

## Bovine Trichomoniasis

Nicky Buller

Animal Health Laboratories  
Department of Agriculture and Food Western Australia  
3 Baron-Hay Court  
South Perth, WA 6151  
[nicky.buller@agric.wa.gov.au](mailto:nicky.buller@agric.wa.gov.au)

Bruce Corney

Biosecurity Queensland  
Department of Agriculture, Fisheries and Forestry  
Brisbane, QLD  
[bruce.corney@daff.qld.gov.au](mailto:bruce.corney@daff.qld.gov.au)

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### Part 1. Diagnostic Overview

#### *Summary*

*Bovine trichomoniasis is a disease of the reproductive tract caused by Tritrichomonas foetus, a flagellate protozoan. In cows, infection leads to embryonic and early foetal death, abortion, foetal maceration, pyometra and transient or permanent infertility. Asymptomatic infection occurs in bulls, which become persistent carriers and the main reservoir of infection. Young bulls are less susceptible to infection. Heifers have a higher incidence of the disease compared to cows, as the latter may remain immune for up to three years post-infection.*

*The disease is found in all cattle-producing countries in the world. In some parts it still causes major economic loss due to abortion, infertility and the culling of carrier bulls. The disease is found in extensively farmed beef cattle, but in intensively farmed beef and dairy cattle the use of artificial insemination has reduced the incidence of the disease. As a result, it is seldom seen in cattle in New Zealand or southern parts of Australia, but it still occurs in northern Australia.*

*Bovine trichomoniasis is diagnosed by detection of the parasite in direct smears or culture, or by PCR of preputial washings, vaginal material or aborted tissues. Other trichomonads may be present in samples and need to be differentiated from T. foetus through either morphology or PCR.*

*The disease has occurred in Australia since 1946 and in New Zealand since 1937. Cattle and buffalo semen imported into Australia must be free of T. foetus.*

*Breeding centres must demonstrate that bulls are free of T. foetus.*

*The OIE states that for international trade requirements detection of T. foetus by either direct examination or culture is the current prescribed test. Although PCRs are available, further validation is needed before they can be accepted as a prescribed test.*

#### **Aetiology**

The causative agent of bovine trichomoniasis is *Tritrichomonas foetus* (Riedmüller, 1928),<sup>1</sup> which belongs to the Class Parabasalia, from the classification system of Dyer, where protozoa with the “9+2” flagellum belong to the phylum Zoomastigina.<sup>2,3</sup> The protozoan is 8-18 µm long, 4-9 µm wide, pyriform (pear-shaped) but with

considerable pleomorphism. It is characterised by three anterior flagella of length 11-17  $\mu\text{m}$  and one posterior flagellum 16  $\mu\text{m}$  in length. An undulating membrane with 2-5 waves is positioned lengthways along the organism and extends to form the posterior flagellum. The axostyle is prominent with a chromatic ring at the point of emergence from the posterior end of the organism (Figures 1, 2 and 3).

*T. foetus* consists of only one trophozoitic form and has a simple lifestyle. The trophozoites can adopt a spherical shape, known as a pseudocyst, and internalise (or retract) their flagella.<sup>4</sup> The formation of pseudocysts occurs during times of stress either inside the body of the host or in culture media and also during adhesion to and subsequent phagocytosis of sperm cells.<sup>5</sup> The role of the pseudocyst form is not fully known but it may be involved in transfer to a new host or attachment to host cells.<sup>4</sup> Pseudocysts can generate multinucleated organisms that, under favourable environmental conditions, release single organisms. Pseudocysts also occur in preputial samples at a rate of 55% compared to pear-shaped parasites at 20%. *T. foetus* does not form cysts and cannot survive outside the host.<sup>5</sup>

Three serotypes of *T. foetus* have been described worldwide and all have been reported in Australia: var *brisbane*, var *belfast* and var *manley*, with the former two being the most common in Australia.<sup>6,7,8</sup>

*T. foetus* is indistinguishable from *T. suis* (found in nose and gut of pigs) according to morphology, pathogenic potential in subcutaneous mouse assays, by DNA fingerprinting using restriction fragment length polymorphism and variable-length DNA repeats, and by comparison of the 16S rRNA gene.<sup>9</sup> Serological properties shared by the two organisms also suggest they are the same species.<sup>10</sup> *T. foetus* causes chronic large bowel diarrhoea in cats; however, sequence information from the TR7/TR8 variable-length repeat within the internal transcribed spacer region<sup>11</sup> and within the elongation factor-1 alpha (EF-1 $\alpha$ ) gene<sup>12</sup> suggests a bovine genotype and a feline genotype.

Other trichomonads may be present in the bovine genital tract: *Pentatrichomonas hominis*, *Tetratrichomonas buttreyi*, *Tetratrichomonas pavlova*, *Tritrichomonas enteris* and *Pseudotrichomonas* species.<sup>13,14</sup> *Trichomonas tenax* occurs infrequently in humans in the oral gingival or in tracheobronchial sites, and *P. hominis* occurs infrequently in the intestinal tract. These species only transiently infect heifers.<sup>15</sup> The organisms do not persist in the reproductive tract, specific antibodies of IgA type is not detected, and only a mild inflammatory response is produced compared to infection with *T. foetus*.<sup>15</sup>

Mechanisms leading to infertility and abortion are related to the parasite's ability to infect the mucosal surfaces of the reproductive tract, to bind to spermatozoa and to release a cysteine protease that induces cell death. Adherence of *T. foetus* to spermatozoa results in loss of motility, agglutination and release of lysozymes that digest the sperm.<sup>5,16</sup> In the cow, *T. foetus* moves through the reproductive tract where it adheres to and infects vaginal epithelial cells, uterine epithelial cells, oviduct cells, and in pregnant cows, the placenta. Adherent *T. foetus* cells release a cysteine protease (CP30) that produces cytopathic effects in oviduct cells and in epithelial cells of the vagina and uterus resulting in apoptosis.<sup>17,18</sup> The cysteine protease is capable of cleaving IgG<sub>2</sub> and evading the host immune response.<sup>19</sup>

## Clinical Signs

In trichomoniasis, overt clinical signs are not seen. Evidence of the disease in a herd is chronic infertility, returns to service after four to five months (with a higher incidence in heifers compared to cows), aborted fetuses (at any time during gestation from two-months onwards but more commonly from three to five months), and persistent vaginal discharge post coitus.<sup>20</sup>

In cows, clinical signs vary from mild vaginitis or endometritis, to acute inflammation of the entire reproductive tract. Infection during pregnancy leads to early embryonal death, abortion and, sporadically, pyometra.<sup>20</sup>

Return to service within three to five weeks post-coitus indicates infection and related failure to become pregnant, or early termination.<sup>20</sup> Reduced calving rates of approximately 18% are greatest in the first two years in cows experiencing infection for the first time, with decreasing production loss in subsequent years.<sup>21</sup>

Cows recovering from infection are generally resistant to the infection for one to three years although this varies between animals.<sup>20</sup>

No clinical signs are present in infected bulls.<sup>20</sup>

## Epidemiology

The major route of transmission of *T. foetus* is through coital contact between a bull and a heifer or cow, although contaminated artificial insemination (AI) equipment may also transmit the infection.<sup>20</sup>

An infected bull is the main reservoir of infection and is a major risk factor for ongoing disease in a herd. The infective dose for three- to seven-year old bulls is  $10^6$  organisms, but some can be infected with as little as  $10^2$  organisms, indicating a likely difference in host susceptibility. Older bulls (four, five and six years of age) are more susceptible to infection when naturally mated to infected heifers, whereas younger bulls (three year olds) are less susceptible.<sup>22,23</sup> The exclusive use of young bulls may reduce the incidence of the disease in cows in extensively managed herds.<sup>23</sup>

The infective dose for cows is usually  $10^3$  organisms.<sup>22</sup> Infection in previously non-infected cows is most likely to occur when the time interval between services is less than 20 minutes. Once cows are infected, *T. foetus* can be isolated two weeks after mating.<sup>22,24</sup> Passive transmission (transmission of the organism from infected to non-infected cows via a non-infected bull) can occur but the transmission rate is low.<sup>25</sup>

Survival of *T. foetus* in the uterus occurs for up to 22 months. It causes inflammation, which either prevents conception, or leads to abortion. Cows may recover from infection, usually within 90 days and over a number of oestral cycles, but reinfection is common.<sup>24</sup> IgG<sub>1</sub> opsonic antibodies are produced in the vaginal mucus and antibodies of class IgG<sub>2</sub> are produced in the serum and may result in immunity for up to nine months post-infection, but there is a progressive loss of immunity over the following 20 months during which the animal can become re-infected.<sup>21,24</sup> Immunity does not develop with age.

The immune response in infected bulls is poor, and consistent with a carrier status. Agglutinating antibodies are not detected in the preputial cavity.<sup>26</sup> The immunological response is not reliable for diagnostic purposes, hence serology is limited in its use for detection of *T. foetus*, due to poor sensitivity and specificity.<sup>26</sup> The antibody titre in serum may be 1:32 without infection but increases to 1:512 in heifers 11 weeks post-

infection, and to 1:128 in bulls.<sup>26</sup> Non-specific agglutinins against *T. foetus* have been reported from a variety of animals including frogs, birds, rabbit and horse.<sup>25</sup>

Immunisation with a vaccine containing killed cells of *T. foetus* prevents genital infection in most bulls up to the age of five years; however, it does not effectively prevent or cure infection in bulls older than five and a half years.<sup>27</sup> Vaccinated bulls challenged with *T. foetus* produce a systemic and genital immune response due to IgG<sub>1</sub> and IgG<sub>2</sub> antibodies, but unvaccinated bulls, when challenged, do not produce an antibody response and persistent infection occurs.<sup>28</sup> Vaccination reduces the rate of abortion by only 30%.<sup>29</sup>

Serotyping of the protozoan is no longer performed, but all three serotypes of *T. foetus* have been reported in Australia. In north-eastern Australia a study found a distribution of 80% for *T. foetus* var *brisbane* and 20% for *T. foetus* var *belfast*. In NSW var *belfast* predominated with a low incidence of var *manley*. Serotypes var *belfast* and var *brisbane* were the only serotypes detected in WA.<sup>6,7,8,30,31,32</sup>

The host range for *T. foetus* includes cattle (*Bos taurus* and *B. primigenius indicus*), horses, roe deer, cats and pigs.<sup>3,33</sup> Other mammals such as domestic cats, horses and roe deer can be hosts to *T. foetus* and experimental infection has been established in rabbits, golden hamsters, guinea pigs, dogs, pigs and goats.<sup>3</sup>

### Occurrence and Distribution

Bovine trichomoniasis was first reported in France in 1888. It has a worldwide distribution especially in countries where natural service is commonly practised. It has been reported from Argentina, Austria, Canada, Czech Republic, Denmark, Germany, Hungary, Italy, Japan, Kenya, Mexico, Norway, Serbia and Montenegro, Slovakia, Spain, South Africa, Switzerland, Poland, Puerto Rico, Romania, Russia, United Kingdom and United States, although nowadays, with the use of AI, the disease is seen rarely in many countries.<sup>3,14,25,29,34</sup>

In Australia, bovine trichomoniasis was reported in 1948<sup>35</sup> to have occurred on King Island in 1946 and in Queensland in 1950.<sup>36</sup> It is widespread in extensively managed beef herds in the northern parts of Australia. In the Northern Territory, the disease occurs in the districts of Darwin, Victoria River, Elsey, Gulf and Barkly Tablelands, with a low incidence around the Alice Springs district. The last survey, in the mid-eighties, reported 65.6% of herds infected (1,008 bulls were tested on 41 stations in the Victoria River district), with a prevalence in infected herds of 2.9-33.3% (an average of 11.7%).<sup>37</sup> In Queensland, a 1974 study indicated 17.5% of herds were infected.<sup>31</sup> In Western Australia (WA), a 1977 survey of *T. foetus* through abattoir sampling of bull genitalia indicated a prevalence of 1.8% in pastoral areas and 28.2% in the Kimberley region.<sup>38</sup> *T. foetus* was not found in samples from the south-west of WA.<sup>32</sup> The disease is rarely seen in intensively managed herds in the southern parts of Australia due to the use of AI.

In New Zealand the disease was first reported in 1937.<sup>39</sup> It has been reported infrequently since then, from a beef herd in the Tokomaru Bay area in 1982<sup>40</sup> and from the Hawke's Bay/Gisbourne regions in 1996.<sup>41</sup>

No control programs exist in New Zealand or Australia and the disease is not notifiable.

### Gross Pathology

In infected bulls, gross pathological changes are not seen.<sup>42</sup> Of importance to note for collection of samples, is that *T. foetus* is present in all regions of the penis and prepuce with a concentration in the glans penis, and occasionally the anterior urethral orifice, but does not occur in the epididymes, ampullas, seminal vesicles, pelvic urethra or testes.<sup>42</sup>

In cows, the uterus is the main organ affected. Transient infection occurs in the oviducts, cervix, uterus and vagina.<sup>20,24</sup> The placenta is oedematous and often shows a degree of autolysis. Aborted fetuses vary in gestational age from two months to full term. Gross lesions may not be apparent but in some aborted fetuses a moderately enlarged liver is present. However, microscopic lesions occur in the placenta and foetal lungs.<sup>43</sup> Trichomonads have been observed in the placental stroma, and in lung and airways of aborted fetuses and these tissues should be targeted for sample collection. A pyogranulomatous bronchopneumonia is seen, histopathologically, in many fetuses.<sup>43</sup> Pregnant animals may show gross evidence of impending abortion through haemorrhagic placentomes, partial detachment of the cotyledons and placenta, and pale yellow foetal fluids containing a fine colloidal suspension.<sup>24</sup> Genital tracts and fetuses may appear normal despite the infection. Pyometra is occasionally apparent and a pale yellow, semi-solid material may be present in the uterine horns, cervix and oviducts. *T. foetus* occurs in the uterus, oviducts, foetal fluids and placentomes<sup>24</sup> and these areas should be targeted for culture.

### Diagnostic Tests (General)

Diagnosis of trichomoniasis takes into account clinical history of the herd, which includes signs of early abortion, repeated returns to service and irregular oestrous cycles, and is confirmed by detection of the protozoan by culture and/or polymerase chain reaction (PCR).

Two main methods for sampling the prepuce of bulls are used; the aspiration method of Bartlett (1949)<sup>20</sup> and the metal brush scraping method of Stuka and Katai (1969) as quoted by Tedesco *et al.*, (1979).<sup>44</sup> Both methods give the same diagnostic sensitivity if samples are cultured within two hours of collection.<sup>44</sup> In the Bartlett method, the pipette is scraped against the glans penis and the adjacent preputial membrane before aspirating the material. Sampling from the right side of the prepuce results in a four times more likelihood of a positive result compared to a sample taken from the left side.<sup>45</sup> This may be due to the position of the glans penis as the highest numbers of *T. foetus* are found on the portion of the glans penis excluding the galea glandis, whereas relatively low numbers occur on the remainder of the penis and prepuce.<sup>46</sup> The protozoan does not reside in the lower urethra. The vagina is sampled for heifers and cows. Both collection methods are described in Part 2. Tissues from aborted fetuses are collected and samples may be inoculated into transport media in the field.

Successful growth and identification of *T. foetus* from samples is influenced by transport conditions and temperature. Both culture and PCR outcomes are adversely affected if temperatures exceed 37°C for 24 hours or more during transport.<sup>47</sup> Transport temperatures of 10-20°C temporarily inhibit replication of *T. foetus* but the organism recovers once incubated at the correct temperature. Inoculated media should not be chilled or refrigerated as this will kill the protozoan. A study found that transport in phosphate buffered saline containing foetal calf serum (PBS/FCS) at 4°C and culture within 24 hours into growth medium, or transport in PBS/FCS at 37°C and

culture into growth medium at 24 or 48 hours, was satisfactory, whereas transport at 4°C in modified Plastring's medium<sup>36,48</sup> resulted in non-viable cells.<sup>49</sup> Optimum transport in Plastring's medium is at 37°C. Transport at the higher temperatures requires the presence of serum (or milk).<sup>49</sup> Inoculated Diamonds<sup>50</sup> or InPouch™ TF media must be transported at 22-37°C. Positive results are obtained at this temperature range for up to four days, whereas cultures kept at 4°C for five days will be negative. All results are negative if samples are kept at -20°C for three hours.<sup>51</sup> As a general rule, transportation at 25°C followed by incubation at 37°C in the laboratory, is ideal.

A number of media have been developed for transport and culture of *T. foetus*; Trichomonas Medium (Oxoid), modified Plastring's medium,<sup>36,48</sup> modified Diamonds medium<sup>50,51</sup> and InPouch™ TF medium (BioMed Diagnostics). They all support the growth of *T. foetus*; however, with some differences<sup>52</sup> that need to be taken into account when testing for *T. foetus*. Growth in Diamonds medium and modified Plastring's medium show the quickest growth with the highest concentration of cells at two to four days, compared to the InPouch™ TF medium, where the highest number of cells occurs later. Cells survive longer in the InPouch™ TF medium and modified Plastring's medium, as cells are present at day seven, whereas loss of viable cells occurs from day five in Diamonds medium.<sup>52</sup> Culture (tested using modified Plastring's liver infusion medium) has a sensitivity of 72% (95% probability: 58.07-86.38%) and a specificity of 95.37% (95% probability: 94.07-96.65%) when applying Bayesian methods in the absence of a gold standard.<sup>53</sup> These results are similar with the InPouch™ TF medium, which has a culture sensitivity of 67.8% and specificity of 98.8% at 95% confidence intervals for a single sample after infection of one week's duration.<sup>54</sup> Sensitivity and specificity increase to 80.0% and 98.1%, respectively, when samples are collected over three consecutive weeks.

*T. foetus* protozoa are visually detected in growth medium using a wet preparation examined under a light microscope or by dark ground illumination. Examination using phase contrast can assist with identification based on morphology. The identification of *Trichomonas* to species level is done using morphology after staining by Lugol's iodine and Diff-Quick method<sup>55</sup> (see Part 2).

Other trichomonads may be found in the normal bovine reproductive tract and need to be differentiated from *T. foetus*. These non-pathogenic trichomonads may be found in soil, water, urine, faeces, intestine or rumen of animals and it is important to avoid contamination from soil and faeces when collecting samples.<sup>14,56</sup>

A number of PCRs have been reported for detection of *T. foetus* in bulls and cows. A real time PCR using a 3' minor groove binder-DNA probe targeting the conserved regions of the internal transcriber spacer-1 has been developed<sup>57</sup> but not submitted to SCAHLS or approved by the OIE for international trade requirements<sup>56</sup>. The probe is 2,500-fold and 250-fold more sensitive than culture followed by microscopy for smegma and mucus respectively, and detects a single cell of *T. foetus* per tube from smegma or vaginal mucus. It is 500-fold more sensitive than conventional PCR.<sup>57</sup>

### Diagnostic Tests For International Trade

The OIE provides a number of documents encompassing bovine trichomoniasis that should be consulted for international trade. These deal with the export and import of cattle for breeding, bulls for breeding by natural and AI, and bovine semen;<sup>58</sup> diagnostic tests for detecting *T. foetus* in bovine samples;<sup>56</sup> prescribed list of tests for

international trade;<sup>59</sup> and collection and processing of bovine semen.<sup>60</sup> In the Terrestrial Manual,<sup>56</sup> writing in blue indicates the prescribed tests for the international movement of animals and animal products, and those that are optimal for determining the health status of animals.

For cows and heifers exported for breeding, and bulls exported for breeding by natural service or AI, or semen sent for export, a negative test result from culture or direct microscopic examination of vaginal mucus, or preputial specimens, is required.<sup>60</sup> Vaginal mucus and preputial specimens must be inoculated into one of the recommended culture media and delivered within 24 hours to the laboratory, protected from exposure to daylight and maintained within a temperature range of >5°C and <38°C. The inoculated media should be cultured for up to seven days and samples examined at intervals over this time.<sup>56</sup>

For collection of semen for AI, three consecutive weekly cultures are required for bulls older than six months, or those that could have had contact with cows prior to pre-entry selection into an AI centre. A negative culture result must be obtained for all three samplings. One test (result negative) is required from bulls that are less than six months of age, or that have been kept with bulls only prior to pre-entry selection.<sup>60</sup> Pre-entry selection requires bulls to be kept in isolation for seven days prior to entry into an AI centre.

### **Guidance on Safety and Containment Requirements**

Normal procedures apply when working with *T. foetus* in a PC2 laboratory.

## **Part 2 – Test Methods**

### **Collection of samples from the bull**

As stated in OIE guidelines, for AI requirements, animals less than six months old, or kept since that age in a single sex group, should be tested once using a preputial specimen. Animals aged six months or older that may have had contact with females should be tested three times at weekly intervals using a preputial specimen.<sup>60</sup>

It is important when collecting samples to avoid faecal contamination and the possible introduction of intestinal trichomonads into the sample. Contamination is minimised by removal of extraneous hair and material around the preputial orifice or vulva. The area must not be cleaned with disinfectants as this may reduce diagnostic sensitivity.<sup>56</sup> Contamination between animals should be avoided through the operator changing gloves between animals, by using single-use syringes, pipette, mare infusion pipettes or Tricamper. If single-use equipment is not available then disinfection of equipment and thorough rinsing in sterile water should be done after each animal.

#### *Equipment and materials*

- Bartlett pipette, mare infusion pipette or Tricamper from (Department of Agriculture, Fisheries and Forestry, Queensland)
- Rubber bulb or 20 mL syringe to place onto pipette
- 10 mL phosphate buffered saline (PBS)
- 10 mL Trichomonas medium
- Gloves

Place the bull into a crush and fasten a hind leg to the crush. A plastic pipette (made from a 540 mm length of strong plastic tubing with an 8 mm outside diameter and a 1.5 mm wall) is attached to a 90 mL rubber bulb, or a mare infusion pipette is attached to a 20 mL syringe, and introduced into the full length of the preputial sac. The bulb is squeezed and material is collected from the ventral fornix and dorsal surfaces of the penis and surrounding preputial mucosa. The flat bevelled edge of the pipette tip is moved back and forth over the surface of the glans penis and the preputial mucosa while keeping the bevelled edge against the mucosal surface.<sup>20,61</sup> The highest concentration of *T. foetus* cells is in the glans penis, not including the galea, and this correlates to the area containing the most smegma.<sup>46</sup> It is important not to introduce fluid into the preputial cavity as this increases the collection of extraneous debris and bacteria which impacts on culture and survival of *T. foetus* and sample integrity for microscopic examination.<sup>20</sup> During the collection the bulb is compressed and relaxed 15-20 times and is collected for a half to one minute. Placement of the end of the pipette is controlled by hand through the wall of the prepuce. A 0.5-2 mL sample is collected and the pipette and material is rinsed thoroughly in 10 mL of PBS. After standing to sediment the material, 1 mL of the cellular deposit in PBS is used to inoculate the transport medium.<sup>20,61</sup>

If using the Tricamper (60 cm long polyethylene tube with a 75 mm long, 8 mm diameter corrugated scraper head with a 1.5 mm collection bore attached to a 6 mm diameter tubing with a 1.5 mm internal diameter), aspiration is not necessary. The Tricamper is scraped over the surface of the glans penis and preputial mucosa. The collected smegma is rinsed into PBS as with other collection devices and 1 mL placed into culture medium. If collecting for PCR, the collected material is rinsed into 5 mL of physiological saline.<sup>57</sup> After use, the pipettes are rinsed thoroughly in water followed by an overnight rinse in 30 mL sodium hypochlorite in 1L of water, then rinsed in distilled water and dried thoroughly.

### **Collection of samples from heifers and cows**

A mare infusion pipette or a 380-400 mm long piece of tubing, as described for collection of samples from bulls, is fitted with a 20 mL syringe and filled with 5-7 mL of sterile normal saline. The pipette is introduced into the vagina and the bulb or syringe compressed several times to flush the vagina. A sample is withdrawn and 1 mL placed into the culture medium.<sup>20</sup> The best time to collect samples from the vagina of heifers and cows is 12-19 days post-coitus. In cases of pyometra, *T. foetus* is nearly always present. Other collection times are just after abortion or just prior to oestrus.

The Tricamper can also be used to collect vaginal mucus by gently inserting into the vagina and moving backwards and forwards a few times. The collected mucus is rinsed into culture medium, or into 5 mL of physiological saline if collecting for PCR.<sup>57</sup>

It is important when collecting samples to avoid faecal contamination and the possible introduction of intestinal trichomonads into the sample. Contamination is minimised by removal of extraneous hair and material around the vulva. The area must not be cleaned with disinfectants as this may reduce diagnostic sensitivity.<sup>56</sup> Contamination between animals should be avoided through the operator changing gloves between animals, by using single-use syringes, pipette, mare infusion pipettes or Tricamper. If

single-use equipment is not available then disinfection of equipment and thorough rinsing in sterile water should be done after each animal.

### Collection of samples from abortion material

*T. foetus* is present in placental fluids, uterine exudate, foetal fluids and foetal stomach contents, and these specimens should be selected for culture.<sup>24,43</sup>

### Transport media and transport conditions

Several transport and growth media are available for *T. foetus*, including modified Diamond's media,<sup>50,51</sup> modified Plastridge's medium,<sup>36,48</sup> InPouch™ TF (Biomed Diagnostics) and Trichomonas medium (Oxoid). Material collected from the prepuce is washed into 10 mL PBS and allowed to stand to sediment the material at the bottom of the container. It is important to use saline at 0.85% as *T. foetus* reacts to changes in osmotic pressure similar to red blood cells and may lyse under adverse concentrations. One mL of sedimented preputial material or 1 mL of vaginal material is placed into the transport medium at collection. Samples are transported to the laboratory at a temperature range of 5-37°C with an optimum temperature of 25°C. *T. foetus* will die at temperatures of <4°C and >37°C, and will be inert at 4-20°C.<sup>49,51,52</sup>

### Culture

Trichomonas medium inoculated with preputial washings and or vaginal mucus is incubated at 37°C for seven days.

Some media such as modified Diamond's medium and Oxoid's Trichomonas medium contain a small amount of agar that helps maintain microaerophilic conditions at the bottom of the vial and confines some contaminating organisms to the upper part of the medium. When preparing a wet preparation, an aliquot should be taken from the bottom of the vial where the concentration of *T. foetus* cells is greatest. A wet preparation is examined for the presence of *T. foetus* at intervals from day one to day seven.<sup>52,56</sup> The 2012 version of the OIE method for Trichomoniasis, chapter 2.4.17<sup>56</sup>, under "culture" states: 'culture media is examined at intervals from day 1 to day 7'; and under "culture procedures—modified Diamond's medium" the OIE method states: 'culture media is examined daily over the seven day period'. The OIE authors have clarified this anomaly and culture media are examined at intervals during the seven-day period allowing the laboratory to determine the appropriate days for examination.

Under culture experiments cells grown in modified Diamond's medium<sup>50,51</sup> grow more rapidly and have the highest concentration ( $10^4$  cells/mL) at days two, three and four. Viability decreases from day five in Diamond's medium and some strains may not be seen at day five.<sup>52</sup> Cell growth in the InPouch™ TF and modified Plastridge's medium<sup>36,48</sup> is slower (and slightly less) and peaks at days two to day six or seven, but survival is longer. Cells survive up to seven days in the InPouch™ TF medium and modified Plastridge's medium.<sup>52</sup>

In an experiment using a laboratory strain of *T. foetus*, growth increased over a seven day period when cultured in Oxoid's Trichomonas medium (Thermofisher) at 37°C (P. Ashley, unpublished data 2013). However, it must be noted that this was a laboratory strain and no preputial material was added to the medium. A field strain from naturally infected animals may behave differently.

**Table 1. Summary of times for optimal growth and survival for different media**

Culture medium	Optimal growth (days)	Time period for survival in culture (days)
Modified Diamonds <sup>50,51</sup>	2-4 <sup>52</sup>	1-4/5 <sup>52</sup>
Modified Plastridges <sup>36,48</sup>	2-7 <sup>52</sup>	1-7 <sup>52</sup>
InPouch	2-6/7 <sup>52</sup>	1-7 <sup>52</sup>
Trichomonas medium (Oxoid)	2-7	1-7

No effect on culture sensitivity occurs when samples are stored at room temperature (taken as 22°C) for 4 or 24 hours before incubation at 37°C.<sup>54</sup>

Specificity and sensitivity of culture tested using experimentally infected bulls demonstrates a sensitivity of 76.0% and a specificity of 98.5% when two cultures are sampled on consecutive weeks.<sup>54</sup>

### **Wet preparation for the identification of *T. foetus***

#### *Principle of the test*

*T. foetus* protozoa are visually detected in growth medium using a wet preparation examined under a light microscope. The identification of *Tritrichomonas* to species level can be done by morphology. A PCR is available for differentiation of *T. foetus* from other trichomonads.

#### *Method*

A drop (approximately 25 µL) of medium from a culture of *T. foetus* is placed onto a glass slide labelled with the specimen identification and covered with a coverslip. The material is examined using a light microscope with the iris of the condenser diaphragm almost closed to improve the contrast of unstained objects. The light intensity from the bulb is decreased to aid contrast. Dark ground illumination or phase contrast microscopy also can be used. The slide is scanned on low power (×10 objective) in a grid pattern. Ten to 15 traverses of the coverslip should occur in the five minutes of examination. *T. foetus* exhibits a jerky, rolling movement. The protozoan is 8-18 µm long and 4-9 µm wide, slightly longer and slimmer than segmented neutrophils (10-15 µm), and smaller than epithelial cells (15-20 µm), both of which may be seen in the wet preparation. The diameter of a leucocyte is lightly less than the length of *T. foetus*, excluding the flagella and the portion of the axostyle extending posteriorly beyond the protoplasm of the 'body'. Suspect organisms are examined under high power (×20 or ×40 objective) and inspected for an undulating membrane and flagella (three anterior and one posterior). In an environment not conducive to optimal growth, the trichomonads may become spherical (pseudocells) and appear larger, more refractile and less granular than a leucocyte, although pseudocells may also occur naturally.

### **Microtitre plate for the examination of bulk numbers of samples**

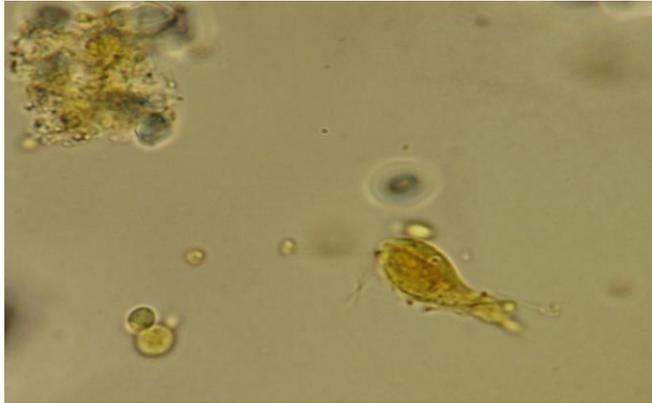
#### *Principle of the test*

Examination of bulk numbers of samples can be performed using a 96 well microtitre plate. The protozoa are visually detected in an aliquot of growth medium transferred to a microtitre plate well and examined using an inverted microscope. The identification of *Tritrichomonas* to species level can be done by morphology. A PCR is available for differentiation of *T. foetus* from other trichomonads.

*Method*

A 45 µL aliquot from the bottom layer of each culture broth is transferred to individual wells in a flat-bottomed 96-well microtitre plate. The plate is examined using an inverted microscope.

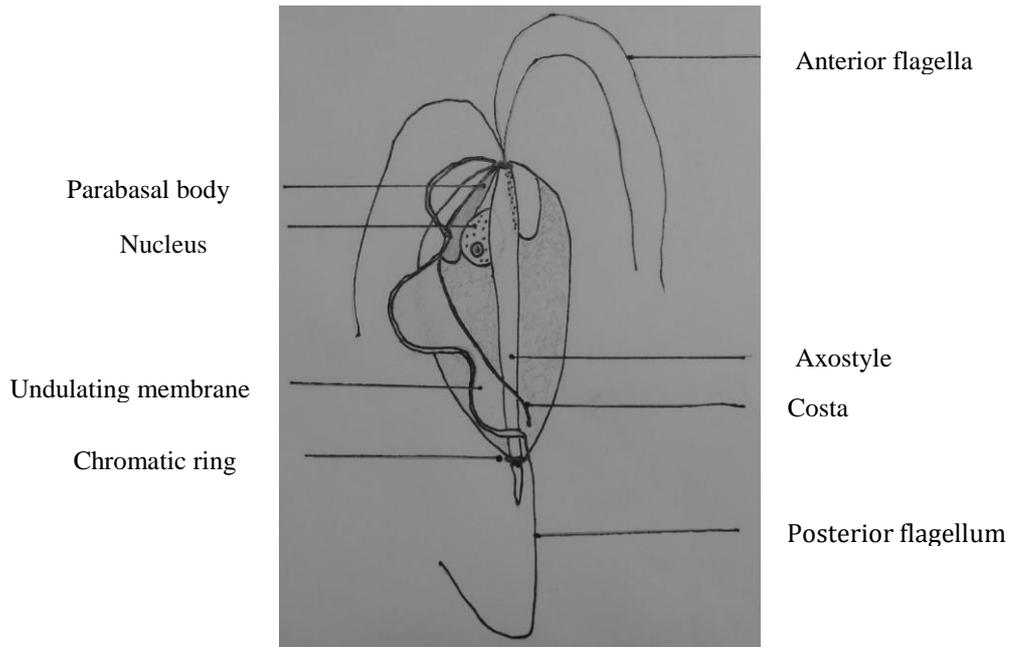
Note that samples in microtitre trays should not be allowed to become cold as the decreased temperature reduces motility of the protozoan making their detection more difficult.



**Figure 1. Wet preparation of *T. foetus* stained with iodine** (Copyright © Western Australian Agriculture Authority, 2012)



**Figure 2. Unstained wet preparation of *T. foetus* with yeast cells** (Copyright © Western Australian Agriculture Authority, 2012)



**Figure 3. Schematic showing parts of *T. foetus* cell** (Copyright © Western Australian Agriculture Authority, 2012)

### Examination of stained smear for *T. foetus*

In most cases, a wet preparation examined under a light microscope (or using dark field) is suitable for the detection of *T. foetus*; however, samples may contain other flagellates that are morphologically similar to *T. foetus*. A number of stains may be used including Leishman's, Giemsa and Wright's stain, silver stain, iron-haematoxylin and others; however, some of these methods are cumbersome and time consuming and not all methods stain every component of the protozoan. Giemsa gives inconsistent results. A staining method incorporating iodine and animal serum with a commercial Wright-Giemsa base staining kit (Diff-Quick) provides consistent staining results for *T. foetus*.<sup>55</sup>

### Staining method for *T. foetus*<sup>55</sup>

#### *Principle of the test*

Other protozoans morphologically resembling *T. foetus* may be present in preputial samples.<sup>13,14</sup> These include *Pentatrichomonas hominis*, *T. enteris*, *Monocercomonas ruminantium* and others. *P. hominis* is found in the intestine of cattle and humans and has four to five anterior flagella, whereas *T. enteris* has three anterior flagella. This staining method optimises visualization of the key anatomic structures of the protozoan to aid in accurate identification. The use of iodine and iodide improves the staining of the flagella and costa. The use of serum improves the adhesion of parasites to the slides and may improve the binding of the dyes to the parasite structures.<sup>55</sup>

#### *Test procedure*

Take 1 mL of a two-day-old culture of *T. foetus* and centrifuge at 16,000 *g* for 10 seconds. Discard the supernatant and re-suspend the pellet in 50 µL culture medium in which the parasites were grown. Add 10% serum if not already in the medium.

Place 10 µL onto a glass microscope slide and using a glass 'spreader', or a second microscope slide, make a thin smear using a standard blood smear technique.

Air dry. Fix the smear for one minute in methyl alcohol from the Diff-Quick kit (or dip 15 times into a Coplin jar containing methyl alcohol). Use tissue paper to blot the excess fixative solution. Stain for one minute with Lugol's iodine (see Part 3) (or 15 dips into a Coplin jar containing Lugol's iodine). Rinse slides briefly. Stain for one minute in Solution I (1.0 g/L xanthene dye in buffer) from the Diff-Quick kit. Drain off excess. Stain for 30 seconds (or 10 dips for 30 seconds in the solution in a Coplin jar) with Solution II (1.25 g/L thiazine dye in buffer). The procedure must be adhered to otherwise precipitation may occur on the slides. Wash with distilled water until the wash water is clear. Dry slides and examine under oil with a ×100 oil-immersion objective lens.

#### *Interpretation of results*

The flagella of *T. foetus* stain purple. The undulating membrane has 2-5 waves and stains purple. The nucleus stains dark purple. The axostyle stains translucent purple, the costa stains purple, and the cytoplasm stains blue and contains translucent vacuoles.

**Table 2. Morphological features of Trichomonads<sup>3</sup>**

Organism	Anterior flagella	Posterior flagella	Undulating membrane	Host
<b>Tritrichomonas</b>				
<i>T. foetus</i>	3	1	2-5	Cattle
<i>T. enteris</i>	3	1	3	Cattle
<i>T. vaginalis</i>	4	0	4	Human
<b>Tetratrichomonas</b>				
<i>T. buttreyi</i>	3 or 4 variable length	3-5	1	Pigs, cattle
<i>T. pavlovi</i>	4	1	2-4	Calves
<b>Pentatrichomonas</b>				
<i>P. hominis</i>	4	1	3 waves	Human, primates, cats, dogs, cattle

#### *Tritrichomonas* species<sup>3</sup>

*T. foetus* is pyriform, 8-18 µm×4-9 µm (dimensions are variable between references), with three anterior flagella and a posterior flagellum that is almost as long as the anterior flagella. The undulating membrane of two to five waves extends almost the full length of the body, and continues posteriorly as the posterior flagellum. A costa, or chromatic basal rod, extends along the base of the undulating membrane. The

axostyle is thick and hyaline with a chromatic ring at the point of emergence from the body. A cylindrical or club-shaped parabasal body lies between the nucleus and the costa.

*T. enteris* is 6-12  $\mu\text{m}$   $\times$  5-6  $\mu\text{m}$ . It has three anterior flagella of equal length and an undulating membrane which extends three-quarters the length of the body, and a free flagellum extends beyond it. The axostyle is slender, straight and bent like a spoon around the nucleus; it extends a quarter of a body length beyond the body. It is found in cattle (it may be a synonym of *P. hominis*).

*Tetratrichomonas* species<sup>3</sup>

*T. buttreyi* is found in the caecum of pigs, and in the intestine and rumen of cattle.<sup>3</sup> It has also been found in the preputial smegma of virgin bulls.<sup>62</sup> It is ovoid to ellipsoidal and 4-7  $\mu\text{m}$   $\times$  2-5  $\mu\text{m}$  in size. Three to four anterior flagella vary in length from a short stub to more than twice the length of the body. The flagella end in a knob or spatulate structure.

*T. pavlovi* is found in the digestive tract of calves. It is pyriform in shape and 11-12  $\mu\text{m}$   $\times$  6-7  $\mu\text{m}$  in size.<sup>3</sup> Four anterior flagella are present and they are the same length as the body. An undulating membrane of 2-4 waves extends almost the length of the body. The axostyle is weak and slender. Many food vacuoles are seen in the cytoplasm. There is no chromatic ring. It grows in *Trichomonas* media but dies out on subculture. *T. pavlovi* may be a synonymous with *P. hominis*.

*Pentatrichomonas* species<sup>3</sup>

*Pentatrichomonas hominis* is pyriform and measures 8-20  $\mu\text{m}$   $\times$  3-14  $\mu\text{m}$ . Five anterior flagella are present although some organisms have three and others four. Four anterior flagella are grouped together and the fifth is separate and to the posterior. A sixth flagellum runs alongside the undulating membrane and extends beyond it as a free trailing flagellum. The undulating membrane runs the length of the body. The axostyle is hyaline and thick, with a sharply pointed tip. There is no chromatic ring. It is a commensal of the vertebrate digestive tract.

*Pseudotrichomonas* species<sup>3</sup>

Found in pond water, they are 10-14  $\mu\text{m}$  in length, with three anterior flagella and one associated with the undulating membrane, which runs the length of the body. There is no free posterior flagellum, but there is a slender axostyle that protrudes posteriorly. It forms cysts.

### Histopathology

Microscopic lesions occur in the placenta and in lung of the aborted foetus, although some infected animals do not show any macroscopic or microscopic lesions.<sup>24,43</sup> The endometrium is eroded in the interplacentomal areas, and neutrophils and macrophages occur in the stratum compactum. A concurrent salpingitis and cervicitis may be present in some animals with these lesions. The inflammatory exudate on the uterine lumen, surface of the endometrium and in the endometrial glands, contains *T. foetus* cells. Oedema and a diffuse, mild, predominantly mononuclear cell inflammatory response, occurs in the stroma of the chorion where *T. foetus* cells invade. A mild to moderate focal necrosis is seen in the chorionic epithelium. Inflammatory cells, mainly neutrophils and some macrophages are seen between the cotyledon and caruncle. Oviducts contain inflammatory cells in the folds and sub-

epithelial tissue.<sup>24</sup> A pyogranulomatous bronchopneumonia is seen often in the foetus, and macrophages and occasional giant cells may contain phagocytosed parasites. *T. foetus* cells are seen in the lung and airways, in abomasal crypts and sometimes in intestinal crypts of aborted foetuses.<sup>43</sup> Compared to bacterial-induced abortions, there is a lack of extensive necrosis.

Trichomonads are stained easily with haematoxylin and eosin (H&E) in non-autolysed tissues but are better visualized, especially in autolysed tissues, using Giemsa or Bodian's silver protargol stain (See Part 3).<sup>62</sup> The latter is best for demonstrating the flagella and undulating membrane.<sup>63</sup>

### **Polymerase Chain Reaction (PCR) for specific detection of *T. foetus***<sup>57</sup>

A real time PCR using a 3' minor groove binder-DNA probe (TaqMan<sup>®</sup> MGB) targeting the conserved regions of the internal transcribed spacer region has been developed<sup>57</sup> but not submitted to SCAHLS or approved by the OIE for international trade requirements. The MGB probe has a 2500-fold or 250-fold increase in sensitivity compared to culture/microscopy of smegma or vaginal mucus, respectively. It has equal or greater sensitivity when testing crude DNA (prepared from boiled clinical material) compared to purified DNA (extracted using a commercial reagent QIAamp DNA mini kit). The sensitivity is equivalent to conventional PCR (using TFR3-TFR4 primers<sup>64</sup>) when testing purified DNA, but is more sensitive than conventional PCR when testing crude DNA.<sup>57</sup> A single cell per tube from smegma or mucus can be detected. Sensitivity of the assay can be increased by culturing clinical material for two days. Two days culture in the InPouch<sup>™</sup> TF medium and modified Plastringe's medium at 37°C was found to provide optimal numbers of *T. foetus*.<sup>57</sup>

Samples are transported to the laboratory at ambient temperature within 48 hours of collection. Samples (clinical material in 5 mL sterile physiological saline) for PCR are stored at 4°C until tested.<sup>57</sup>

Purified DNA is extracted from preputial smegma, or vaginal mucus, in 5 mL of saline using the QIAamp DNA mini kit (Qiagen, Australia) according to the manufacturer's protocol with the exception of a final elution volume of 50 µL rather than 400 µL.<sup>57</sup>

Crude DNA is prepared from 1 mL of preputial smegma, or vaginal mucus in saline, centrifuged for 5 minutes at 12,000 *g*. The supernatant is discarded. The pelleted material is resuspended in 500 µL sterile distilled water and heated at 95°C for 10 minutes. The suspension is centrifuged for 30 seconds at 2,000 *g*. A 5 µL aliquot of supernatant is used in the PCR reaction.<sup>57</sup>

A 25µL reaction volume contains Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies), 900 nM TFF2 and TFR2 primers and 80 nM TRICHP2 fluorescent 3' minor groove binder-DNA probe (Table 3). RealMasterMix probe mix (Eppendorf) can be substituted for Platinum Quantitative PCR SuperMix-UDG. Thermal cycling is conducted on a Rotor-Gene (Qiagen Australia) or equivalent. The thermal profile is 50°C for 2 minutes, 95°C for 2 minutes and 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 45 seconds. Fluorescence is acquired at the end of each annealing/extension cycle and normalised on a no-template control. A positive result is indicated by passing the threshold of 0.1. The detection limit is 0.003 pg/tube.

**Table 3. Primers and probe for PCR**

Primer or probe	Primer sequence 5'-3'
TFF2	GCG GCT GGA TTA GCT TTC TTT
TFR2	GGC GCG CAA TGT GCA T
TRICHP2	6-FAM-ACA AGT TCG ATC TTT G-MGB-BHQ

### PCR for differentiation from other Trichomonads<sup>64,65,66</sup>

The differentiation of *T. foetus* from other trichomonads can be confirmed by PCR. Primers TFR1 and TFR2 (Table 4) amplify conserved areas of the internal transcribed spacers ITS1 and ITS2 and the intervening 5.8S gene of members of the Trichomonadidae family.<sup>64</sup> Primers TFR3 and TFR4 (Table 4) are specific for *T. foetus* and produce an amplicon of 347 bp.<sup>64</sup> TFR3 is complementary to the 5' end of the 28S rRNA gene, and the TFR4 primer is located at the border of the 18S rRNA gene and the ITS1 region. *T. foetus* produces amplicon from both primer sets, whereas other trichomonads only produce amplicon with TFR1 and TFR2.

DNA is extracted using the DNeasy tissue kit (Qiagen). A range of PCR protocols have been reported with a number of different master mix concentrations. The primer concentration has ranged from 0.2-1.0 µM final concentration, 200 µM dNTPs, 1.75-2.5 mM MgCl<sub>2</sub> and 0.375-2.5 U *Taq*. Thermocycling protocols have used an annealing temperature of 60-67°C. In the comparison for the real time MBG probe PCR,<sup>57</sup> the following concentrations were used: a 15 µL reaction volume containing 500 nM each TFR3 and TFR4 primers,<sup>64</sup> 1x PCR reaction buffer containing MgCl<sub>2</sub> (Roche Diagnostics), 200 µM dNTPs, 1 U *Taq* DNA polymerase (Roche Diagnostics) and 1 ng of *T. foetus* DNA. Amplification is carried out on a GeneAmp PCR system model 2700 (Applied Biosystems) or similar, with an initial denaturation at 94°C for 90 seconds, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 67°C for 30 seconds and extension at 72°C for 90 seconds with a final single extension at 72°C for 15 minutes. The 347 bp produced is visualized in a 2% agarose gel using SybrSafe stain (Invitrogen), or ethidium bromide.

Amplicon generated by primers TFR1 and TFR2 is digested with restriction enzyme *Hpy*CH4IV to differentiate *T. foetus* from other trichomonads.<sup>65</sup> *T. foetus* produces a product of 371 bp, *P. hominis* 338 bp, and *Tetratrichomonas* species products of 388-391 bp. Digestion results in 220 and 151 bp fragments for *T. foetus*; 161, 135 and 42 bp fragments from *P. hominis*, and *Tetratrichomonas* species generate fragments of 180-181, 162-168, and 43-45 bp.

Other primers (Table 4) designed to the conserved regions in the 18S rRNA and the 8.8S rRNA and amplifying the ITS1 region have been reported that allow differentiation and identification of *T. foetus*, *Tetratrichomonas* species and *P. hominis* based on size differences of amplicon.<sup>67</sup> The master mix is composed of 1 pmol of each primer, 2.0 mM dNTP, 1.75 mM MgCl<sub>2</sub>, 1xPCR buffer II, 0.375 U *Amplitaq*, 1 µL template in a 20 µL reaction volume.

**Table 4. Primers for detection of *T. foetus* and differentiation from other trichomonads**<sup>64,65,66,67</sup>

Target	Primer name	Primer sequence 5'-3'	Amplicon bp
Trichomonads	TFR1	TCG TTC AGT TCA GCG GGT CTT CC	372
	TFR2	GGT AGG TGA ACC TGC CGT TGG	
<i>T. foetus</i>	TFR3	CGG GTC TTC CTA TAT GAG ACA GAA C C	347
	TFR4	CCT GCC GTT GGA TCA GTT TCG TTA A	
<i>T. foetus</i>	F	GTA GGT GAA CCT GCC GTT G	157
	R	ATG CAA CGT TCT TCA TCG TG	
<i>P. hominis</i>	F+R		142
<i>Tetratrichomonas</i> species	F+R		170-175

### Maintenance of *T. foetus* for quality control cultures

*T. foetus* can be maintained for long periods in Plastridge's medium when incubated at 25°C (Paul Duffy, Department of Agriculture, Fisheries and Forestry, Queensland, personal communication, 2012). Cultures survive for up to one month in Trichomonas medium (Oxoid) at 25°C before requiring subculturing (Paul Ashley, Animal Health Laboratories, Department of Agriculture and Food WA, personal communication). If maintained at 37°C in Plastridge's medium, subculturing must be done once or twice a week.

*T. foetus* can be stored at -80°C. The medium and freezing procedure are described in preservation of isolates under Part 3.

### Quality Control for Media

Quality control checks, such as sterility and pH, are performed according to NATA (National Association of Testing Authorities) requirements. A culture of *T. foetus* is used to check growth.

## Part 3 Media and Reagents

### Media for cultivation of *T. foetus*

#### Trichomonas medium (Oxoid, Thermofisher)

Trichomonas medium (Oxoid, Thermofisher)	18.75 g
Distilled water	500 mL
Inactivated horse serum (CSL)	40 mL
Chloramphenicol (SR78)	3 mL

Suspend powder in water in an appropriately sized Schott bottle and autoclave at 121°C for 30 minutes. Cool to 50°C in a waterbath. Inactivate 40 mL of horse serum

at 56°C for 30 minutes. Aseptically add 3 mL of ethanol to 1 vial of Chloramphenicol Supplement Code SR78 and mix to dissolve. Add supplement and horse serum to Trich base while stirring under hood. Keep bottle warm in a container of warm water and dispense in 4 mL volumes in sterile 5 mL tubes. This medium contains liver digest.

### **Tritrichomonas foetus medium (TFM)**<sup>36,48</sup>

(Based on modified Plastridge's medium)

Neutralised liver digest (Oxoid, cat # LP0027)	12.5 g
Tryptose (Oxoid, cat # LP0047)	5 g
Bacto agar (Oxoid, cat # LP0011)	1.5 g
Sterile inactivated bovine serum (Sigma, cat # B 9433)	500 mL
Penicillin (Sigma, cat # P 3032)	0.75 g
Streptomycin (Sigma, cat # S 9137)	0.082 g
Distilled water	500 mL

#### *Preparation of stock solutions*

##### 1. Basal medium

In a 1 L bottle add 12.5 g neutralised liver digest and 5.0 g tryptose to 500 mL distilled water. Adjust pH to 7.4 using HCL or NaOH. Add 1.5 g Bacto agar and heat to dissolve before autoclaving at 121°C for 15 minutes.

##### 2. Antibiotic solution

Add 0.75 g penicillin and 0.082 g streptomycin to 100 mL distilled water and dissolve. Filter sterilise through a 0.2 µm cellulose acetate filter.

#### *Preparation of TFM*

Aseptically combine basal medium, sterile inactivated bovine serum, and antibiotic solution according to Table 5. Dispense into sterile McCartney bottles or sterile 5 mL serum bottles.

**Table 5. Volumes for preparation of TFM**

Component	100 mL	500 mL	1000 mL
Basal medium	50 mL	250 mL	500 mL
Sterile inactivated bovine serum	50 mL	250 mL	250 mL
Antibiotic solution	1 mL	5 mL	10 mL

Optional: Nystatin (200 units) can be added to each 1 mL of the medium immediately prior to use.

Store media at -20°C.

Notes: 50% foetal calf serum heat-inactivated at 56°C for 1 hour, or 50% adult bovine serum heated to 65°C for 30 minutes removes natural agglutinins to *T. foetus*. Adult bovine serum inactivated at 65°C for 30 minutes is superior to media containing serum inactivated at 56°C for 30 minutes.<sup>52</sup>

### **Modified Diamond's medium**<sup>50,51,52</sup>

Trypticase Peptone	2 g
Yeast extract	1 g
Maltose	0.5 g

L-cysteine hydrochloride	0.1 g
L-ascorbic acid	0.02 g
Make up to	90 mL water
Add:	
K <sub>2</sub> HPO <sub>4</sub>	0.08 g
KH <sub>2</sub> PO <sub>4</sub>	0.08 g
Adjust pH to 7.2–7.4 with NaOH or HCl	
Add:	
Agar	0.05 g
Autoclave for 10 minutes at 121°C	
Add :	
inactivated bovine serum	10 mL
Penicillin G	100,000 units
Streptomycin sulphate	0.1 g

Aseptically dispense into 10 mL aliquots and store at 4°C.

Note: Bovine serum is inactivated by heating at 56°C for 30 minutes. The addition of agar confines contaminating organisms to the upper portion of the culture medium and assists in maintaining microaerophilic conditions at the bottom of the medium, where the largest concentration of *T. foetus* cells occur.

#### **InPouch™ TF**

Available from BioMed Diagnostics.

#### **Stains for *T. foetus*<sup>55</sup>**

##### **Lugol's iodine**

Potassium iodide	10 g
Iodine powder	0.5 g
Distilled water	100 mL

Dissolve the potassium iodide in water and then add the iodine.

##### **Diff-Quick**

This is a Romanowski stain. It is available commercially.

##### **Bodian's silver protargol stain<sup>62,63</sup>**

This is available as a kit from American MasterTech

##### *Method*

Cut paraffin-embedded tissue to a thickness of 7 µm. Deparaffinise slide using xylene or xylene substitute and hydrate through alcohols. Rinse in tap water and then distilled water. Place protargol solution (from kit) into a 50 mL plastic centrifuge tube, add slide, cap loosely and heat in a microwave oven until the solution is hot but not boiling. Tighten the cap, agitate the centrifuge tube and place inside a microwave without heating, for 15-20 minutes. Repeat the heating and incubating cycle until the tissue becomes dark tan to brown. Rinse slide in distilled water. Place slide in hydroquinone solution (from kit) for 10 minutes. Rinse slide in three changes of distilled water. Place slide in gold chloride solution (from kit) for 2-4 minutes. Rinse

in distilled water. Place slide in 2% oxalic acid (from kit) for 6 minutes to give the tissue a pale violet tint. Do not over treat as this will destroy the silver-protein reaction. Rinse slide in distilled water. Place slide in 5% sodium thiosulphate for 6 minutes. Rinse slide in 3 changes of distilled water. Dehydrate through three changes of fresh absolute alcohol. Clear tissue through three changes of xylene, or xylene substitute. Coverslip and mount.

### Freezing protocol for *T. foetus*

#### *Materials*

Gibco RPMI Medium 1640 (# 31800-022, Invitrogen)

Cryovials 1 mL (Nunc)

-80°C freezer

#### *Preparation of RPMI stock*

Prepare RPMI medium 1640 from label. Measure out 5% less distilled water than desired to later allow adjustment of pH. Add powdered medium to water at room temperature (15-30°C). Do not heat water. Stir gently. Rinse out all powder from inside the packet. Add 2.0 g of NaHCO<sub>3</sub> per L. Dilute to the desired volume with water. Stir until dissolved but do not over-stir. Adjust pH of medium to 7.2 (which is 0.2 below desired final working pH). Use 1% NaOH or 1M HCl. After pH has been adjusted, keep container closed until the medium is filtered. Sterilise immediately by membrane filtration. pH units will usually rise to 7.4 after filtration.

#### *Preparation of freezing medium*

Foetal calf serum	10%
RPMI 1640 (with L glutamine)	80%
Dimethylsulphoxide (DMSO)	10%

#### *Method*

Grow *T. foetus* in culture medium for 2-4 days, until cells look healthy.

Centrifuge at 1500 g for 10 minutes.

Add 1 mL of deposit to 2 mL of freezing medium.

Put the cryovial into a thick (3-4 cm wall thickness on all sides) styrofoam box and place into a minus 80°C freezer for 24 hours. Remove cryovial and store in freezer box in the minus 80°C freezer or in liquid nitrogen.

Alternatively, use a specially designed freezing container that cools the samples at -1°C/minute. For example, the Nalgene™ Cryo 1°C Freezing Container; Catalogue Number 5100-0001. Isopropyl alcohol is added to the container to the level as marked on the container. The Nalgene Freezing Container is placed into the minus 80°C freezer for 80 minutes. The cryovial is removed and stored in the freezer box in the minus 80°C freezer, or in liquid nitrogen.

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