CONTAGIOUS BOVINE PLEUROPNEUMONIA (INFECTION WITH *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES*)[1]

SUMMARY

Contagious bovine pleuropneumonia (CBPP) is a disease of ruminants (Bos *and* Bubalus *genuses) caused by* Mycoplasma mycoides *subsp.* mycoides (Mmm). *It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges in bovines. Definite diagnosis requires the isolation or detection of the aetiological agent. The main problems for control or eradication are the frequent occurrence of subacute or subclinical infections, the persistence of chronic carriers after the clinical phase and the lack of extensive vaccine coverage.*

Detection 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.

For cultivation of the pathogen, the tissues are ground in buffered solution and inoculated into selective broth and solid media with antibiotics or other inhibitors to prevent the growth of cell-walled bacteria. The growth of Mmm *can take up to 10 days, depending upon the type of sample and the mycoplasma titre.*

In broth, growth is visible as a homogeneous cloudiness which forms swirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical 'fried-egg' appearance. The biochemical characteristics of Mmm *are the following: sensitivity to digitonin, reduction of tetrazolium salts, fermentation of glucose, absence of arginine hydrolysis, and the absence of or very slight phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis may be confirmed by immunological tests, such as the growth inhibition and immunofluorescence tests (both use hyperimmune sera). The polymerase chain reaction is a rapid, specific, sensitive and easy-to-use test.*

Serological tests: For diagnosis, the modified Campbell & Turner complement fixation test remains a suitable test for certifying individual animals prior to movement, including for international trade. The competitive enzyme-linked immunosorbent assay is also a suitable test for certifying individual animals prior to movement. An immunoblotting test has undergone evaluation and is highly specific and sensitive.

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

A. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an infectious and contagious respiratory disease of *Bovidae* caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) with a major impact on livestock production and a potential for rapid spread. As a result, CBPP-infected countries are excluded from international trade of live animals.

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[1] After taxonomic revision of the *Mycoplasma mycoides* cluster by Manso-Silvan *et al*. (2009) the designations Small Colony (SC) and Large Colony (LC) are no longer used.

Mmm is a mycoplasma, i.e. a wall-less bacteria (mollicute), belonging to the so-called "mycoides cluster" that groups five mycoplasma species that are ruminant pathogens (Manso-Silvan *et al*., 2009). These five mycoplasmas share phenotypic and genotypic characteristics that cause cross-reactions in conventional diagnostic techniques. The closest relative to *Mmm* is *M. mycoides* subsp *capri* (*Mmc*), which is usually found in goats.

In natural conditions, *Mmm* affects only the ruminants of the *Bos* genus, i.e. mainly bovine and zebu cattle but also the yak (*Bos grunniens*) and water buffaloes (*Bubalus bubalis*) (Santini *et al*., 1992). *Mmm* has been isolated from sheep and goats in Africa, in Portugal and in India (Srivastava *et al*., 2000). 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. Small ruminants and wild animals do not play a role in the epidemiology of the disease, and CBPP is not a zoonotic agent.

The incubation period for naturally infected animals can range from 3 weeks to 6 months. The clinical manifestations in cattle range from hyperacute through acute, subacute and chronic forms.

CBPP is manifested by anorexia, fever and respiratory signs, such as dyspnoea, polypnoea, cough and nasal discharges during the acute stage of the disease when the causative agent can spread rapidly; in the chronic stage there may be long-term persistence of the agent. Typical lesions include a unilateral pneumonia associated with pleurisy. During the chronic stage of the disease, clinical signs wane and infected animals are more difficult to identify. In these cases, lungs may contain typical encapsulated lesions called sequestra. These 'silent' carriers may be infectious and thus responsible for unnoticed persistence of the infection in a herd; they play an important role in the maintenance and in the epidemiology of the disease.

CBPP has been unequivocally identified in Europe since the 18th century and it gained a world-wide distribution during the second half of the 19th century through cattle trade. CBPP was eradicated from many countries at the beginning of the 20th century, mostly through stamping-out strategies (UK, USA) or by vaccination campaigns followed by stamping-out strategies (Australia). Today, CBPP remains enzootic in many Sub-Saharan African countries, while in Europe the last CBPP cases were observed in Portugal in 1999. The situation in some Asian countries is unclear. Please consult WOAH WAHIS interface[2] for latest disease situation.

In the field, CBPP might be confused with other diseases causing respiratory problems such as pasteurellosis or other mycoplasmosis. The absence of confirmatory diagnosis may lead to antibiotic treatments being used in the case of CBPP outbreaks.

There is no known risk of human infection with *Mmm*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.* In CBPP-free areas, it is advisable to manipulate *Mmm* in biosafety level (BSL) 2 laboratories, while BSL1 would be sufficient in enzootic zones. Good laboratory practices should be used for all procedures.

B. DIAGNOSTIC TECHNIQUES

Clinical diagnosis of CBPP is unreliable as initial signs may be slight or non-existent and may be indistinguishable from any severe pneumonia. Therefore, CBPP should be investigated by pathological, microbiological, molecular or serological diagnostic methods. As the pathological lesions of CBPP are distinctive, and pathognomonic, abattoir surveillance for CBPP involving lung examination is a practical method for disease monitoring.

It is recommended to isolate and identify the causative organism in order to confirm an outbreak. Table 1 lists the laboratory methods used for the diagnosis of CBPP.

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2   http://www.woah.org/en/animal-health-in-the-world/the-world-animal-health-information-system/the-world-animal-health-information-system/

Table 1. Laboratory methods currently used for diagnosis of CBPP and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination*
Detection of the agent[a]						
In-vitro culture isolation (followed by species identification tests)	+	–	–	+++	–	–
Direct molecular test (PCR)	–	–	–	++	–	–
Detection of immune response						
CFT	+++	++	+++	++	+++	–
Immunoblotting	++	++	++	++	++	–
C-ELISA	+++	++	+++	++	+++	–

*NB: at present, there is no test described in the table that allows evaluation of the immune status
of an animal after vaccination, with the current T1 strains.

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; CFT = complement fixation test;
C-ELISA = competitive enzyme-linked immunosorbent assay.

[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Samples

A key to isolation success lies in collecting good quality samples. *Mmm* 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. 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 lung samples should be collected from lesions at the interface between diseased and normal tissue.

When collecting nasal swab samples, a transport medium should be used to protect the mycoplasmas and prevent proliferation of cell-walled bacteria and fungi (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% heat-treated serum (horse or pig), 0.3% agar, 500 International Units [IU]/ml penicillin, 0.2 g/litre thallium acetate).

After collection, all samples must be kept refrigerated at 4°C and sent to the laboratory within 24 hours. For longer periods they should be frozen at or below –20°C.

1.2. *In-vitro* culture

The presence of the pathogen varies greatly with the stage of development of the lesions and a negative result is not conclusive, particularly if the animal was treated with an antibiotic.

Mmm needs selective media to grow, but it is not considered a fastidious mycoplasma. There are several media compositions used in different reference laboratories but, essentially, they should contain: a basic medium such as heart-infusion broth or PPLO broth (pleuropneumonia-like organisms), 1–2.5% yeast extract, 10–20% inactivated horse serum, 0.1% glucose, 1% tryptose, and 0.0024% DNA. To avoid growth of other bacteria, the media can also contain an antibiotic of the penicillin family (for example, 500 IU/ml penicillin G) as mycoplasmas are naturally resistant. The media should be used both as broth and solid. All culture media prepared should be subjected to quality control and must support growth of low passage *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are performed correctly.

After grinding with broth, the tissue samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and onto five solid media plates. To avoid contaminating bacteria and to reduce the number of tubes and plates per sample, the supernatant of the ground sample may be filtered through a 25-mm filter with 0.45 pore size. The pleural fluid can be inoculated directly without prior dilution or filtration as, when infected, it is almost a pure culture of *Mmm*. To ensure the best conditions for mycoplasma growth, the tubes and plates are incubated at 37°C in a 5% CO_2 atmosphere and should be inspected daily for up to 10 days. After this time if there is no growth, the sample is considered negative. Positive samples in liquid medium show a homogeneous cloudiness, usually within 2–4 days. They frequently present a silky, fragile filament called a 'comet'. During the following days a uniform opacity develops which forms swirls 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, biochemical tests, the indirect fluorescent antibody (IFA) test or polymerase chain reaction (PCR) can be performed to identify the colonies.

1.3. Biochemical and immunological identification tests

Biochemical tests were used routinely in the past but have now been superseded by other tests, namely the PCR. Biochemical tests alone do not allow identification of a precise *Mycoplasma* species because of overlapping of the few phenotypic traits that can be evaluated. Therefore, molecular tests such as PCR are recommended for identification of isolates. The biochemical methods are given below for historic reasons.

Following subculture, 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, the organism can be identified using biochemical tests (Freundt *et al*., 1979).

Mmm needs cholesterol to grow and is, therefore, sensitive to digitonin (like all members of the order Mycoplasmatales), does not produce 'film and spots', ferments glucose, reduces tetrazolium salts (aerobically and anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic activity.

For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO 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 tetrazolium reduction, as well as a pH indicator (e.g. phenol red). (NOTE: a pH indicator should not be added to a medium containing triphenyl tetrazolium 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) (Freundt *et al*., 1979), fluorescent antibody test (FAT), IFA, agar gel immunodiffusion test (AGID) (Provost, 1972), or dot immunobinding on a membrane filter (MF-dot) test (Brocchi *et al*., 1993).

At present, few laboratories use immunochemical tests on a routine basis for identification of *Mmm* due to the development of PCR-based tests that are more specific, sensitive, rapid and easy to perform and standardise.

1.4. Molecular identification and typing methods – polymerase chain reaction (PCR)-based tests

See Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* for further details of implementation and validation of PCR-based tests.

PCR has become the method of choice for the rapid and specific identification of *Mmm* when the organism is isolated from a clinical sample. To avoid cross-contamination and carryover-contamination, strict separation of laboratory rooms, used for PCR preparation and handling of reactions, is needed. Various authors have developed a PCR system for *Mmm* identification and there is no preferred one, though the more sensitive, nested PCR should be avoided because of the higher risk of PCR product carryover, resulting in false positives. As the DNA target is not from a sample, where the number of cells vary and PCR inhibitors can be present, but from an isolate the sensitivity is not a critical point. Primers complementary to DNA regions CAP-21, *lppA* gene and 16S rRNA gene of the genome of *Mmm* have been designed by different authors and used in PCR systems, followed by restriction endonuclease analysis of the amplified product (amplicon) (PCR-REA) or by a second amplification (nested-PCR) (Table 2).

Table 2. Conventional PCR systems used for Mmm *identification*

Target DNA region*	Amplicon (bp)	Restriction enzyme	Hydrolyses products (bp)	Specificity	Reference
CAP-21 (position: 181628)	574	*Asn*I	379, 178 220, 178, 153	*Mmm* *Mmc*	Bashiruddin *et al.,*1994
(position: 443115)	275		43	*Mmm*	Dedieu *et al.*, 1994**
Gene *lppA* (nested)	717	–		*Mmm*	Miserez *et al.*, 1997***
(position: 20061)	503				

**Mmm* PG1 sequence Gene Bank accession number: NC005364
**Primers sequence: MSC1: ATA-CTT-CTG-TTC-TAG-TAA-TAT-G; MSC2: CTG-ATT-ATG-ATG-ACA-GTG-GTC-A
***Primers sequence: SC3NEST1-L: ACA-AAA-AGA-AGA-TAT-GGT-GTT-GG and SC3NEST1-R: ATC-AGG-TTT-ATC-CAT-TGG-TTG-G; SC3VII: ATT-AGG-ATT-AGC-TGG-TGG-AGG-AAC and SC3IV-S: TCT-GGG-TTA-TTC-GAA-CCA-TTA-T

As an example of one PCR system that is used in routine for *Mmm* identification, the PCR-REA procedure adapted from Bashiruddin *et al.,*1994, is provided below.

1.4.1. DNA extraction

Any accepted method for DNA extraction would be appropriate. A simple and effective method is to select a single colony, resuspend in 100 µl of PCR-grade water, boil for 15 minutes to lyse the cells and release the DNA, and centrifuge at 8000 *g* for 1 minute. The supernatant with the DNA will be used in the PCR reaction after diluting 1/10 in PCR-grade water. Alternatively 500 µl of a 4-day broth culture, from a single colony, is centrifuged at 8000 *g* for 1 minute, the pellet is resuspended in 100 µl of PCR-grade water and boiled and centrifuged as above. After diluting 1/10, 1 µl of the supernatant is used as template in the PCR.

1.4.2. PCR amplification

i) Preparation of the master mix

Synthetic oligonucleotides should be dissolved in TE buffer (Tris-EDTA [ethylene diamine tetra-acetic acid]) to a 100 µM concentration. This stock solution is stable at –20°C for at least 4 years. A working solution is prepared from the stock solution by a 1/2 dilution, to obtain a final concentration of 50 µM. In a final volume of 25 µl the PCR reaction should contain the following:

	Concentration	Volume in one reaction (total volume 25 µl)	Final concentration in the reaction
H$_2$O PCR-grade	–	16 µl	–
Reaction buffer without MgCl$_2$	10 ×	2.5 µl	1×
MgCl$_2$	25 mM	3 µl	3 mM
dNTPs	10 mM	1 µl	400 µM
Primer MM450*	50 µM	0.5 µl	1 µM
Primer MM451**	50 µM	0.5 µl	1 µM
Taq pol.	5U/µl	0.5 µl	2.5 U
template DNA	1 µl		

*Sequence: 5'-GTA-TTT-TCC-TTT-CTA-ATT-TG-3'
**Sequence: 5'-AAA-TCA-AAT-TAA-TAA-GTT-TG-3'

ii) Amplification conditions

One µl of template DNA of tested sample, or positive control (DNA from *Mmm*-type strain PG1) or negative control (water) is added to the mix. The amplification is performed under the following conditions: 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; and hold at 4°C indefinitely.

iii) Detection of amplified products

After amplification, the reactions should be opened in a separate room where the PCR products are detected by 1.5% agarose gel electrophoresis for 2 hours at 90 V. Positive reactions and the positive control, and not the negative control, should present an amplicon of 574 bp visible under UV light after staining with a suitable fluorescent dye.

1.4.3. Restriction endonuclease analysis (REA)

An enzymatic restriction of the PCR product is carried out in 10 µl reaction volume containing: 2 µl of PCR reaction, 5 U *Asn*I (2 µl), 1× buffer, at 37°C for 1 hour. The result is analysed in a 2% agarose gel electrophoresis for 1 hour at 100 V and the products visualised as above.

Identification is based on the sizes of the restriction products as presented in Table 2.

NB: Direct detection and identification of *Mmm* by PCR in clinical samples has not yet been fully evaluated and so the conventional PCR may be inadequate because of the presence of PCR inhibitors, fewer microorganisms in the sample and the presence of other bacteria, with a concomitant reduction in sensitivity and specificity. These problems should be avoided through the use of real-time PCR assays for *Mmm* detection, as fluorescence resulting from specific genomic amplification is detected and measured as the amplicon is being synthesised, and a 2–3 log increase in sensitivity, compared with conventional PCR, is obtained (Lorenzon *et al.,* 2008). When using samples such as pleural fluid, the PCR can be performed after boiling the sample and centrifuging to recover DNA in the supernatant. For lung fragments, the PCR is applied after DNA extraction procedures using an extraction kit or a phenol/chloroform extraction.

1.4.4. *Mmm* strain typing

Mmm strain typing has greatly benefited from advances in DNA sequencing. There are now a number of *Mmm* genomes that are available online[3]. However, the comparison of full *Mmm* genomes is not straightforward because of multiple genome rearrangements or DNA insertions. To overcome this problem while keeping the very fine differentiation of strains, an extended

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3   https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=656088

multilocus sequence typing (MLST) approach has been developed that allows unique identification of strains, phylogenetic analysis as well as molecular dating (Dupuy *et al.,* 2012).

2. Serological tests

Serological test results for CBPP should not be analysed and interpreted individually but in groups of animals from the same herd or region because false positive or false negative results may occur in individual animals. Tests on single animals can be misleading, either because the animal is in the early stage of disease, which may last for several months, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive. False-positive results can occur (2%), 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.

The complement fixation test (CFT) and enzyme-linked immunosorbent assays (ELISAs) are recommended for screening and eradication programmes. The highly specific immunoblotting test is useful as a confirmatory test but is not fit for mass screening.

2.1. Complement fixation (recommended for determining disease free status)

The modified Campbell & Turner CFT remains the recommended procedure and it is widely used in countries where infection occurs (Provost *et al*., 1987). The CFT is most conveniently carried out in a microtitre format and has been harmonised within countries of the EU (European Commission, 2001).

With a sensitivity of 63.8% and a specificity of 98% (Bellini *et al*., 1998), the CFT 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.

2.1.1. Reagents

For CFT validation and accreditation, the quality control and standardisation of all the reagents is a critical issue as well as the pipettes and tips that are used to dispense them. To facilitate the accreditation procedure (ISO/IEC[4] 17025) appropriate antigen and sera controls can be obtained from WOAH Reference Laboratories for CBPP.

The features of the following reagents, should be taken into account, as they have an impact in the final result.

i) Ultra-pure sterilised water. The quality of the water is critical for the development of the reaction.

ii) *Positive reference sera:* for harmonisation purposes a positive bovine reference standard serum (PRS) is available from the WOAH Reference Laboratories in Portugal and Italy, and should be used in the diagnostic laboratory for routine use and antigen titration. The PRS has been obtained from a naturally CBPP-infected bovine, and is negative against *Brucella*, *Mycobacterium paratuberculosis*, *Chlamydia*, *Coxiella burnetii*, *Leptospira*, 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, and should also be negative for adventitious viruses. The PRS presents 0% haemolysis in a dilution of 1/160 and 50% haemolysis in a dilution of 1/320. The PRS should be stored at –20°C in aliquots, this would prevent repetitive freeze–thawing, which causes deterioration of the serum.

iii) *Negative reference sera:* the negative control serum (NRS), also available from WOAH Reference Laboratories in Portugal and in Italy, is a serum from a healthy bovine from a bovine spongiform encephalopathy (BSE)-free source. It is CBPP free and should be negative against the above microorganisms. The NRS should be stored in aliquots at –20°C.

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4   ISO/IEC: International Organisation for Standardisation/International Electrotechnical Commission.

iv) *Antigen:* the test must use an antigen that has been prepared from a suspension of a selected *Mmm* strain essayed previously and presenting the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. The antigen is previously checkerboard titrated and used at a dose of 2 complement fixing units (CFU) and should be standardised to give 50% fixation, at a dilution of 1/320 of PRS. It must be stored at 4°C ± 3°C and should not be frozen. It is produced, standardised and delivered by WOAH Reference Laboratories. The use of an antigen standardised against the WOAH reference sera promotes international harmonisation of diagnostic testing.

v) *Buffer:* veronal buffered saline (VB), pH 7.2 ± 1, is the standard diluent for the CFT. The VB can be prepared from tablets commercially available or it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium chloride ($MgCl_2 \times 6\ H_2O$ – 0.501 g), and calcium chloride ($CaCl_2 \times 6\ H_2O$ – 0.18 g) in 1 litre of distilled water. A concentrated stock solution is used diluted 1/5 in double-distilled water, before use.

vi) *Haemolysin* (*amboceptor*): the haemolysin is a hyperimmune rabbit serum to SRBC (sheep red blood cells). The quantity used is 6 haemolytic doses read at 50% end-point (HD_{50} [50% haemolysing dose]). The SRBC are obtained by aseptic puncture of the jugular vein. They can be preserved in Alsever's solution or in sodium citrate. They are used in a 6% suspension. Haemolysin is also available commercially.

vii) *Haemolytic system* (*HS*): HS is prepared by diluting haemolysin in VB to give a dose of 12 HD_{50}. An equal volume of 6% SRBC antibody suspension is added, and the system is sensitised in a water bath at 37°C for 30 minutes with periodic shaking.

viii) *Complement* (*C'*): 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. A commercially produced complement can be used, according to the manufacturer's instructions. 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 30 minutes, an appropriate quantity of sensitised SRBC is added to each dilution. The titration is read after incubation for a further 30 minutes. 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.

2.1.2. Test procedure (using microplates)

The most critical factors for CFT performance are the control of the reagents and that it is carried out by trained and qualified personnel. Temperature and incubation times should also be carefully controlled. The entire procedure should be performed in a temperature-controlled room at 21°C ± 3°C.

Contaminated or haemolysed sera should not be tested, as this will interfere with the reaction. Undiluted test sera samples and appropriate working standards should be inactivated for 30 minutes in water bath at 56°C ± 2°C. This will destroy the complement of the sera and reduce or eliminate the anti-complementary activity (ACA).

Usually, only one serum dilution (1/10 in VB) is tested routinely but serial dilutions must be done for the positive control (PRS). Using standard 96-well microtitre plates with round (U) bottoms, the technique is performed as follows:

i) Dispense 25 µl of test serum inactivated and diluted 1/10 in VB in the wells of the first and second rows. The first row is an anti-complementary control for each serum.

ii) Volumes of 25 µl of antigen at a dose of 2 CFU are added to each well except the anti-complementary controls, to which 25 µl of VB should be added to compensate for lack of antigen.

iii) Add 25 µl of C' at a dose of 2.5 units. Shake gently and incubate at 37°C for 30 minutes with periodic (at least twice during the incubation period) or continuous shaking.

iv) Add 25 µl of HS. Shake gently and incubate at 37°C for 30 minutes. Microplates must be shaken twice during the incubation period.

v)   It is necessary to set up the following controls:

a)   C': 0.5 units, 1 unit and 2.5 units.

b)   HS: 75 µl of VB + 25 µl of HS.

c)   Antigen: 25 µl of 2 CFU of antigen + 25 µl of C' at 2.5 units + 25 µl of HS = 25 µl of VB.

These controls, along with the positive control serum (PRS) and the negative control serum (NRS), should always be used in each microplate or in a series of microplates where the same batches of reagents are used.

vi)   *Interpretation of results*

After centrifugation of the microplates at 125 g for 5 minutes, the analysis is carried out based on the percentage of complement fixation observed.

Positive result: 100% fixation at 1/10;

Doubtful results: 25, 50 or 75% fixation at 1/10.

vii)   *Validation of results*

Expected results for the plate controls are as follows:

a)   PRS: expected titre.

b)   NRS: 100% haemolysis

c)   Anticomplementary control of serum samples: 100% haemolysis

d)   Antigen control: 100% haemolysis

e)   Complement units' control: 100% haemolysis for 2.5 units

f)   HS control in absence of complement: 0% haemolysis

It is recommended that any CFT result, even partial (25, 50 or 75%), at a serum dilution of 1/10 be confirmed by additional investigations, such as immunoblotting or post-mortem examination and bacteriological tests according to contingency.

2.2. Competitive enzyme-linked immunosorbent assay

A competitive ELISA (C-ELISA) developed by the WOAH Collaborating Centre for the diagnosis and control of animal diseases in tropical countries[5] (Le Goff & Thiaucourt, 1998), has been validated internationally in accordance with WOAH standards (Amanfu *et al*., 1998). The performance of this C-ELISA method has also been validated by the French Committee for Accreditation in 2009.

Compared with the CFT, the C-ELISA has equal sensitivity and greater specificity. Advice on standard protocols and the availability of reagents can be obtained from the WOAH Reference Laboratories for CBPP, or the WOAH Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis (see Table given in Part 4 of this *Terrestrial Manual*).

Validation tests (Amanfu *et al.,* 1998; Le Goff & Thiaucourt, 1998) that have been carried out in several African and European countries indicated: 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 CFT 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 (Niang *et al.,* 2006).

To enhance its repeatability and the robustness, this C-ELISA is now provided as a ready-made kit that contains all the necessary reagents, including precoated plates kept in sealed bags. This kit can be obtained commercially and availability can be checked through the WOAH Reference Laboratory in France. The kit has been especially designed to be robust and offers a good repeatability. Sera are analysed in single wells. The chromogen is TMB (tetramethyl benzidine) in a liquid buffer and the reading

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5   See online list for contact details: https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3

is at 450 nm. The 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 has been set at 50% and should be valid in every country. However, each laboratory should establish its own measurement uncertainty in agreement with ISO 17025 requirements; see chapter 2.2.4 *Measurement uncertainty.*

2.3. Immunoblotting test

An immunoblotting test (IBT) is an immunoenzymatic test that has been developed to confirm doubtful CFT or C-ELISA results. A field evaluation indicated a higher specificity than the CFT enabling the detection of CFT false positives (Gonçalves *et al.* 1998).

2.3.1. Reagents

i) Antigen strips (see Section B.2.3.2 below).

ii) *Positive control serum:* serum from a naturally CBPP infected animal, used at a dilution of 1/100 in the IBT. This control presents 50% fixation at 1/80 dilution in the CFT and shows an immunoblotting profile with five specific *Mmm* antigenic bands of 110, 98, 95, 62/60 and 48 kDa. This serum is available from the WOAH Reference Laboratory in Portugal.

iii) *Dilution buffer:* phosphate-buffer saline (PBS), pH 7.2, containing 0.1% skim milk and 0.1% egg albumin. Skim milk quality is a critical issue, as it will influence the sensitivity of the reaction. Standardised skim milk should be used instead of common non-fat dry milks (Gaurivaud & Poumarat, 2012).

iv) *Peroxidase-conjugated anti-bovine IgG* (*H + L chains*): this is commercially available and should be previously checkerboard titrated against the positive control serum, to select the appropriate dilution at which the five specific bands are clearly visible (Gaurivaud & Poumarat, 2012).

v) *Substrate:* substrate is prepared by adding 30 mg 4-chloro-1-naphthol, dissolved in 10 ml methanol, to 50 ml PBS (pH 7.2) and 30 µl H_2O_2. Alternative substrates, such as BCIP/NTB (5-bromo-4-chloro-3-indolyl phosphate combined with nitrotetrazolium blue chloride), are available.

2.3.2. Preparation of antigen strips

i) The strain used to prepare the antigen is critical. Antigen should be prepared from a tested *Mmm* strain that must present the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. The antigen is a suspension of *Mmm* cells in PBS, pH 7.2, obtained from a 48-hour culture.

ii) The 5–15% polyacrylamide gel gradient used historically (Gonçalves *et al.* 1998) is not optimal for the repeatability of western blotting. The use of 7.5% polyacrylamide gel as recommended by Schubert *et al.* (2011) and Gaurivaud & Poumarat (2012), allows a good separation of all the five antigenic proteins and also a good repeatability of the pattern between electrophoresis runs.

iii) The separated proteins are transferred to a 14 × 14 cm 0.45 µm nitrocellulose membrane at 70 V in transfer buffer (20% methanol, 193 mM glycine, 25 mM Tris/HCl, pH 8.3). The effectiveness and homogeneity of the transfer have to be checked. This can be easily carried on with commercial staining solutions or kits that allow reversible staining and imaging of the blot prior to use.

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 three times for 15 minutes in 0.1% (v/v) Tween 20 in PBS, the nitrocellulose membrane is further washed in PBS. The membrane is then dried and one strip cut and tested for the presence of the five specific bands identified at 110, 98, 95, 62/60 and 48 kDa.

v) The nitrocellulose membrane is cut into strips, 0.4 cm wide and used for antibody testing. Each batch of strips should be tested, with a positive and negative reference serum, in order

to evaluate the clear presence of the five specific antigenic proteins and the absence of background. The resolution of the 95 and 98 kDa proteins should be carefully checked.

2.3.3. Test procedure

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

i)   Sera samples to be tested are diluted 1/3 in dilution buffer (see Section B.2.3.1.iii).

ii)   Each test sample together with the positive and negative control sera is placed in contact with the antigens strips and incubated at 37°C for 2 hours with continuous agitation. Strips are then washed at room temperature three times for 15 minutes each in 0.1% (v/v) Tween-20 in PBS, and further washed once in PBS.

iii)   An appropriate dilution of peroxidase-conjugated anti-bovine IgG (H + L chains) is incubated with the strips for 1 hour at room temperature with continuous agitation. Wash as above.

iv)   Substrate (see Section B.2.3.1.v) is added to the strips, which are then left in the dark with continuous agitation and examined periodically until the protein bands are visible (maximum 30–40 minutes). The reaction is stopped with distilled water.

v)   *Results analysis:* The strips are dried and examined for the presence of the core IgG immunoblot profile of the five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa that should be seen in the positive control serum. Samples presenting a similar immunobloting profile are considered to be CBPP positive.

NB: The IBT is rather difficult to standardise as many factors can influence the final banding pattern (Gaurivaud & Poumarat, 2012). Of major concern is the *Mmm* culture stage and the strain that has been chosen. Recent *Mmm* strains of European origin lack the 98 kDa band. This could lead to dubious results in animals infected by such strains. The CBPP WOAH Reference Laboratory in Portugal can provide antigen strips as well as the positive and negative control sera, upon request.

C. REQUIREMENTS FOR VACCINES

1. Background

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 completely satisfactory and cost effective. Today, the only vaccines commonly used are produced with attenuated *Mmm* strains.

Various attenuated *Mmm* strains have been used in the past and have been abandoned, such as the KH3J or V5 strains. Two strains are now used for preparing CBPP vaccines: strain T1/44, a naturally mild strain isolated in 1951 by Sheriff & Piercy in Tanzania (Sheriff & Piercy, 1952), and its derivative strain T1sr (Wesonga & Thiaucourt, 2000; Yaya *et al*., 1999). 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 (Mbulu *et al*., 2004); however, as the vaccine is to be injected subcutaneously, this should not create a serious risk (Hubschle *et al*., 2002).

T1/44 and T1sr vaccines can effectively protect herds when vaccinations are regularly performed (i.e. once a year for T1/44 and twice a year for T1sr). They can be used for CBPP control on a wider scale (national or regional), but they cannot lead to CBPP eradication when used alone.

The target species are the susceptible hosts of the *Bos* and *Bubalus* genuses.

New attenuated vaccines should have less residual virulence than T1/44 while maintaining or improving the same duration of immunity (1 year) and immunogenicity.

New inactivated vaccines should induce a significantly longer immunity (>1 year), should not hamper the detection of CBPP outbreaks and, ideally, should be compatible with other antigens to produce multivalent vaccines.

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 as manufacturers may be obliged to meet either European Pharmacopeia, United States (of America) Department of Agriculture or other national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

At present, only two *Mmm* attenuated strains are recommended for CBPP vaccination: T1/44 and T1sr. T1/44 was attenuated by passaging a mild field strain 44 times in embryonated eggs. This ensured an attenuation of the strain while keeping its immunogenic properties. However some residual virulence has been observed in this strain and the percentage of reactors varies greatly from one region to another. These reactions are usually observed in primo-vaccinated animals. It has been observed that immunogenicity declines as attenuation increases. Strain T1sr is a direct derivative of T1/44, adapted to streptomycin resistance by four serial passages in growth medium with increasing concentrations of streptomycin. T1sr has no residual virulence but induces shorter immunity (6 months)

The master seed used for vaccine production should be as close as possible to the original vaccinal strains. Grand parental stock of these strains are kept at the WOAH Reference Laboratory in France and at the WOAH Collaborating Centre for Quality Control of Veterinary Vaccines in Ethiopia.

The genome sequence of strain T1/44 was published in 2016 (Gourgues *et al.,* 2016).

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

It is recommended to cultivate the master seed in a suitable medium that does not contain any preservative such as antibiotics so as to allow it to be shown that the master seed stock is pure. The freedom from extraneous agents should be tested according to international or national guidelines. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

The purity and identity of the master seed is difficult to establish by conventional microbiological techniques. Notably, the morphological aspects of mycoplasma colonies on solid medium is not typical and may vary according to the composition of the medium (percentage of serum or agar for example). In addition it has been noted that T1/44 master seed could lead to colonies of different aspects maybe in relation to hypervariable antigens. Hence, the purity of the master seed should be established after a filter-cloning procedure and specific identification of at least 10 individual clones. T1 strains can be identified by a specific PCR (Lorenzon *et al.,* 2000). T1sr clones can be differentiated from T1/44 by their ability to grow in the presence of streptomycin.

2.1.3. Validation as a vaccine strain

Although *Mmm* strains can now be subtyped by exquisite molecular markers, there is as yet no evidence that a single vaccine strain could not protect against all circulating *Mmm* strains.

i) Innocuity

As noted above, strain T1/44 has a known residual virulence that may vary according to local conditions. Post-vaccinal reactions are characterised by a localised inflammatory reaction that develops at the site of injection (Willems' reaction). It may be noticed as early as 1 week post-injection. In many cases this local reaction will wane naturally but in some instances it may become extensive and lead to the death of the animal if no suitable antibiotic treatment is administered. The percentage of reactors being unpredictable, preliminary trials with a few animals is not a complete guarantee of inocuity.

After reconstitution, the master seed 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. Inocuity 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.

ii) Immunogenicity

For CBPP there is no susceptible laboratory animal allowing an easy control of potency. There is also no strict correlation between antibody titres after vaccination and actual protection. The only way to control the potency of a vaccine is to perform a natural challenge in the susceptible host by the 'in-contact' method. The potency of the grand parental stock has been assessed. Primo-vaccination with the minimum required dose gave a 40–60% protection rate. Higher protection rates have been obtained after repeated vaccinations.

2.2. Method of manufacture

2.2.1. Procedure

Vaccine bulk cultures must be obtained with a maximum of three successive passages of the master 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 similar titres in all the vials when the freeze drying process is finished.

2.2.2. Requirements for ingredients

A suitable sterile liquid medium allowing the growth of the vaccine strain at the requested titre is needed. It is usually composed of a 'base' that can be autoclaved, which is then supplemented to allow for mycoplasma growth. The base consists of a meat digest. It can be prepared in-house or bought in powder form (for example, brain–heart infusion, PPLO broth, etc.). The supplement usually consists of animal serum (often horse serum at 10% final concentration), fresh yeast extract (5–10%) and other ingredients such as glucose, glycerol, DNA, and fatty acids. However there is no specific requirement regarding the composition of the medium but rather regarding the guarantee that all ingredients comply with quality assurance as mentioned in chapter 1.1.8, with special focus on products of biological origin as well as any national requirement.

It is usually recommended that no preservative, such as penicillin or similar antibiotic, be added to the medium. However, strain T1sr has to be grown in a medium containing 1 mg/ml streptomycin so as to prevent reversion to streptomycin-sensitivity.

2.2.3. In-process controls

It is recommended to assess the purity of the product during the production process. For example, purity can be assessed rapidly, before freeze-drying by observation under the microscope at ×40 with phase-contrast trans-illumination. Mycoplasmas will appear as very small grey dots, sometimes forming chains, having a Brownian agitation. Classical bacteria will appear as much bigger and bright, sometimes with a noticeable mobility.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use have to be performed according to recommendations of this *Terrestrial Manual* (chapter 1.1.9) or according to National regulations.

ii)   Identity

See Section C.2.1.2.

iv)   Stability

Freeze-dried products are considered stable when stored at –20°C (i.e. several years).

Stability may be shorter when freeze dried products are stored at 4°C. This stability may vary for a number of reasons, such as the quality of the vials, the stoppers and the freeze-drying process. It remains the responsibility of the vaccine producer to prove that its product still has the requested titre at the expiry date.

To this end it is recommended that the residual moisture of the product does not exceed 3%.

v)   Batch potency

NB: 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.

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 into 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 of at least of 10^7 live mycoplasmas per vaccine dose. Manufacturers must ensure that their production processes are able to yield homogeneous batches with minimal variations from one vial to another. In this case, three vials are enough to assess the batch titre. Otherwise, sampling frames must be put in place to ensure that the sample number is sufficient to validly represent the vaccine batch. The manufacturer must also ensure that the minimum titre is retained until the expiry date if the product is kept at the correct temperature.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.2.1 and C.2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

i)   Target and non-target animal safety

Safety has to be ascertained for target animals solely (i.e. the *Bos* genus).

ii)   Reversion-to-virulence for attenuated/live vaccines and environmental considerations

T1/44 strains are known to induce post-vaccinal reactions in some animals vaccinated for the first time. Adequate warnings must be included in the leaflet describing this vaccine, including the recommended antibiotic treatment to be put in place. Local subcutaneous

reactions should not be considered as a hazard to naïve cattle as there is normally no shedding, however, in the absence of reliable data, surveillance should be put in place in cattle herds where post-vaccinal reactions have been observed.

iii) Precautions (hazards)

CBPP vaccines based on T1/44 or T1sr strains and satisfying quality controls are harmless to humans.

2.3.3. Efficacy requirements

As a result of the very high cost of efficacy testing in cattle for CBPP only indirect assurance will be sought. Vaccine producers must ensure that each batch has been issued from a reference grand-parental stock of known origin and characteristics, that it has been produced according to good manufacturing practices and, more specifically, that the final product has not been obtained with procedures that may have induced a drift from the original master seed.

It has also to be reminded that complete protection of cattle herds are obtained only after repeated vaccinations.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to actual CBPP vaccines.

2.3.5. Duration of immunity

Not required to actual CBPP vaccines.

2.3.6. Stability

As part of the approval procedure, the manufacturer should be required to demonstrate the stability of the vaccine's titre at the end of the claimed shelf-life period. Storage temperature shall be indicated for the freeze-dried product and also for the final product once reconstituted in the appropriate diluents. Warnings should be given if product is damaged by improper vaccine temperatures.

REFERENCES

AMANFU W., SEDIADIE S., MASUPU K.V., BENKIRANE A., GEIGER R. & THIAUCOURT F. (1998). Field validation of a competitive ELISA for the detection of contagious bovine pleuropneumonia in Botswana. *Rev. Elev. Med. Vet. Pays Trop.*, **51**, 189–193.

BASHIRUDDIN J. B., NICHOLAS R. A. J., SANTINI F. G., READY R. A., WOODWARD M.J. & TAYLOR T. K. (1994). Use of the polymerase chain reaction to detect *Mycoplasma* DNA in cattle with contagious pleuropneumonia. *Vet. Rec.,* **134**, 240–241.

BELLINI S., GIOVANNINI A., DI FRANCESCO C., TITTARELLI M. & CAPORALE V. (1998). Sensitivity and specificity of serological and bacteriological tests for contagious bovine pleuropneumonia. *Rev. sci. tech. OIE Int. Epiz.,* **17**, 654–659.

BROCCHI E., GAMBA D., POUMARAT F., MARTEL J.L. & DE SIMONE F. (1993). Improvements in the diagnosis of contagious bovine pleuropneumonia through the use of monoclonal antibodies. *Rev. sci. tech. Off. int. Epiz.,* **12**, 559–570.

DEDIEU L., MADY V. & LEFEVRE P.C. (1994). Development of a selective polymerase chain reaction assay for the detection of *Mycoplasma mycoides* subsp. *mycoides* SC (contagious bovine pleuropneumonia agent). *Vet. Microbiol.,* **42**, 327–339.

DUPUY V., MANSO-SILVAN L., BARBE V., THEBAULT P., DORDET-FRISONI E., CITTI C., POUMARAT F., BLANCHARD A., BRETON M., SIRAND-PUGNET P. & THIAUCOURT F. (2012). Evolutionary history of contagious bovine pleuropneumonia using next generation sequencing of *Mycoplasma mycoides* Subsp. *mycoides* "Small Colony". *PLoS ONE,* **7**(10):e46821.

EUROPEAN COMMISSION (2001). Health & Consumer Protection Directorate-General. Diagnostic tests for contagions bovine pleuropneumonia. Report of the Scientific Committee on Animal Health and Animal Welfare. Adopted 17 October 2001. SANCO/AH/R25/2001.

FREUNDT E.A., ERNO H. & LEMCKE R.M. (1979). Identification of mycoplasmas. *In:* Methods in Microbiology, Vol. 13, Bergen T. & Norris J.R., eds. Academic Press, London, UK, 377–434.

GAURIVAUD P. & POUMARAT F. (2012). Serodiagnosis of contagious bovine pleuropneumonia by immunoblotting. Euroreference, winter 2012, N°8,
http://www.ansespro.fr/euroreference/Documents/ER08-Meth-PeripneumoEN.pdf

GONÇALVES R., REGALLA J., NICOLET J., FREY J., NICHOLAS R., BASHIRUDDIN J., DE SANTIS P. & PENHA GONÇALVES A. (1998). Antigen heterogeneity among *Mycoplasma mycoides* subsp *mycoides* SC isolates*: discrimination of major surface proteins. Vet. Microbiol.*, **63**, 13–28.

GOURGUES G., BARRÉ A., BEAUDOING E., WEBER J., MAGDELENAT G., BARBE V., SCHIECK E., JORES J., VASHEE S., BLANCHARD A., LARTIGUE C. & SIRAND-PUGNET P. (2016). Complete Genome Sequence of *Mycoplasma mycoides* subsp. *mycoides* T1/44, a Vaccine Strain against Contagious Bovine Pleuropneumonia. *Genome Announc.*, **4**, e00263-00216.

HUBSCHLE O., LELLI R., FREY J. & NICHOLAS R.A.J. (2002). Contagious bovine pleuropneumonia and vaccine strain T1/44. *Vet. Rec.*, **150**, 615.

ISO/IEC (2005). ISO/IEC 17025:2005. General Requirements for the Competence of Testing and Calibration Laboratories. International Organization for Standardization (ISO), www.iso.org.

LE GOFF C. & THIAUCOURT F. (1998). A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia (CBPP). *Vet. Microbiol.*, **60**, 179–191.

LORENZON S., DAVID A., NADEW M., WESONGA H. & THIAUCOURT F. (2000). Specific PCR identification of the T1 vaccine strains for contagious bovine pleuropneumonia. *Mol. Cell. Probes*, **14**, 205–210.

LORENZON S., MANSO-SILVÁN L. & THIAUCOURT F. (2008). Specific real-time PCR assays for the detection and quantification of *Mycoplasma mycoides* subsp. *mycoides* SC and *Mycoplasma capricolum* subsp. *capripneumoniae. Mol. Cell. Probes*, **22** (5-6), 324–328.

MANSO-SILVÁN L., VILEI E.M., SACHSE K., DJORDJEVIC S.P., THIAUCOURT F. & FREY J. (2009). *Mycoplasma leachii* sp. nov. as a new species designation for *Mycoplasma* sp. bovine group 7 of Leach, and reclassification of *Mycoplasma mycoides* subsp. *mycoides* LC as a serovar of *Mycoplasma mycoides* subsp. *capri. Int. J. Syst. Evol. Microbiol.*, **59** (Pt 6), 1353–1358.

MBULU R.S., TJIPURA-ZAIRE G., LELLI R., FREY J., PILO P., VILEI E.M., METTLER F., NICHOLAS R.A. & HUEBSCHLE O.J. (2004). Contagious bovine pleuropneumonia (CBPP) caused by vaccine strain T1/44 of *Mycoplasma mycoides* subsp. *mycoides* SC. *Vet. Microbiol.,* **98**, 229–234.

MISEREZ R., PILLOUD T., CHENG., NICOLET J., GRIOT C. & FREY J. (1997). Development of a sensitive nested PCR method for the specific detection of *Mycoplasma mycoides* subsp. *mycoides* SC. *Mol. Cell. Probe,* **11**, 103–111.

NIANG M., DIALLO M., CISSE O., KONE M., DOUCOURE M., ROTH J.A., BALCER-RODRIGUES V. & DEDIEU L. (2006). Pulmonary and serum antibody responses elicited in zebu cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* SC by contact exposure. *Vet. Res.*, **37**, 733–744.

PROVOST A. (1972). Recherches immunologiques sur la péripneumonie. XIV. Description de deux techniques applicables sur le terrain pour le diagnostic de la maladie. *Rev. Elev. Med. Vet. Pays Trop.*, **25**, 475–496.

PROVOST A., PERREAU P., BREARD A., LE GOFF C., MARTEL J.L. & COTTEW G.S. (1987). Péripneumonie contagieuse bovine. *Rev. sci. tech. Off. int. Epiz.*, **6**, 565–624.

SANTINI F.G., VISAGGIO M., FARINELLI G., DI FRANCESCO G., GUARDUCCI M., D'ANGELO A.R., SCACCHIA M. & DI GIANNATALE E. (1992). Pulmonary sequestrum from Mycoplasma mycoides var. mycoides SC in a domestic buffalo; isolation, anatamo-histopathology and immuno-histochemistry. *Veterinaria Italiana*, **4**, 4–10.

SCHUBERT E., SACHSE K., JORES J. & HELLER M. (2011). Serological testing of cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* Small Colony using four different tests reveals a variety of seroconversion patterns. *BMC Vet. Res.*, **7**, 72.

SHERIFF D. & PIERCY S.E. (1952). Experiments with an avianised strain of the organism of contagious bovine pleuropneumonia. *Vet. Rec.,* **64**, 615–621.

SRIVASTAVA N.C., THIAUCOURT F., SINGH V.P., SUNDER J. & SINGH V.P. (2000). Isolation of *Mycoplasma mycoides* small colony type from contagious caprine pleuropneumonia in India. *Vet. Rec.*, **147**, 520–521.

WESONGA H.O. & THIAUCOURT F. (2000). Experimental studies on the efficacy of T1sr and T1/44 vaccine strains of *Mmm*SC against a field isolate causing contagious bovine pleuropneumonia in Kenya – Effect of a revaccination. *Rev. Elev. Med. Vet. Pays Trop.*, **53**, 313–318.

YAYA A., GOLSIA R., HAMADOU B., AMARO A. & THIAUCOURT F. (1999). Essai comparatif d'efficacité des deux souches vaccinales T1/44 et T1sr contre la péripneumonie contagieuse bovine. *Rev. Elev. Med. Vet. Pays Trop.*, **52**, 171–179.

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NB: There are WOAH Reference Laboratories for contagious bovine pleuropneumonia
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for contagious bovine pleuropneumonia

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.