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GIARDIA IN MOUNTAIN GORILLAS (*GORILLA BERINGEI BERINGEI*), FOREST BUFFALO (*SYNCERUS CAFFER*), AND DOMESTIC CATTLE IN VOLCANOES NATIONAL PARK, RWANDA

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ABSTRACT: Mountain gorillas (*Gorilla beringei beringei*) are critically endangered primates surviving in two isolated populations in protected areas within the Virunga Massif of Rwanda, Uganda, the Democratic Republic of Congo, and in Bwindi Impenetrable National Park in Uganda. Mountain gorillas face intense ecologic pressures due to their proximity to humans. Human communities outside the national parks, and numerous human activities within the national parks (including research, tourism, illegal hunting, and anti-poaching patrols), lead to a high degree of contact between mountain gorillas and wildlife, domestic animals, and humans. To assess the pathogen transmission potential between wildlife and livestock, feces of mountain gorillas, forest buffalo (*Syncerus caffer nanus*), and domestic cattle (*Bos taurus*) in Rwanda were examined for the parasites *Giardia* and *Cryptosporidium*. *Giardia* was found in 9% of mountain gorillas, 6% of cattle, and 2% of forest buffalo. Our study represents the first report of *Giardia* prevalence in forest buffalo. *Cryptosporidium*-like particles were also observed in all three species. Molecular characterization of *Giardia* isolates identified zoonotic genotype assemblage B in the gorilla samples and assemblage E in the cattle samples. Significant spatial clustering of *Giardia*-positive samples was observed in one sector of the park. Although we did not find evidence for transmission of protozoa from forest buffalo to mountain gorillas, the genotypes of *Giardia* samples isolated from gorillas have been reported in humans, suggesting that the importance of humans in this ecosystem should be more closely evaluated.

Key words: *Cryptosporidium*, disease transmission, ecosystem health, *Giardia*, mountain gorilla, One Health, Rwanda.

INTRODUCTION

Mountain gorillas (*Gorilla beringei beringei*) are a critically endangered, charismatic species living in a restricted range in Africa in proximity to humans. Of the world's remaining 880 mountain gorillas, approximately 480 live in the Virunga Massif (Gray et al. 2013), which spans the borders of Rwanda, Uganda, and the Democratic Republic of Congo and includes Volcanoes National Park in Rwanda. The park is bordered by dense human communities, which average 300 people/km^2 (Gray and Kalpers 2005) and practice subsistence crop and animal agriculture. Additionally, there is considerable human activity inside the park for tourism, research, illegal hunting and harvest, and anti-poaching patrols. Livestock are grazed on lands abutting the park boundary while wildlife, including forest buffalo (*Syncerus caffer nanus*), which live inside the park and share habitat with gorillas, routinely enter and exit the park. The buffalo use domestic livestock habitat for crop-raiding and come into direct contact with cattle (*Bos taurus*) and mountain gorillas, as has been documented over the past 20 yr (Plumptre and Harris 1995; Plumptre et al. 1997). This overlap in habitat usage by large herbivores may allow for parasite transmission across host groups over time.

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The protozoal parasites *Giardia duodenalis* (syn. *Giardia intestinalis*, *Giardia lamblia*) and *Cryptosporidium parvum* (Xiao and Fayer 2008) are significant fecal-oral pathogens for both humans and animals, infect a wide range of hosts, and cause disease in their hosts (Fayer et al. 2004; Gillespie and Chapman 2006). Both *Giardia* and *Cryptosporidium* have been identified in humans, domestic cattle, and mountain gorillas. Studies investigating *Giardia* and *Cryptosporidium* transmission have occurred in the geographically distant and separate Bwindi Impenetrable National Park mountain gorilla population in southwestern Uganda (Nizeyi et al. 1999; Graczyk et al. 2002). Only one previously published study has examined the presence of gastrointestinal parasites in the Virunga Massif mountain gorilla population (Sleeman et al. 2000). Furthermore, whether or not these parasites infect other wildlife in the Virunga Massif is not known, nor is it known whether other wildlife could serve as a potential reservoir for transmission of these parasites to or from mountain gorillas.

The environmentally resistant cyst or oocyst stage allows these parasites to survive in soil, feces, and water for up to 1 yr before infecting a new host (Olson et al. 1999; Slifko et al. 2000). In an ecosystem such as the Virunga Massif, all mammals, humans included, may be sharing parasites through contaminated soils and vegetation (Olson et al. 1999; Dumètre et al. 2012) as a result of overlapping habitat and shared foraging grounds. For *Giardia* in particular, the genotypic assemblages A and B have zoonotic potential (Cacciò and Ryan 2008), creating the possibility for cross-species transmission in this ecosystem.

Understanding the transmission cycle of parasites within this ecosystem is critical for identifying beneficial interventions to reduce pathogen spread between species and to improve human and animal health in this region. We assessed the importance of forest buffalo in the *Giardia* and *Cryptosporidium* transmission cycles between cattle and mountain gorillas. Using fecal samples from mountain gorillas, domestic cattle, and forest buffalo collected from areas known to have range overlap, we determined the prevalence, spatial distribution, and genotypic diversity of *Giardia* and *Cryptosporidium* in order to elucidate whether forest buffalo may transmit protozoa between mountain gorillas and domestic cattle.

MATERIALS AND METHODS

Study site and sample collection

This study was conducted in September 2010 in the Volcanoes National Park, which comprises Rwanda's protected portion of the Virunga Massif and covers approximately 130 km^2. Samples were collected in the Shingiro, Nyange, and Kinigi sectors that border the park in which buffalo and other wildlife were anecdotally known to exit the park frequently to forage in farmlands. Fecal samples were collected from each study species (mountain gorillas, forest buffalo, and cattle) in each of the three sectors with a goal of 50 samples from each species per sector, for a total of 150 samples from mountain gorilla, cattle, and forest buffalo based on prior prevalence data. In total, 130 samples were collected from mountain gorilla, 135 samples were collected from cattle, and 55 samples were collected from forest buffalo. Collection was conducted on a single day per gorilla family group, cattle sector, or buffalo sector to avoid oversampling any given individual. All fecal samples were collected and stored on ice in the field, or in a refrigerator in the laboratory, until initial processing within 24 hr of collection. At each sampling site a GPS location was recorded for spatial analysis.

Mountain gorillas live in family groups whose members and home range have been well characterized. Fifteen family groups that had a portion of their home range adjacent to one of the three sectors of this study were sampled. Park rangers collected all feces deposited in the night nests of each individual mountain gorilla in the targeted family group. Multistage cluster sampling by sector and family group was conducted to obtain up to 50 samples per sector while collecting a proportionate number of samples per family group. We collected 30 mountain gorilla samples in Nyange and 50 each in Kinigi and Shingiro. We counted the number of cattle per

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farm or household. If a sector had >50 cattle in the area, simple random sampling was used to determine which cows in that sector were to be sampled. The number of cattle samples collected was 48 in Nyange, 50 in Kinigi, and 37 in Shingiro. For cattle samples, all farms within the three sectors were visited and cattle were either observed defecating and a sample was collected or feces were collected from night quarters where cattle were individually housed. Body condition score (Department for Environment Food and Rural Affairs 2001), sex, breed, age class, and pregnancy status were determined for all cattle sampled, as these have been considered risk factors for protozoal infection and increased shedding in cattle (Fayer 2004; Thompson 2004). The population size of forest buffalo was unknown at the time of this study; to collect samples, trackers followed fresh trails into the forest in each of the three sectors and collected any fresh buffalo feces observed. The number of buffalo samples collected was 11 in Nyange, 28 in Kinigi, and 16 in Shingiro.

Parasite identification

Fresh fecal samples were processed using standard diagnostic methods similar to previously published studies (e.g., Gaydos et al. 2008), with slight modification. Briefly, 3 g of feces were mixed with 12 mL of 0.1% Tween® 80, sieved through disposable gauze, centrifuged for 10 min at 1,000 × G at 4 C, and the supernatant discarded. The fecal slurry, consisting of the top half of the remaining pellet with fine particulate matter, was analyzed. For direct fluorescent antibody (DFA) tests to observe parasites and determine prevalence, 50 μL of 10% formalin were mixed with 5 μL of fecal slurry and air-dried onto hydrophobic DFA slides for microscopy. Slides were stained with EasyStain™ DFA tests (BTF Bio, Pittsburgh, Pennsylvania, USA) and examined under epifluorescent illumination using fluorescein isothiocyanate (FITC) and 4′ 6-diamidino-2-phenylindole (DAPI) emission filter sets. Particles with apple-green fluorescence in a round to oval shape (6–15 μm diameter) with bright, highlighted edges under FITC, and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI, were classified as *Giardia* cysts. Particles with apple-green fluorescence in an oval or spherical shape (3–7 μm diameter) with bright, highlighted edges under FITC, and light blue internal staining with a green rim or up to four distinct, sky-blue nuclei under DAPI, were classified as *Cryptosporidium* oocysts (US Environmental Protection Agency 2005). For a subset of samples selected for preservation, 1 mL of fecal slurry was heated to 72 C for 30 min and resuspended in 1 mL of RNAlater® (Qiagen, Valencia, California, USA) and stored at 4 C for future DNA isolation.

Molecular characterization

Samples positive for *Giardia* by fluorescent microscopy for which we had preserved fecal slurry were processed further to isolate *Giardia* cysts by immunomagnetic separation using Dynabeads® G-C Combo (Invitrogen Life Technologies, Grand Island, New York, USA). For *Giardia*-positive samples for which no preserved fecal slurry was available, fecal material was scraped from the DFA slide for DNA isolation (Gaydos et al. 2008). Parasite DNA was isolated using DNeasy® Blood & Tissue kits (Qiagen), with three consecutive freeze-thaw cycles in liquid nitrogen and boiling water to break apart the cyst walls more effectively.

Molecular characterization of *Giardia* cysts was accomplished through nested PCR amplification and sequencing of a 432-base pair (bp) region of glutamine dehydrogenase (GDH), as described previously (Read et al. 2004), using external forward primer GDHeF (5′-TCAACGTYAAYCGYGGYTTCCGT-3′), internal forward primer GDHiF (5′-CAGTACAACTCYGCTCTCGG-3′), and reverse primer GDHiR (5′-GTTRTCCTTGCACATCTCC-3′). Additionally, a nested PCR amplification and sequencing of a 384-bp region of β-giardin was completed as described previously (Cacciò et al. 2002) using external forward primer G7 (5′-AAGCCCGACGACCTCACCCGCAGTGC-3′), internal forward primer G376 (5′-CATAACGACGCCATCGCGGCTCTCAGGAA-3′), and reverse primer G759 (5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′). For both GDH and β-giardin, PCR amplification conditions were as described except that HotStarTaq® DNA polymerase (Qiagen) was used, and the reactions included 0.4 μg/ml bovine serum albumin. PCR products were treated with ExoSAP-IT® (Affymetrix, Santa Clara, California, USA) according to the manufacturer's instructions before sequencing. Type and subtype were determined by comparison of determined sequences to previously described reference sequences. All sequence data were deposited in GenBank (accession numbers JX839873–JX839886).

Statistical analysis

Prevalence for each species was calculated as the number of positive fecal samples/total collected number of fecal samples. Additionally,

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prevalence was calculated per sector for each species and per age class (preweaned versus weaned), body condition score, and sex of cattle to characterize demographics and possible risk factors for *Giardia* infection. In order to evaluate the spatial distribution and clustering of *Giardia* cases, spatial analysis using a multinomial Bernoulli modeling approach (Kulldorff and Inc 2006; Jung et al. 2010) was conducted using SaTScan™ v.9.1.1 (Kulldorff and Inc 2006) to determine the most likely circular cluster of cases within the distribution of a single species and also within the distribution of all samples collected from all three species: mountain gorillas, cattle, and buffalo. A value of $P<0.05$ was considered statistically significant.

RESULTS

Prevalence of *Giardia* and *Cryptosporidium*

Using epifluorescent microscopy, *Giardia* was detected in mountain gorillas, domestic cattle, and forest buffalo (Table 1). Overall, 9% (11/130) of mountain gorilla feces were positive for *Giardia*; the parasite was detected in fecal samples from 33% of the gorilla groups (5/15 groups surveyed). Of these five infected groups, three were habituated for research activities and two were habituated for tourism. All positive groups were identified as typically residing within the Kinigi sector of the park. Similarly, 6% (8/135) of cattle fecal samples were positive for *Giardia*; all infected cows were age 1 yr or older. Of the cattle for which demographic data was available, 7/114 females and 1/19 males were positive for *Giardia*. No pregnant females ($n=14$) were positive for *Giardia*. Three of the *Giardia*-positive cattle resided in Kinigi, four in Nyange, and one in Shingiro sectors. Of forest buffalo samples, 2% (1/55) were positive for *Giardia*; the one positive forest buffalo sample was collected in Kinigi sector. *Cryptosporidium*-like particles were observed in 1% (1/130) of mountain gorilla, 3% (4/135) of cattle, and 36% (20/55) of buffalo samples. Due to low numbers of these *Cryptosporidium*-like particles (<50 oocyst-like particles per sample), there was insufficient genomic material for molecular characterization and genotyping purposes for *Cryptosporidium* confirmation.

All of the *Giardia*-positive gorilla samples were collected in the Kinigi sector of the park (Fig. 1); SaTScan analysis was conducted to evaluate whether this trend indicated a statistically significant cluster. *Giardia*-positive mountain gorilla fecal samples produced a space-cluster in the Kinigi sector with a radius of 5.24 km (relative risk=31.94, $P<0.0001$). No significant clustering was observed among *Giardia*-positive cattle or buffalo samples. When evaluating all species using a Bernoulli multinomial approach, the *Giardia*-positive cluster was still significant and located in the Kinigi sector as the gorilla-only cluster, which also encompassed the infected buffalo sample (relative risk=5.25, $P<0.0001$).

Molecular characterization

Because the genetic diversity of *Giardia* among host species is an important factor for consideration of shared parasitism, we identified the *Giardia* genotype of each positive sample using molecular tools (Table 2). Due to insufficient DNA recoverable from low numbers of cysts, particularly from slide scrapings, PCR products were obtained for only six of 11 gorilla samples, one of eight cattle samples, and none of one buffalo sample. Nevertheless, sequence analysis of the GDH locus of the *Giardia* detected in the mountain gorilla samples revealed that five samples were of the B-IV subtype, covering three family groups, and one sample from a different family group was of the B-III subtype. Sequence analysis of the β-giardin locus confirmed the B-IV subtype in the five samples and assemblage B in the one sample. Sequence analysis of the cattle sample with both GDH and β-giardin loci revealed that it was *Giardia* assemblage E, which is known to be host specific to cattle.

DISCUSSION

Mountain gorillas surviving in the Virunga Massif face a variety of ecologic

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TABLE 1. *Giardia* prevalence in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle (*Bos taurus*) in three sectors of Volcanoes National Park, Rwanda, as determined by direct fluorescent antibody testing.

	Mountain gorilla			Domestic cattle			Forest buffalo		
Sampled group	*n*	No. positive	Prevalence (%)	*n*	No. positive	Prevalence (%)	*n*	No. positive	Prevalence (%)
Overall	130	11	9	135	8	6	55	1	2
Sector									
Kinigi	50	11	9	50	3	6	28	1	4
Shingiro	50	0	0	37	1	3	16	0	0
Nyange	30	0	0	48	4	8	11	0	0
Age									
Preweaned (<1 year)	—[a]	—	—	24	0	0	—	—	—
Postweaned (>1 year)	—	—	—	111	8	7	—	—	—
Body condition score									
1	—	—	—	8	1	13	—	—	—
2	—	—	—	46	2	4	—	—	—
3	—	—	—	63	4	6	—	—	—
4	—	—	—	15	1	7	—	—	—
Sex									
Male	—	—	—	19	1	5	—	—	—
Female (pregnant)	—	—	—	14	0	0	—	—	—
Female (not pregnant)	—	—	—	100	7	7	—	—	—

[a] (—) = Data not collected.

pressures including sharing their limited habitat with humans (Woodford et al. 2002; Cranfield 2008). While gastrointestinal disease is not a primary cause of gorilla mortality (Cranfield 2008), and in many cases may be asymptomatic, it is important to consider the long-term effects that gastrointestinal parasites may have for general health, survival, and reproduction (Gillespie et al. 2008). Furthermore, continued interaction and contact with humans and livestock may result in altered transmission rates and virulence of gastrointestinal parasites for mountain gorillas, as has been observed with other primates (Gillespie et al. 2004; Wheeler 2010) and may well provide a route of transmission for other pathogens.

We found that *Giardia* prevalence in mountain gorilla has increased to 9% (11/130) compared to the 3% (2/70) prevalence detected in 1997 (Sleeman et al. 2000), although previous investigations by the Mountain Gorilla Veterinary Project in 2003 estimated the prevalence to be 10% (L. Gaffikin et al., unpubl. data). We determined that *Giardia* prevalence in domestic cattle in the region has decreased to 6% (8/135) compared to a 16% prevalence measured in our earlier assessment (unpubl. data). Although preweaned cattle are more susceptible to *Giardia* infections and shed higher number of cysts (Thompson 2000), none of the *Giardia*-positive domestic cows evaluated in this study were younger than 1 yr. *Cryptosporidium* has been found in both cattle and mountain gorillas previously (Nizeyi et al. 1999; Nizeyi et al. 2002), but in this study we confirmed no *Cryptosporidium* infections. Additionally, we detected a low level of *Giardia* in forest buffalo, with a prevalence of only 2% (1/55). This represents a first report for *Giardia* in this host.

The cross-sectional design of our study, coupled with a limited volume of examined feces, may have led to an underesti-

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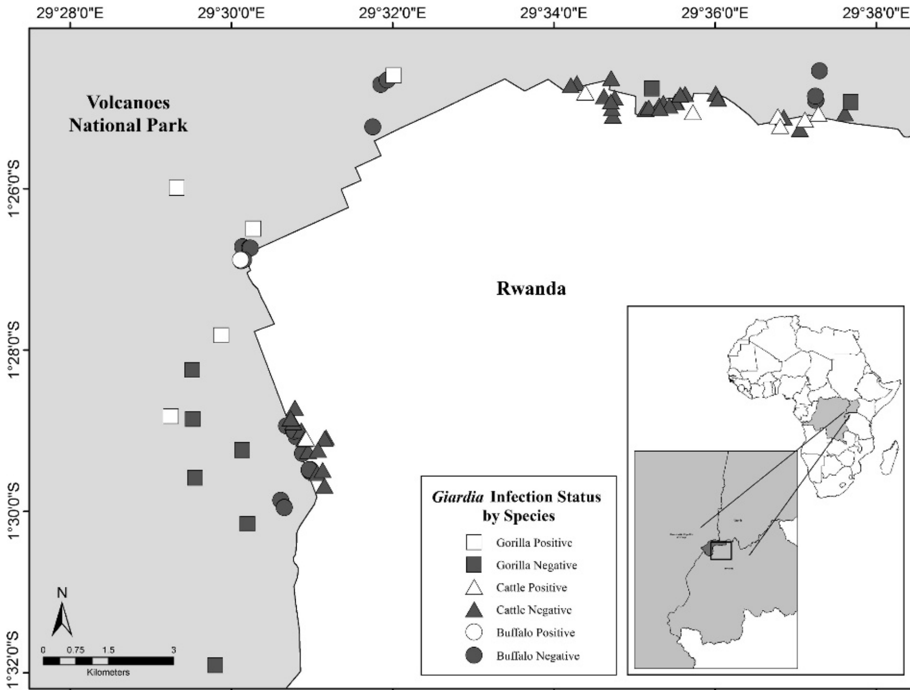

FIGURE 1. Map of project sampling sites in Volcanoes National Park, Rwanda. Squares indicate samples from mountain gorilla (*Gorilla beringei beringei*), triangles indicate samples from cattle (*Bos taurus*), and circles indicate samples from forest buffalo (*Syncerus caffer*). White icons indicate a sample found positive for *Giardia duodenalis* by fluorescent microscopy and grey icons indicate a sample negative for *G. duodenalis*.

mate of the prevalence of *Giardia* and *Cryptosporidium* in our study species, especially considering that infected animals can be low or intermittent shedders. Additionally, it is possible that we oversampled buffalo, as individuals were not visualized prior to collection, and this could have led us to underestimate parasite prevalence in this species. While the range and ecology of forest buffalo may still permit the transport of protozoa between cattle and mountain gorillas, this transmission cycle was not proven in this study.

TABLE 2. *Giardia* genotypes in mountain gorilla (*Gorilla beringei beringei*) and domestic cattle (*Bos taurus*) in Volcanoes National Park, Rwanda using glutamate dehydrogenase (GDH) and β-giardin loci to determine assemblage and subtype genetic information.

Species	*n*	Assemblage result by GDH	Assemblage result by β-giardin
Mountain gorilla	5	B-IV	B-IV
Mountain gorilla	1	B-III	B
Domestic cattle	1	E	E

The low *Giardia* prevalence in cattle we observed in this study may indicate that livestock management changes implemented in recent years are reducing the parasite prevalence in the cattle. Zero-grazing is a livestock management strategy that mandates restrictions on cattle range, with cut grass replacing pastured grazing. In 2006 the Rwandan government implemented a "one cow per family" program with the goals of reducing chronic childhood malnutrition, increasing household food security, and generating alternative income; this required implementation of the zero-grazing livestock management system (Kim et al. 2012). In addition to the reduced pressures on land use in low carrying-capacity regions, this control strategy has also been shown to reduce a

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number of cattle-specific and zoonotic diseases including East Coast fever (*Theileria parva*) and brucellosis (McDermott and Arimi 2002; Bazarusanga et al. 2007). A zero-grazing management system is most effectively implemented with small landholders who own few cattle per household (Lukuyu et al. 2009), and this characterizes the farms surrounding the Volcanoes National Park. While we did not specifically set out to examine the benefits of zero-grazing on the health of cattle in this area, the decrease in *Giardia* prevalence since zero-grazing was implemented may suggest that decreased parasitism is an added benefit of this strategy.

We detected two separate genotypic assemblages of *Giardia* infecting our study species: the multihost species genotype assemblage B in mountain gorillas and the cattle-specific assemblage E in cattle. Within assemblage B, two subtypes were found: B-III and B-IV. Our findings differ from those of a previous study reporting assemblage A in mountain gorillas, humans, and cattle in Bwindi Impenetrable National Park, which suggested cross-transmission between the species may have been occurring (Graczyk et al. 2002). The SSU-rDNA locus used in that study has low phylogenetic resolution and is prone to misclassification bias (Traub et al. 2005; Johnston et al. 2010); in contrast, we employed two loci sensitive enough to distinguish between assemblages. Our discovery of assemblage B is interesting because it has been observed in a wide range of mammalian species including humans (Appelbee et al. 2005; Xiao and Fayer 2008). While there is limited knowledge of *Giardia* diversity in mountain gorillas, it has been established that assemblage B infects nonhuman primates (NHP); a survey of isolates from 31 captive NHP species, including western and eastern gorillas, documented assemblage B in 48% of the samples (Levecke et al. 2009). Furthermore, assemblage subtype B-IV has been implicated in human-to-wild NHP (red colobus monkey, *Procolobus badius tephrosceles*, and guenon monkey, *Cercopithecus ascanius*) transmission in Uganda, particularly when coupled with a high degree of ecologic overlap (Johnston et al. 2010).

While this study may not have directly identified the transmission cycle of *Giardia* into the mountain gorilla population, the genotypic subtype results suggest that humans may play a role. Of all *Giardia* sequences, 83% were characterized as assemblage subtype B-IV and 17% were characterized as assemblage subtype B-III; a survey of 1,658 human *Giardia* isolates from the ZOOP.NET-database (www.rivm.nl/pubmpf/giardia/database/isolates/list) comparing the geographic distribution of B-III and B-IV in humans found that in Africa, B-III was detected in 81% of the samples and B-IV was found in 19% of the samples. In North American samples the opposite trend was found, with 14% of samples characterized as B-III and 86% of samples characterized as B-IV. The distribution in other geographic regions fell somewhere in-between (Sprong et al. 2009).

Others have postulated that the local community and park workers may be introducing gastrointestinal parasites into gorilla populations, in part due to the suboptimal sanitation in communities and the daily forays of park workers into gorilla habitat (Graczyk et al. 2002). Given the relative prevalence of the subtypes of *Giardia* we observed, it might also be appropriate to examine tourists and researchers for their potential role in the transmission cycle of *Giardia* in this area.

While many studies emphasize that human interaction with mountain gorillas is a significant risk factor for zoonotic disease transmission (Graczyk et al. 2002; Chapman et al. 2005; Goldberg et al. 2007), it is important to consider that the conservation status of the mountain gorilla is highly dependent upon contact with humans. This contact includes implementation of behavioral research programs involving daily human observation, anti-poaching

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patrols to protect the integrity of the park and its wildlife community, and gorilla ecotourism, which generates significant revenue for both the park and surrounding communities while educating global citizens about these endangered animals. Disease-prevention measures have been implemented to reduce disease transmission from humans to gorillas directly or via environmental transmission (Cranfield 2008), but these have been mainly enforced for those in daily contact with the animals, such as researchers or park rangers. Tourism brings individuals from across the world to an ecosystem with an endangered species sensitive to human pathogens. Only a few studies have been conducted on the role that tourism plays in zoonotic disease transmission in primate-based ecotourism endeavors (Muehlenbein and Ancrenaz 2009; Muehlenbein et al. 2010).

Giardia was found in all species we examined—mountain gorilla, cattle, and forest buffalo—in and around the Volcanoes National Park in Rwanda. Compared to studies conducted 7–13 yr earlier, *Giardia* prevalence has not changed for mountain gorillas but its prevalence in cattle was lower in this study, perhaps due to livestock management changes implemented in the interim. Additionally, our genetic subtype analysis suggests that tourism or research may play a larger role in transmission of these gastrointestinal parasites and, thus, should be included in future studies involving gastrointestinal parasites. These future studies should more deeply examine the role of human-primate contact on disease transmission to endangered populations.

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