Validation of molecular diagnostic tests for detection of *Tritrichomonas* in Swedish cats

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Abstract

Tritrichomonas foetus is a protozoan parasite that infects the gastro-intestinal tract of cats. Symptoms of infection often include loose stool and diarrhea, and asymptomatic chronic infection is not unusual if the infection goes untreated. Transmission between cats happens through the faecal-oral route, and even if the cat is asymptomatic, it can still transmit the parasite. Molecular diagnostic testing, such as PCR, has improved the detection of *T. foetus.* The aim of this study was to validate and compare two different PCR systems and different methods of sample preparation with variation in faecal sample size. The chosen method was optimized and tested in a field study on 50 Swedish domestic cats. The results show that sample preparation influences the performance of the PCR analysis. Smaller quantities of faeces showed lower Cq-values in general. Both PCR systems that were tested successfully detected *T. foetus* DNA throughout all dilution levels. The in-house system generally showed slightly lower Cq-values, as well at higher primer and probe concentration.

One positive sample was detected in the field study, resulting in a prevalence of 2% out of the 50 cats. The positive sample originated from a male cat, between 1-5 years of age living in a multi-cat household. There were no statistically significant associations between infection status and the categories that were investigated. Due to the small sample size, no real conclusion can be drawn that there is no association. The results of this study show that PCR methods are useful for detecting *T. foetus* and support the continued use of these methods in veterinary and agricultural diagnostics. Although a low prevalence was seen, the findings confirms that the parasite indeed is present in Sweden. Further studies, including a larger and more geographically representative sample size, are needed to estimate the true prevalence and understand the risk factors with feline trichomoniasis.

Keywords: domestic cats, tritrichomonas foetus, PCR, molecular diagnostics

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Abbreviations

Abbreviation	Meaning
PCR	Polymerase Chain Reaction
LOD	Limit of Detection
N/A	Not Available
DNA	Deoxyribonucleic acid
Cq	Cycle threshold
MQ	Milli-Q
T.F	Tritrichomonas foetus – system F
T. V	Tritrichomonas foetus – system V

1. Introduction

Parasitic infections are a well-known problem in animals affecting their health and welfare. The parasite *Tritrichomonas foetus* is an obligate, single-celled protozoan that infects the reproductive system of bovines and the gastrointestinal tract of felines (Dąbrowska et al. 2020b). The parasite was first described in bovines as a sexually transmitted infection that can result in infertility or abortion (Yaeger & Gookin 2005). The bovine strain does not currently exist in Sweden, and any case of infection is subjected to mandatory reporting.

In felids *T. foetus* was first discovered in the 1990s, when it was identified as the cause of clinical issues in domestic cats, with diarrhoea as the main symptom (Yao & Köster 2015a; a). Although diarrhoea is the most common clinical sign, hosts can also be asymptomatic chronic carriers of the parasite. It was first recognised in the USA, but further studies have shown that the protozoan is relatively common in many parts of the world, with a prevalence of around 10-20% in European countries (Profizi et al. 2013a; Dąbrowska et al. 2020a).

T. foetus is transmitted between cats via the faecal-oral route. Although *T. foetus* does not survive well in the environment, it can persist for several days in faeces (Gookin et al. 2017).The risk of *T. foetus* outbreaks appears to be higher in areas with high housing density, such as catteries and breeding facilities (Gookin et al. 2004a; Kerek et al. 2025). The feline and bovine *T. foetus* are morphologically identical. There are minor differences among the loci, and larger differences in protease inhibitors of the different isolates. Based on these molecular differences there has been debate about separating them into two distinct species (Yao & Köster 2015b).

The diagnostic methods available for detecting *T. foetus* include microscopic examination of faecal smears and/or cultivation in specific media. More recently, polymerase chain reaction (PCR) has been used. PCR is now a widely applied method for detecting and diagnosing a wide range of pathogens of medical interest, including parasites in livestock and companion animals.

Although non-lethal, *T. foetus* causes clinical symptoms that can affect health and welfare in both the short and long term. It is therefore important to diagnose it with high sensitivity and accuracy to prevent further spread. In addition, emerging antiparasitic resistance in *T. foetus* is a cause for concern given the increasing global microbial resistance (Kerek et al. 2025).

The purpose of this study is to validate various PCR protocols for detecting *T. foetus* in faecal samples from domestic cats.

2. Background

2.1 Biology and life cycle

The parasitic *Tritrichomonas foetus* belongs to the family *Trichomonadidae* which is known to cause illness in felines and bovines, although significant genetic differences between the infectious isolates have recently led to debate about reclassifying them as separate species (Walden et al. 2013). In cats, the parasite can cause various gastro-intestinal symptoms, particularly chronic diarrhoea (Yao & Köster 2015a). The cell of *T. foetus* is pyriform in shape, has an undulating membrane, and three anteriorly positioned flagella (Germot et al. 1996; Frey & Müller 2012). Its only known life-stage is trophozoite form, and it has not been found to develop cysts. Reproduction occurs through binary fission, as it does not produce eggs. Both size and shape make it similar to *Giardia intestinalis*, although *T. foetus* trophozoites are larger with a length between 10-25 μm and a width of 3-15 μm. Another distinguishing feature is the motility as *T. foetus* exhibits a jerk-like motion (Yao & Köster 2015a). Instead of typical mitochondria, *T. foetus* has hydrogenosomes which allows it to survive in the oxygen-depleted environment of the gastro—intestinal tract (Makiuchi & Nozaki 2014; Tolbert & Gookin 2016). *T. foetus* primarily obtains its nutrition from bacteria (Manning 2010). Some veterinarians recommend a diet high in fibre to help control and relieve the symptoms in infected cats (*Tritrichomonas foetus infection in cats* 2025). The most common treatment is ronidazole at 30 mg per kg for 14 days, which is currently a licensed medication in Sweden and has been reported to cause neurological signs (Gookin et al. 2010; Hinney et al. 2019).

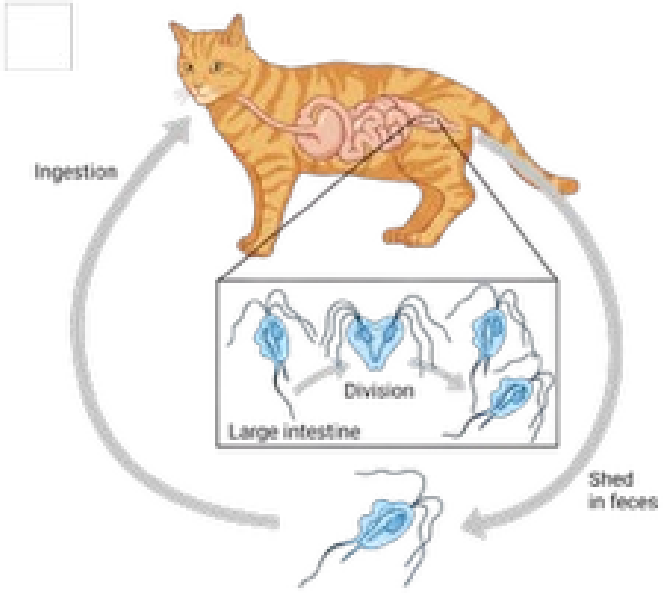


Figure 1 The life cycle of T. foetus in the domestic cat (Lappin 2016)

2.2 Infection and causes

The symptoms of feline trichomoniasis result from changes in the intestinal lumen. Breakdown of the protective mucus and glycoconjugates, which form a protective layer over the epithelial cell and the lamina propria, allows parasitic invasion. Inside the epithelial cells, *T. foetus* is able to avoid the immune response which allows it to cause chronic issues (Tolbert & Gookin 2016). Common symptoms include diarrhoea or faeces of looser consistency, but cats can also be asymptomatic carriers (*Tritrichomonas blagburni* 2025). The faeces produced by infected cats are often mixed with mucus, and sometimes blood. As the infection is localised in the colon, most cats infected with the parasite maintain relatively good health and body condition. The duration of which symptoms are visible after trichomoniasis can range from 1 day, up to 8 years (Gookin et al. 2017).

2.3 Diagnostic testing

There are currently diagnostic methods available to test and diagnose *T. foetus* in domestic cats. These methods include both molecular and microscopic techniques. The survival of live trophozoites in the environment as described in the literature is not very long. A study observing the duration that live, cultivation grown *T. foetus* trophozoites could survive in different environments concluded that the parasites could survive for at least 180 minutes in urine, up to 180 minutes in wet cat food, and 15 minutes on filter paper. No survival was observed on cat litter (Rosypal et al. 2012). Both faecal smear and cultivation are recommended for use

with freshly voided faeces. PCR can typically be performed days after voiding, provided the samples are kept refrigerated or frozen.

2.3.1 Faecal smear

A faecal smear is a type of wet mount, which uses a microscopic method to diagnose *T. foetus*. Freshly voided faeces are collected, and a small portion is dispersed in one to two drops of saline solution (0.9% NaCl) and mounted on a microscope slide. The slide is then examined for live trophozoites under the microscope at 10x magnification and confirmed at 40x (Gookin et al. 2003).

2.3.2 Cultivation

Cultivation of live trophozoites can be performed using InPouch cultivation bags or in Diamond's medium. The sensitivity of these methods is estimated to be between 85% and 95% (Rae & Crews 2006). InPouch cultivation is a system produced by Biomed Diagnostics Inc. The method requires 0.025-0.05g of fresh faeces which is inoculated into the medium and results can be read after 15 minutes. The InPouch can be incubated for up to 12 days at 37°C. In the InPouch cultivation bag, other protozoa such as *Giardia intestinalis* and *Pentatrichomonas hominis* may also grow, making differentiation between species difficult (*InPouch TF Feline - Biomed Diagnostics, Inc.* u.å.). To diagnose *T. foetus* using Diamond's medium, direct examination can be performed with an inverted microscope, or by collecting 0.1 mL of the cultivation fluid from the bottom of the tube, and mounting it on a microscope slide (Rae & Crews 2006). A PCR test is recommended to confirm a positive *T. foetus* result with both cultivation methods.

2.3.3 Polymerase chain reaction

The PCR method detects specific DNA strands using unique primers and probes. Oligonucleotides, also called primers, bind to their complementary bases in the target DNA. A forward and a reverse primer each bind to one of the DNA strands and are designed to match a sequence of the target (*DNA Primer - an overview | ScienceDirect Topics* u.å.). DNA strand copying is achieved by cycling the sample through different temperatures in a PCR machine. A high temperature (often 95°C) separates the two DNA strands. The temperature is then lowered for the primers to bind to their targets, and lowered further for the polymerase to extend the primers and begin the copying (Kubista et al. 2006). The development of PCR methods has provided opportunities to diagnose and address many problems in the agricultural sector. It has enabled scientists and veterinarians to detect parasitic infections with a small amount of material, quickly and with high sensitivity. One important application of PCR is the detection of parasitic infections in farm and production animals. Parasites such as the barber's pole

worm *Haemonchus contortus* in sheep, which feeds on blood and can cause anaemia and reduced productivity (Besier et al. 2016), and the large bloodworm *Strongylus vulgaris* in horses, which can cause colic through damage to blood vessels, can both be detected through PCR (Andersen et al. 2013). This has allowed farmers and pet owners to achieve better disease monitoring and control. Detection of these infections at an early stage can increase animal welfare because it helps veterinarians provide the correct treatment and helps farmers to apply targeted treatment to the animals that need it. Targeted deworming reduces unnecessary drug-use and helps slow the development of drug resistance in parasites. Thus, PCR contributes to healthier animals and more sustainable farming practices. It can also be used in plant- and soil agriculture by detecting pathogens that infect crops, water or soil (Khan et al. 2007; Zhang et al. 2025).

2.4 Prevalence

The first finding of *T. foetus* was in the 1900s, when three cows suffering from infertility were examined. The name *Tritrichomonas foetus* was not proposed until 1928 when Riedmuller studied cases of aborted foetuses and linked *T. foetus* to nine of them. The first recorded prevalence of *T. foetus* in the United States occurred in 1932 when trichomonads were found in two cows suffering with infertility. Microscopic examination of vaginal smears was used as the diagnostic method (Rae & Crews 2006).

In felids, *T. foetus* infection was determined as a cause of gastro-intestinal symptoms in the early 2000s. The prevalence of the parasite has been confirmed in many parts of the world, including Europe, Asia, Oceania and North America. True prevalence in these regions is difficult to estimate due few studies, risk for selection bias in the studies, as well as too few case reports (Yao & Köster 2015a).

In Europe, prevalence levels in cats vary considerably across studies, ranging from 5% to 31%. This variation may be explained by differences in study populations, diagnostic methods, sample sizes or the living arrangements of the cats. Higher prevalence is usually observed in cats from multi-cat housing such as catteries, and at cat shows where populations often originate from multi-cat households.

Table 1 Prevalence of T. foetus in cat populations including country, study population, diagnostic method and sample size.

Country	Study population	Diagnostic method	Samples	Prevalence	Reference

UK	Cats with diarrhea	PCR	111	14%	(Gunn-Moore et al. 2007)
France	Cattery-housed	Cultivation (InPouch)	140	14.3%	(Profizi et al. 2013b)
-	International cat show	Faecal smear, cultivation and PCR	117	31%	(Gookin et al. 2004b)
Poland	Veterinary clinic	PCR	1606	10.6%	(Jańczak et al. 2025)
Italy	Multiple housings	PCR	267	5.2%	(Veronesi et al. 2016)
Norway	Cat shows	PCR	52	21%	(Tysnes et al. 2011)

3. Materials and methodology

3.1 Structure of the study

This study is structured with a background describing the origin and spread of the parasite *T. foetus* both worldwide and in Sweden. It also discusses information about how the parasitic infection affects the domestic cat as its host animal, what symptoms can be seen and what treatments are available. The information about the background of the parasite is gathered from online scientific resources and papers, most commonly Google Scholar and Scopus. Information and sources found were handled objectively. The study includes practical laboratory work, including the use of laboratory equipment, different solutions and reagents and handling of positive DNA. A field study was made to test the methods and PCR system. Artificial intelligence tools were used to correct spelling and grammar, and for identifying synonyms.

3.2 Sampling

This study used 50 faecal samples from domestic cats in Sweden. The samples were obtained from cats of all ages, with or without the typical symptoms shown during trichomonas infection. Cats were included regardless of breed. The cats in the study came from regular cat owners, catteries or breeders. Faecal samples were collected by retrieving approximately two teaspoons of fresh faeces with minimal sand contamination and stored in a sealed plastic bag. The data that was collected included information about the cat's age, sex, location and if it came from a single cat or multi-cat living space. Information was not available for all cats. The samples analysed were no more than 7 days old. The samples were either kept in the fridge at maximum 8°C for a maximum of one week or stored in the freezer at minimum -20°C before DNA extraction. After extraction, the samples were analyzed in the PCR machine for 7 days. During this time the extracted samples were kept in the fridge at a maximum temperature of 8°C.

3.3 DNA-extraction

Experiments comparing different amounts of faecal material was conducted to evaluate faecal sample quantity and how it would affect DNA extraction and PCR amplification. The first experiment compared using two teaspoons to one teaspoon of faecal material. This was done by using a wooden spatula to collect the desired amount of faeces and putting it inside a cup. The faeces were then mixed with PBS before being transferred to a bead-beating tube. A second experiment was conducted to determine the least amount of faeces required for successful extraction and amplification. For this, one teaspoon was compared to

swabbing with Q-tips. For the swab method, a cotton tip (Q-tip) was rubbed on the faecal material until the tip was visibly coated with faecal material. The Q-tip was then immersed in PBS solution and agitated until the faecal material had dispersed. The solution was then transferred to a bead-beating tube.

The materials used for the extraction process was ready prepared extraction kits made by TANbead. Extraction of DNA from the samples were done by following Vidilab's standard routines for PCR-analysis. One ml of the samples was transferred to a 1.2 ml bead-beating tube. The samples were centrifuged, whereas the supernatant was then discarded by aspiration using a multi canal pipette. A sample containing Milli-Q (MQ) type-1 water and positive extracted DNA was used as a positive extraction control, whereas the negative control only consisted of MQ type-1 water. A solution was added to the samples, where a lid was then placed and secured tightly on top of the plate, for it to be shaken at 30 Hz. After finishing, Proteinase K was added. New lids were then fitted for it to be vortexed, then incubated at 90°C in sand. Afterwards, the vortex and incubation process was repeated once more. After, the plates were centrifuged one last time before being aspirated and added into extraction plates with lysis buffer. The DNA was then extracted with the TANbead 9600 extraction robot (Vidilab, 2024).

3.4 PCR-method

For this study, DNA amplification was done using the Bio-Rad thermal cycler CFX Opus (*CFX Opus Real-Time PCR System | Bio-Rad* u.å.). The program was run according to Vidilab's standard annealing temperature profile for PCR analysis. This included; denaturation at 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, followed by 57°C for 45 seconds.

3.4.1 Primers and probes

The two systems of primers and probes tested and compared in this study were a single tube nested system (Gookin et al. 2002) and an in-house system. In house system was created using the Eurofins PCR primer design tool based on the GenBank sequence M81842.1 (*PCR Primer Design Tool* 2026). The system published by Gookin et al. (2002) was in this study named T.F and the in-house system was named T.V and they will be called that in this report.

Table 2 Oligonucleotide sequences of the primers and probes used for the T.V and T.F systems.

Oligo	Sequence
T.V Forward	5'-AGA GGA AGG AGA AGT CGT AAC-3'
T.F Forward	5'-CCT GCC GTT GGA TCA GTT TCG TTA A-3'

T.V Reverse	5'-GCT AAT CCA GCC GCT TAT C-3'
T.F Reverse	5'-CGG GTC TTC CTA TAT GAG ACA GAA CC-3'
T.V Probe	5'-[FAM] ACC TGC CGT TGG ATC AGT TTC GTT A [BHQ1]-3'
T.F Probe	5'- [FAM] CGC CGT TTT AGC TTG CTA GAA CA [BHQ1]-3'

Multiple experiments were made to define which PCR system was the most efficient, and at what concentration of primers and probes.
A test was made to evaluate which of the systems was most efficient. The test also showed what concentration of primers and probe gave the best result.

3.4.2 Optimization

The optimal concentrations of the primers and probes were determined by testing the two systems with different concentrations. Three concentrations were tested for both systems (T.F and T.V).

Table 3 Shows the different concentrations of primers and probes that were tested.

Concentration	**Forward primer** (µM)	**Reverse primer** (µM)	**Probe** (µM)
Low	10	10	2.5
Medium	15	15	5
High	20	20	7.5

An experiment was conducted to assess the effect of the varying primer and probe concentrations. This involved testing the master mix of the two systems T.F and T.V at low, medium and high concentrations by adding them to separate wells in duplicate, spiked with the positive control at different dilutions, to evaluate sensitivity.

Table 4 Shows the positive control serially diluted and then ran in duplicates different primer and probe concentrations for the T.F and T.V systems.

T.F low	**T.F medium**	**T.F high**	**T.V low**	**T.V medium**	**T.V high**
$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$
$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$	$5{\cdot}10^{-3}$
$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$
$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$	$5{\cdot}10^{-5}$
$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$
$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$	$5{\cdot}10^{-6}$
neg	neg	neg	neg	neg	neg
neg	neg	neg	neg	neg	neg

3.5 Validation

To evaluate the analytical sensitivity of the qPCR method, dilution series of *T. foetus* DNA was prepared to determine the LOD indicating the lowest concentration at which DNA is detected above a threshold in ≥95% of X replicates. For the dilution in water, both DNA from: i) the bovine strain of *T. foetus,* and ii) MQ type-1 water was used. The dilution followed a logarithmic pattern, with each step reducing the concentration of DNA by a factor of ten. The initial dilution was prepared by mixing 6µL of DNA with 6µL type-1 H_2O, resulting in a total volume of 12µL and a dilution of 1:2 (0.5 · original concentration).

To prevent contamination, pipette tips were discarded after each step, and the dilution was performed in sterile conditions inside a fume hood.

Table 5 A tenfold dilution series of the positive control.

Tube	**Volume water**	**Volume from previous tube**	**Dilution factor**	**Relative concentration**
1	6	6 (conc. DNA)	1:2	0.5 x C
2	90	10	1:20	$5 \cdot 10^{-2}$
3	90	10	1:200	$5 \cdot 10^{-3}$
4	90	10	1:2 000	$5 \cdot 10^{-4}$
5	90	10	1:20 000	$5 \cdot 10^{-5}$
6	90	10	1:200 000	$5 \cdot 10^{-6}$
7	90	10	1:2 000 000	$5 \cdot 10^{-7}$
8	90	10	1:20 000 000	$5 \cdot 10^{-8}$

3.6 Statistical analysis

A statistical analysis of the field study was performed using the Fisher's exact test formula, analysing the associations of test outcome (positive or negative result) against all remaining categories combined.

Fisher's exact test formula:

$$p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{a!\,b!\,c!\,d!\,n!}$$

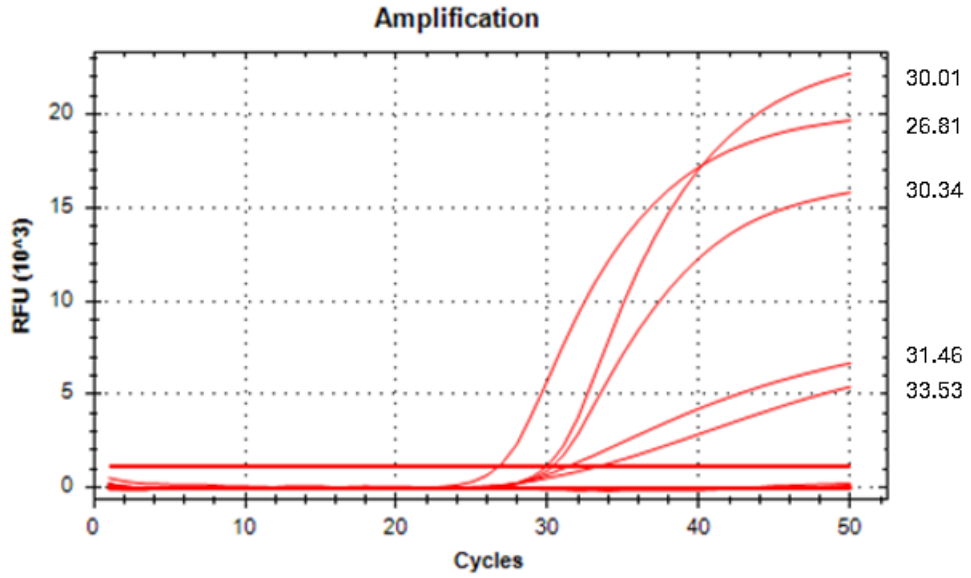
4. Results

4.1 Sample preparation tests

In the first experiment, faecal samples spiked with diluted positive *T. foetus* DNA (1:200) were prepared using either one teaspoon or two teaspoons of faeces. Samples that were prepared with one teaspoon generally showed slightly lower Cq-values compared to samples prepared with two teaspoons. Cq-values for one teaspoon ranged between 26.81 and 30.01, whereas samples with two teaspoons showed Cq-values between 30.34 and 33.53. One of the samples prepared with two teaspoons did not show amplification, and showed as N/A.

The amplification curves show detectable fluorescence beginning at approximately cycle 24-35 in positive samples (Figure 2). Samples prepared with one teaspoon generally showed earlier start of amplification and steeper curves compared to samples prepared with two teaspoons.

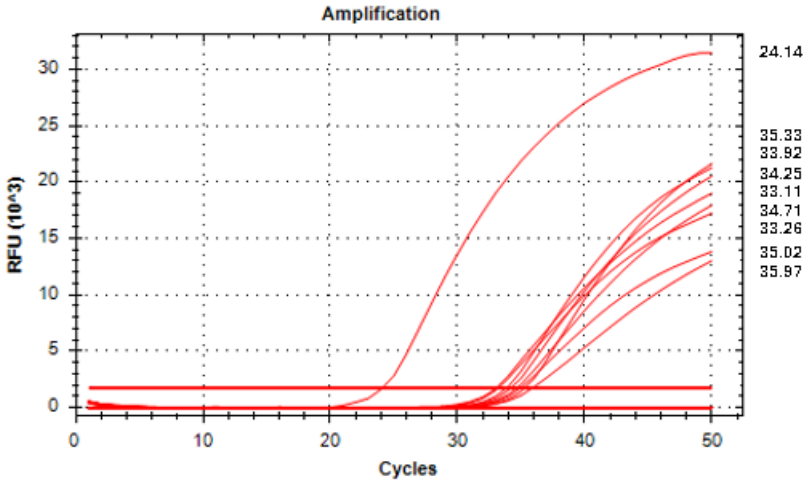
Figure 2 Amplification curve for the first sample-preparation test.



A second experiment compared preparation using one teaspoon of faeces with Q-Tip swabs, collected from positive faecal material. Amplification was detected using both preparation methods (Figure 3). Different Cq-values and amplification curves were observed between the two methods. Cq-values for one teaspoon ranged between 33.11-35.02 whereas samples with Q-Tip swabs showed values

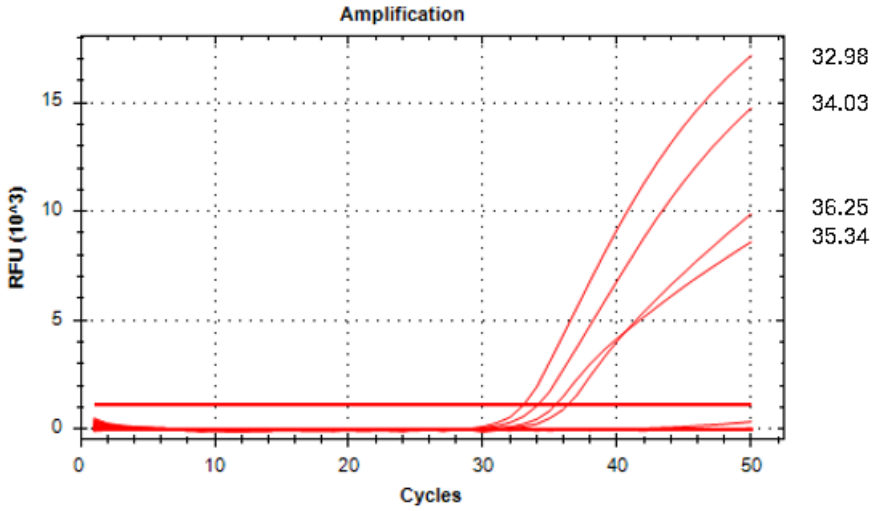
that ranged between 33.26-35.97 The amplification curve shows detectable fluorescence beginning at approximately cycle 31-36 (Figure 3).

Figure 3 Amplification curve for the test of comparing one teaspoon with Q-Tip's.



A third preparation experiment evaluated direct insertion of Q-Tip swabs into lysis buffer without any prior washing and centrifugation steps before the DNA extraction. Amplification was observed in all samples (Figure 4) with detectable fluorescence beginning at cycle 31-36. Cq-values ranged between 32.98 to 36.25.

Figure 4 Amplification curve for the Q-Tip's test directly in lysis buffer.



4.2 Primer and probe optimisation

The performance of the two qPCR systems, T.F and T.V, was evaluated using different dilution levels of positive DNA (Table 4). The primer and probes were evaluated in low, medium and high concentrations. Both systems successfully amplified the diluted *T. foetus* DNA at all different concentration levels. For the highest DNA concentrations, Cq-values ranged between approximately 24-25 cycles for both systems. At lower DNA concentrations ($5 \cdot 10^{-6}$), Cq-values increased to approximately 34-36 cycles. The T.V system generally showed lower Cq-values compared to the T.F system across concentration levels. The medium and high concentrations showed more consistent amplification results compared to the low concentrations. Two samples resulted in N/A, meaning failed amplification in those wells. No significant differences were observed in amplification efficiency, although the T.V system in high concentration generally showed the lowest Cq-values (Table 6).

Table 6 Displays the result (Cq value) obtained from qPCR analysis of the different system concentrations.

T.F low	**T.F medium**	**T.F high**	**T.V low**	**T.V medium**	**T.V high**
24,9	24,49	N/A	24,82	24,23	24,03
24,71	N/A	24,03	24,94	24,2	24,1
32,28	31,41	31,31	32,09	31,13	31,01
32,14	31,42	30,97	32,03	31,36	31,47
35,98	35,4	35,19	35,75	34,78	34,35
36,09	34,75	34,44	35,09	34,68	34,02
N/A	N/A	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	N/A

4.3 Field study - Application of optimised method

The optimised method was applied to a field study of faecal samples collected from 50 cats. One out of 50 samples tested positive with a Cq value around 33, corresponding to a prevalence of 2%. The remaining 49 samples tested negative for *T. foetus*. Information regarding the age, sex, housing and living arrangement was collected. Unknown information was labeled as unknown. The positive sample originated from a male cat between one and five years of age, living in a multi-cat living arrangement and originated from Place C. Among the cats for which living arrangement data was available, one positive sample was identified in the multi-cat group. No positive sample was observed within the group of single-cat housing. No positive samples were detected among cats under 1 year,

or older than 5 years of age. No positive samples were detected among female cats in the study.

Table 7 qPCR results of analyzed samples according to age, sex, housing location and living arrangement. P-values are calculated using Fisher's exact test formula.

Variable	*Total (n)*	*Positive (n)*	*Negative (n)*	*p-value*
Age group				
<1 year	6	-	6	1.000
1-5 years	8	1	7	0.160
>5 years	2	-	2	1.000
Unknown	34	-	34	1.000
Sex				
Male	22	1	21	0.440
Female	13	-	13	1.000
Unknown	16	-	16	1.000
Housing				
Place A	12	-	12	1.000
Place B	34	-	34	1.000
Place C	5	1	4	0.100
Living arrangement				
Single cat	6	-	6	1.000
Multi-cat	14	1	13	0.280
Unknown	30	-	30	1.000
Faeces				
Loose	12	-	12	1.000
Diarrhea	4	-	4	1.000
Normal	34	1	33	0.680

5. Discussion

5.1 Methodology evaluation

5.1.1 Sample preparation

The first experiment showed that samples prepared using one teaspoon of faecal material generally produced lower Cq-values compared to those prepared with two teaspoons. The purpose of the experiment was to determine whether increasing amount of faecal matter would result in lower Cq-values, which was not supported. One explanation is that larger sample increases inhibiting substances which reduces PCR efficiency and delays amplification.

Using one teaspoon of sample and Q-Tip swabbing both showed successful amplification, although the differences in Cq-values might indicate that there are differences in efficiency between the two preparation methods. Direct faecal preparation showed slightly lower Cq-values compared to Q-Tips. Swabbing with Q-Tips may instead have practical advantages, as less faecal sample is required, and preparation time is reduced.

Direct insertion in lysis buffer without any pre-preparation of the faecal sample resulted in successful amplification in all samples. This suggests that pre-processing of the samples may not be necessary, which could improve practicality. However, this method produced higher Cq-values which may be due to inhibiting factors.

5.1.2 Primer and probe optimisation

The results from the primer and probe optimisation experiments show increased Cq-values as the dilution increased, as expected. Samples with higher positive DNA concentration showed earlier amplification (Cq 24-25), while samples with more diluted DNA showed later amplification at Cq 34-36 (Table 6.)

Both PCR systems detected *T. foetus* at all dilution levels. The T.V system generally produced lower Cq-values across all concentration levels, although the differences were relatively small. Lower Cq-values might mean higher sensitivity or improved amplification performance. Although, the significance of the small variation observed, may be limited.

Higher concentrations of primers and probe produced more consistent and lower Cq-values than lower concentrations, which may mean that reduced concentration decreased sensitivity. Amplification was observed at all concentrations, showing

that both systems performed across different concentrations. Some wells failed to show amplification, resulting in N/A values. This may be due to human error, such as pipetting mistakes. The remaining results indicate that there were no reaction-inhibiting factors, and an error in the system is unlikely to have occurred.

5.1.3 Limitations

The limitations identified in this study include the use of only 50 faecal samples, which is a relatively small proportion and likely does not accurately represent the prevalence in Sweden's cat population. The geographical restriction to catteries in a local area can introduce selection bias. This also means the study does not reflect regional variations in *T. foetus* that might exist across Sweden. Samples were stored under different conditions, which may have affected sample quality and DNA detection efficiency. During one week at 8°C, DNA degradation may have occurred in the samples potentially affecting detection sensitivity and thus the results. It is unclear whether the same applies to samples kept in the freezer.

Differences between the two PCR systems were evaluated only descriptively, and as no statistical analysis was performed, the differences cannot be considered significant. Some of the experiments used samples spiked with diluted parasite DNA from bovines as positive samples, rather than felid faecal samples containing whole *T. foetus*. Therefore, the results may not fully represent biological variation. Variation in faecal inhibitors between samples may also have affected the results.

5.2 Field study

Currently, there is limited published data on prevalence in Sweden, making further evaluation difficult. This field study included faecal samples from 50 cats. The results show that only one cat tested positive for *T. foetus*, indicating very low prevalence (2%). Nevertheless, this is consistent with expectations. According to Eva Osterman-Lind at SVA (personal communication), of approximately 2500 samples analysed at their facility, only 4% were positive. Therefore, the result from this field study falls within the expected range. Statistical analysis was limited due to the presence of only one positive sample. A Fisher's exact test was performed to assess the association between positivity and category. No significant associations were observed, as all p-values were above 0.05.

Previous European studies have reported higher prevalence in cat populations, ranging from 5 to 31% (Table 1.). This may be due to differences in study populations, sampling sizes and sampling strategies. Despite the low prevalence

observed in this study, the positive sample came from a multi-cat household consistent with previous studies (Gookin et al. 2004b).

Although prevalence in this study was low, the detection of one positive sample demonstrates that *T. foetu*s is present in Sweden. The positive sample, which originated from a multi-cat household, supports previous studies suggesting that close contact between multiple cats may increase transmission of the infection.

As shown in Table 5, the positive sample was detected in a male cat aged between one and five years, which lived in a multi-cat environment. Because only one sample was positive, no conclusions can be drawn regarding any association between infection and categories such as age, sex or living arrangements. The suggestion that multi-cat housing carries an increased risk may be supported by these results, but no firm conclusion can be made. Statistical analysis of risk factors is therefore limited. As the prevalence was very low, pool testing might be an option to keep down costs.

The sample size in this study is relatively small and cannot be considered representative of the overall *T. foetus* prevalence in Sweden. Prevalence may vary depending on factors such as geography, living arrangements and lifestyle. In addition, the infection is not currently classified as a notifiable disease in Sweden. As a result, there is no comprehensive national data available to determine the total number of tested and confirmed positive cases. Since the feline-infecting strain of the parasite is considered relatively new, estimating the true prevalence is difficult.

The fact that infected cats can be asymptomatic carriers may lead to underdiagnosis. As the main symptoms are diarrhoea or loose stool, which can occur in many other infections or situations, this may result in incorrect treatment. Awareness of the parasite and diagnostic practices may vary between veterinary clinics and cat owners, which may lead to underdiagnosis. An accurate diagnostic method is important to limit the use of medication. Medication eventually enters our water systems, and overuse can negatively affect the environment, such as water quality, which can have adverse effects on aquatic organisms.

5.3 Further research

Additional validation experiments using larger sample sets would strengthen the conclusion. Further research could also include optimisation of the thermal cycling, and investigation of differences between temperature profiles. Further studies on the prevalence of *T. foetus* in Sweden should be conducted to establish a representative estimate of the current situation. As the prevalence is higher in

other European countries, further studies could identify possible risk factors and the true prevalence. This could contribute to earlier detection and more effective infection management, resulting in healthier cats and reduced economic losses for catteries and breeders.

6. Conclusion

The results of this study show that both PCR systems were able to detect *T. foetus* DNA in both dilution tests, and a positive sample from the field study. Optimization of the primer and probe concentrations improved amplification performance. The in-house system generally produced slightly lower Cq-values.

Application of the optimized method in the field-study identified one positive sample, resulting in a prevalence of 2%. Due to the low prevalence, pool testing may be usable for practicality and to keep down diagnostic costs. The sample size was limited to 50 samples, thus there cannot be any real conclusions drawn regarding geographical and national prevalence or risk factors. The findings confirm that the parasite is present in Sweden. The findings support the use of molecular diagnostic methods for detection of *T. foetus* and highlight the need for further studies using larger sample size to estimate the true prevalence.

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Lay summary

Tritrichomonas foetus is a one-celled parasite that infects the gastrointestinal tract of domestic cats. The symptoms caused by an infection include long-term diarrhoea and loose stool, which can negatively impact the cat's health and wellbeing. Infection is more often seen in younger cats, living in multi-cat housing such as catteries and breeders. The infection is spread through the faecal-oral route and is most often seen in places where the cats live close together. Cats can become asymptomatic carriers, meaning they can spread the parasite without showing any symptoms. The symptoms are also similar to many other gastrointestinal diseases, making diagnosing sometimes difficult.

The aim of this study was to compare and validate PCR systems, which is a molecular diagnostic method used to detect DNA in samples. Another aim was to evaluate methods on how to prepare faecal samples before PCR analysis through several laboratory experiments.

The results indicate that sample preparation method may affect PCR performance, as well as sensitivity in detection. Based on the amount of faeces, the results differed slightly. One of the PCR systems displayed lower Cq-values. A field study was performed to evaluate the PCR system in practice. The study included faecal samples from 50 cats. Out of these 50 samples, only one tested positive, corresponding to a prevalence of 2%. This result suggests that the parasite is present in Sweden, but at a low level. Since the sample size was small and geographically limited, the results cannot be considered to represent the real situation in Sweden's cat population.

In conclusion, this study found that one of the PCR systems performed better, and that faecal sample size impacts PCR performance and sensitivity. There is a gap in knowledge about *Tritrichomonas foetus* infection in Swedish cats, risk factors, and its true prevalence.

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