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# Host Range of *Cyclospora* Species: Zoonotic Implication

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**Abstract.** *Cyclospora* is an intracellular, gastrointestinal parasite found in birds and mammals worldwide. Limited accessibility of the protozoan for experimental use, scarcity, genome heterogeneity of the isolates and narrow panel of molecular markers hamper zoonotic investigations. One of the significant limitation in zoonotic studies is the lack of precise molecular tools that would be useful in linking animal vectors as a source of human infection. Strong and convincing evidence of zoonotic features will be achieved through proper typing of *Cyclospora* spp. taxonomic units (e.g. species or genotypes) in animal reservoirs. The most promising method that can be employ for zoonotic surveys is next-generation sequencing.

**Keywords:** *Cyclospora*, animals, zoonosis, epidemiology, genotyping, next-generation sequencing.

## INTRODUCTION

*Cyclospora* spp. (Apicomplexa: Eimeriidae) are intestinal protozoan parasites of vertebrate and invertebrate animals. *Cyclospora cayetanensis* is the only species within the genus *Cyclospora* known to infect humans. Cyclosporiasis is endemic mostly to tropical and subtropical regions and people living or traveling to such regions may be at increased risk for infection (Ortega *et al.* 1994; Sherchand and Cross 2001). In early reports associated with human diarrhea, *C. cayetanensis* was characterized as a coccidian-like body, blue-green alga, large *Cryptosporidium* or small *Isospora*-like organism. More recently, it was found to be a new-emerging pathogen worldwide (Ortega *et al.* 1994). Unlike typical zoonotic infections, sporadic cases of cyclosporiasis and large outbreaks have been mostly linked to various kinds of contaminated fresh produce (e.g., basil, cilantro, mesclun lettuce, raspberries) as well as contaminated water. This parasite still raises questions regarding host specificity, epidemiology, and transmissions to humans. Besides, little is known about the possible role of animals as potential reservoirs (Marangi *et al.* 2015). Also, there is not enough information on the infective dose, when sporulation takes place, and how external circumstances affect the persistence of infectious stages in water and soil. Newly published data imply that the *Cyclospora* heterogeneity level in humans is much more higher than previously expected in various regions (Hofstetter *et al.* 2019). There are no animal models, or *in vitro* culture systems to facilitate *C. cayetanensis* research. Efforts to achieve experimental infection of several animals have been unsuccessful, suggesting host specificity (Eberhard *et al.* 2000).

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To date, on the basis of morphometric and molecular studies *Cyclospora* spp. presence has been detected in several animals species, including mammals and birds (Zerpa *et al.* 1995; García-López *et al.* 1996; Pérez Cordón *et al.* 2008; Pérez Cordón *et al.* 2009). These findings may have been detections of oocysts passing through gastrointestinal tract (Almeria *et al.* 2019). Some studies suggested that free-living nematodes, insects, or rotifers could play a role in the spread of *Cyclospora* (Ortega *et al.* 2010). One of the significant limitations in zoonotic investigations is the lack of precise molecular tools that would be useful in linking animal vectors as a source of infection (Barratt *et al.* 2019). Unfortunately, genome size and genetic heterogeneity of the *C. cayetanensis* may also complicate zoonotic surveys (Cinar *et al.* 2015). The purpose of this review is to elucidate problems of *C. cayetanensis* zoonotic examinations and characterize possible utility of new advanced molecular approaches facilitating such interpretations in future.

### *C. cayetanensis* animal reservoirs and zoonotic surveys

Despite the multitude of publications on *C. cayetanensis* infection detected in humans and environmental samples worldwide, little is known about the presence of the parasite in animals. The genus *Cyclospora* comprises 21 species described from distinct hosts such as arthropods, reptiles, insectivores, and rodents and oocysts of these *Cyclospora* species are morphologically distinct from the smaller *Cyclospora* oocyst found in species isolated from primates, including humans (Tab.1). The diagnosis of *C. cayetanensis* in animals is much more problematic than in humans because animals are hosts of many intestinal coccidian species. Moreover, most of the infections by intestinal protozoa are chronic, so the number of excreted oocysts in animals' faces is low. A low number of oocysts in animal fecal samples might results in difficulties such as detailed measurements of oocysts, sporulation study and observation of sporozoites. Thus, morphological studies of this genus may have been inadequate. Eberhard and coworkers (1999) described three *Cyclospora* species in monkeys based on the microscopic and molecular description. In this study, parasite isolates were morphologically indistinguishable among each other and from *C. cayetanensis,* so the data of morphological description alone are insufficient to study the source of human infection (Eberhard *et al.* 1999). Microscopy alone was also done in dogs. It is known, that dogs are the hosts of the other naturally acquired Apicomplexa species like *Hammondia heydorni* and *Neospora caninum.* Shedding of unsporulated oocyst of these species may have been mistakenly recognized as *C. cayetanensis* and consequently, domestic animals were taken into consideration as an origin of human cyclosporasis (Yai *et al.* 1997; Romero *et al.* 2000). Similar issues may handicap studies of both domestic and wild animals, where it was suggested that animals might be a source of *C. cayetanensis* infection (Smith *et al.* 1996; Legesse and Erko 2004). For the same epidemiological reason, reports linking finding oocysts at the same time in the same area should not be used as proof of a zoonotic source (Zerpa *et al.* 1995). Extensive studies for *C. cayetanensis* were done in domestic animals (pigs, cows, goats, horses, guinea pigs, cats, chickens, ducks, pigeons, and turkeys), wild monkeys as well as dogs in endemic regions of Haiti, Kenya, and Brazil where a high prevalence of *C. cayetanensis* was previously reported in humans. Surprisingly none of the animal species tested were parasitized by *Cyclospora* sp. and it was demonstrated that animals not participated in cyclosporiasis transmission (Eberhard *et al.* 1999; Carollo *et al.* 2001; Eberhard *et al.* 2001). In other studies, both microscopy and molecular methods were used to describe zoonotic coccidia in Galliformes ground-feeding birds in Mexico and Peru and in wild waterfowl Anseriformes birds from Poland. Only samples from wild Taiga bean goose (*Anser fabalis*) and Mute swan (*Cygnus olor*) were subsequently confirmed by nested PCR using fragment of the small-subunit ribosomal RNA concluding that these waterfowls were infected by *C. cayetanensis* (Zerpa *et al.* 1995; García-López *et al.* 1996; Majewska *et al.* 2001; Pieniazek *et al.* 2001). Although *C. cayetanensis* was confirmed by both methods in these birds, it is unknown whether the protozoan can establish infection, or was passing through the gastrointestinal tract using birds as mechanical vectors (Almeria *et al.* 2019). In order to determine new *C. cayetanensis* animal reservoirs, a four-year survey was done in Poland (Majewska *et al.* 2001; Majewska *et al.* 2004). Over three thousand fecal samples from farmed, domestic, wild, and zoo animals were studied. In the study fresh fecal samples were collected from wild animals belonged to 13 birds (639 fecal samples), 6 insectivores (72 fecal samples) and 11 rodents (255 fecal samples) species. Furthermore, 1731 fecal samples tested in this work were also obtained from domestic sources (horses, cattle, dogs, cats, turkeys, chickens and geese) from different regions and 711 fecal samples

**Table 1.** All known *Cyclospora* species and their host based on morphology

| Species | Host | Oocyst morphology (mm) | Author |
|---|---|---|---|
| | ARTHROPODA | | |
| *C. glomericola* | *Glomeris* sp. | 25–36 x 9–10 | Schneider 1881 |
| | REPTILIA | | |
| *C. viperae* | *Vipera aspis,*<br>*Coluber scalaris,*<br>*Natrix viperinus,*<br>*Coronella austriaca* | 10.5 x 7.5 | Phisalix 1923 |
| *C. babaulti* | *Vipera berus* | 17 x 10 | Phisalix 1924b |
| *C. tropidonoti* | *Natrix natrix*<br>*N. stolata* | 17 x 10 | Phisalix 1924c |
| *C. scinci* | *Scinus officinalis* | 10 x 7 | Phisalix 1924d |
| *C. zamenis* | *Coluber viridiflavus viridiflavus* | 17 x 10 | Phisalix 1924e |
| *C. niniae* | *Ninia sebae sebae* | 14.6 x 12.3 | Lainson 1965 |
| *C. shneideri* | *Anilius scytale scytale* | 19.8 x 16.6 | Lainson 2005 |
| | INSECTIVORA | | |
| *C. caryolytica* | *Talpa europaea*<br>*Parascalops breweri*<br>*T. micrura coreana,* | b.d.<br><br>16–19 x 13–16<br>15–18 x 8–10 | Schaudinn 1902 |
| *C. talpae* | *Talpa europaea* | 15–18 x 10–12 | Pellérdy and Tanyi 1968 |
| *C. megacephali* | *Scalopus aquaticus* | 18.5 x 15.7 | Ford and Duszyński 1988 |
| *C. ashtabulensis* | *Parascalops breweri* | 18.0 x 14.3 | Ford and Duszyński 1989 |
| *C. parascalopi* | *Parascalops breweri* | 16.5 x 13.6 | Ford and Duszyński 1989 |
| *C. duszynskii* | *Scalopus aquaticus* | 10–12 × 9–11 | McAllister *et al.* 2018 |
| *C. yatesi* | *Scalopus aquaticus* | 12–18 × 10–17 | McAllister *et al.* 2018 |
| | RODENTIA | | |
| *C. angimurinensis* | *Chaetodipus hispidus* | 21.9 x 19.3 | Ford *et al.* 1990 |
| | PRIMATES | | |
| *C. cayetanensis* | *Homo sapiens* | 7.7-9.9 | Ortega *et al.* 1994 |
| *C. cercopitheci* | *Cercopithecus aethiops* | 8 x 10 | Eberhard *et al.* 1999a |
| *C. colobi* | *Colobus guereza* | 8 x 9 | Eberhard *et al.* 1999a |
| *C. papionis* | *Papio anubis* | 8 x 10 | Eberhard *et al.* 1999a |
| *C. macacae* | *Macaca mulatta* | 8 x 10 | Li *et al.* 2015 |

from 196 species of various mammals, birds and reptiles were collected from animals kept in captivity at the Poznan Zoological Garden. Some questions arose regarding potential *C. cayetanensis* reservoirs in wild insectivores and rodents. Morphological description of the oocysts found in lesser white-toothed shrew (*Crocidura suaveolens*), common vole (*Microtus arvalis*) and fat dormouse (*Glis glis*) suggested the presence of *C. cayetanensis,* but not other *Eimeria* parasites specific for these animals. Typical *Eimeria* for soricid and murid hosts such as, *Caryospora* sp., *E. firestonei, E. milleri, E. leucodontis* or *E. gliris* have different morphology of their excreted oocysts (Majewska *et al.* 2001; Pieniazek *et al.* 2001). Additionally, all positive samples from these three widespread mammalian species were confirmed by molecular method. BLAST results of the SSU-rDNA sequences revealed that sequences of the *Cyclospora* from lesser white-toothed shrew, common vole and fat dormouse were identical with the deposited sequences of the same molecular

**Table 2.** *Cyclospora cayetanensis* identified in primates using molecular markers nPCR – nested PCR; qPCR – quantitive PCR; SSCP – single stranded conformation polymorphism

| Host | Molecular method | Targed | Primers | Author |
|---|---|---|---|---|
| Baboon (*Papio anubis*) | nPCR, sequencing | 18S rDNA | CYCF1E 5'-GGAATTCCTACCCAATGAAAACAGTTT-3' CYCR2B 5'-CGGGATCCAGGAGAAGCCAAGGTAGG-3' CYCF3E 5'-GGAA TTCCTTCCGCGCTTCGCTGCGT-3' CYCR4B 5'-CGGGATCCCGTCTTCAAACCCCCTACTG-3' 1FPL 5'-GCGGATCCGCGGCCGCTGGTTGATCCTGCCAG-3'1520RPL 5'-GCGGATCCGCGGCCGCYGCAGGTTCACCAC-3' | Lopez *et al.* 1996 |
| Chimpanzee (*Pan troglodytes*) | qPCR, SSCP | ITS | CCITS2F 5'-GCAGTCACAGGAGGCATATATCC-3' CCITS2R 5'-ATGAGAGACCTCACAGCCAAAC-3' | Marangi *et al.* 2015 |
| Cynomolgus monkey (*Macaca fascicularis*) | qPCR, sequencing, SSCP | ITS | CCITS2F 5'-GCAGTCACAGGAGGCATATATCC-3' CCITS2R 5'-ATGAGAGACCTCACAGCCAAAC-3' | Marangi *et al.* 2015 |
| Rhesus (*Macaca mulatta)* | nPCR Multiplex PCR, RFLP | 18S rDNA | F1E 5'-TACCCAATGAAAACAGTTT-3' R2B 5'-CAGGAGAAGCCAAGGTAGG-3' F3E 5'-CCTTCCGCGCTTCGCTGCGT-3' R4B 5'-CGTCTTCAAACCCCCTACTG-3' CRP999 5'-CGTCTTCAAACCCCCACTGTCG-3' CC719 5'-GTAGCCTTCCGCGCTTCG-3' PDCL661 5'-CTGTCGTGGTCATCTGT.CCGC-3' ESSP841 5'-GTTCTATTTTGTTGGTTTCAGGACCA-3' | Chu *et al.* 2004 |

marker of *C. cayetanensis* from human (Pieniazek *et al.* 2001). Besides distinct oocysts morphology of the animal host specific coccidia and *C. cayetanensis*, additional molecular methods are needed to evaluate zoonotic sources of human cyclosporiasis. Results of a morphological study of feces from several mammalian species including carnivores, Artiodactyla, and nonhuman primates from a zoological garden in Spain demonstrate that it is impossible to determine the species of *Cyclospora* solely on the basis of their morphology (Pérez Cordón *et al.* 2008). *C. cayetanensis* has been detected mostly in primates using two molecular markers (18S rDNA and ITS), emphasizing their role as potent vectors for human cyclosporosis in different parts of the world (Lopez *et al.* 1999; Chu *et al.* 2004; Marangi *et al.* 2015) (Tab. 2).

## Drawbacks of *Cyclospora* molecular diagnostic in animals

As a result of the limitation of the microscopic assay, molecular-based methods have been developed for the detection of *Cyclospora* in various type of samples to assess infection risk (Chacín-Bonilla 2008; Kitajima *et al.* 2014; Lalonde *et al.* 2016). To establish a reliable zoonotic outcome, microscopic analysis must be supported by molecular results. Because the pathogen is usually present in very low numbers in fecal samples, the detection is a very challenging task. In humans, molecular assays for *Cyclospora* detection are primarily dependent on the quality and purity of the genetic material, so *a priori* choice of DNA extraction method to isolate parasite genetic material from animals is a cru-

cial step as well (da Silva *et al.* 1999; Qvarnstrom *et al.* 2015; Paulos *et al.* 2016; Qvarnstrom *et al.* 2018). To date, the data of differences in usability of commercially available DNA extraction kits in animals fecal samples is restricted. To overcome current molecular genotyping problems, three genetic loci such a region within the small subunit ribosomal RNA gene (SSU rRNA), the 70 kilodalton heat shock protein (HSP70) gene, and the ribosomal internal transcribed spacer (ITS) (Sulaiman *et al.* 2013; Olivier *et al.* 2001) were primarily developed to improved detection *C. cayetanensis* DNA in human fecal samples. According to the literature, SSU-rDNA and ITS gene fragments were used for *Cyclospora* typing in animals (Relman *et al.* 1996; 30 Zhao *et al.* 2013). However, some caution should be required as the *C. cayetanensis* populations may be heterogeneous. The pathogen is a sexually reproducing organism and any isolate may have genetically heterogeneous sequences. Through this process, sporozoites in a single sporocyst are thought to be genetically identical, while the sporocysts in a single oocyst can be genetically distinct (Shirley *et al.* 1996; Mzilahowa *et al.* 2007). Therefore, one *Cyclospora* oocyst is heterozygous, possessing up to two alleles for any given marker and amplicons may vary in their sequences. New genotyping information for *C. cayetanensis*, derived from mitochondrial genome markers, should be helpful in animal source tracking studies. Next-Generation Sequencing (NGS) is the best technique for such studies (Nascimento *et al.* 2019; Houghton *et al.* 2020, Cinar *et al*. 2020).

**NGS shotgun, metabarcoding and commercially available diagnostic test**

Progress on the improvement of emerging molecular tools to *Cyclospora* DNA detection has been observed but it is mostly fronted for humans (Qvarnstrom *et al.* 2018). Recent advances in modern sequencing technologies and availability of efficient software led to complete *C. cayetanensis* mitochondrial and apicoplast genomes (Cinar *et al.* 2015, Cinar *et al.* 2016, Ogedengbe *et al.* 2015; Cama and Ortega 2018). The new NGS strategy used on deep sequencing platforms gains from the increasing availability, speed, and decreasing costs. In general, it is based on two approaches. The first is shotgun metagenomics, which profiles the entire microbial diversity consisting of both pathogenic and neutral microbiome of the host. This technique demands the knowledge of partial or whole *Cyclospora* reference genome, which is then compared to the shotgun data following quality processing, curation, and assembly datasets. The shotgun method may be promising to identify and develop novel target loci of *C. cayetanensis* (Qvarnstrom *et al.* 2015). The whole genome of *C. cayetanensis* is estimated to be 44 megabase pairs with ~7500 genes (Liu *et al.* 2016). *Cyclospora* mitochondrial genome is ~6200 base pairs (bp) in length, whereas the circular apicoplast genome is ~34,000 bp and encodes complete machinery for protein synthesis. The second NGS approach is based on metabarcoding of the small ribosomal RNA subunit (18S), which targets predefined domains using specific primers. This NGS system seems to be extremely useful in terms of the development of new *Cyclospora* diagnostic assays (Qvarnstrom *et al.* 2015; Nascimento *et al.* 2016; Liu *et al.* 2016). Cinar and coworkers described NGS molecular typing of *C. cayetanensis* identifying potential genomic markers such as single nucleotide polymorphisms (SNP) and insertion-deletions that could theoretically be used for *Cyclospora* detection and pathogen subtyping in clinical samples (Cinar *et al.* 2020). These promising results were obtained by typing the mitochondrial genome. It was suggested that the diversity of *C. cayetanensis* and could be used to link outbreaks or even single infection cases to a source. Multicopy and linear mitochondrial genomic sequences observed in *C. cayetanensis* may also be used for the detection and genotyping of other *Cyclospora* species (Cinar *et al.* 2015; Qvarnstrom *et al.* 2018).

The development of rapid diagnostic molecular tests has improved the detection of various protozoan pathogens thanks to higher throughput capacity (Verweij and Verweij 2014). Besides user-friendly software and equipment independence, the ultimate goal of such tests should be better affordability, sensitivity and specificity. Currently, the BioFire FilmArray panel is the only commercially available product capable of detecting *C. cayetanensis* in addition to 22 enteropathogenic agents (including four protozoan species). Buss and coworkers described the sensitivity and specificity of this test during a cyclosporiasis outbreak in the USA (Buss *et al.* 2013). In another study, over one and half thousand clinical stool samples were analyzed, showing that the sensitivity and specificity of this test for *C. cayetanensis* was 100% (Buss *et al.* 2015; Murphy *et al.* 2019). Up to now, no reports were published on the use of this commercial test to analyze samples from animals.

## CONCLUSION

The results of many studies indicated that *Cyclospora* is a very rare pathogen in animals, so any zoonotic surveys became a very challenging task. To establish a proper outcome, morphometric analysis, together with sporulation test should be use as primary method. Animals can harbor many coccidian (the largest group of apicomplexan protozoa) species and microscopy alone is limited by poor resolution needed for proper *Cyclospora* classification (Li *et al.* 2015; Giangaspero and Gasser 2019). To avoid any identification issues of *C. cayetanensis* in animals, molecular data must support the microscopic results. Several molecular based detection methods along with two molecular markers have been developed to identify *C. cayetanensis* in various animals so far (Lalonde and Gajadhar 2011). The most frequent gene fragments used for *Cyclospora* identification in animals were the SSU-rDNA and ITS loci. The major drawback comes from the primers used to obtain *Cyclospora* SSU-rDNA amplicons, because such primers may also cross-amplify other *Eimeria* DNA in animals. Generally, narrow panel of genetic markers for zoonotic investigations suppress final zoonotic conclusions (Giangaspero and Gasser 2019). While the advances in development of molecular methods have been significant in the last decade, there are no data supporting genetic diversity among *Cyclospora* isolates in animal hosts, which may reflect the protozoan infectivity and non-zoonotic/zoonotic interactions. However, as a consequence of *Cyclospora* heterogeneity in the probes, no independently validated genotyping approaches have been developed for zoonotic purposes. The NGS technology may be invaluable for this strategy generating complex data usable for tracing *Cyclospora* zoonotic sources of human infection. NGS shotgun or metabarcoding of nuclear and mitochondrial data for comparative genomic analyses will become an important approach for unculturable *C. cayetanensis* in future, but the genome size is a hindrance to routine use in whole genome sequencing (Guo *et al.* 2019). Strong and convincing evidence of *Cyclospora* zoonotic features will be achieved through proper typing of *Cyclospora* spp. taxonomic units (e.g. species or genotypes) in animal reservoirs (Tang *et al.* 2015). Also assessment is needed whether the parasite is able to establish infection by examination of tissue biopsies as well as estimation of *Cyclospora* population structure in animals.

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