CHAPTER 3.4.14.

TRICHOMONOSIS

SUMMARY

Description and importance of the disease: *Trichomonosis is a venereal disease of cattle caused by* Tritrichomonas foetus*, a flagellate protozoan parasite.*

Trichomonosis is asymptomatic in bulls, however in cows the disease is characterised by infertility, abortion, embryonic and early fetal death, fetal maceration, pyometra and vaginal discharge. The disease has a world-wide distribution and, at one time, was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. The widespread use of artificial insemination in many areas of the world has contributed to reduced prevalence. Nevertheless, trichomonosis is still of importance in countries with extensive farming practices where artificial insemination is not used.

Transmission of the disease is primarily by coitus, but mechanical transmission by insemination instruments or by gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C. Bulls over 3–4 years old are the main reservoir of the parasite as they tend to be long-term carriers, whereas most cows and young bulls (less than 3 years old) may clear the infection spontaneously. For these reasons samples from bulls are usually preferred for diagnosing and controlling the disease in herds.

Identification of the agent by in-vitro ***culture and microscopy:*** Tritrichomonas foetus *is a flagellate, pyriform protozoan parasite, approximately 8–18 μm long and 4–9 μm wide, with three anterior and one posterior flagellae and an undulating membrane. The organisms move with a jerky, rolling motion and are seen in culture tests of preputial samples of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or sometimes in aborted fetuses.* Tritrichomonas foetus *can be cultured* in vitro*, and may be viewed in a wet mount or stained slide. The standard diagnostic method for bulls involves the appropriate collection, examination and culture of smegma from the prepuce and penis, while in cows the preferred sample is vaginal mucus. Smegma can be collected by a variety of means including preputial lavage, brushing or scraping the preputial cavity and glans penis at the level of the fornix with a dry insemination pipette. A number of* in-vitro *culture media exist, including a commercially available field culture test kit, which supports trichomonad growth and allows direct microscopic examination.*

Identification of the agent by molecular methods: *Bovine trichomonosis may also be detected by polymerase chain reaction (PCR) amplification. Both conventional and quantitative real-time PCR have been used successfully in the identification of* T. foetus *and the diagnosis of trichomonosis. Both methods have been used either in combination with culture or alone. The conventional PCR has increased sensitivity when combined with culture, while the quantitative PCR has been successfully used on clinical samples. The quantitative real-time PCR has been validated both in the USA and Canada and it is now available as a commercial kit. This assay is also used routinely in Australia.*

Serological tests: *Attempts have been made at developing immunological tests, including an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms, and these were used as herd tests. However, these tests lack sensitivity and are not used for individual diagnosis of trichomonosis.*

Requirements for vaccines: *A partially efficacious, killed whole-cell vaccine is commercially available as either a monovalent, or part of a polyvalent vaccine containing* Campylobacter *and* Leptospira*.*

A. INTRODUCTION

Description and importance of the disease: Trichomonosis is a bovine venereal disease caused by the flagellate protozoan parasite, *Tritrichomonas foetus*.

Causal pathogen: *Tritrichomonas foetus* belongs to the genus *Tritrichomonas* in the family Trichomonadidae. *Tritrichomonas foetus* is pyriform, 8–18 μm long and 4–9 μm wide, with three anterior and one posterior flagellae, and an undulating membrane. Live organisms move with a jerky, rolling motion, and can be detected by light microscopy. Phase-contrast dark-field microscopy or other methods must be used to observe the details needed for identification. Detailed morphological descriptions, including electron microscopy studies, have been published (Warton & Honigberg, 1979). *Tritrichomonas foetus* has only the trophozoite stage and multiplies by longitudinal binary fission; sexual reproduction is not known to occur. Three serotypes are recognised based on agglutination (Skirrow & BonDurrant, 1988): the *'belfast'* strain, reported predominantly in Europe, Africa and the USA (Gregory *et al*., 1990); the *'brisbane'* strain described in Australia (Elder, 1964) and the *'manley'* strain, which has been reported in only a few outbreaks (Skirrow & BonDurrant, 1988)

The habitual hosts of *T. foetus* are cattle (*Bos taurus, B. indicus*). The bovine gastrointestinal tract hosts a number of other, commensal trichomonads e.g. *Pentatrichomonas hominis*, *Tetratrichomonas buttreyi*, *Tetratrichomonas pavlova*, *Tritrichomonas enteris* and *Pseudotrichomonas* species, which often contaminate preputial samples (Taylor *et al*., 1994). The number of flagellae, observed under phase contrast illumination or after staining, is an important morphological characteristic that can help differentiate *T. foetus* from other bovine flagellated parasites. However, non-*T. foetus* trichomonads are often difficult to distinguish from *T. foetus* based on culture and morphology (Taylor *et al*., 1994).

Tritrichomonas suis, a commensal of pigs, and bovine *T. foetus* are indistinguishable morphologically, serologically and antigenically. The use of modern molecular techniques such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA analysis (RAPD), variable length repeats (VLR), and internal transcribed spacers 1 and 2 (ITS1 and 2) polymerase chain reaction methods (PCR), supports the view that these two species are identical (Tachezy *et al*., 2002). More recently, *T. foetus* and *T. suis* were found to be identical at 9/10 loci and the use of the *T. suis* senior synonym has been suppressed in favour of *T. foetus* (Šlapeta *et al*, 2012).

Tritrichomonas foetus has been reported in domestic cats, horses and roe deer. Other species, such as goats, pigs, dogs, rabbits and guinea-pigs, have been experimentally infected (Levine, 1973). *Tritrichomonas foetus* has also been isolated from cats with diarrhoea and is now commonly known as the 'cat genotype' *T. foetus* (Šlapeta *et al*., 2012). *Tritrichomonas foetus* has also been reported to cause infections in humans including meningoencephalitis and peritonitis in immunocompromised and immunosuppressed individuals (Yao, 2012).

Transmission of infection occurs by coitus, or by gynaecological examination of cows using contaminated instruments. It may also occur via artificial insemination (AI) as semen from infected bulls may be passively contaminated by *T. foetus* present in the preputial cavity. Therefore, all bulls must be routinely checked for absence of *T. foetus* infection. Where AI along with diagnostic monitoring and culling of infected bulls is used, Trichomonosis has been controlled; however, it is still prevalent in the Americas, Australia, South Africa and Eastern European countries where extensive farming is still practised and natural mating is allowed.

The site of infection in bulls is primarily the preputial cavity (BonDurant, 1997), and little or no clinical manifestation occurs. For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years of age, infection may be transient. One of the proposed measures for controlling *T. foetus* infection in a herd is the use of bulls under 3–4 years of age rather than bulls older than 3–4 years (Yao, 2013).

Tritrichomonas foetus is present in small numbers in the preputial cavity of infected bulls, with some concentration in the fornix and around the glans penis (BonDurant, 1997). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which, in pregnant animals, results in invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Skirrow & BonDurrant, 1988). Cows usually clear their infection within 90 days and acquire a short-lived immune protection to *T. foetus* for a period of at least a year and in some cases up to three years (BonDurant, 1997).

Differential diagnosis: Diseases such as campylobacteriosis, leptospirosis, brucellosis, neosporosis, chlamydiosis, bovine viral diarrhoea, infectious bovine rhinotracheitis and anaplasmosis, that may cause clinical signs including infertility, vaginitis, pyometra, abortions and vaginal discharge, should be excluded.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of trichomonosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[(a)]						
Microscopy for morphological identification	+++	++	+++	+++	++	–
Conventional PCR on clinical samples	+	++	–	++	–	–
Conventional PCR in combination with culture	++	+++	–	+++	+	–
Real-time PCR	+++	+++	+++	+++	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of *T. foetus* in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (Buller & Corney, 2013; Yao, 2013).

The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion and, in recently infected cows, they are plentiful in the vaginal mucus 12–20 days after infection. In the infected bull, *T. foetus* organisms are present on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is recommended to allow at least 1 week after the last service before taking a preputial sample.

1.1. Sampling techniques and transport conditions

A number of techniques for collecting preputial samples from bulls or vaginal samples from cows have been described. It is important to avoid faecal contamination, as this may introduce intestinal protozoa that may be confused with *T. foetus* (Taylor *et al.*, 1994). Contamination of samples should be minimised by removal of extraneous material and soiled hair from around the preputial orifice or vulva; however, cleansing of the area, particularly with disinfectants, is to be avoided, as this may reduce diagnostic sensitivity.

Samples collected from bulls, cows and aborted fetuses are tested by conventional methods (direct examination and culture) or by molecular methods. The sampling techniques are the same in both cases; however, samples for culture are inoculated into transport medium or culture medium, whereas those for molecular biology can be collected either in medium or in phosphate buffered saline (PBS) or normal saline.

1.1.1. Collection of samples: bulls

Samples can be collected from bulls by three methods, namely scraping, brushing or washing (Buller & Corney, 2013; Yao, 2013):

i) Scraping the preputial and penile mucosa with an artificial insemination pipette connected to a syringe or a bulb via a silicone rubber tube, is a common technique.

ii) Special brushes made of metal or plastic, may be used to collect smegma from the penis and prepuce (Yao, 2013). Plastic disposable brushes, with a hole in the tip (hollow 'brush' or 'rasper') are commercialised for this purpose. They are easy to use and fast. The tool is gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma is rinsed into ~5 ml PBS or normal saline, or medium.

iii) Preputial lavage is still a common technique. A strong plastic tubing attached to a rubber bulb is inserted into the full length of the preputial cavity and the latter is washed with 20–30 ml PBS pH 7.2 or normal saline Collecting washes from the artificial vagina after semen collection is not recommended due to low diagnostic sensitivity (Gregory *et al.*, 1990).

Sampling techniques for bulls have been compared by several laboratories (reviewed in Yao, 2013). The results indicate that all three methods, i.e. brushing, scraping and lavage, provide similar analytical sensitivity, irrespective of the diagnostic method implemented afterwards, i.e. culture or PCR. Thus the sampling techniques should be chosen based on the context and local conditions.

Several laboratories have examined the effect on diagnostic sensitivity of repeated sampling (reviewed in Yao [2013]). For optimal sensitivity (95% or more) the first sampling should be done after a 1-week sexual rest and bulls should be sampled and tested three times at weekly intervals.

1.1.2. Collection of samples: cows

Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or a brush. The collected mucus is rinsed into ~ 5 ml PBS or normal saline (if PCR is intended) or in medium.

1.1.3. Samples from aborted fetus

Abortions due to *T. foetus* may occur at any time during gestation from 2 months onwards, but most frequently at 3–5 months (Buller & Corney, 2013). When abortions in that period occur and *T. foetus* infection is suspected, the placenta and fetal fluids should be sampled, together with the lungs of the aborted foetus (Buller & Corney, 2013). The abomasal content is also reported to contain high numbers of *T. foetus* (Rhyan *et al.*, 1988).

Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium containing antibiotics should be used (e.g. a thioglycolate broth media with antibiotics [Bryan *et al.*, 1999]) or a commercial plastic pouch. PBS or normal saline is not a good transport medium if the samples are to be cultured (Bryan *et al.,* 1999).

During transportation, the organisms should be protected from extremes of temperature, especially if cultures are to be undertaken in the laboratory. The temperature should remain above 5°C and below 37°C during transport (Bryan *et al.*, 1999). Transport at 25°C followed by culture at 37°C is considered optimal for the survival and growth of *T. foetus* (Buller & Corney, 2013).

In conclusion, the choice of sampling and diagnostic techniques should take into account several factors including the transport conditions and possibilities, and the expected duration of transport.

1.2. Identification of *T. foetus* by direct examination or in culture

1.2.1. Direct detection of parasites

Direct detection of parasites by microscopy immediately after sample collection, or on reception of the sample in transport medium, can be attempted (see below for examination and identification criteria). However, the organisms are often too few to allow for direct detection from the original samples. Thus cultures should be prepared to allow multiplication of the parasites above the detection limit (around 10^4/ml; Bryan *et al.,* 1999).

1.2.2. Culture

Samples should be inoculated into culture media as soon as possible after collection and thereafter incubated at 30°C–37°C, for 48–72 hours or longer, depending on the culture medium used, before being examined (see Section B.1.2.2.1). Inoculated media should not be chilled or refrigerated as this would affect survival of *T. foetus*.

1.2.2.1. Culture media

Several culture media can be used. Diamond's trichomonad medium has been widely used for decades with some modifications over time (Bryan *et al*., 1999). However, other culture media can be used, such as the liver infusion broth medium (Lun *et al*. 2000) also named TFM medium, Clausen's and Oxoid's media. Commercial culture kits are also available.

a) Composition of three commonly used culture media

i) Modified Diamond's medium (Bryan *et al.,* 1999; Lun *et al.,* 2000)

The modified Diamond's medium consists of: 2 g trypticase peptone, 1 g yeast extract, 0.5 g maltose, 0.1 g L-cysteine hydrochloride, and 0.02 g L-ascorbic acid and is made up with 90 ml distilled water containing 0.08 g each of K_2HPO_4 and KH_2PO_4, and adjusted to pH 7.2–7.4 with sodium hydroxide or hydrochloric acid. Following the addition of 0.05 g agar, the medium is autoclaved for 10 minutes at 121°C, allowed to cool to 49°C, and then 10 ml inactivated bovine serum (inactivated by heating to 56°C for 30 minutes), 100,000 units crystalline penicillin C and 0.1 g streptomycin sulphate are added aseptically. The medium is aseptically dispensed in 10 ml aliquots into sterile 16 × 125 mm screw-top vials and refrigerated at 4°C until use.

The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophillic conditions at the bottom where the trichomonads occur in largest numbers.

ii) *Tritrichomonas foetus* medium (TFM) (based on modified Plastridge's medium)

The TFM consists of: 12.5 g neutralised liver digest, 5 g tryptose dissolved in 500 ml distilled water. The pH is adjusted to 7.4 with sodium hydroxide or hydrochloric acid. Following addition of 1.5 g Bacto agar, the medium is autoclaved for 15 minutes at 121°C. An antibiotic solution containing 0.75 g penicillin and 0.082 g streptomycin is prepared in 100 ml distilled water. To prepare 1 litre TFM, 500 ml basal medium is combined to 500 ml sterile inactivated bovine serum and 10 ml antibiotic solution. This medium can be stored at –20°C.

Where a combination of convenience and sensitivity is required, a combined specimen transport and culture kit may be used (BonDurant, 1997; Borchardt *et al.*, 1992). The kit consists of a clear flexible plastic pouch with two chambers. The upper chamber contains special medium into which the sample is introduced. Field samples for direct inoculation into the culture pouch would normally be collected by the preputial scraping technique (BonDurant, 1997). Samples collected by preputial washing require centrifugation before introduction of the sediment into the upper chamber. Following mixing, the medium is forced into the lower chamber, and the pouch is then sealed and incubated at 37°C. Microscopic examination for trichomonads can be done directly through the plastic pouch (Borchardt *et al.*, 1992).

The quality of the water used is important and an antifungal can be added to the media to control yeast growth.

Quality control checks including sterility checks should be carried out on all batches of media.

It is important to make sure that the culture media are used before their established expiry date, as many media are not stable. In-house made media should normally be kept for no longer than 1 month at 5°C ± 3°C.

b) Growth characteristics in the different media

Lun *et al*. (2000) tested the three media described above, i.e. Diamond's, liver infusion broth medium and a commercial culture kit. They found that the three media supported the growth of *T. foetus* at 37°C, sixteen isolates from various geographical origins were tested. The growth characteristics of these isolates were different in the three media. For isolates grown in Diamond's the growth kinetics appeared less variable among isolates, the peak concentration tended to be reached earlier (by day 2–4 post-inoculation) and was generally higher (above 10^7 organisms per ml) than with the other two media. However, the parasites died faster after reaching the peak concentration in Diamond's medium. This has implications for monitoring of cultures (see Section B.1.2.3).

1.2.2.2. Culture inoculation

It is desirable to process samples collected by preputial wash or vaginal wash by centrifugation. If the sampling conditions in the field do not permit the use of a centrifuge, the samples should be decanted for 15–20 minutes on the bench. A volume of approximately 1 ml or less of the sediment or the pellet from the centrifugation step is then inoculated into culture media.

When using commercial sampling devices with a disposable brush, the latter is aseptically cut off directly into culture medium after sampling.

1.2.2.3. Culture conditions

Cultures should be maintained at a temperature within the range 30°C–37°C. In no case should the incubation temperature rise above 37°C.

A known isolate of *T. foetus* should be cultured in parallel with the test samples, as a control.

It is advisable to start examining culture media microscopically at day 2–4 following inoculation, which in most media will correspond to the peak concentration. If Diamond's medium is being used, a single examination at day 2–4 may be performed as the parasites are rapidly dying out in this medium from day 5 onwards (Lun *et al*. 2000). With other media supporting slower growth, such as liver broth infusion and even more so with commercial medium, it is recommended to examine the cultures at intervals until day 7 post-inoculation (Bryan *et al*., 1999; Lun *et al*., 2000). A single examination at day 7 is an alternative option with those media that support slower growth of *T. foetus* than Diamond's (Section B.1.2.3).

In conclusion, it should be emphasised that the different culture media described above are equally successful provided the culture procedures follow the general requirements and are adapted to the type of medium. Laboratories should evaluate what is most suitable for them in their own context, given their facilities and expertise, and considering the climatic, logistical and economic considerations.

1.2.3. Summary of times for optimal growth and survival for different media

The optimal growth for modified Plastridge's medium, *Trichomonas* medium and commercial kit media is after 2–7 days, and the survival time in culture is 1–7 days; for modified Diamond's

medium optimal growth is after 2–4 days and survival time in culture is 1–4/5 days (Buller & Corney, 2013).

1.2.4. *Tritrichomonas foetus* detection and identification by microscopic examination

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from a drop of the sample or culture, or through the wall of a plastic pouch. When a drop of culture medium is examined, it should be carefully taken from the bottom of the tube, where the trichomonads are likely to concentrate due to the microaerophilic conditions.

The organisms may be seen under a standard compound microscope using a magnification of ×40 or ×80 initially, then ×100 or ×400. *Tritrichomonas foetus* is motile and normally exhibits jerky movements in wet preparations. Sufficient time, i.e. 2–5 minutes, should be devoted to examination of each slide, to allow the detection of parasites in low numbers and observation of the morphological features.

The pear-shaped organisms have three anterior and one posterior flagellae and an undulating membrane that extends nearly to the posterior end of the cell. They also have an axostyle that usually extends beyond the posterior end of the cell (Table 2).

Phase-contrast microscopy is very valuable in revealing these features. A rapid, Giemsa-based staining procedure may also be used (Buller & Corney 2013; Lun & Gajadhar, 1999). Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.

The staining of trichomonads is best performed using Lugol's iodine, which enhances the morphological features of the flagellae and the membrane (Lun & Gajadhar, 1999). Briefly, 1 ml of the culture containing the parasites is concentrated at high speed e.g. 16,000 ***g*** for 10 seconds. The supernatant is removed and the pellet is resuspended homogenously in the culture medium containing 10% bovine serum. A thin smear is prepared on a microscope slide using 10 µl of the suspension. The slide is air-dried and fixed for 1 minute in methyl alcohol and then stained in Lugol's iodine for 1 minute. The slide is subsequently stained with the rapid Giemsa method following the manufacturer's instructions. The slide is then washed thoroughly to remove any remaining stain and air dried before being examined at × 100 under oil-immersion (Buller & Corney, 2013). The staining allows the differentiation of *T. foetus* from other trichomonads as shown in Table 2.

Table 2 Morphological features of trichomonads (Buller & Corney, 2013)

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

1.2.5. Overall sensitivity and specificity of culture and identification

Any estimate of the diagnostic sensitivity and specificity of the culture and identification test will be dependent on the efficacy of sample collection, handling and processing, as well as the composition and quality of the culture medium.

In bulls, a commercial pouch kit has a sensitivity of 92% (95% confidence interval, 84–96%), while the same device used in experimentally infected young cows had an apparent sensitivity of 88% through a 10-week period after infection (Kittel *et al.*, 1998).

Estimates for Diamond's and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99% (Skirrow & BonDurrant, 1988). Until recently, it has been assumed that the specificity of the culture test was 100%, but this is likely to be an overestimation.

Diagnostic results with samples from bulls using either Diamond's medium or a field kit have shown that the two methods give comparable results (Borchardt *et al.*, 1992; Bryan *et al.*, 1999; Kittel *et al.*, 1998). It should be emphasised that not every sample taken from a particular bull known to be infected, will necessarily give a positive culture result. Even when conditions of sampling, transport, culture and identification are optimal, more than one negative sample should be obtained before there is reasonable assurance that the animal is not infected. This is the basis for most breeding regulations to specify that bulls above 6 months of age should be tested three times at weekly intervals before concluding on absence of *T. foetus* infection. For bulls below 6 months, or that have been kept with bulls only, one negative test is considered sufficient.

In females, as the infection is usually cleared within 90–95 days, it may be difficult to isolate organisms from individuals in the late stages of their infection.

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted foetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids (Rhyan *et al.*, 1988). In addition, immunohistochemical techniques and molecular methods can be used to demonstrate *Tritrichomonas foetus* organisms in tissues of aborted foetuses.

1.2.6. Immunohistochemical techniques

Immunohistochemical techniques using monoclonal antibodies have been described for revealing *T. foetus* organisms in formalin-fixed tissues (Rhyan *et al.*, 1995). These techniques can be used to identify *T. foetus* in tissues from aborted fetuses (e.g. placenta and lungs).

1.3. Molecular methods – detection of nucleic acids

Several molecular methods have been described for the detection of *T. foetus* DNA prepared from cultures or directly from clinical samples. These include conventional and real-time PCR methods targeting conserved regions of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions (ITS). A PCR diagnostic test offers a number of potential advantages, including increased analytical sensitivity, faster diagnostic turnaround time, and the fact that the organisms in the collected sample are not required to be intact and viable.

1.3.1. DNA Extraction

The preferred samples for DNA extraction are smegma and vaginal mucus in PBS or normal saline, transport medium and cultures.

1.3.1.1. Heat lysis

One ml of sample (vaginal mucus or smegma) is centrifuged for 5 minutes at 12,000 ***g***. The supernatant is removed and the pellet is resuspended in 500 µl RNase/DNase-free water. The suspension is heated at 95°C for 10 minutes. The lysate is centrifuged at 2000 ***g*** for 3 minutes and 5 µl of the supernatant is tested.

1.3.1.2. Magnetic beads-based extraction

There is a variety of magnetic beads kits commercially available on the market. This methodology uses a robotic system; it is sensitive and suitable for high throughput set up, where numerous samples are submitted for *T. foetus* testing.

1.3.1.3. Spin column method

This method uses spin columns and is more suitable for manual extraction of a small number of samples. There is a number of spin column kits available on the market and they all perform similarly.

The sensitivity of conventional PCRs is enhanced when using purified DNA extracts obtained by the use of commercial kits, e.g. magnetic beads or spin column methods. The real-time PCR can be used for all three extraction methods. Increased sensitivity has been observed by McMillen & Lew (2006) when using the heat lysis method, which was not suitable for conventional PCR.

1.3.2. Conventional PCR

Felleisen *et al.* (1998) described a conventional PCR that uses primers TFR3 and TFR4 to differentiate *T. foetus* from other commensal trichomonads, which are faecal contaminants of clinical samples submitted for the diagnosis of bovine trichomonosis.

The primers used are TFR3 and TFR4, which target a 347bp region and have the following sequences:

TFR3 5'-CGG-GTC-TTC-CTA-TAT-GAG-ACA-GAA-CC-3'

TFR4 5'-CCT-GCC-GTT-GGA-TCA-GTT-TCG-TTA-A-3'

The PCR is usually conducted for 40 cycles with a 30 second denaturation at 94°C, 30 seconds annealing at 67°C and 90 seconds extension at 72°C for 90 seconds. The final extension is performed at 72°C for 15 minutes (Felleisen *et al.*, 1998).

The use of two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2) and the *T. foetus*-specific set of primers TFR3 and TFR4 (Felleisen *et al.*, 1998) allowed the differentiation of *T. foetus* from other commensal trichomonads of the gastrointestinal tract that are often faecal contaminants of the bovine reproductive system (Campero *et al.*, 2003). *Tritrichomonas foetus* DNA is amplified by both sets of primers, while the DNA of commensal trichomonads is only amplified by the TFR1 and TFR2 primers.

The sequences of the generic primers TFR1 and TFR2, which amplify a 372 bp region of the ITS1 and ITS2 region of the trichomonad group, are as follows:

TFR1 5'-GTA-GGT-GAA-CCT-GCC-GTT-G-3'

TFR2 5'-ATG-CAA-CGT-TCT-TCA-TCG-TG-3'

The PCR is conducted for 30 cycles with a 30 second denaturation at 94°C, followed by 20 seconds annealing at 58°C and a 30 second extension at 72°C. The final extension was performed at 72°C for 20 minutes.

The TFR3 and TFR4 primers have also been used in conjunction with DNA chelating fluorescent dyes under real-time PCR conditions for research purposes rather than diagnostic screening for trichomonosis (Casteriano *et al.*, 2016).

Grahn *et al.* (2005) have also used the TFR1 and TFR2 primers in combination with a fluorophore, whereas the forward primer (TFR1) is labelled with a 6FAM fluorophore, resulting in increased sensitivity of the conventional PCR.

Hayes *et al.* (2003) have also used RFLP of the amplicon generated by primers TFR1 and TFR2 to differentiate various trichomonads including *T. foetus*.

A loop mediated isothermal amplification assay (LAMP) targeting *T. foetus* 5.8S rDNA has been reported with slightly higher sensitivity than the TFR3/TFR4 PCR and demonstrated increased specificity (Oyhenart *et al*., 2013). Due to the simplicity of the assay over PCR or real time PCR, the LAMP assay may be a cheaper alternative for low skill operators, however the assay has not been field tested at this stage.

1.3.3. Real-time PCR or quantitative PCR

A Minor Groove Binder (MGB) probe real-time PCR method described by McMillen & Lew (2006), based on the ITS-1 region within the same rDNA regions as the TFR3-TFR4 PCR primers, was shown to be highly specific and sensitive compared with culture/microscopy and the previously described PCR methods (McMillen & Lew, 2006). Assay sensitivity was evaluated with 10-fold dilutions of known numbers of *T. foetus* cells, and compared with that for microscopy following culture and the TFR3-TFR4 PCR assay. The probe-based real-time PCR assay detected a single cell per assay directly from non-cultured smegma or vaginal mucus and was 2500-fold and 250-fold more sensitive than microscopy following selective culture respectively and 500-fold more sensitive than culture followed by the TFR3-TFR4 PCR assay. When compared with TFR3-TFR4 amplification of *T. foetus* DNA from cultures, the real-time PCR was consistently 10-fold more sensitive for smegma samples at 0, 2 and 5 days post-culture. For vaginal mucus, both PCRs demonstrated equivalent sensitivities at days 0 and 2 with 10-fold increase in sensitivity for real-time PCR at day 5 post-culture. The sensitivity of the TFR3-TFR4 PCR assay was 10-fold lower compared with the real-time PCR assay when testing purified DNA extracted from clinical specimens. Furthermore, the sensitivity of the real-time PCR assay improved 500-fold when using crude cell lysates, which were not suitable as template for the conventional PCR assay (McMillen & Lew, 2006). Initial evaluations of this real-time PCR method showed that from 159 Australian diagnostic specimens, 14 bulls were positive by real-time PCR (directly from clinical specimens: smegma and vaginal mucus) with only three confirmed by selective culture/microscopy detection (Fisher's exact test $p<0.001$) (McMillen & Lew 2006). The real-time PCR was designed with a MGB probe. The substitution of the MGB probe with other probes e.g. black hole quenchers or TAMRA will result in false positive results. The real-time PCR uses the following primers and probe:

TFF2 (20μM) 5'-GCG-GCT-GGA-TTA-GCT-TTC-TTT-3'

TFR2 (20μM) 5'-GGC-GCG-CAA-TGT-GCA-T-3'

TrichP2 (5μM) 5'-6FAM-ACA-AGT-TCG-ATC-TTT-G-MGB-3'

The real-time PCR is performed using a 25 μl reaction containing commercially available mastermix, 900 nM of TFF2 and TFR2 primers and 80 nM of TichP2 probe. When using a uracyl DNA glycosilase (UDG) containing mastermix, the real-time PCR is conducted at 50°C for 2 minutes to activate the UDG and prevent any carryover contamination, followed by incubation at 95°C for 2 minutes, and then 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 45 seconds. The fluorescence is acquired in the green channel at the end of each annealing/extension step.

Robust evaluation of the commercial version of this real-time PCR assay has been undertaken as described by Effinger *et al.* (2014) using real-time PCR post-culture. This evaluation used 833 cultured smegma samples, which were tested by five laboratories in the USA using the commercial real-time PCR kit different DNA processing methods, PCR equipment and PCR cycling conditions. Pools consisting of one *T. foetus* positive and four negative smegma samples were also processed and 96% of the positive samples were detected in these pools. For individual sample testing an overall agreement of 95.89% was attained between the five participating laboratories. A recent study undertaken in Canada showed that real-time PCR detection of *T. foetus* in pooled preputial samples in PBS was more sensitive than culture methods. The study also demonstrated that there was no difference in the detection of *T. foetus* from known infected bulls between direct real-time PCR (from PBS), culture/microscopy or culture/real-time PCR.

Another real-time PCR method, which targets the gene encoding beta-tubulin 1 from *T. foetus,* has been developed. It is available on the market as commercial kits. Despite the high degree of conservation of beta tubulin genes in trichomonads, the test is claimed to be specific and it has broad range detection of *T. foetus* isolates. However, there is only limited and unpublished data on the evaluation of these kits.

PCR diagnostic frameworks are best determined for a particular region and country taking into account transport time to the diagnostic laboratory, temperature of the sample, and optimised DNA extraction and PCR methods used at the diagnostic laboratory. Currently, the best option is to use published protocols (adapted to the particular laboratory) or commercial *T. foetus* real-time PCR kits applied to transport medium or PBS, which guarantees fast preliminary results especially when using boiled lysates.

1.4. Combination of culture and PCR on 5-day cultures

Combining PCR methods and culture has been reported to yield higher sensitivity or improved specificity, and has been suggested as the most cost-effective and practical approach to assess bulls before breeding (Michi *et al*., 2016). However, it has also been reported that testing clinical samples from bulls directly by PCR is more sensitive than culture followed by PCR (McMillen & Lew, 2006), even though most laboratories process cultures as a priority.

The culture and PCR combination should be implemented in some specific situations where specificity is a problem, due to occurrence of other trichomonads that may result in false positives in culture. In view of the difficulty of distinguishing *T. foetus* from other trichomonads based on morphology, it is recommended that, whenever the facilities exist for DNA-based methods, cultures with trichomonads be systematically tested by PCR to confirm the presence of *T. foetus*.

2. Serological tests

Bulls do not develop prominent immune responses to *T. foetus*. Some immunological tests have been developed for the diagnosis of bovine trichomonosis, such as mucus agglutination test and intradermal test (Rhyan *et al.*, 1999) and an antigen-capture enzyme-linked immunosorbent was described more recently (BonDurant, 1997). However, these tests appear very limited in use due to low sensitivity or specificity, and thus they are not recommended for the detection of *T foetus* in individual animals.

Infected cows develop specific IgG1 and IgG2 antibodies that are present in the vaginal mucus and in serum, but these are not exploited for diagnostic purposes.

C. REQUIREMENTS FOR VACCINES

Whole cell vaccines for cows have been shown to offer protection and are available commercially (Corbeil, 1994) as either a monovalent vaccine or part of a polyvalent vaccine also containing *Campylobacter* and *Leptospira* spp. (BonDurant, 1997). These products have shown efficacy in the female but not in the bull.

One example of a method of whole cell vaccine production is by growing *T. foetus* (culture VMC-84) in modified Diamond's medium (Corbeil, 1994) and freezing the culture at –20°C for 60 minutes. After thawing, a suspension of 5×10^7 organisms/ml in PBS is added to the CL-vaccine.

REFERENCES

BonDurant R.H. (1997). Pathogenesis, diagnosis and management of trichomoniasis in cattle. *Vet. Clin. North Am. Food Anim. Pract*., **13**, 345–361.

Borchardt K.A., Norman B.B., Thomas M.W. & Harmon W.M. (1992). Evaluation of a new culture method for diagnosing *Tritrichomonas foetus. Vet. Med.,* **87**, 104–112.

BRYAN L.A., CAMBELL J.R. & GAJADHAR A.A. (1999). Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch™ TF media. *Vet. Rec*., **144**, 227–232.

BULLER N. & CORNEY B. (2013). Bovine Trichomonosis. Australian New Zealand Standard Diagnostic Procedure. http://www.agriculture.gov.au/SiteCollectionDocuments/animal/ahl/ANZSDP-Bovine-trichomoniasis.pdf (assessed 1 August 2017).

CASTERIANO A., MOLINI U., KANDJUMBWA K., KHAISEB S., FREY C.F. & ŠLAPETA J. (2016). Novel genotype of *Tritrichomonas foetus* from cattle in Southern Africa. *Parasitology,* **143**, 1954–1959.

CAMPERO C.M., RODRIGUEZ DUBRA C., BOLONDI A., CACCIATO C., COBO E., PEREZ S., ODEON A., CIPOLLA A. & BONDURANT R.H. (2003). Two-step (culture and PCR) diagnostic approach for differentiation of non-*T.foetus* trichomonads from genitalia of virgin bulls in Argentina. *Vet. Parasitol*. **112**, 167–175.

CORBEIL L.B. (1994). Vaccination strategies against *Tritrichomonas foetus*. *Parasitol. Today*, **10**, 103–106.

EFFINGER L., PEDDIREDDI L., SIMUNICH M., OBERST R., O'CONNELL C. & LEYVA-BACA I. (2014). Pooling of cultured samples and comparison of multistate laboratory workflows with the MagMAX sample preparation system and VetMAX quantitative polymerase chain reaction reagents for detection of *Tritrichomonas foetus*-colonized bulls. *J. Vet. Diagn. Invest*., **26**, 72–87.

ELDER J.K. (1964). Examination of twelve strains of *Trichomonas foetus* (Reidmuller) isolated in Queensland and the description of a new serotype, *T. foetus* var. *brisbane. Queensl. J. Agric. Sci.,* **21**, 193–203.

FELLEISEN R.S.J., LAMBELET N., BACHMANN P., NICOLET J., MULLER N. & GOTTSTEIN B. (1998). Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences*. J. Clin. Microbiol.*, **36**, 513–519.

GRAHN R.A., BONDURANT R.H., HOOSEAR K.A., WALKER R.L. & LYON L.A. (2005). An improved molecular assay for *Tritrichomonas foetus*. *Vet. Parasitol*., **127**, 33–41.

GREGORY M.W., ELLIS B. & REDWOOD D.W. (1990). Comparison of sampling methods for the detection of *Tritrichomonas foetus. Vet. Rec*., **127**, 16.

HAYES D.C., ANDERSON R.R. & WALKER R.L. (2003). Identification of trichomonadid protozoa from the bovine preputial cavity by polymerase chain reaction and restriction fragment length polymorphism typing. *J. Vet. Diagn. Invest*., **15**, 390–394.

KITTEL D.R., CAMPERO C., VAN HOOSEAR K.A., RHYAN J.C. & BONDURANT R.H. (1998). Comparison of diagnostic methods for detection of active infection with *Tritrichomonas foetus* in beef heifers. *J. Am. Vet. Med. Assoc*., **213**, 519–522.

LEVINE N.D. (1973). The Trichomonads. *In:* Protozoan Parasites of Domestic Animals and of Man, Levine N.D., ed. Burgess Publishing Company, Minnesota, USA, 88–110.

LUN Z.-R. & GAJADHAR A.A. (1999). A simple and rapid method for staining *Tritrichomonas foetus* and *Trichomonas vaginalis. J. Vet. Diagn. Invest*., **11**, 471–474.

LUN Z.-R., PARKER S. & GAJADHAR A.A. (2000). Comparison of growth rates in *Trichomonas foetus* isolates from various geographic regions using three different culture media. *Vet. Parasitol*., **89**, 199–208.

MCMILLEN L. & LEW A.E. (2006). Improved detection of *Tritrichomonas foetus* in bovine diagnostic specimens using a novel probe-based real time PCR assay. *Vet. Parasitol.*, **141**, 204–215.

MICHI A.N., FAVETTO P.H., KASTELIC J. & COBO E.R. (2016). A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. *Theriogenology,* **85**, 781–791.

OYHENART J., MARTÍNEZ F., RAMÍREZ R., FORT M., & BRECCIA J.D. (2013). Loop mediated isothermal amplification of 5.8S rDNA for specific detection of *Tritrichomonas foetus*. *Vet. Parasitol*., **193**, 59–65.

RHYAN J.C., STACKHOUSE L.L. & QUINN W.J. (1988). Fetal and placental lesions in bovine abortion due to *Tritrichomonas foetus*. *Vet. Pathol.,* **25**, 350–355.

RHYAN J.C., WILSON K.L., BENGESS D.E., STAOKHOUSE L.L. & QUINN W.J. (1995). The immunohistochemical detection of *Tritrichomonas* foetus in formalin-fixed paraffin-embedded sections of bovine placenta and fetal lung. *J. Vet. Diagn. Invest.,* **7**, 98–101.

RHYAN J.C., WILSON K.L., WAGNER B., ANDERSON M.L., BONDURANT R.H., BURGESS D.E., MUTWIRI G.K. & CORBEIL L.B. (1999). Demonstration of *Tritrichomonas foetus* in the external genitalia and of specific antibodies in preputial secretions of naturally infected bulls. *Vet. Pathol*., **36**, 406–411.

SKIRROW S.Z. & BONDURRANT R.H. (1988). Bovine trichomoniasis. *Vet. Bull.,* **58**, 591–603.

ŠLAPETA J., MÜLLER N., STACK C.M., WALKER G., LEW-TABOR A. & TACHEZY J. (2012). Comparative analysis of *Tritrichomonas foetus* (Riedmüller, 1928) cattle genotype and *Tritrichomonas suis* (Davaine, 1875) at 10 DNA loci. *Int. J. Parasitol*., **42**, 1143–1149.

TACHEZY J., TACHEZY R., HAMPL V., SEDINOVA M., VANACOVA S., VRLIK M., VAN RANST M., FLEGR J. & KULDAA J. (2002). Cattle pathogen *Tritrichomonas foetus* (Riedmuller, 1928) and pig commensal *Tritrichomonas suis* (Gruby & Delafond, 1843) belong to the same species. *J. Eukaryot. Microbiol*., **49**, 154–163.

TAYLOR M.A., MARSHALL R.N. & STACK M. (1994). Morphological differentiation of *Tritrichomonas foetus* from other protozoa of the bovine reproductive tract. *Br. Vet. J*., **150**, 73–80.

WARTON A. & HONIGBERG B.M (1979). Structure of trichomonads as revealed by scanning electron microscopy. *J. Protozoot.,* **26**, 56–62.

YAO C. (2012). Opportunistic human infections caused by *Tritrichomonas* species: a mini-review. *Clin. Microbiol. News,* **34**, 127–131

YAO C. (2013). Diagnosis of *Tritrichomonas foetus*-infected bulls, an ultimate approach to eradicate bovine trichomoniasis in US cattle? *J. Med. Microbiol.,* **62**, 1–9.

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for trichomonosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991 AS TRICHOMONIASIS. MOST RECENT UPDATES ADOPTED IN 2018.