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Eco-epidemiology of diarrheal disease with an emphasis on *Cryptosporidium* in and around Ranomafana National Park, Madagascar

By

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Abstract

Eco-epidemiology of diarrheal disease with an emphasis on *Cryptosporidium* in and around Ranomafana National Park, Madagascar
By Jonathan R. Bodager

Purpose: Pathogens transmitted between wildlife, livestock, and humans pose a serious threat to human health and wildlife conservation. Although much is known about the basic biology of zoonotic pathogens, the ecology and epidemiology of their transmission between species has received less attention. Likewise, for clinical syndromes such as diarrheal disease, the contribution of novel zoonotic transmission relative to human-to-human transmission on overall disease burden is unknown. This project examined the eco-epidemiology of diarrheal disease in rural Madagascar, with an emphasis on *Cryptosporidium*, one of the most common diarrhea-causing zoonotic parasitic genera in the world.

Methods: In July and August 2011, 278 fecal samples were collected from humans, livestock, and wildlife from Ambodiaviavy and Ankialo, two communities near Ranomafana National Park, Madagascar. Human subjects (n=135) were surveyed for socio-demographic (sex, age, profession, etc.) and health data (diarrheal illness, medication usage, water usage, etc.). DNA was extracted from samples, screened for *Cryptosporidium* by 18S PCR of the SSU, rRNA gene and subtyped using RFLP and genomic sequencing. Chi-square tests of association were used to examine relationships among factors from the survey instrument.

Results: A greater number of diarrheal symptoms occurred in Ambodiaviavy than Ankialo (23.1% vs. 4.2%) with 67% of cases younger than 18 years. Other behavioral practices varied across communities, i.e. - drinking boiled water (Ambodiaviavy = 52.3% reporting 'often'; Ankialo = 58.6% reporting 'never'). There were a total of 41 (14.7%) positive *Cryptosporidium* samples (20.2% from Ambodiaviavy and 12.5% from Ankialo). Species prevalence of *Cryptosporidium* was: 0.8% of humans, 4.0% lemurs, 33.3% peri-domestic rodents, 29.0% bovine, and 23.5% of porcine (one positive canine, n=1). Subtyping revealed a diverse array of *Cryptosporidium* species.

Conclusion: Survey data indicates differing behavioral practices between communities that were not linked to increased diarrhea but suggest varying risks of zoonotic transmission across the human-animal interface. One human, infected with *C. suis*, suggests a potential risk for human-pig transmission in Ankialo, whereas, large numbers of infected cattle in Ambodiaviavy suggest a potential risk for human-cattle transmission. Public health efforts should focus on improving sanitation and hygiene and on rational modifications of daily practices to avoid zoonotic transmission.

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Background

Zoonotic pathogens represent three-quarters of all emerging disease globally (Jones *et al.* 2008). This pathogen emergence places a large burden on the health of humans, domestic animals, wildlife, and overall ecosystem homeostasis (Daszak *et al.*, 2000; Gillespie *et al.*, 2008). Although there is still a great deal to learn about how and why pathogens are transmitted between humans, livestock, and wildlife; evidence is, however, increasing that anthropogenic disturbance of tropical forest ecosystems may be altering human, livestock, and wildlife ecology in ways that facilitate interspecific disease transmission and emergence (Gillespie *et al.*, 2005; Gillespie and Chapman, 2006; Goldberg *et al.*, 2008; Rwego *et al.*, 2008). For clinical syndromes such as diarrheal disease, the contribution of novel zoonotic transmission relative to human-to-human transmission on overall disease burden is unknown. To improve our understanding of this interplay, this project examined the ecology and epidemiology of diarrheal disease in rural Madagascar with an emphasis on the zoonotic protozoan pathogen *Cryptosporidium*.

Studying Global Diarrheal Illnesses

Diarrheal illnesses kill approximately 2.2 million people globally each year, most of whom are children in developing countries (WHO, 2004). Because of this, the Bill and Melinda Gates Foundation decided to fund a 3 year project called the Global Enterics Multicenter Study (GEMS). This study, coordinated by the University of Maryland along with several international partners (including the CDC), has been tasked with conducting population based surveillance of the burden of severe diarrheal disease in children less than 5 years of age at sites across Africa and Asia (personal communication, Michele Parsons). The project is a case-control study that matches severe diarrhea patients from hospitals or ambulatory

facilities with community controls (study is still underway). Our study, on the other hand, differs from the GEMS study because it is an investigation of diarrheal illness and possible causative factors among randomly selected individuals of all age groups, healthy and ill, in rural Madagascar. The small but representative sample size of our study, along with the detailed survey data and cross-species sampling, provides us with the opportunity to integrate behavior analysis and pathogen screening across multiple ecological interfaces; human, animal and wildlife. Additionally, with this level of detail, there is the unique possibility of determining pathogens that are carried asymptomatically by individuals and animals in the region.

Madagascar

In Madagascar, diarrheal diseases are considered one of the highest public health priorities for the country (WHO, 2009). There is still, however, uncertainty as to what is causing the diarrheal illnesses in this country. Building on the 20+ year productive relationship with the International Center for Tropical Environments, headed by Dr. Patricia Wright, the current study is based in and around Ranomafana National Park, a 43,500 hectare World Heritage Site, notable for its high levels of species endemism and diversity, and long history of diverse tropical research (Wright, 1992; Wright, 1997). Specifically, behavioral associations with diarrheal disease were studied in two rural communities, Ambodiaviavy and Ankialo, which are located in different areas of the park and have distinct cultural practices. The majority of households in the area own domestic animals and have household members that participate in farming activities and often interact with the associated forest areas. For these reasons, Ranomafana National Park and the surrounding rural areas represent an ideal location for this study.

Cryptosporidium

This project emphasized the protozoan pathogen *Cryptosporidium*, one of the most common diarrhea-causing parasitic genera in the world (Tzipori *et al.*, 2008). Evidence of its effect on human populations was seen in the massive outbreak of *Cryptosporidium* in Milwaukee, Wisconsin in 1993, in which more than 400,000 people were affected (Mac Kenzie *et al.*, 1994). In particular, *Cryptosporidium* poses a high risk to immunocompromised individuals, elderly, and children. In humans and livestock, these parasites cause diarrhea and other enteric disorders that can contribute to nutritional deficiencies and impaired weight gain (Savioli *et* al., 2006; Nime *et al.*, 1976; Meisel *et al.*, 1976). The emergence of *Cryptosporidium* as a significant human pathogen and its known zoonotic potential make it a threat to global public health (Thompson *et al.*, 2008).

First identified in humans in 1976 (Nime *et al.*, 1976; Meisel *et al.*, 1976), *Cryptosporidium* is a waterborne zoonotic pathogen with a thick protective walled oocyte that allows it to survive outside of a host in hostile environmental conditions for long periods of time (Smith, 1992). The ubiquitous nature of *Cryptosporidium*, coupled with its ability to infect a diverse number of species, allows for numerous transmission routes (Xiao & Ryan, 2004). Transmission routes include: person-to-person, animal-to-person, waterborne and foodborne transmission (Xiao and Ryan, 2004).

Over time, the protozoan parasite has been found in many humans and animals, with a total of 16 accepted species and approximately 50 *Cryptosporidium* genotypes having been described (Xiao *et al.*, 2004a; Feng *et al.*, 2007). Isolations of these various species lead to the understanding of *Cryptosporidium* as a waterborne zoonotic infectious disease with global public health implications (Thompson *et al.*, 2008). The most common disease causing

species in humans are *C. parvum* and *C. hominis*, accounting for greater than 90% of human cases, with the other 10% being accounted for by several other known species (such as *C. suis*, *C. parvum* bovine, *C. canis*, *C. meleagridis*, and *C. felis*) (Xiao *et al.*, 2001; Xiao & Ryan, 2004).

Cryptosporidium causes the disease known as cryptosporidiosis with the primary symptom of acute watery diarrhea (Tzipori & Widmer, 2008). Symptoms can last much longer and cause serious illness and death in those with weakened immune systems, such as HIV/AIDS, cancer, and transplant patients taking immunosuppressive drugs, and those with other inherited immunosuppressing disorders (Current & Garcia, 1991). There is currently no known cure for cryptosporidiosis. Treatment includes supportive care with oral and intravenous hydration and parenteral nutrition (Current & Garcia, 1991). Anti-protozoan medications, such as nitazoxanide have been FDA-approved for the treatment of diarrhea caused by *Cryptosporidium*, however, it's effectiveness in immunocompromised individuals remains uncertain (CDC, 2012).

Despite the adverse impact it has on human and livestock populations, the prevalence and pathogenicity of *Cryptosporidium* in wildlife remains largely unknown. Several studies have found the pathogen in non-human primates (Salyer *et al.*, 2012; Salzer *et al.*. 2007; Nizeyi *et al.*, 1999). Screening of *Cryptosporidium* in lemurs in Ranomafana National Park has never been conducted and this study will begin to provide a baseline prevalence, which can be used to investigate the human-lemur zoonotic transmission potential.

The interaction between this parasite and its human, domestic animal, and wildlife hosts requires further analysis. Collection of baseline data on diarrheal illness and presence of

Cryptosporidium in Madagascar via the survey instrument and fecal sample collection will help to inform public health action and conservation efforts.

Project Aims

The specific aims of this project were to determine if specific ecological and behavioral factors were associated with diarrheal disease in rural Malagasy communities using *Cryptosporidium* as a focal pathogen. The results of this study will contribute to a large ongoing public health study in the area.

Objectives

- *Objective 1*: To determine behavioral associations with human diarrheal disease via survey instrument.
    - H_0: There are no behavioral associations with human diarrheal disease in the study community.
- *Objective 2*: To determine if *Cryptosporidium* is present in the study community in and around Ranomafana National Park, Madagascar.
    - H_0: There is no *Cryptosporidium* present in the study community in and around Ranomafana National Park, Madagascar.
- *Objective 3*: To investigate sources of human, livestock (cattle and pigs), peri-domestic rodent, and wildlife (lemurs and small mammals) *Cryptosporidium* infection via molecular characterization.
    - H_0: *Cryptosporidium* species are genetically distinct between humans, livestock, peri-domestic rodents and wildlife.

Relevance to the Field of Environmental Health

Working to understand how *Cryptosporidium* infection is associated with reported diarrhea and behavioral practices in and around Ranomafana National Park, Madagascar will lead to rational public health and conservation intervention strategies in the region. This project will also add to our knowledge of *Cryptosporidium* as a zoonotic pathogen (transmission patterns, host dynamics, and human risk factors) and could promote improved guidelines for avoiding infection.

Methods

Study Area and Population

The study area is located at Ranomafana National Park, Madagascar, a 43,500 hectare World Heritage Site (21*15.135, 047*25.151), that is well known for its high levels of species endemism and diversity (Wright, 1992; Wright, 1997). The study population included several lemur species (*Microcebus rufus, Eulemur rubriventer, Hapalemur aureus, Propithecus edwardsi, Prolemur simus, and Avahi peyrierasi*), rodents (*Eliurus minor, Eliurus tanala, Rattus rattus, Mus musculus*), fossa (*Cryptoprocta ferox*), bovine (*Bos indicus*), porcine (*Sus domesticus*), canine (*Canis familiaris*), feline (*Felis catus*), and humans. Two communities located on the edge of the park were selected as the focus of this study: Ambodiaviavy (21*15.849, 047*29.087, population = 363) and Ankialo (21*08.062, 047*20.638, population = 361). The communities are located in different areas of the park and have distinct cultural practices.

Survey Instrument

In July and August 2011, surveys were conducted at household and individual levels in both communities: Ambodiaviavy (n=65, total households=10) and Ankialo (n=70, total

households=10). Informed consent of human subjects was obtained prior to specimen collection and survey. Subjects were anonymously given unique identifications (i.e. – 2-1-HS-4, designating the community (2), household (1), human specimen (HS), and individual (4)). The two surveys that were conducted were: 1.) survey of each individual within a household and, 2.) household-level survey of each of the ten households sampled by community. The individual survey was comprehensive and made inquiries on over 70 different variables including: demographic information, health status, medication usage (antimicrobial use, traditional remedies, etc.), water usage (open vs. closed source, drinking boiled water, etc.), interaction with animals and wildlife, diarrhea risk-associated behaviors (eating food known to be contaminated with fecal material, etc.) and work. Further information was collected at the household level with 40 questions that made inquiries about animals owned, where animals are kept, latrine use, and more. Correlation analyses were used to determine relationships between reported diarrhea and associated behaviors to determine behaviors that could increase risk for diarrheal illness. Chi-square tests of association were performed using SAS version 9.2 software (SAS Institute Inc., Cary, NC).

Fecal Sample Collection

All survey participants were asked to provide a fecal sample for examination of diarrheal pathogens and 89% (n=120) complied. Concurrently, domestic animals of participants (bovine, canine, feline, and porcine, n=82) were sampled and baited live-traps were set inside participant homes overnight. The following morning, fecal samples were collected from peri-domestic rodents trapped (n = 48). Wildlife (lemurs, rodents, and fossa n= 28) within Ranomafana National Park were opportunistically sampled non-invasively. All fecal samples were preserved upon collection in RNAlater ® [Cat# 76104] (Qiagen Inc., Valencia, CA).

Nucleic Acid Extraction

Total nucleic acid was extracted from fecal samples (n=278) preserved in RNAlater® using methods described (da Silva et al., 1999). Using the FastDNA® SPIN Kit for Soil [Cat# 6560-200] (MP Biomedicals, LLC, Solon, OH), fecal samples were individually placed inside Lysing Matrix E Tubes and then washed twice with 800 µl of de-ionized water to remove RNAlater®. Washing consisted of four steps: rinsing with de-ionized water, re-suspending, centrifuging for 6 min at 14,000 rcf, and then discarding of supernatant. Once washed, the tubes were then filled with 978 µl of sodium phosphate buffer and 122 µl of MT Buffer and re-suspended before placing in Fast Prep® Cell Disrupter Model FP120A Instrument [Product No. 6001-120] (Qbiogene, Carlsbad, CA) for 30 seconds at a speed of 5.5. The lysing tubes were then centrifuged for 30 seconds at 14,000 rcf. The supernatant was then transferred to clean 1.5 ml microcentrifuge tubes. Two-hundred and fifty microliters of PPS (Protein Precipitation Solution) solution was added to each tube and then inverted by hand 10 times. Each sample was then centrifuged at 14,000 for 5 min in order to form a pellet of any remaining solid fecal matter. The supernatant, now containing the nucleic acid, was transferred to a 15 ml falcon tube along with an addition of 1 ml of Binding Matrix Suspension. After inverting falcon tubes by hand for 2 minutes (allowing the nucleic acid to bind to the matrix), the samples were place on the lab bench to settle for 5 minutes. The matrix-nucleic acid mixture was continually added to the SPIN Filter Tubes and centrifuged at 13,400 rcf for 1.5 minutes until the entire matrix was caught in the filter. The filter was then cleaned with 500 µl of SEWS-M (Salt/Ethanol Wash Solution) which was centrifuged through the matrix at 13,400 rcf for 2 minutes. After air drying for 5 minutes, 100 µl of DNase/Pyrogen Free Water was gently stirred into the matrix and centrifuged at 13,400 rcf for 1 minute. The filter was then discarded and the final nucleic acid, found in the remaining

liquid in the catch tube, was stored in -20º C freezer for working use and with archive storage at -80º C.

18s Polymerase Chain Reaction

Once extracted, DNA was screened using 18S polymerase chain reaction (PCR). Four primers were used: forward primer (F1) for primary PCR [5'-TTC TAG AGC TAA TAC ATG CG-3'], reverse primer (R1) for primary PCR [5'-CCC ATT TCC TTC GAA ACA GGA-3'], forward primer (F2) for secondary PCR [5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3'], reverse primer (R2) for secondary PCR [5'-CTC ATA AGG TGC TGA AGG AGT A-3'] that amplify the small subunit (SSU) rRNA gene of *Cryptosporidium*. Nested PCR was carried out in 0.2 ml PCR tubes. The primary PCR was a 50 µl mixture consisting of 28.1 µl of sterile water, 5 µl of 10x Perkin-Elmer PCR buffer (100 mM Tris-HCL [pH 8.3], 500 mM $MgCl_2$, 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin) [Product No. N808-0129, PE Applied Biosystems, Foster City, CA], 8 µl of deoxynucleoside triphosphates (1.25 mM) [Product No. U1240, Promega Corp., Madison, WI], 2 µl bovine serum albumin (10mg/ml), 3 µl of $MgCl_2$ (25 mm) [Product No. A351F, Promega Corp., Madison, WI], 1.3 µl each of F1 and R1 primers (40 ng/µl), 0.3 µl of GoTaq® DNA Polymerase [Product No. M3005, Promega Corp., Madison, WI] and 1 µl of sample nucleic acid. The secondary PCR was a 50 µl mixture consisting of 26.5 µl of sterile water, 5 µl of 10x Perkin-Elmer PCR buffer, 8 µl of deoxynucleoside triphosphates (1.25 mM), 3 µl of $MgCl_2$, 2.6 µl of both F2 and R2 primers (40 ng/µl), 0.3 µl of GoTaq Polymerase and 2 µl of the primary PCR reaction. Samples were subjected to a preincubation at 94º C for 3 minutes; 35 PCR cycle replications, each consisting of 45 seconds of denaturation at 94º C, 45 seconds of annealing at 55º C, and 1 minute of elongation at 72º C; and a final extension (or elongation) at 72º C for 7 minutes

using Veriti® 96-Well Thermal Cycler [Model 9902] (Applied Biosystems, Foster City, CA). 20 µl of the PCR products were electrophoresed on 1.5% SeaKem® LE Agarose, [Cat# 50004] (Lonza, Rockland, ME) gels with 20 µl of the PCR product, were stained with ethidium bromide, and gel image captured under UV exposure. All PCR reactions were run in duplicate. Reaction mixtures containing sterile water, appropriate PCR reagents, and no nucleic acid were used as negative controls. Positive controls, kindly provided by the Waterborne Disease Prevention Branch, Centers for Disease Control and Prevention, were included with every PCR run. Figure 1 shows a representative gel containing the positive control and one sample that was suspect positive for *Cryptosporidium* based on the intense bands approximately 800 base pairs in length.

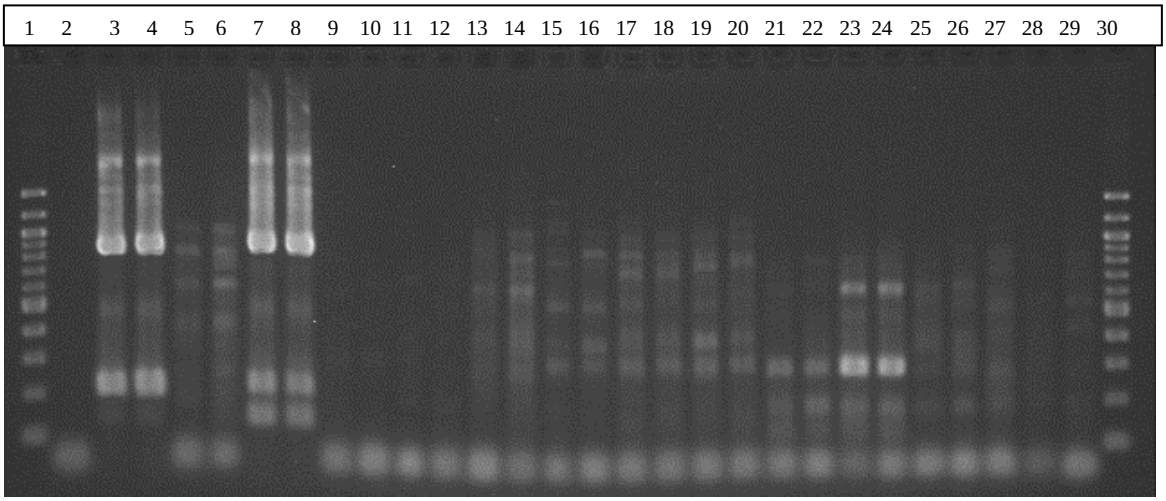

Figure 1. Detection of *Cryptosporidium* oocysts in fecal material by 18s PCR of the SSU rRNA gene. Lane 1: 100bp ladder; Lane 2: Negative Control; Lane 3, 4: Positive Control; Lane 7, 8: suspect positive for *Cryptosporidium*.

Restriction Fragment Length Polymorphism

Analysis of suspect positive specimens was conducted using Restriction Fragment Length Polymorphism (RFLP) screening as outlined (Xiao *et al.* 1999). Restriction was completed

using two digestion enzymes: *Ssp*I and *Vsp*I. The restriction was carried out in 1.5 ml microcentrifuge tubes. The *Ssp*I reaction mixture consists of 22 µl of sterile water, 4 µl of Buffer *Ssp*I, 4 µl of *Ssp*I Enzyme [Product No. R0132L, New England BioLabs, Beverly, MA], and 10 µl of secondary PCR reaction. The *Vsp*I reaction mixture consists of 24 µl of sterile water, 4 µl of Buffer D, 2 µl of *Vsp*I Enzyme [Product No. R6851, Promega, Madison, WI], and 10 µl of secondary PCR reaction. All samples were incubated for 5 hours or overnight in a 37ºC water bath. 40 µl of restriction digests were electrophoresed on 2.0% SeaKem® LE Agarose gels stained with ethidium bromide, and image captured under UV exposure. Figure 2 shows representative gels after digestion by *Ssp*I (Gel A) and *Vsp*I (Gel B) enzyme. Samples were identified by calculating band fragment sizes, against the 100bp ladder. Fragment sizes for each enzyme were then cross referenced to the predicted expected fragment sizes for species of *Cryptosporidium*.

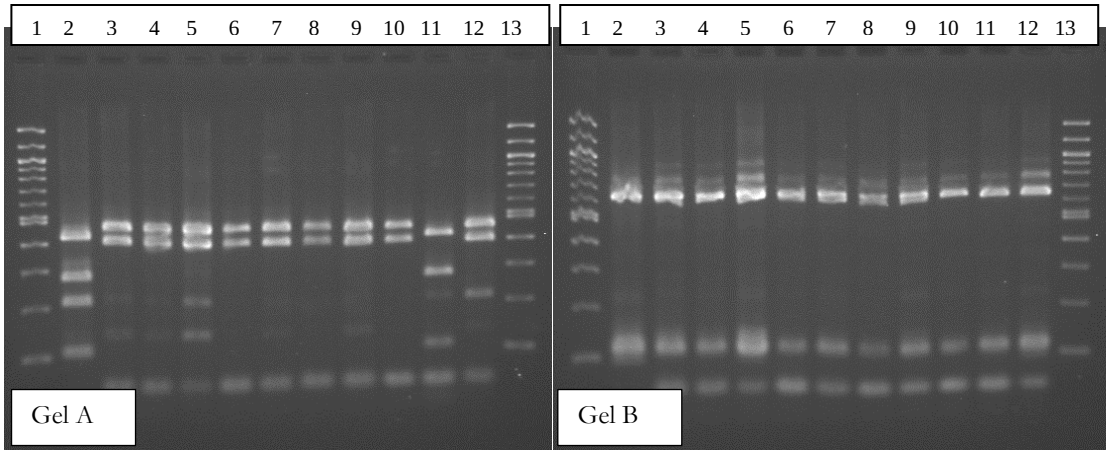

Figure 2. RFLP species level identification of *Cryptosporidium* using *Ssp*I (Gel A) and *Vsp*I (Gel B) enzymes. Lane 1: 100 base pair ladder; Lane 2: positive control *C. canis*; Lane 3-10, 12: Suspect *C. suis*; Lane 11: Suspect *C. canis*.

Genomic Sequencing

Further analysis of *Cryptosporidium* subtypes is currently being conducted by the Waterborne Disease Prevention Branch, CDC, following procedures described (Xiao & Ryan 2008, Alves *et al.* 2003, and Sulaiman *et al.* 2005). Briefly, the secondary PCR product was cleaned and the DNA was sequenced using a two-directional procedure for increased accuracy. The following primers were used: secondary PCR primers, forward (F2) [5'-TCC GCT GTA TTC TCA GCC-3'] and reverse (R2) [5'-GGA AGG AAC GAT GTA TCT-3']; and the intermittent sequencing primer (R3)[5'-GAG ATA TAT CTT GTT GCG-3']. Analysis of the GP60 gene of the secondary PCR products occurred with the use of an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, Calif.). The GCG program (Genetics Computes Group, Madison, WI) was used with manual adjustment to align sequences from the isolates with one another and with published sequences.

Ethical Considerations

This project was submitted to the Institutional Review Board (Emory University) and was deemed exempt from requiring approval as the subcontract of an approved project at Stony Brook University, New York.

Results

Descriptive Statistics for All Study Subjects

There were a total of 135 humans surveyed across the communities of Ambodiaviavy (n = 65) and Ankialo (n = 70) (Table 1). Participants were from one of ten households randomly selected in each community. All survey participants were analyzed for potential behaviors associated with risk for diarrheal disease and infection with *Cryptosporidium*. Mean participant

age of the two communities were similar (Ambodiaviavy = 23.5 years, SD = 18.6; Ankialo = 21.5, SD = 16.3), with over 50% of study participants being under the age of 18 (Table 1). Most individuals reported making no income (60% in both communities) and the majority of income earners reported making less than 10,000 AR a week (1.00 USD is approximately equal to 2,125.00 AR) (Table 1). Few individuals reported high levels of education, with only two individuals from Ankialo having completed 'some university' and one individual from Ambodiaviavy having completed 'some technical school'. There was a difference in the number of active students between surveyed communities (26.2% of Ambodiaviavy vs. 41.1% of Ankialo). Those reporting farming as a primary profession were equal across communities (Ambodiaviavy = 46.2% and Ankialo = 44.3%) (Table 1).

Comparison of Risk Factors for Diarrhea

Data from the human survey were used to investigate potential risk factors for diarrhea related to water use, meal-time practices, defecation practices, and other potentially associated behaviors overall and comparing surveyed communities (Tables 2, 3, 4, 5). Because there was only one human sample positive for *Cryptosporidium* (Ankialo community), no correlation analyses to compare infection status with associated behaviors occurred. Behaviors of individuals were directly compared to reported diarrhea and few significant associations were found. There was no statistical association present between reported diarrhea and the following risk factors: sex, traditional remedy use, tending pigs, working in the field, drinking boiled water, fetching water from an open or closed source, defecating in a water closet or not in a toilet, eating food known to be contaminated with rodent feces, eating animals with visible sores, washing hands before eating, and sleeping with a mosquito net (Table 2). The majority of the individuals reporting diarrhea were under the age of 18

(67%, p = 0.4062, Table 2). There was a significant difference between reported diarrhea in Ambodiaviavy and Ankialo (23.1% vs. 4.2% reported diarrhea symptoms in last four weeks, p = 0.0018, Table 2 and 3). Additionally, a significant association existed between reported medication use and diarrhea with 94.4% indicating the use of medications in the last four weeks (Fisher exact P = 0.0234, Table 2).

In order to better understand the risk factors associated with diarrhea, comparisons were made across communities. There were several significant differences in water use and defecation practices between communities (Table 3). A majority of individuals in Ambodiaviavy reported 'often' drinking boiled water (52.3%), while the majority of individuals in Ankialo reported 'never' drinking boiled water (58.6%, p <0.0001, Table 3), even though the majority of reported diarrhea cases were in Ambodiaviavy. Other significant differences across communities include fetching water from an open source, fetching water from a closed source, and defecating in a water closet (p = 0.0217, p = 0.0232, and Fisher exact p <0.0001, respectively, Table 3). Individuals from Ambodiaviavy fetch water from a closed source more often than Ankialo (55.4% vs. 34.3% respectively) (Table 3). Of the individuals in Ambodiaviavy, 76.9% report 'never' defecating in a water closet, as opposed to 31.9% in Ankialo who 'never' use a water closet. Although not statistically significant across communities, there were high rates of individuals reporting that they 'often' do not defecate in a toilet in both Ambodiaviavy and Ankialo (84.6% vs. 70.0% respectively, p = 0.1388, Table 3).

Table 4 shows an across community comparison of meal-time practices. In Ankialo, 84.3% of individuals indicated that they eat food known to be contaminated with rodent feces, which is significantly higher than the 53.8% who report the same practice in Ambodiaviavy

(p = 0.0005, Table 4). Other factors related to meal-time behaviors were not significantly different across communities, including eating uncooked meat, eating animals with visible sores, and washing hands before eating (Table 4).

Upon comparing other risk factors across communities, significantly more individuals in Ambodiaviavy report 'often' sleeping with mosquito nets (50.8% vs. 5.9%, Fisher exact P<0.0001, Table 5) and significantly more individuals report use of medications and traditional remedies in Ambodiaviavy in the last four weeks (p = 0.0425 and p=0.0174, respectively, Table 5). There was no significant difference across communities when comparing work in the field and crop raids (Table 5).

Medication Usage across Communities

Medication use was first compared across communities and then broken down into two categories: 1.) antibiotics (sub-categories: Amoxicillin, Cotrimoxazole, and Metronidazole) and, 2.) anti-parasitic medications; and then was analyzed against predictors (i.e. - sex, reported diarrhea, and drinking boiled water). A large number of individuals reported using an antibiotic medication in the last four weeks, with no significant difference between the communities (49.2% usage in Ambodiaviavy and 42.9% usage in Ankialo, p = 0.4578, Table 6). The antibiotic used most often in both Ambodiaviavy and Ankialo was Cotrimoxazole (33.8% and 31.4% respectively) with no significant difference in usage across communities (P = 0.7646, Table 6). There was, however, more use of the antibiotic Metronidazole in Ambodiaviavy (12.3% of individual reported using vs. 1.4% in Ankialo, Fisher's exact P = 0.0144, Table 6).

Table 7 provides a comparison of medications used and various predictors across all communities. There was no significant difference between male and female use of antibiotics, with 53.2% of users being male and 46.8% of users being female ($p = 0.5074$, Table 7). A significant number of those reporting diarrhea took an antibiotic in the past four weeks (15 of 18 individuals, or 83.3% reported using an antibiotic, $p = 0.0006$, Table 7). Additionally, a significant number of all individuals reporting antibiotic use also reported 'never' drinking boiled water (61.3%, $p = 0.0069$, Table 7).

Interaction with Lemur Population

The majority of individuals responded 'No' to having interactions with the local lemur population. There were, however, four different individuals who reported eating one of the three lemurs species (Red-fronted Brown Lemur, N=1; Black-and-white Ruffed Lemur, N=1; and Brown Mouse Lemur, N=2). The Brown Mouse Lemur has an IUCN Status of least concern, the Red-fronted Brown Lemur's status is near threatened, and, most importantly, the Black-and-white Ruffed Lemur's status is critically endangered (Table 8).

Analysis of Samples Positive for Cryptosporidium

There were a total of 278 fecal samples screened for the presence of *Cryptosporidium* (Table 9). Fecal samples consisted of 120 human (n=59 from Ambodiaviavy and n=61 from Ankialo, Table 10), 82 domestic animal (n= 62 bovine, n = 17 porcine, n=1 canine, and n=2 feline), 48 peri-domestic rodents, and 28 wildlife (n=25 lemur, n=2 wild rodents and n=1 fossa) (Table 9). Table 10 shows the number of fecal samples collected, the number positive for *Cryptosporidium*, and the prevalence of *Cryptosporidium* by species in Ambodiaviavy,

Ankialo, and Ranomafana National Park. A total of 41 samples (14.7%) were positive for the pathogen, with 20.2% of the subjects in the Ambodiaviavy community testing positive, 17 (12.5%) of the subjects in the Ankialo community, and 1 (0.8%) of the subjects from Ranomafana National Park (Table 10). Screening revealed a prevalence of *Cryptosporidium* of 0.8% in humans, 4.0% in lemurs, 33.3% in peri-domestic rodents, 29.0% in bovine, and 23.5% in porcine (and one positive canine, n=1) (Table 9).

Ambodiaviavy had a significant number of cattle test positive for *Cryptosporidium*, whereas Ankialo had zero cases of the pathogen in their cattle, despite similar sample sizes (51.4% vs. 0%, respectively, Table 10). One fourth of pigs tested in Ankialo were positive for the pathogen, while in Ambodiaviavy none were positive; however, only one pig sample was collected in Ambodiaviavy (25% vs. 0%, respectively, Table 10). Of the peri-domestic rodents sampled, four were positive for *Cryptosporidium* in Ambodiaviavy while twelve were positive in Ankialo (22.2% vs. 40.0%, respectively, Fisher exact P = 0.3431, Table 10).

Nine households in Ambodiaviavy and six households in Ankialo were associated with positive cases of *Cryptosporidium* (at least one positive human or animal) (Table 11). Significantly more individuals in Ambodiaviavy live in households in which at least one cow tested positive for *Cryptosporidium* (92.3% vs. 0% in Ankialo, Fisher exact P<0.0001, Table 12). Of the individuals living in Ankialo, 34.3% live in households whose pigs tested positive for *Cryptosporidium* (Note: sample size differed greatly between communities, Table 10) (Table 12). There was a significant difference between Ambodiaviavy and Ankialo in the number of individuals living in households that had at least one peri-domestic rodent test positive (30.8% vs. 50.0% respectively, p = 0.0231, Table 12).

Only one human sample was positive for *Cryptosporidium* (subtype: *C. suis*, a common pig subtype). This individual was a 43 year-old, male from the Ankialo community. None of animal samples associated with this individual's household were positive, the household does not own pigs, and other members of household also did not report tending pigs (household #9, Ankialo, Table 11). This individual did, however, report interacting with a wild pig, specifically, this individual reported trapping, cooking, and eating wild pigs. The positive human individual reported taking three different medications: Paracetamol (analgesic), Levamisole (anthelminthic), and Calcium lactate (anti-acid). This individual did not report diarrheal symptoms but did report a fever and nausea/vomiting. This individual did report behaviors that could promote increased risk of *Cryptosporidium* infection and/or diarrheal symptoms such as: eating food contaminated with rodent fecal matter, eating uncooked meat, eating animals with visible sores, Additionally, one domestic dog and one lemur tested positive for the pathogen (positive lemur species: *Microcebus rufus*, Table 13). This lemur sample was collected at the Camp Site location, which is one kilometer from the Ankialo community. Five other species of lemur were sampled, but all were negative for the pathogen: *Eulemur rubriventer*, *Avahi peyrierasi*, *Prolemur simus*, *Hapalemur aureus*, and *Propithecus edwardsi* (for common name, IUCN status, and sample details, see Table 13).

Subtyping of Cryptosporidium Positive Samples

There was a diverse array of *Cryptosporidium* subtypes present in the study population (Table 14). The one positive human sample was identified as the subtype *C. suis* (Table 15). The positive lemur sample was subtype *C. hominis* (Table 15). Intriguingly, the majority of the bovine samples were subtype *C. suis* (n=13), with the others being *C. muris* and *C. parvum*

genotype A (Table 15). Porcine samples were of the subtype *C. suis* (n=3) or *C. parvum* (n=1). The rodent population had the most diverse array of subtypes with five identified and one unknown (Table 15). Finally, there was one canine sample screened and it was positive for the subtype *C. canis*. The subtyping data is currently being confirmed by the CDC and results will be updated and integrated in future manuscripts.

Discussion

Our results demonstrated a greater number of reported diarrhea cases in the Ambodiaviavy community than in the Ankialo community, with the majority of those cases occurring in children under the age of 18. We found little to no association between diarrhea and food, water, and other behavioral practices. In nearly all instances, behaviors that may promote increased diarrhea occurred more often in Ankialo, the community with significantly less reported diarrhea. One difference to note was the reported use of medications. Virtually all individuals reporting diarrhea in the past four weeks also reported using medication (94.4%) in the past four weeks (namely antibiotics). This finding suggests that the majority of those with diarrheal illness use antibiotics as treatments. Further investigation of the other diarrhea causing pathogens in these communities would help to identify whether or not the high antibiotic use by community members is appropriate.

In order to further investigate the disparity in reported diarrhea, survey data was compared across the communities. The two communities differed significantly in their use of water. Intriguingly, practices associated with improved sanitation and hygiene were primarily associated with Ambodiaviavy, whose population reported the most instances of diarrhea. Individuals in Ambodiaviavy boil their water more often and report rarely fetching water

from open sources (they often fetch from closed sources). Defecation practices, however, could be associated with the high levels of reported diarrhea in Ambodiaviavy because 76.9% indicated never defecating in a water closet while the majority of Ankialo residents report the use of a water closet. Since *Cryptosporidium* is a waterborne pathogen (Karanis *et al.*, 2007), defecation in the environment could lead to shedding of the pathogen into nearby water sources and promote increased transmission of the zoonotic pathogen. Although only one individual was positive for *Cryptosporidium*, the water use results collected in this study could prove to be useful in future studies of other waterborne pathogens in Ranomafana National Park.

Another community-level finding that was important was the reported use of mosquito nets. Significantly more individuals in Ambodiaviavy use mosquito nets (50.8% vs. 5.9%). The reason for this trend is not clear, however, further investigation into mosquito-borne pathogens would provide important insight.

Our laboratory results demonstrate a very low prevalence of *Cryptosporidium* in the human population, with no humans testing positive in the Ambodiaviavy community and only one human (male, adult) testing positive in the Ankialo community. This result is similar to the results of the Kightlinger *et al.*, 1995 study which looked at intestinal nematodes in children from 18 communities around Ranomafana National Park. They also screened the 1,292 children in their study for *Cryptosporidium* via Kinyoun carbol fuchsin staining and no samples were positive for *Cryptosporidium* (Kightlinger *et al.*, 1995). Recent studies, however, have demonstrated high rates of *Cryptosporidium* infection in similarly impoverished rural communities with limited health care access (Salyer *et al.*, 2012; Getaneh *et al.*, 2010; Ayalew *et al.*, 2008; Raccurt *et al.*, 2006). Our findings, along with those of Kightlinger *et al.* 1995,

suggest that *Cryptosporidium* may not be a major diarrhea-causing pathogen in the impoverished rural populations near Ranomafana National Park. Further screening of humans for *Cryptosporidium* should be conducted in this area.

Cattle from the Ambodiaviavy community (51.4%) were infected with *Cryptosporidium* at a much higher rate than those from the Ankialo community (0%), despite similar sample sizes. There was no evidence of the effects of *Cryptosporidium* infection on the consistency of fecal samples collected from cattle. This pattern of infection could; however, still indicate a negative impact on the cattle in the Ambodiaviavy community. The humans who reported tending cattle in this community were not associated with an increased prevalence of reported diarrhea. This result, along with the fact that 92.3% of individuals in Ambodiaviavy live in households where cattle tested positive for *Cryptosporidium*, could be caused by host-specificity of the cattle *Cryptosporidium* subtype and potentially human immunity to the *Cryptosporidium* subtype found in these cattle.

The overall community-level difference in the prevalence of *Cryptosporidium* in cattle is not clear. Perhaps differing cattle production practices exist or ecological differences were the cause. Further screening of local communities with an emphasis on cattle production practices may shed light on the significant difference in infection rate across communities.

Several peri-domestic rodents associated with each community were found to be positive for *Cryptosporidium*. Interestingly, the number of individuals living in households associated with positive rodents differed significantly, 30.8% in Ambodiaviavy and 50.0% in Ankialo. Rodents are well known carriers of zoonotic pathogens (Meerberg *et al.*, 2009; Daszak *et al.*, 2000; Childs *et al.*, 1998) and transmission of these pathogens can occur via contamination with rodent fecal material (Meerberg *et al.*, 2009). With this in mind, reports of eating food

known to be contaminated with rodent feces occurred at an overall high rate (approximately 70% across all individuals surveyed), with a much higher rate in Ankialo (84.3%) than in Ambodiaviavy (53.8%). Although *Cryptosporidium* found in rodents rarely infect humans, evidence of this practice is cause for concern because of the potential for other zoonotic pathogen transmission across the human-rodent interface. Prevention efforts in the area should include education about the risks of eating food contaminated with rodent fecal material.

Twenty-five percent of pigs in Ankialo were positive for *Cryptosporidium*. Within Ankialo, there were three positive pigs which were subtype *C. suis*, and one positive human, which also had the subtype *C. suis*. The human did not have any positive pigs associated with his household and the individual's family did not own pigs. Interestingly, the individual did report trapping, cooking, and eating wild pigs. This could account for this individual's exposure to *C. suis*. Additional screening of wild pig samples could shed light on this relationship.

Another explanation for the *C. suis* infected human could simply be the local interaction with *C. suis* infected pigs that are present in Ankialo. In general, this suggests transmission from pigs to people within this particular community. The higher prevalence of subtype *C. suis* in pigs than in humans also suggests that pigs may be reservoirs for *C. suis* in this community. Since *C. suis* can cause illness in humans (Xiao & Feng, 2008), there may be an increased risk of transmission for individuals living with pigs in Ankialo.

There was one lemur positive for *Cryptosporidium* in the sample population. This was not unexpected as non-human primates have been found positive for the pathogen in several studies (Salyer *et al.*, 2012; Salzer *et al.*, 2007; Nizeyi *et al.*, 1999). The positive lemur sample

was identified as *C. hominis*, which is known subtype amongst non-human primates (Xiao & Ryan 2004, Xiao *et al.* 2004a). After analyzing the human surveys for lemur interaction, we found the majority of individuals indicating 'No' interaction. There were, however, four different individuals who reported eating one of the following three lemur species: Red-fronted Brown Lemur, Black-and-white Ruffed Lemur, and Brown Mouse Lemur. According to IUCN, the Black-and-white Ruffed Lemur (*Varecia variegata)* is a critically endangered species. This is a significant finding because it was previously thought that the local human population never consumed lemurs (personal communication, Dr. Patricia Wright). We therefore suggest that further efforts are made to communicate the health and conservation risks of consuming wild lemurs in all communities nearby Ranomafana National Park. It is of the upmost importance that local health officials and wildlife conservationists expand their efforts to protect the critically endangered Black-and-white Ruffed Lemur.

One limitation encountered was a mixing of two collected rodent samples from the same cage. Occasionally, multiple rodents get into a trap simultaneously and fecal samples can be mixed. This occurred with only one of the rodent samples in the population. The sample tested positive for *Cryptosporidium* and was counted as only one positive. Missing survey responses from individuals were rare with only one or two missing values found in the analyzed variables. As with most survey instruments, the potential for recall bias exists in this study. Several survey questions asked individuals to recall actions taking place in the previous four weeks. One limitation, in particular, is that individuals may have described diarrheal symptoms differently. For example, one individual may describe a medically loose stool as diarrhea while another may have truly had diarrhea. Future surveys and pathogen screening can build on the methods used in this study to provide an improved understand of

relationship between reported diarrhea, human behavior, and the presence of zoonotic pathogens.

Conclusion

Interactions between humans, animals and wildlife are increasing rapidly throughout the world. The collection of baseline pathogen data and the expansion of our knowledge of zoonotic pathogen transmission are of increasing importance as the human-animal interface expands. Our study contributed to this effort through the investigation of the waterborne zoonotic pathogen *Cryptosporidium*. Working to understand how *Cryptosporidium* infection is associated with reported diarrhea and behavioral practices in and around Ranomafana National Park, Madagascar, has provided us with the opportunity to effectively promote rational public health and conservation intervention strategies as well as to encourage future studies in this region.

To briefly summarize this study's findings, there were a greater number of reported cases of diarrhea in Ambodiaviavy than Ankialo and one potential explanation for this could be poor defecation practices in Ambodiaviavy. Further pathogen screening in the area is needed. The low rate of human *Cryptosporidium* infection in this study population could suggest little to no presence of the pathogen in the human populations surrounding Ranomafana National Park. There is, however, concern over the potential zoonotic transmission between humans and pigs (*C. suis*) in the Ankialo community. Furthermore, the large number of cattle infected with *Cryptosporidium* is an important consideration for the Ambodiaviavy community. The difference in the infection rates of both pigs and cattle across communities should be further investigated. The high rates of individuals consuming food contaminated

with rodent fecal matter are troubling in both communities; however, it is of greatest concern in Ankialo. Finally, the consumption of the critically endangered Black-and-white Ruffed Lemur is disconcerting and efforts should be made to prevent such practices. Through these principal findings, we hope to encourage immediate health and conservation efforts in the region.

Finally, it is important to note the benefit of the methodology used in this study. Because large amounts of total nucleic acid were extracted from the study samples, there is the potential for future pathogen screening of all of the fecal samples collected. Our goal is to continue to expand on this baseline analysis by screening additional pathogens and then integrating those results with the survey data. Expanding our knowledge of other pathogens in this region will promote effective and efficiency health and conservation interventions.

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Tables

Table 1. Characteristics, education and employment comparison of human subjects overall and by community.

CHARACTERISTIC	OVERALL N=135	Ambodiaviavy N=65	Ankialo N=70
Sex - Female (%)	59 (43.7)	31 (47.7)	28 (40.0)
Age, mean (SD)	22.4 (17.4)	23.5 (18.6)	21.5 (16.3)
Age, Number by Group (%)			
Child (0-10 years)	40 (29.6)	18 (27.7)	22 (31.4)
Adolescent (11-17 years)	34 (25.2)	18 (27.7)	16 (22.9)
Young Adult (18-30 years)	22 (16.3)	8 (12.31)	14 (20.0)
Middle Adult (31-50 years)	25 (18.5)	12 (18.5)	13 (18.6)
Late Adult (51 years or older)	14 (10.4)	9 (13.9)	5 (7.1)
Employed (%)	60 (44.4)	31 (47.7)	29 (41.4)
Weekly Income – Malagasy AR (%)			
0 AR (No Income)	81 (60.0)	39 (60.0)	42 (60.0)
1 to 5,000 AR	22 (16.3)	12 (18.5)	10 (14.3)
5,001 to 10,000 AR	19 (14.1)	9 (13.9)	10 (14.3)
10,0001 to 15,000 AR	6 (4.4)	0	6 (8.6)
15,0001 to 20,000 AR	4 (3.0)	3 (4.6)	1 (1.4)
20,0001 and above AR	3 (2.2)	2 (3.1)	1 (1.4)
Education Level			
None (%)	30 (22.2)	20 (30.8)	10 (14.3)
Some Primary (%)	75 (55.6)	32 (49.2)	43 (61.4)
Completed Primary (%)	8 (5.9)	4 (6.2)	4 (5.7)
Some Secondary (%)	19 (14.1)	8 (12.3)	11 (15.7)
Some Technical School (%)	1 (0.7)	1 (1.5)	0
Some University (%)	2 (1.5)	0	2 (2.9)
Current Student Population (%)	46 (34.0)	17 (26.2)	29 (41.4)
Public (% of students)	39 (84.8)	16 (94.1)	23 (79.3)
Private (% of students)	7 (15.2)	1 (5.9)	6 (20.7)
Farming as Primary Profession (%)*	61 (45.2)	30 (46.2)	31 (44.3)

Note: *Several individuals noted farming as one of multiple primary professions

Table 2. Comparison of all individuals reporting diarrhea. Asterisks represent p-values <0.05.

Predictors vs. reported diarrhea (%)	**All Individuals Reporting Diarrhea N = 18**	**Chi-square Value**	**p-value**
Community			
Ambodiaviavy	15 (83.3)		*0.0018
Ankialo	3 (16.7)		
Sex			
Male	11 (61.1)	0.1957	0.6582
Female	7 (38.9)		
Age, Mean (SD)	20.8 (18)		
Age, Number by Group			
Child (0-10 years)	4 (22.2)		0.4062
Adolescent (11-17 years)	8 (44.5)		
Young Adult (18-30 years)	2 (11.1)		
Middle Adult (31-50 years)	2 (11.1)		
Late Adult (51 years or older)	2 (11.1)		
Medication Use	17 (94.4)		*0.0234
Traditional Medicine Use	12 (66.6)	1.4760	0.2244
Tends Porcine			
Never	16 (88.9)		0.3888
Sometimes	0		
Often	2 (11.1)		
Tends Bovine			
Never	10 (55.5)		0.6860
Sometimes	3 (16.7)		
Often	5 (27.8)		
Work in the fields			
Never	5 (27.8)	5.4942	0.0641
Sometimes	7 (38.9)		
Often	6 (33.3)		
Drink Boiled Water			
Never	9 (50.0)		0.2466
Sometimes	1 (5.6)		
Often	8 (44.4)		
Fetch water from an open source			
Never	14 (77.7)		0.6298
Sometimes	1 (5.6)		
Often	3 (16.7)		
Fetch water from a closed source			
Never	5 (27.8)		0.1336
Sometimes	1 (5.6)		
Often	12 (66.6)		
Defecate in a water closet			
Never	10 (55.6)		0.7248
Sometimes	0		

Often	8 (44.4)		
Defecate, not in a toilet			
Never	0		0.7205
Sometimes	3 (16.6)		
Often	15 (83.4)		
Eat food contaminated with rodent feces	11 (61.1)	1.2431	0.5371
Eat animals with visible sores	12 (66.6)	1.4835	0.2232
Wash hands before eating			
Never	3 (16.6)		0.9436
Sometimes	6 (33.3)		
Often	9 (50.0)		
Sleep with a mosquito net			
Never	9 (50.0)		0.0902
Sometimes	0		
Often	9 (50.0)		

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests

Table 3. Comparison of water use and defecation practices across communities. Asterisks represent p-values <0.05.

Predictors (%)	**Ambodiaviavy N = 65**	**Ankialo N = 70**	**Chi-square Value**	**p-value**
Community reporting diarrhea	15 (23.1)	3 (4.2)		*0.0018
Drink Boiled Water				
Never	26 (40.0)	41 (58.6)	26.7446	*<0.0001
Sometimes	5 (7.7)	20 (28.6)		
Often	34 (52.3)	9 (12.8)		
Fetch water from an open source			7.6565	*0.0217
Never	48 (73.8)	40 (57.1)		
Sometimes	8 (12.3)	6 (8.6)		
Often	9 (13.9)	24 (34.3)		
Fetch water from a closed source				
Never	22 (33.8)	40 (57.1)	7.5279	*0.0232
Sometimes	7 (10.8)	6 (8.6)		
Often	36 (55.4)	24 (34.3)		
Defecate in a water closet				
Never	50 (76.9)	22 (31.9)		*<0.0001
Sometimes	0	9 (13.1)		
Often	15 (23.1)	38 (55.0)		
Defecate, not in a toilet				
Never	2 (3.1)	6 (8.6)		0.1388
Sometimes	8 (12.3)	15 (21.4)		
Often	55 (84.6)	49 (70.0)		

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests

Table 4. Comparison of meal-time practices across communities. Asterisks represent p-values <0.05.

Predictors (%)	**Ambodiaviavy N = 65**	**Ankialo N = 70**	**Chi-square Value**	**p-value**
Eat food known to be contaminated with rodent feces	35 (53.8)	59 (84.3)	15.2198	*0.0005
Eat animals with visible sores or blisters	31 (47.7)	41 (58.6)	1.6027	0.2055
Eat uncooked meat				
Never	24 (36.9)	19 (27.5)		0.2293
Sometimes	40 (61.6)	50 (72.5)		
Often	1 (1.5)	0		
Other household members eat uncooked meat				
Never	33 (50.8)	27 (39.1)		0.3794
Sometimes	31 (47.7)	41 (59.4)		
Often	1 (1.5)	1 (1.5)		
Wash hands before eating				
Never	9 (13.8)	14 (20.6)	1.6136	0.4463
Sometimes	23 (35.4)	26 (38.2)		
Often	33 (50.8)	28 (41.2)		

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests

Table 5. Comparison of other risk factors across communities. Asterisks represent p-values <0.05.

Predictors (%)	**Ambodiaviavy N = 65**	**Ankialo N = 70**	**Chi-square Value**	**p-value**
Sleep with a mosquito net				
Never	28 (43.1)	61 (89.7)		*<0.0001
Sometimes	4 (6.1)	3 (4.4)		
Often	33 (50.8)	4 (5.9)		
Work in the fields				
Never	18 (27.7)	20 (29.0)	3.8794	0.1437
Sometimes	17 (26.2)	9 (13.0)		
Often	30 (46.1)	40 (58.0)		
Tends Porcine**				
Never	61 (93.8)	40 (57.1)		*<0.0001
Sometimes	0	9 (12.9)		
Often	4 (6.2)	21 (30.0)		
Tends Bovine				
Never	44 (67.7)	23 (32.8)	19.9443	*<0.0001
Sometimes	9 (13.8)	9 (12.9)		
Often	12 (18.5)	38 (54.3)		
Crops raided in last four weeks	24 (36.9)	21 (30.0)	0.7269	0.3939
Used medication in last four weeks	52 (80.0)	45 (65.3)	4.1151	*0.0425
Used a tradition remedy in last four weeks	41 (64.1)	30 (43.5)	5.6533	*0.0174

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests. ** Only one porcine sample was collected from Ambodiaviavy.

Table 6. Individuals reported medication use by community. Asterisks represent p-values <0.05.

Predictors (%)	**Ambodiaviavy N = 65**	**Ankialo N = 70**	**Chi-square Value**	**p-value**
Antibiotics	32 (49.2)	30 (42.9)	0.5513	0.4578
Amoxicillin	9 (13.8)	9 (12.9)	0.0285	0.8659
Cotrimoxazole	22 (33.8)	22 (31.4)	0.0897	0.7646
Metronidazole	8 (12.3)	1 (1.4)		*0.0144
Ampicillin	2 (3.1)	1 (1.4)		0.6085
Erythromycin	0	1 (1.4)		n/a
Tetracyclin	0	1 (1.4)		n/a
Other antibiotics	1 (1.5)	0		n/a
Anti-parasitic medications	5 (7.7)	3 (4.3)		0.4810
Nivaquine (anti-malaria)	1 (1.5)	0		n/a
Quinine (anti-malaria)	2 (3.1)	0		n/a
Mebendazole (anti-worm)	2 (3.1)	3 (4.3)		n/a
Levamisole	0	1 (1.43)		n/a

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests

Table 7. Characteristics of individuals using medications. Asterisks represent p-values <0.05.

Predictors (all communities)	Antibiotic use (%)				Anti-parasitic use (%) N = 8
	Overall N = 62	Amoxicillin N = 18	Cotrimoxazole N = 44	Metronidazole N = 9	
Sex					
Male	33 (53.2)	10 (55.5)	22 (50.0)	5 (44.4)	3 (62.5)
Female	29 (46.8)	8 (44.5)	22 (50.0)	4 (55.6)	5 (37.5)
Chi-square	0.4394	0.0046	1.0518	0.0022	
p-value	0.5074	0.9457	0.3051	0.9630	0.2963
Diarrhea					
Yes	15 (24.2)	2 (11.1)	8 (18.2)	8 (88.8)	2 (25.0)
No	47 (75.8)	16 (88.9)	36 (81.8)	1 (11.2)	6 (75.0)
Chi-square	11.7028		1.3279		
p-value	*0.0006	1.0000	0.2492	*<0.0001	0.2891
Drink Boiled Water					
Never	38 (61.3)	11 (61.1)	26 (59.1)	6 (66.7)	4 (50.0)
Sometimes	5 (8.1)	2 (11.1)	3 (6.8)	0	2 (25.0)
Often	19 (30.6)	5 (27.8)	15 (34.1)	3 (33.3)	2 (25.0)
Chi-square	9.9602	1.2753			
p-value	*0.0069	0.5285	*0.0418	0.3901	0.7948

Notes: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests.

Table 8. Reported interactions with lemur populations by community.

Human Interactions with Lemurs	**Ambodiaviavy N= 65**	**Ankialo N = 70**
Red-fronted Brown Lemur (*Eulemur rufifrons*)		
Contact with nest or bedding	0	1
Prepared for cooking	0	1
Ate	0	1
Black-and-white ruffed lemur (*Varecia variegata*)		
Contact with nest or bedding	0	0
Prepared for cooking	0	0
Ate	1	0
Brown Mouse Lemur (*Microcebus rufus*)		
Contact with nest or bedding	1	0
Prepared for cooking	2	0
Ate	2	0

Note: Brown Mouse Lemur IUCN Status = least concern, Red-fronted Brown Lemur's status = near threatened, and the Black-and-white Ruffed Lemur's status = critically endangered. Information from IUCN (International Union for Conservation of Nature) webpage (http://www.iucnredlist.org/).

Table 9. Total prevalence of *Cryptosporidium* across species.

Sample Type	**Samples Collected**	**Positive for *Cryptosporidium***	**% Positive**
Wildlife	**28**	**1**	**3.6**
Lemur	25	1	4.0
Rodent (wild)	2	0	0
Fossa	1	0	0
Domestic Animals	**82**	**23**	**28.0**
Bovine	62	18	29.0
Porcine	17	4	23.5
Canine	1	1	100
Feline	2	0	0
Peri-domestic Rodents	**48**	**16**	**33.3**
Human	**120**	**1**	**0.8**
Totals	**278**	**41**	**14.7**

Table 10. Prevalence of *Cryptosporidium* in species on a community level.

	Ambodiaviavy			**Ankialo**			**Ranomafana National Park**		
Sample Type	**# of Samp.**	**# Pos.**	**% Pos.**	**# of Samp.**	**# Pos.**	**% Pos.**	**# of Samp.**	**# Pos.**	**% Pos.**
Wildlife	**-**	**-**	**-**	**-**	**-**	**-**	**28**	**1**	**3.6**
Lemur	-	-	-	-	-	-	25	1**	4.0
Rodent (wild)	-	-	-	-	-	-	2	0	0
Fossa	-	-	-	-	-	-	1	0	0
Domestic Animals	**37**	**18**	**48.6**	**45**	**4**	**8.9**	**-**	**-**	**-**
Bovine	35	18	51.4	27	0	0	-	-	-
Porcine	1	0	0	16	4	25.0	-	-	-
Canine	1	1	100	0	0	0	-	-	-
Feline	0	0	0	2	0	0	-	-	-
Peri-domestic Rodents	**18**	**4**	**22.2***	**30**	**12**	**40.0**	**-**	**-**	**-**
Human	**59**	**0**	**0**	**61**	**1**	**1.6**	**-**	**-**	**-**
Totals	**114**	**23**	**20.2**	**136**	**17**	**12.5**	**28**	**1**	**3.6**

Notes: * = Fisher P value was insignificant = 0.3431; Other p-values were not necessary for this table due to either lack of sample size or zero percent prevalence; ** = positive lemur sample was found at a Camp Site approximately 1 km from Ankialo.

Table 11. Number of positive *Cryptosporidium* cases associated with households.

	Household Identification Number	Bovine	Porcine	Rodent	Human	Canine	**Total**
Ambodiaviavy	**1**	2	-	0	0	-	**2**
	2	1	-	0	0	-	**1**
	3	1	-	0	0	-	**1**
	4	2	-	3	0	-	**5**
	5	2	-	1	0	-	**3**
	6	3	-	0	0	-	**3**
	7	1	-	0	0	-	**1**
	8	1	-	-	0	1	**2**
	9	5	-	0	0	-	**5**
Ankialo	**3**	0	0	7	0	-	**7**
	5	0	1	1	0	-	**2**
	7	0	1	1	0	-	**2**
	8	0	0	1	0	-	**1**
	9	0	-	0	1	-	**1**
	10	0	2	0	0	-	**2**

Notes: Households were excluded if no subjects were positive for *Cryptosporidium*. (-) = no samples collected from species in household.

Table 12. Comparison of individuals living in households associated with at least one case of *Cryptosporidium* in bovine, porcine, rodents, and humans. Asterisks represent p-values <0.05.

Species	Ambodiaviavy N=65 (%)	Ankialo N=70 (%)	Chi-square Value	p-value
Bovine	60 (92.3)	0	-	<0.0001
Porcine	0	24 (34.3)	-	-
Rodents	20 (30.8)	35 (50.0)	5.1628	*0.0231
Humans	0	8 (11.4)	-	-

Note: When expected cell counts were less than 5, two-tailed P values were calculated using Fisher's exact tests. No correlation analysis across community occurred for porcine because there was only one porcine sampled in Ambodiaviavy.

Table 13. Number of fecal samples collected from lemurs by species and location within Ranomafana National Park (N=25).

Lemur Species	Common Name	IUCN Status**	Location	Number Sampled	Positive for *Cryptosporidium*
Microcebus rufus	Brown Mouse Lemur	Least Concern	Camp Site Centre ValBio	3 1	Positive* Negative
Eulemur rubriventer	Red-bellied Lemur	Vulnerable	Sakaroa	5	Negative
Avahi peyrierasi	Peyrieras' Woolly Lemur	Data Deficient	Ambatolahidimy	1	Negative
Prolemur simus	Greater Bamboo Lemur	Critically Endangered	Talatakely-RNP	4	Negative
Hapalemur aureus	Golden Bamboo Lemur	Endangered	Talatakely-RNP	2	Negative
Propithecus edwardsi	Milne-edward's Sifaka	Endangered	Valohoaka-RNP Talatakely-RNP	6 3	Negative Negative

Note: *There was only 1 positive lemur sample at this location. **Information from IUCN (International Union for Conservation of Nature) webpage (http://www.iucnredlist.org/). Status rankings used by IUCN: extinct, extinct in the wild, critically endangered, endangered, vulnerable, near threatened, and least concern.

Table 14. Subtype of *Cryptosporidium* positive specimens.

Sample Type (Gender: M/F or U: unknown)	**Species**	**Location**	**Associated Household by Location**	**RFLP Interpretation**
Bovine (F)	*Bos indicus*	Ambodiaviavy	1	*C. muris*
Bovine (F)	*Bos indicus*	Ambodiaviavy	1	Unknown
Bovine (F)	*Bos indicus*	Ambodiaviavy	3	*C. parvum geno A*
Bovine (F)	*Bos indicus*	Ambodiaviavy	6	*C. suis*
Bovine (F)	*Bos indicus*	Ambodiaviavy	6	*C. suis*
Bovine (F)	*Bos indicus*	Ambodiaviavy	7	*C. suis*
Bovine (F)	*Bos indicus*	Ambodiaviavy	9	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	2	*C. parvum geno A*
Bovine (M)	*Bos indicus*	Ambodiaviavy	4	*C. muris*
Bovine (M)	*Bos indicus*	Ambodiaviavy	4	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	5	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	5	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	6	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	8	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	9	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	9	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	9	*C. suis*
Bovine (M)	*Bos indicus*	Ambodiaviavy	9	*C. suis*
Canine (M)	*Canis familiaris*	Ambodiaviavy	8	*C. canis*
Human (M)	*H. sapiens*	Ankialo	9	*C. suis**
Lemur (M)	*Microcebus rufus*	Camp Site	NA	*C. hominis**
Porcine (F)	*Sus domesticus*	Ankialo	7	*C. suis*
Porcine (M)	*Sus domesticus*	Ankialo	5	*C. suis**
Porcine (M)	*Sus domesticus*	Ankialo	10	*C. parvum*
Porcine (M)	*Sus domesticus*	Ankialo	10	*C. suis*
Rodent (F)	*Rattus rattus*	Ankialo	8	*C. parvum*
Rodent (F)	*Rattus rattus*	Ankialo	Unknown	*C. suis*
Rodent (F)	*Mus musculus*	Ankialo	3	*C. tyzzeri*
Rodent (F)	*Rattus rattus*	Ankialo	3	*C. mouse geno*
Rodent (F)	*Rattus rattus*	Ankialo	3	Unknown
Rodent (M)	*Rattus rattus*	Ankialo	3	*C. baileyi*
Rodent (M)	*Mus musculus*	Ankialo	5	*C. tyzzeri*
Rodent (M)	*Rattus rattus*	Ankialo	7	*C. baileyi*
Rodent (M)	*Rattus rattus*	Ankialo	Unknown	*C. baileyi*
Rodent (M)	*Rattus rattus*	Ankialo	3	Unknown
Rodent (M)	*Rattus rattus*	Ankialo	3	*C. mouse geno*

Rodent (M,F)	*Rattus rattus*	Ankialo	3	*C. baileyi*
Rodent (U)	*Rattus rattus*	Ambodiaviavy	5	*C. mouse geno*
Rodent (U)	*Rattus rattus*	Ambodiaviavy	4	*C. mouse geno*
Rodent (U)	*Rattus rattus*	Ambodiaviavy	4	*C. mouse geno*
Rodent (U)	*Rattus norvegicus*	Ambodiaviavy	4	Unknown

Note: This is preliminary subtyping results based on RFLP analysis. Genomic sequencing is ongoing at the CDC and will be used to confirm results. *Denotes sequencing that has been completed and confirmed.

Table 15. Frequency of *Cryptosporidium* subtypes in study population.

Subject Species	**Subtype of *Cryptosporidium***	**Number of each Subtype**
Human	*C. suis*	1
Bovine	*C. suis*	13
	C. muris	1
	C. parvum genotype A	2
Porcine	*C. suis*	3
	C. parvum	1
Rodent	*C. suis*	1
	C. parvum	1
	C. tyzzeri	2
	C. mouse genotype	5
	C. baileyi	4
	Unknown	3
Lemur	*C. hominis*	1
Canine	*C. canis*	1

Note: This is preliminary subtyping results based on RFLP analysis. Genomic sequencing is ongoing at the CDC and will be used to confirm results.