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Molecular characterization and zoonotic potential of *Cryptosporidium* species in free-ranging poultry in rural Madagascar

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Environmental Science Department

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Abstract

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Zoonotic pathogens are a major global health concern. With increasing deforestation and human encroachment, zoonotic pathogens have begun to pose a serious threat to human health and overall ecosystem health. Despite the preliminary understanding of zoonosis, much is left to learn about the ecology, epidemiology, and transmission of these infectious diseases. On the island of Madagascar, diarrheal disease is a major public health burden and the leading cause of death for children under five years of age. *Cryptosporidium* is a protozoan parasite that can infect the majority of mammals and birds. Previous studies have examined the zoonotic potential of *Cryptosporidium* in mammals in rural developing landscapes, but the role of poultry has not been fully examined. To investigate poultry's role in the transmission of *Cryptosporidium*, from May to August 2012, fecal samples were collected from chickens, ducks, and geese in southeastern Madagascar. DNA was extracted from the samples using *18S* PCR of the SSU rRNA gene and species of *Cryptosporidium* were defined using Restriction Fragment Length Polymorphism (RFLP). There was a presence of *Cryptosporidium* in these three villages in humans, livestock, poultry, and rodents. Poultry had the highest percentage of infection. RFLP evidence suggests that species of *Cryptosporidium* were found in their expected species, with some suspect results. It appears that poultry do not represent a source of zoonotic *Cryptosporidium* for humans in this region of Madagascar. These findings highlight that although *Cryptosporidium* is prevalent in poultry in this rural system, the species of *Cryptosporidium* occurring in poultry and humans in this system are distinct.

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Acknowledgements

Permission to conduct this research was provided by the Government of Madagascar and the Stony Brook University Internal Review Board and Institutional Animal Care and Use Committee. This study was supported by the Jim and Robin Herrnstein Foundation and the Emory University Global Health Institute. I would like to thank the staff of ICTE, MICET, Centre ValBio and Dr. Patricia Wright for their research support and all their help. I would like to thank Michele Parsons of the Enteric Branch at the Centers for Disease Control and Prevention for training me, helping me interpret my RFLP results, and her assistance throughout the duration of this project. I especially would like to thank Dr. Thomas Gillespie for serving as my mentor and thesis committee chair, and providing me with the opportunity to train and work at Emory University, Rollins School of Public Health, the CDC, and Centre Valbio in Madagascar. Thanks is also due to Dr. Sarah Zohdy who coordinated fieldwork during my time in Madagascar, assisted me with work in the Rollins laboratory, and provided feedback on many aspects of my work. I would like to thank my team members Jonathan Bodager, Kristin Derfus, Dr. Cassidy Rist, Emily Headrick, Morgan Mercer, Paul Kennedy, Ashlee Espenson, Dr. Fidisoa Rasambainarivo, and Randi Griffin. Lastly I would like to thank Emory University, the Environmental Science Department, Rollins School of Public Health, and SIRE.

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Abstract

Zoonotic pathogens are a major global health concern. With increasing deforestation and human encroachment, zoonotic pathogens have begun to pose a serious threat to human health and overall ecosystem health. Despite the preliminary understanding of zoonosis, much is left to learn about the ecology, epidemiology, and transmission of these infectious diseases. On the island of Madagascar, diarrheal disease is a major public health burden and the leading cause of death for children under five years of age. *Cryptosporidium* is a protozoan parasite that can infect the majority of mammals and birds. Previous studies have examined the zoonotic potential of *Cryptosporidium* in mammals in rural developing landscapes, but the role of poultry has not been fully examined. To investigate poultry's role in the transmission of *Cryptosporidium*, from May to August 2012, fecal samples were collected from chickens, ducks, and geese in southeastern Madagascar. DNA was extracted from the samples using *18S* PCR of the SSU rRNA gene and species of *Cryptosporidium* were defined using Restriction Fragment Length Polymorphism (RFLP). There was a presence of *Cryptosporidium* in these three villages in humans, livestock, poultry, and rodents. Poultry had the highest percentage of infection. RFLP evidence suggests that species of *Cryptosporidium* were found in their expected species, with some suspect results. It appears that poultry do not represent a source of zoonotic *Cryptosporidium* for humans in this region of Madagascar. These findings highlight that although *Cryptosporidium* is prevalent in poultry in this rural system, the species of *Cryptosporidium* occurring in poultry and humans in this system are distinct.

Introduction

Disease burdens of developing and industrialized countries differ significantly (Kowalewski and Gillespie, 2009). Three fourths of emerging infectious diseases are zoonotic. A zoonotic disease is a disease that can be transmitted between animals and humans (Jones *et. al,* 2008). *Cryptosporidium*, a zoonotic protozoan that causes diarrhea, is responsible for a large number of human infections in developing countries. In poor and developing countries, diarrhea is a major cause of death (WHO, 2009). *Cryptosporidium* infects a wide range of wild animals, domestic animals, and humans (Appelbee *et. al*, 2005). It is one of the most common diarrhea causing parasitic genera in the world, and has been classified by the National Institute of Health as a category B priority pathogen (Tzipori *et. al*, 2008). In humans and livestock, *Cryptosporidium* causes cryptosporidiosis, a gastrointestinal illness characterized by diarrhea, malnutrition, and impaired weight gain (Savioli *et. al* 2006; Nime *et. al* 1976; Meisel *et. al* 1976). Within a global context, diarrheal diseases are the second highest cause of death for children under the age of five (Kerridge *et. al,* 2013). Specifically in Madagascar, diarrheal disease burden is intensified (WHO, 2009). Understanding how *Cryptosporidium* is transmitted in Madagascar can help reduce this major public health concern.

Cryptosporidium is a common protozoan parasite of humans, domestic animals, and wildlife. Due to the wide host range, cryptosporidiosis has been considered a zoonotic disease, but the role of animals in transmission of this disease, especially poultry, is unclear. Even though *Cryptosporidium meleagridis* (an avian *Cryptosporidium*) is the third most common *Cryptosporidium spp*. infecting humans, there have been no reports where a zoonotic source had been found (Silverlas *et. al,*

2012).

Despite this lack of information on poultry's role in the transmission of *Cryptosporidium* between humans and other animals, there is observational and anecdotal data to suggest that in rural areas of Madagascar where poultry interactions are unique, there is potential for its zoonotic role. Village chicken systems in rural Madagascar are of great importance (Figure 1). They are referred to as "backyard poultry." Instead of housing chickens in pens, chickens roam free between households and other areas of the village. The free ranging behavior of chickens, and the time spent inside households are disease risk factors. Poultry production constitutes the majority of animal farming in rural Madagascar (FAOSTAT, 2008). It is a common practice in rural African villages to raise chickens for meat and eggs for households. A unique feature of Madagascar is that families have an area of the house for chickens, and it is a common practice to keep chickens inside the house at night (Kitalyi, 1998). In villages in the southeastern rainforest corridor in Madagascar, livestock and domestic animals are typically kept in the household at night and released during the day.

Current research conducted in the villages of the southeastern rainforest corridor has shown that the interaction between livestock and domestic animals and their interactions with humans, animals, waterways, and feces suggest a strong potential for zoonotic transmission especially for poultry (Wegner, 2013). In order to strengthen our knowledge of the transmission of zoonotic pathogens, this study is designed to examine *Cryptosporidium* in rural villages in Ifanadiana district of southeastern Madagascar with an emphasis on poultry.

Madagascar and Diarrheal Diseases

The World Bank estimated that more than one third of the world's population lives on less than USD two dollars per day, including Madagascar. Extreme poverty restricts access to diagnosis and treatment, contributing to the lack of detection of emerging or re-emerging zoonoses (Macpherson, 2005). Madagascar is one of the poorest countries in the world. The GDP per capita income is 462 USD (United Nations, 2010). Diarrhea is widespread in impoverished countries, and is a leading cause of malnourishment and malnutrition in children of developing areas (WHO, 2014). As mentioned above, cryptosporidiosis is characterized by diarrhea. Globally, diarrhea kills approximately 760,000 children under the age of five per year. According to the World Health Organization, there are 1.7 billion cases of diarrheal diseases every year, and it is the second leading cause of death for children under five (WHO, 2013). In Madagascar, 2.2 million people die annually (WHO, 2006). Seventeen percent of children less than five years of age die from diarrheal diseases, and two percent of neonates die from diarrheal disease as well. Diarrheal diseases are the fourth leading cause of death for all ages in Madagascar (WHO, 2006). For diarrheal disease, the contribution of novel zoonotic transmission of human-to-human transmission on overall disease burden is unknown (Tzipori *et. al,* 2008). In order to prevent gastrointestinal illnesses that are foodborne or waterborne, we must be accurate in our assumptions that we know the causative agents or pathogens and that we know the risk factors associated with its spread (Denno *et. al*, 2007).

Cryptosporidium

Cryptosporidiosis has been considered a zoonotic disease because it is not always

host specific and can infect a wide range of organisms. The role of animals, especially poultry, in rural villages in Madagascar is unknown. To the best of my knowledge, there have not been studies to examine the role poultry play in transmitting *Cryptosporidium* in rural villages in Madagascar. *Cryptosporidium* is waterborne and characterized by a protective walled oocyte, which enables it to survive outside the host in unfavorable environments for up to several weeks (Smith, 2010). Parasites have unique features that enable them to survive for long periods in unfavorable environmental conditions, unlike bacteria and viruses. This ability makes many parasites difficult to control or detect. Transmission stages of waterborne parasites are highly resistant to external environments, and to disinfectant chemicals (Gajadhar and Allen, 2004). As mentioned above, *Cryptosporidium* is able to infect a diverse range of species and has numerous transmission routes (Xiao and Ryan, 2004). These transmission routes include: person-to-person, animal-to-person, waterborne and foodborne transmission routes (Xiao and Ryan, 2004). The oocysts are infectious when shed. This allows for direct person-to-person transmission (Huang and White, 2006). Unlike other common forms of diarrhea, there has not been the development of an effective treatment. Despite efforts to develop models and evaluation of approximately 1,000 chemotherapeutic agents, treatment to clear the host of *Cryptosporidium* is nonexistent (Mead and Arrowood, 2014).

Disease risk of poultry

In avian species, cryptosporidiosis can cause respiratory or enteric infections. There have been reports of over 30 species of *Cryptosporidium* in birds. *Cryptosporidium* has been found in chickens, turkeys, quail, pheasants, peafowl, junglefowl, ducks, geese,

parrots, finches, lovebirds, and budgerigars (Lindsay and Blagburn, 1990). *Cryptosporidium meleagridis* and *C. baileyi* originated in avian species (Streter and Varga, 2000). There have been cases of *C. hominis*, *C. parvum*, *C. muris*, and *C. andersoni* in poultry (Ryan *et. al*., 2003, Zhou *et. al*., 2004 and Ng *et al*., 2006). Poultry are an indigenous and integral part of the farming system. They are economically beneficial to poor communities because they have short life cycles with quick turnovers, low input but outputs at interhousehold and intrahousehold levels, land is not a limiting factor, and they convert low quality feed to high quality protein. Village chickens are the basis for increasing food production and income in rural African communities. For developing countries, smallholder poultry production is an important source of food security and helps to alleviate poverty in rural areas. Smallholder poultry production is a large part of rural villages. Chickens produced in small farms are eaten locally, either from the original household or by consumers in the market. Most of these markets are live bird markets, meaning the chicken is sold alive (Paul *et. al*, 2013). 97% of Ethiopia's 49.3 million chickens are backyard, indigenous poultry. The backyard poultry include small groups of mixed ages that feed by scavenging. Chickens may be exposed to disease causing agents because they commonly mix with chickens in multiple households. Due to their scavenging behavior, they come into frequent contact with different household flocks, which can facilitate the spreading of diseases such as New Castle Disease (Chaka *et. al,* 2013). Similar to Ethiopia, 83% of Madagascar's poultry is backyard poultry (Almeida *et. al*, 2009). Poultry farming for fighting and meat birds is a large part of Malagasy culture. The indigenous Malagasy chicken has dual geographic and genetic origin. It is an important livestock species in Madagascar. Malagasy chickens have been

linked to viral infection, such as vesicular stomaties and avian influenza (Razafindraibe *et. al,* 2008). A limited number of birds have been identified as having *C. meleagridis*, which is the third most important species for human cryptosporidiosis. *Cryptosporidium meleagridis* is the only species that can infect both birds and mammals. There is a wide occurrence of *C. meleagridis* in domestic birds. In some developing countries such as Peru and Thailand, *C. meleagridis* accounts for ten to twenty percent of cryptosporidiosis in humans (Xiao and Feng. 2008). In Madagascar, there are 34.4 million domestic poultry, with the majority in smallholder production systems in rural populations (FAOSTAT, 2008). Some recent studies show that avian *Cryptosporidium* cannot be transmitted to mammals and mammal *Cryptosporidium* cannot be transmitted to avian species; however, other literature refutes this claim. Richter *et. al* experimented with oocysts from ducklings, calves, and mice and found that avian *Cryptosporidium* could not be transmitted to mammals and mammal *Cryptosporidium* was not able to be transmitted to the chickens, ducks, and geese (Ritcher *et. al,* 1994). The paper cites several authors with similar results (Current *et. al*., 1986; O'Donogue *et. al,* 1987; Lindsay *et. al* 1989,1990), and concludes that grazing of waterfowl and cattle on the same land poses no mutual health risks of *Cryptosporidium* infection (Ritcher *et. al,* 1994).

Objectives and Hypothesis

This project aims to strengthen our understanding of the zoonotic potential of poultry-associated *Cryptosporidium* for humans in southeastern Madagascar. It is part of a longitudinal research project, and the results will contribute to an ongoing study of diarrheal pathogens in southeastern Madagascar from a one-health perspective.

Cryptosporidium was examined in three distinct villages, specifically looking at the role of free ranging poultry. It was predicted that the majority of positive samples for *Cryptosporidium* would come from chicken, ducks, and geese. In addition, the project aims to investigate the sources of *Cryptosporidium*, to see if *Cryptosporidium* commonly found in chickens is found in humans or other animals in the villages. Samples from chicken, geese, duck, humans, rodents, and bovine (zebu) were collected for analysis. A major goal is to discover the specific species of *Cryptosporidium* to better understand its zoonotic role. Because a human has tested positive in these villages and livestock for *C. suis* (pig genotype), it is predicted that the species of *Cryptosporidium* will be capable of zoonosis and poultry *Cryptosporidium* will be found in mammals and vice versa. It was predicted that there would be overlap between species found in poultry and species of humans, livestock, and rodents. This study should add to our knowledge of transmission patterns, host dynamics, and human risk factors.

Methods

Study Site

The study location is in the southeastern countryside of Madagascar. The villages border Ranomafana National Park (between 21°02'–21°25'S and 47°18'–47°37'E), a 43,500 hectare UNESCO World Heritage Site (Figure 2). The average monthly precipitation is 508mm for the wet season (January to March) and 143mm for the dry season (June to October) (Dunham *et. al*, 2011). There are cyclones in Madagascar from December to April. Warm phases are associated with heavy rains (Dunham *et. al,* 2011).

Three villages were selected from a district of 65 villages. The villages were chosen to create heterogeneity, and were chosen based on their drastic differences. Ten households were randomly selected in each village. The villages were: Ankialo, Ambodiaviavy, and Ambatolahy. They differ in location, culture, tribe, economic status, agriculture, landscape, proximity to forest, and number and composition of livestock owned (Wegner, 2013).

The study population included chicken, duck, geese, bovine (zebu), rodents, and porcine. Three communities located on the boundary of the Ranomafana National Park were selected for study: Ambodiaviavy, Ankialo, and Ambatolahy. A list of households was provided by staff at Centre ValBio, the world renowned research center located in the national park. Random numbers were assigned to each household, and ten households in each village were picked for survey and fecal sample analysis. To be eligible for selection, the households had to have at least one child and own livestock or poultry. Additional animals not matched to survey data were analyzed as well.

Fecal Sample Collection

Survey participants were asked to donate a fecal sample for diarrheal disease analysis. Fecal samples were collected from domesticated animals in the household, and baited live-traps were used to collect rodents to obtain fecal samples. Any livestock or poultry owned by the families were examined as well. Fecal samples were collected from wildlife noninvasively. Immediately after collection, fecal samples were preserved in RNA later ® [Cat# 76104] (Qiagen Inc., Valencia, CA). Samples were stored at room temperature until DNA extraction. Fecal Samples were taken back to the Gillespie lab in Rollins School of Public Health in Atlanta, Georgia for further analysis.

DNA Extraction

DNA was collected from all the fecal samples using the FastDNA methods in ® SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH), following protocols that can be found in da Silva *et al*., 1999. Large amounts of nucleic acid were collected so that multiple pathogens could be screened.

18S Polymerase Chain Reaction

After extraction, 18S nested PCR targeting a 214-bp fragment was performed using four primers: 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. PCR was performed following methods outlined in Bodager, 2011. Twenty microliters of the PCR products were electrophoresed on a two percent SeaKem® LE Agarose, (Lonza, Rockland, ME) gels with five microliters of the PCR product, were stained with ethidium bromide, and a gel image was captured under UV exposure (Figure 3). Negative controls were reaction mixtures of sterile water, PCR reagents, and did not contain any nucleic acid. Positive controls were provided by the Waterborne Disease lab of the Centers for Disease Control and Prevention.

Restriction Fragment Length Polymorphism

Analysis of suspect positive specimens was conducted using Restriction Fragment Length Polymorphism (RFLP) screening as outlined in Xiao *et al.* 1999. Restriction was completed using two digestion enzymes: *Ssp*I and *Vsp*I. The restriction was carried out in one point five milliliter microcentrifuge tubes. The *Ssp*I reaction mixture consists of two point seven five microliters of sterile water, zero point five microlitres of Buffer *Ssp*I, zero point five microliters of *Ssp*I Enzyme [Product No. R0132L, New England BioLabs, Beverly, MA], and ten microliters of secondary PCR reaction. The *Vsp*I reaction mixture consists of three microliters of sterile water, zero point five microliters of Buffer D, zero point twenty-five microliters of *Vsp*I Enzyme [Product No. R6851, Promega, Madison, WI], and ten microliters of secondary PCR reaction. All samples were incubated for five hours to overnight in a 37ºC water bath. Five microliters of restriction digests were electrophoresed on a two percent SeaKem® LE Agarose gels stained with ethidium bromide, and the image was captured under UV exposure. 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.*

Statistical Analysis

Logistic regression was performed using poultry or non-poultry as an independent variable and infection as the dependent variable. Logistic regression was also performed to find associations between communities and infection. The percent of positive samples was calculated and the percent of negative samples were calculated for overall samples, species, households, and communities. Logistic regression was performed on fecal composition and infection.

Ethical Considerations

This study was submitted to Emory University's Institutional Review Board and was found to be exempt from requiring approval. Animal samples were collected in accordance with Stony Brooke IACUC.

Results

There were a total of 232 fecal samples screened for *Cryptosporidium* (Table 1). Fecal samples consisted of 70 chicken, 17 duck, six geese, 34 bovine, and 41 pigs, four humans, and 60 rodents (Table 1). Bovine, pigs, humans, and rodents were screened to see if the *Cryptosporidium* infecting them was of avian origin. Table 2 shows the number of fecal samples collected, the number positive for *Cryptosporidium,* and the amount of positive *Cryptosporidium* samples by species in Ambatolahy, Ambodiaviavy, and Ankialo. A total of 31 samples or 14% of the samples tested positive for the pathogen, with 12.6% testing positive for Ambatolahy, seven point eight percent for Ambodiaviavy, and 32.4% testing positive in Ankialo (Table 2). Fecal analysis revealed an overall prevalence of *Cryptosporidium* of chicken 14.3%, duck 23.5%, geese 16.7%, bovine zero percent, pig 14.6%, humans 25%, and rodents 20% (Table 1).

In Ambatolahy, the percentage of positive *Cryptosporidium* samples in chickens was 20%, duck seven point seven percent, and geese zero percent (Table 2). Chickens were housed with porcine, bovine, rodents, and ducks. There was only one household with chickens as the sole animal. Ducks were housed with bovine, porcine, chickens, and rodents. Geese were housed alone, and were only found in one household (Table 3).

For Ambodiaviavy, the presence of *Cryptosporidium* in chickens was ten percent, duck samples were not collected, and geese had 50% positive samples (Table 2). There were no ducks in the household surveyed. Chickens were housed with bovine, rodents, and porcine. Geese were housed with bovine and rodents (Table 3).

There was only one household owning chickens, and there was no *Cryptosporidium* found in the chickens of Ankialo. Similarly, only one household owned

geese and the samples were not found to be positive for *Cryptosporidium.* The percentage of positive *Cryptosporidium* samples in ducks was 75% (Table 2). The chickens were alone and the geese were kept with porcine. The ducks were in households with rodents and porcine (Table 3).

In Ankialo, Bodager found a human with *C. suis.* This is pig *Cryptosporidium*. There were no avian sources of *Cryptosporidium* found in any of the human samples (Table 4). Additional humans were tested in Ambatolahy (Table 4). Only one was found to be positive, but the genotype of *Cryptosporidium* was *C. hominis*. In Ambatolahy, there was a human infected, 20% of chickens were infected, and seven point seven percent of ducks were infected. In Ankialo, where a human tested positive for *Cryptosporidium*, 75% of ducks were infected. In Ambodiaviaviy, where no humans tested positive, ten percent of chickens and 50% of ducks were positive (Table 4).

Logistic regressions were performed to see if there were any associations between poultry and percentage of positive samples, and to see if there were associations between the amount of positives samples and villages. Poultry did not test as statistically significant. The null hypothesis was that there is no association between poultry and positive samples. The alternative hypothesis was that there is an association between poultry and positive samples. The p-value was 0.196 with an odds ratio of 0.494 (Table 5). Chicken, geese, and ducks were combined and bovine, rodents, and pigs were combined to increase sample size. After performing a power analysis, N was found to be too small. Logistic regressions were also performed with an independent variable of villages and dependent variable of infection. The p-value for village was 0.885 with an odds ratio of 1.072 (Table 5). The interaction between villages and species was accounted

for to see if there was an association with the amount of positive samples. The p-value for village and species was 0.813 with an odds ratio of 1.028 (TABLE 5).

Subtyping for specific species through Restriction Fragment Length Polymorphism after *VspI* and *SspI* digests showed expected species (Figure 4-7) and Figure 8-9). RFLP evidence suggests that the *Cryptosporidium* species are the expected species common to the animal. Chickens, ducks, and geese had *C. meleagridis* or *C. baileyi,* bovine had *C. parvum*, rodents had *C. muris* (Figure 10), pigs had *C. suis* and humans had *C. hominis*.

Of the positive samples for poultry with a presence of *Cryptosporidium*, nine or 64.29% were described as being liquid, and two or 13.33% were described as being liquid and bloody. Only two or 13.33% were labeled as normal. One sample or six point sixty-seven percent was labeled as being black and thick. Chicken samples included two normal samples, six liquid samples, and one bloody sample. Duck samples included three liquid samples and a black and thick sample. The one goose sample had a bloody liquid composition (Table 6). These results suggest that perhaps the chickens testing positive were symptomatic for cryptosporidiosis. After running a logistic regression, liquid fecal composition was statistically significant, having a p-value of 0.00816.

Poultry sampled for *Cryptosporidium* lived in households that owned livestock and where rodents were found. Chickens were never the sole animals in a household. They were found to be in houses with bovine, rodents, porcine, and ducks. No chickens were found in houses with geese. Geese were found in households that owned no other animals or with porcine, bovine, and rodents. Geese were not found in households with

ducks. Ducks were never the sole animals in a household. Ducks were in households containing porcine, rodents, chicken, and bovine (Table 7).

Discussion

This study found a presence of *Cryptosporidium* in animal and human samples from the three selected villages. 14% of samples tested were positive for *Cryptosporidium*. Ankialo had the most positive samples, but it was not statistically significant. Poultry species had more positive samples than the other species, but was not statistically significant. There was no avian *Cryptosporidium* in non-avian species, and there was no mammalian *Cryptosporidium* in avian species. A liquid fecal composition of poultry samples tested as statistically significant and may indicate that poultry were symptomatic for *Cryptosporidium.*

The presence of *Cryptosporidium* has been found in primates of Ranomafana National Park (Rasambainarive *et. al,* 2013). The first report of *Cryptosporidium* in the rufous mouse lemur and the critically endangered greater bamboo lemur were found by Rasambainarive *et. al* 2013. The presence of *Cryptosporidium* in wild nonhuman primates can be argued to show an increased contact with humans or domestic livestock (Nizayi *et. al,* 2002). In Ranomafana National Park the presence of these parasites in primates, chickens, ducks, geese, a few humans, pigs, and cattle may be due to parasite spillover. Our results demonstrated that there were 31 cases of *Cryptosporidium* infection. 14% of fecal samples tested were positive for *Cryptosporidium.* Every single species had at least one positive fecal sample except bovine.

There were five positive samples out of 199 humans from Bodager 2011. *Cryptosporidium suis*, commonly found in pigs, was found in the human testing positive

in Ankialo. 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 (Bodager, 2011). No humans tested positive in Ambodiaviavy, one tested positive in Ankialo, and there were four possible human samples testing positive in Ambatolahy (Bodager, 2011). This paper only found one of the four possible human samples from Ambatolahy to be positive for *Cryptosporidium.* These results are similar to the results of Kightlinger *et. al* 1995, which studied intestinal nematodes in children in communities of Ranomafana. Jonathan Bodager's findings along with Kightlinger's suggest that *Cryptosporidium* is not the major diarrhea causing pathogen in these rural communities. Further fecal sample analysis for different diarrheal diseases should be conducted.

The presence of *Cryptosporidium* in the villages was expected due to past research conducted around Ranomafana National Park. Bodager's finding of *C. suis* in a human indicates the role zoonoses plays in these villages. After an extensive literature review, it appears that there have not been any studies of poultry *Cryptosporidium* in Madagascar. Wegner and Bodager's behavioral data suggests zoonotic routes of transmission between poultry and humans and poultry and other animals. RFLP was performed to find the species of *Cryptosporidium* present in the positive fecal samples to determine whether avian *Cryptosporidium* appeared in samples from other species. The RFLP results did not support the hypothesis that there were species of *Cryptosporidium* in chickens from other sources and vice versa. The evidence could only indicate expected species with no zoonotic transmission. There were some cases were poultry samples looked like they had the potential to be from a different species, but it was not strong

enough for an accurate reading. RFLP was useful in finding potential possible sources of poultry zoonosis. The next step would be genomic sequencing of the suspect samples. Because of the way poultry live in households, there is great zoonotic potential. While there were many positive samples for *Cryptosporidium*, there was not strong enough evidence of zoonosis.

Our study showed no association between infection and village. The study did not have the same sample size of each animal in each village. Ankialo had the fewest samples. Because the sample size of species was too small, the species were combined to form binary variables of either poultry or non-poultry.

Although the results do not demonstrate zoonoses in the villages, there have been many studies emphasizing the public health concerns of zoonoses of *Cryptosporidium*. *Cryptosporidium suis* has been identified in a HIV positive patient in Lima, Peru and two other humans in London (Xiao *et. al*, 2002 and Leoni *et. al,* 2006). Recent phylogenetic studies demonstrate that *C. parvum* a bovine genotype and *C. meleagridis* originated in rodents and mammals, but have extended their host range to humans (Xiao *et. al*, 2002). 155 species of mammals have been reported to be infected with *C. parvum, C. baileyi, C. canis, C. felis, C. meleagridis*, and *C. muris* which were once thought to be host specific to chickens, dogs, cats, turkeys, and mice. They have now been found to infect humans (Fayer, 2014). Under laboratory conditions, *C. hominis* infected a lamb, pigs, and calves but did not infect inmmunosuppressed mice (Widmer *et. al*, 2000, Giles *et. al*. 2001, Pereira *et. al.,* 2002 and Akiyoshi *et. al*., 2002). Until recent studies, *C. pavum* was considered the only species to infect humans. Now it is likely that other species can infect humans (Xiao and Feng, 2008). *Cryptosporidium* for nonhuman vertebrate hosts

including *C. baileyi, C. canis, C. felis, C.hominis, C.meleagridis, C. muris,* and *C. parvum* have been found to infect humans (Fayer, 2004). *Cryptosporidium muris*, a mouse genotype, was found in two Indonesian girls (Katsumata *et. al.,* 2000), patients in France (Guyot *et. al.,* 2001), and in AIDS patients in Kenya and Peru (Gatei *et. al* 2006; Palmer *et. al,* 2003). The avian pathogen *C. meleagridisis* has been recognized as a human pathogen and is responsible for 10-20% of human *Cryptosporidium* in Lima, Peru and Bangkok, Thailand (Xiao *et. al,* 2001). *Cryptosprodium parvum* has been found in dogs in Italy (Giangaspero *et. al*., 2006). The primary hosts for *C. hominis* are humans. Excluding *C. parvum*, which is the most common zoonotic species, the remaining species known to infect humans have been primarily found in immunocompromised humans (Fayer, 2004). Immunity plays a strong role in *Cryptosporidium* infection. In resource poor countries, cryptosporidiosis is a disease affecting young children with lower immunity. It is found in 16% of AIDS patients (Huang and White, 2006).

Rodents are also an important source of *Cryptosporidium* oocysts. Rodents share habitats with farm animals, such as poultry, and have the ability to transmit *Cryptosporidium* to farm animals (Meerburg *et. al,* 2009). Rodents are abundant and ubiquitous reservoirs of *Cryptosporidium* for humans and livestock. Dozens of syanthropic and wildlife species of rodents are hosts for *Cryptosporidium* (Feng, 2008).

It was not until recently that *Cryptosporidium* was recognized as a pathogen of poultry (Fayer, 2004). *Cryptosporidium* presence in ducks and geese could be associated with feeding mechanisms (Figure 11). Ducks and geese feed by dabbling for plants with heads submerged in water. *Cryptosporidium* is a waterborne pathogen. Migratory behavior of these birds allows for contact with poor quality waters and a wide range of

pathogens. Migratory herbivorous birds consume undigested plant material in cattle feces. Cattle are reservoir hosts for *C. parvum* (Plutzer and Tomor, 2009). Carcass and waste disposal may have affected the amount of positive *Cryptosporidium* samples found in poultry. Chaka's study found that waste and carcass disposal was done in open areas accessible to scavenging chickens (Chaka *et. al*, 2013). Possible risk factors in the Madagascar highlands for avian influenza and new castle disease relate to poultry farming and commercial practices. There is close contact of different animal species such as geese, ducks, chickens, turkeys, guinea fowls, pigeons, and pigs. There is also contact between poultry and wild bird species. Poultry come into contact with agro-ecological environmental factors that are favorable for the survival of disease such as surface water and rice paddies. Live poultry markets and marketing networks are also risk factors (Andriamanivo *et. al*, 2012). Salmonellosis has caused serious problems in the poultry industry. In developing countries such as Asia and Africa, it has caused impediment to the development of the poultry industry (Rajagopal R and Mini M, 2013).

Bodager's survey analysis found that there were differences in water use and defecation practices between Ankialo, Ambodiaviavy, and Ambatolahy, which could have accounted for the differences in *Cryptosporidium* between villages since it is a waterborne disease and can be transmitted via the fecal oral route. The majority of individuals in Ambodiaviavy reported drinking boiled water in contrast to the villagers of Ankialo, where the majority reported never drinking boiled water. There were differences in the collection of water from an open source or a closed source. Ambodiviavy was shown to use more closed sources of water whereas individuals of Ankialo reported using open water sources (Bodager, 2011). In a study of intestinal parasites, it was

demonstrated that the source of water was statistically associated with infection (Nematian *et. al,* 2004). 76.9% of villagers in Ambodiaviavy reported never defecating in a latrine. 31.9% of individuals in Ankialo reported never using a latrine. *Cryptosporidium* can be foodborne as well as waterborne. 84.3% of individuals in Ankialo said they eat food they know has been contaminated with rodent feces (Bodager, 2011). The 2013 survey data from demonstrated that 15.03% of people reported having diarrhea without blood and one point thirteen percent of people reported having diarrhea with blood (Table 4).

Of the 14 samples testing positive for *Cryptosporidium* in poultry, nine were classified as liquid. Two of the samples were bloody and liquid. There were only two normal samples that were positive for *Cryptosporidium* in the poultry. Avoiding poultry that show signs of diarrhea would be advisable. It was noted that in the villages sampled, there were feces on the ground outside of households, inside, and in the kitchens.

The survey results of the positive individual in Ankialo were examined to see possible links between behavior and infection. The individual indicated trapping, cooking, and eating wild pigs. The individual indicated eating food that had been contaminated with rodent feces, eating uncooked meat, and eating animals with open wounds (Bodager, 2011).

Other notable survey data analysis conducted by Bodager included the statistically significant risk factors. Tending porcine and bovine was statistically significant. As mentioned above, eating food with contaminated rodent feces and defecation practices were found to be statistically significant using chi square tests. Drinking boiled water and reporting diarrhea were statistically significant as well (Bodager, 2011). Further

behavioral analysis, such as human poultry interaction or where poultry are stored at night, is needed.

Previous studies have demonstrated the role poultry plays in interacting with humans, and suggests potential for zoonotic transmission. These villages are unique in their interactions with livestock and domesticated animals. Poultry are housed inside during the night. Sanitation and hygiene is lacking, including open defecation and visible fecal material littering the villages and households. These villages are also extremely poor, with 67% reporting no weekly income. Walking over feces barefoot and handling feces are accepted practices. Poultry constitute the majority of animals in the villages, and they show the highest interactions with other animals, humans, water sources, food, feces, household, and kitchens (Wegner, 2013). Even though there is evidence suggesting the importance of poultry in transmitting *Cryptosporidium*, it has not been previously studied in these villages.

Some limitations of the experiment include not being able to process the samples in the field. Use of RNA later could have led to degradation of samples because they were stored at room temperature. Because multiple animals of the same household were analyzed, we had to account for pseduoreplication. Individual observations were dependent on each other because multiple samples were collected from the same household.18S nested PCR can sometimes pick up fragments of the same length that are not *Cryptosporidium*. This is another reason why RFLP is so important.

The field season was conducted June to August, which is the dry season in Madagascar. The dry season in Madagascar is May through October. There is a correlation between season and prevalence of *Cryptosporidium* infection. In a study of

Cryptosporidium conducted by Siwila *et. al,* the highest prevalence of *Cryptosporidium* was at the beginning and end of the wet season. *Cryptosporidium* infection was most prevalent during the wet season (Siwilia *et. al*, 2011). Conducting research in the dry season may have affected our results, and conducting research in the wet season may lead to evidence suggesting zoonosis.

Vaccines are an import means of control and prevention for diseases of domesticated animals and humans. Because of wild animal reservoirs they would need to be vaccinated as well. As of now, the human and animal health divisions of the biopharmaceutical industry are segregated and there has not been a movement to develop vaccines to prevent the spread of diseases from domestic or wild animals to humans. Vaccinating domesticated animals has the potential to fight zoonotic diseases (Monath, 2013). There is not a vaccination or an effective treatment for *Cryptosporidium*. Climate change may exacerbate the potential for transmission of waterborne parasites (Davidson *et. al*, 2011).

Poor water quality and sanitation contribute to increasing rates of diarrhea in Madagascar. The CDC selected 30 impoverished communities near Antananarivo to launch the Safe Water System. Before this could be enacted, three cyclones hit the east coast of Madagascar. Damage included substantial wind, flooding, landslides, affecting 300,000 people and killing over 200. To prevent disease outbreaks after cyclones, Sûr'Eau (a water treatment system) was distributed as a general water treatment method. Findings suggest that Sûr'Eau is perceived as a cholera prevention rather than a water treatment system (Dunston *et. al,* 2001). Contamination of food and water are a leading cause of malnutrition and mortality in developing regions. WHO estimates that 1.1 billion

people do not have access to clean water for necessary activities and personal hygiene (Marino, 2007). The scarcity and quality of fresh water provide many public health challenges in multiples parts of the world. Even in developed countries such as North America and Europe, there have been disease outbreaks due to *Cryptosporidium* from contaminated water (Gajadhar and Allen, 2004).

Further fecal sample analysis for other diarrheal diseases, specifically zoonotic diarrheal diseases, could enhance this study. Because of the large amounts of nucleic acid extracted from each sample there is potential for future pathogen screening. There is survey data in conjunction with this study that can be analyzed to see if there are behavioral associations. Expanding our knowledge of other pathogens as well as behavioral associations will promote effective and efficient health and conservation interventions. Interactions between humans, domesticated animal, and wildlife are increasing rapidly.

The collection of baseline pathogen data is an important step in strengthening our knowledge of zoonosis. This study played an important role in the contribution to this effort through the investigation of the waterborne zoonotic pathogen *Cryptosporidium.* The study indicated potential zoonoses of poultry, but further genotyping or DNA sequencing is needed. While poultry do harbor a lot of *Cryptosporidium* and have potential to play an integral role in zoonotic transmission, there was not sufficient evidence to support the hypothesis that poultry play a role in zoonotic transmission to humans and livestock. This was the first study of C*ryptosporidium* of poultry in Madagascar. It was found that poultry are infected with *Cryptosporidium* and constitute the majority of animals that were infected in all the villages. Because of the high number

of infected poultry for *Cryptosporidium,* poultry appear to be a model species to study. Although this study did not find avian *Cryptosporidium* in humans or animals, DNA sequencing might reveal a zoonotic connection that RFLP was not able to.

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Tables and Figures

Tables

Table 1. Presence of *Cryptosporidium* in fecal samples from poultry, livestock, humans and rodents in rural villages in Ifanadiana district, Madagascar.

Sample Type	Samples Collected	Positive for *Cryptosporidium*	% Positive
Chicken	70	10	14.3
Duck	17	4	23.5
Geese	6	1	16.7
Bovine	34	0	0
Pig	41	6	14.6
Humans	4	1	25
Rodents	60	12	20
Total	232	34	14.7

Table 2. Presence of *Cryptosporidium* in poultry, livestock, rodents, and humans at the village level in Ambatolahy, Ambodiaviavy, and Ankialo in rural Madagascar.

	Ambatolahy			Ambodiaviavy			Ankialo		
Species	Sample (N)	# Pos	% Pos	Sample (N)	# Pos	% Pos	Sample (N)	# Pos	% Pos
Chicken	30	6	20	40	4	10	-	-	-
Duck	13	1	7.7	-	-	-	4	3	75
Geese	4	0	0	2	1	50	2	0	0
Bovine	16	1	6.3	17	0	0	1	0	0
Pig	8	1	12.5	15	0	0	18	4	22.2
Human	4	1	25	-	-	-	-	-	-
Rodent	20	2	10	16	2	12.5	12	5	41.6
Total	95	12	12.6	90	7	7.8	37	12	32.4

Table 3. Different species of livestock, rodents, and poultry sampled from the same households of Ambodiaviavy, Ankialo, and Ambatolahy, Madagascar.

	Species in Households		
Households	Ambatolahy	Ambodiaviavy	Ankialo
1	Chicken, Bovine, Rodents	Chicken, Bovine Rodents	Geese, Porcine
2	Geese	Chicken, Rodents	Porcine, Rodents
3	Chicken, Rodents	Bovine, Rodents	Porcine, Rodents
4	Chicken, Porcine, Rodents	Chicken, Porcine, Rodents, Bovine	N/A
5	Chicken, Duck, Bovine, Porcine, Rodents	Porcine, Bovine, Rodents	Porcine, Duck, Rodents
6	Porcine, Bovine, Rodents	Bovine, rodents	Porcine, Bovine, Rodents
7	Chicken, Bovine, Rodents	Chicken, Rodents	Porcine
8	Duck, Porcine, Rodents	Geese, Bovine, Rodents	Porcine, Ducks, Rodents
9	Porcine, Rodents	Bovine	Porcine
10	Bovine, Rodents	Chicken	Porcine
11	N/A	Chicken, Porcine	N/A
12	Porcine	Chicken, Porcine	N/A
13	Chicken, Porcine	Chicken, Porcine	N/A
14	Duck, Chicken, Porcine	N/A	N/A
15	Porcine	Porcine	N/A
16	N/A	Porcine	N/A

Table 4. Total Presence of *Cryptosporidium* in poultry and humans in rural villages of Ifanadiana district, Madagascar.

	Ambatolahy			Ambodiaviavy			Ankialo		
Species	Samples (N)	# Pos	% Pos	Samples (N)	# Pos	% Pos	Samples (N)	# Pos	% Pos
Chicken	30	6	20	40	4	10	-	-	-
Duck	13	1	7.7	-	-	-	4	3	75
Geese	4	0	0	2	1	50	2	0	0
Human	4	1	25	59	0	0	136	17	12.5
Total	51	8	15.69	101	5	4.95	142	20	14.08

Table 5. Logistic Regression Analysis of *Cryptosporidium* presence and poultry, villages, and the interaction between species and villages in samples from Ambatolahy, Ambodiaviavy, and Ankialo in rural Madagascar.

Independent Variable	p-value	Odds Ratio	B	Standard Error
Species (Poultry)	0.196	0.4936272	-0.7056	0.5456
Village	0.885	1.0721003	0.06962	0.48210
Species*Village	0.81273	1.0277605	0.02738	0.11538

Table 6. Description of poultry fecal samples found to be positive for *Cryptosporidium* collected in Ambodiaviavy, Ankialo, and Ambatolahy, Madagascar during the 2012 field season.

Sample	Description	Species	Village
1-C-CH-3	Diarrhea	Chicken	1
1-C-CH-6	Normal	Chicken	1
1-1-CH-2	Diarrhea	Chicken	1
1-3-CH-8	Diarrhea	Chicken	1
1-3-CH-10	Diarrhea	Chicken	1
1-3-CH-12	Diarrhea	Chicken	1
2-1-CH-3	Diarrhea	Chicken	2
2-4-CH-5	Normal	Chicken	2
2-7-CH-1	Bloody	Chicken	2
2-7-CH-6	Diarrhea	Chicken	2
1-D-DK-5	Diarrhea	Duck	1
3-9-DK-2	Black	Duck	3
3-9-DK-7	Diarrhea	Duck	3
2-8-GS-1	Bloody	Goose	2

Figures

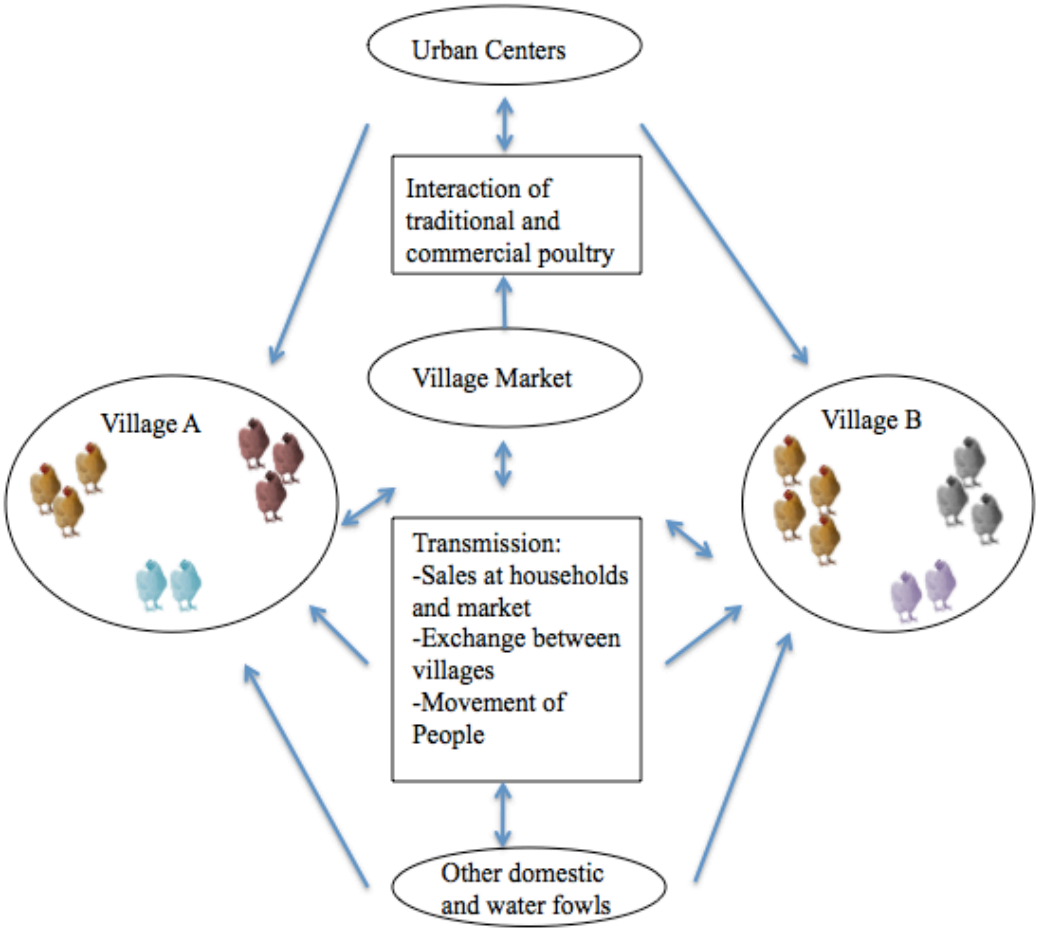

Figure 1. The transmission and interactions of poultry within and between villages and with urban centers or other fowls (modified from Kitalyi, 1998).

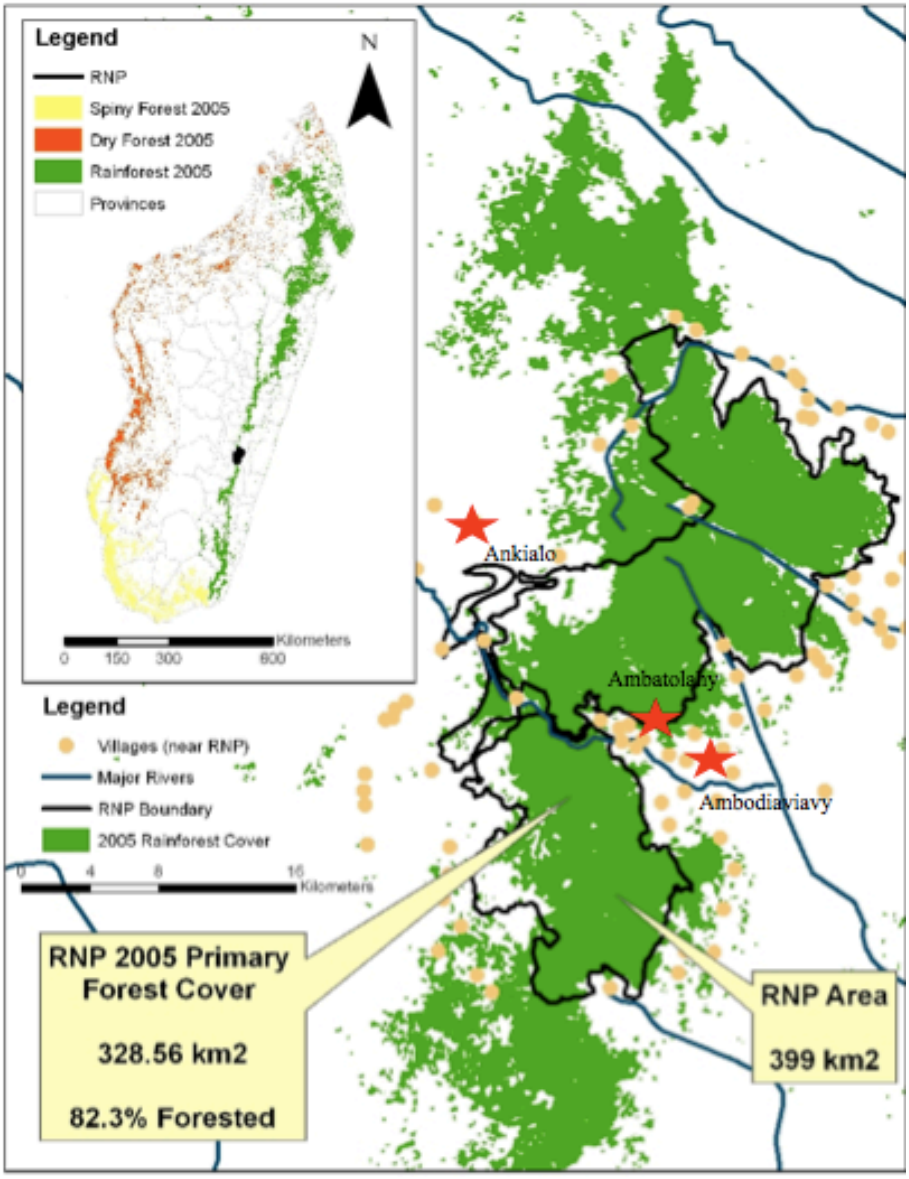

Figure 2. Map of the 2012 field study sights (Ankialo, Ambatolahy, and Ambodiaviavy) in the Ifnadiana District in Southeastern Madagascar (Modified from Brian Gerber).

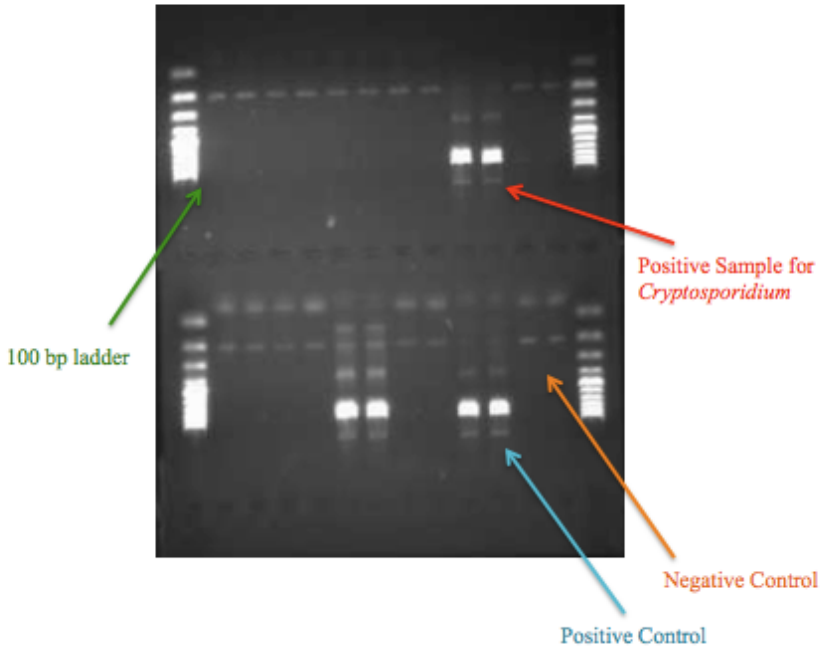

Figure 3. Interpreting bands on UV image after gel electrophoresis to determine whether samples were positive for *Cryptosporidium.*

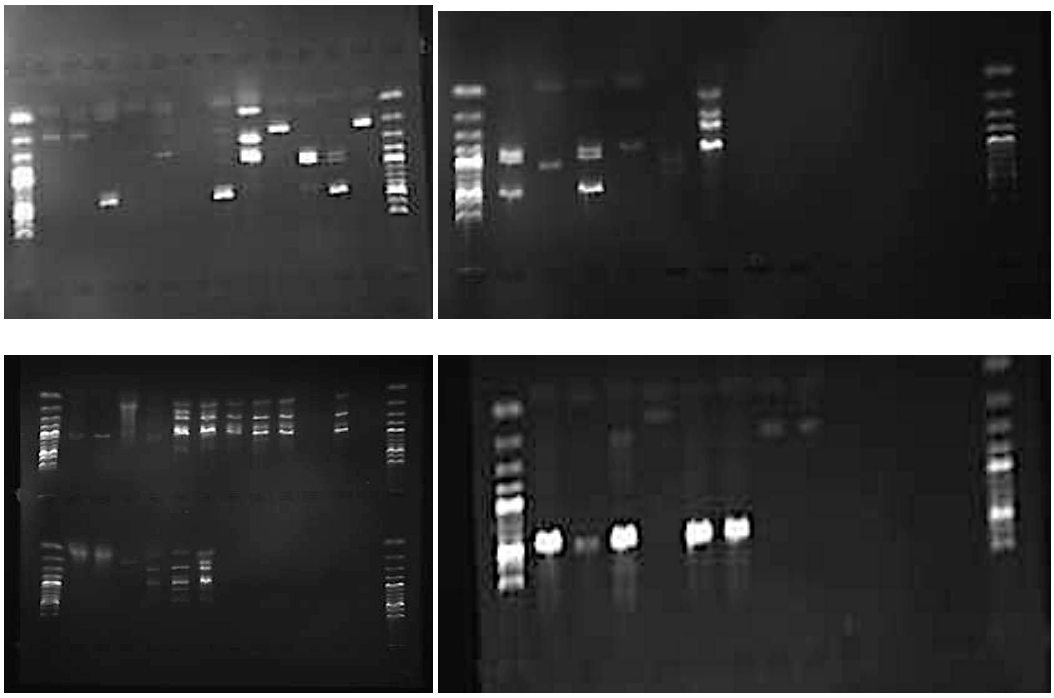

Figure 4-7. Restriction Fragment Length Polymporphism (RFLP) species level identification of *Cryptosporidium* using *SSpI.*

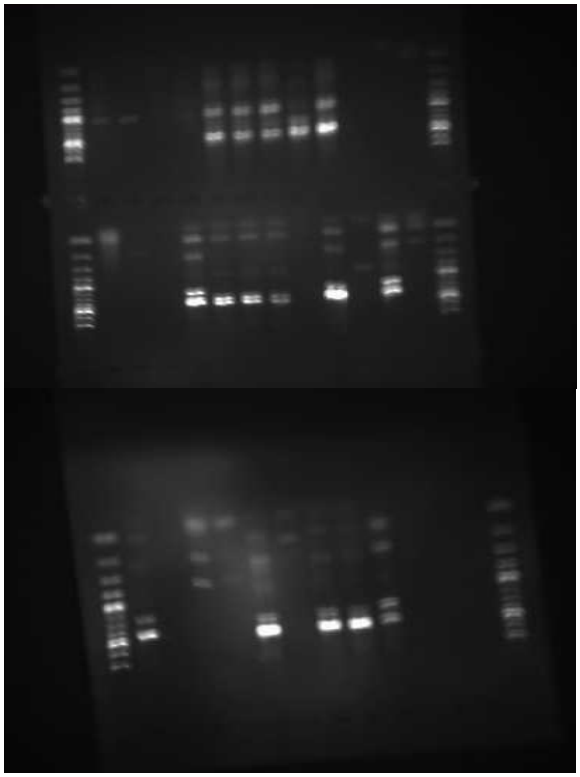

Figure 8 & 9 . Restriction Fragment Length Polymorphism species level Identification of *Cryptosporidium* using *VspI.*

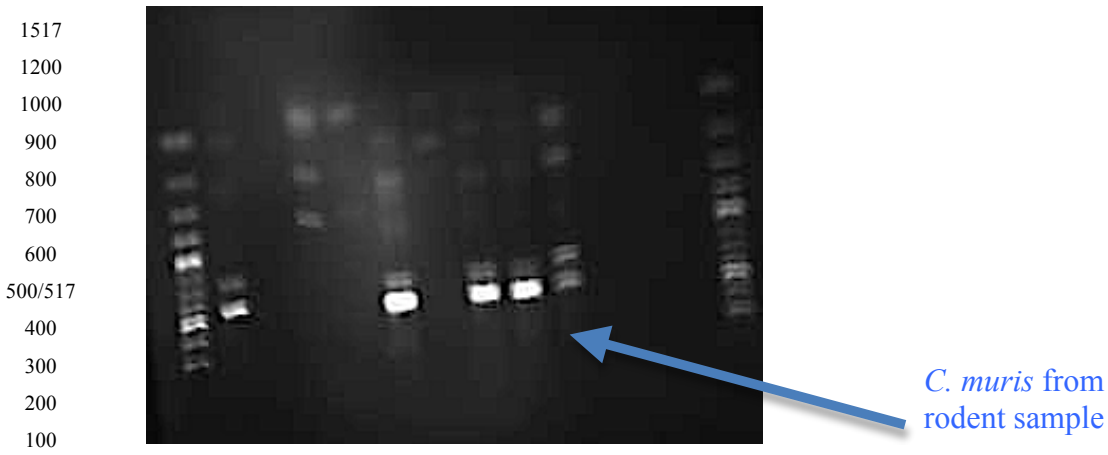

Figure 10. Restriction Fragment Length Polymorphism results demonstrating that rodent samples had the expected species *C. muris*.

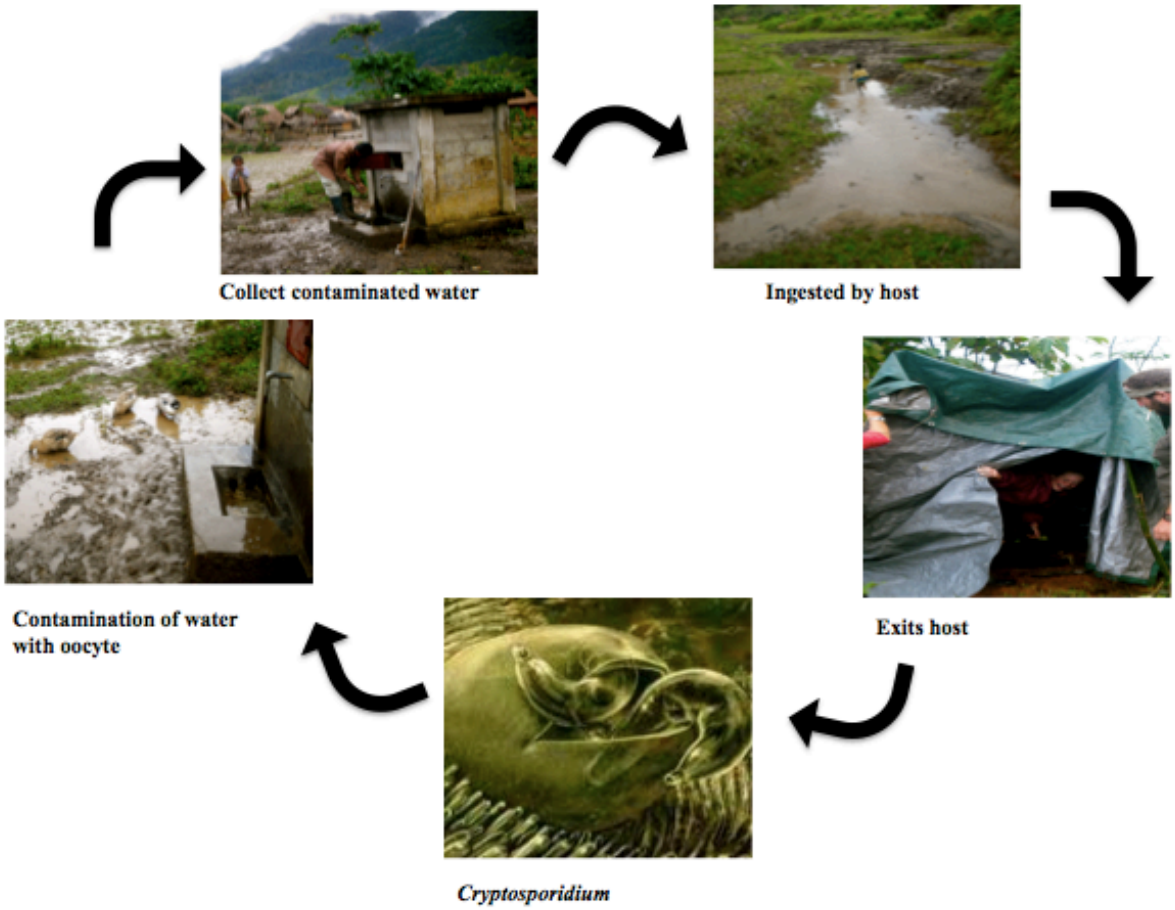

Figure 11. Potential route of zoonotic transmission of *Cryptosporidium* between humans and poultry, based on observed interactions of poultry, humans, and water sources in rural villages in southeastern Madagascar.