Veterinary Parasitology 266 (2019) 12–17

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Comparison of multiplexed-tandem real-time PCR panel with reference real-time PCR molecular diagnostic assays for detection of *Giardia intestinalis* and *Tritrichomonas foetus* in cats

Maira N. Meggiolaro[a], Florian Roeber[b], Victoria Kobylski[b], Damien P. Higgins[a], Jan Šlapeta[a,*]

[a] Veterinary Pathology Diagnostic Services, Sydney School of Veterinary Science, University of Sydney, NSW 2006, Australia
[b] AusDiagnostics Pty. Ltd., Mascot, 2020, NSW, Australia

ARTICLE INFO

Keywords:
Giardia
Tritrichomonas
Giardiasis
Trichomonosis
Diagnostics
MT-PCR
TaqMan
Diarrhoea
Feline

ABSTRACT

Giardia intestinalis and *Tritrichomonas foetus* are frequent enteric protozoan parasites of the gastrointestinal track of domestic cats. Because of different treatment options for the parasites, confirmation of presence of one or both pathogens is necessary. The PCR based assays are suitable for differential diagnosis. We evaluated the performance of Small Animal Diarrhoea panel, a multiplexed-tandem real-time PCR (MT-PCR) assay, that detects DNA of both *G. intestinalis* and *T. foetus*. The sensitivity and specificity were compared to reference real-time PCR assays using 105 faecal samples, 39.05% (n = 41) positive for *G. intestinalis* and 30.48% (n = 32) were positive for *T. foetus*. The faecal samples positive for *T. foetus* had a high proportion of late amplifiers, determined by an arbitrary threshold of C_t-values > 35. On the other hand, only one *G. intestinalis* positive sample was considered a late amplifier. For *G. intestinalis* DNA, the MT-PCR assay had 95.1% sensitivity and 92.1% specificity. For *T. foetus* DNA, the MT-PCR assay had 41.9% sensitivity and 100.0% specificity. To evaluate the interlaboratory reproducibility of the MT-PCR assay, results were compared in two different laboratories and found to be in a very good agreement (Kappa = 0.9). Further analysis of the DNA using conventional PCR determined presence of *G. intestinalis* Assemblage F and *T. foetus* genotype 'feline'. In conclusion, the MT-PCR Small Animal Diarrhoea panel had a good and poor performance against reference assays for *G. intestinalis* and *T. foetus,* respectively. The assay is suitable for detection and differential diagnosis of *G. intestinalis* and moderate to high burdens of *T. foetus* in small animal clinical practice.

1. Introduction

Giardia intestinalis and *Tritrichomonas foetus* are frequent and ubiquitous enteric protozoans affecting domestic cats (Gookin et al., 2017; Gruffydd-Jones et al., 2013; Šlapeta et al., 2015). Both parasites have a world-wide distribution in owned cats (Feng and Xiao, 2011; Yao and Koster, 2015). While *G. intestinalis* is a well-known parasite of the small intestine, and veterinarians are familiar with the disease giardiasis, *T. foetus* is considered to be an emerging infection (Gookin et al., 2017; Gruffydd-Jones et al., 2013). Infection with *T. foetus* is associated with outbreaks of large bowel diarrhoea in < 2 year old cats, yet the epidemiology is not fully understood (Gookin et al., 2004; Hale et al., 2009; Van der Saag et al., 2011).

Both feline giardiasis and trichomonosis present clinically in young animals, with the most consistent clinical sign being diarrhoea (Gookin et al., 2017, 2004). Often, both parasites are present as co-infection (Bell et al., 2010). Because of different treatment options for each disease, confirmation of presence of one or both pathogens is necessary (Bell et al., 2010; Gookin et al., 2006; Gunn-Moore and Lalor, 2011; Šlapeta et al., 2010). For *G. intestinalis* the in-clinic rapid diagnostic coproantigen assays are ideal, with extremely good sensitivity and specificity, and are therefore preferred for in-clinic diagnosis (Barbecho et al., 2018; Uiterwijk et al., 2018). However, no in-clinic rapid diagnostic coproantigen assay is available for *T. foetus*, therefore alternative tests need to be requested or undertaken. Faecal morphological examination is a good option; nevertheless, its sensitivity for both *G. intestinalis* and *T. foetus* are mediocre (Bell et al., 2010; Gookin et al., 2017). The most suitable approaches are molecular PCR based assays with verified high sensitivity and specificity (Gookin et al., 2017). PCR based assays in the form of PCR panels are becoming established tools in veterinary practice. On one hand, they are not in-clinic tests, but turnaround of 1–3 days makes them suitable as part of differential

* Corresponding author.
E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

https://doi.org/10.1016/j.vetpar.2018.12.009
Received 1 November 2018; Received in revised form 11 December 2018; Accepted 12 December 2018

diagnostics in diarrhoea in young cats. Validation and cross validation between laboratories are urgently needed to elucidate the reliability of PCR panels to recognise their limitation and performance against set of reference assays.

The aim of this study was to evaluate the performance of a multiplexed-tandem real-time PCR (MT-PCR) assay for *G. intestinalis* and *T. foetus* on feline DNA samples with presence of *G. intestinalis* and *T. foetus* determined previously using reference real-time PCR assays. To evaluate the reproducibility of the MT-PCR assay, results were compared in two different laboratories.

2. Material and methods

2.1. Feline faecal samples

A total of 105 feline faecal samples submitted to the Veterinary Pathology Diagnostic Services, Sydney School of Veterinary Science (VPDS) during 2017 for diagnostic purposes were used. All samples were collected in sterile containers, without preservatives, and left in 4 °C for not longer than 4 days before DNA isolation.

2.2. Isolation of nucleic acids from faecal samples

Approximately 0.25 g of each faecal sample was added into tubes with glass beads and lysis buffer and then homogenised and disrupted using the high-speed homogeniser, FastPrep® 24 (MP Biomedicals, Australia) at setting 6.0 m/s for 40 s. A magnetic bead based nucleic acid isolation kit, MagAttract Power Microbiome DNA/RNA Kit (27600-4-KF, Qiagen, Australia), adapted for the KingFisher™ Duo (Thermo Scientific™, Australia) was utilised for all DNA isolations. The DNA/RNA was eluted in 100 µL of DNA/RNA free water, following manufacturer protocol. Each batch of 12 samples included one blank control, to monitor for the presence of contamination.

2.3. Multiplexed-tandem PCR (MT-PCR) – Small Animal Diarrhoea panel

Two different versions of the proprietary MT-PCR technology (AusDiagnostics, Australia) were used for the experiments which were (A) High-Plex 24 (Cat.: 9150), consisting of a MT-Processor (liquid-handling system) (AusDiagnostics, Australia) in conjunction with a 384-well real-time PCR thermocycler (DT-Prime real-time PCR detection system) (DNA-Technology, Russia) and permitting 24 samples to be tested per run, and (B) Mini-Plex 12 (Cat.: 9350), consisting of a MT-Processor (liquid-handling system) (AusDiagnostics, Australia) in conjunction with a 96-well real-time PCR thermocycler (CFX96™ real-time PCR detection system) (Biorad, Australia) and permitting 6 samples to be tested per run. Samples were tested on the High-Plex instrument using the Small Animal Diarrhoea – HP 16-well (Cat.: 28174) test panel, or the in the Mini-Plex 12 system, using the Small Animal Diarrhoea – MP 16-well (Cat.: 78174) test panel. For the specific amplification of *G. intestinalis* the 18S gene of nuclear ribosomal DNA was targeted and for *T. foetus* the cysteine protease gene was used. The pathogen targets in both panel versions were identical. This assay includes an initial multiplexed short amplification followed by a single-target amplification, using SYBR Green chemistry. It tests for 14 targets: *Campylobacter* spp., *Dientamoeba fragilis*, *Salmonella* spp., *G. intestinalis*, *Cryptosporidium*, *T. foetus*, *Toxoplasma gondii*, parvovirus/panleukopenia virus, canine distemper virus, canine coronavirus, feline coronavirus, *Clostridium perfringens*, *Clostridium perfringens* enterotoxin (CPE) and *Neospora caninum*. It includes a reference PCR targeting a mammalian gene to test for sample adequacy and an artificial sequence (SPIKE) to check for the presence of inhibitors. The setup procedure in both MT-PCR system versions was as followed: In the first step, 50 µl reaction volumes containing Step 1 RNA mastermix (AusDiagnostics, Cat. 40340RNA), oil and 10 µL of each isolated DNA/RNA was subjected to 15 cycles in the MT-Processor (AusDiagnostics, Australia). After cycling, samples were automatically diluted and aliquoted by the MT-Processor into a 384 well plate (for High-Plex) or a 96 well plate (for Mini-Plex), supplied in the kit, containing step 2 primers for each target. The plate was then transferred to the specific real-time thermocycler and amplification conditions were 95 °C for 10 min, followed by 30 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s A melt curve was generated at the end from 72 °C to 94.8 °C at 0.4 °C intervals and analysed using the Easy-Plex results software (AusDiagnostics, Australia). A sample was recorded as test-positive using the 'auto-call function' of the Easy-Plex software, if the amplicon produced a single melting curve which was within 1.5 °C of the expected melting temperature, the height of the peak was higher than 0.2 dF/dT and the peak width was ≤ 3.8 °C (AusDiagnostics, Australia). Cycle threshold (C_t-values) and take-off values were recorded for each test-positive sample, and quantitative values for each parasite in each sample were determined using an automated comparison with C_t-values data determined for an internal spike-control (SPIKE; tube containing 10,000 copies of a synthesized oligonucleotide template and a specific primer set) for each sample tested (Stanley and Szewczuk, 2005). Each sample was also tested for the presence of amplifiable nucleic acids (i.e. extraction control) using a vertebrate reference gene (NONO).

A total of 105 samples were tested at a Laboratory A (AusDiagnostics) using the High-Plex 24 system (Cat.: 9150) and a subsample (64 samples) was tested at a Laboratory B (VPDS) on the smaller Mini-Plex 12 system (Cat.: 9350). The number of samples tested per run was either 9–24 or 1–5 plus a blank control on the High-Plex and the Mini-Plex system, respectively.

2.4. In-house reference real-time PCR for detection of Giardia intestinalis and Tritrichomonas foetus

All samples (n = 105) were tested in duplicate for *G. intestinalis* real-time PCR using previously described primers and TaqMan probe, (S0799) Giardia-80 F (5′-GAC GGC TCA GGA CAA CGG TT-3′), (S0801) Giardia-127R (5′-TTG CCA GCG GTG TCC G-3′), and (S0800) Giardia-105 T (6-HEX-5′−CCC GCG GCG GTC CCT GCT AG-3′-BHQ1) (Verweij et al., 2003). The TaqMan real-time PCR assay targets a 62-bp fragment of SSU RNA gene of G. intestinalis (GenBank accession no. M54878) (Verweij et al., 2003). The G. intestinalis-specific primers and probe were obtained from Macrogen (Korea). Amplifications were performed with SensiFAST™ Probe No-ROX Kit (BIO-86005, Bioline, Australia) in a total volume of 20 µL per reaction, where the final concentration for primers and probe was 400 nM and 100 nM respectively plus 2 µL of template. The real-time PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Australia) and analysed using BioRad CFX Manager (BioRad, Australia). Each PCR run included known positive control as well as negative control. Positive results were determined if both repeats yielded satisfactory cycle threshold (C_t) values (C_t-values < 38.00). Suspect positive results were determined if one or more repeats yielded C_t-values ≥ 38.00 and negative results were determined if both repeats did not cross the threshold (C_t-values > 40).

All samples (n = 105) were tested in duplicate for *T. foetus* real-time PCR using previously described primers and TaqMan-MGB probe (S0820), TFF2 (5′-GCG GCT GGA TTA GCT TTC TTT-3′), TFR2 (5′-GGC GCG CAA TGT GCA T-3′) and TRICHP2 (6-FAM-5′-ACA AGT TCG ATC TTT G-MGB-BHQ) (McMillen and Lew, 2006). The T. foetus-specific primers and probe were obtained as a premix from Life Technologies (Australia). Amplifications were performed with SensiFAST™ Probe No-ROX Kit (BIO-86005, Bioline, Australia) in a total volume of 20 µL per reaction, where the final concentration for primers and probe was 450 nM and 125 nM respectively, plus 2 µL of template. The real-time PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 45 s on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Australia) and analysed using BioRad CFX

Table 1
Summary of the results from the multiplexed-tandem real-time PCR panel on cat fecal samples.

	MT-PCR positive						
	Giardia intestinalis	*Tritrichomonas foetus*	Coronavirus (FCoV)	Panleukopenia (FPV)	*Clostridium perfringens*	*Campylobacter* spp.	*Salmonella* spp.
Samples (n = 104)	44	13	37	3	100	33	7
Prevalence, %	42.31	12.50	35.58	2.88	96.15	31.73	6.73
Average melt temperature, °C (SD)	92.60 (0.39)	80.12 (0.17)	87.14 (0.17)	80.51 (0.04)	80.84 (0.27)	84.41 (0.40)	87.09 (0.40)

Manager (BioRad, Australia). Each PCR run included known positive control as well as negative control. Positive results were determined if both repeats yielded satisfactory cycle threshold (C_t) values (C_t-values < 38.00). Suspect positive results were determined if one or more repeats yielded C_t-values ≥38.00 and negative results were determined if both repeats did not cross the threshold (C_t-values > 40).

A multiplexed assay including both real-time PCR assays for *G. intestinalis* or *T. foetus* was tested using a subset of 31 samples. All assays were run in duplicate in a duplex assay using a *Sso*Advanced Universal Probes Supermix (Bio-Rad, Australia). Primers and probes concentrations and template volume were kept the same as above. The real-time PCR conditions were as follows: 95 °C for 3 min, followed 40 cycles of 95 °C for 20 s, 60 °C for 45 s on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Australia) and analysed using BioRad CFX Manager (BioRad, Australia). Positive results were determined if both repeats yielded satisfactory cycle threshold (C_t) values (C_t-values < 38.00). Suspect positive results were determined if one or more repeats yielded C_t-values ≥ 38.00 and negative results were determined if both repeats did not cross the threshold (C_t-values > 40).

2.5. Conventional PCR for genotyping of Giardia intestinalis and Tritrichomonas foetus

Giardia intestinalis assemblage identification was determined using primers amplifying GDH and SSU rRNA gene (Feng and Xiao, 2011). To amplify GDH we used GDH1/GDH2 followed by nested primers GDH3/4. To amplify SSU rDNA we used RH11/RH4 followed by nested primers GiarF/GiarR. Oligonucleotides were synthesised by Macrogen Inc. (Seoul, Korea). All PCRs used MyTaq Red Mix (BioLine, Australia) in a final volume of 30 µL where the final concentration for primers was 500 nM plus 2 µL of DNA template. The secondary PCR used 1 µL of the primary PCR. Both nested PCR reactions were run in a T100 PCR cycler (BioRad, Australia). All PCRs were run with a negative control of sterile PCR-grade water. PCR products that yielded an unambiguous single band product of the expected size in 1.5% (w/v) agarose were purified and then directly and bidirectionally sequenced using amplification primers at Macrogen Inc. (Seoul, Korea). All sequences were assembled and aligned with reference sequences representing *G. intestinalis* assemblage A–H using CLC Main Workbench 6.9.1 (CLC bio, a QIAGEN Company, Denmark).

Determination of *T. foetus* genotype was evaluated using PCR amplification of the ITS rDNA region based on primers TFR3/TFR4 as previously described (Felleisen et al., 1998; Šlapeta et al., 2012). Oligonucleotides were synthesised by Macrogen Inc. (Seoul, Korea). All PCRs used MyTaq Red Mix (BioLine, Australia) in a final volume of 30 µL where the final concentration for primers was 500 nM plus 2 µL of DNA template. PCR reactions were run in T100 PCR cycler (BioRad, Australia). All PCRs were run with a negative control of sterile PCR-grade water. PCR products that yielded an unambiguous single band product of the expected size in 1.5% (w/v) agarose were purified and then directly and bidirectionally sequenced using amplification primers at Macrogen Inc. (Seoul, Korea). All sequences were assembled, aligned with reference sequences representing *T. foetus* genotype and analysed using CLC Main Workbench 6.9.1 (CLC bio, a QIAGEN Company, Denmark).

2.6. Statistical analysis

Sensitivity and specificity were calculated using 2 × 2 tables using test assay and reference assay considered to determine 'true' positives. Specificity was calculated as: number of true negatives / (number of true negatives + number of false positives). Sensitivity was calculated as: number of true positives / (number of true positives + number of false negatives). The test strength agreement was calculated using Kappa, standard error (SE) and 95% confidence interval (95%CI) (www.graphpad.com/quickcalcs/).

3. Results

3.1. Multiplexed-tandem real-time PCR (MT-PCR) small animal diarrhoea panel

One sample was inhibited from the original 105 samples, as determined by the SPIKE control, and did not yield any positive result, and thus was excluded. All samples (n = 104) were tested at Laboratory A (AusDiagnostics) using the High-Plex 24 platform. Out of 104 samples, 44 (42.31%) and 13 (12.50%) tested positive for *G. intestinalis* and *T. foetus*, respectively. In addition, FCoV, FPV, *Clostridium perfringens, Campylobacter* spp. and *Salmonella* spp. were detected using the Small Animal Diarrhoea panel (Table 1). The average melt temperature for the produced amplicons was 92.60 °C (SD: 0.39) for *G. intestinalis* and 80.12 °C (SD: 0.17) for *T. foetus*.

To evaluate the reproducibility of results obtained with the Small Animal Diarrhoea panel between two different MT-PCR systems and the two different laboratories, a subsample of 64 samples was compared in Laboratory A and Laboratory B (VPDS) (Supplementary Data). Twenty three (23/64) were called positive for *G. intestinalis* by both laboratories and additional three samples were positive for *G. intestinalis* in Laboratory A (estimated target concentration 28, 35 and 11). For *T. foetus*, five samples (5/64) were called positive in both laboratories, and additional one was considered positive by Laboratory B (estimated target concentration was not determined). All remained samples were negative for either *G. intestinalis* and/or *T. foetus*. The test strength agreement between the two laboratories for both *G. intestinalis* and *T. foetus* was 'very good' (Kappa = 0.900, SE 0.098, 95%CI: 0.708–1.000; Kappa = 0.900, SE 0.056, 95%CI: 0.790–1.000).

3.2. Sensitivity and specificity of MT-PCR for G. Iintestinalis using an in-house reference G. intestinalis real-time PCR

To determine sensitivity and specificity of the MT-PCR for *G. intestinalis*, a previously described *G. intestinalis* TaqMan real-time PCR was used as the reference assay. All samples (n = 105) were tested in duplicate and 39.05% (95%CI: 30.25%–48.62%; n = 41) had threshold (C_t) values < 40.00. There were 40 samples (38.10%, 95%CI: 29.37%–47.66%) with Ct-value values < 35.00, with the average C_t-value of 28.70 (min. 20.36, max. 35.54). One sample (0.95%) was called late amplifier (C_t-values of 39.94 and 37.91) (Supplementary

Table 2
Sensitivity and specificity of the multiplexed-tandem real-time PCR panel for *Giardia duodenalis* and *Tritrichomonas foetus*.

	Sensitivity	Specificity
Giardia intestinalis	95.1%	92.1%
Tritrichomonas foetus	41.9%	100.0%

Note: Using 104 samples analysed using reference real-time PCR assays; 41 positive for *G. intestinalis* and 31 positive for *T. foetus*.

Data).

The MT-PCR from Laboratory A for *G. intestinalis* had 95.1% sensitivity and 92.1% specificity; $n = 105$, one samples was inhibited (Table 2). If those samples that were automatically called as 'check' in MT-PCR (Laboratory A) were considered negative, the sensitivity was 82.9% and specificity was 98.4%. The MT-PCR from Laboratory B for *G. intestinalis* had 85.2% sensitivity and 100.0% specificity ($n = 64$). If those samples that were automatically called as 'check' in MT-PCR (Laboratory B) were considered negative, the sensitivity was 74.1% and specificity was 100.0%.

3.3. Sensitivity and specificity of MT-PCR for T. Foetus using an in-house reference T. Foetus real-time PCR

To determine sensitivity and specificity of the MT-PCR for *T. foetus*, a previously described *T. foetus* real-time TaqMan-MGB PCR was used as the reference assay (Fig. 1). All samples ($n = 105$) were tested in duplicate and 30.48% (95%CI: 22.46%–39.87%; $n = 32$) has a Ct-value < 40. There were 14 samples (13.33%, 95%CI: 7.99%–21.27%) with Ct-value values < 35.00, with the average C_t-value of 30.08 (min. 25.49, max. 33.85). Eighteen samples (17.14%) were called late amplifiers (ten with C_t-values 35 < > 38 and eight with C_t-values 38 < > 40) (Supplementary Data).

The MT-PCR from Laboratory A for *T. foetus* had 41.9% sensitivity and 100.0% specificity; $n = 105$, one samples was inhibited (Table 2, Fig. 1A). The MT-PCR from Laboratory B for *T. foetus* had 35.0%

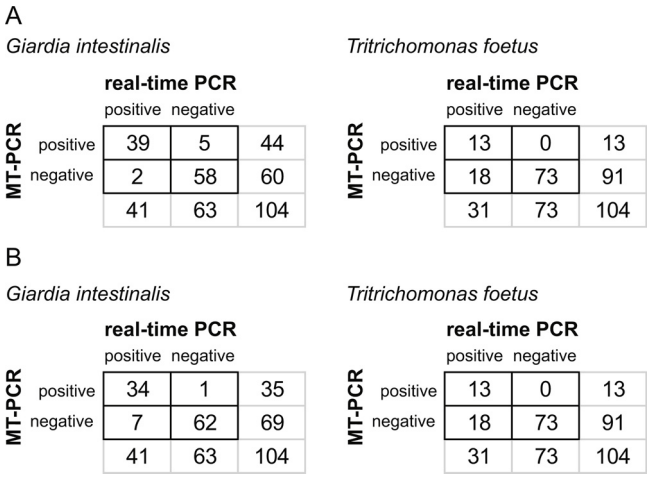

Fig. 1. Comparison of results for *Giardia intestinalis* and *Tritrichomonas foetus* assays. Results from the multiplexed-tandem real-time PCR panel (MT-PCR) were compared to the reference real time PCR assays. The MT-PCR have been performed in Laboratory A and reported either including samples that required manual checking 'check' (A) and those requiring manual check were considered negative (B). Sensitivity and specificity of MT-PCR with the 'check' samples for *G. intestinalis* was 95.1% and 92.1% and for *T. foetus* it was 41.9% and 100.0%, respectively (A). Sensitivity and specificity of MT-PCR without the 'check' samples for *G. intestinalis* was 82.9% and 98.4% and for *T. foetus* it was 41.9% and 100.0%, respectively (B). In total 104 samples were analysed using reference real-time PCR assays; 41 positive for *G. intestinalis* and 31 positive for *T. foetus*.

sensitivity and 100.0% specificity ($n = 64$). If those samples that were automatically called as 'check' in MT-PCR (Laboratory B) were considered negative, the sensitivity was 20.0% and specificity was 100.0%.

3.4. Multiplex real-time PCR assay for detection of Giardia intestinalis and Tritrichomonas foetus

To evaluate the opportunity to multiplex the *G. intestinalis* with *T. foetus* in-house assays, we selected 21 *G. intestinalis* and 20 *T. foetus* samples based on single assay result above; ten of these samples overlapped because they were positive for both parasites (Supplementary Data). Samples positive for *G. intestinalis* had average C_t-values of 26.33 (min. 20.36, max. 33.30) and 52% (11/21) of them were negative for *T. foetus*. Samples positive for *T. foetus* had average C_t-values of 31.90 (min. 25.27, max. 37.32) and 50% (10/20) of them was negative for *G. intestinalis*.

The multiplex assay returned positive result for *G. intestinalis* in all 100% samples ($n = 21$; C_t-values of 28.24, min. 20.51, max. 36.65), that previously tested positive for *G. intestinalis* using a non-multiplexed assay. The multiplex assay returned positive result for *T. foetus* in 85% samples ($n = 17$; C_t-values of 30.18, min. 25.28, max. 38.27) that tested positive for *T. foetus* using a non-multiplexed assay. Three samples that returned negative results for *T. foetus* were those that had a very high C_t-value for *T. foetus* in an independent assay (average 37.04, min. 36.32, max. 37.32) and were negative for *G. intestinalis*. The R^2 (square of the Pearson correlation coefficient) for C_t-values between the *G. intestinalis* and *T. foetus* assays compared to multiplexed assay was 0.83 and 0.56, respectively.

3.5. Presence of Giardia intestinalis Assemblage F and Tritrichomonas foetus genotype 'feline'

Seven *G. intestinalis* real-time PCR positive samples were further analysed to determine *G. intestinalis* assemblage. Using amplification of GDH and SSU rDNA, we determined that all belonged to Assemblage F of *G. intestinalis*. The GDH sequence (487 nt, excluding amplification primers) was 100% identical to a reference gene sequence (EF507597). The SSU rDNA sequence (139 nt, excluding amplification primers) was 100% identical to a reference gene sequence (AF199444), however at this locus and sequenced region, the Assemblage A and Assemblage F of *G. intestinalis* are identical.

Seven *T. foetus* real-time PCR positive samples were further analysed to determined *T. foetus* genotype. The ITS rDNA region (297 nt, excluding amplification primers) was 100% identical to a reference sequence (JX187001) from *T. foetus* genotype 'feline'.

4. Discussion

In small animal practice, *G. intestinalis* is often recognised as the most frequently detected enteric parasite (Bouzid et al., 2015; Palmer et al., 2008). The prevalence ranges between 0–30% and this frequency of detection is highlighted by *G. intestinalis* prominent place in diagnostic PCR faecal panels offered to veterinarians by diagnostic laboratories (Feng and Xiao, 2011; Gizzi et al., 2014; Gruffydd-Jones et al., 2013; Ito et al., 2016; Koehler et al., 2014; Symeonidou et al., 2018; Tysnes et al., 2014).

Faecal PCR panels become a convenient approach for veterinarians, enabling cost effective detection of multiple pathogens simultaneously (Gizzi et al., 2014; Laude et al., 2016; Stanley and Szewczuk, 2005; Stark et al., 2011). The MT-PCR Small Animal Diarrhoea panel was revalidated between two laboratories and against single-plex assays for two main pathogens relevant in differential diagnostics in feline medicine. Both *G. intestinalis* and *T. foetus* are known to be associated with each other, yet the mainstream drug for *G. intestinalis*, metronidazole, is not efficacious for *T. foetus* (Bell et al., 2010; Gookin et al., 2017, 2007). The performance of the MT-PCR Small Animal Diarrhoea panel for *G.*

intestinalis was in good agreement, with excellent sensitivity and specificity compared to the TaqMan assay. The original *G. intestinalis* assay was validated for the human *G. intestinalis* Assemblage A and B (Stark et al., 2011; Verweij et al., 2003). Our study demonstrates that the assay is equally specific for the feline *G. intestinalis* Assemblage F, because all feline samples in this study that were genotyped were confirmed as *G. intestinalis* Assemblage F - the genetically defined subgroup specific for cats (Feng and Xiao, 2011). In clinical diagnostics the *G. intestinalis* assemblage is not determined for cat, dog or human samples (Gizzi et al., 2014; Madison-Antenucci et al., 2016; Stark et al., 2014; Verweij et al., 2004). Most research studies have demonstrated *G. intestinalis* Assemblage F being shed by cats; only in exceptional circumstances were the zoonotic *G. intestinalis* Assemblages A and B detected (Feng and Xiao, 2011; Gruffydd-Jones et al., 2013). Therefore, isolates of *G. intestinalis* causing disease in cats are not considered zoonotic (Gruffydd-Jones et al., 2013). In several circumstances, the *G. intestinalis* call by the automated profile of the MT-PCR Small Animal Diarrhoea panel indicated ambiguity and requested the operator to check the result. A large proportion of such ambiguous *G. intestinalis* results were positive in the single-plex *G. intestinalis* reference assay, enabling adjustments of the automatic call parameters.

Unlike the *G. intestinalis* PCR performance, the *T. foetus* component of the MT-PCR Small Animal Diarrhoea had only poor sensitivity but perfect specificity compared to the reference TaqMan-MGB assay. These differences in the sensitivity are likely the result of the different target genes used by the assays. The TaqMan-MGB assay uses an ITS-1 target, which is multi-copy gene and repeated several times in the genome of the parasite, whereas the MT-PCR assay for *T. foetus* uses the cysteine protease gene which is a single-copy gene. Diagnostics of *T. foetus* is difficult because of its poorly understood pathogenesis, intermittency and load of *T. foetus* shedding (Gookin et al., 2017). The faecal samples positive for *T. foetus* had a high proportion (53%, 16/30) of late amplifiers, determined by an arbitrary threshold of C_t-values > 35. On the other hand, only one (2%, 1/41) *G. intestinalis* positive sample was considered a late amplifier (C_t-values > 35). Such a high proportion of late amplifying samples positive for *T. foetus* suggests the low numbers of *T. foetus* in given samples reached the limit of detection for the *T. foetus* assay (McMillen and Lew, 2006). High sensitivity for *T. foetus* is desired in a clinical situation, where the aim is to quarantine or eliminate the pathogens from individual cat or a cattery. Verification of elimination of *T. foetus* post treatment should be compulsory, however, the probability of undetected occult *T. foetus* shedders may contribute to the maintenance of *T. foetus* in the cat populations, because multiple PCR tests are prohibitive for cat owners due to cost associated with such PCR testing. Availability of probe-based assays for both *G. intestinalis* and *T. foetus* enables multiplexing for simultaneous detection. Both published assays have been validated and are considered reference assays (McMillen and Lew, 2006; Verweij et al., 2003). The *G. intestinalis* assay has previously been implemented in a multiplex assay targeting human enteric pathogens with exceptional sensitivity and specificity in clinical samples (Verweij et al., 2004). Our multiplexed assay confirms exceptional performance of the *G. intestinalis*, yet highlights the difficulties with detection of *T. foetus*.

In conclusion, the MT-PCR Small Animal Diarrhoea had a good and poor performance against reference assays for *G. intestinalis* and *T. foetus,* respectively. The assay is suitable for detection of *G. intestinalis* and moderate or higher burdens of *T. foetus.* To allow for the detection of low-level *T. foetus* infections an ITS based assay may provide the better alternative to the cysteine protease gene.

Conflict of interest

FR, VK were employees of AusDiagnostics, Australia.

Acknowledgements

The authors wish to thank Nichola Calvani and Katrina Gilchrist (University of Sydney) for providing excellent technical help. This work was funded in part by the Veterinary Pathology Diagnostic Services, University of Sydney. The DNA analysis in laboratory A (AusDiagnostics, Australia) was provided *pro bono*. AusDiagnostics, Australia had no role in decision to publish.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2018.12.009.

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