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IP International Journal of Medical Microbiology and Tropical Diseases

Journal homepage: https://www.ijmmtd.org/

Original Research Article

A new single run polymerase chain reaction assay for cyclosporiasis in immunocompromised patients

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ARTICLE INFO

Article history:
Received 21-07-2021
Accepted 27-08-2021
Available online 01-09-2021

Keywords:
Cyclospora cayetanensis
Diarrhea
PCR assay
People living with HIV/AIDS
PLHA
Post–renal transplant
PRT
Modified Ziehl-Neelsen staining

ABSTRACT

Background: *Cyclospora cayetanensis* causes human intestinal cyclosporiasis. It is more common in the immunocompromised patients and mainly seen in people living with HIV/AIDS (PLHA), post-renal transplant (PRT) patients and immunocompromised children (IC). Diagnostic microscopy for the oocysts of the parasite is less sensitive, requiring examination of multiple stool samples. Here we developed a new single run polymerase chain reaction (PCR) assay for the detection of *C. cayetanensis* and it was used to know the hospital based prevalence of cyclosporiasis.

Materials and Methods: A cross-sectional study was conducted from June 2016 to October 2020 in a tertiary care teaching hospital. A new single run amplification PCR-based diagnostic assay was developed for *C. cayetanensis*. Stool samples were collected from 121 PLHA, 135 PRT and 79 immunocompromised children (IC) other than PLHA and PRT. All stool samples were examined for the presence of *C. cayetanensis* oocysts as well as tested with new *C. cayetanensis* PCR assay.

Results: Modified Ziehl-Neelsen staining of the concentrated stool smear did not reveal oocysts of *Cyclospora* species in any stool specimen. However, new PCR assay detected *C. cayetanensis* in 2 stool specimens – one from a PLHA patient and another from a PRT patient, giving a prevalence of 0.6% (2/335), 0.8% (1/121) in PLHA and 0.7% (1/135) in PRT. It was not detected in IC.

Conclusion: Cyclosporiasis is infrequent in southern part of India. The new single run PCR assay developed by us is simple and cost effective molecular assay for the detection of *C. cayetanensis*.

1. Introduction

Cyclospora cayetanensis (*C. cayetanensis*) is the only pathogenic species of the *Cyclospora* genus responsible for human cyclosporiasis.[1] People living in endemic areas or traveling in endemic countries are more likely to become infected. The intestinal parasite is mainly transmitted by the faecal contamination of food or water. It causes an enteric disease and presented with acute or chronic diarrhea. Fever, nausea, vomiting, abdominal pain/cramps and weight loss are common manifestations.[2–4] It is an emerging infectious disease with an increasing number of outbreaks reported from developing and developed countries, including the United States of America and Canada.[5] The prevalence of cyclosporiasis was observed from nil to 41.6%.[6] The global prevalence of *C. cayetanensis* in humans is 3.6%.[7] Cyclosporiasis is more common in the immunocompromised patients and mainly seen in people

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https://doi.org/10.18231/j.ijmmtd.2021.042

living with HIV/AIDS (PLHA), post-renal transplant (PRT) patients and immunocompromised children (IC). A study from north India observed a prevalence of 2.4% among immunocompromised, immunocompetent and healthy individuals.[8]

Conventional diagnosis of cyclosporiasis is made by microscopic examination of the stool smears.[9] The sensitivity of microscopy depends upon the number of oocysts present in a stool sample. No oocytes may be seen in a stool sample due to intermittent shedding of the oocysts, requiring examination of multiple stool samples to diagnose cyclosporiasis.[9] Commercially available antigen detection tests are expensive, have variable sensitivity and therefore not used in resource-limited countries.[10,11] Molecular diagnosis based on polymerase chain reaction (PCR) has higher sensitivity than microscopy. Analysis of the gene encoding 18S ribosomal ribonucleic acid (rRNA) has shown that this locus of the parasitic deoxyribonucleic acid (DNA) is highly conserved and suitable for molecular detection.[12,13] Diagnostic facilities for this ubiquitous parasite in India is limited to major research facilities. There is a need for a sensitive and reliable diagnostic test. We here report a new single run PCR assay developed by us for the detection of *C. cayetanensis* directly from stool samples and it was used to know the hospital based prevalence of cyclosporiasis.

2. Materials and Methods

This cross sectional study was conducted from June 2016 to October 2020 at Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. It was approved by the Institute Ethics Committee (Human studies). The PLHA patients were recruited from antiretroviral treatment (ART) clinic, and PRT patients from Department of Nephrology, and immunocompromised children (IC) from Department of Pediatrics. A total of 335 immunocompromised patients were recruited in the study - 121 PLHA group, 135 PRT group, and 79 IC group. Six children on ART were included in the PLHA group, and six PRT children were included in the PRT group. These 12 children were not included in the IC group. The clinico-demographic details of the participants were recorded in a pre-structured proforma. The immunocompromised participants were categorized into two groups, based on the presence or absence of diarrhea.[14] Group 1 includes the patient presented with diarrhea and group 2 includes the patient presented without diarrhea.

2.1. Microscopic examination for oocysts of C cayetanensis

Stool samples were concentrated by Sheather's sucrose floatation technique.[15] Modified Ziehl-Neelsen staining of stool smears was performed using 1% concentrated sulphuric acid and observed microscopically for the presence of oocysts of *C. cayetanensis*.[16]

2.2. Molecular assay for cyclosporiasis

A new set primer was designed and PCR assay was standardized for *C. cayetanensis*. All stool samples were tested by PCR.

2.2.1. Designing of Primers

A new set of primer was designed using a sequence common to *C. cayetanensis* (Accession number AF111183.1) and targeting its conserved 18S rRNA gene using NCBI-BLASTn with default settings. Sequences were aligned with CLUSTALW to identify common regions suitable for species-specific primers.[17,18] Primer-3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used and custom-synthesized by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The designed Cyclo__Uni_F (5'-TTAGCCGGCGATAGATCATT-3') was used as a forward and Cyclo__Uni_R (5'-TCAAGAACGACAGTAGGGGG -3') was used as a reverse primer respectively. Primers were examined in silico in SnapGene software (v1.1.3, Chicago, USA) (Figure 1).

2.2.2. DNA extraction from stool samples

DNA was extracted from the stool samples using QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The quality and quantity of DNA obtained were evaluated in NanoDrop 2000C (ThermoFisher, Massachusetts, USA). DNA samples were stored at –20 ºC until further use.

2.2.3. Quality control for DNA samples

Purified DNA material of *C. cayetanensis* was obtained from the Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, India. It was used as a positive control DNA template for standardization and validation of the PCR.

2.2.4. Standardization of PCR

PCR was standardized using new primers, positive control DNA material of *C. cayetanensis* as templates and nuclease-free water as negative controls. Gradient PCR was performed to determine the annealing temperature. PCR reaction was performed with 12.5 μl of commercial 2X Taq DNA polymerase master mix (Ampliqon, Odense, Denmark), 2 μl of DNA template, 0.5 μM each of forward and reverse primers with nuclease-free water to make a final volume of 25 μl on Agilent SureCycler 8800 (Agilent Technologies, California, USA). The cycling conditions of PCR were- initial denaturation at 94 ^{0}C for 5 minutes followed by 35 cycles at 94 ^{0}C for 45 seconds, 56 ^{0}C for 45 seconds, and 72 ^{0}C for 45 seconds, followed by a final

extension at 72 ^{0}C for 5 minutes. PCR amplicons were visualized in Biorad gel documentation system after 1.2% agarose gel electrophoresis (Figure 1).

2.2.5. Molecular testing of stool samples

All stool samples were tested for cyclosporiasis by PCR. The stool samples which yielded a 713 base pair (bp) product size were considered positive for *C. cayetanensis*. If 713 bp of band was not seen then the sample was considered negative for *C. cayetanensis*.

2.3. Statistical analysis

Continuous variables were expressed as mean +/- SD. Categorical variables were expressed as frequency/percentage and compared using chi-square and fischer's exact test. A P value of less than 0.05 was considered statistically significant.

3. Results

A total of 335 immnocompromised participants were recruited during the study period. It was divided into three groups - 121 PLHA group, 135 PRT group, and 79 IC group. Two hundred and ten (62.7%) were males, and 125 (37.3%) were females. Males were predominant in PLHA (60.3%) and PRT (74.8%) group, while females were predominant in IC (54.4%) group. Most patients of PLHA and PRT groups were in age from 19 to 45 years. One hundred and forty-nine (44.5%) participants were living in rural area - 45 (37.2%) in PLHA, 58 (43%) in PRT and 46 (58.2%) in IC. Two-third partcipants were using municipal water - 98 (81%) in PLHA, 77 (57%) in PRT and 73 (92.4%) in IC. Majority used municipal water. A little more than half participants had pets in their houses 72 (59.5%) in PLHA, 72 (53.3%) in PRT and 51 (64.6%) in IC. Patients presented with diarrhea were 186 (55.5%) – 76 (62.8%) in PLHA, 44 (32.6%) in PRT and 66 (83.5%) in IC. Only thirteen children presented without diarrhea in the IC group; therefore the IC group is not analyzed further based on diarrhea. The clinico-demographic details of the PLHA and PRT group are depicted in Tables 1 and 2, respectively. Only few participants in all the three groups presented with other clinical signs and symptoms along with diarrhea like nausea/vomiting, abdominal pain, weight loss or malnutrition. Abdominal pain was the most common symptom observed with diarrhea - 9 (7.4%) in PLHA, 8 (5.9%) in PRT and 4 (5.1%) in IC.

Microscopic observation of stool samples from all 335 participants did not reveal any oocysts of *C. cayetanensis*. The PCR test developed by us (Figure 1), on the other hand, revealed two positive results, one in PLHA group and another in PRT group. None of the stool samples from children were positive. The overall prevalence of *C. cayetanensis* was 0.6% (2/335), 0.8% (1/121) in PLHA and 0.7% (1/135) in PRT. The PLHA patient was 46 years old male who presented with diarrhea, abdominal pain, nausea/vomiting and weakness and PRT positive patient was 43 year old, male with no signs and symptoms. Both positive patients were living in urban area, used municipality water and had pets in their houses.

4. Discussion

The coccidian parasite - *C. cayetanensis* causes cyclosporiasis in human, which is linked to large food and water-borne outbreaks throughout the world. Availability of food items imported from different parts of the world has led to globalization of the disease.[1] Cyclosporiasis is a very widespread disease even in developed countries like United States and Canada. In 2017, a total of 1,065 cases were recorded in 40 states of the United States.[19] The prevalence of cyclosporiasis in India is extremely variable and ranging from 0.7% to 22.2%.[3,5] It is not reported much from the southern part of India.

Most of the studies used only microscopy for diagnosis. Very few studies used PCR techniques for the detection of *C. cayetanensis*. We developed a robust and precise single run PCR assay for the detection of *C. cayetanensis.* It was developed using a new set of primers targeting the conserved region of the 18S rRNA gene, though there are limited genomic data available for *C. cayetanensis* making it difficult to develop specific primers.

We collected stool samples from 335 various immunocompromised patients – 121 PLHA, 135 PRT and 79 IC patients. The microscopy did not detect any positive case of cyclosporiasis, the PCR assay detected 2 positive cases, one was PLHA symptomatic patient and another was PRT asymptomatic patients. Here we observed that molecular assays are more sensitive than conventional microscopic methods. None of the IC were positive for cyclosporiasis by either method. It indicates that the overall prevalence of cyclosporiasis is low in southern part of India and observed as 0.6% (2/335), 0.8% (1/121) in PLHA and 0.7% (1/135) in PRT.

A study carried out by Gupta et al. detected *Cyclospora* spp. in one of 250 stool samples from 113 adult PLHA positive patients by microscopy and who was symptomatic.[20] The positive PLHA case in the study was also symptomatic. A few studies have reported prevalence of cyclosporiasis in PRT patients. We detected a single case of cyclosporiasis and a prevalence of 0.7% in the PRT group. The positive PRT patient in this study was asymptomatic and potential to become symptomatic later if left undiagnosed, while timely detection and treatment could prevent later disease and complication. A study conducted in Turkey reported a 10% prevalence of *Cyclospora* spp. in PRT patients.[21] While a similar study from Iran did not detect cyclosporiasis in any of their PRT patients.[22] Yadav et al. from New Delhi, India reported

Table 1: Clinico-demographic details of PLHA patients. Group 1: Patients presented with diarrhea; Group 2: Patients presented without diarrhea

Variables	Total (n=121)	Group 1 (n=76; 62.8%)	Group 2 (n=45; 37.2%)	P value
Age				
≤18	6 (5.0%)	6 (7.9%)	0	
19-45	72 (59.5%)	46 (60.5%)	26 (57.8%)	0.073
46-60	40 (33.1%)	21 (27.6%)	19 (42.2%)	
>60	3 (2.5%)	3 (4.0%)	0	
Gender				
Male	73 (60.3%)	47 (61.8%)	26 (57.8%)	0.659
Female	48 (39.7%)	29 (38.2%)	19 (42.2%)	
Fever	3 (2.5%)	3 (4.0%)	0	
Nausea/Vomiting	4 (3.3%)	4 (5.3%)	0	
Abdominal pain	9 (7.4%)	9 (11.8%)	0	
Weight loss	4 (3.3%)	4 (5.3%)	0	
Weakness	7 (95.8%)	7 (9.2%)	0	
Malnutrition	3 (2.5%)	3 (4.0%)	0	
Residence				
Rural	45 (37.2%)	30 (39.5%)	15 (33.3%)	0.499
Urban	76 (62.8%)	46 (60.5%)	30 (66.7%)	
Water source				
Municipality water	98 (81.0%)	63 (82.9%)	35 (77.8%)	0.488
Filtered water	23 (19.0%)	13 (17.1%)	10 (22.2%)	
Pet animal in house				
Pet animal	72 (59.5%)	45 (59.2%)	27 (60.0%)	0.932
No pet animal	49 (40.5%)	31 (40.8%)	18 (40.0%)	

Table 2: Clinico-demographic details of post-renal transplant patients. Group 1: Patients presented with diarrhea; Group 2: Patients presented without diarrhea

Variables	Total n=135)	Group 1 (n=44; 32.6%)	Group 2 (n=91; 67.4%)	P value
Age				
≤ 18	6 (4.4%)	1 (2.3%)	5 (5.5%)	0.703
19-45	112 (83.0%)	6 (81.8%)	76 (83.5%)	
46-60	17 (12.6%)	7 (15.9)	10 (11.0%)	
Gender				
Male	101 (74.8%)	29 (65.9%)	72 (79.1%)	0.097
Female	34 (25.2%)	15 (34.1%)	19 (20.9%)	
Fever	4 (3.0%)	4 (9.1%)	0	
Nausea/Vomiting	4 (3.0%)	4 (9.1%)	0	
Abdominal pain	8 (5.9%)	7 (15.9%)	0	
Weight loss	4 (3.0%)	3 (6.8%)	0	
Weakness	6 (4.4%)	6 (13.6%)	0	
Malnutrition	2 (1.5%)	2 (4.6%)	0	
Residence				
Rural	58 (43.0%)	25 (56.8%)	33 (36.3%)	0.024
Urban	77 (57.0%)	19 (43.2%)	58 (63.7%)	
Water source				
Municipality water	77 (57.0%)	25 (56.8%)	52 (57.1%)	0.972
Filtered water	58 (42.0%)	19 (43.2%)	39 (42.9%)	
Pet animal in house				
Pet animal	72 (53.3%)	23 (52.3%)	49 (53.9%)	0.864
No pet animal	63 (46.7%)	21 (47.7%)	42 (46.2%)	

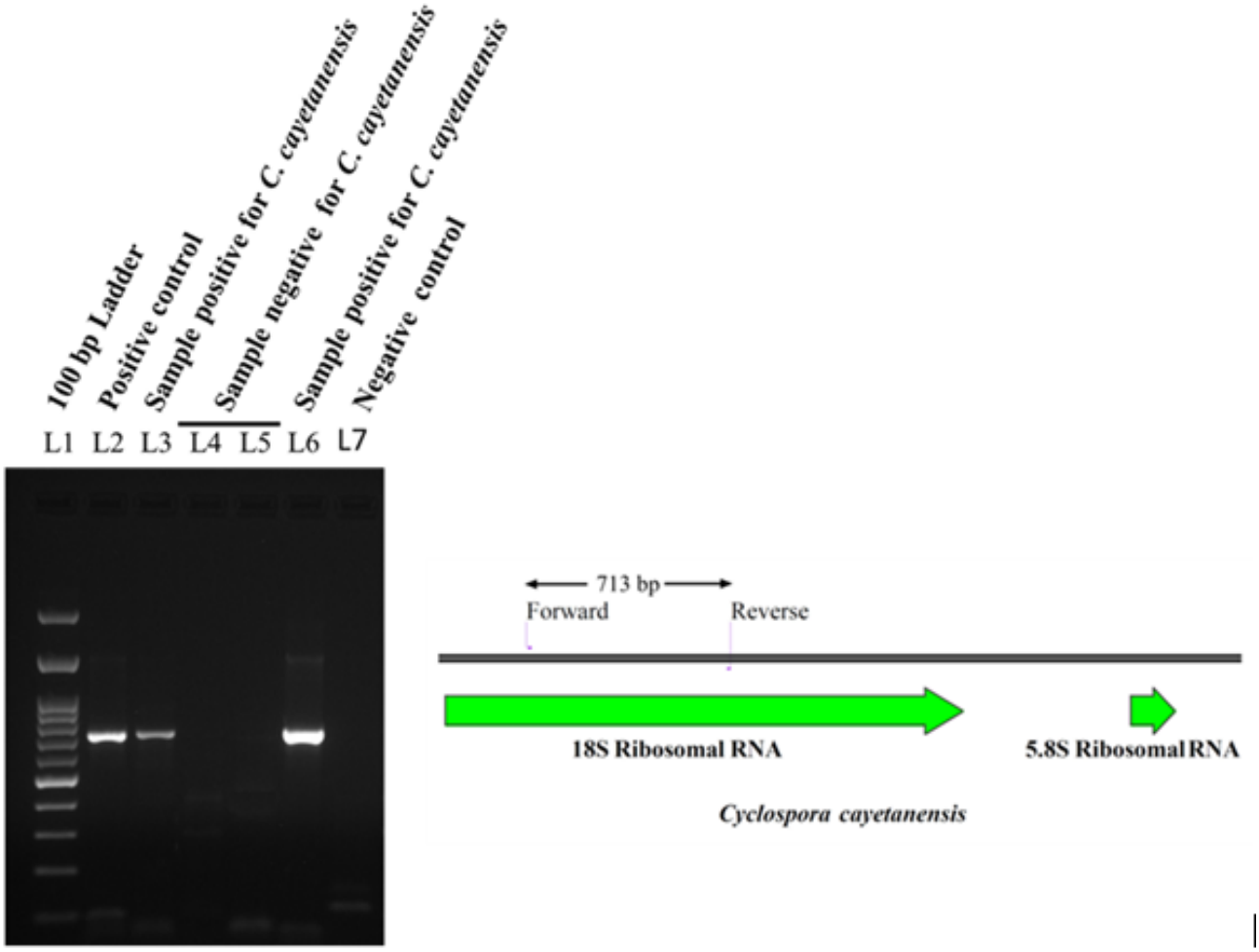

Fig. 1: A. Gel electrophoresis of PCR assay for *C. cayetanensis*; Lane 1 100 bp DNA ladder, Lane 2 positive control (713 bp); Lane 3 & 6 positive samples; Lane 4 & 5 negative samples; Lane 7 negative control. B. Primer-binding sites primer for *C. cayetanensis* by SnapGene software (v1.1.3)

2.4% prevalence of cyclosporiasis in PRT patients and is higher than the current study. They detected it by nested PCR-Restriction Fragment Length Polymorphism (RFLP) which is a two run PCR amplification assay and followed by RFLP.[8] Our PCR is a single run amplification assay and without the need for RFLP. Hence it is a simple, rapid and cost effective molecular assay.

We did not detect a single case of cyclosporiasis in 79 IC patients. It indicates that the low prevalence of cyclosporiasis in southern region of India. A hospital based study from Mexico reported a prevalence of 0.67% among children with diarrhea over a 9-year period, majority diagnosed during the rainy season.[23] Massoud et al. from Egypt reported 17% prevalence in symptomatic and 6% in asymptomatic immune-competent children less than five years old.[24] Hence prevalence of cyclosporiasis is highly variable with geographical area and climate and therefore there is a need for continuous study to monitor its prevalence.

Though less common than other intestinal coccidian parasites, cyclosporiasis is an emerging disease with increasing number of outbreaks reported from different parts of the world.[18,25] Non-availability of simple molecular assay hampers the diagnosis. The development of a reliable, simple and cost effective test can help in making the test available in resource limited countries for its diagnosis.

This is the first study in India where indigenous primers were designed and studied for the detection of cyclosporiasis to the best of our knowledge. Furthermore, this new PCR assay can be used to study genetic diversity or phylogenetic analysis as the product size of the PCR is 713 bp size. The PCR assay has been validated using positive control, in-silico examination, expected product size in gel electrophoresis. Although the prevalence of cyclosporiasis is low in our region but this assay has the potential to diagnosis cases of cyclosporiasis.

5. Conclusion

Cyclosporiasis is infrequent in our region. A new single step PCR assay was developed which is simple, rapid, cost effective and has a potential to study genetic diversities of *C. cayetanensis*.

6. Acknowledgements

The authors thank Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS) Lucknow, India for providing DNA material of *C. cayetanensis* for the study.

7. Source of Funding

The study was supported by Intramural Research Grant from Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India.

8. Conflict of interest

All contributing authors declare no conflicts of interest

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Cite this article: Katiyar M, Gulati R, Parameswaran S, Hamide A, Rajkumari N, Singh R. A new single run polymerase chain reaction assay for cyclosporiasis in immunocompromised patients. *IP Int J Med Microbiol Trop Dis* 2021;7(3):207-212.