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Short communication

Species identification of trichomonads and associated coinfections in dogs with diarrhea and suspected trichomonosis

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ABSTRACT

Trichomonads have been infrequently reported in the feces of dogs where their pathogenicity remains uncertain. It is currently unknown whether *Tritrichomonas foetus* or *Pentatrichomonas hominis* is identified more commonly in dogs with trichomonosis or how often these infections are accompanied by concurrent enteric infectious agents. The objective of this study was to determine the identity of trichomonads present in a series of 38 unsolicited canine diarrheic fecal samples submitted for *T. foetus* diagnostic polymerase chain reaction (PCR) testing between 2007 and 2010. We also examined each fecal sample for an association of trichomonosis with concurrent infection using a convenient real-time PCR panel for nine gastrointestinal pathogens. *P. hominis*, *T. foetus*, or both were identified by PCR in feces of 17, 1, and 1 dogs respectively. Feces from the remaining 19 dogs were PCR negative for *T. foetus*, *P. hominis* and using broader-spectrum Trichomonadida primers. The total number and specific identities of concurrent enteropathogens identified did not differ between fecal samples from dogs that were or were not identified by PCR as infected with trichomonads. These results suggest that *P. hominis* infection is more frequently identified than *T. foetus* infection in diarrheic dogs with trichomonosis and that concurrent enteropathogen infection is common in this population.

1. Introduction

Trichomonads inhabit a variety of vertebrate hosts and consist of both pathogenic and presumably nonpathogenic species (Lopez et al., 2000). These protozoa are obligate parasites of warm, moist, and anaerobic locations within the gastrointestinal and genitourinary tract (Felleisen, 1999). In dogs and cats, trichomonads were initially thought to be only opportunistic commensals. However, in 2003 the pathogen *Tritrichomonas foetus* was identified as a cause of large bowel diarrhea in the cat (Levy et al., 2003). Both *T. foetus* and an unrelated trichomonad, *Pentatrichomonas hominis* have been infrequently reported in dogs and their pathogenicity is unknown (Gookin et al., 2005, 2007; Kim et al., 2010). In particular, *P. hominis* is presumed to be a commensal that may overgrow in dogs with other causes of diarrhea. It is unclear if *T. foetus* or *P. hominis* is more common in dogs with trichomonosis or how often these infections are accompanied by concurrent infections.

The objective of this study was to identify, by means of PCR, the species of *Trichomonas* infecting dogs from which an unsolicited fecal sample was submitted for commercial *T. foetus* diagnostic testing. We further examined each fecal sample for the presence of concurrent infection using a convenience real-time PCR panel for nine gastrointestinal pathogens.

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2. Materials and methods

2.1. Fecal samples and DNA extractions

Voided fecal samples from dogs that were submitted to North Carolina State University for *T. foetus* diagnostic PCR testing were collected over a three-year period (2007–2010). Fecal samples were stored in isopropanol at 4–22 °C for less than 1 week prior to DNA extraction. DNA was extracted from 100 mg of feces using a commercial kit (ZR Fecal DNA Miniprep, Zymo Research, Irvine, CA) in accordance with manufacturer instructions. Fecal DNA from age-matched non-diarrheic dogs, which were previously tested negative for *P. hominis* and *T. foetus* (Gookin et al., 2007) were retrieved from storage at −80 °C for inclusion as controls for real-time TaqMan® PCR assay for concurrent infectious disease.

2.2. Medical records review

Medical records of each dog were solicited from the submitting veterinarians. Where available, data retrieved from the medical record included signalment, description of fecal consistency, and results of fecal diagnostic testing.

2.3. Standard PCR amplifications and DNA sequencing

All extracts of fecal DNA were subjected to PCR amplification of an 876-bp gene sequence of bacterial 16S rRNA as previously described in order to rule out the presence of endogenous PCR inhibitors (Gookin et al., 2007). Each extract of fecal DNA was then subjected to single-tube nested PCR amplification of a 208-bp sequence of the partial internal transcribed spacer region (ITSR) 1 and 5.8S rRNA gene of *T. foetus* using previously published reaction conditions and primer sequences (Gookin et al., 2005). Amplicons migrating at 208-bp based on gel electrophoresis were digested with *Apo*I (New England Biolabs, Beverly, MA) at 50 °C for 90 min. Electrophoresis of 10 μl of each digest in a 3.5% agarose gel containing ethidium bromide was performed to confirm the presence of specific confirmatory bands at 89 and 119-bp (New England Biolabs, Beverly, MA). Each DNA extract was subjected to PCR amplification of a 339-bp sequence of partial 18S rRNA gene of *P. hominis* using previously published reaction conditions and primer sequences (Gookin et al., 2007). Amplification products of the appropriate size were submitted for purification and bi-directional DNA sequencing (MCLAB, San Francisco, CA). Finally, DNA extracts that tested negative for *T. foetus* and *P. hominis* rRNA genes were assayed for the presence of DNA sequences sharing common identity with a larger number of trichomonadida. PCR amplification of 339 to 372-bp sequences of partial ITS1, 5.8S, and ITS2 rRNA gene was performed using primers TFR1 and TFR2 designed by Felleisen (1997). The PCR assay was performed in a 100 uL reaction volume using 1x PCR Buffer II, 2.5 units of *Taq* polymerase, 100 pmol of each primer, 200uM of each deoxynucleoside triphosphate, 10 μg of bovine serum albumin, 6.25 mM MgCl, and 5 μL of DNA. DNA amplification was performed under the following thermocycling conditions: 5 min of initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30-s, annealing at 66 °C for 30-s and extension at 72 °C for 90-s, and a final extension at 72 °C for 15-min.

2.4. DNA extraction and PCR controls

Negative control samples containing DNAse/RNAse-free water were processed in parallel with study samples during DNA extraction and reaction preparation for detection of genomic DNA or amplicon contamination. Positive controls for PCR included approximately 20 ng per reaction of purified bovine *P. hominis* (ATCC 30098, Rockville, MD) or feline *T. foetus* genomic DNA. Amplicons were visualized under UV light following gel electrophoresis of 10 μl of each reaction solution in a 1.5% agarose gel containing ethidium bromide.

2.5. Real-time TaqMan® PCR assays

An extract of fecal DNA from each dog was shipped overnight on dry ice to a reference laboratory (IDEXX, West Sacramento, CA) for real-time PCR assays. Ten real-time PCR assays were used to test for the presence of gene sequences specific for *Clostridium perfringens* enterotoxin A, *Clostridium difficile* Toxin A and B, *Salmonella* spp., *Campylobacter spp.*, *Cryptosporidium* spp., *Giardia* spp., canine enteric coronavirus, canine distemper virus, and canine parvovirus. PCR reactions were performed using a Roche LightCycler 480 (Roche Applied Science, Indianapolis) using 5 μl of extracted DNA and raw data analyzed using the 2nd derivative maximum method to generate crossing points. Real-time PCR was performed concurrently with 7 quality controls including (1) PCR positive controls, (2) PCR negative controls, (3) negative extraction controls, (4) DNA pre-analytical quality control targeting the host ssr rRNA (18S rRNA) gene complex, (5) RNA pre-analytical quality control targeting the host ssr rRNA gene complex, (6) an internal positive control spiked into the lysis solution, and (7) an environmental contamination monitoring control. These controls assessed the functionality of the PCR protocol (1 & 6), absence of contamination in the reagents (2) and laboratory (7), absence of cross-contamination during the extraction process (3), quality and integrity of the DNA and RNA as a measure of sample quality (4 & 5), RT-protocol (5) and absence of PCR inhibitory substances as a carryover from the sample matrix (6).

3. Results

Unsolicited fecal samples from 38 dogs were identified as submitted by veterinarians for *T. foetus* PCR testing from years 2007 to 2010. PCR assays performed on DNA extracted from each sample identified 2 and 18 dogs respectively, as infected with *T. foetus* or *P. hominis*. One of these dogs was positive for amplification of both *T. foetus* and *P. hominis*. PCR amplification products from 17/19 dogs identified as positive for *P. hominis* by PCR were available for bi-directional sequencing. All 17 samples had >98% sequence identity to *P. hominis* (GenBank #HQ149970). Extracts of fecal DNA from the remaining 19 dogs were PCR negative for *T. foetus* and *P. hominis* and amplicons