

CHAPTER 2.7.5.

CONTAGIOUS CAPRINE PLEUROPNEUMONIA

SUMMARY

Definition of the disease: Contagious caprine pleuropneumonia (CCPP) is a disease affecting goats and some wild ruminant species, caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp). In goats it is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnea, cough and nasal discharges. The acute and subacute disease is characterised by unilateral sero-fibrinous pleuropneumonia with severe pleural effusion. Diagnosis is carried out by clinical and necropsy observations that should be confirmed by laboratory tests. As the isolation of Mccp is difficult, molecular techniques should be the methods of choice for laboratory confirmations.

Identification of the agent: Samples to be taken from live animals are broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, and pleural fluid. 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 other bacteria. Growth of Mccp requires very rich media containing high percentages of serum. Isolation is hampered by the very slow growth of Mccp, up to 15 days, and the presence of other mycoplasma species such as *M. ovipneumoniae*.

In broth, growth is visible within 4–15 days but turbidity is always very faint. Mccp sometimes produces 'comets' in unshaken liquid cultures. On agar media, typical 'fried egg' colonies are always very small, 0.1–0.5 mm and differ from *M. ovipneumoniae* colonies that are centerless. Molecular techniques can be used for the rapid and specific identification of Mccp.

Serological tests: For diagnosis, the complement fixation test (CFT) can be used. Goats are often infected by other *Mycoplasma* species that are closely related to Mccp and that will induce cross-reactions in CFT. Alternative tests such as polysaccharide-latex agglutination and competition enzyme-linked immunosorbent assay have been developed.

Requirements for vaccines: CCPP vaccines are inactivated and adjuvanted. The antigen is composed of whole Mccp cells that are concentrated and semi-purified, the minimum content is 0.15 mg of Mccp protein per dose. The adjuvant of choice is saponin with an indicative content of 3 mg per dose. The adjuvant quantity may vary according to batches of saponin.

A. INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp). The acute form of the disease is characterised by unilateral sero-fibrinous pleuropneumonia with severe pleural effusion (Thiaucourt & Bolske, 1996). Synonyms include: pleuropneumonie contagieuse caprine (France), bou-frida (Algeria), and abu-nini (Sudan).

From a taxonomic point of view, Mccp belongs to the so-called "mycoides cluster" (Manso-Silvan *et al.*, 2007) and it received its name only recently (Leach *et al.*, 1993). Its closest relatives are *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma leachii*, which may cross-react with Mccp, but the other members of the mycoides cluster, such as *Mycoplasma mycoides* subsp. *capri* or *Mycoplasma mycoides* subsp. *mycoides*, may also share similarities. Mccp is highly fastidious and faint turbidity in liquid medium or colonies on solid medium may appear only after 5–15 days. Isolation is often unsuccessful and detection may be easier with specific molecular methods such as the PCR (Woubit *et al.*, 2004).

The disease was first described in 1873 in Algeria. Shortly after, in 1881, the disease was introduced to South Africa by a shipment of Angora goats. The disease was eradicated using a policy of slaughter of the infected goats coupled with a traditional vaccination procedure for the in-contact goats (Hutcheon, 1889). This organism was first isolated and shown to cause CCPP in Kenya (MacMartin *et al.*, 1980; MacOwan & Minette, 1976); it has subsequently been isolated in the Chad, Eritrea, Ethiopia, Niger, Oman, Sudan, Tanzania, Tunisia, Turkey, Uganda, the United Arab Emirates, and more recently in Mauritius (Srivastava *et al.*, 2010), China (People's Rep. of) (Chu *et al.*, 2011) and Tajikistan (Amirbekov *et al.*, 2010). CCPP was first reported in mainland Europe in 2004, when outbreaks were confirmed in the Thrace region of Turkey, with losses of up to 25% of kids and adults in some herds (Ozdemir *et al.*, 2005). However, the exact distribution of the disease is not known and it may be much more widespread than the zone represented by the countries where *Mccp* has been isolated, as CCPP is often confused with other respiratory infections and also because the isolation of the causative organism is difficult.

In CCPP outbreaks in mixed goat and sheep herds, sheep may also be infected, as verified by isolation of *Mccp* (Bölske *et al.*, 1995) or detection of antibodies from clinically affected sheep. *Mccp* has also been isolated from healthy sheep (Litamoi *et al.*, 1990) and the role of sheep as a reservoir for the disease has to be considered.

Recently CCPP was confirmed in wild ruminants kept in a wildlife preservation reserve in Qatar. The disease affected wild goats (*Capra aegagrus*), Nubian Ibex (*Capra ibex nubiana*), Laristan mouflon (*Ovis orientalis laristanica*) and Gerenuk (*Litocranius walleri*) with significant morbidity and mortality in these species (Arif *et al.*, 2005). It has also been reported in gazelles in the United Arab Emirates (Nicholas *et al.*, 2008).

CCPP is not a zoonotic infection. There is no known risk of human infection with *Mccp*. 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 CCPP-free countries, *Mccp* should be manipulated under biosafety level 2 (BSL2) (or higher) laboratories.

Differential diagnosis may be difficult in the field as goats may be infected with a number of mycoplasma species that may induce similar signs. However CCPP may be suspected when lesions are restricted to the respiratory tract, affect only one lung and when animals present a conspicuous pleurisy with profuse effusion of pleural fluid. CCPP could also be confused with peste des petits ruminants or pasteurellosis.

B. DIAGNOSTIC TECHNIQUES

The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated, especially where it is enzootic. It must be differentiated from other similar clinico-pathological syndromes such as: peste des petits ruminants, to which sheep are also susceptible; pasteurellosis, which can be differentiated on the basis of distribution of gross lung lesions; and contagious agalactia syndrome (Nicholas & Churchward, 2011; Thiaucourt & Bolske, 1996). The disease caused by *Mccp* is readily contagious and fatal to susceptible goats of all ages and both sexes, rarely affects sheep and does not affect cattle.

Table 1. Laboratory methods currently used for diagnosis of CCPP 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
Agent identification¹						
<i>In-vitro</i> culture²	–	–	–	++	–	–
Molecular tests (PCR)	–	–	–	+++	–	–

1 A combination of agent identification methods applied on the same clinical sample is recommended.

2 Organisms isolated should be subjected to confirmatory molecular, biochemical or immunological methods as described below.

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 immune response						
CFT	++	++	–	++	++	+
Latex agglutination	+	+	–	+++	+	–
C-ELISA	+++	++	–	++	+++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable. PCR = polymerase chain reaction; CFT = complement fixation test; C-ELISA = competitive enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Microscopy of lung exudates, impression smears or sections

Mccp is characterised histopathologically by an interstitial pneumonia with interstitial, intralobular oedema of the lung (Kaliner & MacOwan, 1976). It shows a branching filamentous morphology *in vivo* that can be observed by dark-field microscopy in exudates or tissue suspensions from lesions or pleural fluid. Alternatively, smears made from cut lung lesions can be stained by the May–Grünwald–Giesma method and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. Neither of these techniques provides a definitive diagnosis.

1.2. Molecular identification and typing: polymerase chain reaction

Two polymerase chain reaction (PCR) assays for the specific identification of *Mccp* have been published. The first one (Bascunana *et al.*, 1994) is based on the amplification of the 16S rRNA gene of the mycoides cluster. The PCR product is then analysed by restriction enzyme cleavage for the identification of the *Mccp* amplicon. The second one (Woubit *et al.*, 2004) is based on a specific amplification; the primer sequences (*Mccp*-spe-F/R) are shown hereafter.

Mccp-spe-Forward: 5'-ATC-ATT-TTT-AAT-CCC-TTC-AAG-3'

Mccp-spe-Reverse: 5'-TAC-TAT-GAG-TAA-TTA-TAA-TAT-ATG-CAA-3'

PCR conditions consist of an initial denaturation step of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 15 seconds at 47°C and 15 seconds at 72°C and a final extension step of 5 minutes at 72°C. The expected amplified product is 316 bp long.

The same primers can be used in a quantitative PCR method (Lorenzon *et al.*, 2008).

These PCR techniques can be used directly on clinical materials such as lung tissue and pleural fluid (Bölske *et al.*, 1996), including pleural fluid dried on filter papers. Due to the difficulty in isolating *Mccp*, PCR is the technique of choice for the diagnosis of CCPP. However, isolation of *Mccp* remains the confirmatory test. All mycoplasmas of the mycoides cluster can be assigned a precise phylogenetic position by using a multilocus sequence typing approach (Manso-Silvan *et al.*, 2007).

1.3. Gel precipitin tests to detect antigen in tissue specimens

Mccp releases an antigenic polysaccharide to which a specific monoclonal antibody (MAb) (WM-25) has been produced (Rurangirwa *et al.*, 1987c). This MAb immunoprecipitates in agar gel with the polysaccharide produced by *Mccp*, and is used to identify the causative agent in cases of CCPP, particularly when specimens are no longer suitable for culture because of deterioration during transit. The MAb may be replaced by goat CCPP convalescent sera provided they are precipitating (i.e. they

contain IgMs). This precipitation test may not be completely specific and could give some cross-reactions, notably with *M. leachii*.

1.4. Isolation of mycoplasmas

1.4.1. Selection of samples

The necropsy samples of choice are lung lesions, particularly from the interface between consolidated and unconsolidated areas, pleural fluid, and mediastinal lymph nodes. If microbiological examination cannot be performed immediately, samples or whole lungs can be stored at -20°C for considerable periods (months) with little apparent loss of mycoplasma viability. During transport, samples should always be kept as cool as possible, as mycoplasma viability diminishes rapidly with increasing temperature. Lung samples can be dispatched to other laboratories in frozen form.

1.4.2. Treatment of samples

Swabs are suspended in 2–3 ml of culture medium. Tissue samples are best minced using scissors, and then shaken vigorously, or pulverised in medium using 1 g of tissue to 9 ml of medium. Tissues should not be ground. The suspension is usually prepared with a mycoplasma medium, but if parallel bacteriological examination is to be carried out, a high quality bacteriological medium, such as nutrient broth, may be used to provide a suspension suitable for both forms of examination. Pleural fluid, or a tissue suspension or swab, is serially diluted through at least three tenfold steps (to a nominal 10^{-4}) in the selected mycoplasma medium. Dilutions should also be plated on to solid medium.

1.4.3. Mycoplasma media

The medium used by MacOwan & Minette to culture *Mccp* organisms (MacOwan & Minette, 1976), is termed 'viande foie goat' (VFG), and includes goat-meat liver broth and goat serum. Alternative suitable media are WJ (Jones & Wood, 1988), modified Hayflick's, and modified Newing's tryptose broth (Kibor & Waiyaki, 1986). Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of *Mccp* (Mohan *et al.*, 1990; Thiaucourt *et al.*, 1992).

i) CCPP medium

a) Autoclaved portion (121°C for 15 minutes)

Bacto PPLO (pleuropneumonia-like organisms) broth without crystal violet (Difco) (21 g); deionised water (700 ml).

b) Membrane-filtered portion

Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (200 ml); fresh yeast extract (100 ml); glucose (sterile solution 0.5 g/ml) (2 ml); and sodium pyruvate (sterile solution 0.5 g/ml) (8 ml).

Part B is added to A aseptically. Ampicillin (0.1 g/litre) and thallium acetate (250 mg/litre) can be added to prevent contamination in primary isolations. The final pH of the medium should be 7.4–7.6.

ii) Modified CCPP medium

a) Autoclaved portion (121°C for 15 minutes)

Bacto PPLO broth without crystal violet (17.5 g); glass distilled water (650 ml).

b) Membrane-filtered portion

Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (250 ml); fresh yeast extract (100 ml); 50% glucose (4 ml); 25% sodium pyruvate (8 ml); 5% thallium acetate (4 ml); ampicillin (250 mg); and 0.5% phenol red (4 ml). The pH is adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Part B is added to A aseptically.

1.4.4. Medium production, storage and quality control

Certain medium components, particularly serum, yeast extract and deionised water, should be regularly monitored for growth-promoting capacity before incorporation into mycoplasma media. Low-passage field isolates should be used for this screening purpose.

Broth media may be stored for at least 6 months at -25°C before use, but penicillin or its analogues should not be added until final dispensing. Broth media are dispensed into bijoux (1.8 ml or 2.7 ml) or screw-capped tubes (4.5 ml), and stored for up to 3 weeks at 4°C . Solid media are best made with agarose (0.9% [w/v]), Noble agar (1.5% [w/v]), or purified agar (0.6% [w/v]). Plates, which are poured to a depth of 6–8 mm, should be as fresh as possible when used, and should be stored for no more than 2 weeks at 4°C before use. All culture media should be subjected to quality control and must support the growth of *Mycoplasma* spp. from small inocula. The reference stain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

1.4.5. Cultivation

Cultures are incubated at 37°C . Plates are best incubated in a humid atmosphere of 5% CO_2 , 95% air or N_2 , or in a candle jar with a moisture source. Cultures can also be incubated anaerobically.

Broth cultures are examined daily for evidence of growth – colour change and the appearance of floccular material. Gross turbidity indicates bacterial contamination; cultures showing this should be passed through a $0.45\text{-}\mu\text{m}$ membrane filter before subculture. Broth cultures are subcultured by inoculation of fresh broth medium with one-tenth of their volume, or by streaking agar medium with a loop.

Plate cultures are examined every 1–3 days using a stereo microscope ($\times 5\text{--}50$ magnification) and transmitted and incident light sources. If negative, the plates are discarded after 15 days. Subculture is carried out by the transfer of excised agar blocks bearing isolated colonies to either agar (on which the blocks are pushed, face down) or broth media. Alternatively, an agar plug bearing one colony is drawn into a Pasteur pipette and discharged into fresh broth medium.

Cloning and purification of isolates is performed by repeated transfer of single colonies representing each morphological type seen. Colony morphology varies with the medium used, the mycoplasma species, its passage level and the age of the culture.

In early passage, many mycoplasma species produce colonies of bizarre morphology, often small, centreless, and of irregular shape. This effect is often associated with the use of marginally suitable medium. With passage, such isolates demonstrate conventional 'fried egg' colony morphology, except *M. ovipneumoniae*, which retains centreless colonies. Colonies of *M. mycoides* subsp. *capri* may be up to 3 mm in diameter.

Filtration of broth cultures through $0.45\ \mu\text{m}$ filters before subculture aids purification by excluding cell aggregates.

Cultures suspected of being L-forms of bacteria should be examined for reversion to bacterial form by three to five passages on solid mycoplasma medium from which antibiotics and thallium acetate have been omitted.

Broth media used for primary isolation and which have shown no indication of growth by 7 days, should be subcultured blind.

Broth cultures of each sample, including one blind subculture, should be examined for a minimum of 3 weeks before being discarded. Titrations in broths, if performed in full (to 10^{-10}), are also read at 3–4 weeks and are expressed as colour-changing units per transfer volume. Growth on plates is expressed as colony-forming units (CFU) per ml.

1.5. Identification of mycoplasmas

1.5.1. Polymerase chain reaction

Once the organism has been cultured, verification of *Mccp* can be achieved in 1 day by PCR. See B.1.2 of this chapter.

Recently, PCR and sequencing has been used to establish the molecular epidemiology of CCPP. A multilocus sequence analysis (MLSA) method is also available (Manso-Silvan *et al.*, 2011) that revealed two main lineages comprising five groups from a representative set of *Mccp* strains.

Identification of *Mccp* strains by PCR (and sequencing) has now superseded all other techniques because of its rapidity and reliability. However PCR reactions must be performed with great care to prevent contamination (refer to Guideline 3.2 *Biotechnology in the diagnosis of infectious diseases*).

1.5.2. Biochemical tests

Wild strains should be passaged, and preferably cloned, three times before identification is attempted.

Biochemical tests cannot identify an isolate unequivocally, which at present can only be done by genetic means.

The tests most commonly used are glucose breakdown (*Mccp*: positive), arginine hydrolysis (*Mccp*: negative), 'film and spots' formation (*Mccp*: negative), reduction of tetrazolium chloride aerobically and anaerobically (*Mccp*: +/++), phosphatase activity (*Mccp*: negative), serum digestion (*Mccp*: negative) and digitonin sensitivity (*Mccp*: positive).

1.5.3. Serological identification

Mycoplasmal antigens used in hyperimmune serum production are often contaminated with medium constituents. The antibodies stimulated by these contaminants can cause false-positive reactions in serological identification tests. This problem is avoided by absorption of the antiserum with the medium used to produce the antigen (10 mg lyophilised medium per ml of antiserum), or by growing the mycoplasmas to be used as antigens in medium containing homologous animal components, e.g. growth in VFG medium to immunise goats.

i) Growth inhibition test

The growth inhibition test (GIT) is the simplest and most specific of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum, and detects primarily surface antigens.

Mccp appears to be highly homogeneous serologically and wide zones of inhibition free of 'breakthrough' colonies are observed with antiserum to the type strain, regardless of the source of the test strain (Jones & Wood, 1988). *Mccp* cross-reacts with *M. leachii* (PG50), *M. equigenitalium* and *M. primatum* in the GIT when polyclonal antisera are used, but an MAb specific for *Mccp* in the GIT has been produced (Rurangirwa *et al.*, 1987c). The MAb reagent, WM25, has been reported to be specific for (*Mccp*) isolates by the disc growth inhibition method, which will exclude *M. agalactiae*, *Mcc* and the other members of the 'mycoides cluster' associated with goats, but not bovine group 7 (not usually found in goats): the latter can be excluded, however, by colony indirect fluorescence tests. A small proportion of *Mccp* isolates also cross-react in the GIT with antiserum to *Mcc*. *Mycoplasma leachii* strains can sometimes be found in goats although it is rare. Results should be interpreted carefully as some bovine strains have been misidentified by the GIT using the 'specific' antiserum.

• Test procedure

- a) Broth culture in mid-to-late logarithmic phase is used at three tenfold dilutions, the selection of which is related to the vigour of growth of the isolate on agar.
- b) Agar plates are dried for 30 minutes at 37°C.
- c) Sterile paper disks of 6–7 mm in diameter are impregnated with a drop (10–20 µl) of undiluted antiserum. Disks may be used wet, in which form they can be stored at –20°C, or they can be lyophilised (Dighero *et al.*, 1970), which allows storage at 4°C.
- d) Using a separate plate for each dilution of culture, 1 ml or 2.5 ml volumes are pipetted on to 5 cm or 10 cm diameter plates, respectively. The inoculum is dispersed evenly over the plate, then the excess is removed.
- e) The plates are dried at 20–30°C for 15–20 minutes, preferably under a protective hood, until no visible liquid is present on the surface. Sufficient residual moisture should remain to enable freeze-dried disks to adhere to the agar surface.

- f) Several disks, each impregnated with a different antiserum (selected on the basis of sample source and the biochemical reactions and colony morphology of the isolate), are carefully placed on the agar plates; isolates from CCPP cases should be screened with antisera against *Mccp*, *M. mycoides* subsp. *capri* and *M. ovis-pneumoniae*. A disk containing 1.5% digitonin should also be included on the plates.
- g) The plates are incubated at 37°C for 2–6 days. Initial overnight incubation at 27°C can increase the sensitivity of the test. Inhibition by digitonin is generally readily apparent; however, inhibition by antiserum may be more difficult to interpret, with suppression rather than total inhibition of growth, depending on the species of mycoplasma, colony density and potency of the antiserum. 'Breakthrough' colonies are commonly observed within zones of inhibition. Circular precipitin bands are occasionally seen around disks. Positive inhibition is regarded as a zone of 2 mm or more.

ii) Indirect fluorescence antibody test

The direct and indirect fluorescent antibody tests are the most effective of the various serological methods for identifying most mycoplasmas. They are simple, rapid, and sensitive, yet economical in the use of antiserum. Several forms have been described, the most commonly used and perhaps best being the indirect fluorescent antibody test (IFAT) applied to unfixed colonies on agar. Antiserum against a single strain is sufficient to identify field isolates of that species, and antisera are diluted before use. Cultures do not have to be cloned, but the test is usually applied only after several passages have indicated whether the culture contains more than one species and the growth characteristics of the organism(s) present. This test is not specific for *Mccp* when using rabbit hyperimmune sera.

• **Test procedure**

- a) Two agar plates are predried at 37°C for 30 minutes. Each one is flooded with a different dilution of test broth culture, the dilutions being selected according to the vigour of growth of the strain on agar medium. Alternatively, a drop of undiluted culture is spread over a single 5 cm plate using an L-shaped glass rod.
- b) The plates are incubated at 37°C until the first evidence of growth is observed. If the IFAT cannot be performed immediately, the plates can be stored at 4°C for up to 4 weeks.
- c) Several blocks of approximately 0.5–1 cm² are excised from areas where colonies are numerous, but not confluent. The blocks of each agar culture are cut to the same geometric shape to enable recognition of origin, a different shape being used for each isolate. Several blocks of each isolate are distributed (colony surface facing upwards) on to several different slides, each slide being used for a different mycoplasma antiserum. The colony surface of each block is identified for future reference by undercutting one corner.
- d) Rabbit anti-mycoplasma (ra-m) serum or normal rabbit serum (NRS; as a control on a duplicate block) at a suitable dilution in normal saline or phosphate-buffered saline (PBS), pH 7.2, is gently pipetted on to each agar block until the surface area is totally covered. The optimal dilution of ra-m is determined by chequerboard titration against the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin serum (a-r Ig-FITC) used.
- e) The flooded blocks are incubated on their slides at room temperature for 30 minutes in a humid chamber.
- f) All blocks on one slide are tipped into a 10 ml tube containing approximately 7 ml of PBS.
- g) The plugged tubes are rotated at 18–30 rpm for 10 minutes. The PBS is then decanted and replaced with fresh PBS, and the tubes are rotated again for 10 minutes.
- h) The PBS is decanted and the blocks are placed colony surface facing upwards on their respective slides. Excess moisture is blotted off.
- i) All blocks are flooded with a-r Ig-FITC at optimal dilution.
- j) The blocks are incubated again for 30 minutes at room temperature in a humid chamber, then tipped into tubes containing fresh PBS, and washed twice by rotating, as before.

- k) The blocks, replaced colony surface facing upwards on their respective slides, are examined by an epi-immunofluorescent microscope using the settings recommended by the manufacturer for FITC.
- **Notes on the indirect fluorescent antibody test**
- l) Working dilutions of ra-m and a-r Ig-FITC should be kept at 4°C, which limits their shelf life to approximately 1 week.
- m) Isolates from CAPP should be examined using antisera against *Mccp M. mycoides* subsp. *capri*, positive control cultures should comprise their type strains.
- n) A negative (NRS-treated) control should always be incorporated for each culture.
- o) Interpretation of the IFAT can be difficult. Autofluorescence is produced by some species, particularly acholeplasmas. Even in pure cultures, a proportion of colonies may not stain positively with the relevant antiserum; this is particularly true of *Mcc*. Otherwise, poor results are usually ascribable to the use of an agar culture that has been allowed to grow for too long, or to the use of antiserum that has deteriorated with dilution and age.

2. Serological tests

Serology has not been widely applied to identifying the cause of outbreaks of pleuropneumonia in goats and sheep. Three methods are currently available: CFT, the latex agglutination test and the competitive enzyme-linked immunosorbent assay (ELISA) with a specific MAb (C-ELISA). Goats are frequently infected by mycoplasmas of the mycoides cluster, which may induce cross-reactions with tests such as the CFT that use crude antigenic preparations.

Seroconversion to *Mccp* in experimentally infected animals is observed by the CFT to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter. These various observations indicate that serology should be applied on a herd, not an individual basis, and that whenever possible, paired serum samples collected 3–8 weeks apart, should be examined.

2.1. Complement fixation test (MacOwan & Minette, 1976)

To prepare the antigen, 2 litres of culture of titre higher than 10^9 CFU/ml is centrifuged at 12,000 *g* for 1 hour at 5°C. The deposit is resuspended and washed three times in physiological saline prior to storage in 0.5–1.0 ml volumes at –20°C.

Sterile broth treated as above constitutes a control antigen, and a freeze-dried broth reconstituted at 200 mg/ml constitutes a second control antigen. Prior to testing, the antigen is diluted 1/60 and ultrasonicated for 3 minutes at low power in a container of iced water. The sonicate is centrifuged at 1250 *g* for 30 minutes to remove any debris, and stored at –20°C. If stored for more than 2–3 weeks the antigen should be recentrifuged.

2.1.1. Test procedure

Microtitre plate tests are performed using 0.025 ml volumes, two volumes containing three mean haemolytic doses of complement, and a 1.5% (v/v) final concentration of sheep red blood cells (SRBCs) in U-bottomed microtitre plates as follows:

- i) The following are mixed and incubated at 37°C for 45 minutes:
 - a) 25 µl of doubling dilutions of test serum (heat inactivated at 56°C for 30 minutes) starting with 1/2 dilution;
 - b) 25 µl of antigen containing two units of antigen (the dilution of the antigen must be determined in a chequer-board titration using a known positive serum. One unit of antigen corresponds to the highest antigen dilution yielding the highest titre with the positive reference serum;
 - c) 25 µl of complement (3 haemolytic units).
- ii) 25 µl of sensitised SRBCs, at a final concentration of 1.5% (v/v), is mixed and the plates are incubated at 37°C for 45 minutes.
- iii) The plates are incubated at 4°C for 1 hour to allow the intact SRBCs to sediment.
- iv) Reading the results: The titre will be the highest serum dilution that will fix 50% of the complement, i.e. 50% haemolysis.

2.1.2. Controls

In all CF tests a number of controls are required:

- i) Indicator systems (RBCs + haemolysin) alone to ensure that RBCs do not lyse spontaneously.
- ii) Indicator system with complement only to show that enough complement is present to lyse the cells.
- iii) Indicator system with antigen only and no complement to show that antigen alone does not lyse the cells.
- iv) Indicator system with serum alone and no complement to show that the serum alone does not lyse the cells.
- v) Indicator system with complement and antigen to detect any anticomplementary activity of the antigen.
- vi) Indicator system with the complement and serum to detect any anticomplementary activity of the serum.

NB: as many mycoplasmas, notably those belonging to the mycoides cluster, are expected to induce cross-reactions in the CFT, additional tests should be performed when finding CF positive titres in a CCPP-free country or zone. Suspicious sera should be tested in parallel with antigens prepared with various mycoplasma species and notably *M. capricolum*, *M. mycoides* subsp. *mycoides*, *M. leachii* and *M. mycoides* subsp. *capri*. The antigen yielding the highest titre should indicate which species was infecting the animal/herd.

2.2. Latex agglutination test

Latex beads sensitised with the polysaccharide produced by *Mccp* and present in culture supernatant have been used in a slide agglutination test (Rurangirwa *et al.*, 1987a). This test is presently used routinely in Kenya. It is a very useful test in an outbreak because it can be performed at the bedside using a drop of whole blood.

This test is sensitive at an early stage of the disease as long as IgM persists in the serum. Its specificity is not well characterised. Cross-reactions may occur as *Mccp* polysaccharides are similar to those produced by *M. leachii* and may be found in other bacteria.

2.3. Competitive enzyme-linked immunosorbent assay

A C-ELISA was developed (Thiaucourt *et al.*, 1994) and proved both specific and sensitive. This test has recently been reformatted as a kit containing pre-coated plates and ready-made reagents, including MAb 4/52. It is now a strict competition assay instead of a semi-blocking test as in the original publication. The new kit has been re-validated to establish its cut-off value, 55% inhibition (PI), to obtain a strict specificity of 99.9%. It allows the detection of positive sera in CCPP-infected herds, but its true sensitivity at the individual level has not yet been fully evaluated. As it is highly specific, it can be used to evaluate herd status using targeted sampling of recovered animals in the tested herds should greatly enhance the sensitivity without any specificity problem. In the OIE Reference Laboratory, the uncertainty of measurement for this C-ELISA has been evaluated at ± 8 PI.

This test can be used to evaluate the CCPP vaccine quality as the seroconversion measured 1 and 2 months post-vaccination is proportional to the *Mccp* antigen or saponin content. However, the correlation between C-ELISA titre and protection has not yet been established (Peyraud *et al.*, 2014).

C. REQUIREMENTS FOR VACCINES

1. Background

Successful eradication of CCPP has already been achieved in Southern Africa in 1889 by applying a strategy based on the slaughter of affected animals and the inoculation of all in-contact goats. At that time, inoculation consisted in the subcutaneous injection of preparations containing pleural fluid or affected lungs homogenates. Contrary to what is observed in contagious bovine pleuropneumonia, the subcutaneous injection of live *Mccp* into naïve goats is not followed by any untoward inflammatory reaction. This may have contributed to the success of

the eradication programme in spite of the crude nature of the preparations. However the respective contributions of slaughter and inoculations to the eradication process have not been evaluated.

Few experiments have been performed to develop live vaccines and evaluate their potency. Studies have focused on inactivated preparations containing saponin as an inactivating agent and adjuvant (Rurangirwa *et al.*, 1987b). The optimum dose of antigen was established at 0.15 mg of *Mccp* protein and 3 mg of saponin. In the original publication, the concentrated antigen was freeze dried and reconstituted extemporaneously with a diluent containing 3 mg of saponin per ml. Such a procedure ensured a very long shelf life for the concentrated antigen (>14 months) and the duration of protection exceeded 12 months.

Due to the fastidious nature of *Mccp*, the production of CCPP vaccines is costly. *Mccp* requires very rich media, the yield is limited, the procedure involves a purification process, and inactivated vaccines also require larger amounts of antigen compared with live vaccines.

CCPP vaccines should be safe. The fact that live *Mccp* strains do not induce post-vaccine reactions could be an advantage for this kind of vaccine. For inactivated vaccines containing saponin, the pro-inflammatory effect of the saponin has to be verified as it may vary according to the producers or batches. It is not recommended to vaccinate pregnant animals because of a possible reaction to saponin.

CCPP vaccine efficacy should last at least 1 year and protect vaccinated animals from clinical disease.

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

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Any local isolate of *Mycoplasma capricolum* subsp. *capripneumoniae* can be used because of the homogeneity of this subspecies. The choice of strain will mostly depend on growth characteristics: rapid growth, ease of concentration and purification, etc.

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

Seed lots must be shown to be:

i) Pure

Purity can be tested by randomly selecting 10 *Mccp* clones originating from the seed and verifying the identity of these 10 clones, for example using a specific PCR technique (other tests may not be specific enough). Due to the fastidious nature of *Mccp* strains, any contaminant is likely to overgrow *Mccp* and be detectable by this procedure.

ii) Safe

No inflammatory reaction is normally observed when injecting *Mccp* strains subcutaneously into susceptible goats. It is therefore difficult (if necessary at all) to establish the seed's safety.

iii) Efficacious

Vaccines produced with the selected strain must induce protection when produced according to standard procedures and injected into naïve susceptible goats.

2.1.3. Validation as a vaccine strain

As there is no laboratory animal model for CCPP, seed validation as a suitable vaccine strain will have to be performed on susceptible goats at least once. The vaccine has to be prepared according to standard procedures and injected into susceptible goats. Vaccinated and naïve goats should be put in contact with CCPP-infected animals at least 3 months after vaccination. The expected level of protection should be at least 90% with a number of animals per group yielding statistically significant results.

If the inactivation process or the presentation of the final vaccine differs from the original presentation where protection has been demonstrated, additional tests must be performed to show the immunogenicity of the new final product.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, a working seed is first established by amplifying an aliquot of the freeze dried master seed bank that has been shown to induce protection. There is no specific requirement for the type of medium used provided it ensures satisfactory growth of the *Mccp* strain.

Mccp cells have to be concentrated and purified. Again there is no specific requirement for this step and producers may choose any method deemed necessary, provided the end product is pure and devoid of extraneous products originating from the culture medium. For example, cultures can be centrifuged at high speed (> 12,000 **g**) for 20 minutes, the pellet resuspended in an adequate volume of sterile PBS for washing, and *Mccp* pelleted again.

Washed concentrated *Mccp* antigen can be diluted to adjust the protein content, and freeze dried. The content of each vial is adjusted so that the final quantity of protein should be 0.15 mg per dose once the vaccine is reconstituted. A primary inactivation of the concentrated antigen by saponin can take place at this stage.

The final vaccine is obtained by reconstituting the freeze dried product with the necessary volume of diluents containing 3 mg of saponin per dose. Saponin acts as an inactivating agent for *Mccp* and as an adjuvant.

NB: Any production procedure that modifies the antigen content and characteristics or the adjuvant type warrants a new validation/registration verification.

2.2.2. Requirements for ingredients

No specific requirements. For general requirements, please refer to chapter 1.1.8, with special focus on products of biological origin originating from a country with negligible risk for transmissible spongiform encephalopathies.

2.2.3. In-process controls

The purity of the *Mccp* growth can be assessed regularly by rapid methods such as phase-contrast observation of cultures. It will ensure that there is no cell-walled bacterial contamination (mycoplasmas appearing as tiny grey spots barely visible while cell-walled bacteria appear bigger and brighter).

The absence of medium contaminants in the final concentrated *Mccp* product may be assessed by techniques such as SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) with controls including *Mccp* antigen and diluted production medium. The absence of medium contaminant ensures that the protein dosage refers to mycoplasma antigen and not to contaminants.

The amount of mycoplasma antigen in the concentrated product is evaluated by its protein content. Any suitable technique is acceptable, e.g. the bicinchoninic acid technique, provided the proper controls are included in the testing, for example, bovine albumin standards or reference *Mccp* antigen.

Once the antigen is inactivated by saponin, sterility can be assessed by seeding a sample on suitable media allowing *Mccp* growth.

2.2.4. Final product batch tests

i) Sterility

Standard procedures have to be used for testing sterility on a representative number of vials (see Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination*).

ii) Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, batch safety testing is to be performed.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch release, each of at least three healthy seronegative goats is inoculated by the recommended route of administration with the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to the batch safety test described here.

iii) Batch potency

It is unpractical to test the potency of the batches in susceptible hosts because of the difficulties encountered in reproducing CCPP. For batch release, indirect tests can be used for practicability and animal welfare considerations.

The three naïve goats that are vaccinated for the safety test must demonstrate a persistent specific and high titre seroconversion to *Mccp* antigen for at least 2 months post-vaccination. Unfortunately the serological tests that are used for CCPP have not been evaluated for a correlation between post-vaccination seroconversion and protection. However, the demonstration of a suitable specific seroconversion ensures that the tested product contains the correct antigen and that it can induce an immune response in vaccinated animals. For the time being, a western blot analysis may be used to evaluate this seroconversion. Alternative tests could be CFT or the specific C-ELISA that detects antibodies directed towards a single epitope. The latex agglutination test may not be used as it detects antibodies to a polysaccharide. This polysaccharide can be found in other mycoplasmas of the mycoides cluster and there is also no vaccine requirement for the polysaccharidic content of the vaccine (the possible protective role of the immune response towards polysaccharides has not yet been established).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.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

For the purpose of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by the recommended route of administration in an *in-vivo* test in eight susceptible goats. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after injection. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. Due to the presence of saponin in the vaccine, a transient feverish reaction may be observed as well as a localised swelling.

iii) Precautions (hazards)

Mccp is not pathogenic for humans. Accidental self-injection may induce a local irritation because of the presence of saponin in the vaccine.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy

(protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge and in comparison with nonvaccinated control animals.

Efficacy will be evaluated 3 months post-vaccination by an in-contact method whereby artificially infected goats are put in contact with vaccinated and naïve animals. Protection rate will be based on the observation of clinical signs (days with fever) and of lesions when animals are disposed of, 1 to 2 months after the initial onset of disease in the control group. The protection rate should reach at least 90% (± 10).

Due to the conserved nature of *Mccp* strains, any field pathogenic strain can be used to assess vaccine protection, provided it proves pathogenic in the control group.

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

Not applicable to current CCPP vaccines.

2.3.5. Duration of immunity

As part of the registration/licensing procedure, the manufacturer should be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection. In the case of saponin inactivated CCPP vaccine, this period is estimated at 1 year.

2.3.6. Stability

As part of registration/licensing procedure, the manufacturer should be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated and warnings should be given if product is damaged by freezing or ambient temperature.

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NB: There is an OIE Reference Laboratory for Contagious caprine pleuropneumonia (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for contagious caprine pleuropneumonia