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Research paper

Development and comparative evaluation of different LAMP and PCR assays for coprological diagnosis of feline tritrichomonosis

Joanna Dąbrowska[a], Jacek Karamon[a], Maciej Kochanowski[a], Bruno Gottstein[b], Tomasz Cencek[a], Caroline F. Frey[b], Norbert Müller[b,*]

[a] Department of Parasitology and Invasive Diseases, National Veterinary Research Institute, Partyzantów Avenue 57, 24-100, Puławy, Poland
[b] Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122, 3012, Bern, Switzerland

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ABSTRACT

The protozoan parasite *Tritrichomonas foetus* may cause severe diarrhea in cats all over the world. In order to evaluate the methodology in coprological molecular diagnosis of feline tritrichomonosis, we compared previously published ("old") and newly developed ("novel") loop-mediated isothermal amplification (LAMP) (targeted to the *T. foetus* β-tubulin and the elf1α 1 gene, respectively) as well as an old conventional and an old and novel real-time PCR (all targeted to overlapping regions of *T. foetus* rDNA) assays regarding their diagnostic sensitivities and specificities. Here, the novel real-time PCR yielded the best methodical performance in that a sensitivity with a detection limit of ≤ 0.1 trophozoites (corresponding to *ca.* ≤ 0.13 trophozoites per mg feces) and a maximal specificity for diagnosis of *Tritrichomonas* spp. was achieved. The other test systems exhibited either an approximately 10-times lower sensitivity (≤ 1 trophozoite corresponding to *ca.* ≤ 1.3 trophozoites per mg feces) (conventional PCR and both LAMP assays) or a lower specificity (old real-time PCR). Conversely, the diagnostic performance assessed with clinical fecal samples from cats demonstrated identical sensitivities (8 of 20 samples tested were positive) for the novel PCR and both LAMP assays. Diagnostic sensitivities were significantly higher than those found for the old real-time (5 positive samples) and conventional PCR (6 positive samples), respectively. Accordingly, our data suggested the novel PCR and both LAMP assays to be well suited molecular tools for direct (*i.e.* without including an *in vitro* cultivation step) coprological diagnosis of tritrichomonosis in cats. Interestingly, relative high (novel LAMP, 7 positive samples) to at least moderate (old LAMP, 6 positive samples and 1 sample with equivocal score) diagnostic sensitivities were also achieved by testing clinical samples upon simple visual inspection of colorimetric changes during the LAMP amplification reactions. Accordingly, both LAMP assays may serve as practical molecular tools to perform epidemiological studies on feline (and bovine as well as porcine) tritrichomonosis under simple laboratory conditions.

1. Introduction

Several years ago, infection of cats with the protozoan parasite *Tritrichomonas foetus* supposedly transmitted *via* the fecal-oral route and associated with lymphocytic inflammation, neurophilic colitis, and chronic large bowel diarrhea were identified as an issue of considerable relevance in veterinary medicine (Yao and Koster, 2015). Feline tritrichomonosis occurs worldwide with a prevalence varying from 2 to 59% depending on geographical region investigated (Gookin et al., 2017).

T. foetus is also a sexually transmitted parasite that has been identified as an important cause of infertility and abortion in cattle (Clark et al., 1983). Bovine tritrichomonosis is prevalent in countries such as Southern Africa, or countries from North, Central and South America where natural breeding of the animals is still practiced (Casteriano et al., 2016).

T. foetus was also found in pigs but was regarded as an apathogenic commensal both in the nasal cavity and the intestine. Since *T. foetus* is a non-pathogenic parasite in the porcine host, only preliminary data about the prevalence of the respective infection is available in current literature (Li et al., 2015; Mueller et al., 2015; Pakandl, 1994).

In bovine tritrichomonosis, semen collected for artificial insemination and subjected to testings for *T. foetus*-negativity, preputial washings from bulls and cervicovaginal secretions from cows are samples of diagnostic relevance (Kimsey et al., 1980; Yule et al., 1989). In contrast, feline cases of tritrichomonosis are identified by analysis of fecal

* Corresponding author.
E-mail address: norbert.mueller@vetsuisse.unibe.ch (N. Müller).

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samples (Lim et al., 2010). The simplest methods for detection of *T. foetus* are based on examination of diagnostic samples by light microscopy mostly including a pre-analytical *in vitro* cultivation step for proliferation of the parasite in order to achieve a satisfactory diagnostic sensitivity. Unfortunately, however, microscopical diagnosis may be complicated by contamination of samples with motile moving flagellates (such as apathogenic trichomonad protozoa) which might be misidentified as *T. foetus* (Taylor et al., 1994). Accordingly, testing of samples *via in vitro* cultivation followed by confirmatory PCR specific for *T. foetus* is currently considered the most successful concept for a reliable diagnosis of bovine and feline tritrichomonosis (Frey and Müller, 2012). Conventional and real-time diagnostic PCRs described so far are targeted to either the rRNA gene units (Felleisen et al., 1998; Frey et al., 2017) or the cysteine protease gene (McMillen and Lew, 2006; Meggiolaro et al., 2019) of *T. foetus* and these assays allow a highly sensitive and relatively specific detection of the parasite. As assessed for the real-time PCR developed by Frey et al. (2017), however, false positive results were obtained by testing preputial washings from Swiss Alpine cows that contained a flagellate related to apathogenic *Simplicimonas*-like organism.

In the field of parasitology, loop-mediated isothermal amplification (LAMP) of DNA was tested as a molecular diagnostic alternative for PCR and was already successfully applied for detection of protozoan pathogens such as *Giardia duodenalis* (Plutzer and Karanis, 2009), *Trypanosoma vivax* (Kuboki et al., 2003) and *Toxoplasma gondii* (Lass et al., 2017) in humans or different animal hosts. Moreover, LAMPs based on either the 5.8 s rDNA gene (Oyhenart et al., 2013) or the elongation factor 1 α 1 (elf1α1) gene (Oyhenart, 2018) as target sequences were developed for sensitive and specific detection of bovine tritrichomonosis. LAMP is a simple, rapid, and cost-effective method that basically can be executed without sophisticated equipment needed for DNA amplification (Francois et al., 2011). Reaction is carried out under isothermal conditions in a transparent tube and subsequent inspection of the color change using SYBR®Green as a method of visualizing LAMP amplicons (Njiru et al., 2008).

In the present study, we describe a comprehensive investigation comparing different conventional PCR, real-time PCR and LAMP assays regarding their diagnostic operating characteristics in direct (*i.e.* without involving prior cultivation of the parasite in medium) coprological diagnosis of feline tritrichomonosis. For this purpose, a novel real-time PCR and a novel LAMP assay were extensively evaluated in terms of both their diagnostic sensitivity and specificity, and compared with some of the corresponding published assays (Felleisen et al., 1998; Frey et al., 2017; Oyhenart, 2018).

2. Materials and methods

2.1. Origin of genomic DNA from trichomonad isolates, Giardia duodenalis and Escherichia coli

The origin and preparation of genomic DNA from different trichomonad isolates listed in Table 1 was described by Reinmann et al. (2012) or in the case of the *Simplicimonas*-like isolate by Frey et al. (2017). DNA from *G. duodenalis* clone WBC6 (ATCC 50803) and *Escherichia coli* TOP10 (Thermo Fischer Scientific, Basel, Switzerland) were kindly provided by J. Müller, Institute of Parasitology Bern.

2.2. In vitro cultivation of T. foetus

Trophozoites from the *T. foetus* reference strain (bovine genotype; ATCC 30924) were cultivated *in vitro* in Diamond's medium (Diamond, 1957) supplemented with 11.1% heat-inactivated horse serum and antibiotics (50 IU penicillin–streptomycin). Cultures were incubated at 37 °C for 24 h and subsequently kept at room temperature until the late log-phase was reached. Numbers of parasites were determined microscopically by using a Neubauer counting chamber and subsequently used for genomic DNA preparation or spiking of fecal samples, respectively (see below).

2.3. Spiking of fecal samples with T. foetus trophozoites and DNA extraction

Defined aliquots (150 mg) of a fecal sample from a confirmed (by real-time PCR, see below) negative cat were spiked with 50 µl of suspensions containing serial 1:10 dilutions of cultivated trophozoites from the bovine genotype of *T. foetus* (previously disrupted by vortexing with glass beeds for 10 min and sonification in ice for 30 s) and subsequently subjected to DNA extraction by using the ZR Fecal DNA MicroPrep® kit (Zymo Research, Irvine USA) according to the instructions of the manufacturer. DNA was eluted in 100 µl elution buffer and stored at -20 °C.

2.4. Clinical specimens

A total of 20 fecal samples from chronic diarrheic cats with long-term unsuccessful antibiotics treatment as well as apparent lack of (other) intestinal protozoan and helminthic infections were collected and further examined (by PCR and LAMP, see below) for presence of *T. foetus* at the Institute of Parasitology, National Veterinary Research Insitute (NVRI) in Pulawy, Poland.

2.5. DNA extraction

DNA extraction from *T. foetus* (bovine genotype) cultures with known cell numbers were performed with the DNeasy® Blood and

Table 1
Comparative analyses of specificities of LAMPs and PCRs by testing DNA preparations from different trichomonad species, *Giardia duodenalis* and *Escherichia coli*.

Tested species	TF-βtub-LAMP	TF-elf1α-LAMP	rt-TF-rDNA-1-PCR	rt-TF-rDNA-2-PCR	c-TF-rDNA-PCR
Trichomonas vaginalis	–[a]	–	–	–	–
T. gallinae	–	–	–	–	–
T. gallinarum	–	–	–	–	–
T. mobilensis	+[b]	+	+	+	+
T. foetus (porcine genotype)	+	+	+	+	+
P. hominis	–	–	–	–	–
Simplicimonas-like DNA	–	–	(+)[c]	–	–
Giardia duodenalis	–	–	–	–	–
Escherichia coli	–	–	–	–	–

[a] Negative.
[b] Positive.
[c] Weak-positive but identified as unspecific reaction by demonstration of an untypical melting point of the DNA amplification product (paragraph 3.2).

Tissue Kit (Qiagen, Switzerland) using the protocol for cultured cells. DNA was eluted in 200 µl elution buffer and stored at -20°C. Fecal samples (150 mg) spiked with *T. foetus* trophozoites (see above) as well as clinical samples (150 mg) were performed by using the ZR Fecal DNA MicroPrep® kit according to the instructions of the manufacturer. DNA was eluted in 100 µl elution buffer and stored at -20°C.

2.6. LAMP, real-time PCR and conventional PCR

TF-βtub-LAMP primers were designed using LAMP Primer Explorer version 5 software (http://primerexplorer.jp/e/) based on the of β-tubulin gene sequence of *T. foetus* (GenBank accession no. JX399872.1). The selected TF-βtub-LAMP primer set includes two outer primers (TF-βtub-F3 and TF-βtub-B3) and two inner primers (TF-βtub-FIP (TF-βtub-F1 + TF-βtub-F2) and TF-βtub-BIP (TF-βtub-B1 + TF-βtub-B2) (Fig. S1). *In silico* testing of the primers for specificity by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) demonstrated low sequence identities (94%) to β-tubulin (and other) genes from other related or unrelated organisms (data not shown).

Optimization of the TF-βtub-LAMP assay was carried out by testing different concentrations of inner (10–20 pmol) and outer primers (5–10 pmol) in the reaction mix, and a range of amplification temperatures (50–66 °C) during a time-period between 30 and 120 min. Based on this pre-evaluation, the following protocol for the TF-βtub-LAMP was applied: the total reaction volume was 15 µl and contained 7.5 µl of Isothermal Mastermix (OptiGene, Horsham, UK), 1 µl of each primer (20 pmol TF-βtub-FIB/TF-βtub-BIP and 5 pmol TF-βtub-F3/TF-βtub-B3), 1.5 µl of PCR-grade H_2O (Qiagen, Mat. No. 1073291) and 2 µl of DNA template. Reactions were run for 90 min at 65 °C in the LightCycler® 2.0 Instrument (Roche Diagnostics, Basel, Switzerland). Fluorescence was measured every 30 s with the channel setting F1/1 and fluorescence signals from the amplification products were quantitatively assessed by applying the standard software (version 3.5.3) according to the instructions for the LightCycler® 2.0 Instrument.

The TF-elf1α1-LAMP and corresponding primers were described by Oyhenart (2018) and amplification reactions were essentially performed based on the protocol of the TF-βtub-LAMP (see above) but here reactions were run only for 45 min at 62 °C.

LAMP testings were also performed by applying a protocol allowing visual (by eye) detection of the diagnostic DNA amplification products without relying on a quantification of fluorescence signals by a thermocycler such as the LightCycler® 2.0 Instrument (Wozniakowski et al., 2012). Here, reaction mixes described above were incubated for 90 min at 65 °C (TF-βtub-LAMP) or 45 min at 62°C (TF-elf1α1-LAMP), respectively. After this incubation step, Sybr® Green (10,000x concentrated in DMSO) (SYBR® Green I Nucleic Acid Gel Stain, Invitrogen, Sydney, Australia) was added at a dilution of 1:10. In this assay, SYBR® Green I turned into a yellow-greenish color in positive samples containing LAMP DNA amplification products while negative samples are detectable by their clear orange color (Fig. 1).

Real-time PCRs rt-TF-rDNA-1-PCR (Frey et al., 2017) and rt-TF-rDNA-2-PCR (novel assay, developed in this study) were carried out in the LightCycler® 2.0 Instrument and here amplification reactions (50 cycles) included previously published ("old") primers (here named rt-TF-rDNA-1-PCR-F and rt-TF-rDNA-1-PCR-R) or novel primers (here named rt-TF-rDNA-2-PCR-F and rt-TF-rDNA-2-PCR-R) and fluorescence resonance energy transfer (FRET) DNA hybridization probes (here named rt-TF-rDNA-1/2-PCR-FRET-1 [3′fluorescein labelled probe] and rt-TF-rDNA-1/2-PCR-FRET-2 [5′-LC-Red 640 labelled probe]) (Frey et al., 2017) for detection of the amplification products from both real-time PCRs. In the case of previously developed ("old") rt-TF-rDNA-1-PCR, amplification reactions were exactly done according to Frey et al. (2017). In the case of the novel rt-TF-rDNA-2-PCR, however, the protocol was modified in that 3′truncated derivatives of primers (here named primer rt-TF-rDNA-2-PCR-F and rt-TF-rDNA-2-PCR-R) from the previously described conventional rDNA-PCR (Felleisen et al., 1998; here named c-TF-rDNA-PCR) were used. The reaction mixes of the real-time PCRs contained uracil DNA enzyme and dUTP as substitute of dTTP to prevent carry-over contamination (Longo et al., 1990).

For both real-time PCRs and LAMPs, quantitation of amplification products was achieved by plotting the fluorescence signals *versus* the cycle number (real-time PCRs) or time (LAMPs) at which the signals crossed the baseline (Figs. 1 and 2). Adjustment of the baseline was performed by using the "minimize error" mode. Positive samples were identified by a fluorescence signal which accumulated to values above the baseline within the reaction period.

After completion of the amplification reaction, a melting-curve was performed by continuously increasing the temperature from either 50 °C to 95 °C (real-time PCRs) or 65 °C to 95 °C (LAMPs), and measuring loss of fluorescence signal using corresponding software (v.3.5.3) (Roche Diagnostics). Melting-peaks at about 65.0 °C (rt-TF-rDNA-1-PCR), 57.5 plus 65.5 °C (rt-TF-rDNA-2-PCR), 90.0 °C (TF-βtub-LAMP), and 87.5 °C (TF-ef1α1-LAMP) indicated specific amplification of *T. foetus* DNA.

The c-TF-rDNA-PCR including primers c-TF-rDNA-PCR-F and c-TF-rDNA-PCR-R directed to the rDNA units of *T. foetus* was done according to Felleisen et al. (1998). Here again, the reaction mixes included the UDG-based prevention of carry-over contaminations by amplification products (Longo et al., 1990).

In each LAMP, conventional PCR and real-time PCR run, a positive control containing 1 µl of *T. foetus* DNA (bovine genotype; ATCC 30924) equivalent to about 10 trophozoites and a negative control containing 1 µl of H_2O was included. Furthermore, 1 µl positive control DNA was added to duplicates from clinical sample reactions in order to monitor possible inhibitory effects within the amplification reactions.

Sequences from all primers and FRET-DNA probes and respective names used in the present study are listed in Table S1. This table also includes original names for those primers and probes that had already been published elsewhere (see Felleisen et al., 1998; Frey et al., 2017; Oyhenart, 2018).

3. Results

3.1. Methodical sensitivities of conventional PCR, real-time PCR and LAMP methods

Testing of serial 1:10 dilutions of genomic DNA from *T. foetus* (bovine genotype) by the different DNA amplification assays allowed detection of DNA equivalent to 1 parasite (TF-βtub-LAMP, TF-elf1α1-LAMP and c-TF-rDNA-PCR) or 0.1 parasite (rt-TF-rDNA-1-PCR, rt-TF-rDNA-2-PCR), respectively (Figs. 1 and 2). Furthermore, DNA standard curves revealed $\log_{10}$ slope values that were equivalent for 10-times DNA amplification rates (Figs. 1 and 2). In the case of the real-time PCRs, 10-times DNA amplification was achieved within 4.132 (rt-TF-rDNA-1-PCR) and 4.879 (rt-TF-rDNA-2-PCR) cycles suggesting a higher amplification efficiency of the rt-TF-rDNA-1-PCR as compared to the rt-TF-rDNA-2-PCR. In the case of LAMPs, 10-times DNA amplification took 7.139 min (TF-elf1α1-LAMP) and 14.4 min (TF-βtub-LAMP), respectively. Accordingly, TF-elf1α1-LAMP exhibited approximately the double amplification efficiency as TF-βtub-LAMP.

Methodical sensitivities of TF-βtub-LAMP and TF-elf1α1-LAMP *via* visual colorimetric determination turned out to be $\leq$ 1 parasite for both assays (Fig. 1).

The differential efficiencies of the different diagnostic DNA amplification assays were demonstrated by a 6-times repetitive testing of DNA from feline fecal samples spiked with serial 1:10 dilutions of cultivated *T. foetus* trophozoites (Table 2). Respective analyses revealed that the detection limits were consistently (*i.e.* 6 positive scores in 6 testings) 1 parasite (corresponding to *ca.* 1.3 parasites per 1 mg feces) per reaction in the cases of TF-βtub-LAMP, TF-elf1α1-LAMP and c-TF-rDNA-PCR. Conversely, a 10-times lower detection limit, namely 0.1 parasite (corresponding to *ca.* 0.13 parasites per 1 mg feces) per

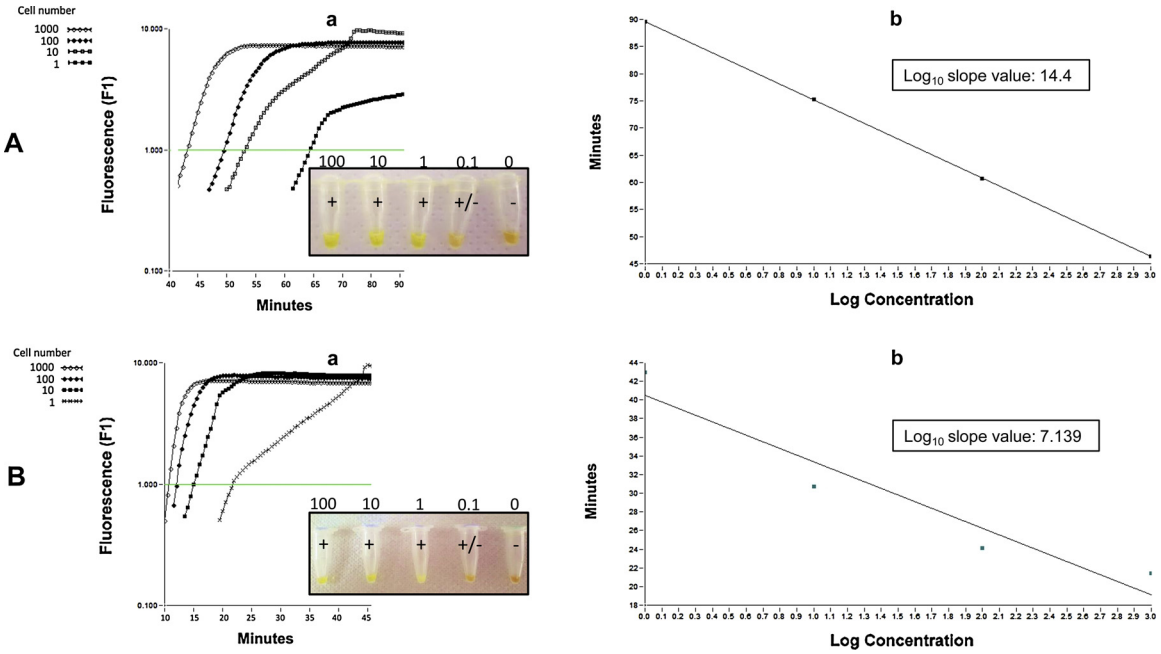

Fig. 1. From TF-βtub-LAMP (A) and TF-elf1α1-LAMP (B), amplification plots (a) and corresponding standard curves (b) representing time threshold values (*i.e.* time-point during the reaction at which the signals crossed the baseline indicated as a horizontal line) from reactions for 1000, 100, 10, and 1 parasite(s) are shown. Reactions for 0.1 parasites and the negative control were negative (not shown). Log_{10} slope values (in min) were equivalent for 10-times DNA amplification rates. TF-βtub-LAMP (insert in Fig. 1A, panel a) and TF-elf1α1-LAMP (insert in Fig. 1B, panel a) analyses of 100, 10, 1, 0.1 and 0 parasite(s) by visual inspection of tubes regarding colorimetric changes during the DNA amplification reaction were scored positive (+), equivocal (+/−) or negative (−) as indicated.

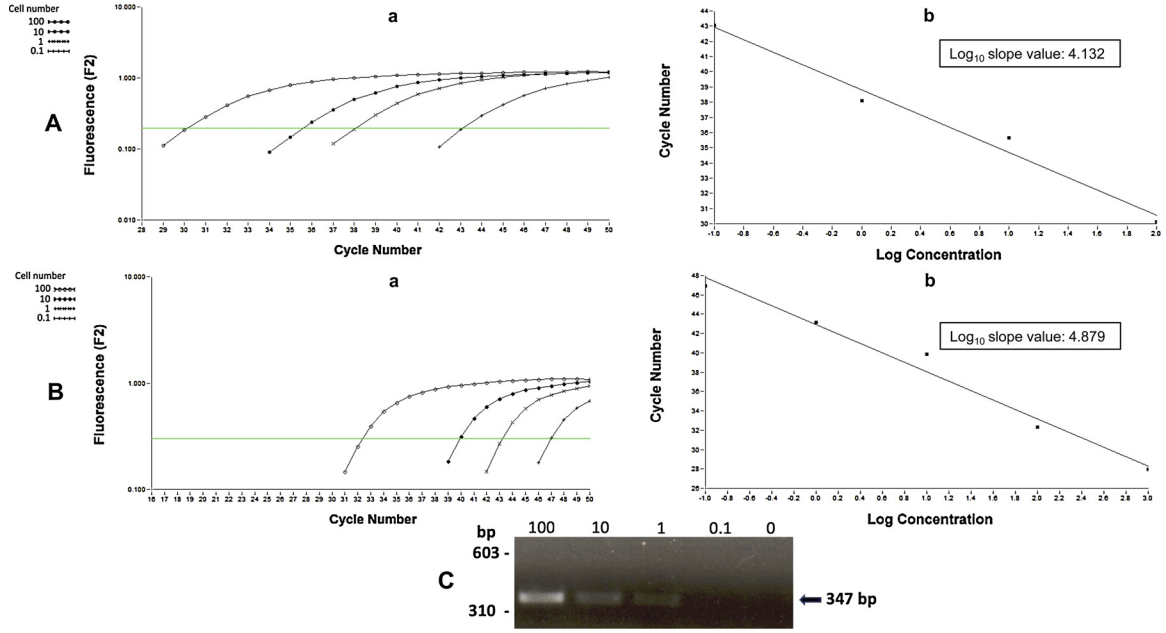

Fig. 2. From rt-TF-rDNA-1-PCR (A) and rt-TF-rDNA-2 -PCR (B), amplification plots (a) and corresponding standard curves (b) representing cycle threshold values (*i.e.* cycle numbers at which the signals crossed the baseline indicated as a horizontal line) from reactions for 100, 10, 1, and 0.1 parasite(s) are shown. Reactions for 0.01 parasites and the negative control were negative (not shown). Log_{10} slope values (number of cycles) were equivalent for 10-times DNA amplification rates. For the c-TF-rDNA-PCR, amplification products from genomic DNA equivalent to 100, 10, 1, 0.1, and 0 (negative control) parasite(s) were analysed by 2% agarose gel electrophoresis. Size markers (on the left) and approximate sizes of amplification products (on the right) are given in base pairs.

reaction was consistently observed for both real-time PCRs.

In conclusion, as compared to LAMP and conventional PCR assays, the two real-time PCRs exhibited an approximately 10-times higher methodical sensitivity in detecting *T. foetus* DNA in feces from cats.

3.2. Diagnostic specificities of conventional PCR, real-time PCR and LAMP methods

The different diagnostic DNA amplification assays were evaluated regarding their specificities by testing the amplification of DNAs (10 ng DNA per reaction) from various trichomonads including human pathogen *Trichomonas vaginalis* and different animal pathogens

Table 2
Comparative analyses of methodical sensitivities of LAMPs and PCRs for detection of *Tritrichomonas foetus*.

No. of *T.foetus* cells	TF-βtub-LAMP (n = 6)		TF-elf1α-LAMP (n = 6)		rt-TF-rDNA-1-PCR (n = 6)		rt-TF-rDNA-2-PCR (n = 6)		c-TF-rDNA-PCR (n = 6)	
	water[a]	feces[b]	Water	feces	water	feces	water	feces	water	Feces
100	100[c]	n.d.	100	n.d.	100	n.d.	100	n.d.	100	100
10	100	100	100	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100	100	100	100
0.1	0	0	0	0	100	100	100	100	0	0
0.01	n.d.[d]	n.d.	n.d.	n.d.	0	0	0	0	n.d.	n.d.

[a] Serial 1:10 dilutions of DNA equivalents from a known number of *T. foetus* trophozoites in water.
[b] DNA preparations from fecal samples containing serial 1:10 dilutions from a known number of *T. foetus* trophozoites.
[c] Percentages of positive results from 6 parallel testings (n = 6) are given.
[d] Not determined.

Tritrichomonas mobilensis, a porcine genotype of *T. foetus* (formerly classified as *Tritrichomonas suis*), *Trichomonas gallinae*, *Tetratrichomonas gallinarum,* and *Pentatrichomonas hominis* as well as an apathogenic *Simplicimonas*-like organism previously identified in vaginal swabs of cattle (Frey et al., 2017) (Table 1). Here, all assays scored negative for *T. vaginalis* and *T. gallinae*, *T. gallinarum,* and *P. hominis* DNA. Overall negativity was also observed for the protozoan enteropathogen *G. duodenalis* and the enterobacterium *E. coli*. As expected, however, all assays amplified DNA from the *Tritrichomonas* genus, namely *T. mobilensis* and the porcine genotype of *T. foetus* thus basically excluding a discrimination between these organisms and the bovine genotype of *T. foetus*. As far as the *Simplicimonas*-like organism is concerned, an amplification signal was exclusively detected by the rt-TF-rDNA-1-PCR. In concordance with previous findings (Frey et al., 2017), however, this result was recognized as false-positive because the amplification product of the *Simplicimonas*-like DNA exhibited a peak melting-point at about 59 °C, whereas the *T. foetus* product had its melting-peak at about 65 °C (data not shown).

In conclusion, all diagnostic DNA amplification assays evaluated are basically suited to perform a specific diagnosis of tritrichomonosis in different hosts.

3.3. Evaluation of diagnostic sensitivities from LAMP, real-time PCR and conventional PCR assays with clinical samples

For the determination of diagnostic sensitivities of the different DNA amplification assays, amplification reactions were performed in duplicates with DNA preparations from fecal samples representing 20 suspected feline tritrichomonosis cases (see Materials and methods). As shown in Table 3, TF-βtub-LAMP, TF-elf1α1-LAMP and rt-TF-rDNA-2-PCR equally performed best in that 8 of 20 samples scored positive. In comparison, rt-TF-rDNA-1-PCR and c-TF-rDNA-PCR clearly exhibited a lower diagnostic sensitivity because positive results were achieved only for 5 and 6 samples, respectively. In all positive PCR and LAMP assays demonstration of DNA melting-peaks at about 65.0 °C (rt-TF-rDNA-1-PCR), 57.5 plus 65.5 °C (rt-TF-rDNA-2-PCR), 90.0 °C (TF-βtub-LAMP), and 87.5 °C (TF-ef1α1-LAMP) indicated specific amplification of *T. foetus* DNA. Parallel testing of the samples in presence of DNA equivalents of approximately 10 *T. foetus* trophozoites (inhibition controls, see Materials and methods) did not provide any evidence for false-negative reactions. Diagnostic sensitivities of TF-βtub-LAMP and TF-elf1α1-LAMP *via* visual colorimetric determination turned out to be moderate in that 6 of 8 positive clinical samples (plus one sample with equivocal score) were unambiguously identified in both assays.

4. Discussion

Routine diagnosis of feline tritrichomonosis can be carried out by simple microscopical demonstration of the parasite in *in vitro* cultures that had been inoculated with fecal material from diseased cats. Here, *in vitro* cultivation of *T. foetus* can be performed by using Diamond's medium (Diamond, 1957) or an InPouch® test system (Megacor, Lindau, Germany) both recommended to achieve a relatively selective growth of *T. foetus* trophozoites starting from fecal samples. However, these two *in vitro* cultivation systems are considered to be of limited coprological practicability *e.g.* because morphological identification of the parasite *per se* and overgrowth of enteric bacterial contaminations make microscopical reading of the culture assays notoriously difficult. For this reason, a biphasic coprological method including time-consuming parasite proliferation by *in vitro* cultivation and subsequent detection of the enriched parasite material by PCR has gained increasing diagnostic importance in many diagnostic facilities. In order to short-cut this laborious diagnostic scheme, we now evaluated different PCR and LAMP assays regarding their suitability as molecular tools for a direct coprological diagnosis of feline tritrichomonosis without relying on an *in vitro* cultivation step.

Direct coprological diagnosis of *T. foetus* infections in cats based on real-time PCR was already successfully demonstrated earlier (McMillen and Lew, 2006; Meggiolaro et al., 2019). Conversely, conventional PCR (Felleisen et al., 1998) and LAMP (Oyhenart et al., 2013; Oyhenart, 2018) were only applied for detection of *T. foetus* in bovine genital fluid and the respective diagnostic approach involved *in vitro* cultivation of the parasite prior to the amplification reaction. In the present study, the conventional PCR (in this study named c-TF-rDNA-PCR) described by Felleisen et al. (1998) was included in our comparative diagnostic evaluation because this test is recommended by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals as one of the basic molecular diagnostic method for the identification of *T. foetus* (OIE, 2018). Conversely, our reasons for evaluating the LAMP methodology complementary to PCR were as follows: (i) in coprology, the PCR methodology must be regarded with caution because the DNA amplification reactions may be affected by inhibitory fecal substances residing in the DNA preparations (Stauffer et al., 2008), and (ii) conversely, LAMP has proven effective in coprological practice because GspSSD LF polymerases mediating the respective isothermal amplification reaction were found to be highly resistant to such inhibitors (Kemleu et al., 2016).

Our study led to the development of a novel real-time PCR (named rt-TF-rDNA-2-PCR) and a novel LAMP (named TF-βtub-LAMP) both allowing reliable detection of *T. foetus* in fecal samples from cats. The development of the real-time PCR was based on 3′truncated primers used for the previously described rDNA-targeted conventional PCR (Felleisen et al., 1998). Furthermore, in contrast to a previously developed TaqMan real-time PCR test (McMillen and Lew, 2006; Meggiolaro et al., 2019), our assay included a FRET hybridization probe DNA detection system that had particularly been adapted to the Light Cycler® Instrument.

Actually, our novel rt-TF-rDNA-2-PCR was developed to replace a

Table 3
Comparative analyses of diagnostic sensitivities of LAMPs and PCRs by testing DNA preparations of fecal sample from 20 diarrheic cats with suspected tritrichomonosis.

Sample Number (n = 20)	TF-βtub-LAMP		TF-elf1α-LAMP		rt-TF-rDNA-1-PCR	rt-TF-rDNA-2-PCR	c-TF-rDNA-PCR
	LightCycler®[a]	Colorimetric[b]	LightCycler®	Colorimetric			
1	+[c]	−[d]	+	−	+	+	+
2	+	+	+	+	+	+	−
3	+	+	+	+	−	+	+
4	+	+	+	+	+	+	−
5	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
7	+	+	+	+	−	+	+
8	+	+/−[e]	+	+/−	−	+	+
9	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−
13	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−
16	−	−	−	−	−	−	−
17	−	−	−	−	−	−	−
18	−	−	−	−	−	−	−
19	−	−	−	−	−	−	−
20	−	−	−	−	−	−	−

[a] LAMP analysis in the LightCycler® Instrument.
[b] LAMP analysis by visual inspection of colorimetric changes during the DNA amplification reaction.
[c] Positive.
[d] Negative.
[e] Equivocal.

previous rDNA-targeted real-time PCR (Frey et al., 2017) (now renamed rt-TF-rDNA-1-PCR) that exhibited unsatisfactory sensitivity (N. Müller, unpublished observation) and specificity (Frey et al., 2017) in our coprological diagnostic application. In more concrete terms, the diagnostic specificity was considered insufficient because we observed an unexpected amplification reaction with DNA from an apathogenic *Simplicimonas*-like organism previously identified in vaginal swabs of cattle (Frey et al., 2017; Table 1). Conversely, the reduced diagnostic sensitivity became obvious in that diagnostic samples positive in conventional PCR occasionally scored negative in the rt-TF-rDNA-1-PCR (N. Müller, unpublished observation). This latter observation was confirmed in the present study where the diagnostic sensitivity of rt-TF-rDNA-1-PCR was lower as compared to all other molecular amplification assays evaluated (Table 3). Although not clarified yet, a limited (but undetectable *via* inhibitory controls) robustness of the rt-TF-rDNA-1-PCR to inhibitory fecal components residing in the DNA preparations might explain the reduced diagnostic sensitivity of this test.

The conventional (Felleisen et al., 1998) and real-time (Frey et al., 2017) PCRs used for detection of *T. foetus* by us so far are targeted to conserved regions of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions (ITS1 and ITS2) (Felleisen et al., 1998; Frey et al., 2017). Therefore, we initially decided to evaluate the respective sequences also regarding their suitability as targets for LAMP (data not shown). In a pilot study (data not shown), however, LAMP performed much better when the β-tubulin gene of *T. foetus* served as target for the amplification reaction. We selected this additional target because the β-tubulin gene turned out to be a widely used DNA marker for parasite detection (Elard and Humbert, 1999; Grant and Mascord, 1996) or species identification as *e.g.* demonstrated for *Trichomonas vaginalis* (Madico et al., 1998; Schirm et al., 2007; Simpson et al., 2007).

In order to assess the suitability of the LAMP methodology in coprological molecular diagnosis of feline tritrichomonosis, we compared the novel TF-βtub-LAMP with a previously described LAMP assay (targeted to the elf1α1 gene, named TF-elf1α1-LAMP) that had been successfully applied for diagnosis of bovine tritrichomonosis (Oyhenart, 2018). Due to the lack of an objective gold standard for the evaluation of coprological diagnosis of *T. foetus* infections, we considered it important to perform an extensive methodical sensitivity and specificity assessment of LAMP in comparison to the different conventional and real-time PCR tests available. An integral view on the individual methodical sensitivities (as determined *via* testing on spiked samples, Table 2) and specificities (as determined *via* testing on different trichomonads species, Table 1) of the different DNA amplification assays identified the rt-TF-rDNA-2-PCR as the best-performing test allowing exclusive detection of *Tritrichomonas* species at a detection limit of ≤ 1 trophozoite (Table 1). Refering to the analyses of diagnostically relevant fecal samples from diarrheic cats (Table 3), however, at least the two LAMP assays exhibited the same performance as rt-TF-rDNA-2-PCR. Accordingly, all three these tests can be regarded as molecular tools that are well suited for direct coprological diagnosis of tritrichomosis in cats. The maximal consistency of the diagnostic performance in these three tests provided high confidence that the respective results (8 positive *versus* 12 negative scores) reflected the effective *T. foetus* infectivity status of the 20 clinical samples tested. This consideration is important because in our study neither reliable *in vitro* cultivation data, genotypic data (*i.e.* sequencing-based confirmation of feline genotype) from the *T. foetus* trophozoites detected in the fecal samples nor extensive anamnestic and clinical data about the samples were available as backup information.

Although supposedly being irrelevant in most clinical settings it is important to note that all assays included in the study scored positive for *T. mobilensis*. This common characteristics of the tests has the advantage that both LAMP and PCR can basically be used for diagnosis of *T. mobilensis* infections of squirrel monkeys and other mammals representing potential hosts of this parasite (reviewed in Frey and Müller, 2012). At the same time, however, these tests have the disadvantage that they cannot discriminate *T. mobilensis* from *T. foetus*.

Interestingly, at least satisfactory diagnostic sensitivities were also achieved by LAMP-based testing of clinical samples through simple visual inspection of colorimetric changes during the isothermal LAMP amplification reactions. This option makes LAMP a valuable methodology for direct dectection of *T. foetus* in fecal samples without relying

on a hig-tech laboratory specialized on molecular diagnostics. Accordingly, the colorimetric LAMP assays described in the present study may serve as practical molecular tools to perform epidemiological studies on feline, bovine and porcine tritrichomosis under simple laboratory conditions.

Declaration of Competing Interest

The authors declare no conflicting interests.

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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.2019.07.014.

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