Transmission Dynamics of Cryptosporidium Infection in a Natural Population of Non-Human Primates at Polonnaruwa, Sri Lanka

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Abstract. Infections from Cryptosporidium parvum are of interest not only to public health, but also to wildlife conservation, particularly when humans and livestock encroach on nature and thereby increase the risk of cross-species transmissions. To clarify this risk, we used polymerase chain reaction to examine the hypervariable region of the C. parvum 18S rRNA gene in feces from three monkey species. Samples were isolated from regions where disease transmission between monkeys, livestock, and humans was likely (soiled habitat) or unlikely (clean habitat). Monkey individuals, their social groups, and different species shared multiple genotypes/isolates of C. parvum. Ecological and molecular analyses suggested that Cryptosporidium infection among Toque macaques in soiled habitats was mainly the bovine genotype C. parvum. Monkeys inhabiting clean habitat, particularly gray and purple-faced langurs, lacked Cryptosporidium species/types associated with bovines. Livestock apparently was a main source of infection for wild primates.

INTRODUCTION

Cryptosporidium infections in Sri Lanka are the main cause of diarrhea in young children,1 and many domestic animals (cattle, goats, buffaloes, and pigs) serve as reservoir hosts.2–4 There are no reports on the role of wildlife in the epidemiology of this disease in Sri Lanka. However, a recent study revealed that three wild primate species, inhabiting the Polonnaruwa Nature Sanctuary, harbor endemic infections of Cryptosporidium.5 They include Toque macaques (Macaca sinica sinica), gray langurs (Sempithecus priam thersites), and purple-faced langurs (Trachypithecus vetulus philbricki). Two of these primates, the Toque macaque and purple-faced langur, are endemic to Sri Lanka. The most significant finding of this study was that the majority of infected monkeys occupied habitat that had been soiled because of promiscuous defecation by humans and livestock. Most areas of the sanctuary around water sources were used as open toilets. Some macaques around these soiled habitats also rummaged for food scraps from human picnicking and had high prevalences of Cryptosporidium and other enteric parasites.5 In contrast, many other primate groups lived predominantly in the forested environment and depended on natural food and clean water; infection among these monkeys was either absent or less prevalent.

Earlier screening for Cryptosporidium oocysts data suggested that these primates could acquire this infection from either humans or livestock.5 Alternative possibilities were not tested, namely the transmission of primate-specific Cryptosporidium isolates among these three primate species of different habitats or of any other Cryptosporidium isolates from other wild mammals of sylvatic origin. Because this infection was asymptomatc in this population, it was not clear whether the monkeys could act as reservoir hosts as do some other wild animals.6

The aim of this study was to explore further the epidemiology of cryptosporidiosis in this population of primates. Specifically, we did a phylogenetic analysis of the hypervariable region of 18S rRNA gene of Cryptosporidium spp. to identify the isolates from the three primate species from soiled and clean habitats.7 This is the first report of molecular typing and phylogenetic analysis of Cryptosporidium spp. in wild non-human primates.8–10

MATERIALS AND METHODS

Study area. The study was conducted in the Polonnaruwa Nature Sanctuary, located in the northeastern dry zone of Sri Lanka,1 The sanctuary is comprised of religious shrines and archeological ruins and is covered by natural dry evergreen forest. The total area encompassing the home ranges of all primate groups involved in this study was 273 ha, and it had been mapped with a grid to help quantify habitat quality and the home range patterns of all primate species. The whole area was divided into soiled or clean habitat depending on the human and animal densities and anthropogenic use.3 Briefly, 33 ha were subjected to high visitation (2–46 persons/ha/d) and frequently used as open toilets; these were classified as soiled habitat. The rest of the area was more forested and rarely used as open toilets; it was classified as clean habitat. However, livestock grazing (0.7–0.9 head/ha) varied locally and seasonally and was a common feature with little discrimination over the two areas. Another unique feature of the study site was its water distribution pattern. The lake was the main water source and drained into the irrigation channel; these were used by humans, domestic animals, and wildlife of the soiled area. On the other hand, monkeys of clean habitat had access to rain-fed water holes, seasonal ponds, or wells.5

Study population. The home range areas of neighboring groups of monkeys of the same species were mutually exclusive to different degrees: home range overlap among the small sized territories (4–6 ha) of the purple-faced langurs was < 10%, but that among the large ranges of gray langurs or macaques (13–48 ha) was considerably more, up to 20–60%. Home range overlap among the three species, however, was almost complete. Toque macaques and gray langurs had terrestrial feeding habits, whereas purple-faced langurs were

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highly arboreal. This primate population has been monitored since 1968; macaques in particular were individually identified, and all species’ demographic histories, ecologies, and behaviors were known. None of these primates were managed or intentionally provisioned.11

The selected study sample consisted of 89 Toque macaques (four groups from soiled habitats and seven groups from clean habitats), 21 gray langurs (one group from a soiled habitat and two groups from clean habitats), and 15 purple-faced langurs (one group from a soiled habitat and two groups from clean habitats). All groups had been previously screened for Cryptosporidium infection using modified Ziehl Neelsen staining techniques and a nested polymerase chain reaction (PCR) assay on the 18S rRNA gene for identification purposes only.5 The numbers of samples selected for sequence analysis from each positive group from all three primates species inhabiting both soiled and clean habitat varied (Table 1).

**Sample collection.** Individually identified macaques and langurs were followed in their home ranges. Fecal samples were collected immediately after defecation, and only the top layer (without soil) of fresh fecal samples (2–8 g) was scooped and stored in sterile plastic containers. Each animal selected for this study was sampled only once, and the fecal analysis was done within 4–5 hours of collection. The fecal specimens from macaques and langurs were concentrated using Sheather sucrose solution as described earlier.4 Oocysts were transferred into microcentrifuge tubes containing laboratory grade water and stored at 4°C until further analysis.

**Genomic DNA extraction.** Oocysts were lysed after several cycles of freeze thawing, and DNA was extracted using Wizard genomic DNA purification kit (Promega Corp., Madison, WI) according to the manufacturer’s instructions. Purified DNA was resuspended in a final volume of 25 μL of provided DNA rehydration buffer.

**Nested PCR assay.** The nested PCR was performed as previously described by using the external primers KLJ1 (5’caca-cataagaaggca-3’) and KLJ2 (5’atggatctacagttgctg3’) in the first round of amplification, followed by a PCR reaction using the internal primer set CBP-DIAGE (5’gcgcgaattc-aagctcgtagttggatttctg-3’) and CBP-DIAGR (5’gcgcgcggc-gtaaggtgctgaaggagtaagg-3’), with additional restriction sites (underlined) for site directional cloning.7 PCR products were separated on 1.2% agarose gels and visualized after ethidium bromide staining. The 434-bp nested PCR fragment was gel purified using a Qiaquick Gel Extraction kit (Qiagen, Valencia, CA).

**Cloning and sequencing.** Purified nested PCR fragments were cloned into a TA cloning vector (Invitrogen, Carlsbad, CA) and subsequently transformed to One Shot Top 10 F’ competent cells. Blue/white selection was applied on LB agar plates containing 100 μg/mL Ampicillin. Plasmid DNA was isolated using the Revprep Orbit from GeneMachines (GeneMachines, Ann Arbor, MI). DNA was sequenced using diluted ABI Big Dye 3.0 chemistry, and sequencing reactions were analyzed on an ABI 3730 Analyzer (Applied Biosystems, Foster City, CA) and edited using VectorNTI (Invitrogen). At least eight clones were sequenced from each amplified PCR product to find out whether isolates contained mul-

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**Table 1**

<table>
<thead>
<tr>
<th>Primate species and social group</th>
<th>Home range includes soiled substrate</th>
<th>No. of clones</th>
<th>No. of genotypes (Percentage)</th>
<th>Home range has clean substrate</th>
<th>No. of clones</th>
<th>No. of genotypes</th>
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<tbody>
<tr>
<td><strong>Toque macaques</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1 Derika</td>
<td>24</td>
<td>5</td>
<td>42_01 (37.5%)</td>
<td>M3 Bliss</td>
<td>20</td>
<td>58_08 (20%)</td>
</tr>
<tr>
<td></td>
<td>(42)</td>
<td>42_02</td>
<td>42_02 (37.5%)</td>
<td></td>
<td>20</td>
<td>58_02 (80%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42_06</td>
<td>42_06 (54.5%)</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>42_12</td>
<td>42_12 (8%)</td>
<td></td>
<td>20</td>
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<tr>
<td>Yanci</td>
<td>9</td>
<td>3</td>
<td>44_13 (22.5%)</td>
<td>Pullet</td>
<td>8</td>
<td>56_08 (80%)</td>
</tr>
<tr>
<td>(44)</td>
<td></td>
<td>44_15</td>
<td>44_15 (55%)</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>44_03</td>
<td>44_03 (22.5%)</td>
<td></td>
<td>8</td>
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</tr>
<tr>
<td>CH-1 Fery</td>
<td>9</td>
<td>3</td>
<td>14_10 (44%)</td>
<td>J Reen</td>
<td>8</td>
<td>5_07 (75%)</td>
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<tr>
<td>(14)</td>
<td></td>
<td>14_05</td>
<td>14_05 (44%)</td>
<td></td>
<td>8</td>
<td>5_08 (25%)</td>
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<td></td>
<td></td>
<td>14_04</td>
<td>14_04 (12%)</td>
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<td>8</td>
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<td><strong>Gray langurs</strong></td>
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<tr>
<td>SG AF1</td>
<td>16</td>
<td>3</td>
<td>82_02 (4%)</td>
<td>LT AF2</td>
<td>12</td>
<td>80_08 (80%)</td>
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<td>(82)</td>
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<td>82_08</td>
<td>82_08 (46%)</td>
<td></td>
<td>12</td>
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</tr>
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<td>82_03</td>
<td>82_03 (50%)</td>
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<td>12</td>
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<tr>
<td>J1F1 (69)</td>
<td>20</td>
<td>2</td>
<td>69_05 (15%)</td>
<td>AM2</td>
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<td>84_06 (80%)</td>
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<td></td>
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<td>69_07</td>
<td>69_07 (85%)</td>
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<td>8</td>
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<tr>
<td><strong>Purple-faced langurs</strong></td>
<td></td>
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<tr>
<td>E8 AF3</td>
<td>8</td>
<td>3</td>
<td>78_08 (10%)</td>
<td>H10 AF4</td>
<td>8</td>
<td>70_08 (75%)</td>
</tr>
<tr>
<td>(78)</td>
<td></td>
<td>78_07</td>
<td>78_07 (15%)</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>78_01</td>
<td>78_01 (75%)</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>AM3 (73)</td>
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<td>1</td>
<td>73_01</td>
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<td>20</td>
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multiple infections. When nucleotide differences were found between clones derived from the same individual, the PCR cloning and sequencing were repeated to rule out PCR artifacts. Altogether, 150 clones were sequenced from 12 monkeys of eight social groups (Table 1). All clone sequences were identical to or very similar to *C. parvum* sequences in GenBank.

**Phylogenetic analysis.** Reference *C. parvum* sequences were human isolate *C. hominis* clone OX1b (AY204230); bovine isolates *C. parvum* type A (AF015772), *C. parvum* type B (clone KSU-1: AF308600),

12 clone Hmb1 (AY204237), and clone HMa1 (AY204238); and isolates from rhesus monkey (AF112569), rabbit (AY120901), and mouse (AF112571). The closely related *Cryptosporidium meleagridis* (AF112574) was used as an outgroup. Many sequences in the data set were identical (Figure 1); only a single representative of each type was used in phylogenetic analysis. Heuristic searches with 100 random taxon addition replications and tree-bisection reconnection were performed with PAUP*4 under minimum evolution (ME), maximum parsimony, and maximum likelihood (ML) criteria. The evolutionary model for ME and ML, chosen by nested log-ratio tests (model test, Posada and Crandall), was HKY+I (0.77) + G (0.74), although a variety of models including uncorrected distance were found to produce very similar topologies. Because of the large amount of stochastic variation in nonparametric bootstrap datasets caused by the small number of polymorphisms in the dataset, bootstrap analyses were largely inconclusive and were not reported.

**RESULTS**

18S rRNA gene sequencing analysis. Initial BLAST search indicated that these 434-bp nucleotide sequences from the hypervariable region of the 18S rRNA were similar to *C. parvum* reference sequences. They were closely related to mainly two reference genotypes, *C. parvum* type A and type B, and a few isolates were related to *C. hominis*. Common sequences were derived from different individuals from the same social group and from different species of monkeys. Most of the monkeys had multiple infections from more than one genotype. The number of isolates derived from each individual differed (Table 1). The sequences derived from this study have been deposited in the GenBank with the following accession numbers: EF446672 (TM42_02), EF446673 (GL82_02), EF446674 (TM05_07), EF446675 (TM42_13), EF446676 (TM14_04), EF446677 (TM56_08), EF446678 (GL82_03), and EF446679 (PL78_01).

**Phylogeny.** The trees found by heuristic searches using MP, ME, or ML were not significantly different and were consistent with visual inspection of polymorphisms (Figures 1 and 2). As can be seen in the ML tree (Figure 1), the *C. parvum* 18S sequences collected in this study can be broadly separated into two types and four major clades and are identical to or very similar to previously reported *C. parvum* sequences collected from other organisms. Specifically, Clade 2 contained the reference *C. parvum* type B found in other wildlife (mouse, deer, chipmunk, and muskrat) and cattle in North America and differed by single base changes from Clade 1, and GL82_02 and TM5_7 (macaque isolate TM42_02) was placed in Clade 1 in all analyses, but this may be because of the inability to properly account for indels in the phylogenetic analyses (Figure 2). Together, these isolates were designated type B because of their similarity to *C. parvum* type B. Clade 4 contained the reference genotypes found in North American cattle, including Genotype 2 type A, and was closely related to the sequences in Clade 3. Macaque isolates TM42_13 and TM14_4 could not be assigned to any clade with certainty (although in the MP tree, they were assigned to Clades 4 and 3, respectively). Together, these isolates and allied reference sequences were designated type A because of their similarity to *C. parvum* type A. The single outlier from the two types was purple-faced langur isolate PL78_01, which differed by a single unique polymorphism from the human isolate *C. hominis* and was clearly different from the other isolates collected in this study.

With the exception of langur isolate PL 78_01, the ancestral
state of C. parvum in Sri Lankan monkeys was represented by the reference sequence C. parvum type B and the other sequences in Clade 2. Isolates TM82_02 and TM5_7 and the Clade 1 and type A isolates were derived from Clade 2. Within type A, the ancestral state was represented by C. parvum type A, other reference sequences, and the monkey isolates of Clade 4. Clades 3 and the other type B isolates were derived from Clade 4.

Every clade contained isolates from multiple species, and in many cases, the sequences from multiple species were completely identical in the region examined. In every case in which more than one sequence was isolated from a single monkey, those sequences were distributed throughout the tree, often in more than one clade or even in both types.

Clade 2 contained isolates collected only from clean habitat, whereas Clades 1 and 4 contained isolates collected only from soiled habitat. Clade 3 contained a mixture of isolates collected from clean and soiled habitat, with isolates from clean and soiled habitat having identical sequence in the region examined. The small number of isolates from langurs in clean habitats was identical, whereas those from macaques in clean habitats are present in every clade except Clade 1.

**DISCUSSION**

*Cryptosporidium parvum* is the most common cause of acute cholera-like diarrheal gastrointestinal disease in humans and livestock. Several host-adapted genotypes of *C. parvum* from hosts such as human, bovine, mouse, ferret, pig, monkey, and bear have been described. Its global importance has been shown by several major waterborne outbreaks, including one in Milwaukee, WI, which caused acute illness in > 403,000 people. Subsequently, most epidemiologic studies were focused mainly on the prevalence of *Cryptosporidium* infection in wild and domestic animals living around the vicinity of water catchments areas to assess their potential as reservoirs of disease to human and livestock. Ground and water contamination with human and domestic animal feces is also a potential threat to wildlife. Humans and mountain gorillas (Gorilla gorilla beringei) share the same habitat in Uganda where *C. parvum* was isolated from mountain gorillas, suggesting a possible anthropo-zoonotic mode of transmission. Our earlier study suggested a similar possibility because the highest prevalence of *C. parvum* infection was found in monkeys from habitat soiled by feces from humans and their livestock.

This study indicated that the three monkey species were infected with four major clades of *C. parvum*. All species and monkey individuals from soiled habitat harbored isolates closely related to bovine or zoonotic *C. parvum* type A. This included some Toque macaques occupying clean habitat but whose wide ranging behavior invited greater risk of exposure to soiled areas. Conversely, all of the isolates from the three gray langurs and one purple-faced langur from clean habitat were more closely related to *C. parvum* type B. Infection from contaminated ground was least likely for the arboreal and folivorous purple-faced langurs.

It is of interest that the predominant isolate recovered from a purple-faced langur of a soiled area was more closely related to *C. hominis* found in humans than it was to either type of *C. parvum* (this purple-faced langur also had two isolates similar to *C. parvum* type A). Although the ancestral origin of this isolate is human genotype 1, it is not clear how this arboreal langur got the infection.

*C. parvum* has been found predominantly in livestock, which serves as a reservoir for infection of both humans and wildlife. On the other hand, it has been suggested that wild mammals may serve as reservoirs of *Cryptosporidium* infection to both domestic animals and humans. In the case of the monkeys at Polonnaruwa, all three species exposed to areas soiled by cattle harbored *Cryptosporidium* isolates identical to or very similar to *C. parvum* type A bovine isolates, whereas monkeys not so exposed lacked this genotype. This would point to cattle as the likely source of *C. parvum* type A for the monkeys. However, further work with additional genetic loci and samples from local humans and livestock will be needed to confirm these findings, because multilocus genotype analysis will give a proper understanding of the host population structure.

The majority of the monkey population harbored a zoonotic strain of the infection, but the risk of transmission of the disease from monkeys to humans was not studied. Notwithstanding, this potential risk should not be ignored, particularly in suburban places where these primates are attracted to crops, home gardens, or food scraps near human habitation. On the other hand, the progressive encroachment of humans and their livestock into natural forested areas is an unequivocal source of parasitic infection for wildlife. The improved disposal of human garbage (to prevent food scraps from attracting wildlife to suburban areas), as well as the prevention of illegal livestock grazing in nature sanctuaries and the installation of sealed public toiled facilities in such areas, would serve in the interest of both human health and wildlife conservation.

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