Improved detection of *Tritrichomonas foetus* in bovine diagnostic specimens using a novel probe-based real time PCR assay

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Abstract

A *Tritrichomonas foetus*-specific 5′ Taq nuclease assay using a 3′ minor groove binder-DNA probe (TaqMan® MGB) targeting conserved regions of the internal transcribed spacer-1 (ITS-1) was developed and compared to established diagnostic procedures. Specificity of the assay was evaluated using bovine venereal microflora and a range of related trichomonad species. Assay sensitivity was evaluated with log10 dilutions of known numbers of cells, and compared to that for microscopy following culture (InPouch™ TF test kit) and the conventional TFR3-TFR4 PCR assay. The 5′ Taq nuclease assay detected a single cell per assay from smegma or mucus which was 2500-fold or 250-fold more sensitive than microscopy following selective culture from smegma or mucus respectively, and 500-fold more sensitive than culture followed by conventional PCR assay. The sensitivity of the conventional PCR assay was comparable to the 5′ Taq nuclease assay when testing purified DNA extracted from clinical specimens, whereas the 5′ Taq nuclease assay sensitivity improved using crude cell lysates, which were not suitable as template for the conventional PCR assay. Urine was evaluated as a diagnostic specimen providing improved and equivalent levels of *T. foetus* detection in spiked urine by both microscopy following culture and direct 5′ Taq nuclease detection, respectively, compared with smegma and mucus, however inconclusive results were obtained with urine samples from the field study. Diagnostic specimens (*n* = 159) were collected from herds with culture positive animals and of the 14 animals positive by 5′ Taq nuclease assay, 3 were confirmed by selective culture/microscopy detection (Fisher’s exact test *P* < 0.001). The 5′ Taq nuclease assay described here demonstrated superior sensitivity to traditional culture/microscopy and offers advantages over the application of conventional PCR for the detection of *T. foetus* in clinical samples.

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Keywords: *Tritrichomonas foetus*; Bovine venereal disease; 5′ Taq nuclease; Minor groove binder; TaqMan®

1. Introduction

Trichomoniasis is a venereally transmitted reproductive disease of cattle caused by the protozoan parasite *Tritrichomonas foetus* (Levine, 1973), and is widely distributed in regions relying on extensive herd management and natural breeding, including areas of the USA, Canada, Spain, Africa, Asia, Australia and Latin America (Behera et al., 1987; BonDurant et al., 1990; Copeland et al., 1994; Erasmus et al., 1989; Griffiths et al., 1984; Kvasnicka et al., 1989; Martin-Gomez et al., 1998; McCool et al., 1988; Perez et al., 1992; Rae et al., 2004; Takizawa and Ito, 1977). Infection can be inapparent in bulls, but can cause early pregnancy abortion in cows. Bulls (particularly over 5 years old) are the primary carrier, and may reinfect previously infected cows during service, while cows clear the infection after a few months (Riley et al., 1995; Yule et al., 1989). Trichomoniasis is similar to...
Diagnostic specimens (preputial smegma or cervicovaginal mucus) are typically collected by washing or scraping with an aspirated AI infusion pipette, however a unique tool with a scraper head or ‘metal brush’ can improve the collection of secretions by reducing contamination levels and improving isolation rates compared to aspiration or washing (BonDurant et al., 2003; Irons et al., 2002; Kittel et al., 1998; Mancebo et al., 2005; Clark et al., 1971; Fitzgerald et al., 1954; Lun et al., 2000; Parker et al., 1999; Tedesco et al., 1977). Diagnosis of infection is typically based on microscopic examination of an inoculated enrichment/transport medium (e.g. InPouch™, BioMed Diagnostics) to identify spindle-shaped trichomonads with characteristic darting motility (Kittel et al., 1998). *T. foetus* is considered easily recognised within 7 days without the need for further selective isolation, but increasing evidence indicates that microscopic examination may be insufficient to confirm the taxa of trichomonads isolated from the prepuce of bulls due to contamination with intestinal or coprophilic trichomonads (Campero et al., 2003; Kittel et al., 1998; Taylor et al., 1994). A 100% diagnostic sensitivity of culture has been demonstrated, however rates of 81–90% of naturally infected bulls and only 58–78% of infected female cattle are more commonly reported (Bryan et al., 1999; Clark et al., 1971; Fitzgerald et al., 1954; Lun et al., 2000; Parker et al., 1999; Reece et al., 1983; Skirrow, 1987; Skirrow et al., 1985). Success of culture in these studies appears to vary with the sampling technique used, the temperature during sample transport, the time in transit, use of different media, the number of repetitive samples taken from bulls (thereby increasing the likelihood of isolation), different isolate growth characteristics and the number of *T. foetus* organisms inoculated (Bryan et al., 1999; Parker et al., 2003). As field samples are often subjected to suboptimal conditions, there has long been a need for the development of a more robust diagnostic test for trichomoniasis (BonDurant, 2005; Bryan et al., 1999).

Diagnostic culture followed by microscopic examination to detect the presence of *T. foetus* organisms is considered the ‘gold standard’ protocol and researchers have developed polymerase chain reaction or DNA based assays to enhance the culture diagnosis success rate (Felleisen et al., 1998; Ho et al., 1994; Nickel et al., 2002). The internal transcribed spacer-1 (ITS-1) based PCR method developed by Felleisen et al. (1998) has found wide acceptance and has been applied to detect *T. foetus* in culture to improve upon microscopic examination and to differentiate *T. foetus* from morphologically similar trichomonads (Campero et al., 2003; Cobo et al., 2003; Hoevers et al., 2003; Parker et al., 2001, 2003). A new ITS-1 PCR using a fluorescently labeled primer has been developed and differentiates *T. foetus* from other trichomonads based on amplicon size, however the diagnostic sensitivity or applicability of this assay has not been demonstrated (Grahn et al., 2005). Results using the Felleisen et al. PCR directly on clinical samples or cultured clinical material have shown either equivalent or improved detection by PCR when compared with conventional culture and microscopy (BonDurant et al., 2003; Felleisen et al., 1998; Mukhufhi et al., 2003; Parker et al., 2001). Some researchers have suggested that the culture or clinical specimen DNA preparations contain inhibitory substances and novel DNA processing procedures have subsequently improved the sensitivity of the PCR test (Chen and Li, 2001; Mukhufhi et al., 2003). The aim of this study was to develop a robust 5′ *Taq* nuclease assay targeting the ITS-1 region of *T. foetus* to circumvent the specificity and processing issues observed using conventional PCR for the improved detection of this pathogen.

5′ *Taq* nuclease assays using fluorescent probes are highly sensitive and specific, and also allow the quantification of target DNA within the sample (Mackay, 2004). 5′ *Taq* nuclease assays have improved the detection of a wide range of protozoan parasites, including *Trichomonas vaginalis* (Hardick et al., 2003; Jordan et al., 2001), *Plasmodium chabaudi* (Cheesman et al., 2003), *Cryptosporidium parvum* and *Giardia lamblia* (Fontaine and Guillot, 2002; Guy et al., 2003). Minor groove binder (MGB) probes demonstrate higher specificity and sensitivity than non-MGB probes in 5′ *Taq* nuclease assays (Kutyavin et al., 2000), and thus are highly suited for routine diagnostic application as demonstrated for the detection of bovine retroviruses and *C. fetus* subsp. *venerealis* from clinical material (Lew et al., 2004a,b; McMillen et al., 2006). This study describes firstly the development of a *T. foetus*-specific 5′ *Taq* nuclease assay, followed by the optimisation of
sampling, transport, and specimen processing protocols for the sensitive and specific diagnosis of bovine venereal trichomoniasis.

2. Materials and methods

2.1. Bacterial and protozoan culture

Isolates of *T. foetus* were obtained from the Animal Research Institute (DPI&F), and the American Type Culture Collection (Table 1). *T. foetus* strains were grown at 37°C in 1.25% neutralised liver digest, 0.5% tryptose, 0.15% bacto agar, 50% sterile inactivated bovine serum, 0.1% P + S solution (0.75% penicillin, 0.082% streptomycin). *Campylobacter* species were grown at 37°C in 1× Brain-Heart Infusion (Oxoid), 0.2% yeast extract, 0.07% Bacto agar for between 1 and 3 days. *Pseudomonas aeruginosa* and *Proteus vulgaris* were grown at 37°C on blood agar plates for 24 h. *Neospora caninum* tachyzoites were cultured invero cells as previously described (De Meerschman et al., 2002).

2.2. Animal samples

Preputial smegma samples were collected from bulls (while restrained in a veterinary crush) using either a bull rasper or aspirated using an artificial insemination (AI) infusion pipette. A bull rasper (polyethylene, 60 cm long with a 75 mm long, 8 mm diameter corrugated scraper head with a 1.5 mm collection bore attached to 6 mm diameter tubing with a 1.5 mm internal diameter, similar to those produced by Elastecnica, Argentina based on Sutka and Katai, 1969) was gently scraped along the surface of the penis and internal prepuce near the fornix. No aspiration was necessary. The collected smegma was rinsed into approximately 5 ml physiological saline. A sterile AI infusion pipette was gently scraped along the surface of the penis and internal prepuce near the fornix, applying gentle suction with a syringe or rubber bulb. The collected smegma was rinsed into approximately 5 ml sterile physiological saline.

Vaginal mucus samples were collected from heifers with a bull rasper during restraint in a veterinary crush. A bull rasper was inserted so that the anterior end reached the vulva. The rasper was moved gently backwards and forwards. No aspiration was necessary. The rasper was removed and the collected mucus rinsed into approximately 5 ml sterile physiological saline.

Urine was investigated to determine its feasibility as an alternative clinical specimen for diagnosis of venereal *T. foetus* infection. Urine from two consecutive voids was collected in a series of sterile collection containers following either palpation of the perineum to induce urination in heifers or subcutaneous or intramuscular administration of a diuretic (Furosemide, Ilium Veterinary Products, Australia).

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichomonas foetus</em></td>
<td>YVL-W</td>
<td>Field isolate (DPI&amp;F, Qld)^a</td>
</tr>
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<td><em>T. foetus</em></td>
<td>BP-4 (30003)</td>
<td>ATCC^b</td>
</tr>
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<td>ATCC</td>
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<td><em>Pentatrichomonas hominis</em></td>
<td>hs-3 (30000)</td>
<td>ATCC</td>
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<tr>
<td><em>Trichomonas vaginalis</em></td>
<td>C-1 (30001)</td>
<td>ATCC</td>
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<td><em>Campylobacter coli</em></td>
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<td>NCTC^c</td>
</tr>
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<td><em>C. fetus</em> subsp. <em>venerealis</em></td>
<td>98-109383</td>
<td>Field isolate (DPI&amp;F, Qld)</td>
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<td>ATCC</td>
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<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em></td>
<td>11168</td>
<td>NCTC</td>
</tr>
<tr>
<td><em>C. hyointestinalis</em></td>
<td>N3145</td>
<td>Field isolate (DPI&amp;F, Qld)</td>
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<tr>
<td><em>C. spatorum</em> subsp. <em>bubulus</em></td>
<td>Y4291-1</td>
<td>Field isolate (DPI&amp;F, Qld)</td>
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<td><em>Pseudomonas aeruginosa</em></td>
<td>27853</td>
<td>ATCC</td>
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<tr>
<td><em>Proteus vulgaris</em></td>
<td>6380</td>
<td>ATCC</td>
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<tr>
<td><em>Neospora caninum</em></td>
<td>50843</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Leptospira borgpetersenii</em></td>
<td>93/94451/3</td>
<td>Field isolate (DPI&amp;F, Qld)</td>
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<td><em>L. interrogans</em> serovar Pomona</td>
<td>Pomona</td>
<td>CCRL^d</td>
</tr>
</tbody>
</table>

^a Animal Research Institute, Department of Primary Industries and Fisheries, Qld, Australia.
^b American Type Culture Collection, Virginia, USA.
^c National Collection of Type Cultures, London, United Kingdom.
^d WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region.
The first container collected was discarded as being the most likely to be heavily contaminated with fecal material, hair, and other debris.

2.3. Diagnostic culture/microscopy

Culture-based diagnosis for *T. foetus* was conducted by inoculating InPouch™ TF media (Biomed Diagnostics) with 0.5 ml of preputial smegma or vaginal mucus in saline followed by transport to the laboratory at ambient temperatures (up to 48 h) and incubated at 37 °C. The media was examined microscopically every 24 h for 5 days for the presence of motile trichomonads.

2.4. PCR template preparation

DNA was extracted from liquid culture, preputial smegma, vaginal mucus or urine samples using a commercial kit (QIAamp DNA mini kit, Qiagen) as per the manufacturer’s protocol, except using a final elution volume of 50 μl rather than 400 μl. DNA concentration was determined following spectrophotometric measurement at A_{260} using a biophotometer (Eppendorf).

Crude cell lysates were prepared for the S' Taq nuclease assay by a heat lysis method. One millilitre of the sample (preputial smegma or vaginal mucus in saline, urine) was centrifuged for 5 min at 12,000 × g, and the supernatant discarded. The pellet or compressed mucus was resuspended in 500 μl sterile dH_{2}O, and heated at 95 °C for 10 min. The suspension was centrifuged for 30 s at 2000 × g, and 5 μl supernatant was assayed by S' Taq nuclease assay.

2.5. PCR amplification and conventional PCR assay

All primers used in this study were synthesised by Prologix Australia Pty Ltd. The 347 bp *T. foetus*-specific product was amplified in a 15 μl reaction volume, using 500 nM TFR3 and TFR4 primers (Felleisen et al., 1998), 1 × PCR reaction buffer with MgCl_{2} (Roche Diagnostics), 200 μM dNTPs, 1 U Taq DNA polymerase (Roche Diagnostics) and 1 ng of *T. foetus* DNA. For smegma, mucus and urine extracts, 2 μl of kit purified DNA was added as template for conventional PCR assays. The reactions were cycled in a GeneAmp PCR system model 2700 (Applied Biosystems Inc.), using the following temperature profile: an initial denaturation at 94 °C for 90 s, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 90 s, including a final single extension for 15 min at the end of the profile. Amplification products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA, pH 8) agarose gels using size markers (Marker XIV, Roche Molecular Biochemicals, Germany) and were visualized under UV illumination by ethidium bromide staining. The presence of *T. foetus* was indicated by the presence of the 347 bp *T. foetus*-specific amplicon.

2.6. Sequencing

The 347 bp *T. foetus*-specific amplicon from strain YVL-W was ligated into a cloning vector (pCR2.1, TOPO-TA cloning kit, Invitrogen Corporation) as described in the manufacturer’s protocol. Plasmids with inserts were sequenced using the T7 and M13 Reverse primers, and BigDye Terminator Mix (Applied Biosystems Inc.), following the manufacturer’s protocols. Sequences were analysed by the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Nathan, Qld 4111, Australia) using an ABI 377 DNA Sequencer.

2.7. S' Taq nuclease assay

Primer and probe combinations for S' Taq nuclease assay using fluorescent 3' minor groove binder-DNA probes were designed for *T. foetus* ITS-1 using Primer Express Version 2 (Applied Biosystems Inc.), and BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm sequence specificity. Primer and probe sequences are: TFF2: GCGGCGTAGATGGTTCTTTT; TFR2: GGCGCGCAATGTGCAT; TRICHP2: 6-FAM-ACAAGTTTGATCTTTG-MGB-BHQ. 5' Taq nuclease assay for *T. foetus* was conducted in a 25 μl volume, using either Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) or RealMasterMix probe mix (Eppendorf), with 900 nM TFF2 and TFR2 primers, and 80 nM TRICHP2 fluorescent 3’ minor groove binder-DNA probe in a Corbett Rotor-Gene RG-3000 (Corbett Research, Australia). The thermal profile was 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 45 s. Fluorescence acquisition occurred at the end of each annealing/extension cycle. A positive result was indicated by the fluorescence (normalised to a no-template control) passing a threshold of 0.1. Assay conditions were optimized using serial dilutions of *T. foetus* purified DNA. Quantitative estimates of target cells/ml were calculated using a standard curve generated with either gDNA or crude cell extracts prepared from serial dilutions of known numbers of
2.8. Sensitivity evaluation and effects of transport

*T. foetus* strain YVL-W cells were counted using a hemocytometer and inoculated into smegma, mucus and urine collected from healthy animals which previously tested negative by both selective culture/microscopy and 5′ *Taq* nuclease assay. Serial log dilutions started at 10^5 cells/ml and were diluted to 1 cell/ml. Genomic DNA was extracted from the spiked specimen using a commercial DNA extraction kit (QIAamp DNA minikit, Qiagen), and assayed by both conventional PCR assay and 5′ *Taq* nuclease assay. Inoculated specimens were also prepared for 5′ *Taq* nuclease assay by heat lysis. InPouch™ TF test kits were inoculated with laboratory-spiked specimens, as for diagnostic culture. DNA was also extracted from aliquots of inoculated InPouch™-TF media using a commercial kit (QIAamp, Qiagen) and assayed by both 5′ *Taq* nuclease assay and conventional PCR assay. Estimates of cell equivalents/assay or cells/inoculum were calculated from the enumerated spiked specimen by determining equivalent cell numbers contained in the final volume used as PCR assay template or for the culture inoculum.

5′ *Taq* nuclease assays were scored positive if the fluorescence (normalised against a no-template control) passed a threshold of 0.1. A positive conventional PCR assay required detection of a 347 bp amplicon. Cultured samples were scored positive on the presence of motile trichomonads with multiple anterior flagella, a posterior flagellum, a visible undulating membrane and characteristic jerky motility upon microscopic examination.

Sample transport was simulated by storing the inoculated InPouch™ TF media and animal samples for up to 5 days at ambient temperatures for smegma or mucus, and at 4 °C for urine. Samples for 5′ *Taq* nuclease assays, conventional PCR assays and selective culture were processed as described above at time 0, and after 2 and 5 days of storage. To examine changes in cell numbers under these storage conditions, quantified cell estimates were compared using 5′ *Taq* nuclease assay results of the 10^4 cells/ml spiked samples.

2.9. Field diagnostic comparison

Smegma and mucus samples were collected from 129 bulls and 30 cows as described above, from herds in north eastern Australia with confirmed culture/microscopy positive animals. Urine was also collected randomly from 71 animals (48 bulls, 23 cows) as described above, to confirm the usefulness of urine for *T. foetus* screening. Specimens were assayed by both *T. foetus* culture/microscopy and the 5′ *Taq* nuclease assay as described above. Fisher’s exact test (Stein and Torrie, 1980) was used to determine the statistical significance of the results obtained with the traditional culture/microscopy methods and the *Taq* nuclease assay.

3. Results

3.1. Assay specificity and sensitivity

The sequence of the species-specific amplicon from *T. foetus* strain YVL-W was 100% homologous with the following GenBank ITS accessions: *T. foetus* (AF339736), *T. suis* (U85966), and *T. mobilensis* (U86612). 5′ *Taq* nuclease assay of genomic DNA from a range of related organisms and bovine venereal microflora (Table 1) did not produce any non-specific amplification.

The sensitivity of the assay using genomic DNA preparations of *T. foetus* as template was consistent at 3 fg DNA and *C*_T values ≥40 are negative (Fig. 1). Cell equivalents were compared to standard curves derived from crude cell lysates of serial dilutions of *T. foetus* cells (Fig. 1). Inter-assay variability was evaluated through eight assays of *T. foetus* genomic DNA samples, with a standard deviation of 1.21 cycles between the *C*_T values obtained for a given sample. Both *T. foetus* DNA and the *T. foetus* cell lyse templates were used as assay positive controls to compensate for any variation in *C*_T values between real time PCR experiments and maintain assay integrity. The 5′ *Taq* nuclease assay detected a single cell equivalent per assay from laboratory-spiked preputial smegma, vaginal mucus and urine (Table 2), with less than a cell equivalent per assay being reliably detected from several heat-lysed specimens.

When techniques used for preparing clinical samples for 5′ *Taq* nuclease assay were compared, processing the samples using the heat lysis method provided as good or better sensitivity than the QIaamp DNA minikit (Table 2). A number of the samples collected from healthy animals contained visible contamination with feces, semen and blood. Microscopic examination revealed high levels of bacteria, semen, blood, pus, epithelial cells and plant material in many genital specimens. The sensitivity limits and quantitative estimates observed using the 5′ *Taq* nuclease assay...
Fig. 1. Cycle threshold (Cₜ) values demonstrating the sensitivity of the *Tritrichomonas foetus*-specific 5' *Taq* nuclease assay for *T. foetus* genomic DNA and crude cell lysates of serially diluted cells. (a) Sensitivity of detection for genomic DNA. Trendline: $R^2$ value = 0.9759; $y = 0.0005 e^{-0.6897x}$. (b) Sensitivity of detection for crude cell lysates. Trendline: $R^2$ value = 0.9863; $y = 4E + 13 e^{-0.7899x}$.

Table 2
Detection limits for *T. foetus* from laboratory-spiked stored smegma, mucus and urine using microscopy following selective culture, conventional PCR assay (Felleisen et al., 1998) and 5' *Taq* nuclease assay

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Storage time (days)</th>
<th>Selective culture/microscopy (cells/inoculum)</th>
<th>Assay post-enrichment culture</th>
<th>Conventional PCR</th>
<th>5' <em>Taq</em> nuclease assay</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td>5' <em>Taq</em> nuclease assay</td>
<td>Conventional PCR</td>
<td>QIAamp</td>
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<td></td>
<td></td>
<td></td>
<td>assay</td>
<td>QIAamp</td>
<td>Heat lysis</td>
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<tr>
<td>Smegma</td>
<td>0</td>
<td>&gt;25000</td>
<td>50</td>
<td>1</td>
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<tr>
<td></td>
<td>5</td>
<td>&gt;25000</td>
<td>50</td>
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<td>1</td>
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<tr>
<td>Mucus</td>
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<td></td>
<td>5</td>
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<td>ND</td>
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</table>

a Smegma and mucus samples for 5' *Taq* nuclease assay were stored at ambient temperatures in physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in InPouch™ TF test kits at 37°C.

b Sensitivity limits for both conventional PCR and 5' *Taq* nuclease assay are given in cell equivalents/assay. Selective culture/microscopy sensitivity limits are indicated as the minimum number of cells/inoculum of InPouch™ TF media required for motile *Tritrichomonas*-like protozoans to be visible on subsequent microscopic examination.

c DNA was extracted from InPouch™ TF media for 5' *Taq* nuclease and conventional PCR assay using a commercial kit (QIAamp).

d Positive assay results were possible for the log dilutions below this concentration, but were not reliably obtained.

e Not done.
did not change in the presence of these potentially inhibitory substances. These same crude heat lysis extracts did not amplify reliably in conventional PCR which thus required kit purified DNA as reliable PCR template (data not shown). Assay of selectively cultured specimens by conventional PCR detected as few as 500 cells inoculated into the InPouch™ TF test kit, substantially improving upon the sensitivity of culture/microscopy.

Culture/microscopy-based detection was markedly less sensitive than PCR, giving positive identification of motile trichomonads following inoculation with a minimum of 2500 cells. Spiking experiments demonstrated that smegma and mucus required the inoculation of high cell numbers to obtain a positive identification by culture/microscopy compared to that observed for urine (Table 2).

### 3.2. Sample storage and transport

Several trends were observed throughout the course of storage. Selective culture/microscopy-based diagnosis of *T. foetus* was most sensitive after 2 days, and several InPouch™ test kits with visible motile trichomonads after 2 days, which were subsequently not visible after 5 days (Table 2). Estimates of the numbers of *T. foetus* cells present in each sample using the 5′ *Taq* nuclease assay were generally lower with longer storage/transport up to 5 days (Table 3), but detection limits did not change (Table 2).

Following enrichment in InPouch™ TF test kits, both the 5′ *Taq* nuclease assay and conventional PCR assay improved upon the detection sensitivity of selective culture/microscopy, although sensitivity was markedly reduced when compared to 5′ *Taq* nuclease assay or conventional PCR assay without enrichment culture (Table 2).

### 3.3. Field diagnostic comparison

Of the 159 samples tested from animals from culture positive herds (129 female, 30 male), 14 samples (13 smegma and 1 mucus) were positive in the 5′ *Taq* nuclease assay with 3 of the smegma samples confirmed positive by microscopic examination of the InPouch™ TF test kits. For 1 herd group of 20 animals, the selective culture/microscopy was performed in an accredited veterinary diagnostic laboratory during routine herd management. Quantitation of *T. foetus* density in the clinical specimens against clinical samples spiked with directly enumerated *T. foetus* was performed by 5′ *Taq* nuclease assay, although the data was unable to be related to numbers of parasites present in the host due to variability in both the volume of genital mucus collected and the site of collection within the genital tract. All 145 negative samples were negative by both 5′ *Taq* nuclease assay and culture/microscopy. The Fisher’s exact test indicated statistically significant results (*P* < 0.001) confirming higher sensitivity of the 5′ *Taq* nuclease assay compared with culture/microscopy. Incomplete urine sampling was undertaken and no correlating urine samples from smegma and mucus *T. foetus* positive animals were collected. All other urine samples collected tested

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Storage time (days)</th>
<th>5′ <em>Taq</em> nuclease assay post-enrichment culture</th>
<th>5′ <em>Taq</em> nuclease assay</th>
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<td></td>
<td>5</td>
<td>148</td>
<td>6994</td>
</tr>
<tr>
<td>Urine</td>
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<td>ND</td>
<td>3343</td>
</tr>
<tr>
<td></td>
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<td>ND</td>
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</tr>
<tr>
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<td>7957</td>
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</table>

a Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4 °C. Samples for culture were stored in InPouch™ TF media at 37 °C.

b DNA was extracted from InPouch™ TF media for 5′ *Taq* nuclease assay via a commercial kit (QIAamp).

c Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml.

d Not done.
negative in both 5’ Taq nuclease assay and culture/microscopy.

4. Discussion

The ‘gold standard’ diagnostic test for trichomoniasis in either male or female cattle is the cultivation of live organisms from smegma or cervicovaginal mucus until parasites propagate to detectable numbers to allow the recognition of T. foetus organisms microscopically (Clark et al., 1971; Reece et al., 1983). Traditional culture/microscopy diagnosis is more effective for smegma (81–91%) than mucus (58–75%) and PCR methods have been developed to differentiate T. foetus from non-pathogenic trichomonads (Bryan et al., 1999; Campero et al., 2003; Clark et al., 1971; Felleisen et al., 1998; Fitzgerald et al., 1954; Lun et al., 2000; Parker et al., 1999; Reece et al., 1983; Skirrow, 1987; Skirrow et al., 1985). Increased specificity and sensitivity for the detection of T. foetus infection in cattle will improve both the accuracy of prevalence studies and the understanding of disease epidemiology, thereby improving disease management practises (Felleisen, 1997; Levy et al., 2003; Rae, 1989). Real time PCR-based techniques have been successfully used for the clinical diagnosis of a wide range of pathogens from a variety of sources, including Campylobacter jejuni from human stools (Iijima et al., 2004; Nogva et al., 2000) and T. vaginalis from female urine and genital secretions and male urine (Hardick et al., 2003; Jordan et al., 2001). These assays provide greater sensitivity and specificity compared to selective culture and direct microscopic examination, and diagnoses can be obtained in significantly less time. As there is no need for post-amplification processing, real time PCR techniques improve the sensitivity and specificity of conventional-PCR assays and are also less labour intensive (Wilhelm and Pingoud, 2003). This is the first report of a novel real time probe-based PCR assay for the specific and sensitive detection of T. foetus directly from clinical samples.

The 5’ Taq nuclease assay for T. foetus described here is based on the ITS-1 region shown previously to be conserved and reliable as a target for conventional PCR (Cobo et al., 2003; Felleisen et al., 1998; Hoevers et al., 2003; Parker et al., 2003). Previous reports using the Felleisen et al. (1998) T. foetus PCR assay have described the amplification of non-specific fragments (BonDurant et al., 2003); the ITS-1 based 5’ Taq nuclease assay described here proved specific and robust with no non-specific detection. The in silico matches of the primers and probe described here with ITS-1 sequences of T. suis and T. mobilensis are consistent with current phylogenetic understanding of the Tritrichomonadinae subfamily which indicate that these organisms belong to the same species (Felleisen, 1997; Kleina et al., 2004; Tachezy et al., 2002). The Felleisen et al. (1998) assay based on ITS-1 has also been adapted to detect T. foetus in cat feces (Gookin et al., 2002), further demonstrating the specificity of this target to detect this species from a range of clinical sources.

Sensitivity of detection was markedly improved compared to culture and microscopy, with the reliable detection of a single organism equivalent per assay from smegma, cervico-vaginal mucus or urine. Our observations concluded that T. foetus loses viability in culture, due to the absence of visible motile trichomonads in InPouch™ TF test kits within a few hours of inoculation. The reason for this death is unclear, although isolate-specific antimicrobial susceptibility may play a significant role. The low sensitivity of microscopic examination following selective culture indicates that this is not the most effective diagnostic tool for T. foetus infection, leading to false negative results. For example, a sheath scrape from an infected bull is reported to contain an average of 141 organisms/ml (Mukhufhi et al., 2003), which is well below the sensitivity limits of selective culture/microscopy. In addition, the presence of non-T. foetus trichomonads cultured from the preputial mucosa of bulls can confound positive identification of T. foetus (Hayes et al., 2003). The poor sensitivity of microscopy following selective culture was confirmed in our field study where only 3 of the 14 infected animals that were detected by 5’ Taq nuclease assay detected as positive by culture/microscopy. This study clearly demonstrates that PCR direct detection from clinical specimens is more sensitive compared with PCR following culture enrichment.

Using purified DNA samples prepared directly from spiked clinical specimens, we demonstrated equal sensitivity of the 5’ Taq nuclease with the conventional Felleisen et al. ITS-1 PCR (1998). The 5’ Taq nuclease assay provided a higher level of sensitivity when using crude heat lysis extracts as template, detecting less than a single cell equivalent compared to a single cell equivalent when purified DNA prepared directly from the clinical sample was used. It is likely that this is due to the potential of 12 copies of ITS1 target per T. foetus genome (Chakrabarti et al., 1992). However, the conventional PCR assay was not amenable to using crude heat lysis extracts as template, limiting its sensitivity to 1 cell equivalent per assay. The detection limit of less than one cell equivalent/assay when assaying heat lysed clinical
specimens by the 5' Taq nuclease assay is approximately 10-fold that observed when assaying purified DNA using the conventional PCR assay. Assay sensitivity has been reported to decline when assaying DNA extracted directly from clinical specimens compared to DNA extracted from cultured specimens (Chen and Li, 2001; Felleisen et al., 1998). This was probably due to the presence of a range of inhibitory substances present in the clinical samples that were not removed during DNA isolation (Al-Soud and Radstrom, 2001; Felleisen et al., 1998; Inglis and Kalischuk, 2003). It is not known if new assays such as that described by Grahn et al. (2005) provide equivalent sensitivity to the assay described here as cell equivalents were not reported. However, based on concentrations of genomic DNA tested, the assay described here demonstrated higher sensitivity (0.003 pg/assay) compared with the detection limit described by Grahn et al. (2005) at 0.1 pg/assay.

5' Taq nuclease assays such as the one described here allow accurate estimation of the number of copies of the target DNA in the clinical sample if performed with appropriate quantitative standards. Such standards were used in this study to estimate T. foetus numbers within the clinical samples, but these estimates were unable to be related to the density of T. foetus in the infected animal due to variability in the volume and physical localisation of genital mucus collected. The development of a rigorously standardised methodology for the collection of genital mucus, allowing collection of a specified volume of mucus from an area that accurately reflects variations in the T. foetus population of infected animals, will allow the assay described in this study to be used to estimate the levels of T. foetus infecting a host.

5' Taq nuclease assays do not require viable organisms for detection, and are capable of detecting target sequences despite high levels of non-target organisms or cells. As viable cells are not needed, transport from the field to the laboratory becomes greatly simplified, without the need for complex transport enrichment media. The presence of a high proportion of contaminating organisms or host cells in a specimen did not provide any impediment to an accurate diagnosis in this study. In addition, the use of a bull rasper has been found to simplify the collection of genital specimens from both male and female cattle (Tedesco et al., 1977), and is expected to provide a suitable specimen for either selective culture/microscopy or 5' Taq nuclease assay as demonstrated in a related C. fetus subsp. venerealis study (McMillen et al., 2006). Urine has been used as a specimen for the diagnosis of human trichomoniasis (Schwebke and Lawing, 2002) and was thus screened here to determine whether T. foetus could be detected in urine submitted to diagnostic laboratories for the detection of other pathogens, i.e. Leptospira spp. Although feasible in vitro, the field sampling in this study was inconclusive to confirm whether urine can be used as diagnostic specimen for T. foetus detection. A similar study describing real time PCR to target C. fetus subsp. venerealis found that urine was not a suitable diagnostic specimen for the diagnosis of the related venereal disease campylobacteriosis (McMillen et al., 2006). As female cattle mostly clear T. foetus within 3 months and bulls can carry the organism for >3 years, our study supports the recommendation to screen smegma from bulls as the most reliable diagnostic sample for the detection of T. foetus within a herd (BonDurant, 2005).

5. Conclusion

The 5' Taq nuclease assay described here is a reliable, sensitive and specific detection method for T. foetus in bovine venereal diagnostic specimens. This assay reliably detected 1 cell equivalent per assay, and was able to readily discriminate between the target organism and other venereally localised trichomonads. Specimen collection from male and female cattle using a bull rasper was found to be simple and efficient, and specimens suspended in physiological saline proved to be stable during transport at ambient temperatures. Diagnostic specimens could be processed by a simple and rapid heat lysis technique with no loss of sensitivity. Marked improvements were observed in both sensitivity and specificity over those obtained with selective culture-based and conventional PCR techniques. Thus real time PCR technologies offer many advantages as a diagnostic tool in clinical and veterinary diagnostic laboratories (Mackay, 2004) and T. foetus detection could be determined simultaneously with the detection of C. fetus subsp. venerealis (BonDurant, 2005; McMillen et al., 2006). Stringent controls (DNA and cell lysates) were used here, however it will be essential to standardise inter-laboratory internal positive and negative control protocols for the monitoring of assay integrity. A multi-centre evaluation of the specimen collection, transport, processing and assay procedures should prove valuable as well as the establishment of certified laboratories to undertake this testing.

Acknowledgments

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