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Short communication

Entamoeba histolytica infection in wild lemurs associated with proximity to humans

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ABSTRACT

Amoebiasis, caused by *Entamoeba histolytica*, affects 50 million people worldwide, and results in 100,000 deaths annually. It is particularly prevalent in developing nations where poverty and poor sanitation contribute to contamination of food and water. *E. histolytica* is also a zoonotic protozoan parasite with the potential to infect non-human primates. Lemurs, primates endemic to Madagascar, are the most threatened mammalian group in the world due to habitat loss. As forests disappear, humans and lemurs come into more frequent contact, and the potential for *E. histolytica* to infect lemurs intensifies. Consequently, we screened 176 fecal samples from seven lemur species at eight sites in the rain forests of southeastern Madagascar for *E. histolytica* to determine if human proximity influenced lemur infection. Of samples examined, 4.0% (from three lemur species) were positive for *E. histolytica*. Of lemurs infected with *E. histolytica*, three (43%) exhibited diarrheal feces. Distance to human settlements explained the variation in *E. histolytica* infection seen in lemurs. These results provide the first evidence of *E. histolytica* in wild lemurs and highlight the need for additional work to better understand the eco-epidemiology of this potential threat to these species.

1. Introduction

Entamoeba histolytica is an enteric protozoan parasite that infects about 50 million people worldwide, causing widespread morbidity and mortality (WHO, 2002). Invasive amoebiasis, caused by *E. histolytica*, has been cited as the third most common cause of death due to parasitic diseases, following malaria and schistosomiasis (WHO, 2002). *E. histolytica* is transmitted by intake of a mature cyst through either fecal-contaminated food and water or direct oral-fecal contact and is particularly problematic in developing nations with poor sanitation and hygiene practices (Ravdin, 1989). The only known reservoirs of this zoonotic parasite are humans and non-human primates (NHPs). The majority of cases are asymptomatic (Ravdin, 1989), but infection with this gastrointestinal parasite in both humans and NHPs may cause hemorrhagic dysentery, liver abscesses and death (Haq et al., 1985; Levecke et al., 2010).

The lemurs, NHPs endemic to Madagascar, are all threatened by habitat loss (Schwitzer et al., 2014). Recent studies suggest that pathogens may be an important conservation consideration for lemur populations (Bodager et al., 2015; Bublitz et al., 2015; Zohdy et al., 2015). Experimental infection of *E. histolytica* in NHPs suggests that infection mimics human amoebiasis (Haq et al., 1985), and evidence from captive lemurs suggests that *E. histolytica* is pathogenic and may be fatal (Berrilli et al., 2011). Previous research has revealed *Entamoeba* spp. in wild lemurs including red-fronted lemurs, Verreaux's sifaka, and ring-tailed lemurs in western Madagascar (Clough, 2010; London and Sauther, 2013); however, these studies were unable to determine if a subset of *Entamoeba* spp. observed represented *E. histolytica*, as opposed to the non-pathogenic *Entamoeba* spp. (i.e., *E. dispar*, *E. moshkovskii*,*E. hartmanni*, *E. coli* and *E. polecki*-like organisms).

We surveyed seven wild lemur species for *E. histolytica* at eight sites in and around Ranomafana National Park (RNP), Madagascar. We hypothesized that lemurs living in closer proximity to human villages would have increased incidence of *E. histolytica*.

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Table 1
Distance to nearest village and percent positive for *Entamoeba histolytica* for lemur fecal samples collected from eight sites in and around Ranomafana National Park, Madagascar.

Site	Site code (disturbance level)	Lemur species sampled	Number of samples	Distance to nearest village* (m)	Positive for *E. histolytica*
Ambatolahy Dimy	M1 (Medium)	1 (Er)	5	900 (934.16)	0
Bevohazo	VL (Very Low)	1 (Er)	3	500 (539.52)	0
Campsite	H1 (High)	1 (Mr)	21	300 (267.34)	14.3% (3/21)
Vohiparara	M2 (Medium)	3(Vv, Er,Ef)	9	1200 (1181.99)	0
Valohoaka-Menarano	VL2 (Very Low)	2(Pe, Er)	13	1300(1304.37)	0
Talatakely	H2 (High)	5(Pe,Ps, Ef,Er,Ha,)	85	700 (707.55)	4.7% (4/85)
Vatoharanana	L (Low)	1 (Vv)	2	4000 (3950.91)	0
Valohoaka	VL3 (Very Low)	5(Pe,Vv, Ef,Er,Mr)	38	2700 (2741.50)	0
Total	8	7 (5–55)**	176		7

* Distance was measured from centroid of sampling site to nearest village (human settlements with > 10 households) and rounded to 100 m.

** Seven species were sampled, with 5–55 samples per species, with species abbreviations as follows, and numbers in parentheses equal to the number of samples per species: Er = Eulemur rubriventer (40), Mr = Microcebus rufus (55), Vv = Varecia variegate (5), Ef = Eulemur rufifrons (30), Pe = Propithecus edwardsi (20), Ha = Hapalemur aureus (18), Ps = Prolemur simus (8).

2. Materials and methods

2.1. Study site and sample collection

RNP (41,000 ha) is a rainforest-dominated protected area hosting 13 species of lemurs (21°02′–21°25′S; 47°18′–47°37′E). Despite its protected status, RNP is threatened by forest conversion for subsistence agriculture (Wright et al., 2012).

Fecal samples were opportunistically collected from 16 habituated and non-habituated lemur groups at eight forest sites that varied in distance from human habitation (Table 1.). Sampled lemurs included seven species (*M. rufus, Eulemur rubriventer, Eulemur rufifrons, Prolemur simus, Propithecus edwardsi, Hapalemur aureus, Varecia variegata*). We sampled the smallest and largest lemurs in the park (*M. rufus* and *P. edwardsi*), as well as the most endangered, *P. simus*. We tracked lemurs until all or most members of each group had defecated, collecting 0.6–0.9 ml of feces from each individual. Samples were collected from the fecal mass center to avoid environmental contamination.

To sample *M. rufus*, Sherman traps (XLR, Sherman Traps Inc., FL) were set in three sites (VL3, H1, and H2; see Table 1) at ≈50 m intervals. Traps were baited with banana around 16:00, and were checked between 20:00 and 20:30. In general, there are higher lemur population densities at sites with the least disturbance, except for *Microcebus*. Density of *Microcebus* was 23/km^2 in H2 (Wright et al., 2012), slightly higher at H1 (C. Karanewsky, personal communication) and half that at VL3 (P. Wright, unpublished data). In sites where lemur abundance was lower (VL3), traps were checked at 22:00 and 05:00 to increase capture success. Captured lemurs were sexed and weighed, and fresh fecal samples were collected as produced. For lemurs captured at night, individuals were released immediately after data collection, while those captured pre-dawn were kept in cotton bags in a cool dry tent until nightfall, then released at site of capture.

We homogenized 1 ml of feces from each sample with an equal volume of RNAlater nucleic acid stabilizing buffer (Ambion, Life Technologies, Grand Island, NY) and stored at −4 °C until transport to the United States.

2.2. Molecular methods

DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH), as outlined in Gomes et al. (1999). Extracts were screened for *Entamoeba* spp. and *E. histolytica* using the one-step nested protocol outlined in Foo et al., 2012, which uses specific forward primers and a single common reverse primer. The primers were designed to target the *Entamoeba* small subunit (SSU)-rRNA gene. For *Entamoeba* spp. detection, the following primers were used: Eg-SS-F1 (*Entamoeba* spp. forward) 5′-TGTGATTAAAACGCTCGTAGTTGAA-3′, and Eg-SS-CR1 (*Entamoeba* spp. reverse) 5′-CTCGTTCGTTACCGGAAT TAACC-3′. 2uL of DNA extract was combined with 18uL of Taq PCR MasterMix (Qiagen), (10uL of Taq MasterMix, 6uL of water, and 2uL of primers − 1uL of *Entamoeba* spp. forward primer and 1uL of *Entamoeba* spp. reverse primer). Thermal cycler settings were: 95 °C for 5 min; (95 °C for 30 s; 55.8 °C for 30 s; 72 °C for 30 s) × 35; 72 °C for 10 min: 4 °C ∞. PCR products were then run on 1% agarose mini-gels using 6X loading dye (Thermo Scientific). All samples were run for 30 min at 80 V. Results were viewed with Molecular Imager Gel Doc™ XR System (Bio-Rad). Bands positive for *Entamoeba* spp. appeared at 750 bp.

Samples positive for *Entamoeba spp*. were subsequently screened for *Entamoeba histolytica* using primers from Foo et al. (2012): Eh-SS-F1 (*Entamoeba histolytica* forward) 5′-GAAGCATTGTITCTAGATCTGA-3′, and Eg-SS-CR1 (*Entamoeba* spp. reverse) 5′-CTCGTTCGTTACCGGAAT TAACC-3′. Cycle sequencer settings were: 95oC for 5 min; (95 °C for 30 s; 52.1 °C for 30 s; 72 °C for 30 s) x 35; 72 °C for 10 min; 4 °C ∞. PCR products were run using gel electrophoresis was run for 30 min at 80 V. *E. histolytica* positive bands were ~300 bp in size.

2.3. Statistical analyses

We collected 176 lemur fecal samples from June to September in 2012 and 2013 from seven of the thirteen lemur species found in eight sites (Table 2) in and around RNP. Each year, fecal samples were collected from what were presumed to be different individuals with the exception of *P. simus*. There are only two *P. simus* remaining in Ranomafana, we sampled the male twice in 2012 and four times in 2013, and the female once in 2012 and twice in 2013. Habituated groups have individuals with unique collars. This made individuals identifiable and reduced the chance of resampling the same individuals. We were unable to distinguish age and sex of many unhabituated lemurs because several species are sexually monomorphic.

The centroids of lemur sites (mean of coordinates) and human villages (> 10 households identified by Google Earth Pro) were calculated and distances from each lemur sampling site to the nearest human

Table 2
Entamoeba histolytica in lemur species sampled in and around Ranomafana National Park, Madagascar. Numbers in parentheses indicate the number of positive samples/total number of samples analyzed.

Lemur species	Positive for *E. histolytica*
Propithecus edwardsi	0% (0/20)
Hapalemur aureus	5.6% (1/18)
Prolemur simus	0% (0/8)
Eulemur rufifrons	0% (0/30)
Eulemur rubriventer	7.7% (3/39)
Microcebus rufus	5.4% (3/56)
Varecia variegata	0% (0/5)
Total	4.0% (7/176)

*Eight samples collected from two individuals. Both individuals positive at one point.