Failure to detect *Tritrichomonas foetus* in a cross-sectional survey in the populations of feral cats and owned outpatient cats on St Kitts, West Indies

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Abstract

Objectives For over two decades, feline trichomonosis caused by *Tritrichomonas foetus* has been recognized as a large-bowel protozoan disease of the domestic cat. It has a wide distribution, but no reports exist in the Caribbean. The objectives of this study were to detect the presence of *T foetus* and its prevalence in the domestic cat on St Kitts, West Indies.

Methods A cross-sectional study was performed between September 2014 and December 2015. This study recruited 115 feral cats from a trap–neuter–return program and 37 owned cats treated as outpatients at the university veterinary clinic. Fresh feces were inoculated in InPouch culture medium, as per the manufacturer's instructions. In addition, PCR was performed using primers for *T foetus*. DNA extraction with amplification using primers of a *Felis catus* NADH dehydrogenase subunit 6 was used as a housekeeping gene for quality control.

Results Only two owned cats had reported diarrhea in the preceding 6 months. None of the 152 samples were positive on InPouch culture microscopic examination. Only 35/69 feral cat fecal DNA samples were positive for the housekeeping gene, of which none tested PCR positive for *T foetus*.

Conclusions and relevance *T foetus* was not detected by culture and PCR in feral cats and owned cats on St Kitts. A high proportion of PCR inhibitors in the DNA samples using a commercial fecal DNA kit can lead to underestimating the prevalence, which should be taken into consideration when a survey on gastrointestinal pathogens depends exclusively on molecular detection.

Keywords: Feline trichomonosis, *Tritrichomonas foetus*, feral cat, cell culture, PCR, St Kitts

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Introduction

Feline trichomonosis presents clinically as chronic large-bowel diarrhea in affected cats. These cats usually shed liquid to semi-formed feces, often with blood and/or mucus. The causative pathogen of the disease is a species of *Tritrichomonas*, although the specific species is debatable. *Tritrichomonas foetus* has been incriminated as the etiological pathogen of feline trichomonosis for almost two decades. However, recently, Walden et al proposed a new species – *Tritrichomonas blagburni* – as the etiological pathogen of feline trichomonosis. Nevertheless, analysis of transcriptomics of the two did not reveal genetic distinctness between them. Furthermore, a proteomic analysis of about 1500 proteins on each using two-dimensional gel electrophoresis coupled with liquid chromatography tandem mass spectrometry revealed an almost identical profile, although 24 proteins did show a more than four-fold difference. It appears more data are needed to confirm the independent species status of *T. blagburni* from *T. foetus*. Consequently, *T. foetus* is used herein.

Feline trichomonosis has a wide geographical distribution. It has been found in 19 countries on four continents. However, it has not been reported in the Caribbean. The main objectives of the current study were, in a cross-sectional study, to detect the presence of *T. foetus* and its prevalence in domestic cats on St Kitts, West Indies.

Materials and methods

Ethics

Animal work was performed under protocols approved by the Institutional Animal Care and Use Committee of Ross University School of Veterinary Medicine. Animal care and use were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Sample collection

At the Ross University Veterinary Clinic (RUVC), a trap–neuter–return programme entitled the ‘Feral Cat Project’ (FCP) was initiated to control the feral cat population on St Kitts. For the FCP cats, sample collection started in September 2014 and ended in July 2015. For each cat general information such as sex, body weight, breed, reason for presentation, FIV status, antibiotics use and its prevalence in domestic cats on St Kitts, West Indies. 7

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Molecular detection of *Tritrichomonas foetus*

DNA was extracted from individual feces using the QIAamp DNA Stool Kit (QIAGEN) following the manufacturer’s instructions. DNA quantity and quality were assayed using the Tecan Infinite M200 Pro. DNA was stored at −20°C.

PCR was used to amplify NADH dehydrogenase subunit 6, a housekeeping gene of the domestic cat (*Felis catus*) and a fragment of the ITS1-5.8S rRNA-ITS2 region of *T. foetus*. The primers for NADH dehydrogenase subunit 6 were 5’-TTAATTCGCCACGAGTAACTCCATA-3’ and 5’-ATGATAACATACAATGTATTTTAAG-3’ (accession no.: KP279629), with an expected product of 528 bp. The primers for *T. foetus* were TFR3 and TFR4, which are specific to *Tritrichomonas* species and amplify a 347 bp DNA fragment of ITS1-5.8S rRNA-ITS2, as previously described. All primers were synthesized by IDT. NADH dehydrogenase subunit 6 was used as a quality control to rule out inhibitors in the feces.
interfering with PCR amplification. The sample was re-run if no NADH dehydrogenase subunit 6 fragment was produced. A repeated failure on this PCR suggested the presence of PCR inhibitors and the sample was excluded in data analysis. PCR mix (25 µl) was used for each reaction with a final concentration of each forward and reverse primer at 1.0 µM. HotStart Taq Plus 2 × Master Mix (QIAGEN) was used for samples with a DNA concentration $\geq 20$ ng/µl, whereas Taq DNA polymerase (TaKaRa; Clontech) was used for samples with a DNA concentration between 5 and 20 ng/µl. Volumes of DNA solutions used for each PCR were 2.0, 4.0, 8.0 and 15.0 µl for samples with DNA concentration $>100.0$, $\geq 50.0$, $\geq 20.0$ and $>5.0$, respectively. PCR was performed in a thermal cycler (Mastercycler Nexus Gradient) for 35 cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 2 mins, with a final extension at 72°C for 10 mins following an initial cycle at 95°C for 2 mins. PCR products were visualized via electrophoresis in 1.2% agarose gel.

**Statistical analysis**

Student’s $t$-test was performed using Microsoft Excel 2016.

**Results**

In total, 115 FCP cats and 37 RUVC-owned outpatient cats were included in the current study. Of 115 FCP cats, 64 were male and 51 female. In both young and adult age groups, the mean body weight of male cats was significantly higher than that of female cats ($P <0.05$; Table 1). Further, the presence of FIV infection in males was three time higher than in females (Table 2). All 37 owned outpatient cats (19 females, 18 males) presented to RUVC for routine medical care. All were younger than 1 year of age, except for five cats that ranged in age from 1–13 years. Of 23 cats with known FIV status, 17 were negative. Only five had a history of taking antibiotics in the 2 weeks immediately prior to the current visit. Two cats had had diarrhea recorded in their medical history, one current and the second in the previous 6 months.

All 115 FCP cats and 37 RUVC-owned outpatient cats were tested by culture in InPouch. None were microscopically positive for *T. foetus* during the entire culture period of 6 days. Furthermore, 69 fecal samples of FCP cats had a DNA concentration $>5.0$ ng/µl. They had an average 260/280 ratio of 2.11 ± 0.22 and were tested by PCR. Surprisingly, only 35 were PCR positive for the feline NADH dehydrogenase subunit 6. Among the latter, none were PCR positive for *T. foetus* (Table 3). The average ratio of 260/280 for the 35 positive and 34 negative PCRs for the feline NADH dehydrogenase subunit 6 were 2.10 ± 0.19 and 2.13 ± 0.25, respectively.

**Discussion**

In this study, we performed a first-of-its-kind in the Caribbean cross-sectional survey of feline trichomonosis on feral and owned outpatient cats on St Kitts. We did

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**Table 1** Age and body mass of feral cats on St Kitts included in a cross-sectional study between September 2014 and July 2015

| Age   | Mean ± SD body mass (kg) |
|-------|--------------------------|
|       | Young (n) | Adult (n)* | ND (n)* |
| Male (n) | 64 | 29 | 23 | 12 | 2.25 ± 0.64 (17) | 3.32 ± 0.77 (6) | 2.65 ± 0.83 (12) |
| Female (n) | 51 | 28 | 10 | 13 | 2.00 ± 0.51 (9) | 2.39 ± 0.25 (4) | 2.16 ± 0.59 (13) |
| Total | 115 | 57 | 33 | 25 |

*P <0.05 between males and females
ND = not determined

**Table 2** Feline immunodeficiency virus (FIV) status of feral cats on St Kitts included in a cross-sectional study between September 2014 and July 2015

| Young | Adult | ND | Total |
|-------|-------|----|-------|
| FIV positive (%) | FIV negative (%) | FIV positive (%) | FIV negative (%) | FIV positive (%) | FIV negative (%) |
| Male (n) | 64 | 2 (6.9) | 27 | 9 (39.1) | 14 | 4 (33.3) | 8 | 15 (23.4) | 49 |
| Female (n) | 51 | 4 (14.3) | 24 | 0 (0.0) | 10 | 0 (0.0) | 13 | 4 (7.8) | 47 |
| Total | 115 | 6 (10.5) | 51 | 9 (27.3) | 24 | 4 (16.0) | 21 | 19 (16.5) | 96 |

ND = not determined
not detect *T foetus* in 115 feral cats or 37 owned outpa- 
tient cats by culture in InPouch. Furthermore, con- 
tventional PCR tested 35 samples of fecal DNA from feral 
cats for *Trichomonas* species ITS1-5.8S rRNA-ITS2; 
none was PCR positive. The 95% confidence interval (CI) 
for a combination of 152 samples with zero positive was 
calculated using the normal approximation method, 
and the upper 95% confidence limit was 0.0%.13 These data 
unequivocally showed that *T foetus* was undetected in 
the populations of feral cats and owned cats on St Kitts, 
which was somewhat surprising given that feline tricho- 
omonosis is widely spread worldwide. By 2015, the 
disease had been recorded in 19 countries on four continents, 
including 13 countries in Europe (Austria, Finland, 
France, Germany, Greece, Italy, The Netherlands, 
Norway, Poland, Spain, Sweden, Switzerland and the 
UK), two in North America (Canada and The USA), two 
in Australia/Oceania (Australia and New Zealand) and 
two in Asia (Japan and South Korea).2 Since then it has 
also been detected in further countries in Asia (Hong 
Kong, China)12 and in South America (Brazil).13,14 So far, 
in total, 21 countries on five continents have recorded 
feline trichomonosis.

It is plausible that the negative finding of the current 
study was due to the fact that the study cats presented 
for routine medical care or elective neutering. It has been 
shown that the prevalence of *T foetus* is highly variable 
among various cat populations in Ontario, Canada: ie, 
0% (95% CI 0.0–7.7%; n = 46) from the humane society; 
0.7% (95% CI 0.0–3.9%; n = 140) from a cat clinic; and 
23.6% (95% CI 13.2–37.0%; n = 55) in cat shows.15 Among 
the 37 owned cats only two had a history of diarrhea in 
the preceding 6 months prior to sampling. A cat with a 
history of diarrhea in the past 6 months was three time 
more likely to be positive for *T foetus*.2 Unfortunately, 
there was no medical history for the 115 feral cats, though 
all were deemed healthy enough to undergo general 
anesthesia by a licensed veterinarian. These negative 
results are consistent with some previous discoveries. 
Gookin et al reported that *T foetus* was not recovered 
from feces of 100 feral cats and 20 healthy indoor cats.3

A cross-sectional survey was carried out in the Czech 
Republic for detecting *T foetus* among 170 cats between 
September 2010 and September 2012. The detection 
methods included InPouch culture followed by PCR 
confirmation of culture positivity for the motile tropho-
zoites of trichomonads. The cats were from: catteries 
(32.7%); private owners (35.7%); inpatients at the Small 
Animal Clinic, Brno (23.4%); and shelters (8.2%). None 
were positive for *T foetus*, although one cat was positive 
for *Pentatrichomonas hominis*.16 The same authors further 
performed a metadata analysis of studies using PCR for 
species identification. In total 1495 cats from nine differ-
ent countries (Australia, the Czech Republic, France, 
Germany, the UK, Italy, Greece, the USA and Switzerland) 
were included in their metadata analysis for the prev-
ance of *T foetus*. They found a prevalence of 1.1% (95% 
CI 0.2–2.0%) and 5.0% (95% CI 3.6–6.4%) in cats without 
or with diarrhea, respectively. Nevertheless, the authors 
pointed out these combined data were very likely to 
underestimate the prevalence of *T foetus*.16 It is noteworthy 
that the high prevalence of *T foetus* recorded in the 
literature was often associated with diarrheic client-
owned cats rather than cross-sectional sampling. Such a 
bias in sampling resulted in an overestimated prevalence 
than a cross-sectional survey would have.2

We were surprised by the 49.3% (n = 34/69) PCR fail-
ure rate in amplification of a feline housekeeping gene, 
NADH dehydrogenase subunit 6, from DNA isolated 
from the feces of the FCP cats. DNA preparations were 
obtained using the QIAamp DNA Stool Kit. The average 
ratio of 260/280 was 2.11 for all DNA samples, indicating 
the high purity of the DNA. It was 2.13 for the samples 
that failed to yield positive PCR results for amplifying 
feline NADH dehydrogenase subunit 6 in repeated effort, 
suggesting that the failure was not due to DNA impurity. 
We had previously used the same kit in preparing DNA 
from feces of African green monkeys (AGM). A similar 
effort was made in amplifying AGM’s housekeeping 
gene, *NAHDH1*. In the case of AGM the failure rate was 
16.4% (n = 11/67),17 three times lower than that of cats in 
the current study. It was reported that the failure rate in 
PCR amplification of bacterial 16S rRNA gene was 26.8% 
(n = 11/41) in one study and 18.3% (n = 11/60) in another 
from feline fecal DNA isolated using the same kit.18,19 In 
all these cases, the high failure rate in amplifying a house-
keeping gene or bacterial 16S rRNA gene suggests the 
presence of PCR inhibitors. Bilirubin, bile salts, heavy

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### Table 3 Test results of *Tritrichomonas foetus* in feral cats and outpatients of a veterinary clinic on St Kitts in 2014 and 2015

|                      | InPouch culture | PCR on feline NADH dehydrogenase subunit 6 | PCR on *T foetus* |
|----------------------|----------------|---------------------------------------------|------------------|
|                      | Tested (n) | Positive (n) | Tested (n) | Positive (n) | Tested (n) | Positive (n) |
| Feral cats           | 115       | 0            | 69        | 35          | 35        | 0            |
| RUVC cats            | 37        | 0            | ND        | ND          | ND        | ND           |

RUVC = Ross University Veterinary Clinic; ND = not determined
metals, hemoglobin degradation products and complex polysaccharides in feces are PCR inhibitors, even when present in low concentration. Too often DNA prepared from feces of human and various animals are tested for the presence of DNA of certain pathogens by PCR. It is assumed a negative result was due to lack of the targeted pathogen’s DNA in the sample. However, high PCR failure rates in both cats and AGM should serve as a warning that some of those negative PCR findings are due to the presence of PCR inhibitors rather than the absence of the pathogen’s DNA; ie, false negative. This should be considered a significant reason why prevalence is easily underestimated.

Conclusions
A cross-sectional study of 115 feral cats and 37 owned outpatient cats was carried out to detect T foetus using culture and PCR. None of the 152 cats were found to be positive among these cat populations on the island of St Kitts. Surprisingly, PCR inhibitors existed in a high proportion of DNA samples using a commercial fecal DNA kit, which may result in the underestimation of prevalence of microbial pathogens in the gastrointestinal tract.

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Conflict of interest
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