Novel genotype of *Tritrichomonas foetus* from cattle in Southern Africa

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SUMMARY

Bovine trichomonosis caused by *Tritrichomonas foetus* is a significant reproductive disease of cattle. Preputial samples were collected using sheath washing technique in bulls in Namibia. Thirty-six trichomonad cultures were characterized using the TaqMan-probe commercial real-time polymerase chain reaction (PCR) diagnostic assay (VetMAX™-Gold Trich Detection Kit) and CYBR real-time PCR assay based on TFR3/4 primers. Diagnostic real-time PCRs and DNA sequencing of the internal transcribed region confirmed presence of *T. foetus* in 35 out of 36 samples. Multilocus genotyping using cysteine proteases (CP1, CP2, CP4, CP5, CP6, CP7, CP8, CP9) and malate dehydrogenase (MDH1) gene sequences demonstrate that the *T. foetus* in Namibia are genetically distinct from those characterized elsewhere. We report the discovery of a novel genotype of *T. foetus* in Namibian cattle, distinct from other *T. foetus* genotypes in Europe, South and North America and Australia. We suggest recognition of a 'Southern African' genotype of *T. foetus*. Identification of the new genotype of *T. foetus* demonstrates the need for wider global sampling to fully understand the diversity and origin of *T. foetus* causing disease in cattle or cats.

Key words: *Tritrichomonas foetus*, cattle, Southern Africa, multilocus genotyping, PCR.

INTRODUCTION

Bovine trichomonosis caused by *Tritrichomonas foetus* can have a significant impact in the reproductive performance of cattle (Ondrak, 2016). The protozoan parasite *T. foetus* is sexually transmitted in cattle resulting in early embryonic deaths, abortion and infertility (BonDurant, 1997; Ondrak, 2016). The parasite is also naturally present as a commensal in pig caecum, stomach and nose (Šlapeta *et al*. 2012; Doi *et al*. 2013; Mueller *et al*. 2015). In domestic cats *T. foetus* causes feline enteric trichomonosis (Levy *et al*. 2003). Comparative analysis of feline, porcine and bovine isolates of *T. foetus* led to the recognition of 'bovine' and 'feline' genotypes for isolates from cattle and pigs, and cats, respectively. The bovine isolates from Argentina, Czech Republic and Australia all shared identical sequences across ten DNA loci (Šlapeta *et al*. 2012). No isolate from African cattle has been compared, despite wide distribution of *T. foetus* in African cattle (Pefanis *et al*. 1988; Madoroba *et al*. 2011).

A recent retrospective study from six Southern African countries demonstrated wide prevalence of major reproductive infection in Southern African cattle (Madoroba *et al*. 2011). *Tritrichomonas foetus* was detected in 142 (4·1%, $n = 3458$) bull sheath washings and scrapings, and *Campylobacter fetus* was present in 1·9% (60/3161) samples (Madoroba *et al*. 2011). The study demonstrated *T. foetus* prevalence of 4·5, 3·8 and 3·3% in bulls from South Africa (90/11999), Namibia (45/1201) and Botswana (7/210), respectively (Madoroba *et al*. 2011). *Tritrichomonas foetus* was not detected in Swaziland ($n = 7$) or Zambia ($n = 41$), most likely due to the low number of samples investigated. Few studies exist about the prevalence of *T. foetus* in Southern Africa. Cattle farming remains important in Southern Africa with many regions where cattle is predominantly serviced naturally providing opportunities for venereal infections such as trichomonosis. Vaccine TrichGuard® (Zoetis, South Africa) and TrichGuard® V5L (Zoetis, South Africa) is marketed in Southern Africa for aid in prevention of *T. foetus* or in combination with *C. fetus* and three species of *Leptospira*, respectively. TrichGuard vaccine is a killed vaccine containing axenic trophozoites in adjuvant against *T. foetus* for cattle (Baltzell *et al*. 2013).

The aim of this study was to culture and identify *T. foetus* from cattle in Namibia and compare their molecular identity to known genotypes of *T. foetus*. To do so, bull samples were collected and cultured.

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Established cultures were characterized using molecular diagnostic techniques and the genotype confirmed by multilocus genotyping.

MATERIALS AND METHODS

Sample collection

Between 2014 and 2015 about 4000 bulls present in herds from central regions of Namibia (Khomas, Hardap, Erongo, Omaheke and Otjozondjupa) were sampled once for *T. foetus* by collecting preputial samples using sheath washing technique (Irons *et al.* 2002). The same samples were inoculated into a Steve's transport medium (Steve's TM, Vrede Veterinary Laboratory, South Africa) and delivered to the Central Veterinary Laboratory of Windhoek (CVL) within 72 h after the sampling.

Culture of Tritrichomonas foetus

Samples in transport medium were examined directly under a standard light microscope (Olympus BX53 equipped with Olympus SC100 high-resolution digital colour camera) using a magnification of 1000× (100× objective and 10× eye piece) for the presence of motile protozoa with three flagella. Giemsa-stained smears were used to confirm trichomonad morphology. Prepared enriched '*Trichomonas* medium' and Thioglycollate medium U.S.P. (Oxoid LTD, Basingstoke, Hampshire, England) were dispensed aseptically into sterile McCartney bottles in 15 mL aliquots and used as culture medium for all the samples collected for isolation of *T. foetus* with microscopic examination of the medium at intervals from day 1 to day 7 after inoculation and incubation at 37 °C. The results were recorded as positive when trichomonad organisms displaying unique morphological characteristics were present, or negative if there was no growth of trichomonads.

DNA isolation

DNA from 36 *T. foetus* positive cultures was extracted using the Maxwell®16 Cell LEV DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with an elution volume of 50 μL. DNA was shipped to the Faculty of Veterinary Sciences, The University of Sydney and stored at −20 °C prior molecular characterisation.

Molecular diagnostics for Tritrichomonas foetus

Diagnostic *T. foetus* real-time polymerase chain reaction (PCR) was based on primers TFR3 [S0001] (5′-CGG GTC TTC CTA TAT GAG ACA GAA CC-3′) and TFR4 [S0002] (5′-CCT GCC GTT GGA TCA GTT TCG TTA A-3′) amplify region of the internal transcribed spacer (ITS) rDNA (Felleisen *et al.* 1998; Mueller *et al.* 2015). The real-time PCR reactions used KAPA SYBR® FAST qPCR (2×) Master Mix (Kapa Biosystems, Inc., MA, USA) on CFX96 Touch™ Real-Time PCR Detection System with the corresponding CFX Manager 3·1 software (BioRad, Australia). The volumes of the real-time PCR reactions were made up to 20 μL, including 2 μL of template DNA (all samples were used at its original concentration or diluted 1:10). The PCR mix included primers at a final concentration of 200 nM. PCR conditions were 3 min at 95 °C, followed by 40 cycles of 3 s at 95 °C and 20 s at 63 °C as previously described by Mueller *et al.* (2015). ddH2O served as a negative control. Positive PCRs were directly sequenced by Macrogen Ltd. (Seoul, Korea; http://dna.macrogen.com/). Oligonucleotides were synthesised by Macrogen Inc. (Seoul, Korea).

Trichomonas foetus DNA Test Kit (VetMAX™-Gold Trich Detection Kit, 4483869, Life Technologies, Thermo Fisher, Australia), a test based on TaqMan probe qPCR, was used according to the manufacturer's instruction on CFX96. The amplification on CFX96 and data were analysed with the corresponding software (BioRad, Australia). The real-time PCR threshold was arbitrarily set to a single threshold at 100 rfu. Data (C_t-values) were interpreted according to the manufacturer's instructions (Life Technologies, Thermo Fisher, Australia).

Conventional trichomonad PCR amplifying ITS rDNA region based on primers TFR1 [S0062] (5′-TGC TTC AGT TCA GCG GGT CTT CC-3′) and TFR2 [S0063] (5′-CGG TAG GTG AAC CTG CCG TTG G-3′) as previously described using MyTaq™ Red Mix (BioLine, Australia) (Felleisen, 1997; Šlapeta *et al.* 2012).

Multilocus genotying of Tritrichomonas foetus

Protein coding genes, cysteine proteases (CP1, 2, 4–9) and cytosolic malate dehydrogenase 1 (MDH1) were amplified from *T. foetus* DNA as previously described (Šlapeta *et al.* 2012; Sun *et al.* 2012). Oligonucleotides were synthesised by Macrogen Inc. (Seoul, Korea). All PCR amplifications were performed with MyTaq™ Red Mix (BioLine, Australia) in a total volume of 30 μL. Primers were added at a concentration of 0·25 μM each. The PCR was run using the following cycling conditions: 95 °C for 15 s, 55 °C for 15 s and 72 °C for 20 s for 35 cycles. All reactions were initiated at 95 °C for 2 min and concluded at 72 °C for 7 min. PCRs were amplified in the Verity PCR cycler (Thermo Fisher Scientific, Australia). Each PCR mix contained 2 μL of the sample DNA. All PCRs were run with negative controls (ddH2O).

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All PCRs that yielded unambiguous single bands of the expected size were directly and bidirectionally sequenced using amplification primers at Macrogen Ltd. (Seoul, Korea).

DNA sequences were verified, assembled and multiple sequence alignments with *T. foetus* and *Tritrichomonas mobilensis* were produced in CLC Main Workbench 6·8·1 (CLC bio, a QIAGEN Company, Denmark; http://www.clcbio.com/). Individual gene alignments were concatenated in CLC Main Workbench. Split networks from distances (K2P) were calculated using NeighborNet and cluster network visualized in SplitsTrees 4 (v 4·14·3; http://www.splitstree.org/) (Huson and Bryant, 2006).

Sequence data and data accessibility

Sequences were assembled, aligned with related sequences and analysed using CLC Main Workbench 6·8·1 (CLC bio, Denmark) and deposited in GenBank (National Center for Biotechnology Information, NCBI) under the Accession Numbers: KX425856-KX425919.

RESULTS

Thirty-six cultures contained trichomonads morphologically consistent with *T. foetus*. All but two (94%, 34/36) tested positive with an average C_t-value of 21·31 (min. 14·53, max. 29·83) using *T. foetus* specific qPCR based on TFR3/4 primers targeting ITS rDNA (Table 1). The C_t-value was not returned for NAM-1 and NAM-19. A diluted (1:10) DNA template confirmed presence of *T. foetus* DNA in 97% (35/36) samples (C_t-value mean 25·02, min. 18·11, max. 34·85), including NAM-1 sample (C_t-value = 30·28). Sample NAM-19 did not return C_t-value in both runs and was considered *T. foetus* negative. Furthermore, NAM-19 remained negative using trichomonad universal primer set TFR1/2.

Duplicate USDA-licensed diagnostic *T. foetus* specific qPCR confirmed presence of *T. foetus* DNA in 35 samples (C_t-values: average 25·41, min. 20·10, max. 30·96) using DNA diluted 1:10 (Table 1). Sample NAM-19 did not return C_t-values.

PCR products from qPCR based on TFR3/4 primers amplifying ITS rDNA were subjected to DNA sequencing to further identify the genotype of *T. foetus* (Table 1). Previously, ITS rDNA single polymorphism has been shown diagnostic between the 'bovine' and 'feline' genotype of *T. foetus* (Šlapeta *et al*. 2010, 2012). All 38 DNA sequences of ITS rDNA revealed a novel genotype with an AA insertion (adenosines) in ITS1 rDNA compared with the 'bovine' or 'feline' genotype of *T. foetus* (Fig. 1). The ITS2 rDNA for all 35 DNA sequences matched the 'bovine' genotype of *T. foetus* with C (cytosine) at the diagnostic residue (Šlapeta *et al*. 2010) (Fig. 1).

The presence of the novel *T. foetus* genotype prompted the characterization of cysteine proteinase 2 (CP2) locus of *T. foetus* previously recognized as the most divergent (22 nt differences) between the known 'feline' and 'bovine' genotypes (Šlapeta *et al*. 2012; Suzuki *et al*. 2016). Amplification of CP2 was successful for 36% (13/35) DNA samples that were considered positive for *T. foetus* using the qPCR assays. NAM-2, NAM-3, 5, 30, 34, 35, 13, 20, 21, 26, 27, 28 and 32 samples amplified CP2 and were those with lower C_t values in diagnostic PCR (Table 1). All obtained 669 nt long CP2 sequences were identical to each other and distinct by 15 nt from the 'bovine' *T. foetus* genotype coding for 9 amino acid differences (Fig. 1;

Table 1. Summary of molecular diagnostics for *Tritrichomonas foetus* isolates

Isolate	qPCR *T. foetus* (VetMAX) 1:10 (C_t)		qPCR *T. foetus* (TFR3/TFR4) 1:1 (C_t)	qPCR *T. foetus* (TFR3/TFR4) 1:10 (C_t)	*T. foetus* (TFR3/TFR4) Sequencing 'Southern African'
NAM-1	28·52	28·3	N/A	30·28	+
NAM-2	25·93	26·4	20·34	22·85	+
NAM-3	21·94	21·81	15·87	19·22	+
NAM-4	29·68	29·58	26·97	30·26	+
NAM-5	24·98	25·62	21·20	25·70	+
NAM-6	26·17	26·69	23·70	27·44	+
NAM-7	25·85	25·76	22·49	26·14	+
NAM-8	26·81	26·53	24·01	27·76	+
NAM-9	27·84	28·15	24·08	27·44	+
NAM-10	31·09	30·82	29·83	34·85	+
NAM-11	29·45	29·51	25·47	29·38	+
NAM-12	26·08	25·98	22·55	26·47	+
NAM-13	N/A	24·1	18·44	22·11	+
NAM-14	26·67	26·55	23·32	25·08	+
NAM-15	25·33	25·29	22·14	26·26	+
NAM-16	28·77	28·65	26·73	30·46	+
NAM-17	27·11	27·05	23·05	26·79	+
NAM-18	27·94	28·07	24·66	28·21	+
NAM-19	N/A	N/A	N/A	N/A	–
NAM-20	25·01	24·7	20·04	24·06	+
NAM-21	23·28	23·22	18·23	22·23	+
NAM-22	25·4	25·65	21·62	25·29	+
NAM-23	23·78	23·4	21·19	26·23	+
NAM-24	26·6	26·24	21·89	25·98	+
NAM-25	24·33	24·37	20·11	24·12	+
NAM-26	23·07	23·05	19·15	22·61	+
NAM-27	19·85	20·34	14·53	18·20	+
NAM-28	22·44	22·33	17·03	20·61	+
NAM-29	27·09	27·09	23·96	28·10	+
NAM-30	20·78	20·91	14·78	18·11	+
NAM-31	29·51	29·41	26·28	29·82	+
NAM-32	24·27	24·58	20·04	28·17	+
NAM-33	27·25	26·98	24·06	24·88	+
NAM-34	24·8	25·1	21·55	27·80	+
NAM-35	24·83	25·09	20·35	23·80	+
NAM-36	29·31	29·4	24·75	27·97	+

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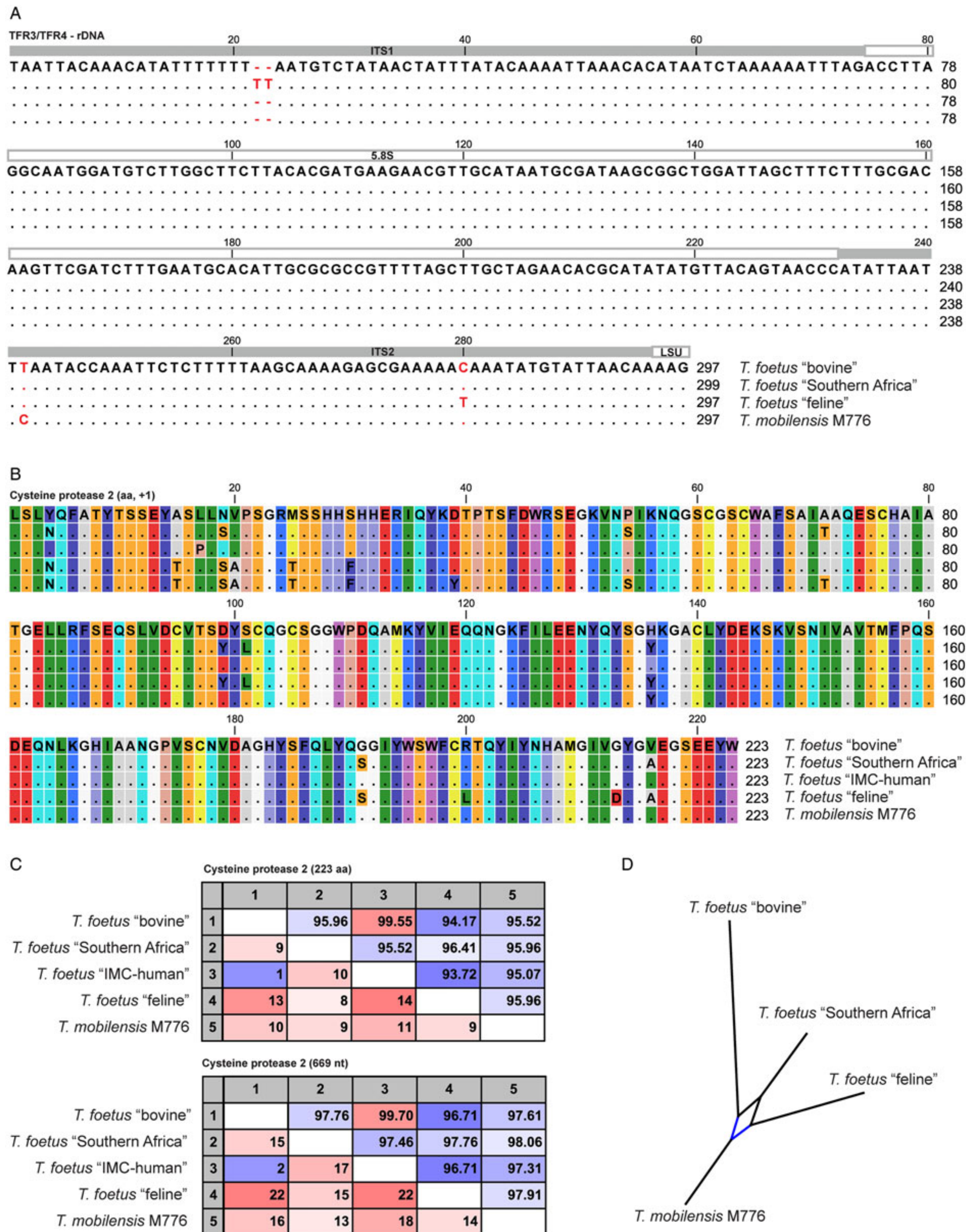

Cysteine protease 2 (223 aa)

		1	2	3	4	5
T. foetus "bovine"	1		95.96	99.55	94.17	95.52
T. foetus "Southern Africa"	2	9		95.52	96.41	95.96
T. foetus "IMC-human"	3	1	10		93.72	95.07
T. foetus "feline"	4	13	8	14		95.96
T. mobilensis M776	5	10	9	11	9	

Cysteine protease 2 (669 nt)

		1	2	3	4	5
T. foetus "bovine"	1		97.76	99.70	96.71	97.61
T. foetus "Southern Africa"	2	15		97.46	97.76	98.06
T. foetus "IMC-human"	3	2	17		96.71	97.31
T. foetus "feline"	4	22	15	22		97.91
T. mobilensis M776	5	16	13	18	14	

Fig. 1. Comparison between *Tritrichomonas foetus* genotypes and *Tritrichomonas mobilensis*. (A) Multiple sequence alignment of internal transcribed spacer region of ribosomal RNA gene of *T. foetus* and *T. mobilensis*. Identical residues presented as dots. Differences highlighted. (B) Multiple sequence alignment at cysteine protease 2 (CP2) of *T. foetus* and *T. mobilensis*. (C) Pairwise comparison of CP2 nucleotide sequences (upper) and amino acid sequences (lower). The top right represents pairwise similarity and bottom left presents number of differences. (D) Split cluster network analysis (K2P model) calculated using NeighborNet using all 10 loci and 4554 alignment positions in the final dataset.

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Table 2. Multilocus comparison of *Tritrichomonas foetus* 'Southern African' genotype with *T. foetus* and *T. mobilensis*

		T. foetus 'bovine' genotype		*T. foetus* 'feline' genotype		*T. mobilensis*	
Gene	Sequence (nt)[a]	Nucleotide difference (and per; identity)	Amino acid difference	Nucleotide difference (% identity)	Amino acid difference	Nucleotide difference (% identity)	Amino acid difference
CP1	503	4 (99·2)	1	1 (99·8)	1	n/a	n/a
CP2	669	15 (97·8)	9	15 (97·8)	8	13 (98·1)	9
CP4	273	2 (99·3)	1	0 (100)	0	1 (99·6)	1
CP5	361	4 (98·9)	1	3 (99·2)	Stop	1 (99·7)	0
CP6	318	7 (97·8)	1	1 (99·7)	0	2 (98·4)	0
CP7	373	1 (99·7)	0	2 (99·5)	0	n/a	n/a
CP8	907	5 (99·5)	2	2 (99·8)	0	n/a	n/a
CP9	289	2 (99·3)	0	2 (99·3)	0	2 (99·3)	0
MDH1	562	1 (99·8)	0	1 (99·8)	0	2 (99·8)	1
ITS1+2	299	2 (99·3)	–	3 (99·0)	–	3 (99·0)	–
Total	4554	38		28		24	

CP, *Tritrichomonas* sp. cysteine protease; nt, nucleotide; MDH, malate dehydrogenase; ITS, internal transcribed spacer.
[a] Polymerase chain reaction (PCR) amplified and sequenced DNA without PCR primers.

Table 2). The remaining DNA samples produced insufficient PCR product for direct sequencing.

Multilocus genotyping based on protein coding genes confirmed unique status of the studied isolates (Table 2). Eight protein coding genes (CP1, CP2, CP4, CP5, CP6, CP7, CP8 and MDH1) and ITS locus have been amplified and sequenced for two isolates NAM-27 and NAM-30. Comparison with *T. foetus* 'bovine' genotype and *T. foetus* 'feline' genotype, the loci were equally (97·8–99·8 and 97·8–99·8%, respectively) identical at the nucleotide sequence with the studied isolates. The least identical (97·8%) locus was CP2 and CP6 between *T. foetus* 'bovine' genotype and studied isolates (Table 2). Amplification of CP8 from NAM-27 yielded an insufficient PCR product for direct sequencing, therefore primers F1/R2 (Sun *et al*. 2012) producing a shorter product were successfully applied.

The closest sister species to *T. foetus* is *T. mobilensis* (Šlapeta *et al*. 2012). Six nucleotide sequences for protein coding genes and ITS are available for *T. mobilensis* spaning over 2472 residues (Table 2). The novel *T. foetus* genotype has the least variable sites (1%, 24/2472) with *T. mobilensis* compared with 25 and 33 with *T. foetus* 'feline' and 'bovine' genotype, respectively. Inferring the phylogenetic relationship between the *T. foetus* genotypes and *T. mobilensis* using split networks revealed splits leading to *T. foetus* (Fig. 1).

DISCUSSION

This study revealed the presence of a novel genotype of *T. foetus* in Southern African cattle. All previously characterized *T. foetus* from cattle (Europe, America, Australia) were identical at the ITS marker and/or using multilocus approach (Šlapeta *et al*. 2012). The Southern African *T. foetus* isolates form a new genetic lineage, the *T. foetus* 'Southern African' genotype. The *T. foetus* 'Southern African' genotype is detectable using either the USDA-licensed diagnostic qPCR or commonly used PCR/qPCR based on the TFR3/4 primer pair (Felleisen *et al*. 1998).

The *T. foetus* 'Southern African' genotype is closely related to *T. foetus* 'bovine' genotype from cattle and pigs, *T. foetus* 'feline' genotype from cats, and *T. mobilensis*. Experimental infections with the non-homologous genotype (cattle with the *T. foetus* 'feline' genotype and *vice versa*) are possible under laboratory conditions, but they do not result in the same disease outcome (Stockdale *et al*. 2007, 2008). Across six protein coding gene loci (2199 nt), the *T. foetus* 'Southern African' genotype is most closely related to *T. mobilensis* with only 20 nt (<1%) single nucleotide polymorphisms. These changes only code for nine amino acid changes in CP2 and a single amino acid change in MDH1. The genetic distance is comparable with the distance of the *T. foetus* 'feline' genotype to *T. mobilensis* (Šlapeta *et al*. 2012). It is therefore worthwhile to consider if the pathobiology of the *T. foetus* 'Southern African' genotype is distinct to that of those studied previously (Stockdale *et al*. 2007, 2008). *In vitro* culture studies demonstrate functional differences between isolates and could be used to compare the *T. foetus* 'bovine' genotype with the 'Southern African' genotype (Tolbert *et al*. 2013, 2014).

It was previously thought that the *T. foetus* 'bovine' and 'feline' genotypes strictly isolated from cattle and cats, respectively, was a strong indicator that they possess distinct epidemiology and evolutionary trajectories (Šlapeta *et al*. 2012). This also implied a single origin of the bovine *T. foetus* that is now challenged with the discovery of the *T.*

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foetus 'Southern African' genotype. Currently, it is impossible to unambiguously resolve the origin of bovine isolates, despite using multiple genes and assuming *T. mobilensis* as the sister to all *T. foetus*. Recent studies of transcriptomes of feline, bovine and porcine *T. foetus* using RNA-seq have demonstrated the opportunities of next-generation sequencing to better understand the biology and identity of closely relating microorganism (Morin-Adeline *et al.* 2014, 2015).

Over the past 6 years *T. foetus* was characterized from several countries using molecular techniques that unambiguously identify the strain and genotype (Šlapeta *et al.* 2010; Doi *et al.* 2013; Mueller *et al.* 2015; Arranz-Solís *et al.* 2016; Suzuki *et al.* 2016). To date, only two main genotypes and close allies have been identified (Šlapeta *et al.* 2012). Identification of this new genotype of *T. foetus* demonstrates the need for wider global sampling to fully understand the diversity and origin of *T. foetus* that causes disease in cattle or cats. The genetic diversity of *T. foetus* has potential implication for efficacy of the killed, whole-cell *T. foetus* vaccine (TrichGuard®) if a different *T. foetus* genotype is used in the vaccine than the one circulating in cattle to be vaccinated. Recent systematic review of the efficacy of the *T. foetus* vaccine identified potential over-estimate of efficacy and limited or no evidence that vaccination decreases infections or open risk in heifers (Baltzell *et al.* 2013).

The outcome of this study is the discovery of a yet unknown 'Southern African' genotype of *T. foetus* in Namibian cattle, distinct from the *T. foetus* 'bovine' genotype from Europe, South and North America and Australia. Identification of the new genotype of *T. foetus* demonstrates the need for wider global sampling to fully understand the diversity and origin of *T. foetus* causing disease in cattle or cats.

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