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Original Contribution

Giardia Infection and Trypanosoma Cruzi Exposure in Dogs in the Bosawás Biosphere Reserve, Nicaragua

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Abstract: Indigenous Mayangna and Miskitu inhabit Nicaragua's remote Bosawás Biosphere Reserve, located in the North Caribbean Coast Autonomous Region. They are sedentary horticulturists who supplement their diet with wild game, hunting with the assistance of dogs. To test whether hunting dogs increased the risk of human exposure to protozoal zoonotic neglected tropical diseases (NTDs), we sampled dogs from three communities varying in population size and level of contact with other communities. We screened dog feces (n = 58) for *Giardia* and *Cryptosporidium* DNA and sera (n = 78) for *Trypanosoma cruzi* antibodies and DNA. *Giardia* DNA was detected in 22% (13/58) of samples; sequencing revealed the presence of both zoonotic genotypes (assemblages A and B) and dog-specific genotypes (assemblages C and D). *Giardia* shedding was associated with community and age. Older dogs and those in the two, more accessible communities had greater odds of shedding parasites. Seroprevalence of *T. cruzi* antibodies, indicating prior exposure, was 9% (7/78). These results contribute to the limited literature on NTDs in indigenous populations, and suggest hunting dogs can both serve as sentinels of environmental NTDs and pose zoonotic risk for their owners and communities.

Keywords: Bosawás, Domestic dogs, *Giardia*, Nicaragua, *Trypanosoma cruzi*, Indigenous health

Introduction

Indigenous peoples frequently encounter barriers to medical care, resulting in poorer health (Gracey and King 2009; Schurer et al. 2015; Stephens et al. 2006). Poor, rural, and remote communities also depend more directly on the land for sustenance, and typically interact more closely with animals in their immediate environment (Aguirre 2009;

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Schurer et al. 2015). Therefore, exposures and infections in companion hunting dogs within indigenous communities may provide insight into strategies to improve human health in these regions with limited medical facilities or surveillance (Fan et al. 1998; Himsworth et al. 2010a, b; Schurer et al. 2015).

In Latin America, neglected tropical diseases (NTDs) disproportionately impact indigenous peoples (Montenegro and Stephens 2006). The UNESCO Bosawás Biosphere Reserve is a large protected area within the autonomous northern territory of Nicaragua. The Bosawás Reserve was established in 1991 to safeguard Nicaragua's remaining tropical rain forest (Smith 2003), and is inhabited largely by indigenous Mayangna and Miskitu people. It is remote, with access largely via waterways, and lacks modern medical facilities. In fact, the autonomous territories in Nicaragua were excluded from a large national health survey (Sequeira et al. 2010).

Infections with *Giardia duodenalis* (synonymous with *Giardia lamblia*), one of the most common intestinal parasites worldwide, can impose a significant chronic disease burden on communities, and, in the absence of adequate medical care, impair the growth and cognitive function of children (Feng and Xiao 2011). Infections may be asymptomatic or cause diarrhea, abdominal cramps, and malabsorption in humans (Thompson et al. 1993). Infective cysts, shed in feces, can accumulate in the environment and remain viable for months. No national or regional disease burden estimate is available for *Giardia* in Latin America (Hotez et al. 2008), and very limited regional surveillance data exist.

Giardia spp. have variable host specificity; thus, molecular characterization is essential for understanding transmission cycles and epidemiology. Of the seven genetic assemblages of *Giardia*, designated A-G, humans are infected primarily with assemblages A and B, and dogs with C and D (Ballweber et al. 2010). However, subtypes of A are reported in companion animals, and high frequencies of assemblage A have been reported in dog feces from indigenous rural communities (Bryan et al. 2011; Himsworth et al. 2010a, b; Schurer et al. 2012). Assemblage B has broad host specificity, and was predominant in one urban study in Nicaragua (Lebbad et al. 2008).

Cryptosporidium spp. infections pose a severe threat to immunocompromised individuals, children, and the elderly (Rossle and Latif 2013). Similar to *Giardia* cyst, the *Cryptosporidium* oocyst can survive for months in the environment. However, *Cryptosporidium* is more host specific than *Giardia*, and *Cryptosporidium parvum,* the livestock-associated species, is most commonly identified in zoonotic infections. There is, however, increasing evidence that *C. canis* plays a role in infections in developing countries (Snelling et al. 2007). A recent study in the Río San Juan Department in Nicaragua found that the prevalence of *Cryptosporidium* infection in schoolchildren aged 4–15 was 36% (Munoz-Antoli et al. 2011). Infected but asymptomatic domestic dogs may have a high prevalence of *Cryptosporidium* shedding (Titilincu et al. 2010), and provide a means for regional surveillance.

Chagas disease, caused by *Trypanosoma cruzi,* contributes to a high disease burden in Latin America (Hotez et al. 2008; Montenegro and Stephens 2006). The majority of the 8 to 9 million identified cases in Latin America occur in poor rural and peri-urban areas. Triatominae insects are the primary route of transmission to humans, although other transmission routes for the protozoan exist, and both sylvatic and domestic transmission cycles are recognized (Coura et al. 2014). In the domestic setting, Triatominae vectors typically dwell in crevices or earthen floors of houses and thus present a challenge for eradication (Coura et al. 1994; Zeledon et al. 2006). In Nicaragua, the presence of vectors in rural communities correlates with seroprevalence of *T. cruzi* (Palma-Guzman et al. 1996; Rivera et al. 1995), although no information is available for Bosawás. Dogs are important reservoirs of infection, and can potentially act as sentinels for human infections (Palma-Guzman et al. 1996; Rivera et al. 1995; Tenney et al. 2014). Given that hunting dogs move between domestic and forest environments, both sylvatic and domestic cycles of *T. cruzi* must be considered in studies of the parasite's epidemiology (Coura et al. 1994, 2014).

As sedentary horticulturalists, the Mayangna and Miskitu supplement their diet through hunting, fishing, and livestock (Koster and Tankersley 2012). In contrast to other neotropical communities, Mayangna and Miskitu rely heavily on dogs for hunting; dogs are utilized in 83% of day trips (Koster 2008a). The dogs encounter diverse wildlife species and, potentially, their pathogens (Koster 2008b). The dogs travel and reside with their owners, sharing sources of food and water, raising questions around disease transmission between dogs and owners and the possible use of dogs as surrogates for pathogen exposure. Given the remoteness of Bosawás, the isolation and marginalized status of the Mayangna and Miskitu people, and the warm humid climate, we hypothesized that dogs in Bosawás would be exposed to *Giardia*, *Cryptosporidium,*

and T. cruzi. The specific aim of this study was to determine pathogen exposure and prevalence in dog populations of three indigenous communities.

Materials and Methods

Study Site

The study was conducted in settlements within Bosawás Reserve. The habitat is humid lowland tropical forest, population density is relatively low, and communities are a mix of Mayangna, Miskitu, and Mestizos. Miskitu-predominant settlements lie along the Río Coco, while Mayangna-predominant settlements are scattered among the smaller rivers. The two Mayangna (Arang Dak and Amak) and one Miskitu (Raiti) communities (Fig. 1) varied in infrastructure, population, and amount of through traffic. Arang Dak (14°30′57″, 84°59′58″; Zone 16P, 715,589.57, 1,605,719.77) is a very small, remote, isolated Mayangna settlement of about 280 people in 35 households situated on the Río Lakus. Raiti (14°35′16″, 85°01′28″; Zone 16P, 712,465.66, 1,614,413.77), on the Río Coco, is a large Miskitu town of over 2000 people in a few hundred households, with paved paths, restaurants, and an infirmary. Amak (14°14′18″, 85°09′08″; Zone 16P, 700,596.03, 1,555,751.68) is a busy, mid-sized Mayangna community of about 1200 people in 150 households located at the confluence of Río Amak and Río Bocay, tributaries of Río Coco.

Study Sample

In June 2013, blood and fecal samples were collected from dogs at centralized locations in Amak, Arang Dak, and Raiti. The sole selection criterion for inclusion into the study was an age of at least six months to ensure that dogs had reached an age at which maternal antibodies were unlikely to be present. A total of 28, 23, and 27 dogs were included from Amak, Arang Dak, and Raiti, respectively. Physical examinations were performed on each dog with age, sex, and, body condition score assessed. An age category [juvenile ($<$ 1 year), young adult (1–3 year), or mature adult ($>$ 3 year)] was determined based on owner information, as well as dentition, tartar deposition, and tooth wear. In total, 36 adults, 28 young adults, and 14 juveniles were examined. Blood was obtained from all 78 participating dogs; feces were obtained from 18 dogs in Arang Dak, 24 in Raiti, and 16 in Amak. Whole blood, serum after centrifugation, and feces were aliquoted into cryotubes with RNA*Later* (Qiagen Inc., Valencia, CA). Samples were stored at room temperature for a maximum of 2 weeks prior to transport and storage at − 80°C. This study was approved as Protocol #17506 by the University of California, Davis Institutional Animal Care and Use Committee.

Isolation, Amplification, and Identification of DNA from Fecal Samples

Cryovials with fecal samples and RNA*Later* were centrifuged for 10 min at 1000×*g* to pellet fecal material, with 100 µL of feces transferred into 90 µL of lysis buffer ATL (Qiagen Inc., Valencia, CA) after centrifugation. Samples were subjected to three rounds of freeze-boil (4 min in liquid N_2, followed immediately by 4 min in boiling water) to rupture any *Giardia or Cryptosporidium* parasites and then incubated overnight at 56°C with an additional 90 µL of lysis buffer ATL and 40 µL of proteinase K. Remaining steps of oocyst/cyst DNA extraction were carried out as per manufacturer instructions (DNeasy Blood and Tissue Kit; Qiagen Inc., Valencia, CA). The samples were then stored at − 20°C until further analysis.

To detect and distinguish host-specific and zoonotic genotypes, *Giardia* isolates were characterized using a semi-nested PCR and DNA sequence analysis of the glutamate dehydrogenase (GDH) gene (Read et al. 2004). *Cryptosporidium* isolates were molecularly characterized with primers designed to target ribosomal DNA from the 18S rRNA gene (Morgan et al. 1997). Amplified PCR products were extracted from gels using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) following manufacturer instructions. Nucleotide sequences were analyzed using Chromas Lite 2.01 (Technelysium Pty Ltd.), nBLAST searches of the GenBank database, and aligned against reference sequences using the ClustalX sequencing alignment program (Thompson et al. 1997). Phylogenetic analysis was used to infer relationships between isolates and reference sequences using MEGA 6.0 (Tamura et al. 2013) as described previously (Adell et al. 2014). The Kimura 2-parameter distance model with 1000 bootstrapped values was used to evaluate genetic divergence, and phylogenetic trees were built using the neighbor-joining method.

T. cruzi DNA and Antibody Detection

Multiple tests were performed for *T. cruzi* detection. The Trypanosoma Detect™ Rapid Test (InBiOS International,

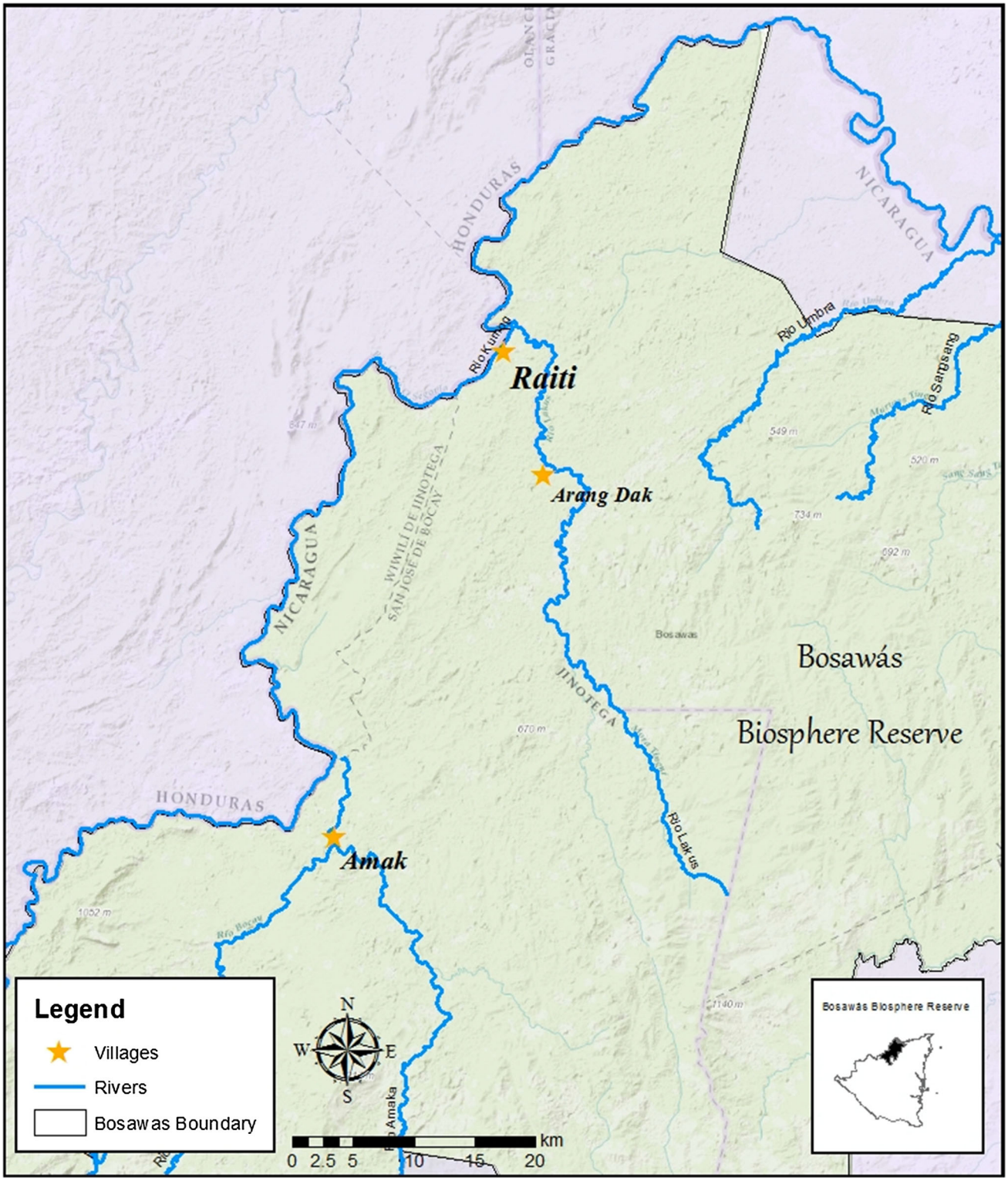

Figure 1. Map of communities within Bosawás Reserve, Nicaragua. The largest village, Raiti, (> 2000 people) lies on the Río Coco, the mid-size Amak (~ 1200 people) is located at the confluence of the Río Amak and the Río Bocay (Río Coco tributaries), and thus experiences a large degree of boat traffic, while Arang Dak is a very remote small village of 280 people on the Río Lakus.

Table 1. Multivariable Logistic Regression for Predictor Variables of *Giardia* Detection in Dog Fecal Samples.

Variable (strata for odds ratio)	*N*	*Giardia* prevalence (%)	Odds ratio (95% CI)	*P* value
Age (years) category of dog				
Juvenile (≤ 1)	13	8	0.17 (0.01–1.37)	0.144
Young adult (1–3)	17	12	0.14 (0.01–0.79)	0.045*
Mature adult (> 3)	28	36	REF	REF
Community				
Amak	16	50	6.72 (1.35–42.95)	0.027*
Arang Dak	18	6	0.40 (0.02–3.62)	0.459
Raiti	24	17	REF	REF

REF = reference group used in statistical analyses, determined from the category with the largest number of samples
*Significant at $P < 0.05$ level. Ages were determined by physical examination and owner reports during interviews

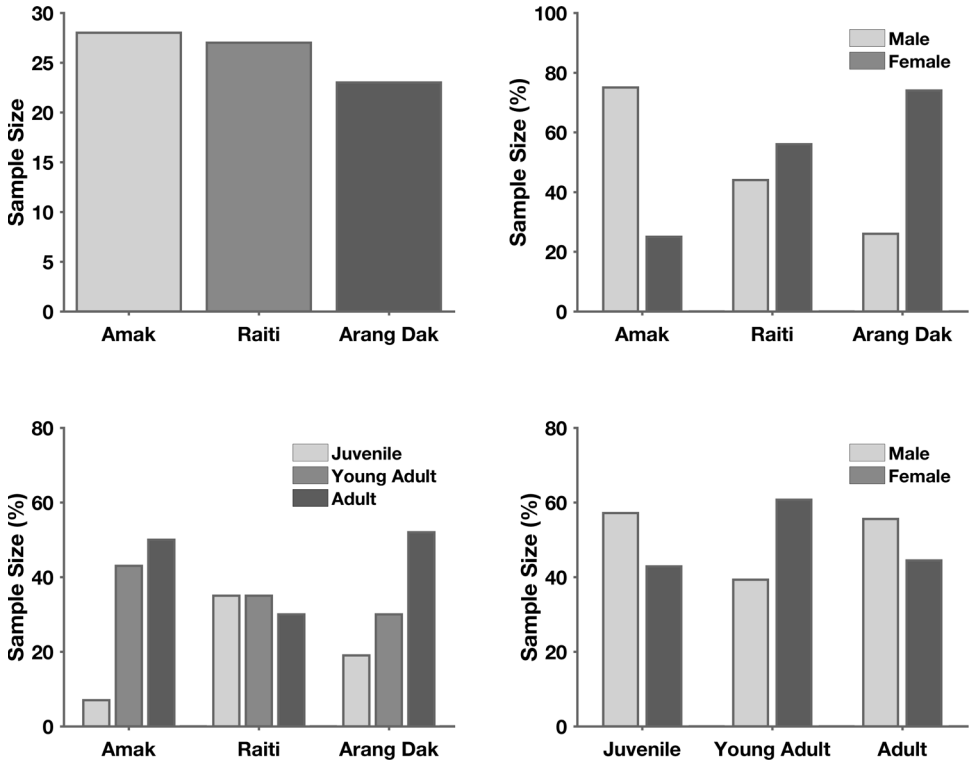

Figure 2. Demographic data of hunting dogs examined in Amak, Arang Dak, and Raiti. Dogs < 6 months old were excluded from the study; otherwise, no exclusion criteria were applied. Ages were determined by physical examination and owner reports during interviews.

Inc., Seattle, WA), a rapid immunochromatographic strip assay based on recombinant antigens, was used as per manufacturer instructions for initial screening (Nieto et al. 2009). Samples that were positive or weak positive on the Rapid Test were sent to the Texas A&M Veterinary Medical Diagnostic Lab for titer determination using the indirect fluorescent antibody test (Nieto et al. 2009), at 1:20, 1:40,

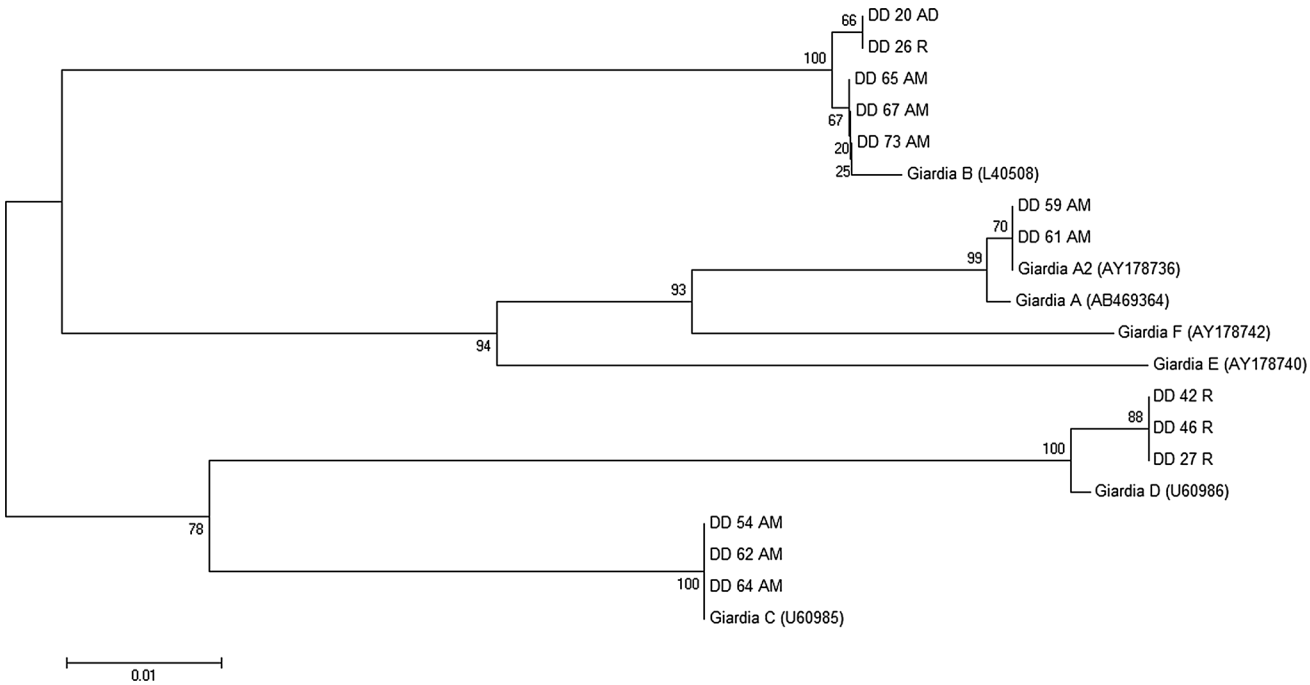

Figure 3. Phylogeny of *Giardia* isolates recovered from dog fecal samples and sequenced using the GDH gene. *Giardia* reference sequences are listed with accession numbers followed by assemblage in parentheses, with evolutionary distance represented by the scale bar. Study isolates are listed with sample ID ending with location identifiers (AD = Arang Dak, R = Raiti, and AM = Amak).

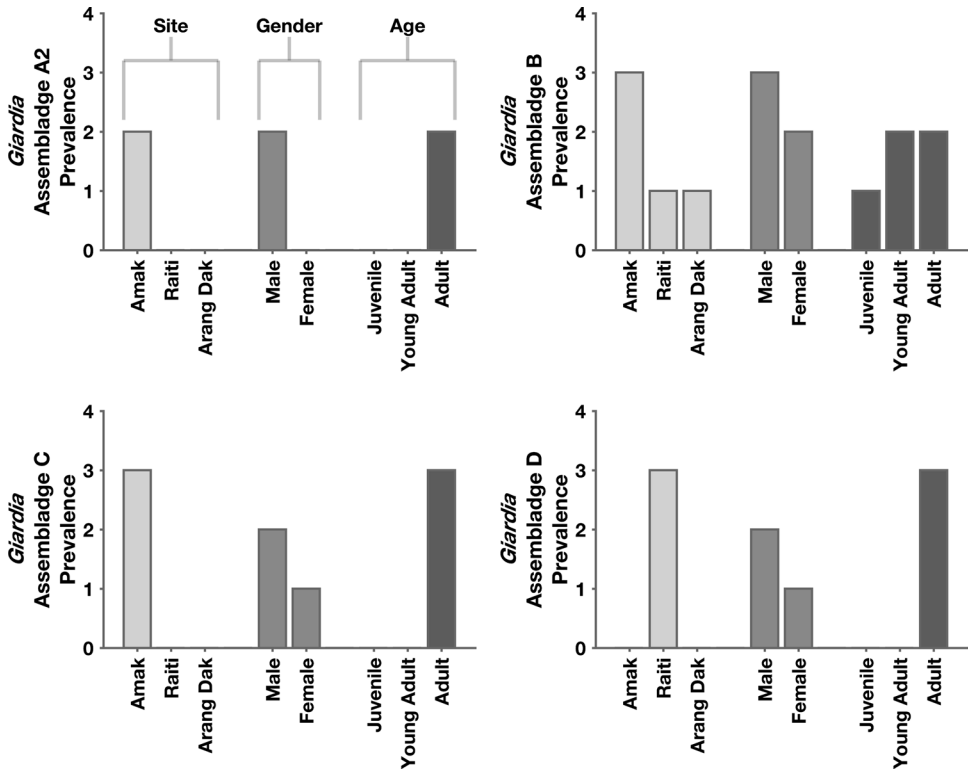

Figure 4. *Giardia* assemblage distribution by location, sex, and age shed in dog feces from Amak, Arang Dak, and Raiti in Bosawás Reserve, Nicaragua. Clean nucleotide sequences were obtained from 13 samples and matched to reference sequences,utilizing Chromas Lite 2.01 nBLAST searches of the GenBank database and the ClustaIX sequencing alignment program.

1:80, 1:160, 1:320, 1:640, and 1:1280 dilutions, standardized with Tcl.

Additionally, whole blood from all dogs was sent to University of Georgia for PCR detection of *T. cruzi*. DNA was extracted from blood using a QIAamp DNA Blood Mini Kit extraction kit (Qiagen Inc., Valencia California), and duplex PCR was performed, which detects *T. cruzi* and *T. rangeli* (Chiurillo et al. 2003). Positive and negative controls for PCR, as well as negative controls for extraction, were included for each PCR run.

Statistical Analysis

Statistical analyses were performed in the R modeling environment (R Development Core Team 2017, stats package). Pearson's Chi-square tests were used to determine if there were differences between age in male and female dogs, differences in age between communities, and differences in sex distribution among communities. In order to examine associations between risk factors and the presence or absence of *Giardia* or *Cryptosporidium* DNA in feces and *T. cruzi* DNA in serum, bivariate logistic regression was used to identify predictor variables significantly associated with outcome variables (P value < 0.2). These predictor variables were then included in multivariable logistic regression models that retained risk factors significant at the $P < 0.05$ level. Odds ratios, 95% confidence intervals (CI) of odds ratios, and P values were used to infer statistical associations. Referent categories were determined by selecting the category with the largest number of samples. For dogs, sex (male as referent category), age group ($>$ 3 years old as referent category), and community (Amak as referent category) were considered for possible association with the occurrence of *Giardia* or *Cryptosporidium* in feces or anti-*T. cruzi* antibodies in serum.

Results

Sample size was limited given the logistical challenges of working in remote communities. No attempt was made to obtain a random sample; in some cases, multiple dogs from the same household were included. Similar numbers of dogs were examined in Amak ($n = 28$), Arang Dak ($n = 23$), and Raiti ($n = 27$) (Fig. 2). Samples represent approximately 50% of the dog population in Arang Dak, but only 10–20% in Raiti and Amak. The sex ratio of our sample differed significantly among communities ($P = 0.001$), with Arang Dak dogs being predominantly female and Amak dogs being predominantly male. The majority of dogs (53%) were $\leq$ 3 years of age. Age category did not differ significantly across sites, or by sex ($P > 0.05$) (Figure 2).

Initial PCR screening detected *Giardia* in 19 of 58 (33%) fecal samples, with 13 samples returned with clean sequences. *Cryptosporidium* was not detected in any of samples. Amak had the highest number of *Giardia*-positive dogs (8 of 16) confirmed by sequencing, followed by Raiti (4 of 24) and then Arang Dak (1 of 18). Multivariable logistic regression (Table 1) revealed associations between the presence of *Giardia* DNA and location, with dogs living in Amak roughly seven times (95% CI 1.35–42.95, $P = 0.02$) more likely to be PCR positive compared to dogs living in Raiti. There was no significant difference between the number of *Giardia*-positive dogs in Arang Dak and Raiti observed (95% CI 0.02–3.62). Adults were most likely to be positive (10 of 28), followed by young adults (2 of 17) and juveniles (1 of 13). Young adult dogs were 86% (95% CI 0.01–0.79, $P = 0.05$) less likely to be *Giardia* positive compared to adult dogs, and juvenile dogs were 83% (95% CI 0.01–1.37, $P = 0.14$) less likely to be *Giardia* positive compared to adult dogs.

Figure 3 shows the phylogenetic relationship of *Giardia* isolates and reference sequences for each assemblage. Among sequences returned, five were closest to assemblage B, three each were closest to assemblage C and D, and two were closest to assemblage subtype A2. Sequences were deposited in GenBank under accession numbers from MH145336 to MH145348. Figure 4 presents the distribution of *Giardia* assemblage by location, sex, and age. Stratified by location, Amak had greatest genetic diversity, with three assemblages detected (A, B, and C), while Raiti had two assemblages detected (B and D), and Arang Dak had one assemblage detected (B). Stratified by sex, male dogs had four assemblages detected (A, B, C, and D) and female dogs had three assemblages detected (B, C, D). Stratified by age, four assemblages were detected (A, B, C, and D) in adults while only one (assemblage D) was detected in young adults and juveniles.

Seven seropositive dogs were confirmed through *T. cruzi* IFA (titer 1:32 or $>$) (Nieto et al. 2009), three from Arang Dak and four from Amak. Therefore, *T. cruzi* seroprevalence was 9% of the total sample. Seroprevalence was not significantly associated with community, sex, or age. *Trypanosoma cruzi* DNA was not detected in any of the whole blood samples via PCR, indicating the absence of

active infection or potentially sample degradation. One dog was both exposed to *T. cruzi* and infected with *Giardia*.

Discussion

We screened serum and fecal samples from hunting dogs across three communities in Bosawás, and assessed potential risk factors for exposure to *Giardia and T. cruzi*. There is very little published information on the health status of dogs in Bosawás (Fiorello et al. 2017), and to our knowledge, this is the first study to investigate protozoal parasites in this population. As such, it provides an important step toward understanding disease risk for indigenous people within Bosawás and baseline context with which to explore the impact of impending environmental changes.

Giardia prevalence among dogs in this study (22%) fell within the range of published findings in Central and South America. Lebbad et al. (2008), utilizing similar PCR protocols, found that 8/100 fecal samples from dogs in Leon, Nicaragua, were positive. Mundim et al. (2007) collected feces from 410 urban dogs in Brazil and found that 29% were positive, using microscopy. A meta-analysis of 169 studies using a variety of detection methods in dogs and cats reported an overall pooled prevalence of 15% with wide variability (Bouzid et al. 2015).

Age has been identified as a predictor of infection by *Giardia* in dogs, with previous research finding that younger dogs have a higher prevalence than older dogs (Fontanarrosa et al. 2006; Mundim et al. 2007). Our finding that older dogs ($>$ 3 years old) had increased odds of shedding *Giardia* may indicate that hunting is a risk factor, given that in Bosawás, dogs most valued for hunting are typically older (Koster and Tankersley 2012). The causal mechanisms increasing risk for older dogs, however, remain uncertain. For example, it may be that hunting increases exposure to pathogens circulating in the environment or that hunting reduces a dog's immune system and ability to fight infection via over-exertion, or some other combination of factors. A better understanding of the reason why older dogs appear to be at greater risk of *Giardia* infection, may provide further insights into disease transmission dynamics in the human population as well.

Controlling for age in multivariable logistic regression, dogs from Amak were found to have significantly increased odds of shedding *Giardia* in feces. Located at the confluence of two major rivers, Amak is a busy, relatively accessible community when compared with the extremely remote Arang Dak and distant Raiti. The increased contact rate with people and their animals traveling from outside of the village, in combination with Amak's close proximity to the forest, may expose hunting dogs to *Giardia* from both traveling humans and their animals as well as from wildlife. A higher seroprevalence of tick-borne diseases was also found in dogs from Amak compared to the other communities (Fiorello et al. 2017). Future studies should aim to more robustly evaluate the hypothesis that living in a village with relatively higher through traffic in combination with close proximity to forested lands increases the risk of *Giardia* exposure and potentially other pathogens.

The detection of sub-assemblage A2 (typically considered host specific to humans) and assemblage B is evidence of zoonotic transmission of *Giardia* between humans and their hunting dogs and supports the potential for dogs as sentinels of zoonotic diseases circulating in the environment. Detection of *Giardia* DNA suggests active infection, although it is possible that dogs could pass *Giardia* parasites through their gastrointestinal tracts after ingesting contaminated water or human feces. Future work in the region should include testing of environmental sources in combination with multi-locus genotyping to further characterize *Giardia* transmission (Ryan and Cacciò 2013).

We did not detect *Cryptosporidium* in the fecal samples, but *Cryptosporidium* from dogs may still be a public health concern in the region. A survey of schoolchildren in Nicaragua found a *Cryptosporidium parvum* prevalence of 36% in a sample size of 272 individuals (Titilincu et al. 2010). Our limited sample size and inability to freeze samples in the field may have resulted in false negatives.

The seroprevalence of *T. cruzi* (9%) was similar or lower than previously reported for other remote communities (Alroy et al. 2015; Pineda et al. 2011; Saldaña et al. 2015), and shelter dogs in Texas (Esch and Petersen 2013). In 2005, the prevalence of *T. cruzi* infection in humans in Nicaragua was estimated to be 1.1%, with 25% of the population at risk of infection (Gurtler et al. 2005, 2007). Seroprevalence in clinical studies has ranged from 13.1% in an endemic region of Nicaragua to 3.2–4.3% in areas of infection control efforts (Palma-Guzman et al. 1996; Sequeira et al. 2010). In the dogs sampled in Bosawás, the prevalence was highest in Arang Dak (13%), the smallest and most remote of villages sampled. This may indicate more rustic housing conditions and closer proximity to the forest are risk factors (Fung et al. 2014; Chaves et al. 2013; Cardinal et al. 2006).

While this research shows the potential value in using dogs as sentinels of exposure to protozoal zoonotic NTDs, it also highlights the challenges associated with working in remote locations to better understand indigenous community health. For example, enrolling villages with known demographics a priori would have been the preferred approach and allow for a more robust study design to replicate factors of interest across numerous villages, such as population size and amount of through traffic, logistics around locating and enrolling villages in relatively unexplored areas such as the Bosawás precluded us from doing so. Therefore, our results are likely less generalizable compared to a study with a more extensive sample. Future research should work to expand the number and diversity of villages sampled. However, while this study was limited to three villages, differences in their demographic and geographic makeup provided a useful dataset to explore factors related to NTDs exposure.

Conclusion

The exposure and infection of dogs with zoonotic NTDs in remote regions of Nicaragua indicate that their owners may also be at risk for infection by both *Giardia* and *T. cruzi*. The diversity of *Giardia* assemblages identified suggests multiple sources of pathogens. The seroprevalence of *T. cruzi* in dogs indicates that the pathogen was present, but the data collected in this study did not allow us to determine if domestic or sylvatic sources pose the greatest risk. Continuing to work with dogs in these remote communities may provide an avenue to better understand transmission cycles and human risk for infection.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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