# Contagious Equine Metritis

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## Part 1. Diagnostic Overview

### Summary

Contagious equine metritis (CEM) is a bacterial infection of the genital tract of mares, which is characterised by an inflammation of the endometrium and results in temporary infertility. Stallions may become infected but show no clinical signs. It is a localised infection restricted to the reproductive tract of the mare.

*Taylorella equigenitalis*, the cause of CEM, was first isolated in Australia in 1977. Thirty isolations were made in 1978 and 1979. However, Australia has been free from *T equigenitalis* since 1980. CEM or the presence of *T equigenitalis* has never been reported in New Zealand.

The primary signs of infection in mares are a slight to copious mucopurulent vaginal discharge 1-3 days after mating and a variable endometritis cervicitis and vaginitis. Mares will often return to oestrus within a few days.[1] The severity of disease is variable and signs may be absent in three to four weeks after infection, although prolonged asymptomatic carriage is established in some infected mares. The sites of persistence of *T equigenitalis* in the mare are urogenital membranes, primarily in the clitoral sinus and fossa and, very infrequently, the uterus. Foals born of carrier mares may also become carriers.

*T equigenitalis* can also infect other equid species such as donkeys.

The transmission of CEM is primarily venereal, although inadequate hygiene during examination or other handling procedure of the genitalia of horses can also transmit the infection. Thorough washing and disinfection combined with local and systemic antibiotic treatment can eliminate *T equigenitalis.* Vaccination has been found to be ineffective.

Prior infection and vaccination are not fully protective, and failure of antibody to persist has meant that control of infection has relied entirely on prevention of transmission through the detection of *T equigenitalis* on swabs of urogenital membranes.

The detection of *T equigenitalis* is based primarily on the culture of the organism from swabs of urogenital membranes. However, the emergence of *Taylorella asinigenitalis*, an organism with similar colonial appearance and cultural characteristics to *T equigenitalis* and which may also infect horses, has necessitated the use of PCR assays for the effective discrimination between the two species. None of these PCR assays has been evaluated by SCAHLS, as *T asinigenitalis* has not been reported in Australia, which has been free from *T equigenitalis* since 1980, and neither organism has been reported in New Zealand. These assays have been included in this ANZSDP to facilitate the rapid diagnosis of CEM in the event of its re-entry into Australia or entry into New Zealand.

## 1. Aetiology

Contagious equine metritis (CEM) is caused by *T equigenitalis*[2], a gram-negative, non-motile, bacillus or small cocco-bacillus, which is often pleomorphic and may exhibit bipolar staining. Another species of *Taylorella*, *T asinigenitalis*, was described in 2001. This species was isolated from male donkeys in the USA. It is not associated with disease, although the experimental work is limited, but can be passed to donkeys and horses during mating.[3] *T asinigenitalis* has a similar colonial appearance, cultural characteristics and gives identical biochemical reactions to *T equigenitalis.*

## 2. Clinical Signs

The primary signs are a slight to copious mucopurulent vaginal discharge and a variable endometritis, cervicitis and vaginitis, usually 1-3 days after mating. Acutely infected mares develop a vaginal discharge, which may vary from frank pus to a mucopurulent fluid that may persist for 3-4 weeks. The discharge may be copious and coat the perineum, mat the hairs of the tail and smear the buttocks, or the discharge may only accumulate on the anterior floor and not produce external signs or only produce a slight excess of vaginal mucus. Usually, only 30-40% of mares served by an infected stallion develop clinical signs and most infected mares recover uneventfully. *T equigenitalis* may persist for 2-10 weeks in some mares but it may also establish a carrier status for many months in others. Chronic carriers may show reduced fertility, irregular oestrus cycles and occasional low-grade vaginal discharge. CEM is also commonly associated with an early and unexpected return to oestrus but rarely with abortion.[4,5,6]

Infected stallions and teasers are asymptomatic and act as reservoirs of *T equigenitalis*. Infected stallions, which mate with numerous mares, act as the principal means of transmission.

## 3. Epidemiology

CEM is a sexually transmitted disease of horses that causes endometritis and temporary infertility in mares. The incubation period is usually 1-3 days but may be up to 12 days. The duration of clinical signs in experimentally infected animals can vary from 4-11 days. *T equigenitalis* usually persists for a relatively short time (2-10 weeks) but may establish a carrier status that can persist for more than 10 months. The isolation of *T equigenitalis* from carrier mares may also be erratic with experimentally infected animals being negative for up to 2

weeks.[5] Re-exposure of mares previously infected with *T equigenitalis* is associated with the development of minimal or no clinical evidence of disease.

The fossa and sinuses of the clitoris of mares are the preferred sites for *T equigenitalis* but it may be recovered from the cervix and endometrium in pure culture.[4] Long-term carriers of the organism in the uterus are not common but have been reported.[6] In stallions the urogenital membranes become contaminated at coitus, leading to a carrier state that may persist for many months or years.[7] *T equigenitalis* may also be spread by the unhygienic examination of mares and unsanitary washing of the stallion's penis.[6]

Serum antibody persists for 3-7 weeks after infection, but often is not detectable for up to 21 days after recovery from acute infection in mares.[8] However, no serological test, alone, can reliably detect infection.

## 4. Occurrence and Distribution

CEM was initially described in the United Kingdom.[9]

The disease was first identified in Australia in Victoria in 1977. The last Australian case was recorded in 1980 and ongoing surveillance has not revealed any further cases. Australia was officially declared CEM-free in 1985.

Other countries in which the disease is present or suspected to occur include Austria, Belgium, Bosnia and Herzegovinia, Croatia, Czech Republic, Denmark, Finland, France, Germany, Guinea-Bissau, Ireland, Italy, Japan, The Netherlands, Norway, Slovakia, Slovenia, Sweden, the former Yugoslavia Republic of Macedonia, and areas of former Yugoslavia (Montenegro and Serbia).

The disease has never been recorded in New Zealand or South Africa.[10]

## 5. Gross Pathology

There are no characteristic gross lesions.

## 6. Diagnostic Test Methods

The diagnosis of CEM is based on the detection of *T equigenitalis* in infected horses. The successful culture of *T equigenitalis* is dependent on the collection of appropriate specimens and the submission of swabs in an appropriate transport medium under suitable transport conditions. The fastidious nature of the organism makes isolation difficult. Once isolated its unreactivity in traditional biochemical tests used for the identification of bacteria make it difficult to reliably identify. This has been further complicated by the discovery of *T asinigenitalis,* which is non-pathogenic but very closely related to *T equigenitalis* from which it cannot be reliably differentiated using traditional microbiological techniques.

PCR can be used to differentiate between *T equigenitalis* and *T asinigenitalis.* PCR has also been used for the detection of *T equigenitalis* from genital swabs.

Serological tests have a role to play in the rapid assessment of spread of infection in an outbreak[5] but cannot be used alone to reliably diagnose CEM. However, the complement fixation test can be used in conjunction with culture for *T equigenitalis* in screening mares 20 days or more after being mated with a carrier stallion. [6]

## Part 2. Test Methods

### 1. Collection of Specimens for the Isolation of *T equigenitalis*

Swabs should not be collected from horses that have been treated with antibiotics during the previous 7 days. The presence of antibiotics may sub-lethally damage *T equigenitalis*, which nonetheless persists on the urogenital membranes but cannot be grown on laboratory media. PCR can also be used to determine the efficacy of antibiotic treatment although PCR will not indicate viability.

Stallions: Swabs should be taken from:

- prepuce,
- urethral fossa, and
- anterior urethra

Non-pregnant mares:

- cervix,
- clitoral sinus, and
- clitoral fossa

Pregnant mares

- clitoral sinus and
- clitoral fossa

Vaginal swabs should be taken from mares with a vaginal discharge.

There are occasions when *T equigenitalis* cannot be cultured from infected animals. It is recommended that several attempts be made to culture *T equigenitalis* over a 3-week period to minimise the chances of not detecting an infected animal.[5] A current Australian Quarantine and Inspection Service requirement for importing live horses is that they are swabbed at 7-9 day intervals on three occasions with negative results before being certified *T equigenitalis*-free. [11]

### 2. Transport of Specimens to the Laboratory

Swabs should be submitted in Amies charcoal transport medium[12] to absorb inhibitory by-products of bacterial metabolism (see Part 3)[13] and kept cool during transport. Do not use Stuarts transport medium. Refrigeration of the swabs is not recommended as *T equigenitalis* survives longer at room temperature than at 4$^{o}$C.[14] However, exposure of swabs to high temperatures should be avoided. Swabs should arrive at the laboratory and be plated out within 48 hours of collection, otherwise negative results of culture are unreliable. Submitters should provide the time and date of collection.

### 3. Culture of *T equigenitalis*

A diagnosis of CEM is made by the isolation of *T equigenitalis* by culture. *T equigenitalis* is a fastidious and slow growing organism. *T equigenitalis* will grow on 7% sheep or horse blood agar as just small dew-drop colonies, which can be easily overgrown by commensal bacteria. To optimise the growth of *T equigenitalis* each swab must be inoculated on to a selective medium based on Eugon or Columbia agar (without added glucose)[12], 5% chocolatised horse blood or sheep blood and 5% lysed horse blood. (The chocolatised blood can be produced by heating the liquid medium, containing blood, at 70-80$^{o}$C for 12 minutes). When cooled to 45-50$^{o}$C, trimethoprim (1 µg/mL), clindamycin (5 µg/mL) and amphotericin B (5 µg/mL) are added to the medium.[15,16] Thymidine, which will inactivate trimethoprim, is present in bacteriological media containing peptone, so it is important to add 5% lysed horse blood as lysed horse blood contains thymidine phosphorylase, which will inactivate thymidine, thus allowing trimethoprim to exert its selective effect. This is the preferred medium for isolating *T equigenitalis* and has been used successfully to isolate both biotypes (streptomycin-sensitive and streptomycin-resistant) of this pathogen and to inhibit the growth of many commensal bacteria (see Part 3; 2).[15,6] The addition of Isovitalex does not have any significant growth promoting effect.[16]

If streptomycin sulphate (200 µg/mL) is added to the above medium, it can be used to culture isolates of *T equigenitalis* resistant to this antibiotic. The advantage of using streptomycin sulphate is that it reduces the growth of contaminants that may obscure the presence of small numbers of *T equigenitalis*.[17] However, as streptomycin-sensitive strains are more commonly detected than streptomycin-resistant strains the use of streptomycin sulphate is not recommneded.[6]

Plates must be incubated at 35-37°C in 5-10% (v/v) $CO_2$ in air. At least 72 hours is normally required before colonies of *T equigenitalis* become visible, after which time daily inspection is needed. Visual detection of colonies may take up to 14 days.[18] A standard incubation time of at least 7 days is advisable before certifying cultures negative for *T equigenitalis*.

Quality control systems to ensure that CEM culture media in use will support the growth of *T equigenitalis* must be in place.

**4. Polymerase Chain Reaction (PCR) Assays**

Conventional and real-time assays have been developed to detect and differentiate these two organisms. Conventional PCR assays for the direct detection of *T equigenitalis* on genital swabs[19,20] may not differentiate between *T equigenitalis* and the closely related *T asinigenitalis*, although a recent assay can be used to directly detect only *T equigenitalis* on genital swabs.[21] A conventional PCR assay has been developed to differentiate between these two organisms [22] and for the direct detection of, and discrimination between these two organisms. [22] Real-time PCR assays have been developed for the direct detection of, and discrimination between these two organisms. [23,24]

The direct detection of *T equigenitalis* in swabs also has the advantage of not being significantly affected by commensal bacteria that are commonly found in

the genital tract of horses that may overgrow *T equigenitalis* colonies when bacteriological culture is undertaken. [23]

### 4.1 Preparation of DNA Samples from Genital Swabs and Cultures

Swabs are agitated in 0.2 mL of 0.1 M PBS (pH 7.4) in a 1.5 mL microfuge tube for 5 s. Bacterial cells are grown on CEM-selective medium at 37°C in 10% $CO_2$ for 38 h. Colonies are scraped off the agar surface. The bacteria and transport medium are then pelleted by centrifugation in a microcentrifuge at 18000 x *g* for 30 s. The supernatant is aspirated and the pellet resuspended in 100 µL nuclease free water (Sigma, St. Louis, MO) prior to heating in a heat block at 95-100°C for 15 min. Insoluble material is pelleted by centrifugation in a microfuge at 18000 x *g* for 1 min. The lysate is used either immediately or after storage at -20°C. [24]

### 4.2 Primers PCR

Primer sets to distinguish between *T equigenitalis* and *T asinigenitalis* are listed below. [22]

| Organism | Primer | Product Size |
|---|---|---|
| *T equigenitalis* | TEQF 5'GGTTTGTGTTAATACCATGGAC 3' | 406 |
| | TEQR 5'TCGCTACCAAGACCCG 3' | |
| *T equigenitalis-like* | TELF 5'AGTTTTAGGATAATACCCTAGGA 3' | 706 |
| (*T asinigentialis*) | TELF 5' TCTCATTAGAGTGCCCTTTTAC 3' | |
| Positive control | POSF 5' GAGTTTGATCCTGGCTCAG 3' | 1500 |
| (Universal primers) | POSR 5' GGTTACCTTGTTACGACTTCAC 3' | |

### 4.3 Conventional PCR

Amplifications are carried out in a 50 µL reaction mixture as follows: 2 µL template DNA (10 ng/mL), 0.5 µM of each of 4 primers (see above), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM of each dNTP, 7.5 mM MgCl, 2 µL dimethylsulfoxide and 2.5 units of *Taq* polymerase. PCR for amplification of bacterial DNA encoding the 16S rRNA (positive control) was performed in a 50 µL reaction mixture containing the following components: 2 µL template DNA (10 ng/ml), 0.75 µM of each of the 2 universal primers (see above), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM of each dNTP, 1.5 mM $MgCl_2$, 2 µL dimethyl sulfoxide, and 2.5 units *Taq* polymerase. [22]

The conditions for amplification of *T asinigenitalis* and *T equigenitalis* DNA and bacterial DNA (positive control) are the same. After an initial denaturation/enzyme activation step (95°C, 10 min), 35 cycles are performed as follows: 95°C for 1 min, 56°C for 1 min; 72°C for 2 min. PCR products are separated electrophoretically in a 2% agarose gel in Tris-acetate buffer (40 mM Tris acetate, 2mM EDTA, pH 8.5) and then digitised after staining in ethidium bromide. [22]

### 4.4 Primers and Probes for Real Time PCR

The primers Tay377for (CCGCGTGTGCGATTGA) and Tay488rev (TTTGCCGGTGCTTATTCTTCA) prime the amplification of 112 bp PCR

product. Specific detection of *T equigenitalis* is with TequiFAM, AAAGGTTTGTGTTAATACCATGGACTGCTGACGG probe and *T asingentialis* with TasiniHEX, AAAGTTTTAGGATAATACCCTAGGATGCTGACGG. [24]

### 4.5 Real Time PCR

Amplifications are performed in a total volume of 50 µL containing 2 µl of bacterial lysate and validated on a real-time PCR machine (MX3000P; Stratagene; La Jolla, CA). Each reaction should contain 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton® X-100 (supplied as a 10 X reaction buffer; Promega, Madison, WI), 2.5 mM $MgCl_2$ 0.2 mM (of each) deoxynuclease triphosphate (Promega), 400 nM of each primer pair (for *Taylorella*), 100 nM of the TequiFAM and TasiniHEX probes and 2.5 U of *Taq* DNA polymerase (Promega). [24] Following initial denaturation of the template and primers at 94°C for 2 min, a total of 40 cycles of the following regimen is used to amplify the specific *Taylorella* signal; denaturation, 5 s at 94°C; primer annealing, 10 s at 72°C.

In-house optimisation and validation in conjunction with appropriate controls is required to determine the "critical threshold" for the assay in the absence of prescribed standards and different real-time PCR platforms. Only those samples that produce a significant increase in fluorescence over background determined by the real-time PCR software are considered positive.

## 5. Identification of *T equigenitalis*

Colonies of *T equigenitalis* may be small, smooth with an entire edge, glossy and yellowish grey reaching 3-4 mm in diameter after about 5 days.[6,14]

*T equigenitalis* is a small, non-sporing, non-motile gram negative bacillus or cocco-bacillus, which may be pleomorphic (up to 6 µm long) and may exhibit bipolar staining.

*T equigenitalis* is strongly oxidase positive, catalase positive, non-motile, asaccharolytic and microaerophilic. Test methodologies are described in Barrow and Feldmann.[25] *T equigenitalis* will not grow under strictly aerobic or anaerobic conditions. Slow growing organisms that fit this description should be tested using a slide agglutination test using antiserum against *T equigenitalis*.[26] However, PCR is required to differentiate between *T equigenitalis* and *T asinigenitalis.*

## 6. Serology

No serological test, by itself reliably detects infection. However, the complement fixation test (CFT) has been successfully used as an adjunct to culture for *T equigenitalis* in screening mares 20 days or more after being bred with a carrier stallion. [6] The CFT is generally negative 12 weeks after infection.

### 6.1 Preparation of *T equigenitalis* Antigen

*T equigenitalis* is grown on 10 Eugon agar plates (as described above but without antimicrobial agents). Growth 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 3000 x *g* 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 h 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 SAT or the CFT, this stock suspension is diluted so that it equals a 10-fold dilution of the density of the suspension for the *Brucella* serum agglutination test. [14,27]

### 6.2 Complement Fixation Test

Methods for a CFT and interpretation of results have been described by Croxton-Smith et al. (1978) [28] and Bryans (1979) [29]. The CFT used throughout Australia for the bovine brucellosis eradication program (Australian Bureau of Animal Health, 1979) can be adapted by replacing the *Brucella 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 reactions at 1:4 should be regarded as suspicious. Reactions at 1:8 or higher should be regarded as positive.

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## Part 3. Reagents and Test Kits for the Diagnosis of Contagious Equine Metritis

### 1. Amies Transport Medium

| | |
|---|---|
| Sodium chloride | 4.0 g |
| Potassium chloride | 0.1 g |
| Calcium chloride | 0.05 g |
| Magnesium chloride | 0.05 g |
| Potassium dihydrogen orthophosphate | 0.1 g |
| Disodium hydrogen orthophosphate | 0.75 g |
| Sodium thioglycollate | 0.5 g |
| Charcoal, Bacteriological | 5.0 g |
| Agar Noble | 3.0 g |
| Milli-Q Water | 500 mL |

Commercially made alternatives may be used.
Example: Amies Transport Media (Oxoid CM425) - Follow manufacturer's directions.

Procedure:
1. Weigh ingredients into individual weighing dishes.
2. To a 1000 mL flask add about ¼ of the water.
3. Add the weighed ingredients, rinsing each weighing boat with a little of the Milli-Q water to ensure all contents are transferred and then add the remaining Milli-Q water.
4. Put a stirrer bar into the flask; cover the opening of the flask with foil and place on the magnetic stirrer. Heat gently while stirring until medium dissolves. The charcoal will not dissolve but remain in suspension.
5. Insert cotton-wool bung & tape over with autoclave tape, cover the head and neck of flask with aluminium foil.
6. Sterilise by autoclaving @ 121°C for 20 mins.
7. Cool to 56°C in water bath or incubator for 1 hour.
8. Place on stirrer and stir gently to prevent charcoal from settling during dispensing. Dispense 3mL into sterile 5mL bottles.
9. Label with media type, batch number and expiry date.
10. Store at 4°C in cold room.

Shelf life**:** 24 months

Nominal pH: 6.8 +/- 0.2 at 25°C after sterilisation

### 2. CEM selective medium

Columbia blood agar base (Commercially made alternatives may be used)

| | |
|---|---|
| 1. Agar | 15.0 g |
| 2. Pantone | 10.0 g |
| 3. Bitone | 10.0 g |
| 4. NaCl | 5.0 g |
| 5. Tryptic digest of beef heart | 3.0 g |
| 6. Cornstarch | 1.0 g |

or

Eugon agar (Commercially made alternatives may be used)

| | |
|---|---|
| 1. Agar | 15.0 g |
| 2. Pancreatic digest of casein | 15.0 g |
| 3. Glucose | 5.5 g |
| 4. Papaic digest of soybean meal | 5.0 g |
| 5. NaCl | 4.0 g |
| 6. L-Cystine | 0.5 g |
| 7. $Na_2 SO_3$ | 0.2 g |
| 8. Chocolatised horse blood or sheep blood | 50 ml |
| 9. Lysed horse blood | 50 ml |
| 10 Trimethoprim | 1 µg/mL |
| 11. Clindamycin | 5 µg/mL |
| 12. Amphoteracin B | 5 µg/mL |

Procedure:

1. Add components of Eugon or Columbia agar to distilled water and bring volume to 1.0 L.
2. Mix thoroughly; gently heat until boiling.
3. Autoclave at 121$^{o}$C for 15 mins.
4. Add chocolatised horse blood (the chocolatised blood can be produced by heating the liquid medium, containing blood, at 70-80$^{o}$C for 12 minutes).
5. Add 50 mL lysed horse blood (Commercially made alternatives may be used)
6. Cool to 45-50$^{o}$C.
7. Add trimethoprim, clindamycin and amphotericin B.
8. Pour into sterile Petri dishes