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Not gone but forgotten: *Tritrichomonas foetus* in extensively-managed bulls from Australia's Northern Territory

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ABSTRACT

Bovine trichomonosis, caused by infection with the protozoan parasite *Tritrichomonas foetus*, is globally recognised as a cause of reproductive failure in cattle. Maintained in clinically normal bulls, *T. foetus* infection results in infertility and abortion in infected cows. In Australia's Northern Territory (NT), logistical limitations associated with extensive livestock production inhibit wide-scale testing and diagnosis, allowing the parasite to persist undetected. In the present study, *T. foetus* was detected in 18/109 preputial cultures collected from bulls on a property in the NT with a history of low birth rates and reproductive failure using real-time PCR testing. Of the *T. foetus*-positive samples, 13/18 were genotyped using the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rDNA unit. Selected samples were further characterised using the protein-coding genes of cysteine proteases (CP-1, 2, 4–9) and cytosolic malate dehydrogenase 1 (MDH-1) to determine if the isolates were 'bovine', 'feline' or 'Southern Africa' genotypes. All samples were 100% identical to the *T. foetus* 'bovine' genotype across all markers. This is the first reported case of trichomonosis in Australian cattle since 1988 and is a reminder that *T. foetus* should be considered whenever reproductive failure occurs in extensive cattle systems.

1. Introduction

The main bacterial, viral and parasitic causes of reproductive failure in cattle in Australia are *Campylobacter fetus venerealis*, Bovine Viral Diarrhoea Virus, *Leptospira* sp. and *Neospora caninum*, all of which are difficult to manage once present in a herd (Christensen & Clark, 1979; McCool et al., 1988; Collantes-Fernández et al., 2014; Lilenbaum & Martins, 2014; Reichel et al., 2018; Oyhenart, 2019). Reproductive failure presents a particularly difficult problem in extensively managed cattle because a reliance on natural mating perpetuates disease transmission and any associated reproduction losses may go undetected, making disease diagnosis challenging (Buller & Corney, 2013).

Bovine trichomonosis, caused by infection with the protozoan parasite *Tritrichomonas foetus*, is a venereal disease that is isolated from clinically normal bulls but causes infertility and abortion in infected cows at any time during gestation (usually during early gestation) due to a transient vaginitis (Buller & Corney, 2013; Collantes-Fernández et al., 2014; Yao, 2015). In most areas of Australia bovine trichomonosis is rarely diagnosed due to a predominance of intensive production systems, seminal testing for the parasite prior to artificial insemination, and culling of infected bulls (Ondrak, 2016). The last published report of *T. foetus* infection in Australia occurred in 1988 in bulls from the Northern Territory (NT) where extensive cattle systems are still common (McCool et al., 1988; Buller & Corney, 2013). Considering the difficulties associated with conducting routine diagnostics in remote areas such as the NT, the lack of reports of *T. foetus* infection in Australian cattle herds for over 30 years, however, should not be interpreted as absence of the organism (Hancock et al., 2015; Mueller et al., 2015; Reichel et al., 2018; Rush et al., 2019).

There are three distinct genotypes recognised within *T. foetus* (Casteriano et al., 2016): (i) the 'bovine' genotype, which is found in the urogenital tract of cattle and causes reproductive failure and is also found as a commensal organism in the gastrointestinal tract of pigs in Europe, the Americas and Australia; (ii) the 'feline' genotype, which is globally distributed and causes large bowel diarrhoea in cats; and (iii) the 'Southern Africa' genotype, which was discovered in the urogenital tract of cattle in Namibia, Africa (Šlapeta et al., 2012; Mueller et al., 2015; Casteriano et al., 2016). The distribution of the Southern Africa *T. foetus*

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genotype in other countries or continents is currently unknown (Casteriano et al., 2016). These three *T. foetus* genotypes can be differentiated with molecular testing by targeting the internal transcribed spacer (ITS) regions 1 and 2 and the 5.8S rDNA unit, the cysteine protease 2 (CP-2) gene, or a multilocus typing approach (Šlapeta et al., 2012; Casteriano et al., 2016).

The aim of this study was to characterise *T. foetus* as a cause of reproductive failure in a herd of extensively managed bulls from Australia's Northern Territory. To do so we used a combination of traditional culture and comprehensive molecular tools to identify *T. foetus* and establish reference strains.

2. Methods

2.1. Sample population

One hundred and nine bulls were sampled for *T. foetus* testing by a private veterinarian following identification of reproductive failure on an extensively managed farm. The term 'reproductive failure' was used in response to a lower than expected branding rate of approximately 50% (where branding rate equals calves branded as a percentage of cows mated) in a large herd of naturally mated heifers, despite approximately one-fifth of the bulls being culled prior to breeding due to poor conformation and low semen quality. All bulls sampled were between 2 and 8 years of age with an average age of 5 years. Some animals had been purchased and transported from a property in the neighbouring state of Queensland at least 12 months previously. The remaining bulls came from the on-farm breeding programme.

2.2. Sample collection

Preputial smegma was collected in October 2020 from 109 bulls (arbitrarily assigned #1–#109) using a Tricamper™ tool (60 cm long polyethylene tube with a corrugated scraper head; Biosecurity Sciences Laboratory, Department of Agriculture and Fisheries, Queensland), according to the collection procedure recommended by Elizabeth Macarthur Agricultural Institute (EMAI, Department of Primary Industries and Environment, NSW). Briefly, whilst the anterior aspect of the preputial sheath was held, the Tricamper™ tool was inserted into the prepuce, with the scraper head adjacent to the penis. The Tricamper™ tool was then moved back and forth in order to scrape across the preputial mucosa and surface of the penis. Holding a finger over the hole in the end of the Tricamper™ tool, in order to retain any preputial fluid that had been sucked into the hollow inside of the tool by capillary action, the tool was then removed from the prepuce. The scraper head was then inserted into a 5 ml container of phosphate-buffered saline (PBS). The head of the Tricamper™ tool was cut off, the lid of the PBS container replaced, and the container shaken vigorously to rinse the preputial smegma off the Tricamper™ head. After a few minutes, when the smegma had settled, approximately 1 ml of supernatant from the smegma solution was used to inoculate 4 ml of Oxoid *T. foetus* enrichment media (TFEM, Department of Primary Industries, Western Australia) with added inactivated horse serum (80 ml/l adjusted to pH 6.0) and chloramphenicol (100 mg/l). The TFEM had been transported frozen from EMAI to the submitter in the week prior to sampling and was allowed to thaw to room temperature before inoculation.

2.3. Sample testing for Tritrichomonas foetus by culture and microscopy

Samples were transported at ambient temperature to EMAI for diagnostic testing. The ambient temperature the samples were subjected to during this time is unknown. Upon arrival at EMAI, approximately 72 h after collection, the samples were incubated at 37 °C and inspected on days 4 and 7 post-arrival for the presence of motile protozoa using an inverted light microscope (Zeiss Telaval 31) at 50× magnification. Individual samples were considered suspect positive if trichomonad organisms were observed with microscopy. Smears were prepared from a drop (25 μl) of each sample and allowed to air-dry before inactivation with iodine and staining with Diff-Quik to confirm trichomonad morphology (Lun & Gajadhar, 1999).

2.4. DNA isolation

DNA was extracted from all 109 TFEM samples using a MagMax Core Nucleic Acid Purification Kit (A32702; Thermo Fisher, Waltham, MA, USA) on a Kingfisher Flex semi-automated magnetic purification system (Thermo Fisher), according to the manufacturer's instructions. DNA was extracted from 300 μl of each sample, aspirated from the base of the culture bottles, and the extracted DNA was eluted into a final volume of 90 μl. To monitor for the presence of PCR inhibition, 2 μl of an internal extraction control (VetMax™ Xeno™ Control DNA; 10,000 copies/μl; Thermo Fisher) was added to each sample. All samples were isolated in conjunction with a negative extraction control.

2.5. Molecular testing for Tritrichomonas foetus

An in-house multiplex real-time TaqMan™ PCR assay, based on McMillen et al. (2006), was used to screen all samples at EMAI for the presence of *T. foetus* DNA using an Applied Biosystems Quant Studio 5 Real-Time PCR system. Multiplexed primer and probe combinations for the 5' *Taq* nuclease assay using fluorescent 3' minor groove binder (MGB) DNA probes were TFF2: GCGGCTGGATTAGCTTTCTTT (900 nM); TFR2: GGCGCGCAATGTGCAT (900 nM); TRICHP2: 6-FAM-ACAAGTTC GATCTTTG-MGB-BHQ (80 nM) and VetMax Xeno™ primer probe mix (0.8 μl) (Thermo Fisher). The assay was conducted using 5 μl sample DNA in a final volume of 20 μl with TaqMan™ Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA). Cycling conditions were as follows: 1 × 95 °C for 10 min; followed by 45 × 95 °C, 15 s and 60 °C, 60 s. Results were analysed at a threshold of 0.1 ΔRn. Samples with a cycle threshold (Ct) value below 40 were considered positive. The assay was repeated using a 1 in 10 dilution of sample DNA in PBS when the internal control indicated PCR inhibition (Xeno™ Control DNA Ct-value > 34), irrespective of the Ct-value of the sample for *T. foetus* DNA. If PCR inhibition was still present on the second run (i.e. Xeno™ Control DNA Ct-value > 34), DNA was re-isolated using a 1 in 2 dilution of original culture with PBS and the PCR assay repeated (Table 1).

2.6. Multilocus Tritrichomonas foetus genotyping

To genotype all suspect positive samples, the ITS regions (ITS1 and ITS2) and the 5.8S rRNA gene were amplified using TFR3 and TFR4 primers in a real-time PCR assay at Sydney School of Veterinary Science (SSVS), The University of Sydney, as previously described (Mueller et al., 2015; Casteriano et al., 2016). A subset of positive samples was further characterised using the protein coding genes for cysteine proteases (CP-1, 2, 4–9) and cytosolic malate dehydrogenase 1 (MDH-1), which include the most divergent markers between the three primary genotypes ('bovine', 'feline' and 'Southern Africa'), *via* conventional PCR (Šlapeta et al., 2012). PCR reactions were run using MyTaq Red Mix (Bioline, Australia) and *Sso*Advanced SYBR Green Supermix (Bio-Rad, Australia) for all protein coding markers and ITS regions, respectively, as previously described (Šlapeta et al., 2012; Casteriano et al., 2016). All reactions included 2 μl of template DNA and ddH_2O as the no-template control. All samples that produced unambiguous bands of the expected size or melt curves consistent with a *T. foetus* product were sent for direct Sanger sequencing using their respective amplification primers by Macrogen Ltd (Seoul, South Korea). Sequencing results were assembled, aligned and compared to reference *T. foetus* sequences representing the 'bovine', 'feline' and 'Southern Africa' genotypes (Šlapeta et al., 2012; Casteriano et al., 2016) using CLC Main Workbench ver. 6.8.1 (CLC Bio, Denmark).

Table 1
Summary of *Tritrichomonas foetus* microscopy and real-time PCR results from the present study

Sample ID	Overall call	Microscopy	TaqMan™ real-time PCR Ct-values	XENO™ real-time PCR Ct- values	TFR3/4 real-time PCR Ct-values	ITS1-5.8S-ITS2 sequence
#14	Suspect positive	Negative	34.26	33.06	na	–
#34	Positive	Negative	33.34	29.86	32.87/32.48	MW322811
#37	Positive	Positive	16.7	31.64	17.26/14.25	MW322812
#39	Positive	Negative	27.73	29.82	26.36/26.74	MW322813
#44	Positive	Positive	16.68/20.96[a]	35.79/34.00[a]	14.49/14.51	MW322814
#49	Positive	Positive	17.59	33.79	19.30/16.61	MW322815
#50	Suspect positive	Negative	38.31	29.46	na	–
#60	Positive	Positive	18.27/22.39[a]	36.65/32.68[a]	15.41/16.08	MW322816
#65	Positive	Positive	19.58	33.42	16.43/20.49	MW322817
#68	Suspect positive	Negative	38.52	32.81	na	–
#70	Positive	Negative	31.05/35.74[a]	35.91/32.87[a]	30.91/33.33	MW322818
#72	Positive	Positive	19.50/24.37[a]	34.36/32.75[a]	17.18/18.23	MW322819
#77	Positive	Positive	21.09	31.80	13.81/19.20	MW322820
#83	Positive	Positive	18.03	33.49	16.24/19.22	MW322821
#88	Suspect positive	Negative	33.23/39.98[a]	34.69/32.52[a]	34.20/na	–
#91	Positive	Positive	17.00	31.01	26.59/16.74	MW322822
#100	Positive	Positive	18.98	29.52	17.96/18.22	MW322823
#103	Suspect positive	Negative	37.35	29.92	na	–

Notes: Samples were considered positive for *T. foetus* if they yielded a positive diagnostic qPCR result and could be identified to the species level by Sanger sequencing of the internal transcribed spacer (ITS) 1 and 2 regions. Samples unsuitable for sequencing were termed 'suspect positive'.
Abbreviations: na, samples where no amplification occurred; Ct, cycle threshold.
[a] Samples that were re-run at a 1 in 10 template dilution due to PCR inhibition, i.e. late amplification of Xeno™ (Ct-value > 34).

2.7. Case definition for Tritrichomonas foetus positivity

Samples were considered positive for *T. foetus* if they yielded a positive *T. foetus* TaqMan™ real-time PCR result (*T. foetus* Ct-value < 40 and Xeno Control DNA Ct-value ≤ 34) and could be identified to the species level by Sanger sequencing of the ITS regions. Samples were considered suspect positive for *T. foetus* where they could not be identified by Sanger sequencing but still yielded a positive *T. foetus* TaqMan™ real-time PCR result (Table 1).

2.8. Animal ethics approval

Animal ethics approval was not required since only routine diagnostic samples, submitted as part of a work up for reproductive failure by an independent veterinarian, were utilised.

3. Results

3.1. Diagnostic testing by microscopy and PCR

Of the 109 samples submitted for *T. foetus* diagnosis, 10 (9.2%) were positive by microscopy and 18 (16.5%) were positive according to the *T. foetus* TaqMan™ real-time PCR assay. All ten microscopy-positive samples were PCR-positive (Table 1). Stained smears from selected cultures revealed morphology consistent with *T. foetus* (Fig. 1). PCR inhibition was eliminated in all samples *via* dilution and/or re-extraction, and negative extraction and no-template PCR controls remained negative, ruling out contamination (Table 1).

3.2. Molecular characterisation for Tritrichomonas foetus genotyping

Of the 18 samples identified as suspect positive according to the *T. foetus* TaqMan™ real-time PCR, DNA sequencing of the ITS1 and ITS2 products (using TFR3/TFR4 primers) to confirm and characterise the isolates was successful in 72% (13/18), including all ten microscopy-positive samples. Sequencing results from all 13 samples (#34, #37, #39, #44, #49, #60, #65, #70, #72, #77, #82, #91, #100) matched the 'bovine' genotype of *T. foetus* (JX187006) with C (cytosine) at the diagnostic residue in ITS-2 rDNA when compared to the 'feline' genotype, as well as the absence of the AA insertion in ITS1 rDNA when compared to the 'Southern Africa' genotype (Šlapeta et al., 2012; Casteriano et al., 2016). The remaining samples (5/18) failed to amplify at the ITS regions, all of which were late amplifiers according to the *T. foetus* TaqMan™ real-time PCR assay (Ct-value > 33.23) (Table 1).

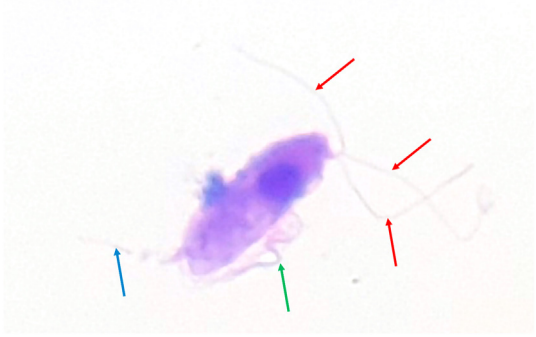

Fig. 1 *Tritrichomonas foetus* trophozoite isolated from a bull in Australia's Northern Territory, demonstrating the organism's pear-shape, the presence of three anterior flagella (red arrows), one posterior flagellum (blue arrow) and the undulating membrane (green arrow). Stained with iodine and Diff-Quik under 1000× magnification.

To further characterise the *T. foetus* isolates, five samples (#37, #49, #82, #91, #100) were used for PCR amplification of the CP-2 locus, which is recognised as the most divergent marker between the known 'feline', 'bovine' and 'Southern Africa' genotypes (Šlapeta et al., 2012; Casteriano et al., 2016). All five 669 nucleotide CP-2 sequences from this study were identical to each other and to the 'bovine' genotype of *T. foetus* (JX187033). The CP-2 sequences matched the 'bovine' genotype of *T. foetus*, but were distinct from the cattle 'Southern Africa' genotype and the 'feline' genotype as well as the CP-2 genotype from its closest relative, *Tritrichomonas mobilensis* (LC054291) from the black-capped squirrel monkey (*Saimiri boliviensis*) (Fig. 2).

Two isolated cultures (#49 and #91) were fully characterised using 4,552 bp across all 10 markers including CP1-2, CP4-9, MDH-1 and ITS. Both isolates were 100% identical to each other and 100% identical to a previously characterised strain, YVL-W from Queensland, Australia (Šlapeta et al., 2012). The cultured *T. foetus* represents the 'bovine' genotype, which is distinct from both the 'Southern Africa' genotype *sensu* Casteriano et al. (2016) and 'feline' genotype *sensu* Šlapeta et al. (2012).

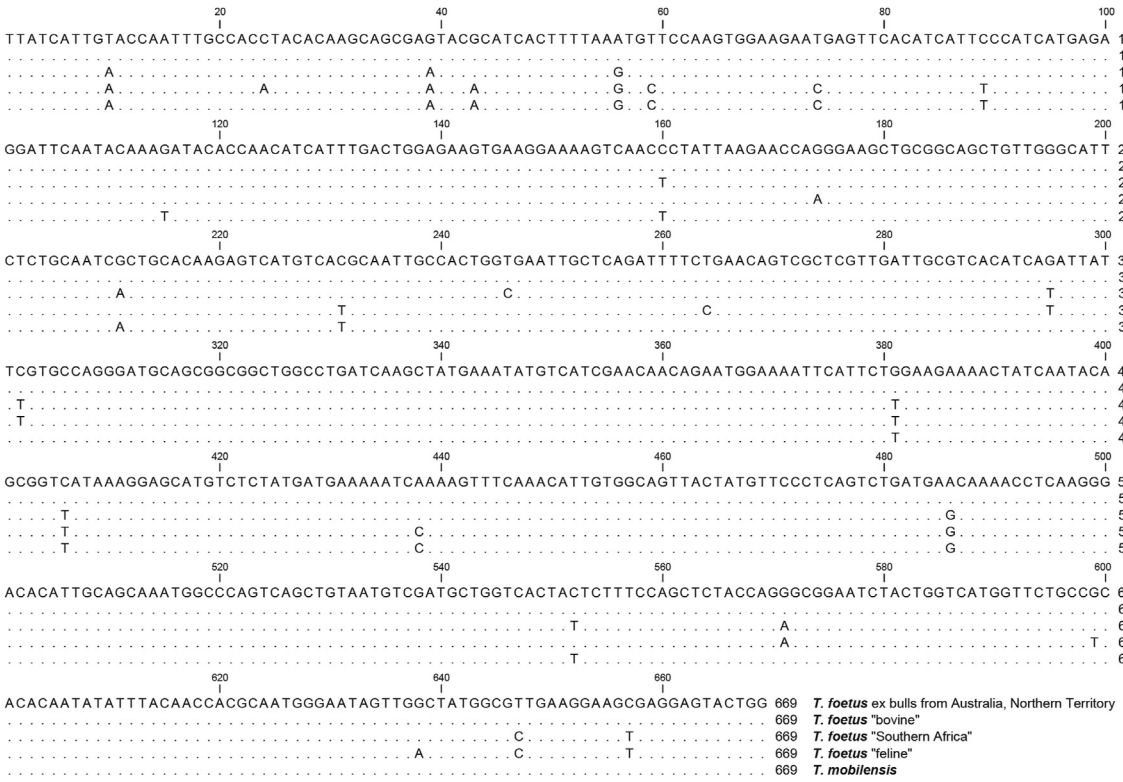

Fig. 2 Nucleotide differences between *Tritrichomonas foetus* isolated from bulls from Northern Territory, Australia at cysteine protease 2 (CP-2). Multiple sequence alignment of three known *T. foetus* genotypes, i.e. 'bovine' (JX187033), 'feline' (JX187027) and 'Southern Africa' (KX425901), and its closest relative, *T. mobilensis* (LC054291), are shown. CP-2 is the most divergent known marker differentiating these genotypes and *T. foetus* from *T. mobilensis*. Identical residues are indicated by dots.

3.3. Long-term storage of isolates

The two fully characterised isolates (#49 and #91) were axenised at SSVS using Modified Diamonds Medium (MDM; ATCC medium 719) with 5% (v/v) deactivated foetal bovine serum (FBS) and 0.1% (w/v) agar and fortified with antibiotics (1×10^6 U/l penicillin, 15 g/l streptomycin and 100 mg/l chloramphenicol). Cultures were maintained in MDM with 5% (v/v) FBS and 1 × Antibiotic-Antimycotic (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml Fungizone™; Gibco). Following axenisation, both *T. foetus* isolates sustained growth without the presence of antibiotics and were successfully transferred back to Oxoid TFEM. Both isolates were then frozen in liquid nitrogen in 5% (v/v) DMSO at EMAI for long-term storage.

4. Discussion

This study identified the *T. foetus* 'bovine' genotype as the likely aetiological agent causing reproductive failure in a herd of extensively managed cattle in the NT, Australia, providing important reference strains for future research. Bovine trichomonosis, as a result of infection with *T. foetus*, has been mostly eradicated in Australia due to the use of artificial insemination and culling of infected bulls (Buller & Corney, 2013). It has long been suspected, however, that the extensive production practices routinely employed in the remote areas of the northern states of Australia have facilitated persistence of the parasite. Between 2015 and 2020 there were only nine diagnostic submissions to the NT veterinary diagnostic laboratory consisting of 21 and 108 samples from buffalo and cattle, respectively, with all samples testing negative for *T. foetus* (Suresh Benedict, Senior Bacteriologist, Northern Territory Department of Primary Industries, personal communication). These results are unlikely to reflect the true prevalence of bovine trichomonosis in cattle due to the limited number of samples submitted. The present study, therefore, represents the first published report of the *T. foetus* 'bovine' genotype in Australian cattle since 1988 (McCool et al., 1988).

A lack of pen-side diagnostics, and the requirement for continuous hot and cold transport chains to maintain the integrity of the culture media, have likely both contributed to *T. foetus* infection persisting undetected in some bulls (Buller & Corney, 2013). Infected bulls have low numbers of *T. foetus* in all regions of their penis, and therefore culturing samples in enriched media to multiply *T. foetus* and increase diagnostic sensitivity is imperative in order to aid both identification by traditional microscopy and detection *via* molecular methods (Mukhufhi et al., 2003; Cobo et al., 2007). The elapsed time between sampling and initial microscopy testing (approximately one week, comprised of three days of TFEM transport at ambient temperature and four days of TFEM incubation at 37 °C), and the fragility of *T. foetus* when removed from the bovine urogenital tract, likely explains the lower number of microscopy-positive samples compared to PCR-positive samples (i.e. 10/109 *versus* 18/109). Although the Oxoid TFEM sent to the submitting veterinarian on this occasion facilitated successful culture, lyophilisation of *T. foetus* culture media prior to shipping to remote regions has been demonstrated to produce comparable culturing results to traditional reconstituted media and could be considered for similar investigations in the future (Rush et al., 2019). While reconstitution of media prior to sampling would resolve issues associated with transportation of frozen media to the submitter, the inoculated media still needs to be maintained between 5 and 37 °C during transport back to the diagnostic laboratory for the parasite to remain viable and multiply (Buller & Corney, 2013). An alternative approach addressing these logistical challenges is the possibility of using Loop-mediated Isothermal Amplification (LAMP) technology for pen-side testing (Oyhenart, 2018; Dąbrowska et al., 2020a). A recently developed LAMP test targeting the elongation factor 1 alpha gene of *T. foetus* demonstrated good sensitivity (90%) and specificity (85%) for *T. foetus* when applied directly on the infected cervical vaginal mucus or smegma samples (Oyhenart, 2018). New approaches without the

need for pre-culture and DNA isolation such as LAMP testing, or coupling LAMP testing with pre-culture using reconstituted lyophilised media, may improve diagnosis and control of *T. foetus* in remote regions such as the NT.

Of the 18 suspect positive samples detected using the McMillen et al. (2006) *T. foetus* TaqMan™ real-time PCR, 13/13 (100%) were definitely identified as the 'bovine' genotype according to Sanger sequencing of the ITS 1 and 2 regions generated by the genotyping qPCR assay (TFR3/4 primers). The absence of the AA insertion at ITS1 differentiated these samples from the 'Southern Africa' genotype, which remains undetected outside of Namibia (Casteriano et al., 2016). Due to the similarity of the two genotypes at the ITS locus, it is advisable whenever *T. foetus* is identified in molecular assays to further characterise additional markers (CP1-2, CP4-9, and MDM-1) to enable differentiation of the different *T. foetus* genotypes. While the pathogenicity and distribution of the 'Southern Africa' genotype remains unknown, it is evident that multiple genotypes occur in cattle demonstrating the importance of culture isolation and characterisation across multiple markers, as was performed in the present study (Casteriano et al., 2016).

The *T. foetus* 'bovine' genotype was reported at high prevalence (87%) in the gastrointestinal tract of domestic pigs managed in close proximity to *T. foetus*-free cattle on a property in NSW, Australia (Mueller et al., 2015). While *T. foetus* is a well-recognised cause of venereal disease in cattle, this result suggests that it also may occur as a gastrointestinal commensal of pigs (Šlapeta et al., 2012; Mueller et al., 2015). Although the concurrent farming of pigs and cattle does not appear to be a risk factor for bovine trichomonosis and did not occur in the present study, the link between the parasite in pigs and cattle remains unknown and warrants further investigation (Jin et al., 2014; Mueller et al., 2015; Collantes-Fernández et al., 2019; Dąbrowska et al., 2020a; Dąbrowska et al., 2020b).

5. Conclusion

This is the first published evidence of trichomonosis in Australian cattle since 1988 and suggests that routine *T. foetus* testing in areas of extensive livestock production where natural mating routinely occurs is warranted, especially when a history of reproductive failure is present. New diagnostic approaches that bring the 'lab to the animal' should be pursued in order to circumvent existing logistical constraints that prevent traditional *T. foetus* surveillance.

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CRediT author statement

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Data availability

Sequences obtained in this study were deposited in GenBank under the following accession numbers: MW291513-MW291530, MW297548-MW297550 and MW322811-MW322823 (Supplementary Table S1).

Declaration of competing interests

The authors confirm that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2021.100012.

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