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Invited article

Occurrence of *Giardia, Cryptosporidium*, and *Entamoeba* in wild rhesus macaques (*Macaca mulatta*) living in urban and semi-rural North-West India

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ABSTRACT

Giardia duodenalis, Cryptosporidium spp., and *Entamoeba* spp. are intestinal protozoa capable of infecting a range of host species, and are important causes of human morbidity and mortality. Understanding their epidemiology is important, both for public health and for the health of the animals they infect. This study investigated the occurrence of these protozoans in rhesus macaques (*Macaca mulatta*) in India, with the aim of providing preliminary information on the potential for transmission of these pathogens between macaques and humans. Faecal samples ($n = 170$) were collected from rhesus macaques from four districts of North-West India. Samples were analysed for *Giardia/Cryptosporidium* using a commercially available direct immunofluorescent antibody test after purification via immunomagnetic separation. Positive samples were characterised by sequencing of PCR products. Occurrence of *Entamoeba* was investigated first by using a genus-specific PCR, and positive samples further investigated via species-specific PCRs for *Entamoeba coli*, *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii*. *Giardia* cysts were found in 31% of macaque samples, with all isolates belonging to Assemblage B. *Cryptosporidium* oocysts were found in 1 sample, however this sample did not result in amplification by PCR. *Entamoeba* spp. were found in 79% of samples, 49% of which were positive for *E. coli*. Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii*, did not result in amplification in any of the samples. Thus in 51% of the samples positive at the genus specific PCR, the *Entamoeba* species was not identified. This study provides baseline information on the potential for transmission of these zoonotic parasites at the wildlife-human interface.

1. Introduction

Giardia duodenalis, Cryptosporidium spp., and *Entamoeba* spp. are intestinal protozoa capable of infecting a range of host species, and are important causes of human morbidity and mortality (Hunter and Thompson, 2005; Kotloff et al., 2013; Stanley Jr, 2003). *Cryptosporidium* spp., mainly *C. hominis* and *C. parvum,* have been responsible for large-scale waterborne epidemics in the developed world, and are amongst the top four causes of moderate-to-severe diarrhoea in young children in the developing world (Checkley et al., 2015; Kotloff et al., 2013; Shirley et al., 2012; Sow et al., 2016). Around 200 million people in Asia, Africa and Latin America are reported to have symptomatic giardiasis (Feng and Xiao, 2011). *Entamoeba histolytica*, the cause of amoebic colitis and amoebic liver disease, is responsible for up to 100 000 deaths annually (Stanley Jr, 2003).

Understanding the epidemiology of these parasites is important, both for public health as well as for the health of the animals they infect. This is made difficult by morphologically identical parasites sometimes having separate pathogenicity, host ranges and life cycles. Thus, molecular characterisation is required to elucidate transmission pathways. For instance, *Giardia duodenalis* is considered a species complex comprised of at least 8 distinct genetic

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groups (Assemblage A to H), with Assemblages A and B found both in humans and a range of animal species (Thompson and Smith, 2011).

Cryptosporidiosis, giardiasis and amoebiasis are all important diseases in India where poverty, lack of hygiene, free roaming animals, high population density, and infrastructure inadequacies regarding water supply and sanitation, facilitate infection (Kaur et al., 2002; Nath et al., 1999, 2015b). Rhesus macaques (*Macaca mulatta*) are one of the most common primates in India, particularly in human-dominated habitats (Kumar et al., 2013). Indeed, in some Indian districts, the close contact between rhesus macaques and human activities means that they are regarded as a nuisance, particularly due to crop raiding activities (Saraswat et al., 2015). Macaque species have been implicated as wildlife reservoirs for zoonotic pathogens such as Kyasanur forest disease, a zoonotic tick-borne viral haemorrhagic fever (Singh and Gajadhar, 2014). Nevertheless, it is unclear whether there is transmission of intestinal protozoans between humans and urban monkeys, and if so, how significant this is for public health and for the conservation of the macaques. This study investigated the occurrence of *Giardia duodenalis, Cryptosporidium* spp., and *Entamoeba* spp. in rhesus macaques in four districts of North-West India, with the aim of using molecular characterisation of isolates to provide preliminary information on the potential for transmission of these pathogens between macaques and humans.

2. Materials and methods

2.1. Animals

2.1.1. Rhesus macaques

Faecal samples ($n = 170$) were collected from free-living rhesus macaques in four non-overlapping locations in North-west India.

Troop 1: Located at Punjab University, Chandigarh. Monkeys move freely throughout the campus, spending large amounts of time feeding, defecating and sleeping near areas used for preparation of human food. Estimated troop size, 300 animals.
Troop 2: Located at Jakhoo Temple, Himachal Pradesh. Primarily based around a forested hilltop temple, however also move freely into the surrounding city of Shimla. Estimated troop size, 200 animals.
Troop 3: Located around a small local temple in the municipality of Kurali, Punjab. This temple also owns a cattle-breeding facility where the troop spends much of its time. There is direct contact between the cows and the moneys, with macaques eating grain provided to the cattle and picking food off the ground contaminated with cattle faeces. Estimated troop size, 100 animals.
Troop 4: Located on the outskirts of a semi-rural town Nada Sahib, Haryana. Co-exists with roughly 30 Tarai grey langurs (*Semnopuithecus hector*). Estimated troop size, 200 animals.

2.1.2. Domestic cattle (Bos indicus)

Faecal samples ($n = 14$) were collected from calves from the breeding facility in Kurali with which Troop 3 was in close contact.

2.2. Sample collection and preservation

Rhesus macaques faecal samples were collected non-invasively, and were identified by being morphologically consistent fresh stools located where these monkeys had been observed immediately preceding collection. Each stool sample was considered to be from a separate individual. Calf faecal samples were collected directly from the stool after the animal had been observed to defecate.

Approximately two grammes of faecal material, collected from the middle of the fecal mass, was placed in an 8 ml aliquot of 2.5% (w/v) potassium dichromate, mixed thoroughly, and transported to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis. One gram of faecal material was transported to the Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, and kept unpreserved at 4 °C for 2 weeks prior to DNA isolation.

2.3. Sample processing

At NMBU, the samples were washed twice with phosphate buffered saline, and then passed through a faecal parasite concentrator with a pore diameter 425 μm (Midi Parasep, Apacor, Berkshire, England) and centrifuged to create a pellet. *Giardia* cysts and *Cryptosporidium* oocysts were isolated using an in-house immunomagnetic separation method (IMS) using Dynabeads™ (GC-Combo, Life Technologies, Carlsbad, CA) as previously published (Robertson et al., 2006). Briefly, 10 μl anti-*Giardia* beads, 10 μl anti-*Cryptosporidium* beads, 100 μl SL buffer A and 100 μl SL Buffer B, were used to generate 55 μl of purified sample from approximately 200 mg of the faecal pellet. Five μl of the resulting purified sample was dried and methanol-fixed to welled slides for detection of *Giardia* cysts and *Cryptosporidium* oocysts using a commercially available *Cryptosporidium/Giardia* direct immunofluorescent antibody test (IFAT; Aqua-Glo, Waterborne Inc., New Orleans), in accordance with manufacturer's instructions. Prior to being screened, dried samples were also counterstained with 4′6 diamidino-2-phenylindole (DAPI), a non-specific fluorescent stain that binds to double-stranded DNA. Stained samples were screened using a fluorescence microscope equipped with appropriate filters (for FITC and DAPI) and Nomarksi optics. Samples were initially screened at ×200, and possible findings examined more closely at x 400 and x 1000. The total number and DAPI staining of cysts and oocysts on the slide was recorded. Due to the large number of *Giardia* positive samples, only those with either over 100 DAPI negative cysts, or over 20 DAPI positive cysts were included in molecular analyses. These criteria resulted in 26 *Giardia* positive samples being included. All *Cryptosporidium*-positive samples were included in molecular analysis.

2.4. DNA isolation

2.4.1. Entamoeba

At PGIMER, DNA was isolated using QIAamp® Fast DNA Stool Mini Kit, with an incubation at 70 °C for 5 min, in accordance with the manufacturer's instructions.

2.4.2. Giardia and Cryptosporidium

For *Giardia*/*Cryptosporidium*-positive samples, DNA was isolated using the remaining 50 μl of purified cysts/oocysts after IMS using the QIAmp DNA mini kit (Qiagen GmbH) at NMBU. The protocols followed the manufacturer instructions with slight modifications; cysts/oocysts were first mixed with 150 μl of TE buffer (100 mM Tris and 100 mM EDTA) and incubated at 90 °C/100 °C (*Giardia*/*Cryptosporidium*) for 1 h before an overnight proteinase K lysis step at 56 °C and spin column purification. DNA was finally eluted in 30 μl of PCR grade water and stored at 4 °C.

2.5. PCR and sequencing

In all cases, the primary PCR consisted of 8.3 μl PCR water, 1 μl forward and 1 μl reverse primer (at a final concentration of

0,4 mM), 0.2 μl BSA (20 mg/l), 12.5 μl of 2× HotStartTaqMaster and 2 μl of template DNA. For each PCR, positive and negative controls were included. PCR products were visualized by electrophoresis on 2% agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, CA). Target genes and PCR conditions are provided in Supplementary Table 1.

2.5.1. Giardia

Conventional PCR was performed on *Giardia* positive samples at the glutamate dehydrogenase (GDH), triosephosphate isomerase (TPI), β-giardin (BG) and small subunit rRNA (SSU rRNA) genes (Caccio et al., 2008; Hopkins et al., 1997; Lalle et al., 2005; Read et al., 2002, 2004; Robertson et al., 2006; Sulaiman et al., 2003). Positive samples were purified using a High Pure PCR Product Purification Kit (Roche, Oslo, Norway) and sent to a commercial company (GATC Biotech, Germany) for sequencing in both directions. Sequences from both directions were assembled and manually corrected by analysis of the chromatograms using the program Geneious™.

2.5.2. Cryptosporidium

Conventional PCR was performed on *Cryptosporidium* positive samples at the SSU rRNA gene (Xiao et al., 1999).

2.5.3. Entamoeba

An *Entamoeba* genus-specific conventional PCR was performed on rhesus macaque samples as previously published (Verweij et al., 2003). Two samples were not analysed due to insufficient faecal material for DNA isolation. A single round multiplex PCR targeting the SSU rRNA gene, and that identifies *E. histolytica*, *E. dispar* and *E. moshkovskii*, was performed on all samples (Hamzah et al., 2006). For samples that tested positive on the genus-specific PCR, a species-specific PCR for *E. coli* was performed as previously described (Tachibana et al., 2009). Four *Entamoeba* genus-specific positive samples were not tested for *E. coli* due to laboratory error.

2.6. Statistics

Prevalence of *Giardia*, *Cryptosporidium* and *Entamoeba* were compared for the four different macaque troops using the Chi-squared test. Proportion of samples that resulted in amplification by PCR was compared using Fischers exact test.

3. Results

3.1. Prevalence of Giardia cysts shed by wild rhesus macaques

Examination of rhesus macaque faecal samples using immunofluorescent microscopy revealed the presence of *Giardia* cysts in 31% (53/170) of samples. Macaques excreted 55 to 6325 cysts per gramme faeces (mean, 555; median, 165). There was a significant difference in the prevalence of *Giardia* cysts between Troops 1, 2, 3 and 4; 45% (25/55), 20% (9/55), 33% (15/46) and 17% (4/24), respectively ($p < 0.05$).

3.2. Giardia genotyping

Of the twenty-six *Giardia* positive samples selected for molecular characterisation, seventeen tested positive at one or more gene, with the SSU rRNA loci being the most sensitive (Table 1).

Table 1
Results of PCR from *Giardia* positive faecal samples from wild rhesus macaques (*Macaca mulatta*) with close human contact.

#	Cysts[a]	DAPI[b]	TPI[c]	GDH[d]	GDH[e]	BG[f]	SSU[g]
1	950	800	–	B (KX787059)	B (KX787059)	B (KX787068)	B (KX787044)
2	1150	600	–	–	–	–	B (KX787042)
3	200	150	–	–	B (KX787061)	–	B (KX787047)
4	130	70	–	–	–	–	Positive
5	190	60	–	B (KX787060)	–	B (KX787069)	–
6	110	50	–	–	–	–	B (KX787043)
7	110	50	–	–	–	B (KX787055)	B (KX787046)
8	80	50	–	–	–	B (KX787056)	B (KX787050)
9	50	40	–	–	–	–	B (KX787045)
10	70	20	–	–	–	–	B (KX787049)
11	40	20	–	–	–	–	–
12	30	20	–	–	–	–	Positive
13	30	20	B (KX787057)	–	–	–	–
14	20	20	–	–	–	–	Positive
15	20	20	B (KX787058)	–	–	–	B (KX787048)
16	160	10	–	–	–	–	–
17	80	10	–	–	–	–	Positive
18	40	10	–	–	–	–	–
19	320	0	–	–	–	–	Positive
20	240	0	–	–	–	–	–
21	170	0	–	–	–	–	–
22	160	0	–	–	–	–	–
23	130	0	–	–	–	–	–
24	130	0	–	–	–	–	–
25	130	0	–	–	–	–	–
26	110	0	–	–	–	–	Positive

TPI, triosephosphate isomerase; GDH, glutamate dehydrogenase; BG, beta giardin; SSU, small subunit rRNA; -, PCR negative; Positive, amplification on PCR however no sequencing results; Assemblage (Accession number) provided where sequence of PCR products was obtained.

[a] Number of *Giardia* cysts used for DNA isolation.
[b] Number of DAPI positive *Giardia* cysts used for DNA isolation.
[c] Sulaiman et al. (2003).
[d] Caccio et al. (2008).
[e] Read et al. (2004) & Robertson et al. (2006).
[f] Lalle et al. (2005).
[g] Hopkins et al. (1997) & Read et al. (2002).

Amplification by PCR was more likely if more than twenty DAPI-positive cysts were used for DNA isolation, 80% (12/15), than if 10 or less DAPI positive cysts were used, 27% (3/11) ($p < 0.05$). There was no observed correlation observed between the total number of cysts and the likelihood of a sample being positive by PCR.

Sequencing of PCR products revealed Assemblage B in all samples. Sequences were submitted to GenBank and Accession numbers are provided (Table 1). Multiple alignment of consensus sequences at the TPI, GDH, BG and SSU rRNA genes showed *Giardia* excreted by macaques to be very similar to each other, 98–99%, with differences primarily due to ambiguous nucleotides. Importantly, there was heterozygosity of alleles within the sequences corresponding to the reverse internal primer at the BG gene and the reverse internal primer at the SSU rRNA genes. BLAST results of macaque sequences at the TPI, GDH and BG genes showed 99% identity to *Giardia* isolates from humans, common marmosets and a beaver. Two samples, 5 and 8 (Table 1), showed 100% identity at the BG gene to a *Giardia* isolate from a sheep and human.

3.3. Prevalence of Cryptosporidium spp. oocysts shed by wild rhesus macaques

Examination of rhesus macaque faecal samples using immunofluorescent microscopy revealed the presence of *Cryptosporidium* oocysts in 1 of 170 samples, with this animal from Troop 3. This sample contained 50 oocysts per gramme of faeces, all of which stained positively with DAPI, however was negative by PCR at the SSU rRNA gene.

3.4. Entamoeba coli and unknown Entamoeba spp. in wild rhesus macaques

Examination of rhesus macaque faecal samples using a genus-specific conventional PCR revealed the presence of *Entamoeba* spp. in 79% (132/168) of samples. There was no significant difference in the prevalence of *Entamoeba* spp. between Troops 1, 2, 3 and 4; 78% (43/55), 69% (31/45), 83% (19/23) and 87% (39/45) respectively ($p = 0.21$).

Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii*, did not result in amplification in any of the samples (0/168). Species-specific PCR for *E. coli* resulted in amplification in 49% (63/128) of samples positive at the genus-specific PCR. Thus, in the other 51% (65/128), no species of *Entamoeba* was identified. There was a significant difference in the prevalence of *E. coli* between Troops 1, 2, 3 and 4; 26% (11/42), 75% (21/28), 56% (10/18) and 45% (21/39), respectively ($p < 0.01$).

3.5. Giardia and Cryptosporidium in calves living in association with wild rhesus macaques (Troop 3)

Examination of faeces from domestic calves living together with Troop 3, revealed *Giardia* spp. cysts in 64% (9/14) of samples. Calves excreted 55 to 19 250 cysts per gramme faeces (mean, 4746; median, 302). Five positive samples were analysed further by PCR, and all five tested positive at one or more loci. Sequencing of PCR products revealed Assemblage A (KX787052, KX787054) in two calves, Assemblage A1 (KX787067) in one calf, Assemblage E (KX787051, KX787063, KX787065) in one calf, and a mixed infection of Assemblage A1 (KX787062, KX787053) and E (KX787064, KX787066) in one calf.

Cryptosporidium spp. oocysts were detected in 36% (5/14) of samples. Calves were excreting 100 to 5000 oocysts per gramme faeces (mean; 1480, median; 700). PCR at the SSU rRNA gene was negative for all 5 samples.

4. Discussion

This study describes a very high prevalence of *Entamoeba* spp., a moderate prevalence of *Giardia duodenalis* Assemblage B, and a very low prevalence of *Cryptosporidium* spp. in wild rhesus macaques in India, some of which have relatively close contact with humans and domestic animals.

The high prevalence of *Entamoeba* spp. in the macaques is consistent with results from studies in other closely related nonhuman primates (Feng et al., 2011; Feng and Xiao, 2011; Tachibana et al., 2009). *E. dispar* was not identified in this study, but has been detected in macaques from China and Nepal (Feng et al., 2013; Tachibana et al., 2013). Macaques were not infected with *E. histolytica* and *E. moshkovskii*, consistent with previous reports from other wild urban dwelling macaques (Feng et al., 2013; Tachibana et al., 2013). Since *E. histolytica*, *E. moshkovskii* and *E. dispar* are commonly reported in humans in India, this suggests that macaques are not a wildlife reservoir for these human pathogens, and that transmission from humans to macaques is not common among the macaque troops investigated (Nath et al., 2015a; Parija and Khairnar, 2005: Parija et al., 2014). Molecular identification of *Entamoeba* spp. in 520 samples from a range of captive nonhuman primate species revealed *E. hartmanni* (51.9%), *E. polecki*-like (42.7%), *Entamoeba histolytica* nonhuman primate variant (36%), *E. coli* (21.5%), *E. dispar* (2.4%) and *E. moshkovskii* (1.9%), as well as unidentified *Entamoeba* spp (18.9%). *E. polecki* and *E. hartmanni* were not tested for in our study as they are not considered pathogenic to humans, however they may be responsible for the unidentified *Entamoeba* spp observed. The reason for different prevalences amongst the macaque troops is not clear and could be due to a combination of various factors including diet, water sources, microbiome, genetics, and interactions with other humans or animals.

The moderately high prevalence of *Giardia* in rhesus macaques in this study is higher than reported for other macaque species, 2.4–9%, where IFA/PCR was used for diagnosis (Sricharern et al., 2016; Ye et al., 2012, 2014). As these studies also investigated macaque populations in close contact with humans, the difference in *Giardia* prevalence may be due to innate differences in the study populations, or, alternatively, due to different levels of food, water or environmental contamination where these population live. The study population in our study may have an increased exposure to *Giardia* due to its high prevalence amongst humans, domestic animals, and environmental water sources in India (Daniels et al., 2015; Laishram et al., 2012). *Giardia* infection has been associated with human contact in other primate species (Gillespie and Chapman, 2008; Graczyk et al., 2002; Salzer et al., 2007).

Macaques in China and Thailand have been reported to be infected with *G. duodenalis* Assemblage A and B, as seen in other nonhuman primates (Levecke et al., 2009; Sricharern et al., 2016; Ye et al., 2012), while in this study macaques around Chandigarh were only found to be infected with Assemblage B. Although this indicates a zoonotic potential for *Giardia* infections in macaques, the results should be interpreted with caution as most of the samples were only positive at one gene and it has been show that some isolates show certain taxonomic grouping at one gene and a different grouping at another gene (Lebbad et al., 2010; Robertson et al., 2006). Furthermore, it is difficult to interpret the zoonotic potential of these isolates, as multi-locus typing data can reveal animal isolates to be distinct from human isolates, despite them appearing similar based on a single locus (Ryan and Caccio, 2013; Sprong et al., 2009). Despite close contact with cattle shedding Assemblage A and E cysts, these genotypes were not found in samples from macaques.

There was only a single macaque faecal sample that was positive

for *Cryptosporidium*, suggesting that this protozoan in not an important parasite in rhesus macaques in this region of India. Since this positive sample contained few oocysts and was from the troop that had intimate contact with the calves shedding *Cryptosporidium* oocysts, it is possible that this sample represents carriage, and not a true infection. *Cryptosporidium* may be more common in very young macaques that are likely under-represented in this study due to the sampling technique relying on stool morphology.

In this study, using IFA as the gold standard, then PCR at different gene loci had the following sensitivities; SSU rRNA (58%), BG (15%), GDH (12%) and TPI (8%). Overall sensitivity of PCR, using all loci, was 65% (17/26) in macaques, and 100% (6/6) in the calves. PCR sensitivity may have been limited by the low number of DAPI positive cysts available for DNA isolation. Alternatively, the allelic sequence heterozygosity observed at the primer binding sites would suggest that the primers used in this study are not optimal for the *Giardia* isolates found in the study population. Similar limitations of PCR have been observed in genotyping canine *Giardia* isolates (Sommer et al., 2015). Not surprisingly, positive DAPI staining of cysts, indicating the presence of nuclear DNA, was associated with increased likelihood of a positive PCR result.

5. Conclusion

Entamoeba coli, unknown *Entamoeba* spp. and *G. duodenalis* Assemblage B were common in urban dwelling rhesus macaques around Chandigarh, India. *Cryptosporidium* spp., *E. histolytica* and *E. moshkovskii* do not appear to be important pathogens in this population. Further molecular investigation is needed to firmly establish the zoonotic potential of *Giardia* infections in macaques.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2016.12.002.

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