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Source: Journal of Feline Medicine and Surgery Open Reports, 4(1)

Published By: SAGE Publishing

URL: https://doi.org/10.1177/2055116918782584

Short Communication

jfms OPEN REPORTS

Journal of Feline Medicine and Surgery Open Reports
1–5

DOI: 10.1177/2055116918782584
journals.sagepub.com/home/jfmsopenreports

This paper was handled and processed by the American Editorial Office (AAFP) for publication in *JFMS Open Reports*

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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

Accepted: 7 April 2018

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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.[1,2] 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.[3–5] However, recently, Walden et al proposed a new species – *Tritrichomonas blagburni* – as the etiological pathogen of feline trichomonosis.[6] Nevertheless, analysis of transcriptomics of the two did not reveal genetic distinctness between them.[7] 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.[8] 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.[2] 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 and feline immunodeficiency virus (FIV) infection status was collected. After anesthesia was administered, colon flushing was attempted using a lubricated Kendall feeding tube and urethral catheter connected to a 5 ml syringe, which was inserted through the anus for 1–2 inches, and 3 ml veterinary saline (Abbott) was infused. The contents of the rectum was collected after being flushed a couple of times. If the stool was too firm to allow harvesting with this technique, feces were collected from the anus or rectum with a gloved finger. About 1 g of feces was harvested from each FCP cat. The collected feces were brought to the Research Laboratory of Ross University School of Veterinary Medicine within 1 h of collection. Approximately 10 drops of the flush were added to the upper chamber of the InPouch TF Feline *T foetus* test kit (BioMed Diagnostics) immediately and the remaining into two 1.7 ml micro-centrifuge tubes. The latter were stored at −80°C. Alternatively, an aliquot, the size of an uncooked rice grain, was inoculated in the upper chamber of the InPouch Kit, and the remaining feces were divided into four aliquots and immediately stored at −80°C.

For the owned outpatient cats presented to RUVC, consent was obtained from each cat's owner prior to sample collection, which occurred between May and December 2015. Patient information was collected prior to sample collection. The information included age, sex, breed, reason for presentation, FIV status, antibiotics use in the past 2 weeks, and current diarrhea and diarrhea in the previous 6 months. During sample collection, the patient was held steady by one person, and another person performed the colon flushing and collected samples were processed as described above.

Cell culture of Tritrichomonas foetus

The inoculated InPouch pouches were transferred to a refrigerated incubator (VWR) set at 37°C immediately after the sample was collected. It was incubated for 6 days, as per the manufacturer's instructions, and was checked daily, microscopically, for the presence of *T foetus*. On day 6, a wet mount slide was made from five drops of culture medium for further confirmation. The sample was defined as negative if no *T foetus* was ever found.

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'-TTAATTCCCACGAGTAACTTCCATA-3' and 5'-ATGATAACATACAATGTATTTATTTTAAG-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.[9,10] All primers were synthesized by IDT. NADH dehydrogenase subunit 6 was used as a quality control to rule out inhibitors in the feces

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	Adult	ND	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	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

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 ⩾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, ⩾50.0, ⩾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

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

not detect *T foetus* in 115 feral cats or 37 owned outpatient cats by culture in InPouch. Furthermore, conventional PCR tested 35 samples of fecal DNA from feral cats for *Tritrichomonas* 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%.[11] 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 trichomonosis 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 trophozoites 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 different countries (Australia, the Czech Republic, France, Germany, the UK, Italy, Greece, the USA and Switzerland) were included in their metadata analysis for the prevalence 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 failure 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, *NADH1*. 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 housekeeping gene or bacterial 16S rRNA gene suggests the presence of PCR inhibitors. Bilirubin, bile salts, heavy

metals, hemoglobin degradation products and complex polysaccharides in feces are PCR inhibitors, even when present in low concentration.[18,20] 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.

Acknowledgements We are very grateful to Kimberly Anne Van Ness, Amanda Landry, Samantha Gerb, Veronica Liquori and Anamercy Martin of Ross University School of Veterinary Medicine (RUSVM) for their help in fecal sample collection and/or DNA extraction.

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was supported by an intramural grant from RUSVM (Feline trichomoniasis, to CY and LK). Publication cost of this article was provided by the Center One of RUSVM. The sponsors played no roles in study design; in the collection, analysis or interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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