Molecular Characterization of Cyclospora-like Organisms from Baboons

Fred A. Lopez, Jenette Manglicmot, Thomas M. Schmidt, Cory Yeh, Huw V. Smith, and David A. Relman

Cyclospora organisms are intestinal pathogens of humans that are increasingly recognized in many parts of the world; yet, the reservoirs and host range remain poorly defined. Analysis of 18S ribosomal DNA (rDNA) suggests that the human-associated Cyclospora species (Cyc-hu) is most closely related to the Eimeria species, which are host species–specific. Recently, oocysts identical to those of Cyc-hu were detected in baboon fecal specimens from Tanzania. The 18S rDNA from 3 of these baboon-associated oocyst specimens was amplified and sequenced. Phylogenetic analysis indicated that these baboon-associated Cyclospora-like organisms (Cyc-bab) are nearly identical to each other and are distinct from Cyc-hu (1.6%–1.7% dissimilar); however, these Cyc-bab organisms are the closest known relatives of Cyc-hu. Together, these primate-associated cyclosporans constitute a coherent clade within the diverse group of Eimeria species. These findings raise important questions about the evolutionary relationships of the eimeriids and Cyc-hu host range and should lead to improved polymerase chain reaction–based diagnostics.

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Human experimentation guidelines of the US Department of Health and Human Services were followed. Protocols were approved by the Human Subjects Panel at Stanford University.

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Present affiliation: Section of Infectious Diseases, Louisiana State University School of Medicine, New Orleans.

Reprints or correspondence: Dr. David A. Relman, Veterans Affairs Palo Alto Health Care System 154T, 3801 Miranda Ave., Palo Alto, CA 94304 (relman@cmgm.stanford.edu).

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Molecular characterization of the 18S rRNA gene provides...
a reliable, quantifiable, and widely accepted method for phylogenetic analysis [14, 15]. In a recent demonstration of its usefulness, Carreno et al. [16] found that Isospora suis may belong to the Sarcocystidae family, rather than the Eimeriidae family, as was previously suspected from considerations based on morphology and sporulation [16]. While 18S rDNA sequences discriminate between members of different genera and among members of the same genus, they may not discriminate well among different members of the same species.

The growing database of ssu rDNA sequences from Eimeria species and the recent availability of Cyclospora-like oocysts from baboons in Tanzania presented us with an opportunity to determine the phylogenetic relationships between these baboon-associated organisms and human-associated Cyclospora and to refine our understanding of the diversity of eimeriids with potential for pathogenicity in humans.

Materials and Methods

Oocyst sources, isolation, and visualization. Formed fecal specimens were collected in Gombe National Park, Tanzania, in early 1995 from 3 baboons (nos. 22, 34, and 40) without signs of intestinal disease [10]. Formol ether concentration and sucrose density flotation were used to concentrate and purify oocysts from these samples [17]. The final concentration of purified oocysts from each of the baboon specimens was ~10,000/mL. Oocysts were characterized by light, Nomarski differential interference contrast, and epifluorescence microscopy. Samples containing oocysts were stored at 30°C.

Human fecal specimens containing Cyclospora-like oocysts were obtained from Kathmandu, Nepal (“Cy9,” 1994; “Et113,” 1995), Bangkok, Thailand (“Sph”), and Jakarta, Indonesia (“Sau”) (courtesy of P. Echeverria and O. Sethabutr, Armed Forces Research Institute for Medical Sciences, Bangkok), Florida (“CDC-1,” courtesy of M. Eberhard and N. Pieniacek, Centers for Disease Control and Prevention, Atlanta), and Lima, Peru (“mix-1,” courtesy of Y. Ortega, University of Arizona, Tucson). The last of these 6 specimens was a mixture of oocysts from several individuals living in close proximity to each other; the other 5 specimens were each collected from different individuals. Oocysts were partially purified by formol ether concentration or sucrose density flotation. The concentration of oocysts in the final suspensions varied from 1000/mL to 10,000/mL.

Oocysts were purified at sites physically separate from the laboratory where further molecular studies were performed. They were stored and prepared for polymerase chain reaction (PCR) in a separate room from that in which PCR ampiclon were handled and analyzed.

Amplification of ssu rDNA from oocysts. Oocysts were prepared for PCR with a mechanical glass bead−disruption method involving 0.2 mL of oocyst suspension and 0.25 mL of glass beads [18]. PCRs incorporated 1–10 μL of the disrupted oocyst suspension, 2 mM magnesium chloride, either 1 or 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), 200 μM each dNTP, and 20 pmol of each primer in a 100-μL volume.

For eimeriid range-restricted 18S rDNA amplification, a nested protocol described previously was used [4], with the following modifications: After a “hot start” and initial denaturation at 95°C for 4 min, an initial set of 35 cycles was run, each consisting of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, using the outer primer pair, CYCF1E (Cyclospora 18S rRNA positions 418–436) and CYCR2B (Cyclospora 18S rRNA positions 1053–1035). A second set of 35 PCR cycles was then performed using an inner primer pair, CYCF3E (Cyclospora 18S rRNA positions 685–704) and CYCR4B (Cyclospora 18S rRNA positions 978–959).

For Eukarya broad range ssu rDNA amplification from baboon oocyst preparations, a heminested PCR approach was used. An initial 40 cycles of PCR were performed with primers 1FPL (Eukarya broad range) and 1520RPL (universal) [19]. The second set of reactions (40 cycles) incorporated 5 μL of the initial reaction product, an annealing temperature of 55°C, and primers 1FPL and CYC2RB (for the 5 region of the 18S rDNA) or primers CYC1FE and 1520RPL (for the 3′ region of the 18S rDNA).

PCR products were visualized after electrophoresis in 1.5% agarose gels and staining with ethidium bromide. Products were purified with either Centricon-100 concentrators (Amicon, Beverly, MA) or Promega Wizard PCR preps (Madison, WI).

DNA sequence determination and phylogenetic analysis. Some PCR products were sequenced directly using internal primers complementary to conserved regions within ssu rRNA genes, the fluorescent Dye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, CA), and an ABI 373A automated DNA sequencer (373A; Applied Biosystems). From each of the 3 baboon specimens, a nearly complete 18S rDNA sequence was assembled from the two overlapping 5′ and 3′ sequence fragments using Sequence Navigator software (Perkin Elmer/Applied Biosystems, Foster City, CA). In addition, some 18S rDNA PCR products were cloned in the TA vector system (Invitrogen, Carlsbad, CA) prior to sequencing. In all cases, products were sequenced completely from both strands, with 2-fold coverage for at least one strand. Assembled, nearly complete 18S rDNA sequences from the 3 baboon-associated Cyclospora specimens have been submitted to GenBank (accession nos.: baboon no. 22, AF061566; baboon no. 34, AF061567; and baboon no. 40, AF061568). These three sequences correspond to Dictyostelium discoideum ssu rRNA positions 22–1840, 20–1840, and 22–1839, respectively.

Alignments of 18S rDNA sequences from the baboon specimens were based on conserved primary and secondary structures of eukaryotic ssu ribosomal RNA. From the assembled, nearly complete 18S rDNA sequences, 1567 nucleotide positions could be unambiguously aligned as judged by these criteria and were selected as outgroups. A maximum parsimony (PAUP 4.0) [20], the least-squares distance method of DeSoete [21], and maximum likelihood (FastDNAmI) [22] algorithms were used to create phylogenetic trees. Bootstrap values for the PAUP-generated phylogenetic tree were calculated from 1000 resamplings. An unrooted, bootstrapped parsimony analysis that included the Saccharomyces cerevisiae 18S rDNA sequence revealed a consensus phylogeny consistent with previously published analyses [4, 23], and one in which the Cryptosporidium species fall outside a clade composed of the other coccidians; therefore, S. cerevisiae was dropped from further analyses, and the Cryptosporidium sequences were used as outgroups. A
Results

Spherical oocysts, 8–10 μm in diameter, were concentrated from the stools of 3 different baboons (nos. 22, 34, and 40). These baboons were asymptomatic at the time of sample collection. The oocysts found in their stools were monotypic and identical to those previously identified in fecal specimens from humans as *Cyclospora* (*C. cayetanenesis*), based on the criteria of morphology, morphometry, autofluorescence under UV light, and sporulation [10]. In addition, oocysts from each of these baboon fecal specimens underwent glass bead disruption, and the resulting suspensions were used in a nested PCR procedure with eimeriid range-restricted 18S rDNA primers [4]. These reactions each generated a band of the expected size (~300 bp) from the 3 baboon specimens (figure 1, lanes 2–4). PCR results alone or with *Cryptosporidium parvum* chromosomal DNA failed to generate visible bands (figure 1, lanes 1, 5). Thus, the baboon-associated oocysts shared both phenotypic and genotypic features with the human-associated *Cyclospora* oocysts. These findings suggested that baboons might serve as natural hosts and reservoirs for the latter.

18S rDNA–based phylogenetic analysis of baboon-associated, *Cyclospora*-like oocysts. Broad range *Eukarya* ssu rDNA PCRs were performed, with the aim of obtaining nearly complete 18S rDNA sequences from each of the Cyc-bab oocyst samples. No products were detected in reactions using *Eukarya* broad range (1FPL) and universal (1520RPL) ssu rDNA primers (results not shown). However, heminested PCRs with 5 μL of final material from this initial reaction, and either of the primer pairs, 1FPL + CYCR2B, or CYCF1E + 1520RPL, generated products of the expected size from all 3 baboon specimens, ~1050 bp and 1400 bp, respectively (data not shown). The PCR products from each of the 3 specimens, corresponding to overlapping 5' and 3' regions of the 18S rDNA, were sequenced directly. The pairs of overlapping sequences from each of the baboon-associated oocyst specimens were then assembled into contiguous 1752-bp (baboon 22), 1750-bp (baboon 34), and 1750-bp (baboon 40) 18S rDNA sequences; 1567 (masked) rDNA sequences from the 3 baboon specimens varied from each other at 0.0%–0.1% (0–1) of all nucleotide positions; thus, on the basis of directly sequenced, amplified ssu rDNA data, only 1 strain (or sequence type, Cyc-bab) could be discerned in these 3 samples. The same conclusion applies to the ssu rDNA sequence analysis of multiple, independent human-associated *Cyclospora* specimens (see below).

The baboon-associated *Cyclospora* species is the closest known relative of the human-associated organism. The Cyc-bab and Cyc-hu sequences varied at 1.6%–1.7% of all available nucleotide positions, suggesting that these 2 primate-associated coccidians are distinct and are as distant as are some pairs of different *Eimeria* species (e.g., *E. mivati* vs. *E. acervulina*, 1.3%). The Cyc-bab and Cyc-hu sequences are 3.3%–4.9% and 4.0%–5.1% dissimilar, respectively, to those of the 10 available *Eimeria* species. There are 28 variable positions between the human- and baboon-associated *Cyclospora* sequences. Most of these positions are clustered in several distinct regions of the 18S rDNA (figure 3).

Multiple eimeriid infection and 18S rDNA sequence variability within a baboon-associated oocyst sample. Multiple *Eimeria* species or strains may infect a single host simultaneously [26]. Given the known difficulty in resolving species or strains by morphology, as well as our data indicating significant 18S rDNA sequence differences among morphologically identical primate-associated cyclosporans, three approaches were taken to examine the possibility of multiple coccidian infection of these baboon stool specimens. First, the sequences of the 5 18S rDNA fragments analyzed independently. Using 921 masked nucleotide positions from the sequence of the amplified 5' region and 1295 masked positions from the 3' region, phylogenetic trees were inferred from the separate sets of data and then compared. All 3 algorithms generated trees from the two 18S rDNA regions with nearly identical topology (see figure 4). This result suggests that the two ssu rDNA fragments amplified from separate nested reactions using
Figure 2. Phylogenetic analysis of Cyclospora-like organisms using parsimony methods (PAUP v4.0d64) and based on 1567 18S rDNA nucleotide positions. Percentage of 1000 bootstrap replications that corroborate topology are presented at selected branch nodes. C. parvum and C. muris were used as outgroup. Animal hosts for eimeriids are indicated at right. Bar = 10 hypothesized nucleotide changes.

Figure 3. Schematic diagram of Cyclospora 18S rRNA gene indicating variable positions between baboon-associated (Cyc-bab) and human-associated (Cyc-hu) oocyst sequences. Differences between human-associated and 3 baboon-associated sequences are indicated along length of gene by following notation: Cyc-hu nucleotide/position number/Cyc-bab nucleotide. Position numbering is based on previously published Cyc-hu 18S rDNA sequence (1747 bp, U40261). Cyc-bab sequences contain extra U between Cyc-hu nucleotide positions 1671 and 1672.
each of the baboon fecal specimens originated from a single strain or closely related strains in each case. Second, comparison of the 5’ and 3’ sequences in the region of overlap (baboon 22, 600 nucleotides in length; baboon 34, 502 nucleotides; and baboon 40, 585 nucleotides) revealed variability at only 0.0%–0.3% of the positions, again suggesting that the two products were amplified from a single dominant strain type in each specimen.

The third approach was a more direct examination of ssu rDNA sequence heterogeneity within a single baboon fecal specimen. The amplified 5’ and 3’ 18S rDNA regions from the baboon 40 specimen were each cloned, and five clones of each were sequenced. Among the 5’ clone sequences, dissimilarity scores ranged from 0.0% to 0.3%, and 3’ sequences of the same organism in each case. Bar is proportional to % differences between sequences.

**Discussion**

Human infection with organisms sharing morphologic and morphometric features with those of the *Cyclospora* genus was first described in 1979 [2]. Our understanding of these organisms and of human infection has evolved considerably since that time, due in part to heightened awareness and to improved abilities to recognize them in clinical specimens. Nonetheless, much of this understanding has been based upon visual detection of these coccidians and phenotypic characterization. As evidence of their global prevalence and unexpected spread accumulates, the need to identify *Cyclospora* reservoirs and modes of transmission has become more acute. Vehicles implicated in *Cyclospora* transmission include water and food [28–34]. However, the widespread outbreaks of human cyclosporiasis in North America beginning in 1996 [28, 29] have revealed our relative ignorance about many of the details concerning these issues and have emphasized the need to develop more precise approaches for parasite detection and identification. The poor sensitivity and specificity of traditional approaches suggest that *Cyclospora* infection and disease have been underestimated.

Despite the emergence of *Cyclospora* in the developed world, its host range remains poorly defined. The description of primate-associated *Cyclospora* oocysts by Smith et al. [10] was significant in suggesting the presence of an additional host and
reservoir for the human-associated *Cyclospora*, as well as a possible animal model for propagation and further study of this enigmatic organism. Cyclosporan oocysts from nonprimate hosts have been described as slightly larger than the primate-associated *Cyclospora* oocysts, but with otherwise identical morphologic and sporulation characteristics [35]. Phenotypic characteristics alone, however, may not be sufficient to differentiate reliably between closely related species of coccidians. The results of our study attest to this point.

Despite identical morphology, morphometry, autofluorescence, and sporulation characteristics, baboon- and human-associated *Cyclospora* oocysts are not genetically identical. 18S rDNA sequence analysis suggests that there is one dominant eimeriid sequence type, Cyc-bab, in the 3 baboons studied and that it is distinct from that found in humans. The degree of 18S rDNA sequence dissimilarity between the 2, relative to that found between some pairs of *Eimeria* species, and their different hosts, suggests that different species designations might be warranted for these 2 primate-associated cyclosporans. These features, and our finding of relative sequence homogeneity within and between oocyst samples from the same host, permit speculation that Cyc-bab and Cyc-hu are not freely shared between baboons and humans and that baboons might not support the development of the human-associated *Cyclospora*. Genetic analysis of additional *Cyclospora*-like oocysts from baboons, other nonhuman primates, and humans—especially those that live in proximity to infected primates—will be needed to support these suggestions, prior to any taxonomic revision or proposal of a new species name.

The extent to which differences in 18S rDNA sequences might predict differences in host specificity is unknown. Efforts to propagate the human-associated *Cyclospora* in nonhuman hosts have failed so far [36]. All of the baboons included in our study were asymptomatic, a common observation among natural hosts that are infected with *Eimeria* organisms, including those described in myriapods, vipers, and rodents [1, 35], would help to resolve this taxonomic issue. Genotypic information must also be integrated with additional phenotypic information, such as antigenic profiles and anatomic niche.

One of the limitations of the current PCR-based assay for *Cyclospora* is that it does not distinguish between *Cyclospora* and *Eimeria* species [4]. The acquisition of new sequence information from close relatives of the human-associated *Cyclospora* enhances our ability to design primers and probes that might be specific for the human pathogen.

Polyparasitism, or naturally occurring infection of a single host by multiple parasite strains or species, is a common feature of the *Eimeria* species. In addition, there is increasing evidence, in general, for coinfection of a host by ≥1 strain of a single species of parasite [37]. Factors that influence the likelihood of these events include variables pertaining to host and environment, as well as stochastic factors. In our analysis, we found no convincing evidence of polyparasitism at the species level in the 3 baboons from whom specimens were available or in the 6 independent human *Cyclospora*-containing fecal specimens, described in this study. The degree of sequence variability we observed between clones is well below that usually found between different coccidian species. These data corroborate previously published findings from an additional 9 human specimens [4]. However, our data are limited by the size of the sequence data set. In addition, strain typing is probably not feasible using a relatively conserved genetic locus, such as the ssu rDNA. Sequence variability of <1% may be explained by a number of possibilities, including multiple, variable rDNA operon alleles. Preliminary data suggest that there may be more than one rDNA operon type in *Cyclospora* (Manglicmot J, Relman DA, unpublished observations).

Coevolution of host and parasite is an emerging theme among diverse types of chronic infection. Examples are found in the relationships between rodents and their ectoparasites (i.e., chewing lice) and between single-stranded DNA viruses (e.g., polyomaviruses, papillomaviruses, and parvoviruses) and their respective hosts [38, 39]. One can test the hypothesis that the inferred phylogenetic histories of host and parasite are congruent using likelihood ratio methods [40]. However, additional data will be needed before this hypothesis can be tested for the eimeriids and their hosts.

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**References**


