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# Identification and morphologic and molecular characterization of *Cyclospora macacae* n. sp. from rhesus monkeys in China

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## Abstract

*Cyclospora* spp. in nonhuman primates are most closely related to *Cyclospora cayetanensis*, an emerging human pathogen causing outbreaks of cyclosporiasis in North America. Studies thus far indicate the possible existence of host specificity in *Cyclospora* spp. In this study, 411 fecal specimens from free-range rhesus monkeys (*Macaca mulatta*) were collected and examined for *Cyclospora* by sequence analysis of the small subunit rRNA gene. A novel *Cyclospora* species was identified in 28 (6.8 %) specimens and named *Cyclospora macacae* based on morphologic and molecular characterizations. The oocyst of *C. macacae* is spherical and measures 8.49±0.55× 8.49

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± 0.49 μm in diameter. Phylogenetic analysis grouped this species together with the other four *Cyclospora* species infecting primates, including *C. cayetanensis* in humans, forming a monophyletic group closely related to avian *Eimeria* species. In addition, *C. cayetanensis* was detected in one specimen, although whether rhesus monkeys can serve as a natural reservoir host of *C. cayetanensis* needs further investigation.

**Keywords**

*Cyclospora*; *Cyclospora macacae*; Rhesus monkeys; SSU rRNA gene

## Introduction

*Cyclospora cayetanensis*, an important human pathogen causing acute diarrhea, is commonly found in some developing countries such as Haiti, Guatemala, Peru, and Nepal and rarely found in developed countries. However, it has caused numerous outbreaks in the USA and Canada since the mid 1990s (Hall et al. 2012; Herwaldt 2000; Ortega and Sanchez 2010; CDC 1996), including recent multi-state outbreaks in summer 2013 in the USA (CDC 2013). Most of these outbreaks are foodborne and associated with imported fresh produce (Hall et al. 2012; Herwaldt 2000; Ortega and Sanchez 2010; Shields and Olson 2003). Ingestion of contaminated water has also been implicated as a risk factor for *C. cayetanensis* infections (Hall et al. 2011; Ortega and Sanchez 2010). Because of the scarcity of human *C. cayetanensis* specimens and lack of animal models, studies in nonhuman primates have been used to improve the understanding of the biology of *Cyclospora* spp. (Eberhard et al. 1999a; Eberhard et al. 2014; Perez Cordon et al. 2008; Smith et al. 1996; Zhao et al. 2013).

Morphologically, oocysts of *C. cayetanensis* are spherical and 8–10 μm in diameter and differ significantly from other *Cyclospora* spp. in insectivores and rodents (Ortega et al. 1994; Ortega et al. 1993). Phylogenetic analysis based on the small subunit rRNA (SSU rRNA) gene suggests that *C. cayetanensis* and other *Cyclospora* spp. in primates are related to members of the *Eimeria* genus (Relman et al. 1996). Thus far, of 19 known species of *Cyclospora*, three species, including *C. cercopitheci*, *C. colobi*, and *C. papionis* from nonhuman primates (African green monkeys, colobus monkeys, and baboons, respectively), are most closely related to *C. cayetanensis* based on morphologic and molecular characterization (Eberhard et al. 1999a; Lopez et al. 1999). Moreover, these four *Cyclospora* species from primates (including humans) are probably host specific, even though their nonhuman primate hosts have overlapping ranges (Eberhard et al. 2001; Ortega and Sanchez 2010).

In November 2010, we collected fecal samples from free-range rhesus monkeys (*Macaca mulatta*) in a public park in Guizhou province in Southwestern China to conduct an epidemiological survey of cryptosporidiosis, giardiasis, and microsporidiosis (Ye et al. 2012). Organisms similar to *Cyclospora* spp. were identified in some specimens. In the present study, we conducted morphologic and molecular characterization of *Cyclospora* sp. in these animals and describe the occurrence of a parasite unique to macaque monkeys. We

have proposed the name *Cyclospora macacae* n. sp. for this newly identified parasite from rhesus monkeys.

## Materials and methods

### Specimens

A total of 411 fecal specimens collected in November 2010 from rhesus monkeys (*M. mulatta*) were used in this study. They consisted of fresh stools picked up from grounds frequented by the animals. The rhesus monkeys were free-range animals in a public park in Guiyang City, Guizhou Province, China. Twenty-three water samples collected from a lake where these monkeys bathed were also examined. Permission for specimen collection was obtained from the park management prior to the execution of the study. The detailed specimen collection was described in a previous publication (Ye et al. 2012). The fresh specimens were stored in 2.5 % aqueous potassium dichromate at 4 °C prior to DNA extraction.

### DNA extraction and PCR

After washing 200 μl of fecal specimens or water concentrates twice with distilled water by centrifugation at 3000×*g* for 10 min, DNA was extracted from them using the FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA). *Cyclospora* spp. in the specimens were genetically characterized by nested PCR amplification of a 680-bp fragment of the SSU rRNA gene (Li et al. 2011). The near full-length (1677 bp) SSU rRNA gene of the unique *Cyclospora* sp. was amplified by nested PCR analysis of three overlapping fragments (fragments 1, 2, and 3) using the following primers. The primers for fragment-1 (680 bp) were previously described (Li et al. 2011). The primers for fragment-2 (580 bp) were CYCP2F1 (5′-TGTAAAACCCTTCCAGAGAAC-3′) and CYCP2R1 (5′-AGAAGTGATGCGGAAACCAAA-3′) in primary PCR and CYCP2F2 (5′-TGTCGTGGTCATCCGGCC-3′) and CYCP2R2 (5′-ACCTGGTGAGTTTCCCCG-3′) in secondary PCR. The primers for fragment-3 (676 bp) were CYCP3F1 (5′-AACCTGGTTGATCCTGCCAG-3′) and CYCP3R1 (5′-TGATCCTTCTGCAGGTTCACCTA-3′) in primary PCR and CYCP3F2 (5′-AATCAAAGTCTCTGGGTTCTGGG-3′) and CYCP3R2 (5′-CGTGTTACGACTTT TGCATCCTT-3′) in secondary PCR. The PCR condition for fragment-2 and fragment-3 was similar to that used in PCR for fragment-1, except that the annealing temperatures of 55 and 60 °C were used, respectively, in primary and secondary PCR for fragment-2 and 53 and 55 °C, respectively, in primary and secondary PCR for fragment-3.

### Sequence analysis

The secondary PCR products were sequenced in both directions on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences obtained were assembled using ChromasPro (http://www.technelysium.com.au/ChromasPro.html), edited using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned using ClustalX (http://bips.ustrasbg.fr/fr/Documentation/ClustalX/). To determine the taxonomic identity of the newly identified *Cyclospora* sp., a neighbor-joining tree based on near-complete sequences of the SSU rRNA gene was constructed using genetic distances from the Kimura

two-parameter model and the software Mega 6 (http://www.megasoftware.net/). The reliability of cluster formation was evaluated by the bootstrap method with 1000 replicates.

### Morphological assessment

Oocysts of the new *Cyclospora* sp. were purified by discontinuous sucrose and cesium chloride gradient centrifugation (Arrowood and Donaldson 1996). The purified oocysts were examined as wet mounts by differential interference contrast microscopy and epifluorescence microscopy with a 330–380-nm ultraviolet excitation filter (Zhou et al. 2011).

## Results

### Occurrence of *Cyclospora* spp. in rhesus monkeys

Of the 411 fecal specimens from rhesus monkeys, 28 (6.8 %) were positive for *Cyclospora* spp. by PCR analysis of the ~680-bp fragment. There were no obvious differences in fecal consistency between positive and negative specimens. DNA sequence analysis identified two types of sequences of the SSU rRNA gene. One sequence was identical to the reference sequence (AF111183) for *C. cayetanensis*, whereas the other 27 sequences were identical to the 621-bp SSU rRNA sequence (KC441080) from a *Cyclospora* sp. recently detected in a crab-eating macaque (*Macaca fascicularis*) in China (Ye et al. 2014). Of the 23 water samples from the lake where the monkeys bathed, four samples (17.4 %) were identified as positive for the new *Cyclospora* sp.

### Sequence characteristics of SSU rRNA gene of *Cyclospora* sp

Efforts were made to acquire the near-complete sequence of the SSU rRNA gene of the newly identified *Cyclospora* sp. Altogether, a 1677-bp sequence, except for the 5′ and 3′ ends of the gene, was obtained and deposited in GenBank with the accession number KP335196. Pair-wise comparisons of the SSU rRNA sequence between *Cyclospora* sp. and known *Cyclospora* spp. in human and nonhuman primates (GenBank accession no. AF111183, AF111184, AF111185, AF111186, and AF111187) showed sequence differences of 1.5, 0.9, 1.0, and 1.0 % from *C. cayetanensis*, *C. colobi*, *C. cercopitheci*, and *C. papionis*, respectively.

### Phylogenetic relationship of *Cyclospora* spp

To establish the relationship between the newly identified *Cyclospora* species and other related apicomplexans, the obtained SSU rRNA sequence (1677 bp) was aligned with reference sequences in GenBank, including those from *Cyclospora*, *Eimeria*, *Isospora*, *Cystoisospora*, and *Toxoplasma*. In a neighbor-joining analysis, the novel *Cyclospora* sp. from rhesus monkeys grouped with other *Cyclospora* spp. from nonhuman primates (*C. colobi*, *C. cercopitheci*, and *C. papionis*) and was relatively distant from the human pathogen *C. cayetanensis* (Fig. 1). These five *Cyclospora* species from primates formed a monophyletic group with 100 % bootstrap support. This primate *Cyclospora* group clustered with a clade containing avian *Eimeria* species. They were more distant from bovine, rabbit, and rodent *Eimeria* species. In addition, the avian *Isospora* species formed a cluster within various *Eimeria* groups and were more related to the rodent *Eimeria* species.

### Morphometrics of oocysts of *Cyclospora* sp

Oocysts of the novel *Cyclospora* sp. were almost perfectly spherical in microscopy. They measured 8.49±0.55×8.49± 0.49 μm in size, with a length/width shape index of 1.02 (*n*=11, Fig. 2a), and showed typical blue autofluorescence under an epifluorescence microscope with a 330–380-nm excitation filter (Fig. 2b). Both unsporulated and partially sporulated oocysts could be seen by microscopy (Fig. 2a).

### Description of *C. macacae* n. sp

Based on the unique genetic feature of the novel *Cyclospora* and the apparent restriction of the parasite to macaque monkeys, we propose to name the *Cyclospora* sp. identified in this study as *C. macacae* n. sp.

Diagnosis—oocysts measure 8.49±0.55×8.49±0.49 μm with a length/width shape index of 1.02 (*n*=11). Two sporocysts are seen in each oocyst.

Host type—Rhesus monkeys (*M. mulatta*).

Other natural hosts—crab - eating macaques (*M. fascicularis*).

Type locality—Guiyang, Guizhou Province, China.

Prevalence—found in 6.6 % of rhesus monkeys sampled.

Site of infection—unknown, oocysts collected from feces.

Material deposited—the SSU rRNA sequence of this species has been deposited in GenBank under accession number KP335196.

Etymology—the species name of *C. macacae* was derived from the genus name of its host (*M. mulatta*) from which this parasite was recovered.

## Discussion

To date, only four *Cyclospora* spp. from primates have been identified in previous studies using a combination of morphologic observations and molecular characterizations, including the human-pathogenic species *C. cayetanensis* and three non-human primate species: *C. cercopitheci*, *C. colobi*, and *C. papionis* from monkeys and baboons (Eberhard et al. 1999a; Ortega et al. 1994). In a few reports, some organisms resembling the primate *Cyclospora* species were also observed in fecal samples of monkeys, baboons, and mandrills, although the taxonomic status of these *Cyclospora*-like organisms has not been clearly established because of the lack of either morphologic or molecular data (Eberhard et al. 2014; Perez Cordon et al. 2008; Smith et al. 1996; Zhao et al. 2013). In this study, we identified a novel *Cyclospora* species from rhesus monkeys in southwestern China and named it *C. macacae* based on morphologic and molecular characterizations.

The oocysts of *C. macacae* under microscopy (spherical, mean 8.5 μm in diameter) are similar to morphologic descriptions of other *Cyclospora* spp. in primates, such as *C.*

*cayetanensis* (spherical, mean 8.6 μm in diameter), *C. cercopitheci* (spherical, mean 9.2 μm in diameter), *C. colobi* (spherical, mean 8.3 μm in diameter), and *C. papionis* (spherical, mean 8.8 μm in diameter) (Eberhard et al. 1999a; Ortega et al. 1994). This observation suggests that all five known *Cyclospora* species infecting primates have small and spherical oocysts, in contrast to those *Cyclospora* species that infect insectivores and rodents, which have large and oblong oocysts (Eberhard et al. 1999a). Although these *Cyclospora* spp. infecting primates are morphologically similar and may not be easily defined at the light-microscopy level, the sequence and phylogenetic analysis based on the SSU rRNA gene can distinguish *C. macacae* from the other four primate *Cyclospora* species. Data from this and previous studies further support the conclusion of host specificity in *Cyclospora* species, although similar primate species, such as two species of macaque monkeys in the case of *C. macacae* and two species of colobus moneys in the case of *C. colobi*, can be infected naturally with the same *Cyclospora* species (Eberhard et al. 1999a; Eberhard et al. 2001; Eberhard et al. 2014; Zhao et al. 2013).

For the specimens used in this study, previous genetic characterization of *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* indicated that the rhesus monkeys in close contact with humans in the public park are commonly infected with human-pathogenic protist species/genotypes or subtypes, an indication of cross-species transmission of enteric pathogens between monkeys and humans (Ye et al. 2012). In this study, we conducted an examination of *Cyclospora* infections in these monkeys and found 27 monkeys infected with *C. macacae* and one monkey infected with *C. cayetanensis*. Here, *C. macacae* has been reported to infect rhesus monkeys for the first time, and it has never been reported in humans thus far. In the lake where these monkeys frequently bathed, 4/23 water samples were identified to contain *C. macacae*, indicating that drinking water or recreational water might have played a role in the transmission of *C. macacae* among monkeys. Surprisingly, *C. cayetanensis* was also detected in one fecal specimen of a rhesus monkey by PCR analysis. *C. cayetanensis* is known to be human pathogenic, and humans are probably the only natural host (Aksoy et al. 2014; Ortega and Sanchez 2010). Attempts have been made to identify nonhuman hosts or reservoirs for *C. cayetanensis* and to establish experimental infection model in multiple laboratory animals (also including rhesus monkeys), but none of the tested animals became infected (Eberhard et al. 1999b; Eberhard et al. 2000). However, results of this and a previous study have identified the presence of *C. cayetanensis* in the fecal samples of two rhesus monkeys in China and Nepal, suggesting that *C. cayetanensis* may under rare occasions infect rhesus monkeys (Chu et al. 2004). Because of limited numbers of specimens and the absence of tissue analysis, whether rhesus monkeys can serve as a natural reservoir host of *C. cayetanensis* needs further investigation.

Our phylogenetic analysis based on the 1677-bp sequence of the SSU rRNA gene showed that *C. macacae* from rhesus monkeys is more closely related to *Cyclospora* spp. from non-human primates than to *C. cayetanensis* from humans, probably reflecting the host differences between humans and lower primates. The tree also places the primate *Cyclospora* group between various *Eimeria* groups, forming a cluster with avian *Eimeria* species rather than to mammalian (rabbit, bovine, and rodent) *Eimeria* species. This is consistent with previous taxonomic descriptions of coccidia (Eberhard et al. 1999a; Li et al. 2007; Morrison et al. 2004; Zhao et al. 2013). In addition to the placement of the primate

*Cyclospora* within the *Eimeria* clade, two avian *Isospora* species are placed there as well, whereas the mammalian *Cystoisospora* species form sister groups with *Toxoplasma gondii* as reported previously (Morrison et al. 2004). However, the true taxonomic placement of *Cyclospora* spp. will require genetic characterization of diverse parasites at other loci.

The identification and description of *C. macacae* in rhesus monkeys extends our knowledge on the taxonomy and transmission of *Cyclospora* spp. in primates. With further characterization, the *C. macacae*-rhesus monkey relationship may serve as a useful model to improve our understanding of the biology of *C. cayetanensis*. In the era of genomics, whole genome sequencing is needed to better understand the genetic relationship between *Cyclospora* spp. and *Eimeria* spp. and among various *Cyclospora* species, and the genetic basis for host specificity of *C. cayetanensis*.

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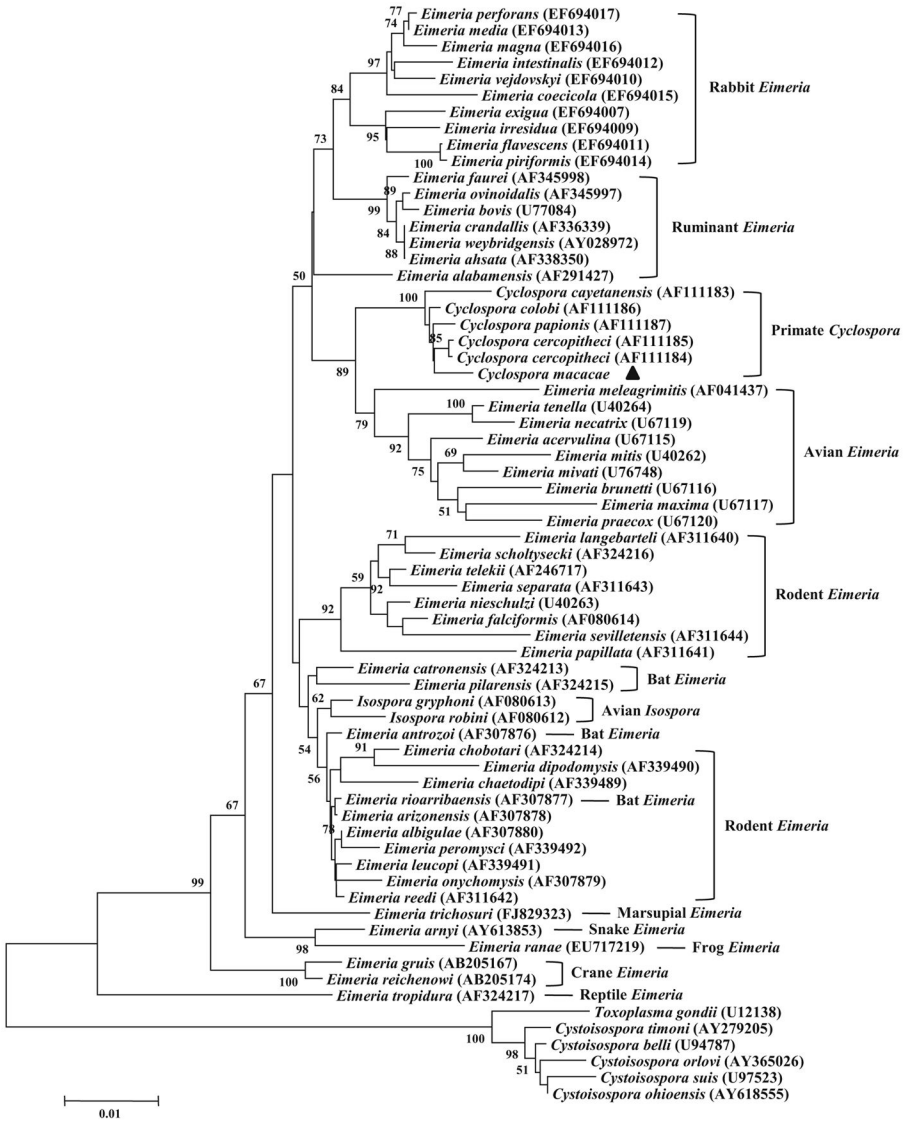


**Fig. 1.**

Phylogenetic relationship among common *Cyclospora*, *Eimeria*, *Isospora*, *Cystoisospora*, and *Toxoplasma* species as inferred by a neighbor-joining analysis of the SSU rRNA sequence, based on genetic distances calculated by Kimura two-parameter model. Bootstrap values greater than 50 % from 1000 replicates are shown. The novel *Cyclospora* species *C. macacae* is indicated by a filled triangle

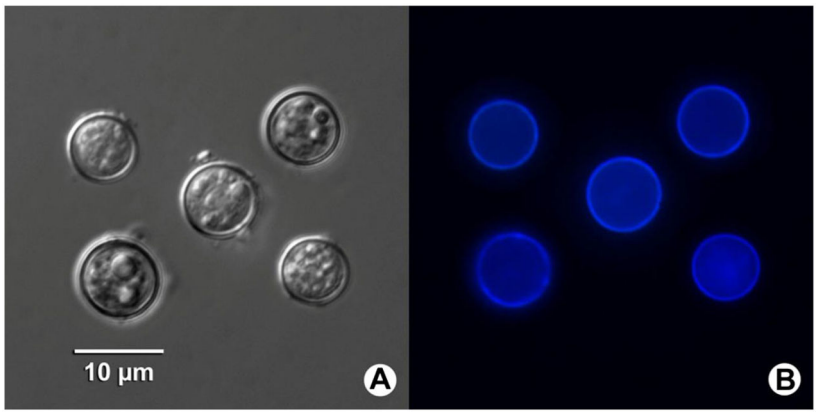


**Fig. 2.**

Photomicrographs of *Cyclospora macacae* oocysts isolated from feces of rhesus monkeys (*Macaca mulatta*) in China. (**a**) Oocysts under differential interference contrast microscopy of wet mount. (**b**) Typical blue autofluorescence of the oocysts is observed under epifluorescence microscopy using a 330–380-nm ultraviolet excitation filter