**CHAPTER 2.4.9.**

**CONTAGIOUS BOVINE PLEUROPNEUMONIA**

**SUMMARY**

Contagious bovine pleuropneumonia (CBPP) is a disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polyppnoea, cough and nasal discharges. Diagnosis depends on the isolation of the aetiological agent. The main problems for control or eradication are the frequent occurrence of subacute or subclinical infections and the persistence of chronic carriers after the clinical phase.

**Identification of the agent:** Samples to be taken from live animals are nasal swabs and/or broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, pleural fluid and synovial fluid from those animals with arthritis. Direct examination of the exudate or smears is possible, but requires great skill.

For cultivation of the pathogen, the tissues are ground in medium containing antibiotics and inoculated into media that contain inhibitors to prevent the growth of contaminating bacteria. The growth of MmmSC takes several days.

In broth, growth is visible within 3–10 days as a homogeneous cloudiness with whirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical ‘fried-egg’ appearance. The biochemical characteristics of MmmSC are the following: sensitivity to digitonin, reduction of tetrazolium salts, fermentation of glucose, absence of arginine hydrolysis, and the absence of phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis is confirmed by immunological tests, such as the growth inhibition and immunofluorescence tests (both use hyperimmune sera). The polymerase chain reaction is now used as a rapid, specific, sensitive and easy to use test.

**Serological tests:** For diagnosis, the modified Campbell & Turner complement fixation test remains the prescribed test for international trade. However, it has significant limitations regarding sensitivity and specificity. The competitive enzyme-linked immunosorbent assay was designated as an OIE prescribed test for international trade by the OIE International Committee in May 2004. An immunoblotting test has undergone evaluation and is highly specific and sensitive.

**Requirements for vaccines:** Attenuated strains now recommended for vaccine production: the T1/44 and T1sr. The minimal recommended titre is $10^7$ mycoplasmas per vaccinal dose, but higher titres of at least $10^8$ are recommended.

**A. INTRODUCTION**

Contagious bovine pleuropneumonia (CBPP) is a contagious disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). CBPP has been known to occur in Europe since the 16th century but it gained a world-wide distribution only during the second half of the 19th century because of increased international trade in live cattle. It was eradicated from many countries by the beginning of the 20th century through stamping-out policies. However the disease persists in many parts of Africa. The situation in Asia is unclear. There have been no reported outbreaks in Europe since 1999. In natural conditions, MmmSC affects only the ruminants of the Bos genus, i.e. mainly bovine and zebu cattle. MmmSC (bovine biotype) has been isolated from buffaloes in Italy (Bubalus bubalus) (36), and from sheep and goats in Africa and more recently in Portugal and in India (37). Among wild animals, one single case has been reported in American buffaloes (Bison bison) and none in African buffaloes (Syncerus caffer) or other wild ruminants. Wild animals do not play a role in the epidemiology of the disease. CBPP is manifested by anorexia, fever and respiratory signs, such as dyspnoea, polyppnoea, cough and nasal discharges. In the case of acute outbreaks under experimental conditions, the mortality rate may be as high as 50% in the absence of antibiotic treatment. When an outbreak first occurs in an area, the mortality will be high but is often lower in the field following the primary outbreak. Clinical signs are not
always evident; subacute or asymptomatic forms occur frequently as the clinical signs in affected animals subside with partial recovery. In this case their lungs show typical encapsulated lesions called ‘sequestra’. These animals may be responsible for unnoticed persistence of the infection in a herd or a region and play an important role in the epidemiology of the disease. Transmission of the disease occurs through direct contact of an infected animal with a naive one. There is no evidence of transmission through fomites as _MmmSC_ does not persist in the environment. In most continents, control strategies are based on the early detection of outbreaks, control of animal movements and a stamping-out policy. In Africa control of the disease is based on vaccination campaigns using attenuated _MmmSC_ strains such as T144 or T1sr. Although the use of antibiotics is theoretically prohibited, they are widely applied in the field. The consequences of these antibiotic treatments in terms of clinical efficacy, emergence of resistant strains, and persistence of chronic carriers have not been evaluated yet. However, recent work has shown that antibiotic treatment of cattle may greatly reduce the transmission to healthy contacts but this requires treatment of all affected cattle in a group (20). The _M. mycoides_ cluster consists of six mycoplasma species or groups of strains, originating from bovines and goats (11, 32, 39). This cluster can be subdivided in two groups, capricolum and mycoides, comprising very closely related species. These six mycoplasmas share serological and genetic characteristics, and this causes taxonomic and diagnostic problems (11) with standard techniques. Specific identification of _MmmSC_ can now be achieved by polymerase chain reaction (PCR) or the use of specific monoclonal antibodies (MAbs). Although _MmmSC_ has been considered to be a very homogeneous biotype, recent molecular techniques, such as enzymatic digestion of whole DNA or Southern blotting using an insertion element as a probe, were able to identify differences among strains. A recently described technique that provides an easier way to perform molecular epidemiology of CBPP is a multi-locus sequence analysis (or typing). This technique allows the three main lineages that correlate with the geographical origins (Europe, Southern Africa, rest of Africa) to be distinguished (24). Quite interestingly, the strains of European origin can be clearly differentiated from African ones (10, 16, 42). Recent European strains form a particular cluster and differ from all other strains by no duplication of a long 17 kb DNA fragment (15) and deletion of a 8.4 kb fragment. They are not able to oxidise glycerol, which may account for an apparent lower pathogenicity (19, 43). However, the oldest European strain kept in collection (1967) appears as an unique strain without the deletion and duplication. African strains seem to be more diverse. The sequence of the complete genome of the reference strain PG1 has been published recently (45). There is no doubt that further technical development will allow for a finer characterisation of strains.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

The causal organism can be isolated from samples taken either from live animals or at necropsy. Samples taken from live animals are nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing and pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs. Blood may also be cultured (21). Samples taken at necropsy are lungs with lesions, pleural fluid (‘lymph’), lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis. The samples should be collected from lesions at the interface between diseased and normal tissue.

The agent can be detected by culture, nucleic acid methods and immunological tests described below. Bacteriological identification of the agent is more complex and can be done by biochemical tests, nucleic acid recognition methods and immunological methods. These methods are described here in general terms; however, it is recommended that the definitive identification be done by an OIE Reference Laboratory.

The presence of pathogens varies greatly with the stage of development of the lesions, and a negative result is not conclusive, particularly after treatment with an antibiotic.

When dispatching samples to the laboratory, it is advisable to use a transport medium that will protect the mycoplasmas and prevent proliferation of other bacteria (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% serum, 0.3% agar, 500 International Units [IU]/ml penicillin, thallium acetate 0.2 g/litre).

The samples must be kept cool at 4°C if stored for a few days or frozen at or below −20°C for a longer period. For laboratory-to-laboratory transfer, lung fragments or pleural fluid can also be freeze-dried.

#### a) Culture

_MmmSC_ needs appropriate media to grow (35). In attempting isolation, 2–3 blind passages may be required. Many attempts to isolate fail because the organism is labile, is often present in small quantities, and is demanding in its growth requirements. The media should contain a basic medium (such as heart-infusion or peptone), yeast extract (preferably fresh), and horse serum (10%). Several other components can be added, such as glucose, glycerol, DNA, and fatty acids, but the effects vary with the strains. To avoid growth of other bacteria, inhibitors, such as penicillin, colistin or thallium acetate, are necessary. The media can be used as broth or solid medium with 1.0–1.2% agar. All culture media prepared should be subjected to quality control.
and must support growth of *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

After grinding in broth containing antibiotics, the lung samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and on to solid medium. The pleural fluid can be inoculated directly without previous dilution. Hermetic sealing of the Petri dishes or the use of incubators with controlled humidity are recommended in order to avoid desiccation. To ensure the best conditions for mycoplasma growth, a CO₂ incubator or candle jar should be used. The tubes and Petri dishes are inspected at day 5 and at day 10. In fluid medium, a homogeneous cloudiness usually appears within 2–4 days, frequently with a silky, fragile filament called a ‘comet’, which is characteristic of *Mmm*SC (or *M. capricolum* subsp. *capripneumoniae*, the cause of contagious caprine pleuropneumonia). During the following days a uniform opacity develops with whirls when shaken. On agar media, the colonies are small (1 mm in diameter) and have the classical appearance of ‘fried eggs’ with a dense centre. At this stage, the indirect fluorescent antibody (IFA) test or PCR can be performed.

b) **Biochemical tests**

For routine field use, the immunological tests and PCR are sufficient, but where these give dubious results, biochemical tests may be used. These biochemical tests should be carried out by a reference laboratory. For this purpose, after two or three subcultures, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original form in the medium without inhibitors. Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical tests (2, 14).

*Mmm*SC is sensitive to digitonin (like all members of the order Mycoplasmatales), does not produce ‘film and spots’, ferments glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties.

For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO [pleuropneumonia-like organisms] broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride solution for tetr İzolium reduction, plus a pH indicator (e.g. phenol red). (Note: a pH indicator should not be added to a medium containing triphenyl tetrázolium chloride.) For demonstration of proteolysis, growth is carried out on casein agar and/or coagulated serum agar.

Once the biochemical characteristics have been checked, one of the following immunological tests can be performed to confirm the identification: disk growth inhibition test (DGIT), fluorescent antibody test (FAT), and the dot immunobinding on a membrane filter (MF-dot) test. The isolation and identification of the CBPP agent can be difficult and time consuming and depends on careful use of the appropriate procedures and media. When possible, classical bacteriology laboratories should set up a special section for work only with mycoplasmas.

c) **Nucleic acid recognition methods**

Radiolabelled or enzyme probes have been developed, but have been superseded by the more convenient and safe PCR technology.

The PCR is sensitive, highly specific, rapid and relatively easy to perform. Primers specific for the *M. mycoides* cluster (38) and for *Mmm*SC (12, 29, 31) have been reported and PCR assays have been developed (5, 12, 29), including a new technique that permits the specific identification of the T1 vaccinal strains (25). Using samples such as lung exudate allows the PCR to be performed directly after differential centrifugations to remove inflammatory cells and pellet mycoplasmas. For lung fragments, the PCR is applied after DNA extraction. The PCR can also be performed on urine or blood. The main advantage of the PCR technique is that it can be applied to poorly preserved samples (contaminated or without any viable mycoplasmas as may occur following antibiotic treatment). If direct detection of DNA from the organ under test fails, specimens should be enriched by culturing them in an appropriate medium for 24–48 hours, followed by attempted detection of DNA from the culture. The PCR has become the primary tool for identification of *Mmm*SC. If a sample is PCR positive in a CBPP-free zone, the test should be confirmed by a second and different PCR; infection can be confirmed by the use of only one immunological test.

One of the problems with PCR is the possible occurrence of contamination if the necessary precautions and quality management system are not implemented correctly in the diagnostic laboratory. Great care must be taken to respect the strict separation between those parts of the laboratory that may be contaminated with PCR products (such as the electrophoresis room) and those parts of the laboratory devoted to preparing the PCR reagents.
The onset of real-time PCR assays should solve this possible troubleshooting as fluorescence resulting from genomic amplification is measured directly without opening the tubes. This technique has already been applied to \textit{MmmSC} detection (17) and further developments are expected in the near future.

d) Immunological tests

The aetiological agent or its antigens can be demonstrated by immunochromatographic tests on infected tissues, tissue fluids and/or cultures of the organism. However, as some of these tests are dependent on a minimum number of organisms being present in the sample, only positive results are taken into account.

i) \textit{Indirect fluorescent antibody test}

The IFA test can be performed on smears from clinical material using hyperimmune rabbit serum against \textit{MmmSC} and labelled anti-bovine IgG. Hyperimmune bovine serum has been used, but may have cross-reactive antibodies. The test is satisfactory when applied to pleural fluid smears, but is less satisfactory with lung smears due to considerable nonspecific fluorescence. However, good results can be obtained using lung smears counterstained with Eriochrome black.

ii) \textit{Fluorescent antibody test}

The FAT is commonly performed on broth and agar cultures. It is slightly less specific than the IFA test.

\textbf{Broth culture}: Place two drops on a microscope slide. Fix for 15 minutes with methyl alcohol, and leave in contact with the labelled hyperimmune serum for 30 minutes at 37°C in a humid chamber. Rinse three times with phosphate buffered saline (PBS, pH 7.2) and examine under an epifluorescence microscope (×80).

\textbf{Colonies grown on solid medium}: Cut a block of agar supporting a number of young colonies and place on a slide with the colonies facing upwards. Place one or two drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes. Place the block into a tube and wash twice for 10 minutes with PBS. Place the block on a slide with the colonies facing upwards and examine as before.

\textbf{Petri dish culture}: The gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel three times with PBS, flood the surface with 1 ml of labelled serum and incubate for 30 minutes in a humid chamber. Rinse four times with PBS and examine directly under the microscope. The FAT in a Petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species.

\textbf{Interpretation of the FAT}: With broth culture, the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out with colonies on agar, because the background appears dark green.

iii) \textit{Disk growth inhibition test}

The DGIT is based on the direct inhibition of the growth of the agent on a solid medium by a specific hyperimmune serum (14). However, cross-reactions within the mycoides cluster are common and great care should be taken to differentiate \textit{MmmSC} (bovine biotype) from \textit{MmmLC} (caprine biotype; LC: large colonies). It is a simple test to perform, but some results require experience to be interpreted: small inhibition zones (less than 2 mm wide), partial inhibition with ‘breakthrough colonies’, false-negative and false-positive reactions (very rare). The quality of the hyperimmune serum used in this test is critical for good results.

iv) \textit{Agar gel immunodiffusion test}

The agar gel immunodiffusion (AGID) test can detect the specific antigen present at the surface of \textit{MmmSC} and the circulating galactan invading the haemolymph system of sick animals (18). Pleural fluid, ground lung fragments or even sequestra can be tested against a hyperimmune serum in two wells cut 5 mm apart in the gel. The gel is composed of Noble agar (12 g) and thallium acetate (0.2 g/litre) in PBS, pH 7.2 (1000 ml). The test is considered to lack sensitivity and is known about its specificity, but it has served as a screening test and only positive reactions should be taken into account. The results are better when the plate is incubated at 37°C and can be read within 24 hours. A simpler field test has been developed using impregnated paper discs instead of wells (34).

v) \textit{Dot immunobinding on membrane filtration}

The MF-dot test can be used for routine identification tests in the laboratory (33). Specific SC biotype specific MAbs have been developed to overcome cross-reactions within the mycoides cluster (8).
vi) **Immunohistochemistry**

*Mmm*SC immunoreactive sites can be detected in lung lesions using the peroxidase–antiperoxidase method on sections of paraffin-embedded material (13). Because the isolation of the agent is not always achieved from chronic cases and after treatment with antimicrobial drugs, this test is only supplementary to the diagnosis of CBPP (6); a negative result is not conclusive.

### 2. Serological tests

Serological tests for CBPP are valid at the herd level only. Tests on single animals can be misleading, either because the animal is in the early stage of disease, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive.

**a) Complement fixation (a test suitable for determining freedom from disease and a prescribed test for international trade)**

The Campbell & Turner complement fixation (CF) test remains the recommended procedure (although the current method is slightly different from the original one), and it is widely used in all countries where infection occurs (35). The CF test, as a micromethod, has been harmonised in the European Union (27). For antigen titration and harmonisation purposes, an international standard positive bovine serum is available from the OIE Reference Laboratory in Teramo, Italy. However, the CF test is still difficult to perform, requiring well-trained and experienced personnel.

- **Reagents**
  - i) **Veronal buffer (VB), pH 7.3.** A concentrated stock solution is used diluted 1/5 in sterilised double-distilled water.
  - ii) **The serum samples, free from erythrocytes, must be inactivated at 56°C for 30 minutes and diluted 1/10 in VB.**
  - iii) **The antigen is a suspension of *Mmm*SC, previously checkerboard titrated and used at a dose of 2 complement fixing units (CF units). It must be kept at 4°C and not frozen. It is produced, tested and delivered by Internationally recognised laboratories.**
  - iv) **The complement (C') is obtained from normal guinea-pig serum. It is freeze-dried and reconstituted with double-distilled water. It must be kept at –20°C after reconstitution. It is titrated by making a close dilution series in VB containing an appropriate quantity of the antigen to be used in the test. After incubation at 37°C for 2 hours, an appropriate quantity of sensitised sheep red blood cells (SRBC) is added to each dilution. The titration is read after incubation for a further hour. The highest dilution giving complete haemolysis of the SRBC equals 1 C’ unit, from which can be calculated the dilution required for 2.5 units in 25 µl.**
  - v) **The haemolysin is a hyperimmune rabbit serum to SRBC. The quantity used is 6 haemolytic doses read at 50% end-point (HD50 [50% haemolysing dose]).**
  - vi) **The SRBC are obtained by aseptic puncture of the jugular vein. They can be preserved in Alsever’s solution or with sodium citrate. They are used in a 6% suspension.**
  - vii) **The haemolytic system (HS) is prepared by diluting haemolysin in VB to give a dose of 12 HD50. An equal volume of 6% SRBC suspension is added, and the system is sensitised in a water bath at 37°C for 30 minutes with periodic shaking.**
  - viii) **The positive bovine standard sera has been obtained from a naturally infected animals negative to antibodies against *Brucella*, bovine viral diarrhoea virus, respiratory syncytial virus, infectious bovine rhinotracheitis virus, adenovirus, bovine herpes virus 4, foot and mouth disease viruses, bovine leukosis virus, and parainfluenza 3 virus. The infected animals are also negative for adventitious viruses.**
  - ix) **The negative control serum (NS) is a healthy bovine serum, negative to the above microrganisms.**

- **Test procedure (using microplates)**
  - i) **Dispense 25 µl of the test serum samples (already diluted 1/10). Add 25 µl of antigen at a dose of 2 CF units.**
  - ii) **Add 25 µl of C’ at a dose of 2.5 units. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.**
  - iii) **Add 25 µl of HS. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.**

It is necessary to set up the following controls:

- **Complement:** 0.5 units, 1 unit and 2.5 units.


**Haemolytic system:** 75 µl of VB + 25 µl of HS.

**Antigen:** 25 µl of 2 CF units of antigen + 25 µl of C’ at 2.5 units + 25 µl of HS = 25 µl of veronal buffer.

Note: the microplates must be shaken vigorously twice during the incubation period. The above-mentioned controls, the PS and the NS are always used in each microplate or in a series of microplates where the same batches of reagents are used.

**iv) Reading and interpreting the results:** After centrifugation of the microplates at 125 g for 2 minutes, the reading is carried out based on the percentage of complement fixation observed.

Positive result: 100% inhibition of haemolysis at 1/10;

Doubtful results: 25, 50 or 75% inhibition of haemolysis at 1/10.

It is recommended that any fixation of complement, even partial (25, 50 or 75%), at a serum dilution of 1/10 should be followed by additional investigations.

The limitations of the CF test are well known. With a sensitivity of 70% and a specificity of 98% (7), the CF test can detect nearly all sick animals with acute lesions, but a rather smaller proportion of animals in the early stages of the disease or of animals with chronic lesions. In addition, therapeutic interventions and improperly conducted prophylactic operations (partial slaughter of the herd) may increase the number of false-negative reactions. However, for groups of animals (herd or epidemiological unit) the CF test is capable of detecting practically 100% of infected groups.

The nature of the pathogenesis of the disease is such that the incubation period, during which antibodies are undetectable by the CF test, may last for several months.

Despite the high specificity of the CF test, false-positive results can occur, of which an important cause is serological cross-reactions with other mycoplasmas, particularly other members of the *M. mycoides* cluster. The validity of the results has to be confirmed by post-mortem and bacteriological examination, and serological tests on blood taken at the time of slaughter.

**b) Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)**

A competitive enzyme-linked immunosorbent assay (C-ELISA) developed by the OIE Collaborating Centre for the diagnosis and control of animal diseases in tropical countries (see Table given in Part 3 of this Terrestrial Manual) (23), has undergone evaluation (3). An indirect ELISA based on the use of a lipoprotein antigen is currently being validated by the IAEA (1, 9). In May 2004, the C-ELISA was designated as an OIE prescribed test for international trade by the OIE International Committee. Compared with the CF test, the C-ELISA has equal sensitivity and greater specificity. Advice on the availability of reagents can be obtained from the OIE Reference Laboratories for CBPP, or the OIE Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis (see Table given in Part 3 of this Terrestrial Manual).

Validation tests (3, 23) that have been carried out in several African and European countries would indicate i) that the true specificity of the C-ELISA has been reported to be at least 99.9%; ii) that the sensitivity of the C-ELISA and the CF test are similar; and iii) antibodies are detected by the C-ELISA in an infected herd very soon after they can be detected by the CFT and C-ELISA antibody persists for a longer period of time (30).

This C-ELISA is now provided as a ready made kit that contains all the necessary reagents including precoated plates kept in sealed aluminium foil. The kit has been especially designed to be robust and offer a good repeatability. As a consequence, sera are analysed in single wells. The substrate has been modified and is now TMB (tetramethyl benzidine) in a liquid buffer and the reading is at 450 nm. The substrate colour turns from pale green to blue in the first place and becomes yellow once the stopping solution has been added. MAb controls exhibit a darker colour while strong positive serum controls are very pale. The cut-off point has been set at 50% and should be valid in every country.

- **Reagents**
  i) Stock antigen is prepared by washing a concentrated suspension of mycoplasma (2 mg/ml) and lysis with sodium dodecyl sulphate at 0.1%. The stock is kept at –20°C until use.
  ii) MAbS are available from the OIE Collaborating Centre for the Diagnosis and Control of Animal Diseases in Tropical Regions (see Table given in Part 3 of this Terrestrial Manual).
  iii) The conjugate DAKO P260 is diluted in PBS according to the manufacturer's instructions, with the addition of 0.5% horse serum and 0.05% Tween 20.
iv) Substrate is made of 1 mM ABTS (2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulphonic acid]) and H$_2$O$_2$ in citrate buffer.

- **Test procedure**
  
i) ELISA plates are coated with a lysed antigen solution in PBS, pH 7.4 (100 µl/well) and incubated overnight at 4°C.
  
ii) The plates are washed once in PBS diluted 1/5 with 0.05% Tween 20.
  
iii) Sera that have not been heat inactivated (diluted 1/10) and MAb diluted in PBS with 0.5% horse serum and 0.05% Tween 20 are left in contact with the antigen for 1 hour at 37°C under moderate agitation in a humid chamber. Heat-inactivated serum will not give satisfactory results.
  
iv) The plates are washed twice and conjugate is added to all the wells (100 µl); the wells are then incubated for 1 hour at 37°C.
  
v) The plates are washed three times and the substrate is added to all the wells (100 µl).
  
vi) Reading is performed at 405 nm when the absorbance in the control MAb has reached 0.8–1.6.

c) **Immunoblotting test**

An immunoenzymatic test designated the immunoblotting test (IB test) has been developed and is of diagnostic value. A field evaluation indicated a higher sensitivity and specificity than the CF test. A core profile of antigenic bands, present both in experimentally and naturally infected cattle are immunodominant. The more accurate picture of the immune status of animals given by this test is due to the possibility of a more precise analysis of the host’s immune response in relation to the electrophoretic profile of $Mmm$SC antigens; thus the test overcomes problems related to nonspecific binding. It should be used primarily as a confirmatory test, after other tests and should be used in all cases in which the CF test has given a suspected false result.

- **Preparation of antigen strips**
  
i) The antigen is prepared by harvesting and washing a suspension of mycoplasma cells obtained from a 48-hour culture.
  
ii) A 4% stacking/5–15% gradient-resolving SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel) is prepared and used to perform electrophoresis of the sample with appropriate molecular weight standards.
  
iii) The separated proteins are transferred to a 14 × 14 cm 0.45 µm nitrocellulose membrane at 70 V constant voltage in transfer buffer (20% methanol in 193 mM glycine, 25 mM Tris/HCl, pH 8.3).
  
iv) The membrane is dried and labelled on the side on which the proteins were electrophoresed. The nitrocellulose membrane is incubated in blocking buffer (PBS containing 5% skim milk, 1 M glycine and 1% egg albumin) for 2 hours at room temperature. After washing at room temperature for three 15-minute washes in 0.1% (v/v) Tween 20 in PBS, the nitrocellulose membrane is then washed again in PBS alone. The sheet is then dried and one strip cut and tested from the edge of the membrane. Specific bands are identified at 110, 98, 95, 62/60 and 48 kDa.
  
v) The nitrocellulose membrane sheet is cut into strips, 0.4 cm wide and each strip is labelled. These strips are the antigen used for blotting.

- **Test procedure**

NB: The strips must be kept with the antigen side up during the procedure.

i) Serum samples for testing are diluted 1/3 and positive and negative control sera are prepared using dilution buffer (PBS containing 0.1% skim milk and 0.1% egg albumin).

ii) An antigen strip is placed in each test sample (and controls) and incubated at 37°C for 2 hours with continuous agitation. Strips are then washed, as above.

iii) Strips are incubated for 1 hour at room temperature in an appropriate dilution of peroxidase-conjugated anti-bovine IgG (H + L chains) in dilution buffer, with continuous agitation. Wash as above.

iv) Substrate is made by adding 30 mg 4-chloro-1-naphthol dissolved in 10 ml methanol to 50 ml PBS and 30 µl H$_2$O$_2$. Substrate is added to the strips, which are then left in the dark with continuous agitation and examined periodically until the protein bands are suitably dark. The reaction is stopped with distilled water.
v) Reading the results: The strips are dried and examined for the presence of the core IgG immunoblot profile of five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. Sera giving a similar immunological profile are considered to be positive.

d) Other tests
A rapid field slide agglutination test (SAT) with either whole blood or serum (41) has been developed to detect specific agglutinins: the antigen is a dense suspension of stained mycoplasmas that is mixed with a drop of blood or serum. Due to a lack of sensitivity, the test detects only animals in the early stages (i.e. acute phase) of the disease. It should be used only on a herd basis. A latex agglutination test has been developed that is easier to interpret than the SAT (4).

For CBPP, the CF test and ELISAs can be used in screening and eradication programmes, but the highly specific IB test should be used as a confirmatory test. However, the IB test is not fit for mass screening and may be difficult to standardise in countries with marginal laboratory facilities so IB testing should be performed in a reference laboratory.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Since the beginning of the 20th century, many vaccines against CBPP have been described (e.g. killed vaccines, and heterologous vaccines), but none of them has proven to be really satisfactory. Today, the only vaccines commonly used are produced with attenuated MmmSC strains.

1. Seed management

a) Characteristics of the seed
Two strains are used for preparing CBPP vaccines: strain T1/44, a naturally mild strain isolated in 1951 by Sheriff & Piercy in Tanzania, and strain T1sr (44, 46). The 44th egg-passage of strain T1, called T1/44, was sufficiently attenuated to protect cattle without post-vaccinal severe reactions, however such reactions may still occur in the field although rarely. Their frequency is unpredictable. Cattle breeds should be assessed for their sensitivity before mass vaccination. It should be noted that when given by intubation, the vaccine can produce CBPP lesions (28); however, as the vaccine is to be injected subcutaneously, this should not create a serious disease problem (22).

The identity of the strain can be verified with the insertion sequence profile or by the specific PCR assay (25).

The master seed strain is kept in freeze-dried form at –20°C. It is deposited at an international laboratory from African Unity, PANVAC.

b) Method of culture
For vaccine production, a system of freeze-dried seed lots originating from master seed cultures is used. These seed lots are kept at –20°C.

The media used for seed cultures are usually the same than for batch production. However there is no specific requirement, they should ensure a correct growth of the vaccine strain.

For vaccine bulk cultures, in order to avoid the risk of inadvertent cloning of vaccine seed, the whole content of a vaccine seed vial should be inoculated directly into a tube filled with production medium. A second tube may be seeded as a dilution from the first one.

2. Method of manufacture

The media used for vaccine production may differ slightly from media for isolation purpose. In the case of a vaccine production, what matters more is the final titre that can be obtained rather than the speed of growth. Furthermore the harvested mycoplasmas should withstand the freeze drying process without excessive titre loss.

Examples of vaccine culture medium are Gourlay’s medium or F66, however modifications of these media are licit and may include addition of buffers.

Vaccine bulk cultures must be obtained with a maximum of three successive passages of the seed. A passage is defined here by a 1/100 dilution of a culture in the exponential phase of growth.
For example, 0.5 ml of culture from the seed are transferred to 50 ml of fresh medium and, when turbidity is observed, these 50 ml are used to seed 5000 ml of medium, which represents the final product when the optimum titre has been reached. Each vaccine producer should then evaluate the speed of growth of the vaccine strain in the medium that is used to optimise the harvest time.

A stabiliser can be added to final cultures before freeze drying. The manufacturer should ensure an homogeneous distribution in the vials and use of a proper freeze dryer to have identical titres in all the vials when the freeze drying process is finished.

3. In-process control

Good Manufacturing Practice should be observed to avoid contaminations at each step of the production and to ensure purity of the final product.

As an example, phase contrast microscopic examination of cultures easily allows the detection of contaminations by bacteria or fungi.

4. Batch control

a) Purity and identity

Suitable media must be seeded with the final product to ensure purity of the final product and absence of contamination with classical bacteria and fungi. All media should remain sterile (35). Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

Absence of contamination by other mycoplasmas must be checked. For example a growth inhibition test with the final product and a hyperimmune serum to MmMSC (preferably raised with T1/44 antigen) can be performed. The presence of mycoplasma colonies within the inhibition zone must be followed by identification of these colonies to rule out the presence of other mycoplasmas than the vaccine strain.

The identity of the vaccine strain present in the final product must be guaranteed by the producer.

For example a specific PCR can be used to identify T1 strains. In addition streptomycin resistance can be used to differentiate T1/44 from T1sr.

b) Titration

The minimum titre is $10^7$ live mycoplasmas per vaccine dose, but higher titres are recommended because of the loss of titre between production plant and actual injection to animals. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination and preferably with the diluent provided by the vaccine manufacturer. Titrations should be performed on at least three vials per batch. This titre must be evaluated with a titration technique that allows a precision of ±0.25 logs. A batch passes the test if three vials chosen randomly have titres above this limit. The manufacturer must ensure that the minimum titre is retained until the expiry date if the product is kept at the correct temperature.

c) Safety

After reconstitution, the vaccine is inoculated subcutaneously into two mice, intraperitoneally into two mice and intraperitoneally into two male guinea-pigs. None of the animals should die within the following month, and the guinea-pigs should not show signs of orchitis. Safety tests should be carried out on (at least two) cattle or zebu cattle. These are inoculated with ten vaccinal doses each, and observed for adverse effects for at least 4 weeks.

d) Potency

Potency tests are not performed routinely with production batches as there is no laboratory animal that would allow this test to be performed at low cost. Potency tests in cattle are also not performed because of the cost. Getting statistically significant protection rates would involve using at least 50 naïve animals.

Potency of the final product is ensured by using a master seed lot of well known origin for which the potency test has already been performed, by strictly following the production standard protocols (avoiding multiple passages) and by ensuring that the final titres are correct.

e) Duration of immunity

Strain T1/44 confers protection for approximately 1 year (21), but the protection conferred by the T1sr strain may only be 6 months long. Serological conversion (CF test) takes place in some animals. The antibodies disappear 3 months after vaccination.
f) **Stability**

Periodic titration of the stored vaccine allows the shelf life to be calculated. Freeze-dried vaccine must be stored at −20°C. At this temperature its storage life is at least 1 year (35). Viability may even be conserved for many years without loss of titre allowing for the constitution of emergency stocks. The titres of these stocks naturally need to be controlled regularly.

g) **Preservatives**

For lyophilisation, stabilisers can be added. For example, dried skimmed milk can be added: 45 g/litre of culture medium. For reconstitution of a freeze-dried vaccine normal sterile saline solution (9 g/litre) is preferably used. Alternatively, a molar solution of magnesium sulphate (248 g per litre) is used at room temperature. This molar solution protects mycoplasmas against inactivation by heat (35). The purity of the salts used is important. When using magnesium sulphate as a diluent for vaccine reconstitution, it is also important to insure that the pH of the final product does not drop below 6.5 as this may induce a loss of titre (26).

h) **Precautions (hazards)**

 Procedures for use in the field and reconstitution of freeze-dried vaccines have been described by Provost et al. (35).

Intense reactions may appear when infected animals are vaccinated, as occurred recently following emergency vaccination campaigns in East Africa. These reactions usually occur within 2–3 days. Local reactions may also appear at the site of injection after 2–3 weeks with strain T1/44. These reactions are known as a 'Willems reaction', and consist of an invading oedema that leads to death if antibiotic treatment, such as tetracyclin or tylosin, is not given. Strain T1sr is completely devoid of residual pathogenicity, which makes it an alternative choice to T1/44, although the duration of immunity is shorter. Concerns were raised about the ineffectiveness of T1sr to control outbreaks in southern Africa leading to its suspension (40).

The general sensitivity of a given bovine population should be first tested by vaccinating sample groups (35).

5. **Tests on the final product**

These tests should be performed after reconstitution of a pool of at least five vials of the freeze-dried vaccine in the recommended diluent.

a) **Safety**

Safety tests should be carried out on cattle or zebu cattle, according to Section C.4.c.

b) **Potency**

The test is carried out according to the protocol described in Section C.4.d. Because CBPP cannot be easily reproduced experimentally, and due to its cost, only one potency test need be performed on each seed lot, providing the titre is satisfactory and that production parameters have not been changed.

**REFERENCES**


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Chapter 2.4.9. - Contagious bovine pleuropneumonia


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**NB:** There are OIE Reference Laboratories for Contagious bovine pleuropneumonia (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).