months when APV is used, and for 6–9 months when OAV is used.

The vaccine should be administered by deep intramuscular injection. The use of nylon 5 ml volume syringes for a 3 ml dose and a gauge 14–15 needle is advised, and the recommended age for primary vaccination is 4–6 months. For routine, prophylactic vaccination, a single dose of OAV at 4–6 months, a booster 3–6 months later, and annual revaccination thereafter, is recommended. Where husbandry practices are such that reaching individual animals at appropriate times is impracticable, annual vaccination of all animals over 4 months of age, preferably before the breeding season, and vaccination of all calves under 1 year of age, 6 months later, is recommended. In the face of an outbreak in vaccinated animals, one dose of APV, followed by one dose of OAV, is recommended.

Leakage of OAV into subcutaneous tissue can occasionally give rise to fibrous lumps at sites of injection. Rarely, abscesses may develop if sterility conditions are not observed, though most animals are resistant to such infections. APV may occasionally cause shock reactions.

ii) For control and eradication

Not applicable.

2.3.3. Stability

The OAV emulsion should be pure white, and should stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible. It can be stored at 4–8°C for 6 months without any significant loss of potency. It must not be frozen. Increase in the content of lanolin improves stability, but also increases the viscosity – a distinct disadvantage. Use of other emulsifying agents such as 'Arlacel' helps to produce thinner, stable emulsions.

3. Vaccines based on biotechnology

Not applicable at present

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NB: FIRST ADOPTED IN 1989; MOST RECENT UPDATES ADOPTED IN 2012.