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# Sporadic cyclosporiasis in symptomatic Cuban patients: Confirmation of positive results from conventional diagnostic methods by molecular assay

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ABSTRACT

In Cuba, there are few studies on cyclosporiasis. Here, we report results from 1247 stool samples from symptomatic patients that were examined by microscopy methods and positive cases confirmed by nested PCR targeting the 18S rRNA gene, followed by sequencing. Seven positive samples, all diagnosed during May–June, were confirmed by the molecular method, indicating an occurrence in this patient cohort of 0.56%.



*Cyclospora cayetanensis* is an obligate, intracellular, coccidian parasite causing cyclosporiaisis in humans, typically presenting with abdominal symptoms. In immunocompetent persons, infection is usually self-limiting but may persist for several weeks; infection in immunocompromised patients can be severe (Giangaspero and Gasser, 2019).

Cyclosporiasis has not been commonly reported in Cuba, but prevalences of between 0.2–4.4% have been reported in symptomatic children (Nuñez et al., 2003). The highest prevalence (4.4%) was from children with diarrhea with low or moderate dehydration or vomiting, presenting at Cerro Pediatric Hospital in Havana between May and August (Núñez et al., 2003). In adult patients with HIV infection or AIDS, prevalences of 3–3.5% have been reported (de Paz et al., 2003; Escobedo and Núñez, 1999).

Compared with some other Latin American countries, these prevalences are relatively low, with prevalences reported to range from 7.9% in Haiti to 41.6% in Peru (Giangaspero and Gasser, 2019). However, some lower prevalences have been reported from some other Latin American countries (for an up-to-date overview, see Chacín-Bonilla, 2017). Although cyclosporiasis outbreaks have not been documented in Cuba, an outbreak among Spanish travelers to Cuba has been reported (Ramírez-Olivencia et al., 2008).

For a broader-based survey, we investigated 1247 stool samples (of which 148 were diarrheic) from 875 adults and 372 children submitted to the National Reference Laboratory of Intestinal Parasitic Infection, 'Pedro Kouri' Institute of Tropical Medicine (IPK) between 3rd January and 28th December 2018. Direct wet mounts were stained with Lugol's iodine and also examined following formalin-ethyl acetate concentration. A modified Ziehl-Neelsen acid-fast stain was used for coccidian parasites (Garcia, 2001).

*C. cayetanensis* oocysts were observed in 7 samples (0.56% occurrence in this cohort); no other parasites were found in these samples. Oocyst numbers were low for all samples, ranging from just a couple of oocysts in the whole slide up to 1 oocyst per field of view. Age and gender distribution are shown in Table 1. None of the cases were related to each other, and all were immunocompetent; none of the patients had HIV infections.

Although three cases were children, adults were also infected, with the oldest patient 54 years of age, indicating all age groups in Cuba may be infected. However, children are generally more

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**Table 1**
Positive cases of cyclosporiasis by age, gender, month of diagnosis, and symptoms.

| Age (years) | Date of diagnosis | Symptoms |
|---|---|---|
| Female patients | | |
| 4 | May 7th | Abdominal pain, acute diarrhea, and flatulence |
| 11 | June 10th | Abdominal pain, acute diarrhea, weight loss, and fever |
| 28 | June 19th | Weight loss, abdominal pain, anorexia, chronic diarrhea, and flatulence |
| Male patients | | |
| 3 | May 3rd | Acute diarrhea, weight loss, and flatulence |
| 21 | May 23rd | Abdominal pain, acute diarrhea, weight loss, and flatulence |
| 36 | May 29th | Abdominal pain, chronic diarrhea, fatigue, and flatulence |
| 54 | June 28th | Abdominal pain, chronic diarrhea, and vomiting |

commonly reported as being infected (Chacín-Bonilla, 2010) and this seems to be the case in our study also, with 43% of the cases being in children, whereas children made up only 30% of the overall cohort investigated.

Clinical manifestations in our patients were similar to those commonly reported (Table 1). However, as no testing for bacterial or viral pathogens was conducted, we cannot exclude that other etiological agents could have been associated with the symptoms reported here. All our cases were diagnosed during May and June (Table 1), suggesting a seasonal association. All patients were from Havana, where the average temperature during the period of diagnosis was 27.3 °C, the humidity was 74%, and rainfall was 98 mm. The seasonal trend of increased prevalence of cyclosporiasis has been previously described from various nations and often coincides with warm periods of maximal rainfall. Such seasonality probably reflects the requirement for moist, warm conditions for oocyst sporulation, along with other region-specific factors that may increase likelihood of transmission (Chacín-Bonilla, 2010). Cyclosporiasis has also been more frequently observed during the early rainy season in Guatemala, with 90% of cases occurring between May and July (Bern et al., 1999), and similar situations have also reported from Mexico (Orozco-Mosqueda et al., 2014) and Honduras (Kaminsky et al., 2016). In Haiti, however, which is closer to Cuba (being less than 700 km distant), most cases of *Cyclospora* infection were reported to occur in the coolest months between January and April (Eberhard et al., 1999); the authors argue that high temperatures may affect oocyst survival, and therefore cooler periods provide better conditions for transmission. However, as most infections in the Haiti study were asymptomatic with very low oocyst excretion (Eberhard et al., 1999), this lack of similarity in seasonality may indicate a more profound difference in the situation or reflect the differences in study type; although oocyst excretion was also low in our cases, all were symptomatic and we did not investigate asymptomatic individuals as was done in the Haitian study. Furthermore, the temperature and rainfall conditions in Havana during the period when the cases were diagnosed are not dissimilar to those recorded in the cooler months of the year at the study site in Haiti, indicating, perhaps, the importance of local weather conditions.

It is also worth noting that there are differences in the rate of occurrence between these two studies. Although this may be due to a variety of factors, including the cohorts being investigated, the superior healthcare facilities in Cuba and social differences between the two countries are likely to be contributory factors. We suggest that in Cuba, targeted implementation of *Cyclospora* surveillance during early summer could provide better information on risk factors for infection.

In our study, for all cases in which *C. cayetanensis* oocysts were observed, a diagnostic PCR was used for confirmation. For these samples, following 8–10 freeze/thaw cycles in liquid nitrogen alternated with a 95 °C water bath, DNA was extracted from stool samples (200 mg) using the QIAamp DNA Stool Kit (QIAGEN Inc., Valencia, California) following the manufacturer's protocol.

Conditions of the two reactions of a nested PCR to amplify a 510 bp of the fragment of *C. cayetanensis* 18S rRNA gene, using primers described by Liu et al. (2014), were optimized using serial dilutions of reagents. Optimized concentrations and conditions were: 1.0 U of GoTaq® DNA Polymerase (Promega), 0.4 μM concentrations of primers, and 3 mM $MgCl_2$, in a total volume of 25 μL, with 5 μL DNA as template, 5 μL of 5× PCR buffer (Applied Biosystems, USA), and 0.2 mM of each deoxynucleoside triphosphate (Applied Biosystems, USA). Reactions were performed for 35 cycles in a BIO RAD T100™ thermocycler. The amplification product was visualized on 2% agarose gels stained with 0.5 μg/mL ethidium bromide in a U:Genius UV equipment (Syngene, UK). Ultrapure water was used as negative control. Serial dilutions (21 ng–0.2 pg/μL) of *C. cayetanensis* DNA determined the limit of detection to be 0.2 pg/μL, which is similar to that reported by Shin et al. (2016) in a multiplex-touchdown PCR ($2 \times 10^1$ copy).

For all sevens samples tested here, the correct size of band was observed. This not only confirms and supports the positive microscopy results, but also, for the first time in Cuba, enables implementation of molecular diagnosis of cyclosporiasis. The amplified DNA was purified using QIAquick® PCR Purification kit (QIAGEN Ltd.) and sequenced in both directions with the Beckman Coulter Genomics sequencing system (Essex, United Kingdom). Although sequencing is expensive for developing countries, it is an important quality control procedure when implementing a PCR diagnostic tool. Sequences were obtained from three samples and aligned using BioEdit v7.0.1 package, then compared with sequences in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences showed 96% homology with isolates from China, Nepal, and Haiti, and were deposited in GenBank (Accession numbers: MK533706 - MK533708).

A phylogenetic tree was constructed based on the neighbor-joining algorithm (Fig. 1). *C. cayetanensis* SSU-RNA reference sequences used were KJ569532, KY770756, FJ009121, and GQ292774; *Eimeria jerfinica* KU192975 was used as an outgroup. Distance-based analyses were conducted using Kimura 2-parameter distance estimates, including alignments obtained from ClustalW using the Molecular Evolutionary Genetics Analysis version 6.06 (MEGA 6) (Tamura et al., 2013). Bootstrap proportions were calculated by analyzing 2000 replicates of the phylogenetic tree; a monophyletic grouping was indicated.

As the 18S rRNA gene is highly conserved, limited sequence variability was expected. PCR at the internal transcribed spacer regions between the small- and large-subunit ribosomal RNA genes would be expected to contain greater sequence variability and be more useful for examining epidemiological associations between isolates.

In conclusion, cyclosporiasis is endemic in Cuba but infrequently reported, and appears to be seasonal. Although traditional microscopic techniques are highly valuable in the diagnostic laboratory, particularly in countries such as ours, PCR at the 18S rRNA gene is a good tool for confirming infection. However, heterogeneous markers may be more suitable for investigations of outbreaks.

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## Transparency Declarations

There are no conflicts of interest to declare.

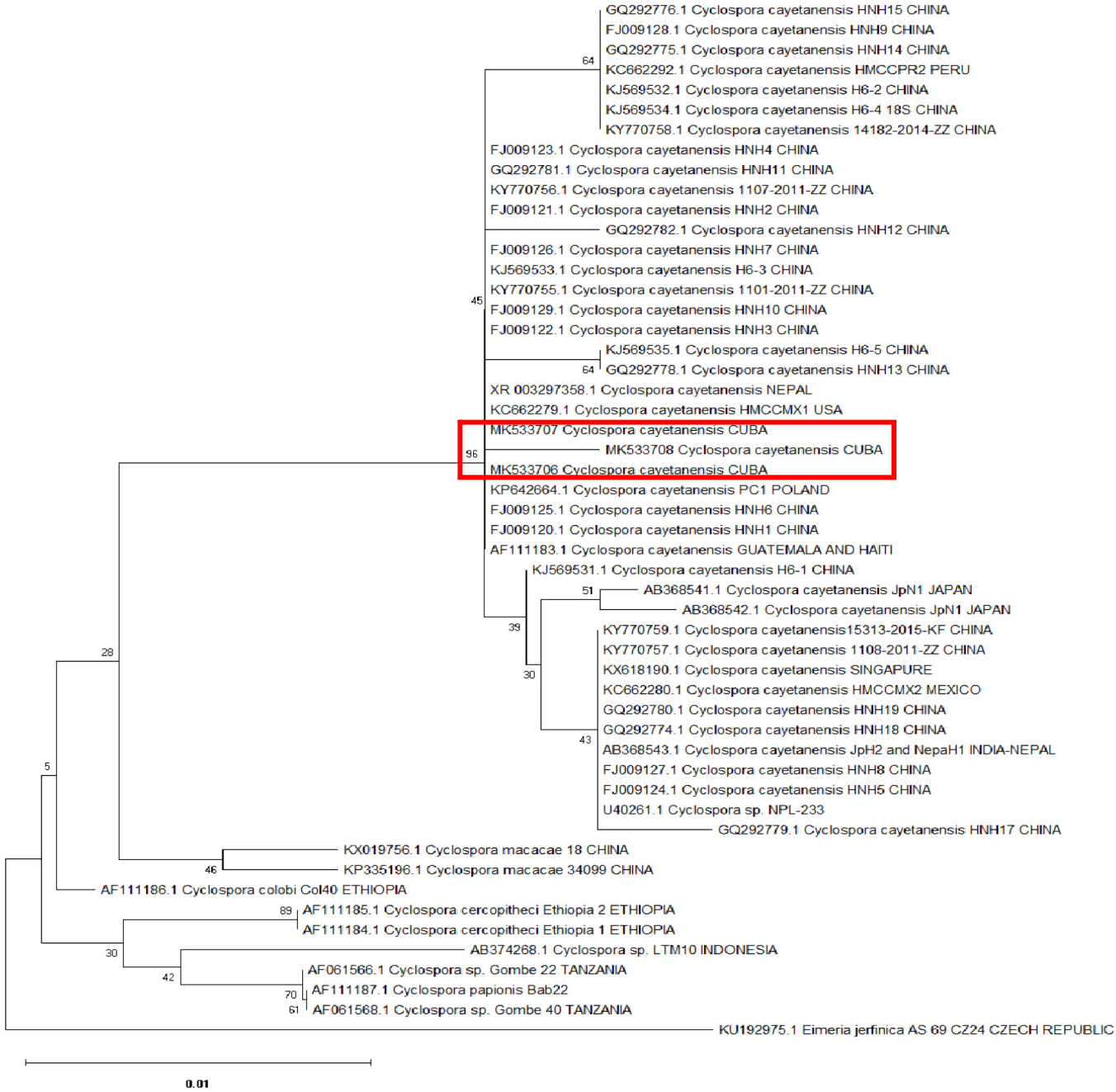


**Fig. 1.** Phylogenetic position of three Cuban *Cyclospora cayetanensis* isolates based on SSU rRNA DNA sequences. NCBI accession numbers are included. Cuban sequences are in the red box. Evolutionary distances computed using the Kimura 2-parameter method clustered by the NJ method. Evolutionary analyses were conducted in MEGA6 and further bootstrap analysis of 2000 replicates.

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