**Prevalence of intestinal parasites in shelter cats with diarrhea from Colorado, USA, and optimization and application of a qPCR assay for detection of *Cyclospora cayetanensis***



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May – August 2014   
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Summary

*Objectives* The objectives of this study were to optimize a *Cyclospora cayetanensis* quantitative PCR (qPCR) assay and apply it to feces of cats with diarrhea from a shelter in Colorado, USA to determine whether or not this human parasite was the cause of the diarrhea and thus if it carries zoonotic potential, as well as to assess which other fecal parasites can be found in shelter cats with diarrhea.

*Methods* The optimization of a published SYBR Green based qPCR of a fragment of the internal transcribed spacer 2 (ITS-2) gene of *C. cayetanensis* followed by a melting curve analysis were performed as published. Feces were analyzed using microscopic examination for parasite eggs, cysts and oocysts after using Sheather’s sugar centrifugation. For detection of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts a commercial IFA was used (MERIFLUOR® *Cryptosporidium/Giardia*, Meridian Biosciences).

*Results* Fecal samples from a total of 60 shelter cats with acute diarrhea were evaluated. Enteric parasites were detected in 8 of 60 cats (13.3%) and included *Cryptosporidium* spp. (6.7%), *Giardia* spp. (3.3%) and *Isospora felis* (3.3%). DNA of *C. cayetanensis* was not amplified from any sample.

*Conclusion and relevance* The parasites detected in this study were similar to those in other similar studies and *C. cayetanensis* was not detected. Since all cats were negative for *C. cayetanensis*, it is not likely the diarrhea was caused by this parasite, but more research needs to be done to investigate whether *C. cayetanensis* has the zoonotic potential to infect cats.

Introduction

Diarrhea is commonly found in shelter animals and can be caused by different factors such as stress, dietary changes and enteric parasites. The most commonly found parasites in shelter animals with diarrhea are *Giardia* spp*., Cryptosporidium* spp., *Ancylostoma* spp., and *Strongyloides stercoralis* 1–3*.*

*Giardia duodenalis* is a common intestinal protozoal parasite of both humans and animals which can be subclinical or cause diarrhea, vomiting and weight loss. Infection occurs either by ingesting contaminated water or food or by fecal-oral route via direct contact between humans and animals 4. Cats can harbor both host-specific (assemblage F) and zoonotic strains of *G.duodenalis* (assemblage A). Prevalence rates of *G. duodenalis* in cats in the United States range from 2 to 15% 5.

*Cryptosporidium* spp. are protozoal parasites that can also cause gastrointestinal disease in both humans and animals. Cats usually harbor the host-specific *Cryptosporidium felis* and humans harbor *C. hominis* and *C. parvum*. In most countries studied *C. hominis* and *C. parvum* are responsible for more than 90% of the human cases of cryptosporidiosis. The rest of the cases are attributable to *C. meleagridis, C. canis* and *C. felis* 6,7. Prevalence of *Cryptosporidium* spp*.* in cats range from 3.2 to 5.4% in the United States 2,8. As with *Giardia* spp*.*, the most likely route of contamination is by consuming contaminated drinking water or food 9. The infective oocysts are very resistant against extreme environmental conditions and can therefore survive for months 10. Other parasites commonly harbored by cats can produce significant clinical illness in people after contact with cat feces.

*Cyclospora cayetanensis* is an intestinal protozoal parasite that is most likely to be transmitted by ingestion of contaminated food or water. The oocysts of this parasite are environmentally resistant and the agent is known to be endemic in some developing countries, such as Haiti, Peru and Nepal. Humans appear to be the only natural host and when infected, the parasite can cause prolonged diarrhea, nausea, and abdominal cramps 11. Infected individuals shed unsporulated oocysts in their feces and within 7-15 days the oocysts sporulate and then are infectious to other susceptible hosts 12. *Cyclospora cayetanensis* has not been diagnosed in cats or other animals before. Humans seem to be the only natural host for this parasite 13. However, in this study a PCR test for *Cyclospora cayetanensis* was optimized and applied to cat feces to determine whether this parasite has the zoonotic potential to infect cats besides humans. This explains why this protozoa is included in the study, even when it has never been detected in cats before.

Materials and methodsStudy population

Fecal samples from 60 cats, age ranging from 16 weeks – 3 years old, housed in a shelter in North Central Colorado were included in the study. All cats selected were having acute diarrhea of at least 2 days duration. Fecal samples were collected at day 0 in the months July, August, September and October of the year 2014. Animal characteristics (age, sex, breed, stool consistency) were recorded. Stool consistency was estimated using a standardized fecal scoring system created by Nestlé PURINA (7=watery puddles, 6=texture but no shape, 5=moist piles, 4=moist log shape, 3=normal). Medical information regarding vaccinations, deworming history, current treatments or underlying systemic infections were included.

Fecal assays

Fecal samples collected at day 0 were examined for parasites and parasitic eggs by microscopic examination after using Sheather’s sugar centrifugation technique as previously described by O’Handly et al 14. For *Giardia*spp. cysts and *Cryptosporidium* spp. oocysts a commercial immunofluorescence assay (IFA) (MERIFLUOR® *Cryptosporidium/Giardia*, Meridian Biosciences, Cincinnati, OH) was used. For detection of *Isospora felis* only microscopic examination after Sheather’s sugar centrifugation technique was performed.

Fecal concentration

Approximately 3 grams of feces were washed with 3-4 ml of phosphate buffered saline solution containing ethylenediaminetetraacetic acid (PBS-EDTA) and filtered through gauze. The filtrate was placed on top of 7 ml of Sheather’s sugar solution and then centrifuged at 800×g for 10 min. The interface was being transferred into a clean 15 ml tube and centrifuged at 1500×g for 10 min. The supernatant was discarded and 10-12 ml of PBS-EDTA was added, mixed with the sediment and centrifuged at 1500×g for 10 min. Again the supernatant was discarded and 7 ml of PBS-EDTA was added, mixed with the sediment and centrifuged at 1500×g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS-EDTA and the filtrate was being transferred into an Eppendorf tube and saved at -20°C.

Fecal flotation

Between 2 and 3 grams of feces were washed with 3-4 ml of distilled water and filtered through gauze. The filtrate was centrifuged at 1500×rmp for 2-3 min. The supernatant was decanted and mixed with 12-13 ml of Sheather’s sugar solution (ı = 1.26) in a 15 ml centrifuge tube and centrifuged at 1500×rpm for 10 min. Approximately 2-3 ml of sugar solution was added to give a positive meniscus and a coverslip was placed on top of the solution and left for 10 min at room temperature. The coverslip was placed on a microscope slide and the slide was examined microscopically for *Giardia* spp*.* cysts and *Cryptosporidium* spp*.* oocysts.

Immunofluorescent assay

10 µl of the fecal concentration filtrate were pipetted onto microscope slides supplied in an in vitro direct immunofluorescence assay (DFA) capable of simultaneous detection of *Giardia* spp*.* cysts and *Cryptosporidium* spp*.* oocysts (Merifluor Crypto/Giardia kit, Meridian Diagnostic Corporation, Cincinnati, OH). The assay was then performed and interpreted following the manufacturer’s instructions.

DNA extraction

DNA was extracted from the fecal samples as described by da Silva et al 15. PCR amplification was performed on the IFA positive samples as published using the beta-giardin, triose phosphate isomerase, and glutamate dehydrogenase genes for *Giardia* and the heat shock protein-70 gene for *Cryptosporidium* 16–20. All fecal samples were tested for the presence of *C. cayetanensis* by using a validated PCR protocol.

Validation of Cyclospora cayetanensis PCR

The assay used was already published by Kitajima et al 11 and was optimized for use in human samples. In this study this PCR was optimized for the conditions in the laboratory at Colorado State University and validated for use in feline fecal samples. First the primers used in the assay were compared by BLAST analysis in the GenBank in order to evaluate the specificity. A forward and a reverse CCITSS2 primer were used. The sequences of the primers are as follows:

Forward: 5´-GCA GTC ACA GGA GGC ATA TAT CC-3´ (23bp).   
Reverse: 5´-ATG AGA GAC CTC ACA GCC AAA C-3´ (22bp).

All fecal samples were concentrated using Sheather’s sugar concentrating technique. DNA was extracted 15 from all samples and the hsp-70 PCR was performed as published by Lalle et al 18. An annealing gradient ranging from 53-60°C was performed in order to determine the optimal annealing temperature. An assay titration ranging from 1000 to 0.1 fM of DNA per reaction was conducted to achieve the lowest reliable detection limit of DNA per reaction in this laboratory. Specificity was determined by performing a melting curve analysis using DNA from *Toxoplasma gondii, Cryptosporidium parvum* and *Giardia duodenalis* in a real time PCR. A primer titration was performed in which different concentrations of primers were added to the reaction in order to determine which concentration of forward and reverse primer were optimal. Primer concentrations of 50, 100, 300 and 600 nM were used and combined as followed:

|  |  |  |  |
| --- | --- | --- | --- |
| Primer concentration (nM) | Primer concentration (nM) | Primer concentration (nM) | Primer concentration (nM) |
| F’(50) R’(50) | F’(100)  R’(50) | F’(300)  R’(50) | F’(600)  R’(50) |
| F’(50)  R’(100) | F’(100)  R’(100) | F’(300)  R’(100) | F’(600)  R’(100) |
| F’(50)  R’(300) | F’(100)  R’(300) | F’(300)  R’(300) | F’(600)  R’(300) |
| F’(50)  R’(600) | F’(100)  R’(600) | F’(300)  R’(600) | F’(600)  R’(600) |

In order to express the reproducibility and repeatability of the assay respectively inter and intra assay variability were measured. To measure inter assay variability five replications of each dilution were made in four different real time PCR plates. To determine intra assay variability three replications of each dilution were made in one plate. Once the PCR assay was validated it was used for application to the feline fecal samples. Furthermore a positive control assay was performed to validate the PCR protocol. *Cyclospora cayetanensis* DNA was acquired from the RIVM in The Netherlands. It was originating from a Dutch patient that tested positive for *Cyclospora cayetanensis.*

Application of the validated protocol to cat samples

The assay was used to identify and molecularly characterize the *Cyclospora cayetanensis* positive feline isolates. *Cryptosporidium* spp*.*, *Giardia* spp*.* and *Cyclospora cayetanensis* PCR positive samples were analyzed in forward and reverse direction using an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, California). The sequence data from these isolates were compared by BLAST analysis with sequences from the nucleotide database from the GenBank. A multiple alignment with the sequences on the target genes of this study and reference sequences obtained from the GenBank was performed. The phylogenetic analysis was performed with Neighbor-Joining algorithm using MEGA 4.0 or Geneious 6.1. Bootstrap proportions were assessed by the analysis of 1000 replicates.

Statistical analysis

Overall prevalence for parasite infections in diarrhea in shelter cats was defined as the percentage of fecal samples that tested positive for any parasite by any of the diagnostic tests. Specific parasite prevalence rates were also included.

ResultsValidation of the SYBR Green based qPCR for Cyclospora cayetanensis

In this study a SYBR Green based qPCR of a fragment of the internal transcribed spacer 2 (ITS-2) gene of *C. cayetanensis* as previously described and developed by Kitajima et al (2014) was optimized for the conditions at Colorado State University. An assay titration was performed with an initial concentration of 10mM of DNA per reaction. Then a 10-fold serial dilution of the synthesized oligonucleotide (AF301386) was made ranging from 1000-0.1 fM of DNA per reaction. It was found that the lowest reliable level of detection was 1fM. The use of 100fM of DNA per reaction was considered optimal (table 1). A standard curve was constructed and showed that the assay was linear over a range of four dilutions with an r2 value of 0.926 and a reaction efficiency of 102% (fig. 1). To determine specificity of the oligonucelotide primers a melting curve analysis for the *C. cayetanensis* oligonucleotide was performed. The primers were set up with DNA template from *T. gondii, C. parvum* and *G.* duodenalis. No amplification of DNA other than DNA of *C. cayetanensis* was observed in this reaction. It showed a single melting temperature (Tm) peak between 81.1°C and 81.8°C, which is considered to be the Tm for the oligonucleotide of *C. cayetanensis* (fig. 2). Optimal annealing temperature was set at 57°C, while optimal primer matrix concentrations ranged between 50-100nM per reaction. The reaction showed to be reproducible and repeatable by measuring inter and intra assay variability, which gave consisted values.



Table 1Dilution series that show how many times *Cyclospora cayetanensis* DNA was detectable out of five reactions using different titrations

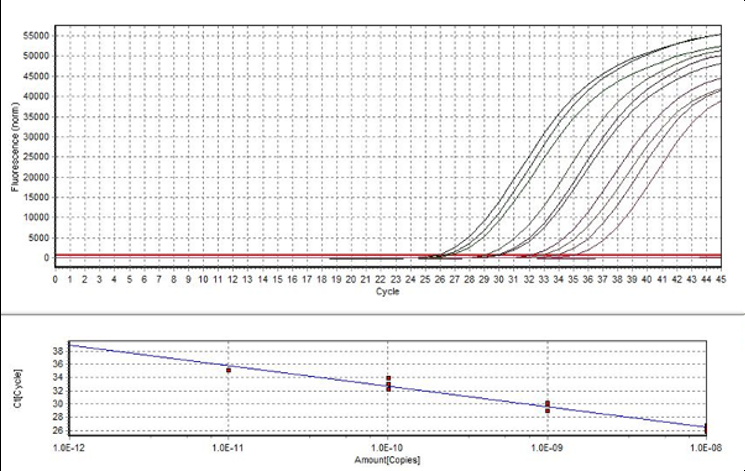


Figure 1Amplification and standard curve of the *Cyclospora cayetanensis* dilution series by the IST-2 PCR

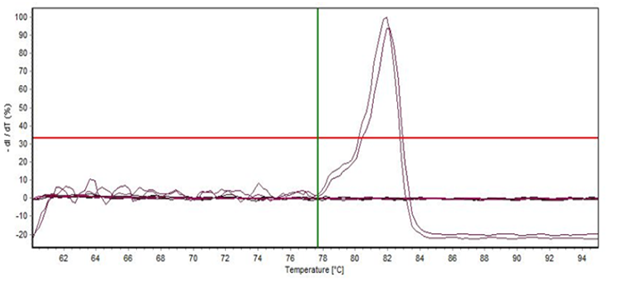


Figure 2Melting curve analysis showing only *Cyclospora cayetanensis* is being amplified in the real time PCR assay

Detection of fecal enteric parasites

60 fecal samples from cats with diarrhea were investigated for the following fecal enteric parasites: *Isospora felis, Cryptosporidium* spp., *Giardia* spp. and *Cyclospora cayetanensis*. In eight out of 60 samples (13.3%) parasites were detected. All fecal samples were first examined using microscopic detection for parasites and parasitic eggs. In two out of 60 samples (3.3%) *Isospora felis* was found. No further steps were taken to genotype this parasite. All 60 fecal samples were examined by making use of a commercially available IFA for detection of *Cryptosporidium* spp. and *Giardia* spp. Two out of 60 samples (3.3%) tested positive for *Giardia* spp. Positive samples were tested using the b-giardin and the glutamate dehydrogenase genes of *Giardia* spp. to sequence the isolates. One isolate of *Giardia* was typed as assemblage F, which is the cat-specific assemblage.

All fecal samples were examined with PCR using the 18S rRNA assay for detection and genotyping *Cryptosporidium* spp. This protozoa was found in four out of 60 samples (7.6%). The assay typed only to *Cryptosporidium* spp. in all four isolates and two of them were typed as *C. felis*. The validated SYBR Green based qPCR for detection of *Cyclospora cayetanensis* was applied to all fecal samples, however DNA of this parasite was not amplified in any of the samples (0%).

Discussion

*Cyclospora cayetanensis* is a parasite known to infect humans and cause symptoms such as diarrhea. This parasites shows a lot of similarities with zoonotic protozoan parasites such as *Cryptosporidium* spp. and *Eimeria* spp. and is therefore expected to be zoonotic as well 21,22. However, *C. cayetanensis* has never been isolated from animals and has only been described as a potential zoonosis 23. Besides, it is unknown how widespread *C. cayetanensis* is in the world, but it is known to be endemic in some developing countries such as Peru, Nepal, Indonesia and Guatemala. Small outbreaks in humans in North America have been reported since 1995, but were associated with consumption of fresh imported vegetables 11,24. Since this parasite is not known to be infective to cats and the prevalence of *C. cayetanensis* is low in North America, it is unlikely that the shelter cats used in this study that were housed in North Colorado were at risk of getting infected with *C. cayetanensis*.

All fecal samples were microscopically examined for parasites and parasitic eggs. However, this is not a very sensitive way of detecting oocysts of *C. cayetanensis,* because recognition of this parasiterequires sporulated oocystes, which can take one or two weeks after being excreted in feces. Besides, it is known that *C. cayetanensis* is usually shed in low numbers in feces, which makes it easy to be overlooked during microscopic examination, especially when the investigator is not experienced in recognizing the parasite. Furthermore, a single negative fecal sample usually is not sufficient to conclude the parasite is not present. Three or more samples, collected on subsequent days, are needed since the oocysts can be excreted intermittently 25,26. In this study, all fecal samples were microscopically examined only a few hours or days after collection and all investigators were inexperienced with working with this parasite.

The PCR protocol that was validated in this study was based on a SYBR Green qPCR developed and validated by Kitajima et al (2014) and was based on DNA extraction of *C. cayetanensis* that was derived from waste water samples while this study focused on fecal samples from cats. The primers used in this study were thus tested specifically for the species they were tested against by Kitajima et al. Besides, the positive control used in this study was based on a human stool sample containing human-specific *C. cayetanensis* oocysts. Since there is a limited amount of information available about the sequence of *C. cayetanensis* at GenBank, more specificity testing is needed to determine whether this protocol is specific enough to also detect a non-human *Cyclospora* DNA in fecal samples from cats24.

The fecal parasites detected in this study were similar to those found in other studies performed on shelter cats with diarrhea2,3,8–10. However, it is known that other parasites, such as nematodes, hookworms and viruses, as well as pathogenic bacteria like *Campylobacter* spp. and *Clostridium* spp. commonly cause diarrhea in shelter cats as well3,9. In this study only four different fecal parasites were evaluated, therefore it is not excluded that other pathogens were present in these cats which caused the diarrhea. Besides, it is known *Giardia* is being shed intermittently, therefore it is indicated to collect several fecal samples over a time of 4-5 days9. In this study only one fecal sample per cat was evaluated and therefore it is likely that some cats infected with *Giardia* were overlooked using this protocol.

For detecting and genotyping *Cryptosporidium* spp. all fecal samples were examined with PCR using the 18S rRNA assay. However, this rRNA PCR assay is not optimal for genotyping *Cryptosporidium* spp. since it only amplifies a highly conserved region of the RNA gene of *Cryptosporidium* isolates6. However, the heat shock protein-70 (hsp-70) gene for *Cryptosporidium* was able to type two of the four *Cryptosporidium* positive isolates as *C. felis.*

The protocol for DNA extraction and detection of *C. cayetanensis* described in this study is a reliable and specific method for diagnosing an infection with this parasite in fecal samples. However, further investigation is needed in order to determine whether *C. cayetanensis* is zoonotic and thus a potential risk to cats or not.

Conclusions

The SYBR Green quantitative based PCR (qPCR) was successfully optimized and validated for the detection of *Cyclospora cayetanensis* in feline fecal samples. The results showed that the protocol that was set up by Kitajima et al (2014) is reproducible and the following values are considered optimal for the conditions at Colorado State University: assay titration is considered optimal when 100fM of DNA per reaction is used. The annealing gradient for this protocol is optimal at 57°C. The primers did not amplify DNA from *T. gondii, C. parvum* or *G. duodenalis* in real time PCR, which shows the primers are specific for amplifying *C. cayetanensis*. Optimal primer titration ranged from 50-100nM of each primer per reaction. The validation results showed that the protocol that was set up at CSU is reproducible and repeatable by performing an inter and intra assay variability as it gave consisted values.

For the detection of fecal parasites and parasitic eggs in 60 shelter cats with acute diarrhea several fecal examinations were performed: microscopic detection of parasites and parasitic eggs after Sheather’s sugar centrifugation, commercially available IFA’s for detection of *Cryptosporidium* spp. and *Giardia* spp. and the validated SYBR Green qPCR for detection of *C. cayetanensis.* The parasites found in this study were similar to those in similar studies. Fecal enteric parasites were detected in 8 of 60 cats (13.3%). The parasites detected included *Cryptosporidium* spp.(6.7%), *Giardia* spp. (3.3%) and *Isospora felis* (3.3%). After genotyping the *Cryptosporidium* spp. positive samples four out of two samples typed as *C. felis*. One out of two *Giardia* spp. positive samples was sequenced as *G. duodenalis assemblage F*, which is the cat-specific assemblage. *C. cayetanensis* was not amplified from any of the fecal samples, therefore it is unlikely the diarrhea was caused by this parasite. However it stays unclear whether *C. cayetanensis* has the zoonotic potential to infect cats besides humans and therefore more research needs to be done to confirm or exclude this hypothesis.

Acknowledgements The authors like to acknowledge Jennifer Hawley for technical assistance and Denise Hoek together with the Institute of Risk Assessment Sciences for providing a *Cyclospora cayetanensis* isolate.

**Conflict of interest**

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

Funding

This study was funded by Merial Ltd, Duluth, GA, USA and the Center for Companion Animal Studies at Colorado State University, Fort Collins, CO, USA.

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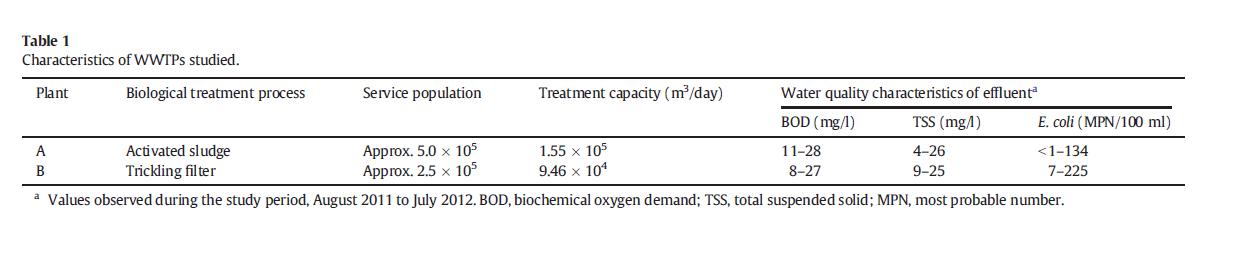
Attachments (1)

Kitajima et al (2014) material & methods

The protocol this study was based on

Collection of wastewater samples

Between August 2011 and July 2012, influent and effluent wastewater grab samples were collected monthly from two WWTPs located in southern Arizona. Plant A utilized a conventional activated sludge process and Plant B utilized a biological trickling filter process or “biotower”. In addition, both plants used chlorination for disinfection. The characteristics of each plant are described in Table 1. All samples were collected in sterile plastic bottles, stored on ice, and transported to the laboratory, where they were processed within 12 h of collection.



Concentration of protozoa in wastewater samples

A total of 48 wastewater samples (12 influent and 12 effluent samples from each of the two plants) were collected, and protozoa in the samples were concentrated using an electronegative filter method, which is capable of concentrating viruses as well as protozoa in water samples, as described previously (Haramoto et al., 2012), with slight modifications. Briefly, the wastewater samples (100 ml influent and 1000 ml effluent) were passed through the electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Billerica, MA) attached to a glass filter holder (Advantec, Tokyo, Japan), followed by an acid rinse and elution of viruses from the filter (Katayama et al., 2002). To recover the protozoan (oo)cysts retained on the filter after the elution of viruses, the filter was detached from the glass filter holder, aseptically cut in half, and one half of the filter was vigorously vortexed in the presence of a ball-shaped stirring bar and 10 ml of an elution buffer containing 0.2 g/l Na4P2O7·10H2O (Kanto Chemical, Tokyo, Japan), 0.3 g/l EDTA(C10H13N2O8)·3Na·3H2O (Wako Pure Chemical Industries, Osaka, Japan), and 0.1 ml/l Tween 80 (Research Organics, Cleveland, OH) in a 50-ml plastic tube. The water portion of the sample was recovered in another 50-ml plastic tube. The same procedure was repeated twice with 10 and 5 ml of the elution buffer, and approximately 25 ml of the resulting protozoa-concentrated sample was obtained.

Immunomagnetic separation

The 25-ml protozoa-concentrated sample was centrifuged at 2000 ×g for 10 min at 4 °C, the supernatant was carefully removed, and the pellet was suspended with 10 ml of phosphate buffered saline (PBS). The tube was centrifuged again at 2000 ×g for 10 min at 4 °C and the resulting pellet was suspended in 10 ml of PBS. To purify *Cryptosporidium* oocysts and *Giardia* cysts, the sample was subjected to immunomagnetic separation (IMS) using the Dynabeads GC-Combo (Life Technologies,

Carlsbad, CA) following the manufacturer's protocol with slight modifications. In brief, Dynabeads® anti-*Cryptosporidium* and Dynabeads® anti-*Giardia* were added to the sample, followed by a rotation for 1 hour at room temperature. Subsequently, the Dynabeads-organism complexes were pelleted using a Dynabeads® MPC®-1 magnet, and the supernatant (approx. 10 ml) was recovered for the *Cyclospora* assay. To wash the Dynabeads-organism complexes, the pellet was resuspended in 10 ml

of PBS. The Dynabeads-organism complexes were pelleted again using the Dynabeads® MPC®-1 magnet, and the supernatant (approx. 10 ml) was also recovered for the *Cyclospora* assay, which was combined with the previously obtained supernatant and resulted in obtaining a total of approx. 20 ml sample for the *Cyclospora* assay. *Cryptosporidium* oocysts and *Giardia* cysts were acid-dissociated from the Dynabeads according to the manufacturer's protocol with slight modification. Briefly, 50 μl of 0.1 NHCl (Kanto Chemical) was added to the tube and the pellet was resuspended by vigorous

vortexing. The Dynabeads were pelleted again using the Dynabeads® MPC®-S magnet, and the supernatant (50 μl) was collected in a tube containing 10 μl of 1.0 N NaOH for neutralization. This (oo)cyst dissociation procedure was repeated once more using 50 μl of 0.1NHCl and the supernatant was recovered in the same tube to obtain purified (oo)cysts with a final volume of 110 μl. Next, the 20ml sample for the *Cyclospora* assay was centrifuged at 2000 ×g for 10min at 4 °C, and the pellet was resuspended in PBS to obtain a *Cyclospora* oocyst suspension with a volume of 1.5 ml.

Immunofluorescent assay (IFA) of Cryptosporidium oocysts and Giardia cysts

Half of the IMS-purified sample of oocysts and cysts was passed through a hydrophilic polytetrafluoroethylene membrane (pore size, 1.0 μm; diameter, 25 mm; Advantec), followed by fluorescent staining of the protozoan (oo)cysts on the membrane using the EasyStain (BTF, North Ryde, Australia). A BX60 fluorescence microscope (Olympus, Tokyo, Japan) was used to count the numbers of *Cryptosporidium* oocysts (round-shaped with a diameter of 4–6 μm) and *Giardia* cysts (oval-shaped with a diameter of 5–8 μm and a width of 8–12 μm) with the B excitation (wavelength of 450–490 nm). The particles that also fluoresced under the G excitation (wavelength of 546 nm) were considered as algae because chlorophyll in the algae fluoresces under this wavelength, and thus, they were excluded from the count. The concentration of (oo)cysts in the original water sample was calculated from (oo)cystpositive samples assuming 100% recovery of (oo)cysts during detection processes, such as the concentration of water samples and IMS.

Protozoan (oo)cyst recovery test

Four influent (50ml each) and four effluent (500 ml each) wastewater samples collected from a WWTP were inoculated with ColorSeed, which contains *Cryptosporidium* oocysts and *Giardia* cysts stained with a Texas Red dye; one vial of ColorSeed containing 100 oocysts and 100 cysts was inoculated to each wastewater sample. The wastewater samples were passed through the electronegative filter (cat. no. HAWP-047-00;MerckMillipore), and the filter was vigorously vortexed in the presence of a ball-shaped stirring bar and 10 ml of the elution buffer (components described above) in a 50-ml plastic tube. Approximately 10 ml of the resulting protozoa-concentrated sample was obtained, and subsequently, the IMS purification was performed following the procedures described above. The number of *Cryptosporidium* oocysts and *Giardia* cysts in the samples were counted with the BX60 fluorescence microscope (Olympus) under the B and G excitations (wavelength of 450–490 and 546 nm, respectively).

Protozoan nucleic acid extraction

The other half of the IMS-purified sample of oocysts and cysts (approx. 55 μl) was used for the nucleic acid extraction of *Cryptosporidium* and *Giardia*. For the *Cyclospora* assay, a 200-μl portion of the 1.5 ml sample was used for the nucleic acid extraction. The samples were subjected to ten cycles of freeze–thaw (−80 °C for 10 min and 56 °C for 5 min), followed by nucleic acid extraction and purification using the QIAamp DNAmini kit (Qiagen, Hilden, Germany) to obtain a DNA extract of 200 μl and further concentration of the extracted DNA using the Amicon Ultra-0.5 (Merck Millipore) to obtain a final volume of approx. 30 μl.

PCR detection of protozoa

***Cryptosporidium spp.***

Nested PCR targeting the 18S rRNA gene of *Cryptosporidium* spp. was carried out as described previously (Xiao et al., 1999, 2000). Briefly, the first PCR was performed in 50 μl of reaction volume containing 5 μl of DNA, 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co., Otsu, Japan), and 15 pmol each of primers (forward, 5′-TTCTAGAGCTAATACATGCG-3′; reverse, 5′-CCCATTTCCTTCGAAACAGGA-3′) to generate approximately1325 bp products (Xiao et al., 1999, 2000). PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension reaction at 72 °C for 1 min, and then a final extension at 72 °C for 7 min. The second PCRwas performed in 50 μl of reaction volume containing 2 μl of the first PCR product, 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co.), and 15 pmol each of primers (forward, 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′; reverse, 5′-AAGGAGTAAGGAACAACCTCCA-3′) to generate 819 to 825 bp products (depending on the species) (Xiao et al., 1999). PCR amplification was performed under conditions identical to those for the first PCR.

***G. intestinalis***

Seminested PCR targeting the GDH gene of *G. intestinalis* was carried out as described previously (Read et al., 2004). Briefly, the first PCR was performed in 50 μl of reaction volume containing 5 μl of DNA, 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co.), and 15 pmol each of forward primer GDHeF (5′-TCAACGTYAAYCGYGGYTTCCGT-3′) and reverse primer GDHiR (5′-GTTRTCCTTGCACATCTCC-3′). PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification with denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, extension reaction at 72 °C for 1 min, and then a final extension at 72 °C for 7 min. The second PCR was performed in 50 μl of reaction volume containing 2 μl of the first PCR product, 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co.), and 15 pmol each of forward primer GDHiF (5′-CAGTACAACTCYGCTCTCGG-3′) and reverse primer GDHiR to generate approximately 432 bp products (Read et al., 2004). PCR amplification was performed under conditions identical to those for the first PCR.

***C. cayetanensis***

A previously published primer set for *C. cayetanensis*-specific single round PCR amplifying 116-bp fragments in the internal transcribed spacer 2 (ITS-2) gene (Lalonde and Gajadhar, 2008) was implemented in SYBR Green-based quantitative PCR (qPCR) format. Briefly, 5 μl of DNA was mixed with 20 μl of PCR mixture containing 12.5 μl of SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Co.) and 10 pmol each of forward primer CCITS2-F (5′-GCAGTCACAGGAGGCATATATCC-3′) and reverse primer CCITS2-R (5′-ATGAGAGACCTCACAGCCAAAC-3′). The qPCR amplification was performed using a Thermal Cycler Dice Real TimeSystemTP800 (TaKaRa Bio Co.) programmed with the following conditions: 95 °C for 30 s, and 45 cycles of 95 °C for 5 s and 55 °C for 30 s. At the end of 45 amplification cycles, a melting curve analysis was performed to confirm specific amplification of the target gene. Using a 10-fold serial dilution of standard plasmid DNA, efficiency (E) of the PCR was calculated according to the formula E = 10−1/S − 1, where S represents the slope of the linear regression curve. The qPCR reactions for the wastewater samples were performed in duplicate (i.e. two PCR tubes per sample) and considered positive only when both tubes fluoresced with sufficient intensity and the average cycle threshold (CT) value was not more than 40, as recommended by the guidelines described elsewhere (Bustin et al., 2009).

PCR inhibition test

A nested PCR to test the presence of PCR inhibition was carried out using salivirus/klassevirus plasmid DNA as an internal control. Briefly, the first PCR was performed in 50 μl of reaction volume containing 5 μl of the extracted DNA after IMS, 1.0 × 103 copies of salivirus/klassevirus plasmid DNA constructed in our previous study (Haramoto et al., 2013), 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co.), and 15 pmol each of forward primer SAL-L1 (5′-CCCTGCAACCATTACGCTTA-3′) and reverse primer SAL-R1 (5′-CACACCAACCTTACCCCACC-3′) (Shan et al., 2010). PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension reaction at 72 °C for 1 min, and then a final extension at 72 °C for 7 min. The second PCR was performed in 50 μl of reaction volume containing 2 μl of the first PCR product, 25 μl of Premix Ex Taq Hot Start Version (TaKaRa Bio Co.), and 15 pmol each of forward primer SAL-L2 (5′-ATTGAGTGGTGCAYGTGTTG-3′) and reverse primer SAL-R2 (5′-CAAGCCGGAAGACGACTAC-3′) to generate approximately 414 bp products (Shan et al., 2010). PCR amplification was performed under conditions identical to those for the first PCR. The nested PCR products of the internal control were separated by electrophoresis on 2% agarose gel and visualized under a UV lamp after ethidium bromide staining.

Equivalent original wastewater volumes tested by the PCR assays for protozoa

As described above, 100 ml of influent and 1000 ml of effluent samples were filtered with the electronegative filter, half of the filter was used for the protozoan analysis. For *Cryptosporidium* spp. and *G. intestinalis*, half of the IMS-purified sample (approx. 55 μl) was applied to DNA extraction, while for the *Cyclospora* assay 200-μl of the 1.5-ml sample which originated from the supernatant of IMS was used for DNA extraction. Subsequently 200 μl of the DNA extracts were concentrated to approximately 30 μl, of which 5 μl was used for the PCR detection of each type of protozoa. Therefore, the original water volumes tested for *Cryptosporidium* spp. and *G. intestinalis* was equivalent to approx. 4.2 ml of influent and approx. 42 ml of effluent, and approx. 1.1 ml of influent and approx. 11 ml of effluent for *C. cayetanensis*.

Nucleotide sequencing and phylogenetic analysis

The (semi)nested PCR products were separated by electrophoresis on 2% agarose gel and visualized under a UV lamp after ethidium bromide staining. PCR products of expected size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Both strands of the product were sequenced using an Applied Biosystems 3730xl DNA Analyzer (Life Technologies). Genetyx software version 9.1.0 (Genetyx, Tokyo, Japan) was used to perform multiple sequence alignment and to generate a phylogenetic tree using the neighbor-joining method with bootstrap values of 1000 replicates. The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers AB808741 to AB808755.

Statistical analyses

Student's t-tests were performed with Microsoft Excel for Mac 2011 (Microsoft Corp., Redmond, WA) to determine whether the log10 reductions at Plants A and B were statistically different. Differences were considered statistically significant if the resultant P value was 0.05 or lower.

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Attachments (2)

Table with parasites that were detected in the cats used in this study  
NP# = number of patient  
FF = fecal flotation  
IFA = immunofluorescense assay  
GDH PCR = glutamate dehydrogenase PCR (for Giardia)  
BG PCR = beta-giardin PCR (for Giardia)  
NPD = no parasites detected  
NDD = no DNA detected

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **ID #** | **Name** | **Day 0 FF** | **Day 0 IFA** | **Day 0 Crypto PCR** | **Day 0 Giardia GDH PCR** | **Day 0 Giardia BG PCR** | **Cyclospora PCR** | **Day 10 FF** | **Day 10 IFA** |
| **NP1** | Spartacus | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP2** | Domino | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP3** | Betty Sue | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP4** | Ulrich | NPD | NPD | Crypto spp. | NDD | NDD | NDD | NPD | NPD |
| **NP5** | Thistle/Nero | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP6** | Etch | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP7** | Pippa | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP8** | Yuki | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP9** | Duchess | NPD | NPD | Crypto spp. | NDD | NDD | NDD | Isospora felis | NPD |
| **NP10** | Sophie/Ember | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP11** | Zeena | NPD | NPD | Crypto spp. | NDD | NDD | NDD | NPD | NPD |
| **NP12** | Yellic | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP13** | Kitkat/Arya | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP14** | Pocket | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP15** | Jaxson | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP16** | Grayman | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP17** | Nox | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP18** | Larry | Isospora felis | NPD | NDD | NDD | NDD | NDD | NPD | Isospora felis |
| **NP19** | Kate | NPD | NPD | NDD | NDD | NDD | NDD | n/a | n/a |
| **NP20** | Ewan | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP21** | Morgan | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | Giardia (2cysts)  Crypto(1 oocyst) |
| **NP22** | Dark Knight | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP23** | Kizzyman | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP24** | Henry | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP25** | Slink | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP26** | Zoltara | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP27** | Warren | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP28** | Flash | NPD | NPD | NDD | NDD | NDD | NDD | NPD | Crypto(1 oocyst) |
| **NP29** | Lacey | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP30** | Gepetto | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP31** | Rondy | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP32** | Matty | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP33** | Silly Milly | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP34** | Pebbles | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP35** | Goldie | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP36** | Gaia | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP37** | Stormy | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP38** | Grey Cat | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP39** | Bebe | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP40** | Kappa | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP41** | Lola | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP42** | Manning | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP43** | Mitty | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP44** | Gill | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP45** | Garrett | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP46** | Norris | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP47** | Emerson | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP48** | Pixie | NPD | Giardia | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP49** | Chloe | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP50** | Marissa | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP51** | Orion | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP52** | Big S | NPD | Giardia | NDD | assemblage F | assemblage F | NDD | NPD | NPD |
| **NP53** | Sunset | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP54** | Rick | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP55** | Beth | NPD | NPD | Crypto spp. | NDD | NDD | NDD | NPD | NPD |
| **NP56** | Merlin | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP57** | Crystal | NPD | NPD | NDD | NDD | NDD | NDD | Isospora felis | NPD |
| **NP58** | Tundra | Isospora felis | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP59** | Gypsy | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |
| **NP60** | Nala | NPD | NPD | NDD | NDD | NDD | NDD | NPD | NPD |