Optimization of a species-specific polymerase chain reaction assay for identification of *Pentatrichomonas hominis* in canine fecal specimens

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**Objective**—To determine the optimum reaction conditions and detection limits of PCR assay for identification of *Pentatrichomonas hominis* in DNA extracted from canine feces.

**Sample population**—DNA extracted from feces of 4 dogs with diarrhea from which trichomonads were observed, 81 dogs that had feces submitted to a diagnostic laboratory, and 19 dogs residing in a laboratory animal facility.

**Procedures**—Optimum reaction conditions and absolute and practical detection limits of 2 *P hominis* 18S species-specific primer pairs were determined by use of an in vitro cultivated canine isolate of *P hominis* in the presence and absence of canine feces. The optimized PCR assay was applied to amplification of *P hominis* 18S rRNA genes from DNA extracted from the feces of dogs.

**Results**—Under optimized conditions, a primer pair was identified as able to detect as few as 1 *P hominis* organism/180-mg fecal sample. The PCR assay identified *P hominis* in diarrheic feces of 4 dogs in which trichomonads were seen by light microscopy. The *P hominis* genes were not amplified from other fecal samples examined.

**Conclusions and Clinical Relevance**—Molecular identification of *P hominis* in feces of 4 dogs with trichomonosis and diarrhea reported here validates the identity of this species in such infections. Sensitive and specific PCR amplification of *P hominis* 18S rRNA genes from DNA extracted from feces will directly facilitate studies examining pathogenicity of this trichomonad and enable differentiation of *P hominis* from other known or novel species of trichomonads that may infect the gastrointestinal tract of dogs. (Am J Vet Res 2007; 68:xxx–xxx)

Trichomonads are obligate protozoan symbionts found in the digestive and urogenital tract of vertebrates. They are characterized morphologically by multiple anterior flagella and a single recurrent flagellum that functions as an undulating membrane. Trophozoites reproduce by binary fission and undergo direct transmission from host to host without formation of environmentally stable cysts.

Genera of the order Trichomonadida consist of commensal and pathogenic species. *Pentatrichomonas hominis* inhabits the large intestine of a number of mammalian hosts and is considered to be a commensal. Trichomonads are occasionally identified in the feces of dogs with diarrhea, where they are presumed to be an opportunistic overgrowth of *P hominis*. However, morphologic or molecular means to confirm the identity of these trichomonads is rarely undertaken, and literature on *P hominis* infection is limited.

Trichomonads other than *P hominis* have been recognized in the gastrointestinal tract of many species including nonhuman primates, pigs, horses, dogs, and cattle. Recently, intestinal trichomonads associated with diarrheal disease in domestic cats were presumed to be *P hominis*. Only by means of molecular characterization and experimental infection studies was the trichomonad identified as *Trichomonas foetus* and determined to be a primary cause of the diarrhea.

Development of a specific means to identify *P hominis* in dogs with trichomonosis and diarrhea would facilitate recognition of novel and potentially pathogenic species of trichomonads that infect the gastrointestinal tract. Recently, Crucitti et al described the use of 2 sensitive and highly specific oligonucleotide probes for the identification of *P hominis*. However, the authors did not determine the detection limits of PCR assay for detection of *P hominis* in biological specimens, particularly feces, and experiments to optimize performance of the PCR assay in biological samples were not reported. Fecal samples are considered to be among the most complex specimens for direct PCR testing because of...
the presence of inherent PCR inhibitors, which are often coextracted along with pathogen DNA. The purpose of the study reported here was to determine the optimum reaction conditions and detection limits of PCR assay for identification of *P. hominis* in DNA extracted from canine fecal specimens.

**Materials and Methods**

**Protozoa**—A bovine isolate of *Pentatrichomonas hominis* was obtained from the ATCC (ATCC 30098) and cultured at 37°C in medium. An isolate of *P. hominis* was cultured from the voided feces of a 13-week-old Cocker Spaniel with loose stools in which trichomonads were identified by light microscopy. Approximately 0.1 g of feces was suspended in 10 mL of PBS solution (pH 7). A 100-µL aliquot of this solution (ie, 0.001 g feces) was inoculated into a culture tube containing 10 mL of antibiotic-fortified (10^6 U of penicillin, 1.5 g streptomycin, and 2 mg of amphotericin B/L) medium and incubated at 37°C with subsequent passage into modified Diamond medium. Logarithmic-phase cultures were centrifuged for 5 minutes at 1,500 × g. Genomic DNA was extracted from a 200-µL aliquot of pelleted organisms by use of a DNA kit in accordance with manufacturer instructions. Identity of the canine isolate was confirmed by partial rRNA gene unit sequence analysis.

**Oligonucleotides**—Three *P. hominis* 18S species-specific primers (Th3, Th4, and Th5) were synthesized on the basis of their published sequences. The effectiveness of each primer pair for *P. hominis* was established previously with a PCR assay by use of genomic DNA isolated from pure cultures of 17 microorganisms including trichomonads and normal or pathogenic flora of the urogenital and gastrointestinal tracts.

**PCR conditions**—The PCR assay was performed in a 100-µL reaction volume of 1X PCR buffer II with 2.5 U of *Taq* polymerase, 100 pmol of each primer, 200µM of each deoxynucleoside triphosphate, 10 µg of bovine serum albumin, and 5 µL of DNA template/reaction. Reaction conditions for each primer pair were optimized for MgCl₂ concentration (range, 2 to 5mM in increments of 0.5mM) and annealing temperature (range, 47°C to 68°C in increments of 3°C) and were performed by use of DNA extracted from feces of a dog with naturally occurring *P. hominis* infection from which we have previously cloned the 18S rRNA gene (GenBank accession No. AY758392).

Amplification of DNA was performed in a thermocycler with the following profile: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at optimal degrees celsius for 1 minute, and extension at 72°C for 2 minutes for 50 cycles and a final extension at 72°C for 5 minutes. Amplicons were viewed by UV illumination after electrophoresis of 10 µL of the reaction solution in 1.5% agarose gel containing ethidium bromide. Reaction products were purified and sequenced by a commercial laboratory.

**PCR detection limits**—In vitro cultivated *P. hominis* organisms in the logarithmic phase of growth were washed 3 times by centrifugation for 5 minutes at 1,500 × g in sterile PBS solution and counted on a hemocytometer by use of a light microscope. Organisms were serially diluted in PBS solution, and genomic DNA was extracted from 200-µL aliquots containing 0.01, 0.1, 1, 10, 100, 1,000, or 10,000 organisms (n = 2 each) by use of a DNA kit in accordance with manufacturer instructions. Twenty-microliter aliquots containing 0.01, 0.1, 1, 10, 100, 1,000, or 10,000 organisms (n = 2 each) were added to 180-ng samples of formed canine feces obtained from a dog with negative culture results for *P. hominis*. The DNA was extracted from spiked fecal samples by use of a commercial DNA stool kit with minor modifications. For each dilution, DNA was eluted in a 200-µL volume. Five microliters of DNA was added to each PCR, resulting in an equivalent number of *P. hominis* organisms, ranging from 0.0025 to 250 organisms.

**Feces in which trichomonads were identified by light microscopy**—Voided fecal samples were obtained from 4 dogs having loose stools in which trichomonads were observed by light microscopic examination of a wet-mount slide. Fecal samples were shipped from referring veterinarians to the authors’ laboratory in 70% isopropyl alcohol. One sample was obtained from a litter of 4-week-old German Shepherd Dogs. The remaining dogs were a 13-week-old female Cocker Spaniel, a 13-week-old castrated male Yorkshire Terrier, and an 8-week-old male Boston Terrier.

**Feces submitted to a veterinary diagnostic laboratory for parasitologic analysis**—A convenience population of fecal samples submitted to a veterinary diagnostic laboratory from 81 dogs was collected over a 6-month period and stored at −80°C prior to DNA extraction. Dogs ranged in age from 3 months to 14 years (median, 5 years) old and comprised 46 males and 35 females, representing 46 breeds.

**Feces obtained from dogs housed in a laboratory animal resources facility**—Feces were collected by loop inserted into the rectum or after voiding from 19 dogs housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited laboratory animal resources facility at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC, and stored at −80°C prior to DNA extraction. Dogs ranged in age from 3 months to 9 years (median, 3 years) old and comprised 8 males and 11 females, representing 7 breeds.

**Detection of inhibitors and controls**—To avoid PCR contamination, DNA extractions, reaction preparation, thermal cycling, electrophoresis, and detection of amplicons were performed in separate areas of the laboratory. During DNA extractions, tubes containing feces and PBS solution (no *P. hominis*) and PBS solution alone were processed in parallel with other study samples to detect any genomic DNA contamination. Negative control tubes were included in each PCR experiment to detect any amplicon contamination. Instead of sample DNA, these tubes received either 5 µL of sterile water, 5 µL of DNA extracted from feces containing no *P. hominis*, or 5 µL of DNA extracted from sterile PBS solution. A single positive control tube was included in each PCR experiment, and this tube received 0.5 µL (20 ng) of purified genomic DNA from a bovine isolate of
P. hominis (ATCC 30098) and 4.5 µL of sterile water instead of sample DNA.

Prior to PCR assay for P. hominis, all samples of DNA extracted from feces were first subjected to PCR amplification of an approximate 876-bp gene sequence of bacterial 16S rRNA. By performing PCR assay for bacterial 16S rRNA gene, anticipated to be abundant in DNA extracted from feces, the possibility that a negative PCR result for P. hominis could be attributed to the presence of endogenous PCR inhibitors in the extracted DNA was ruled out for each sample. Reaction conditions for 16S rRNA gene amplification were as follows: a 100-µL reaction volume of PCR buffer II containing 2.5 units of Taq polymerase, 100 pmol each of primers 515F (5′ GTGCCAGCAGCGCCGCGGTAA 3′) and 1391R (5′ GACGGGCGGTGAGTYGCA 3′), 200 µM of each deoxynucleotide triphosphate, 10 µg of bovine serum albumin, and 5 µL of DNA template. Amplification of DNA was performed at the following temperature profile: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute, and extension at 72°C for 2 minutes for 50 cycles, followed by a final extension for 5 minutes at 72°C.

Results

Optimized PCR conditions—A 785-bp and 339-bp product were amplified by primer pairs Th4-Th5 and Th3-Th5, respectively, by use of purified genomic DNA from a bovine isolate of P. hominis (ATCC 30098) and DNA extracted from the feces of German Shepherd Dog puppies having trichomonad diarrhea from which the P. hominis 18S rRNA gene has been cloned and sequenced (GenBank accession No. AY758392). By use of DNA extracted from feces of the P. hominis–infected puppies as template, PCR assay with either primer pair amplified the target sequence robustly over a wide range of annealing temperatures (47°C to 64°C). For each primer pair, an annealing temperature of 68°C generated a single PCR product having 100% sequence identity with P. hominis (GenBank accession No. AY758392). At an annealing temperature of 68°C, the target sequence of primer pairs Th4-Th5 and Th3-Th5 could be amplified at MgCl₂ concentrations ranging from 2.5 to 5.0 mM and 2.0 to 5.0 mM, respectively. For each primer pair, an MgCl₂ concentration of 2.5 mM generated the most robust PCR product. No amplification products were observed when PCR assay was performed with purified T. foetus, G. lambia, T. hominis spp, or canine fecal DNA as template.

Absolute and practical detection limits of PCR assay for detection of P. hominis—The absolute detection limit of PCR assay for P. hominis was determined by use of 200-µL aliquots of serially diluted canine isolates of P. hominis, ranging from 10,000 to 0.01 organisms. The absolute detection limit was most sensitive for primer pair Th3-Th5 at 100 organisms (2.5 organisms/reaction) in all of the reactions (10/10 replicates; Table 1). The practical detection limit of PCR assay for P. hominis was determined by use of 180-mg samples of canine feces spiked with 20-µL aliquots of serially diluted canine isolates of P. hominis, ranging from 10,000 to 0.01 organisms. The practical detection limit was most sensitive for primer pair Th3-Th5 at 100 organisms/180-mg fecal sample (2.5 organisms/reaction) in all of the reactions (10/10 replicates; Figure 1; Table 1). Sequence analysis of the respective 339-bp and 785-bp products that were amplified by PCR assay of spiked canine fecal samples revealed 100% sequence identity with P. hominis (GenBank accession No. AY758392).

PCR amplification of 16S rRNA genes—An approximate 876-bp gene product of bacterial 16S rRNA was amplified from DNA extracted from feces of all 4 dogs having loose stools in which trichomonads were observed by light microscopy; 76 of 81 dogs from which feces were submitted for parasitologic testing, and 17 of 19 dogs residing in a laboratory animal resources facility. All DNA samples failing to amplify bacterial 16S

Table 1—Absolute detection limits of PCR assay for the detection of Pentatrichomonas hominis as determined by the number of positive results obtained with DNA extracted from aliquots of serially diluted P. hominis.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>200-µL aliquot*</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th4-Th5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Th3-Th5</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

*10 replicate reactions at each dilution were performed.

Table 2—Practical detection limits of PCR assay for the detection of P. hominis in canine feces as determined by the number of positive results obtained with DNA extracted from fecal samples after spiking with P. hominis.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>180-mg sample*</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th4-Th5</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Th3-Th5</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

See Table 1 for key.
in canine feces was reported for children 3 to 5 years old. Both primer pairs performed equally well in amplifying DNA from fecal samples (180 to 220 mg) from 4 dogs having loose stools in which trichomonads were identified by light microscopy, extraction reagents only (no feces; DNA extraction contamination control), and MW markers. See Figure 1 for remainder of key.

Detection of P. hominis in canine feces—By use of primer pair Th3-Th5 under optimized conditions, a 339-bp product having 100% sequence identity with the canine isolate of P. hominis (GenBank accession No. AY758392) was amplified from DNA extracted from the feces of each of the 4 dogs having loose stools wherein trichomonads were observed by light microscopy (Figure 2). The P. hominis 18S rRNA gene was not amplified from DNA extracted from any fecal samples that were submitted for parasitologic testing (n = 76) or those obtained from dogs residing in the laboratory animal resources facility (17).

Discussion

In our study, we optimized and compared the performance of 2 highly specific primer pairs designed by Crucitti et al34 to amplify a partial 18S rRNA gene sequence of P. hominis. Both primer pairs performed exceptionally well in amplifying P. hominis DNA from canine feces over a wide range of annealing temperatures and MgCl2 concentrations. However, under optimized conditions, primer pair Th3-Th5 appeared to be more sensitive and was able to detect as few as 1 organism/180-mg fecal sample. The optimized PCR assay appeared to be more sensitive for identifying P. hominis in spiked feces compared with cultured organisms alone, perhaps because our PCR conditions were tailored for performance in fecal samples.

Trichomonads are reported frequently in parasitic surveys of adults and children in developing countries, where their pathogenicity is unknown and their identity assumed to be P. hominis.35-37 Trichomonads are similarly detected in the feces of dogs. On the basis of microscopic examination or microbiological culture, the prevalence of trichomonads has ranged from 0% to 30% in reported healthy young dogs from Australia, Yugoslavia, and Japan,27,32,33 whereas in New Jersey, 19.8% of 273 stray dogs with either diarrheic or fresh feces were identified with trichomonal infection.3 Molecular studies to determine the identity of these trichomonads were not reported. In our study, we did not amplify the 18S rRNA gene of P. hominis from any of the 17 dogs residing in a laboratory animal resources facility or from 76 dogs having feces submitted to a veterinary diagnostic laboratory for parasitologic testing. Thus, P. hominis was not identified via PCR assay as a prevalent commensal in these dogs.

Large numbers of trichomonads are occasionally observed in the feces of young dogs with diarrhea and are dismissed as an opportunistic overgrowth of P. hominis.3,35 Importantly, molecular identification of P. hominis in the feces of 4 dogs with trichomonosis and diarrhea in our study validates the identity of this species in such infections. However, the contribution of P. hominis to diarrhea in these dogs is unclear. Because fecal samples were preserved in alcohol prior to submission to our laboratory, a light microscopic examination of trichomonad morphology was not performed.

Although the specific indication for submission of feces for diagnostic testing was not investigated for dogs in our study, P. hominis was not identified as a common cause or consequence of gastrointestinal disease for which feces are routinely submitted. A distinct possibility is that P. hominis is an infection of young dogs, of which few were represented in the laboratory animal and tertiary-care populations tested here, or that trichomonads were present in insufficient numbers to detect. In our study, all 4 dogs in which P. hominis infection was identified were < 13 weeks old and had trichomonads present in numbers sufficient to detect with a light microscope. Previously reported ages of dogs with trichomonosis and diarrhea have ranged from 7 weeks to 6 months.5,6,8,9 In 1 study,34 puppies that were experimentally infected with canine isolates of trichomonads spontaneously cleared infection after 35 days and became resistant to reinfection thereafter, suggesting the possibility of acquired immunity. In the same study,34 trichomonads could not be found in adult dogs, nor were adult dogs susceptible to experimental infection. Thus, additional studies to examine the prevalence of P. hominis in populations of young dogs are warranted. For 1 study35 in humans, the highest frequency distribution of P. hominis was reported for children 3 to 5 years of age. The sensitive and specific PCR amplification of P. hominis 18S rRNA genes from DNA extracted from canine feces will directly facilitate future studies to determine the pathogenicity of this trichomonad and enable the differentiation of P. hominis from other known or novel species of trichomonads that may infect the gastrointestinal tract.

a. Quality-control strain Pentatrichomonas hominis ATCC 30098, American Type Culture Collection, Rockville, Md.

b. Medium 2154, American Type Culture Collection, Rockville, Md.
c. QIAamp DNA mini kit, Qiagen, Valencia, Calif.
d. Integrated DNA Technologies, Coralville, Iowa.
e. AmpliTaq Gold DNA polymerase, Applied Biosystems, Foster City, Calif.
g. iCycler iQ, Bio-Rad Laboratories Ltd, Hercules, Calif.

h. QIAquick PCR purification kit, Qiagen, Valencia, Calif.

i. Automated DNA Sequencing Facility, University of North Carolina, Chapel Hill, NC.

j. QIAamp DNA stool mini kit, Qiagen, Valencia, Calif.

References


