

CHAPTER 2.4.16.

TRICHOMONOSIS

SUMMARY

Bovine venereal trichomonosis is caused by Tritrichomonas foetus, a flagellate protozoan parasite. It is world-wide in 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 herds or 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 are the main reservoir of the disease as they tend to be long-term carriers, whereas most cows 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: *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. Smegma can be collected by a variety of means including preputial lavage 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, but a commercially available field culture test kit¹ allows for trichomonad growth and direct microscopic examination.*

Alternative tests: *Bovine trichomonosis may also be detected by polymerase chain reaction amplification. In the past, an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms has been used as a herd test. Similarly, an intradermal test using a trichloroacetic acid precipitate of the organism has been used in herds.*

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

Bovine venereal trichomonosis is caused by the flagellate protozoan parasite, *Tritrichomonas foetus*. The normal hosts of *T. foetus* are cattle (*Bos taurus*, *B. indicus*). Non-pathogenic species of trichomonads occur in the intestine of cattle; *T. suis* of pigs is indistinguishable morphologically, serologically and, with modern molecular analysis, genetically from *T. foetus* (Felleisen, 1997; Soulsby, 1982). Further genetic characterisation is required to determine the taxonomic status of isolates from cattle and pigs.

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). It is important to differentiate *T. foetus* from other contaminant flagellated protozoa

1 InPouch™ TF Test, BioMed Diagnostics, White City, Oregon, United States of America (USA).

that may be present in samples from the bovine reproductive tract (BonDurant *et al.*, 1999; Campero *et al.*, 2003; Parker *et al.*, 2003b; Taylor *et al.*, 1994). Under phase contrast illumination, the number of flagellae observed is an important characteristic as this can help to differentiate *T. foetus* from some bovine flagellates that appear similar. A staining technique has been described that can be used to more clearly observe the morphology and facilitate a definitive identification (Lun & Gajadhar, 1999).

Trichomonas foetus multiplies by longitudinal binary fission; sexual reproduction is not known to occur, and environmentally resistant stages of the parasite have not been observed. However, *T. foetus* may be cultured *in vitro* using a commercially available kit (see footnote 1) or an in-house prepared medium such as Diamond's medium (Diamond, 1983). The organism may also be detected by molecular methods such as polymerase chain reaction (PCR) (Campero *et al.*, 2003; Felleisen *et al.*, 1998).

In a few early studies, three serotypes were recognised based on agglutination (Skirrow & BonDurrant, 1988): the 'belfast' strain, reportedly predominated in Europe, Africa and the USA (Gregory *et al.*, 1990); the 'brisbane' strain in Australia (Elder, 1964); and the 'manley' strain, which has been reported in only a few outbreaks (Skirrow & BonDurrant, 1988). Further work needs to be done in the area of comparing the growth characteristics, genetic and antigenic variation and pathogenesis of isolates of *T. foetus* from different areas before 'strain' and 'serotype' designations can be reliably established.

Transmission of infection occurs by coitus, by artificial insemination, or by gynaecological examination of cows. The site of infection in bulls is primarily the preputial cavity (BonDurant, 1997; Parsonson *et al.*, 1974), 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 old, infection may be transient.

Trichomonas foetus is present in small numbers in the preputial cavity of bulls, with some concentration in the fornix and around the glans penis (Hammond & Bartlett, 1943). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which can be followed in animals that become pregnant by 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; Fitzgerald, 1986; Skirrow & BonDurrant, 1988). Cows usually clear their infection and generally become immune, at least for that breeding season (BonDurant, 1997; Fitzgerald, 1986; Soulsby, 1982).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

1.1. Agent identification by direct examination or culture (the prescribed test for international trade)

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 organisms 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 (European Union, 1988; Kittel *et al.*, 1998; Mukhufhi *et al.*, 2003; Parker *et al.*, 1999; Schonmann *et al.*, 1994).

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 generally recommended to allow at least 1 week to pass after the last service before taking a preputial sample.

1.1.1. Sample collection

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 can be collected from bulls by scraping the preputial and penile mucosa with an artificial insemination pipette (Parker *et al.*,

1999; Schonmann *et al.*, 1994) or brush (Ostrowski *et al.*, 1974; Parker *et al.*, 1999), by preputial lavage (Schonmann *et al.*, 1994) or by washing the artificial vagina after semen collection (Gregory *et al.*, 1990). The latter technique is not recommended as its sensitivity may be lower (Gregory *et al.*, 1990). Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or brush (Kittel *et al.*, 1998; Mancebo *et al.*, 1995).

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 thioglycollate broth media with antibiotics [Bryan *et al.*, 1999; Thomas *et al.*, 1990] or the field culture plastic pouch). During transportation, the organisms should be protected from exposure to daylight and extremes of temperature, which should remain above 5°C and below 38°C (Bryan *et al.*, 1999).

1.1.2. Culture

Where organisms are too few to allow for direct detection and accurate identification, cultures should be prepared. Culture of *T. fetus* is usually required because, in most cases, the number of organisms is not large enough to make a positive diagnosis by direct examination. Several media can be used. Diamond's trichomonad medium or commercial culture kits are the media of choice (Bryan *et al.*, 1999; Eaglesome & Garcia, 1992; Parker *et al.*, 2003a; Ribeiro, 1990). However, other culture media that may be used include Clausen's and Oxoid's media (Eaglesome & Garcia, 1992). Inoculation of samples into culture media should be done as soon as possible after collection. For samples collected by preputial wash it is necessary to process the sample by centrifugation. The sediment is then inoculated into culture media. Some protocols recommend direct viewing of the aspirate or sediment before inoculation but this does not increase diagnostic sensitivity. It is also important to make sure that the culture media are used before their established expiry date, as many media are not stable. The quality of the water used is important and an antifungal can be added to the media to control yeast growth.

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from the sample or culture, or through the plastic wall of the InPouch™ kit (see footnote 1) using the specially provided plastic clip. The motile organisms may be seen under a standard compound microscope using a magnification of 100 or more. An inverted microscope may be useful for examining tubes containing culture medium. Culture media should be examined microscopically at intervals from day 1 to day 7 after inoculation (Bryan *et al.*, 1999; Lun *et al.*, 2000). The organisms may be identified on the basis of characteristic 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. Phase-contrast microscopy is very valuable in revealing these features or a rapid-staining procedure may also be used (Lun & Gajadhar, 1999). Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.

1.1.2.1. Culture procedures

i) Modified Diamond's medium

Glassware used for culture should be washed in distilled water (avoiding the use of detergents). 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 K₂HPO₄ and KH₂PO₄, 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 x 125 mm screw-top vials and refrigerated at 4°C until use. Media should be cultured for up to 7 days, samples being examined at daily intervals (BonDurant, 1997; Lun *et al.*, 2000). The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophilic conditions at the bottom where the trichomonads occur in largest numbers.

ii) Commercial culture test kit

Where a combination of convenience and sensitivity is required, the culture kit (see footnote 1) may be used (BonDurant, 1997; Borchardt *et al.*, 1992; Parker *et al.*, 2003a; Schonmann *et al.*, 1994; Thomas *et al.*, 1990). 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; Schonmann *et al.*, 1994). 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). Diagnostic results with samples from bulls using either Diamond's medium or the field kit have shown that the two methods give comparable results but there are some advantages (in convenience and in test results) with the kit (Borchardt *et al.*, 1992; Bryan *et al.*, 1999; Kittel *et al.*, 1998; Parker *et al.*, 2003a; Schonmann *et al.*, 1994).

1.1.2.2. Overall sensitivity and specificity of the culture and identification test

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, the sensitivity of the InPouch™ TF kit has been estimated to be 92% (95% confidence interval, 84–96%) (Parker *et al.*, 1999; 2003a). Estimates for Diamond's and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99% (Parker *et al.*, 2003a; 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.

Not every sample taken from a particular bull, known to be infected, will necessarily give a positive culture result. Even with optimum conditions of sampling, transport, culture and identification, more than one negative sample should be obtained before there is reasonable assurance that the animal is uninfected. To estimate the probability that an animal is uninfected, negative predictive values should be calculated using an estimate of diagnostic test sensitivity and the animal's pretest probability of infection (Parker *et al.*, 1999). The infection in females is usually cleared within 90–95 days, so it may be difficult to isolate organisms from animals in the late stages of their infection. In experimentally infected young cows, using the InPouch™ TF method of culture, an apparent sensitivity of 88% was achieved through a 10-week period after infection (Kittel *et al.*, 1998).

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted fetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids. Additionally, immunohistochemical techniques and DNA methods can be used to demonstrate tissue-invasive *T. foetus* organisms in aborted fetuses.

1.2. Polymerase chain reaction

Molecular-based techniques that use PCR technology have been developed for the identification of *T. foetus* (Campero *et al.*, 2003; Cobo *et al.*, 2007; Felleisen *et al.*, 1997; 1998; Parker *et al.*, 2001). 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 viable. A real-time PCR has been described (McMillen & Lew, 2006) that described a low detection limit, however insufficient field samples were tested to adequately evaluate its performance. A diagnostic PCR assay includes both a specific extraction technique and DNA amplification using PCR techniques with specific primers. The sensitivity and specificity of the assay will be affected by the choice of extraction, choice of PCR conditions and the choice of primers. PCR assays are capable of detecting very low numbers of parasites from laboratory cultures of the organism with no preputial material present and in the presence of preputial material (Felleisen *et al.*, 1998; Parker *et al.*, 2001). However, in the presence of preputial material, a higher number of parasites is required to yield a positive PCR result; this is most likely due to inhibition by components of the preputial smegma. Several DNA extraction techniques have been described (Felleisen *et al.*, 1998; Huby-Chilton *et al.*, 2009; Parker *et al.*, 2001) and typically, the sensitivity of the diagnostic test will be influenced by the efficiency of the extraction method and the procedures to overcome contaminating inhibitors. Diagnostic specificity of the PCR test depends in part, on the specificity of the primers. A set of primers based on the 5.8s rRNA sequence demonstrated good diagnostic specificity in samples from negative animals (TFR3 and TFR4; Felleisen *et al.*, 1998) and are the primer set most frequently cited in published literature. These primers do, however produce amplification products from some closely related flagellates (*Tritrichomonas suis*, *T. mobilensis* and a trichomonad from cats) that are indistinguishable from those of *T. foetus* (Felleisen *et al.*, 1998; Gookin *et al.*, 2002). These species also cannot be differentiated by microscopy and it is possible that some of these organisms are synonymous with *T. foetus*. These primers can be used to differentiate between *T. foetus* and a non-

T. foetus trichomonad sometimes found in preputial samples (BonDurant *et al.*, 1999; Campero *et al.*, 2003; Parker *et al.*, 2003b).

The diagnostic sensitivity and specificity of these assays has yet to be determined in an adequate sample of positive and negative animals, although research to date suggests good specificity (Campero *et al.*, 2003; Rhyan *et al.*, 1995). The diagnostic sensitivity of PCR tests has been estimated to be similar to that of the InPouch™ TF culture kit (Campero *et al.*, 2003) but with very few animals. PCR techniques are an attractive alternative to microscopy in that they have a faster turnaround time, and they also allow the detection of dead organisms. The validation of PCR techniques should be continued and a large number of known positive and negative samples should be tested. DNA-based techniques have potential as an ancillary or primary test (BonDurant *et al.*, 1999; Campero *et al.*, 2003; Felleisen *et al.*, 1998; Mukhufhi *et al.*, 2003; Parker *et al.*, 2001) and play a key role in differentiating trichomonad protozoa recovered from bovine samples from the reproductive tract.

Several different approaches have been taken to continue on from earlier work that used the one set of primers (TFR3 and TFR4; [Felleisen *et al.*, 1998]) specifically diagnose *T. foetus*. The use of two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2; [Felleisen, 1997]) and one set specific to *T. foetus* (TFR3 and TFR4; [Felleisen *et al.*, 1998]) to differentiate between organisms considered to be faecal contaminants of the bovine reproductive system and *T. foetus* (Campero *et al.*, 2003). Alternatively, the generic primers (TFR1 and TFR2; [Felleisen, 1997]) were used to amplify DNA and then different protozoal species were differentiated using RFLP analysis (Hayes *et al.*, 2003). In a third study, another set of primers was designed to amplify different sized amplicons from trichomonad protozoa, allowing different species to be distinguished (Grahm *et al.*, 2005). Recently, single strand conformation polymorphism (SSCP) analysis demonstrated efficiency in distinguishing bovine *T. foetus* from other trichomonads (Huby-Chilton *et al.*, 2009). PCR on pooled aliquots of sedimented samples has shown evidence of enhanced diagnostic efficiency (Kennedy *et al.*, 2009). It has also been demonstrated that a PCR assay can be used to detect *T. foetus* DNA in formalin fixed endometrial and aborted fetal tissue (BonDurant *et al.*, 2003).

1.3. Immunological tests

Several immunological tests have been used in the past or have been recently developed for the diagnosis of bovine trichomonosis (Kerr & Robertson, 1941; Pierce, 1949; Rhyan *et al.*, 1999; Soto & Parm, 1989). However, they are limited in use and are not recommended for the detection of *T. foetus* in individual animals. In the 1940s, mucus agglutination tests and intradermal diagnostic tests were developed, but problems with sensitivity and specificity restrict their usefulness. Other immunological tests based on the antigen-trapping enzyme-linked immunosorbent assay (ELISA) have been developed (BonDurant, 1997; Gault *et al.*, 1995). Immunohistochemical techniques using monoclonal antibodies have been shown to reveal *T. foetus* organisms in formalin-fixed tissues (Rhyan *et al.*, 1995).

1.3.1. Immunohistochemistry on tissues

There are no specific macroscopic or microscopic lesions in the aborted fetus, and identification of the organisms is necessary for diagnosis. An immunohistochemical technique using a monoclonal antibody (MAb) to detect *T. foetus* in formalin-fixed paraffin-embedded placenta and fetal lungs from bovine abortions has been reported (Rhyan *et al.*, 1995). Immunohistochemical staining is done using a commercially available labelled streptavidin/biotin system² and an MAb (34.7C4.4) to *T. foetus*. In the procedure, deparaffinised 4 µm sections are incubated with the MAb following blocking with non-immune goat serum. After three rinses in buffer, the sections are incubated with biotinylated goat anti-mouse and anti-rabbit immunoglobulin for 30 minutes at 37°C. Following three additional rinses in buffer, peroxidase-labelled streptavidin is applied for 30 minutes at 37°C, and the enzyme activity is diluted with 3% AEC (3-amino-9-ethylcarbazole) in N,N dimethylformamide. Sections are counterstained with Gill II haematoxylin for 3 minutes, rinsed, and blued in buffer for 1 minute. This method has been used to diagnose abortions caused by *T. foetus*.

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 (BonDurant *et al.*, 1993).

2 DAKO Corporation, Carpinteria, California, USA

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 phosphate buffered saline is added to the CL-vaccine.

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