CHAPTER 2.7.6.

CONTAGIOUS CAPRINE PLEUROPNEUMONIA

SUMMARY

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats caused by Mycoplasma capricolum subspecies capripneumoniae (Mccp). This organism is closely related to three other mycoplasmas: M. mycoides subsp. mycoides large colonies (LC), M. mycoides subsp. capri, and M. capricolum subsp. capricolum. Unlike true CCPP, which is confined to the thoracic cavity, the disease caused by the latter three mycoplasmas is accompanied by prominent lesions in other organs and/or parts of the body besides the thoracic cavity.

Typical cases of CCPP are characterised by extreme fever (41–43°C), high morbidity and mortality rates in susceptible herds affecting all ages and both sexes, and abortions in pregnant goats. It appears to be transmitted by an infective aerosol. After approximately 2–3 days of high fever, respiratory signs become apparent: respiration is accelerated and painful, and in some cases is accompanied by a grunt. Coughing is frequent, violent and productive. In the terminal stages, animals are unable to move – they stand with their front legs wide apart, the neck is stiff and extended, and sometimes saliva continuously drips from the mouth. Post-mortem examination reveals fibrinous pleuropneumonia with massive lung hepatisation and pleurisy, accompanied by accumulation of straw-coloured pleural fluid.

The disease has been shown recently to affect wild ruminants such as the wild goats (Capra aegagru), Nubian Ibex (Capra ibex Nubian) and Laristan Mouflon (Ovis orientalis laristanica) and Gerenuk (Litocranius walleri). Clinical disease and seropositivity have been reported in sheep in contact with affected goats, but the role of sheep as reservoirs of infection is unclear.

Identification of the agent: Definitive diagnosis requires culture of the causative organism from lung tissue samples and/or pleural fluid taken at post-mortem. After cloning and purification, isolates can be identified by several biochemical, immunological and molecular tests. Isolating the causative agent is a difficult task. Recently polymerase chain reaction based tests have been described and shown to be specific, sensitive and can be applied directly to clinical material, such as lung and pleural fluid.

Serological tests: Serological tests have been applied for the diagnosis of CCPP in outbreaks in Eritrea and Turkey. Such tests are best used on a herd basis rather than for diagnosis in individual animals. The complement fixation test remains the most widely used serological test for CCPP, although the latex agglutination test is being increasingly used in the diagnostic laboratories as well as a pen side test; it can be used to test whole blood as well as serum. Indirect hemagglutination is also used. A specific competitive enzyme-linked immunosorbent assay has been developed, but is not widely available. As with the other serological tests, it does not detect all reactors, but its specificity and suitability for large-scale testing make it an appropriate test for epidemiological investigations.

Requirements for vaccines and diagnostic biologicals: Vaccine against CCPP caused by Mccp is available commercially.

A. INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia where the total goat population is more than 500 million (1). The first description of the disease was in 1873 in Algeria. Shortly after, in 1881, the disease was introduced in the ‘Cape Colony’ of South Africa by a shipment of Angora goats (15, 16). The disease was eradicated using a policy of slaughter of the infected goats coupled with a traditional vaccination procedure for the in-contact goats. Classical, acute CCPP is caused by
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Mycoplasma capricolum subsp. capripneumoniae (Mccp) (23), originally known as the F38 biotype. This organism was first isolated and shown to cause CCPP in Kenya (24–27); it has subsequently been isolated in the Sudan, Tunisia, Oman, Turkey, Chad, Uganda, Ethiopia, Niger, Tanzania, Eritrea and the United Arab Emirates. CCPP was first reported in mainland Europe in 2004, when outbreaks were confirmed in Thrace, Turkey, with losses of up to 25% of kids and adults in some herds (35). 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 affected, as verified by isolation of Mccp (5) or detection of antibodies from clinically affected sheep (19). Mccp has also been isolated from healthy sheep (21) 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 (2). Disease indistinguishable from naturally occurring CCPP has been experimentally reproduced with Mccp by several groups of workers.

B. DIAGNOSTIC TECHNIQUES

The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated, especially where it is endemic. 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 what has been called 'mastitis, arthritis, keratitis, pneumonia and septicemia syndrome or more often as contagious agalactia syndrome (47). As the longer name implies, the pneumonia is accompanied by prominent lesions in other organs. 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.

1. Identification of the agent

a) Microscopy of lung exudates, impression smears or sections

Mccp is characterised histopathologically by an interstitial pneumonia with interstitial, intralobular oedema of the lung (18, 34). 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 method of May–Grünwald–Giesma and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. Neither of these techniques provides a definitive diagnosis.

b) Nucleic acid recognition methods

Two polymerase chain reaction (PCR) assays for the specific identification of Mccp have now been published. The first one (3) 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 (50) is based on a specific amplification. These PCR techniques can be used directly on clinical materials such as lung tissue and pleural fluid (6). 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 which may be used for identification purposes (30).

c) Gel precipitin tests to detect antigen in tissue specimens

Mccp releases an antigenic polysaccharide (41) to which a specific monoclonal antibody (MAb) (WM-25) has been produced (42, 43). 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.

d) Isolation of mycoplasmas

i) 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 mycoplasm 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.

ii) 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 pulsed in medium \(^1\) 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.

iii) Mycoplasma media

The medium used by MacOwan & Minette to culture Mcp organisms (25), is termed ‘viande foie goat’ (VFG), and includes goat-meat liver broth and goat serum. Alternative suitable media are WJ (17), modified Hayflick’s, and modified Newing’s tryptose broth (19) Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of Mcp (31, 32). Examples of suitable media are as follows (6, 47, 49):

- **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.

- **Modified CCPP medium**
  
  A. Autoclaved portion (121°C for 15 minutes): Bacto PPLO broth without crystal violet (Difco) (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.

Modified Newing’s tryptose broth (19) and agar plates (Gourlay’s medium) (11) are routinely used for isolation and maintenance of Mcp in Kenya.

iv) 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 strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

v) 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 moist source.

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

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1 For example, with the Stomacher 80, A.J. Seward, London, United Kingdom (UK).
passed through a 0.45-µm 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 (x5–50 magnification) and transmitted and incident light sources. If negative, the plates are discarded after 14 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, including *M. capricolum* subsp. *capricolum* (*Mcc*), 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. *mycoides* Large Colony (*MmmLC*) and *Mcc* may be up to 3 mm in diameter.

Filtration of broth cultures through 0.45 µm 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.

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 10⁷), 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.

e) Identification of mycoplasmas

i) Polymerase chain reaction

Once the organism has been cultured, verification of *Mccp* can be achieved in 1 day by PCR. There are now various PCR tests that can be applied for an identification of *Mccp* strains. The first test (3) is based on amplification of a segment of the 16S rRNA gene. The amplified fragment is common to the mycoides cluster. However, when the amplicon is digested with endonuclease *PstI*, a unique cleavage pattern of three fragments for *Mccp* is observed when the enzyme digests are analysed in agarose gel electrophoresis and stained with ethidium bromide (6). The second test is based on a specific amplification and can allow confirmation of CCPP within a few hours (50).

Recently, PCR and sequencing has been used to establish the molecular epidemiology of CCPP. These tests can be performed on dried samples, such as pleural fluid on filter papers. The sequencing allows a precise identification of the species (the cleavage site for the 16S rRNA and a specific detection for the ‘locus H2’) (22, 37).

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 contaminations.

ii) 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 serological or genetic means. Intraspecies variation in some biochemical reactions is often considerable (13), but some tests perform a useful function both as a preliminary screening system and in providing supportive data for serological findings.

The tests most commonly used are glucose breakdown, arginine hydrolysis, ‘film and spots’ formation, reduction of tetrazolium chloride (aerobically and anaerobically), phosphatase activity, serum digestion and digitonin sensitivity. The first three of these tests are performed routinely in isolation and cultivation procedures. Glucose breakdown is indicated by acid (yellow) changes, and arginine hydrolysis by alkaline (red) changes in broth media, using phenol red as indicator. Arginine use cannot be assessed
on conventional medium for isolation and culture as the media for testing the arginine deiminase pathway should contain high concentrations of arginine and no glucose. Film and spots describes an apparent wrinkling of the agar surface due to the deposition on it of an iridescent film of lipid, together with the development of black spots within the medium in the vicinity of ageing colonies. This phenomenon, produced by three mycoplasmas of small ruminants, is demonstrated on agar media containing 20% or more serum, preferably of horse or pig origin. Supplementation of the medium with 10% egg yolk emulsion improves the sensitivity of the test.

The remaining biochemical tests require specific media or reagents. The test for tetrazolium reduction provides corroborative evidence of the mycoplasmal nature of \textit{M. agalactiae} isolates, as this organism is neither glycolytic nor arginine-hydrolysing. Serum digestion (9) distinguishes members of ruminant mycoplasmas, and phosphatase production (7) separates \textit{Mcc} from other members of this cluster. Digitonin sensitivity distinguishes members of the order Mycoplasmatales from those of the order Acholplasmatales (10). A diagnostic medium is available that enables the specific detection of \textit{Mccp} growing on agar medium: colonies show a red coloration (35).

iii) \textbf{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.

Because of the close serological relationships between members of the ‘\textit{M. mycoides} cluster’, isolates from cases of CCPP should, preferably, be identified by at least two of the three tests described below.

- \textbf{Growth inhibition test}

The growth inhibition test (GIT) is the simplest and most specific, but the least sensitive, of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum, and detects primarily surface antigens (8).

\textit{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 (17). \textit{Mccp} cross-reacts with Leach’s bovine group 7 (PG50), \textit{M. equigenitalium} and \textit{M. primatum} in the GIT when polyclonal antisera are used, but an MAb specific for \textit{Mccp} in the GIT has been produced (42). The MAb reagent, WM25, has been reported to be specific for (\textit{Mccp}) isolates by the disc growth inhibition method, which will exclude \textit{M. agalactiae}, \textit{Mcc} and the other members of the ‘\textit{M. 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 (4). A small proportion of \textit{Mccp} isolates also cross-react in the GIT with antiserum to \textit{Mcc}. Group seven of Leach 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.

- \textbf{Test procedure}

i) 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.

ii) Agar plates are dried for 30 minutes at 37°C.

iii) 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 (8), which allows storage at 4°C.

iv) 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.

v) 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.

vi) 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 \textit{Mccp}, \textit{MmmLC}, \textit{Mcc}, \textit{M. mycoides} subsp. \textit{capri} (\textit{Mcc}) and \textit{M. ovipneumoniae}. A disk containing 1.5% digitonin should also be included on the plates.
vii) 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.

• Growth precipitation test
The growth precipitation test detects soluble cytoplasmic and extramembranous antigens released by growing cultures and allowed to diffuse through solid mycoplasma growth medium towards mycoplasma antiserum during growth (20). As with the gel precipitin test, there are strong cross-reactions within the mycoides cluster. If growth inhibition is performed using MAb WM25², which is specific for Mcp, both specific inhibition and a growth precipitin line are achieved simultaneously.

• Indirect fluorescence antibody test
The direct and indirect fluorescent antibody tests are the most effective of the various serological methods for identifying most mycoplasmas (38). 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 (IFA) test 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.

• Test procedure
i) 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.

ii) The plates are incubated at 37°C until the first evidence of growth is observed. If the IFA test cannot be performed immediately, the plates can be stored at 4°C for up to 4 weeks.

iii) 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.

iv) 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 lg-FITC) used.

v) The flooded blocks are incubated on their slides at room temperature for 30 minutes in a humid chamber.

vi) All blocks on one slide are tipped into a 10 ml tube containing approximately 7 ml of PBS.

vii) 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.

viii) The PBS is decanted and the blocks are placed colony surface facing upwards on their respective slides. Excess moisture is blotted off.

ix) All blocks are flooded with a-r lg-FITC at optimal dilution.

x) 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.

xi) The blocks, replaced colony surface facing upwards on their respective slides, are examined by an epimmuno-fluorescent microscope using the settings recommended by the manufacturer for FITC.

• Notes on the indirect fluorescent antibody test

² Available from Kenya Agricultural Research Institute (KARI), P.O. Box 57811, Nairobi, Kenya.
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.

Isolates from CCPP should be examined using antisera against Mccp, MmmLC, Mmc (M. mycoides subsp. capri) and Mcc, positive control cultures should comprise their type strains, namely Mccp, Y goat, PG3 and California kid, respectively.

A negative (NRS-treated) control should always be incorporated for each culture.

Interpretation of the IFA test 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.

f) Other identification tests

Metabolism inhibition (46) and tetrazolium reduction inhibition (44) are other tests sometimes used in the identification of caprine mycoplasmas. A gene probe, F38-12, capable of distinguishing Mccp has been developed (45).

A polysaccharide-specific antigen detection latex agglutination test has been developed to detect CCPP antigen (29). In this test, latex beads are coated with polyclonal immunoglobulin IgG (rabbit) directed against Mccp polysaccharide and used to detect the antigen in the serum of goats with CCPP. This test is proposed to be inexpensive, easy to carry out in the field and useful for detecting CCPP in its earliest stages.

2. Serological tests

Serology has not been widely applied to identifying the cause of outbreaks of pleuropneumonia in goats and sheep. Endemic infections with MmmLC and Mmc can produce a background of positive titres to these organisms in a proportion of apparently healthy animals (17), and under experimental conditions seroconversion to M. mycoides can occur in goats with no clinical signs of disease. Acute cases caused by Mccp rarely show positive titres to the organism before death (27, 33, 47), perhaps because antibodies are ‘eclipsed’ by circulating mycoplasma antigens (33). Seroconversion to Mccp in experimentally infected animals is observed, by the complement fixation (CF) test and indirect haemagglutination (IHA) test, to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter (33). 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.

a) Complement fixation test (the prescribed test for international trade) (25)

The CF test in various forms remains the most widely used serological test for diagnosis of contagious bovine pleuropneumonia (12, 36). In CCPP, the CF test was used for detection of Mccp infection (25) and it has been found to be more specific, though less sensitive, than the IHA test (33). Its main disadvantage is the high level of technical expertise required to perform the test (12).

One method of performing the test is as follows. To prepare the antigen, 2 litres of culture of titre higher than $10^9$ CFU/ml is centrifuged at 40,000 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 sediment 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.

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:

- 25 µl of doubling dilutions of test serum (heat inactivated at 56°C for 30 minutes) starting with 1/2 dilution;
• 25 µl of antigen (the dilution of the antigen must be determined in a chequerboard titration using a known positive serum);
• 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 unlysed cells to settle.

iv) Reading the results: The titre will be the highest serum dilution that will fix 50% of the complement, i.e. 50% haemolysis.

• 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.

b) 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 (14, 39). This test is presently used routinely in Kenya. It is a very useful test in an outbreak because it can be performed at the penside using a drop of whole blood.

Both CF test and IHA test findings emphasise the difficulties inherent in the serological diagnosis of CCPP when using whole cell or membrane preparations as antigen. The use of the more defined antigen, the polysaccharide elaborated by Mccp, provides greater specificity, as there is no cross-reactivity with sera against the other three principal caprine mycoplasmas.

c) Competitive enzyme-linked immunosorbent assay
A competition ELISA has been developed (48) and proved both specific and sensitive. However due to erratic and low demand the production of this test has been stopped until a new format is developed to ensure longer stability of reagents.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS
The first experimental vaccine against Mccp was a live high passage Mccp (28). When inoculated intratracheally, it proved innocuous and protected goats against experimental challenge. However, more recent work has concentrated on inactivated forms of vaccine. The current form used in Kenya (where inactivated Mccp, vaccines have been in use for several years) contains inactivated Mccp suspended in saponin, has been described as having a shelf life of at least 14 months and the optimal dose of 0.15 mg provides protection for over 1 year (40).

1. Seed management
a) Characteristics of the seed
The master seed was isolated from the lungs of a sick goat. In Kenya the strain used is called ‘Yatta’ and it has been confirmed to be Mccp by GIT and PCR, after 15 passages in culture. The master seed should also be pure and free from other contaminants.

b) Method of culture
The master seed is established and stored in a freeze-dried form in 1 ml ampoules. The working seed is prepared by amplifying the master seed in modified Newing’s broth to make a bulk of 4 litres. Its growth is arrested at the growth phase before filament formation.
This culture is tested for sterility before it is distributed in aliquots of 20 ml volumes and stored frozen at –20°C.

c) Validation as a vaccine

The master seed should be prepared from lung specimens or pleural fluids of a goat that dies of pneumonia, showing all the clinical signs of CCPP. The isolate must be confirmed by GiT or by PCR to be Mccp. It must be checked for sterility, safety, potency and extraneous agents.

2. Method of manufacture

For vaccine production, a working seed is first established by amplifying an aliquot of freeze-dried master seed in modified Newing’s broth (19), to make a bulk of 4 litres of culture. This culture is aliquoted in 20 ml volumes and frozen at –20°C. The vaccine is grown in 5 litre pots containing 4 litres of modified Newing’s broth. Each pot is sampled aseptically for sterility testing before 20 ml of working seed is inoculated into each 4 litres of medium. These pots are incubated at 37°C for 4–6 days depending on how fast the mycoplasma grows. After the filaments sediment, the antigen is harvested by centrifugation at 2600 g from pots that are not contaminated. (The filaments are thin and white and sometimes unite to form a resemblance of an inverted pine tree. This occurs on days 4–6 and it is at this point that they become heavy and sediment.) The pellet is resuspended in sterile normal saline and centrifuged to remove the remnants of the growth medium. The pellet is resuspended again in a small volume of sterile normal saline to make a viscous suspension.

Saponin is added to inactivate the mycoplasma at 3 mg saponin to 1 ml of antigen. It is left agitating overnight at 4°C using a magnetic stirrer.

NOTE: Saponin also acts as an adjuvant. Three samples are taken aseptically from this suspension and tested for sterility, for protein estimation and for the innocuity test.

3. In-process control

During production, the following tests are carried out to ensure that the product remains pure and safe. These tests are carried out by quality assurance staff. Each pot is sampled aseptically before inoculation to test for sterility.

After maturity of culture, only pots that have not shown signs of contamination are pooled for centrifugation; the contaminated pots are decontaminated and discarded after autoclaving.

After inactivation with saponin, a sample is taken aseptically for sterility, another one for innocuity and another one for the protein estimation test.

a) Sterility test

This test is aimed at verifying the absence of fungal and bacterial contaminants. Two tubes of thioglycollate are inoculated with 1 ml of the sample each. The tubes contain about 15 ml of broth. These are incubated at 37°C to eliminate aerobics, microaerophilics and anaerobics. The other medium used is soybean casein digest broth. Four tubes are inoculated with 12 ml of sample each.

Two tubes are incubated at 37°C and another two at room temperature (25°C) to eliminate bacterial and fungal contaminants. All media are incubated for 14 days with controls. Absence of any growth shows that the sample is not contaminated.

b) Innocuity test

This test aims to demonstrate the absence of living mycoplasma in the vaccine: 0.3 ml of inactivated antigen is added to 2.7 ml of modified Newing’s broth in a tube and tenfold dilutions are made from 10⁻¹ to 10⁻⁹. A positive control is set up using a viable culture of M. capripneumoniae and a negative control using three uninoculated tubes of medium. All are incubated at 37°C for 12 days. If there is no growth in the tubes inoculated with test sample, the vaccine passes the test.

c) Protein content estimation

The protein content is measured by the bicinchoninic acid method by comparison with a bovine albumin standard.

4. Batch control

a) Sterility
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The sterility test on the final batch is carried out as described in Section C.3.a except that four bottles of each batch are pooled together and samples of the pool are used.

b) Safety

Every batch of CCPP vaccine must be proven to be safe in laboratory animals. Two guinea-pigs are each inoculated by the intramuscular route in the hind leg and another two guinea-pigs are inoculated by the peritoneal route with 0.5 ml containing five doses of vaccine.

If the vaccine is safe, the guinea-pigs should not show any sign of disease for a 14-day observation period and, on post-mortem, there should be no abscesses on the site of inoculation and in the peritoneal cavity, respectively. If any vaccine-related deaths occur during the observation period, the vaccine fails. If on post-mortem examination, abscesses are observed at the site of inoculation and the peritoneum, the vaccine also fails.

c) Potency

Research is in progress at the Kenya Agricultural Research Institute, Muguga, Kenya, to develop a test for potency.

d) Duration of immunity

The vaccine protects goats for 14 months. It is, however, recommended to boost immunity after 1 year.

e) Stability

If stored at 4°C, the vaccine has a shelf life of 1 year. Before use the vaccine should be shaken thoroughly, for even distribution of antigen.

f) Preservatives

At present preservatives are not used in the vaccine.

g) Precautions

Side-effects of the CCPP vaccine include development of a swelling at the site of inoculation and fever for 1–2 days following vaccination, accompanied with inappetence. The swelling may last between 1 and 14 days and is due to the saponin adjuvant. Accidental self-injection causes severe irritation.

5. Tests on the final product

a) Safety

Every batch of vaccine should be tested for safety in laboratory animals as described in C.4.b.

b) Potency

Once the potency test has been developed, every batch of vaccine will be required to be tested for potency.

REFERENCES


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