AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Contagious Equine Metritis

Bacteriology

B. Rogerson

Victorian Institute of Animal Science, Department of Agriculture,
475–485 Mickelham Road, Attwood, Vic. 3049, Australia.

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First published as: *Contagious Equine Metritis*, by B. Rogerson, Australian Bureau of Animal Health (1980).

Revised by B. Rogerson (1986) and published by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 40, *Contagious Equine Metritis*.

1. Introduction

Contagious equine metritis (CEM) is a venereally transmitted bacterial infection of the genital tract. It is characterised in mares by an early return to oestrus following a vaginal discharge within a few days of mating. Stallions show no clinical signs.

Contagious equine metritis was initially recognised in England (Crowhurst, 1977) and has been found in many countries including Australia (Hughes *et al.*, 1978).

The organism responsible is a small gram negative coccobacillus for which the name *Taylorella equigenitalis* has been validated (International Committee on Systematic Bacteriology, 1984).

Methods are given here for the isolation and identification of the organism and for serological procedures which may be of value in screening, in an event of an outbreak of this highly contagious disease.

Mares acutely infected with CEM usually develop a characteristic mucopurulent discharge 36–72 hours after mating. They will often return to oestrus within a few days (Timoney *et al.*, 1977). The severity of the disease is variable and clinical signs may be absent within three to four weeks of infection (Platt *et al.*, 1978). After the initial infection subsides or following inadequate treatment some mares remain carriers with the organism localised in the clitoral fossa and the crevices on the dorsal aspect of the glans clitoris (sometimes referred to as the 'sinuses').

Stallions show no clinical signs of the disease but the organism may localise in the urethral fossa, the anterior urethra and the folds of the internal aspect of the prepuce.

Transmission of the disease is primarily venereal, but personnel handling infected horses could spread infection from stallion to stallion during washing, or from mare to mare during gynaecological examination.

2. Collection of Specimens for Bacteriology

Mares in oestrus or exhibiting a uterine discharge should be swabbed from the cervix or endometrium. Such swabs from a mare with CEM are likely to be relatively free of other bacteria and are therefore suitable for culture on media containing no antibiotics (see 4.2.).
A combined swab from the vestibule of the urethra, the clitoral fossa and the clitoral sinuses should also be taken.

Mares not in oestrus (including pregnant mares) require only the combined swab described above. Stallions should be swabbed from the prepuce, anterior urethra and most importantly from the urethral fossa.

3. Transport of Specimens to the Laboratory

Swabs must be placed in a transport medium immediately after collection and transported to the laboratory as quickly as possible since the number of viable organisms decreases rapidly. Any undue delay may effectively reduce initially low numbers to zero.

A charcoal medium such as Amies formula (see 7.1.) is preferred, however this renders the specimen unsuitable for direct microscopic examination and a duplicate swab not in Amies medium is required for this purpose. When the delay between collection and examination is short (<48 hours) Stuart's transport medium (see 7.2.) can be used satisfactorily.

Refrigeration of the swabs in transport medium is undesirable as the organism has been shown to survive longer at room temperature than at 4°C.

4. Bacteriology

4.1. Examination of Smears

A smear prepared from swabs of the cervix or endometrium or preferably, if present, the actual discharge should be stained by Gram and examined for the presence of bacteria and leucocytes. When present in large numbers, Gram-negative coccobacilli may be seen within the neutrophils and extracellularly.

A positive smear may be considered as suspicious, but should be confirmed by culture. Smears prepared from the clitoral region and from material collected from stallions are uninformative because the causative agent cannot be recognised among the normal flora.

4.2. Culture

T. equigenitalis is slow growing and requires a medium containing blood for primary isolation, although recrystallised haemin (X factor) can be used in place of blood (Swaney and Sahu, 1978).

The preferred culture medium for initial isolation is a chocolatised agar prepared from Eugon agar (BBL) to which has been added 5–10% defibrinated sheep or horse blood. Eugon agar may be replaced by Columbia agar (BBL or Oxoid).

Swerczek (1980) considers that Eugon agar (Difco) is unsuitable for primary isolation. Inhibition of growth of *T. equigenitalis* by other organisms has been observed on primary isolation plates from clitoral fossa swabs. This, like Swerczek's observation, may be due to local acid producted by these other organisms as a consequence of their fermentation of carbohydrates in the medium.

In order to reduce contamination by commensal flora, especially when dealing with clitoral swabs or any swab from stallions, streptomycin (400 µg/mL) and nystatin (Mycostatin — Squibb, 100 units/mL) or amphotericin B (Fungizone — Squibb 5 µg/mL) should be incorporated in the medium for primary isolation.

The occurrence of a strain of the organism sensitive to streptomycin has been reported in the United States of America (Swerczek, 1978). For this reason it is advisable to inoculate a plate of the chosen selective medium not containing streptomycin with the swab from the cervix or endometrium. This less selective medium may also be inoculated from swabs of the clitoris or from the stallion but a high incidence of contamination of these plates can be expected.

A basic chocolate eugon agar with lysed horse blood (5%) ISOVITALEX supplement (Baltimore Biological Laboratories), clindamycin (5 µg/mL), trimethoprim ((1 µg/mL) and amphotericin B (5 µg/mL) has been proposed by Timoney *et al.* (1982). This medium, it is claimed, will support the growth of streptomycin resistant and sensitive strains of *T. equigenitalis* with adequate control of contamination. Atherton (1983) favours this medium but considers the ISOVITALEX to be unnecessary.

Plates should be incubated at 37°C in a humid atmosphere containing 5–10% carbon dioxide in air and examined after four days, and again after six days incubation before being discarded.

Ward *et al.* (1984) suggest that in cases of high risk mares, it may be advisable to incubate for up to 14 days.

4.3. Identification

Growth of *T. equigenitalis* first appears as small, nearly beige coloured colonies after 48 hours incubation, reaching 3–4 mm in diameter after five days. The colonies are smooth with an entire edge.

Using a wire loop with care, it is often possible to push an intact colony across the surface of the agar.

Microscopically, *T. equigenitalis* appears as a small non-sporing, non-encapsulated, Gram-negative coccobacillus. Older cultures and subcultures become more pleomorphic.

T. equigenitalis is catalase-positive, oxidase-positive, non-motile, asaccharolytic and microaerophilic. It will not grow under strictly aerobic or anaerobic conditions.

Identification may be confirmed by a slide agglutination test using rabbit hyperimmune antiserum against *T. equigenitalis* (Rommel *et al.*, 1978).

Rabbit antiserum may be conjugated with fluorescein (for conjugation methods refer to Nairn, 1976) to further assist in identification. A light suspension of the bacteria to be examined is spread over an area of about 2 cm^2 at the centre of a microscope slide. After drying in air the preparation is fixed in acetone (C_3H_6O) for 10 min.

The fixed, dried preparation is covered with a working dilution of the conjugated antiserum and incubated at 37°C in a moist chamber for 30 min.

The working dilution is determined by treating similarly prepared slides of a control organism with serial dilutions of the antiserum. The highest dilution which gives maximum fluorescence is chosen as the working dilution. The treated slides are washed with four changes of phosphate buffered saline (PBS), mounted under 1:1 glycerol: PBS and examined microscopically using a mercury vapour lamp and dark ground condenser (for filter combinations refer to Nairn, 1976).

This technique has proved useful to compare the morphology of fresh isolates with a control organsim. It may also be applied to the examination of direct smears of uterine discharge for the presence of *T. equigenitalis*, but in this case four washes with PBS may not be adequate to remove background fluorescence.

5. Serology

5.1. Serum Agglutination Test and Antiglobulin Test

Methods for both tests and their interpretation have been described by Benson *et al.* (1978).

The following more detailed procedure for preparing the antigen has been followed with satisfactory results.

Growth from 10 three-day-old eugon agar plates (as used for isolation but without antimicrobial agents) is harvested in 10 mL of sterile normal saline and refrigerated overnight in a McCartney bottle containing glass beads. The suspension is homogenised by shaking with the beads, transferred to a centrifuge tube and washed twice with saline at 7000 rpm for 30 min. The washed cell deposit is resuspended in 12 mL of saline using glass beads to assist the mixing and held in a boiling waterbath for two hours with occasional shaking.

After a further wash with saline the cells are resuspended in 12 mL of 0.5% phenol saline [0.5% w/v (0.5 mol/L) phenol (C_6H_6O) in 0.85% (0.15 mol/L) sodium chloride]. For use in the agglutination test this stock suspension is diluted so that it equals in density the suspension used for the brucella serum agglutination test (SAT) (Alton *et al.*, 1988).

5.2. Complement Fixation Test

Methods for a complement fixation test (CFT) and interpretation of results have been described by Croxton-Smith *et al.* (1978) and Bryans (1979). The CFT used throughout Australia for the bovine brucellosis eradication program (Australian Bureau of Animal Health, 1979*a*) can be adapted by replacing the *B. abortus* antigen with *T. equigenitalis* antigen of a concentration equal to a 10-fold dilution of that used for the SAT. Using this method it is recommended that any reaction at 1:4 should be regarded as suspicious. Reactions at 1:8 or higher should be regarded as positive.

5.3. *Passive Haemagglutination Test*

A method and interpretation of results for the passive haemagglutination test (PHT) are described by Fernie *et al.* (1979).

As described, this test requires production of a sonicated antigen and a source of turkey erythrocytes which could pose problems for a routine serology laboratory. However, sheep erythrocytes may be substituted and need not be formalised if prepared fresh as required (Rogerson *et al.*, 1984).

5.4. *Enzyme-linked Immunosorbent Assay*

The enzyme-linked immunosorbent assay (ELISA) test has been applied to CEM by Dolan *et al.* (1984) who compared it with the CFT and concluded that the ELISA was more sensitive, detecting antibody over a longer period post infection.

Many workers doubt the usefulness of serology as an aid to diagnosis of CEM (Eaglesome and Garcia, 1979; Crowhurst *et al.*, 1979; Frank *et al.*, 1979; Anon., 1979).

The major failing of serology is because antibodies are only produced during the active phase of the disease in the mare and frequently drop to undetectable levels within a few weeks of infection, even though many mares remain carriers of the organism (Rogerson *et al.*, 1984). Infected stallions show no immunological response.

The ELISA would appear to be the most sensitive test but carrier mares may still go undetected.

The most useful application of serology is during outbreaks when the disease is spreading rapidly, and has been applied successfully to determine the infection status of stallions by testing the sera of mares recently covered by them.

Routine testing of mares from two to five weeks after service could also provide evidence of continued absence of infection in harems at risk (e.g. where a higher number of non-resident mares are brought in for service).

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7. Appendixes

7.1. Appendix 1 — Amies Transport Medium (Amies, 1967)

Water, distilled	1 L
Agar	4.0 g
Heat until dissolved then add, while hot	
Sodium chloride, NaCl	3.0 g
Potassium chloride, KCl	0.2 g
Disodium hydrogen phosphate,	
Na_2HPO_4 (anhydrous)	1.15 g
(or $Na_2HPO_4.12H_2O$)	2.9 g
Potassium dihydrogen phosphate, KH_2PO_4	0.2 g
Sodium thioglycollate ($C_2H_3NaO_2S$)	1.0 g
$CaCl_2$, 1.0% solution (0.09 mol/L freshly prepared	10.0 mL
$MgCl_2$.$6H_2O$, 1.0% solution (0.05 mol/L)	10.0 mL
Stir until dissolved, then add:	
Charcoal, pharmaceutical neutral	10.0 g

Distribute into 6.0 mL screw-cap bottles or vials, stirring meanwhile to keep the charcoal evenly suspended. Screw down the caps firmLy. Autoclave at 120°C for 20 min. Invert the bottles just before the medium sets in order to distribute the charcoal uniformly. Retighten caps if necessary. Store in a cool place. The final pH is 7.2–7.4.

7.2. Appendix 2 — Stuarts (1959) Transport Medium (Cruickshank et al., 1975)

7.2.1. Anaerobic Salt Solution

Thioglycollic acid (Difco)	2 mL
Sodium hydroxide, NaOH (1 mol/L)	12–15 mL
Sodium glycerophosphate, 20% aqueous, $C_3H_7Na_2O_6P$ (0.93 mol/L)	100 mL
Calcium chloride 1% (0.09 mol/L) aqueous	20 mL
Distilled water (Cl_2 free)	900 mL

Mix the ingredients, adding sufficient sodium hydroxide to bring the pH to 7.2.

7.2.2. Agar Solution

Agar	6 g
Distilled water (Cl_2 free)	1 L

Dissolve by steaming.

7.2.3. Preparation of Complete Medium

Anaerobic salt solution	900 mL
Agar solution	1 L
Methylene blue, 0.1% aqueous	4 mL

Melt the agar and add the salt solution. Adjust the pH to 7.3–7.4. Add the methylene blue and distribute in bijou bottles, filling nearly to capacity. Autoclave at 121°C for 15 min and immediately tighten caps. When cool the medium should be colourless.