Intestinal Protozoa in Wildlife: parasite transmission at the wildlife-human-domestic animal interface

Philosophiae Doctor (PhD) Thesis

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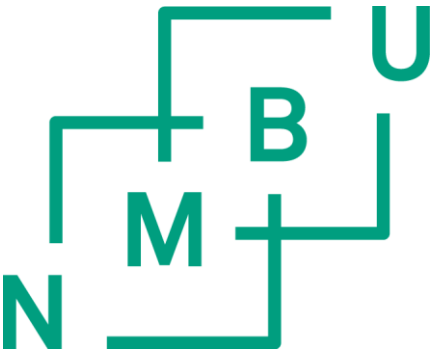

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To my dearest Lilly,

may science and a caring heart guide you through life.

1. Contents

2. Acknowledgements

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3. Abbreviations

bg: Beta (β) giardia

DAPI: 4´, 6-diamidino-2-phenylindole

ELISA: Enzyme-linked immunosorbent assay

gdh: Glutamate dehydrogenase

IFAT: Indirect fluorescent antibody test

IGT: Immunochromatographic tests

KZ: Kristiansand Zoo

mZN: modified Ziehl-Neelsen stain

NHP: Nonhuman primate

No.: Number

OPG / CPG: oocysts per gramme, cysts per gramme

PCR: Polymerase chain reaction.

SSU rRNA: Small sub-unit ribosomal RNA

TCRC: Tchimpounga Chimpanzee Rehabilitation Centre

tpi: Triosephosphate isomerase

4. Scientific Papers

Paper I

John J. Debenham, Rebeca Atencia, Fred Midtgaard, and Lucy J. Robertson. 2015. Occurrence of *Giardia* and *Cryptosporidium* in captive chimpanzees (*Pan troglodytes*), mandrills (*Mandrillus sphinx*) and wild red colobus monkeys (*Procolobus kirkii*). *Journal of Medical Primatology***,** 44(2): 60-65

Paper II

John J. Debenham, Kristoffer Tysnes, Sandhya Khunger, and Lucy J. Robertson. 2017. Occurrence of *Giardia*, *Cryptosporidium*, and *Entamoeba* in wild rhesus macaques (*Macaca mulatta*) living in urban and semi-rural North-West India. *International Journal for Parasitology: Parasites and Wildlife*, 6: 29-34.

Paper III

Ola Brynildsrud, Kristoffer Tysnes, Lucy J. Robertson, and **John J. Debenham**. (Submitted). Phylogenetic analysis of *Giardia duodenalis* sequences in primates: evidence of potential zoonotic and anthropozoonotic transmission. *Parasites and Vectors*.

Paper IV

John J. Debenham, Fred Midtgaard, and Lucy J. Robertson. (Submitted). Low occurrence of *Giardia* and *Cryptosporidium* in domestic cattle and wild herbivores in and around Mikumi National Park, Tanzania. *Veterinary Parasitology: Regional Studies and Reports*.

Paper V

John J. Debenham, Freya Cools, Fred Midtgaard, and Lucy J. Robertson. 2016. Five species of coccidia (Apicomplexa: Eimeriidae), including four new species, identified in the feces of blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania. *Journal of Parasitology*, 102(2): 233-238

Paper VI

John J. Debenham, Hanne Landuyt, Karin Troell, Kristoffer Tysnes, and Lucy J. Robertson. 2017. Occurrence of *Giardia* in wild Swedish red foxes. *Journal of Wildlife Diseases*. doi: 10.7589/2017-01-002.

5. Summary

The interfaces between humans, wild animals, and domestic animals are changing. This process is driven by a multitude of factors, including changes in land use, habitat loss and fragmentation, climate change, ecotourism, commercial use of bush-meat, and a massive growth in the human population. It is said that this process is leading to the emergence of new infectious diseases, and that most of these have their origins in wildlife. Somewhat overlooked, is the importance that this may have on wildlife conservation, as previously isolated wild populations are now exposed to the pathogens of humans and their animals.

Giardia, *Cryptosporidium*, *Entamoeba*, and *Eimeria* are genera of protozoan parasites that cause widespread human and animal disease. *Giardia* and *Cryptosporidium*, in particular, contain species that are able to infect a wide range of host species, and thus are good candidates for crossing the human-domestic animal-wildlife boundaries. Despite this, the role of these parasites in wildlife populations remains largely unknown, both in respect to the impact they may have on wildlife conservation, and also whether wildlife populations may act as reservoirs for human and domestic animal disease.

In this thesis I present a number of studies that investigate the epidemiology of these protozoa in wildlife. Due to their close taxonomic relationship to humans, and thus the inferred susceptibility to similar pathogens, a large focus was on nonhuman primates (NHPs). Nonhuman primates were found to be infected with the same types of *Giardia* as humans, however, there was a large variation in prevalence between populations. In contrast, whereas *Entamoeba* was a common parasite in NHPs with close human contact, no human pathogenic species were detected. *Cryptosporidium* was not found to be a common pathogen in the NHPs studied.

In red foxes, *Giardia* was a common parasite, and these isolates were the same as those commonly found in humans, and different to those often identified in other canids, particularly domestic dogs. This suggests that a human-fox, rather than the hypothesised dog-fox, transmission cycle is more likely, reinforcing the role that this ubiquitous predator may play in zoonotic parasites.

When studying wild and domestic ungulates in Mikumi National Park, Tanzania, neither *Giardia* nor *Cryptosporidium* were common parasites, suggesting that either these are largely naïve populations, or that the environmental conditions are not conducive to the life cycles of these parasites. Coccidian infections with *Eimeria* spp. were common in wild blue wildebeest. However, these were host-specific species, and thus transmission to domestic livestock was considered unlikely.

Finally, a small study on the prevalence of *Cryptosporidium* and *Giardia* in Norwegian reptiles was conducted, and revealed that *Giardia* was absent whilst *Cryptosporidium* was present in low numbers. Unfortunately, molecular characterisation of these isolates was unsuccessful, and thus the zoonotic or anthropozoonotic potential remains unknown. Indeed, difficulty with molecular characterisation of wildlife isolates was a constant hurdle throughout this thesis.

Together, these studies provide new information on the role of *Giardia*, *Cryptosporidium*, *Entamoeba*, and *Eimeria* in the wildlife populations studied. It is clear that certain zoonotic or anthropozoonotic transmissions are possible, such as for *Giardia* in urban-living rhesus macaques in India, and these situations should be monitored for public health and wildlife conservation.

6. Sammendrag (Norwegian summary)

Forholdet mellom mennesker, ville dyr og husdyr er i endring. Denne prosessen drives frem av en rekke faktorer, blant annet endringer i bruk av landområder, tap av habitat og habitatfragmentering, klimaendringer, økoturisme, kommersiell utnyttelse av skogsdyr for kjøtt, og en massiv vekst i den menneskelige befolkningen. Det sies at denne prosessen fører til fremveksten av nye smittsomme sykdommer, og at de fleste av disse har sin opprinnelse i ville dyr. På den andre siden kan dette også være viktig for bevaring av dyreliv, da tidligere isolerte villdyrpopulasjoner nå er utsatt for patogener fra mennesker og deres husdyr.

Giardia, *Cryptosporidium*, *Entamoeba* og *Eimeria* er parasittiske protozooer som forårsaker alvorlig sykdom hos både mennesker og dyr i store deler av verden. *Giardia* og *Cryptosporidium* inkluderer arter som er i stand til å infisere et bredt spekter av verter, og er dermed gode kandidater til å kunne krysse grensene mellom mennesker, husdyr og ville dyr. Til tross for denne erkjennelsen er disse parasittenes rolle i ville dyrepopulasjoner stort sett ukjent, både med hensyn til effekten de kan ha på bevaring av dyreliv, og om hvorvidt ville dyr kan fungere som reservoar for sykdom hos mennesker og husdyr.

I denne doktorgraden presenterer jeg studier som undersøker epidemiologien til disse protozooene hos ville dyr. På grunn av deres nære taksonomiske forhold til mennesker, og dermed følsomheten de har for lignende patogener, har jeg valgt å fokusere mest på primater. Et av mine hovedfunn var at primater smittes med de samme *Giardia*-typene som mennesker, men med en stor variasjon i prevalens mellom populasjonene. *Entamoeba* ble funnet å være en vanlig parasitt hos primater med nær menneskelig kontakt, men det ble ikke påvist noen arter som forårsaker sykdom hos mennesker. Derimot ser *Cryptosporidium* ikke ut til å være et vanlig patogen hos primatene som ble studert.

Hos rødrev var *Giardia* en vanlig parasitt, og ved hjelp av molekylærbiologiske metoder fant jeg at disse isolatene var de samme som vanligvis finnes hos mennesker, og forskjellig fra de som er vanlig hos andre hundearter. Dette betyr at overføring av parasitter mellom menneske og rev, i stedet for den antatte overføring mellom hunder og rev, er mer sannsynlig. Dette styrker rollen som disse utbredte rovdyrene kan utgjøre for spredning / overføring av zoonotiske parasitter.

Blant hovdyr i Mikumi Nasjonalpark i Tanzania ble *Giardia* og *Cryptosporidium* ikke funnet til å være vanlige parasitter, noe som tyder på at disse populasjonene ikke er utsatt for disse parasittene, eller at miljøforholdene ikke støtter livssyklusen til parasittene. Infeksjoner med *Eimeria* spp. var vanlig hos gnu, men fordi artene som ble funnet er regnet som vertsspesifikke, tyder dette på at overføring til eller fra husdyr kan regnes som usannsynlig.

Til slutt ble det gjennomført en mindre studie om forekomsten av *Cryptosporidium* og *Giardia* hos norske reptiler, der det ble funnet at *Giardia* var fraværende mens *Cryptosporidium* var tilstede i lave tall. Det var dessverre ikke mulig å gruppere disse isolatene ved hjelp av molekylærbiologiske metoder, og dermed forblir det zoonotiske eller antropozoonotiske potensialet ukjent. Molekylær gruppering av parasittene fra ville dyr var en konstant utfordring gjennom denne doktorgraden.

Sett under ett gir disse studiene ny informasjon om rollene til *Giardia*, *Cryptosporidium*, *Entamoeba* og *Eimeria* i de studerte dyrepopulasjonene. Overføring av visse zoonotiske eller antropozoonotiske sykdommer vil være mulig, for eksempel av *Giardia* hos urbane makaker i India. Disse forholdene bør overvåkes med tanke på human helse og konservasjon av ville dyr.

7. Introduction

7.1 Background

Throughout human history, our relationship with nature has been dynamic, driven by a multitude of forces including the climate, available foods, disease, and technology. The speed of this change over the past 200 years has been unprecedented, as the industrial revolution has led to an exponential rise in the population of *Homo sapiens*, and a transformation in most landscapes around the globe. Integral to this, has been a shift in the way we view and interact with wildlife.

The interfaces between humans, wild animals and domestic animals are in constant evolution, with changes from clearing of forests for farming, intensification of agriculture, urbanisation of some wildlife species, habitat fragmentation, sharing of water sources, ecotourism, commercialisation of the bush-meat industry, climate change, and mass extinctions. Many of these changes have apparently made this interface more porous, potentially leading to an increase in the transmission of pathogens between these groups. Whilst it may be argued that humans were closer to nature when living as hunters and gatherers, pathogen transmission then was much more geographically restricted than it is today. This changing interface can have consequences for human health, as evidenced by rabies, plague, AIDS, tularaemia, and tuberculosis (Rhyan and Spraker, 2010; Gao et al., 1999). Interestingly, zoonotic transmission often utilizes a domesticated animal "bridge" e.g. horses for Hendra virus, dogs for echinococcosis, or livestock for African trypanosomiasis (Field et al., 2010; Funk et al., 2013; Salb et al., 2008). However, despite this, and the realisation that the health of humans, domestic animals, and wild animals are all integrally linked, our knowledge and understanding of wildlife health are still limited.

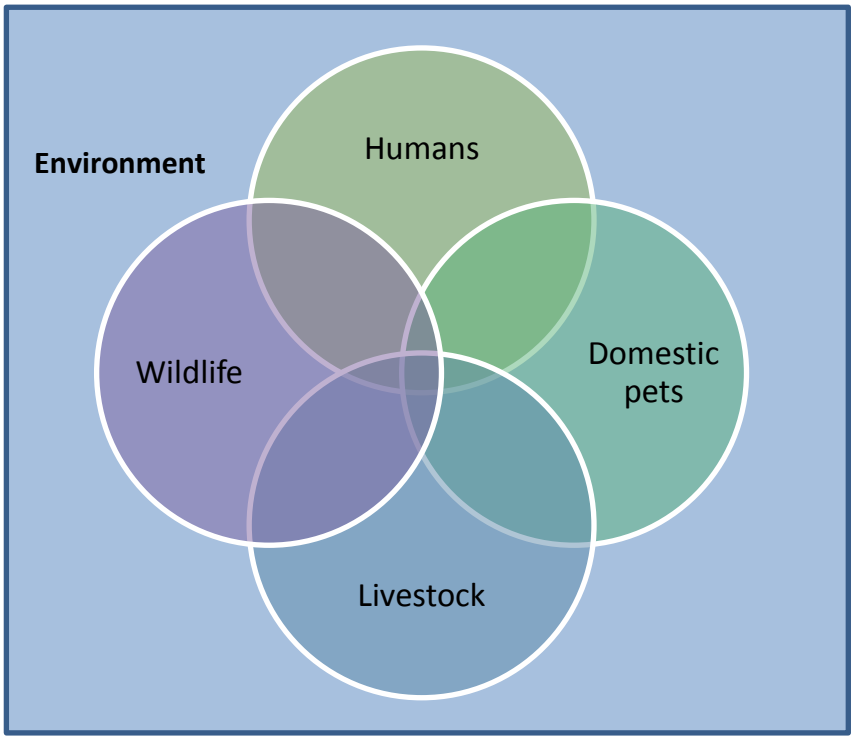

Figure 1. Disease epidemiology can involve interactions between humans, wildlife, livestock, and domestic pets.

Of particular concern in the field of wildlife health are pathogens that do not exhibit host specificity, i.e., that are able to infect species across different taxa. These can have huge impacts on entire sections of an ecosystem, as seen with chytridiomycosis in amphibians (Lips, 2016). Additionally, however, such "host-promiscuous" pathogens are also more likely to be able to transfer between humans, domestic animals, and wild animal populations. Good examples of such pathogens are the ubiquitous parasites *Giardia*, *Cryptosporidium*, and *Entamoeba*, the first two of which are listed under the World Health Organization's "Neglected Diseases Initiative" (Savioli et al., 2006). In order to understand, and thus control, these diseases, we require information on their epidemiology and ecology (Lymbery and Thompson, 2012). Such information is also important for wildlife conservation and captive animal management (Thompson et al., 2010).

Giardia, *Cryptosporidium*, and *Entamoeba* are intestinal protozoa capable of infecting a range of host species, and are important causes of human morbidity and mortality (Kotloff et al., 2013; Hunter and Thompson, 2005; Stanley Jr, 2003; Thompson et al., 2010; Ryan et al., 2017). Although it is recognised that *Giardia* and *Cryptosporidium* are major causes of

human and livestock diarrhoeal disease, information on their significance and potential impact on wildlife populations is scanty. In contrast, *Eimeria* (another genus of intestinal protozoa) is known to be very host-specific, and thereby provides a useful comparative species when studying the transmission and epidemiology of diseases between wildlife and domestic species or humans. In domestic species, *Eimeria* causes a disease called coccidiosis, and can impact many individuals within a population. It is known that this causes significant economic losses in animal production systems, however, there are relatively few data on the impact of coccidiosis in wildlife species.

Although no objective measure, an indicator of the significance of these parasites can be seen by the attention paid to them by the scientific community. Notably, a PubMed search for '*Eimeria'* resulted in 5 095 scientific articles, '*Giardia*' resulted in 7 667 articles, '*Cryptosporidium*' resulted in 7 980 articles, and '*Entamoeba*' resulted in 8 457 articles, as of 8th of May, 2017.

7.2 Nomenclature and taxonomy

Giardia

Giardia is a genus of flagellate protozoans, within the phylum Metamonada, order Diplomonadida. There are six species of *Giardia* (Table 1) based on cyst and trophozoite morphologies. Nomenclature and taxonomy within *Giardia duodenalis* remain debated issues. *Giardia duodenalis* (syn. *Giardia intestinalis*, *Giardia lamblia*) is considered a species complex comprised of at least 8 distinct genetic groups, termed Assemblages A to H. Assemblages A and B infect humans as well as a range of other mammals, whilst Assemblages C to H have a more limited host range (Table 1).

The divisions of *G. duodenalis* into a species complex is based primarily on protein and DNA polymorphisms, with considerable genetic variation between Assemblages (Figure 2). This has led to some authors referring to the different Assemblages as unique species (Table 1; Ryan and Caccio, 2013; Thompson and Smith, 2011; Thompson and Ash, 2016). Furthermore, genetic and protein variation within Assemblages have led to the identification of a number of sub-Assemblages e.g. AI, AII, AIII and AIV. Some of these sub-Assemblages are reported to have distinct epidemiological patterns -e.g., human isolates belong to Assemblage AI and AII, however not AIII. On the basis of multilocus genotyping,

some authors have even begun to further classify isolates within these sub-Assemblages e.g. AI-I, AI-II (Ryan and Caccio, 2013).

It is important to note that rules or guidelines on the establishment of new sub-Assemblages do not exist, thus calling into question the legitimacy of the current nomenclature within this genus.

Table 1. Host ranges of various *Giardia* species and *Giardia duodenalis* Assemblages. Proposed species names for different Assemblages are presented in brackets.

***Giardia* species / sub-species**	**Host**
Giardia agilis	Amphibians
Giardia ardeae	Birds
Giardia psittaci	Birds
Giardia microti	Rodents
Giardia muris	Rodents
Giardia duodenalis Assemblage A (*G. duodenalis*)	Humans and other mammals
Giardia duodenalis Assemblage B (*G. enterica*)	Humans and other mammals
Giardia duodenalis Assemblage C (*G. canis*)	Canids
Giardia duodenalis Assemblage D (*G. canis*)	Canids
Giardia duodenalis Assemblage E (*G. bovis*)	Ungulates
Giardia duodenalis Assemblage F (*G. cati*)	Cats (other felids?)
Giardia duodenalis Assemblage G (*G. simondi*)	Rats
Giardia duodenalis Assemblage H	Pinnipeds

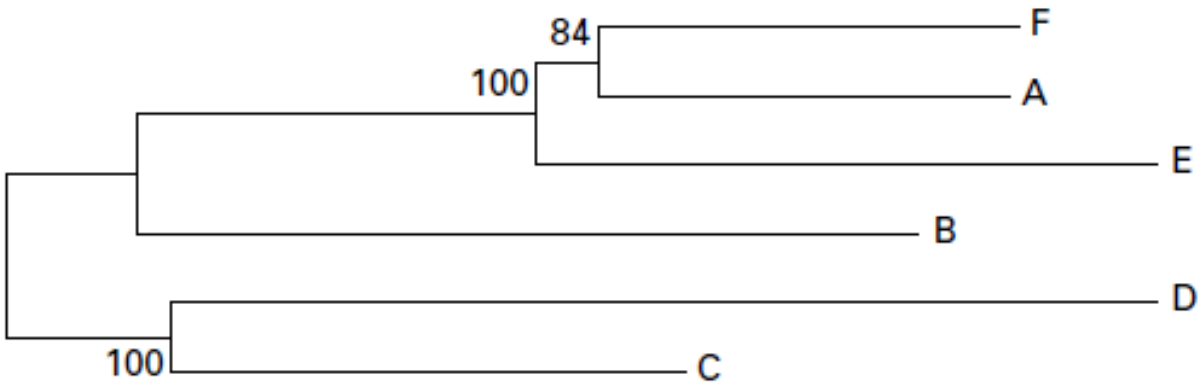

Figure 2. A nucleotide consensus sequence phylogram of *Giardia duodenalis* Assemblages generated from concatenated sequences; SSU rRNA, beta giardin, triose phosphatase, and glutamate dehydrogenase genes (Wielinga and Thompson, 2007).

Eimeria

Eimeria is a genus within the phylum Apicomplexa, most of the members of which are obligate parasites (Arisue and Hashimoto, 2015). Members of this phylum are morphologically characterised by the presence of the apical complex; an assembly of organelles that allows the parasite to invade its host cells (Arisue and Hashimoto, 2015). Other important parasites within the phylum Apicomplexa include *Toxoplasma gondii*, the cause of toxoplasmosis, and *Plasmodium* spp., the cause of malaria. Within the *Eimeria* genus there are over 1 000 described species. In contrast to *Giardia*, *Cryptosporidium*, and *Entamoeba*, most *Eimeria* spp. are very host-specific.

Eimeria cause the disease coccidiosis, which results in significant economic burdens for the livestock and poultry industries. Coccidiosis in wildlife has received scant attention, and whilst it is generally considered to be of limited clinical significance, it is occasionally reported as a cause of mortality in some wildlife species (Morgan et al., 2014; Newman et al., 2001). Also, due to their host specificity, the genetic relationships of *Eimeria* spp. infecting various hosts offer an interesting perspective to study the co-evolution of parasites and their hosts.

Entamoeba

Entamoeba is a genus of protozoa within the phylum Amoebozoa, members of which are characterised by movement through the use of pseudopodia. Pathogenicity to humans of species within this genus varies markedly, from highly pathogenic to non-pathogenic commensal organisms. The pathogenicity of most *Entamoeba* spp. in animal hosts remains unknown. Species within this genus are morphologically quite similar, varying primarily in cyst size, number of cyst nuclei, and the shape of chromatoid bars (Clark et al., 2006).

The genus *Entamoeba* contains many species, six of which are known to infect humans; *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba polecki, Entamoeba coli*, and *Entamoeba hartmanni* (Fotedar et al., 2007). Amongst these, *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically indistinguishable, but exhibit significant genetic diversity and have differing pathogenicities and epidemiologies (Clark et al., 2006). Sometimes these are collectively referred to as the *Entamoeba* complex (Nath et al., 2015). Morphologically identical / similar *Entamoeba* spp. are also found in animals, e.g., *E. hartmanni* and *E. polecki*. This highlights the limited use of microscopy in

determining the species within this genus of protozoa, something which is far more accurately performed using molecular diagnostics (Fotedar et al., 2007).

Cryptosporidium

Cryptosporidium is a genus of oocyst-forming protozoa within the phylum Apicomplexa. Whilst a number of *Cryptosporidium* spp. have been reported in humans, *Cryptosporidium parvum* and *Cryptosporidium hominis* are the two most common species, and of these, *C. parvum* is recognised as being zoonotic (Checkley et al., 2015). Other zoonotic species include *Cryptosporidium meleagridis*, *Cryptosporidium ubiquituum*, and *Cryptosporidium cuniculus* (Table 2). Genetic subclasses exist within some of these species.

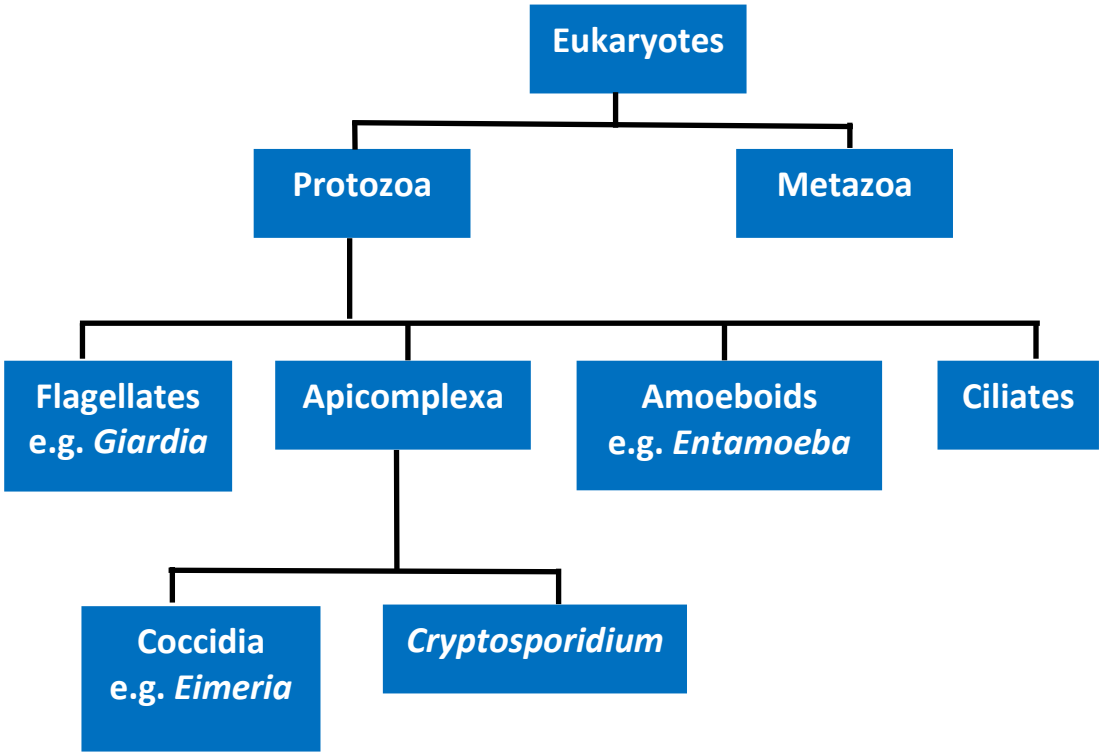

Figure 3. Simplified phylogeny of protozoa showing the relationships between *Eimeria*, *Giardia*, *Cryptosporidium*, and *Entamoeba*.

Table 2. Overview of the current valid *Cryptosporidium* spp.

***Cryptosporidium* species**	**Main host**
C. andersoni	Cattle
C. baileyi	Birds (and humans)
C. bovis	Cattle
C. canis	Dogs (and humans)
C. cuniculus	Rabbits (and humans)
C. erinacei	Hedgehogs and horses
C. fayeri	Marsupials
C. felis	Cats (and humans)
C. fragile	Toads
C. galli	Birds
C. hominis	Humans
C. macropodum	Marsupials
C. meleagridis	Birds and humans
C. molnari	Fish
C. muris	Rodents (and humans)
C. parvum	Ruminants and humans
C. ryanae	Cattle
C. scrofarum	Pigs
C. serpentis	Snakes and lizards
C. suis	Pigs (and humans)
C. tyzzeri	Rodents
C. ubiquitum	Ruminants, rodents, primates
C. varanii	Lizards
C. viatorum	Humans
C. wrairi	Guinea pigs
C. xiaoi	Sheep and goats

(Ryan et al., 2014a; Cacciò et al., 2005; Plutzer and Karanis, 2009; Fayer, 2010)

7.3 Infection and transmission

The life cycles of *Entamoeba*, *Cryptosporidium*, *Giardia*, and *Eimeria* are all similar in that they are all direct (no intermediate host) and involve the production of environmentally resistant (oo)cysts that are infective, either through direct contact or through contaminated food / water, to the next host (Figures 4 - 7). The length of survival of the infective stages in the environment is affected by the temperature, humidity, substrate type / texture, and UV exposure. These factors are thought to be partially responsible for the different epidemiological patterns observed in different environments around the world.

The ubiquitous nature of *Giardia*, *Cryptosporidium*, and *Entamoeba* highlights their success in transmitting infection between hosts. Some of the important factors contributing to this success are (Cacciò et al., 2005; Shirley et al., 2012; Feng and Xiao, 2011; DuPont et al., 1995):

1. A low infective dose , sometimes as few as one (oo)cysts.
2. Infected hosts excreting large numbers of (oo)cysts, sometimes over one million OPG / CPG of faeces.
3. (Oo)cysts are infective immediately after excretion.
4. (Oo)cysts are able to survive for long periods in suitable environments.

Giardia

The life cycle of *Giardia* involves two stages; the infective cyst and the replicating trophozoite within the host (Figure 5). Upon ingestion of an infective cyst, the acidic conditions of the stomach stimulate the excystation of the trophozoite which is then able to infect the intestines. Replication is (almost) entirely via asexual reproduction of the trophozoite on the surface of enterocytes or in the intestinal lumen. As the trophozoites move further down the intestine they, in turn, form environmentally resistant cysts that are excreted in the faeces. This is a non-invasive infection, with symptoms caused by effects on the intestinal surface.

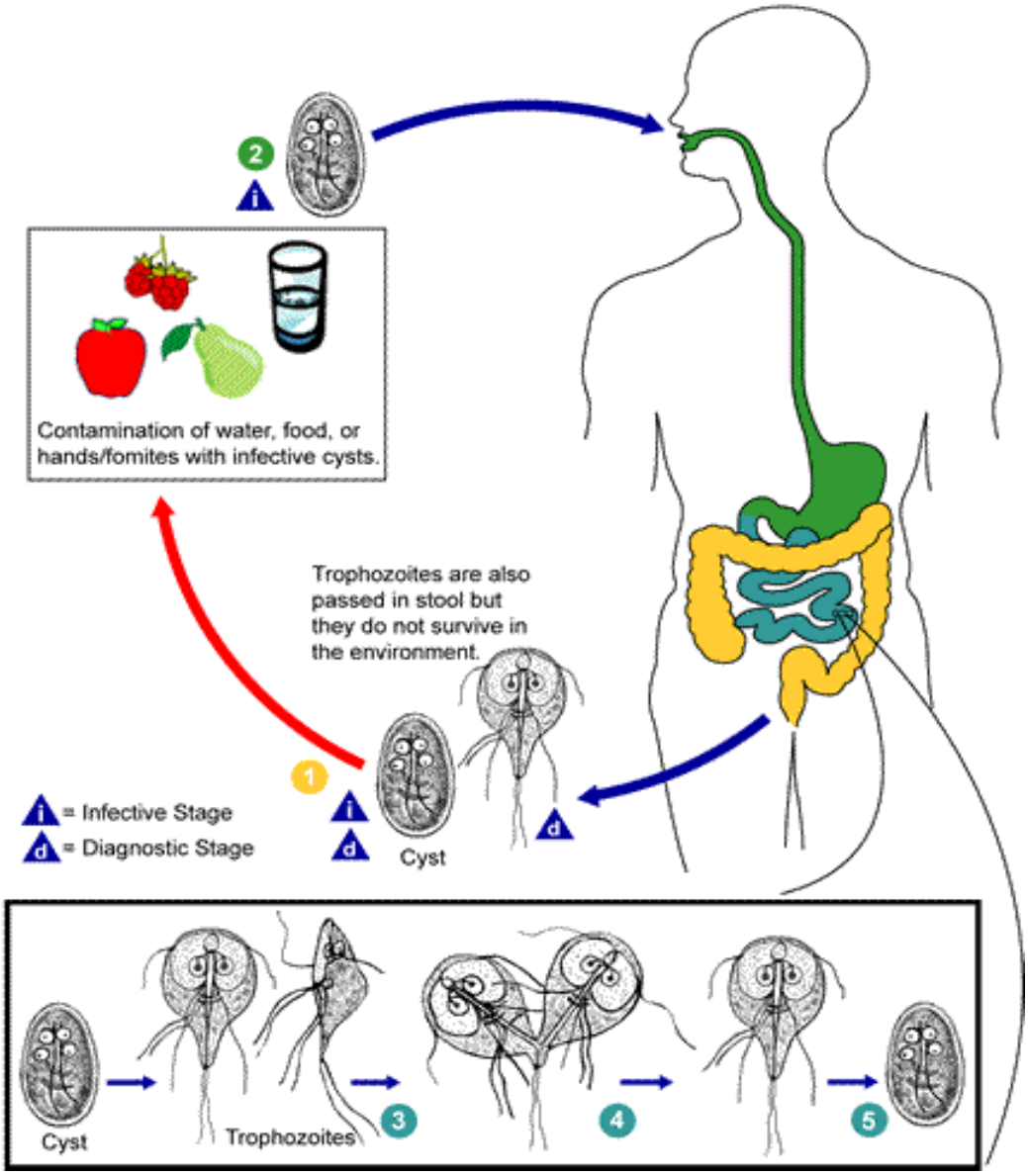

Figure 5. Life cycle of *Giardia* (CDC, 2016c).

Cryptosporidium

Cryptosporidium is an epicellular parasite with a more complex life cycle that involves both sexual and asexual reproduction (Figure 6). The life cycle begins with oral ingestion of the oocysts, which release four infective sporozoites after exposure to the environment within the gastrointestinal tract. Sporozoites are internalised in the enterocytes, specifically into the extracytoplasmatic space, and develop into trophozoites which undergo merogony. These undergo asexual reproduction and re-infect more enterocytes, until they develop into micro- and macrogamonts, which, in turn, undergo sexual reproduction. This results in either the production of a thin-walled oocyst, which may then auto-infect the host, or a thick-walled oocyst which is excreted into the environment to be infective to another host. As with *Giardia* cysts, these oocysts are immediately infectious.

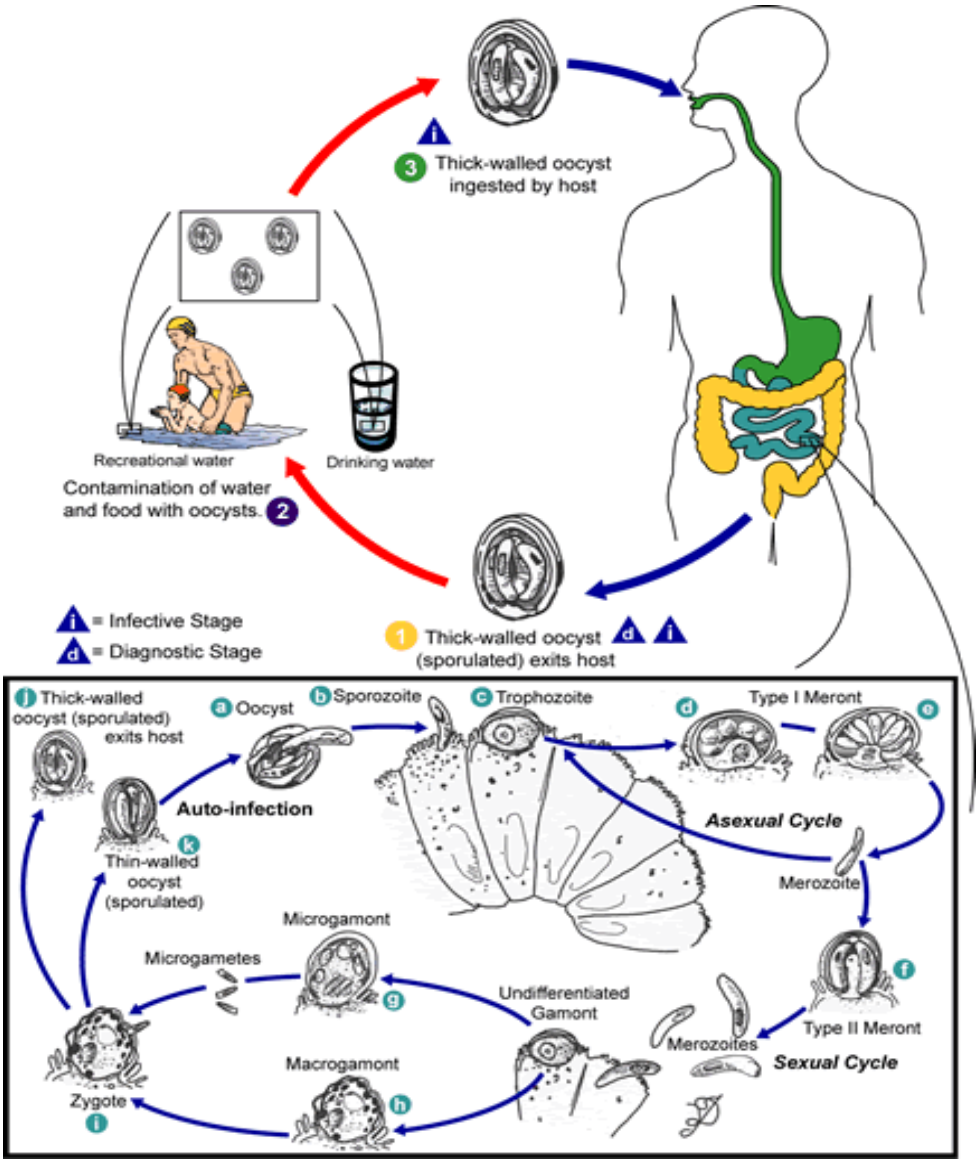

Figure 6. Life cycle of *Cryptosporidium* (CDC, 2016b).

Entamoeba

Entamoeba have a relatively simply life cycle, existing either as an infectious cyst or the amoeboid trophozoite stage (Figure 7; Stanley Jr, 2003). Infection usually results from consumption of food or water containing cysts due to faecal contamination (Stanley Jr, 2003). Within the colon, trophozoites can adhere to the epithelial cells, where they then cause cell death (Stanley Jr, 2003). Trophozoites are also able to invade through the mucosa, causing inflammation in the submucosa. In some hosts, trophozoites then enter the portal system through which they travel to the liver, lungs, or even brain, and can cause the development of abscesses in these organs (Stanley Jr, 2003).

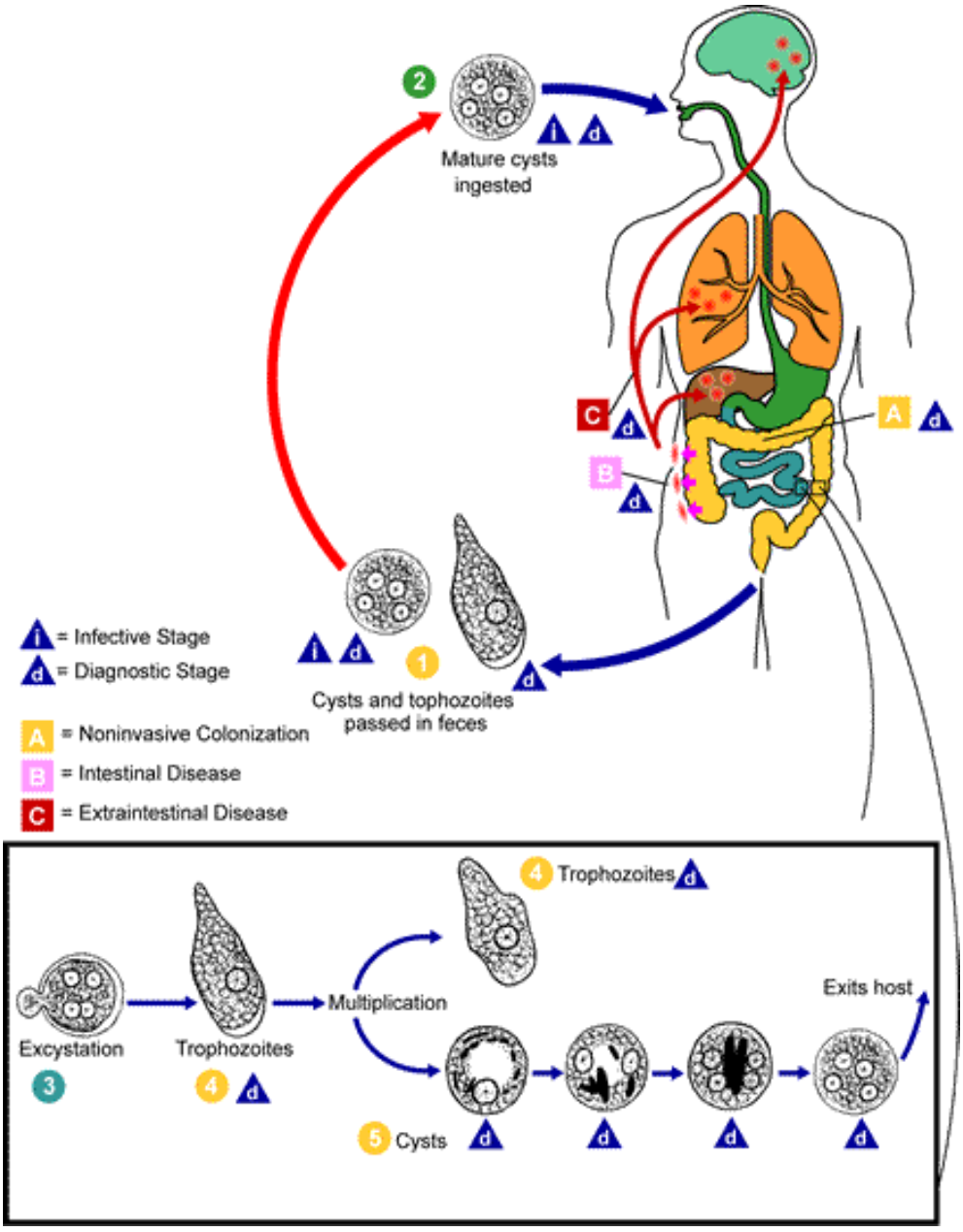

Figure 7. Life cycle of *Entamoeba* (CDC, 2016a).

Eimeria

The life cycle of *Eimeria* spp. begins with the excretion of unsporulated oocysts. After a variable amount of time, roughly 2 - 14 days, these sporulate into sporulated oocysts, which are then infective. This process is dependent on temperature, humidity, and oxygenation. These are then ingested, often with contaminated food / water, and undergo first asexual, then sexual reproduction within the gastrointestinal system, resulting in the excretion of more unsporulated oocysts which are passed out in the faeces (Figure 4).

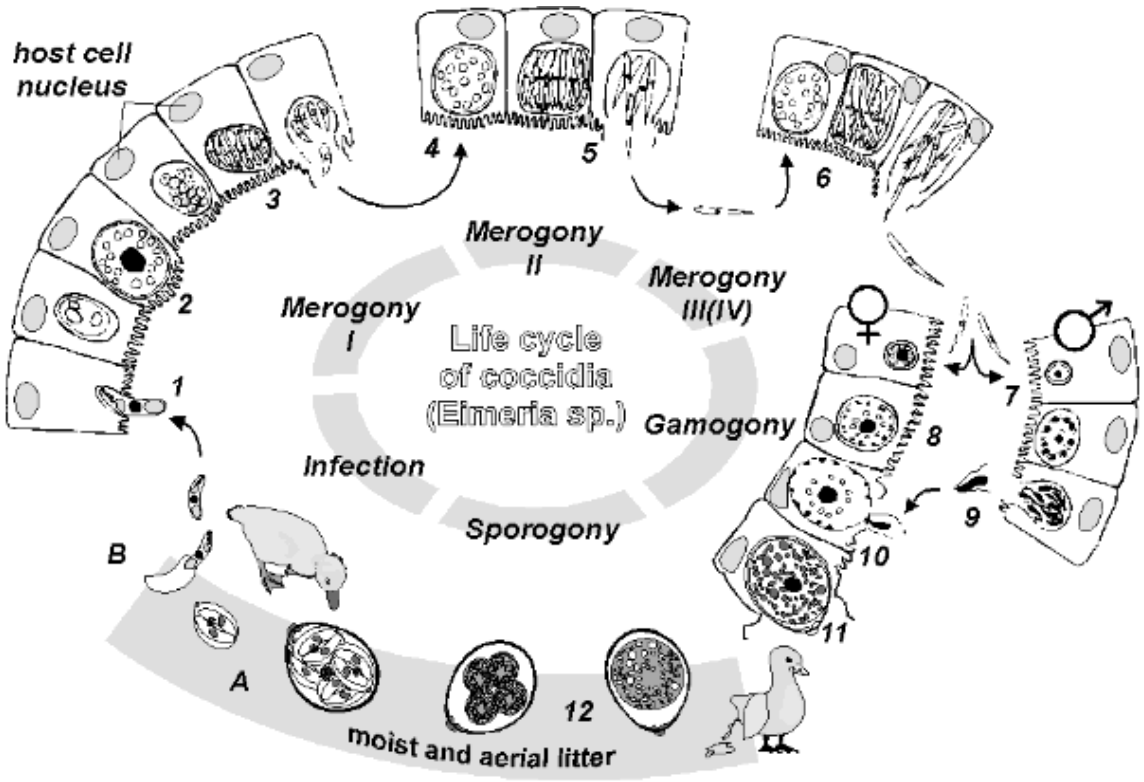

Figure 4. Life cycle of *Eimeria* spp. (Heimann, 2017).

7.4 Diagnosis

These protozoa are usually identified by either detecting (oo)cysts, detecting their antigens, or detecting their DNA in a sample. Generally, PCR and IFAT have higher sensitivities than antigen detection or direct microscopic techniques (Table 3). However, several antigen detection methods utilising enzyme immunoassay have quite high sensitivities and specificities (Bouzid et al., 2013; Garcia and Shimizu, 1997). Detection of host antibodies (serology) may also be used to determine exposure.

Table 3. Advantages and disadvantages of different detection methods for *Cryptosporidium*.

	Advantages	**Disadvantages**
Microscopy	Low technology / cost	Low sensitivity / specificity
	Widely available	Skilled technicians
Antigen detection	No operator skill required	Costly
		Variable sensitivity
IFAT	Excellent sensitivity / specificity	Expensive equipment
	Can quantify	Skilled technicians
Nucleic acid	Excellent sensitivity / specificity	Expensive instruments
amplification	Can speciate, subtype, and quantify	Skilled technicians

(Checkley et al., 2015; Clark, 1999)

Microscopy

Ova and parasite (O & P) examination via microscopy of wet-mount faecal preparations after concentration remains the main tool used to diagnose intestinal parasitic disease in much of the developing world (Ryan et al., 2017). This technique identifies parasites by direct visualisation. In fresh samples, direct wet-mounts may be used to see the motility of *Giardia* and *Entamoeba* trophozoites, and this can be a useful technique in providing rapid answers to clinical disease in cases where large numbers of parasites are excreted. It must, however, be performed on fresh specimens as trophozoites degenerate rapidly without preservation, and in the majority of infections there is no excretion of trophozoites (Fotedar et al., 2007).

Stains may be added to the sample to aid in identification, such as Lugol's iodine for *Giardia* or *Entamoeba* cysts, or acid-fast stains like modified Ziehl-Neelsen (mZN) or auramine phenol for *Cryptosporidium* oocysts. These help to highlight morphological

features of the parasite, allowing it to be more easily distinguished from background debris (Figure 8). By using solutions of different densities, parasite (oo)cysts and eggs may also be separated from the rest of the faecal debris, a technique known as faecal flotation or faecal sedimentation. Faecal flotation is commonly used for the qualitative and quantitative detection of *Eimeria*.

There are also a number of commercially available antibodies with fluorescent tags that bind to the parasite (oo)cyst walls, allowing them to be more easily visualised when viewed under a fluorescent microscope. This technique is known as immunofluorescent antibody testing (IFAT), and is considered as the gold standard in the detection of *Cryptosporidium* and *Giardia*.

One of the limitations of microscopy is its reliance of the skill of the microscopist and, with the exception of IFAT techniques, generally has a lower sensitivity.

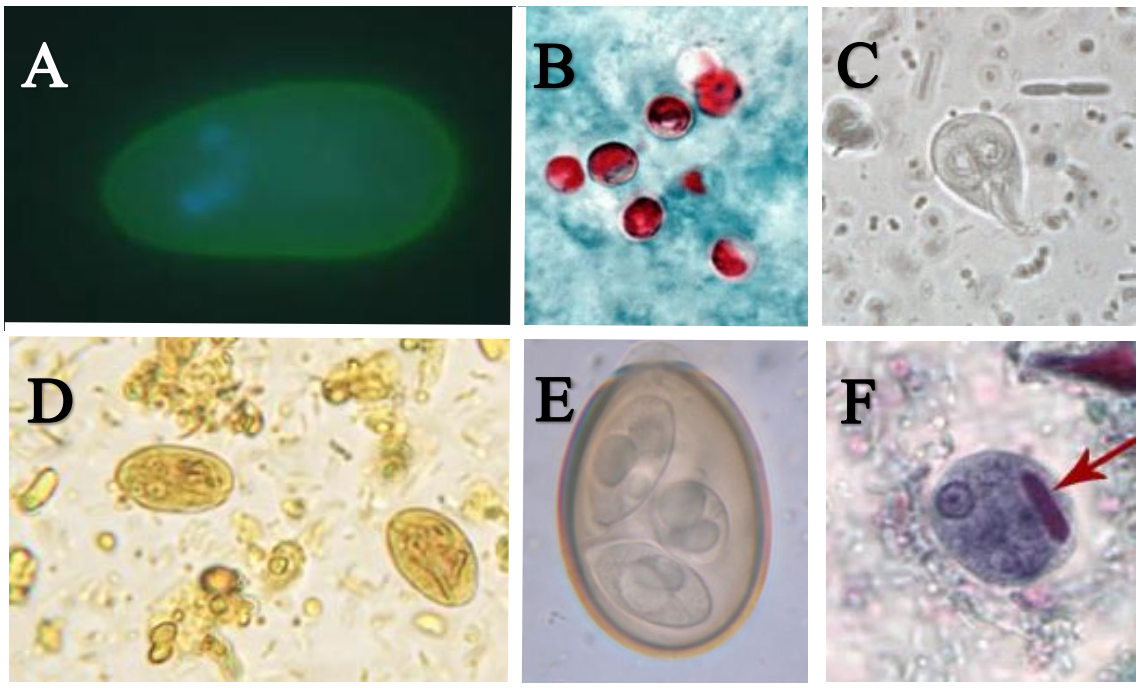

Figure 8. Different forms of microscopy to diagnose enteric protozoa. A) *Giardia* cyst with immunofluorescent antibody and DAPI staining; B) *Cryptosporidium* oocysts with acid-fast stain; C) *Giardia* trophozoite on direct smear; D) *Giardia* cysts with Lugol's iodine stain; E) *Eimeria* oocyst under faecal flotation with saturated saline; F) *Entamoeba* complex cyst stained with trichrome.

Antigen detection techniques

These techniques offer the benefit of rapid, high throughput results, and have been developed for *Giardia*, *Cryptosporidium*, and *Entamoeba*. They include enzyme-linked immunosorbent assays (ELISA) and immunochromatographic tests (IGT). Many different commercial tests have been developed, with quite a range of reported specificities and sensitivities (Figure 9; Ryan et al., 2017; Checkley et al., 2015; Johnston et al., 2003). Importantly, these tests are often developed for parasite species that are pathogenic for humans; *G. duodenalis*, *E. histolytica*, *C. hominis*, and *C. parvum*, and as such, have unknown applicability for other species within these genera.

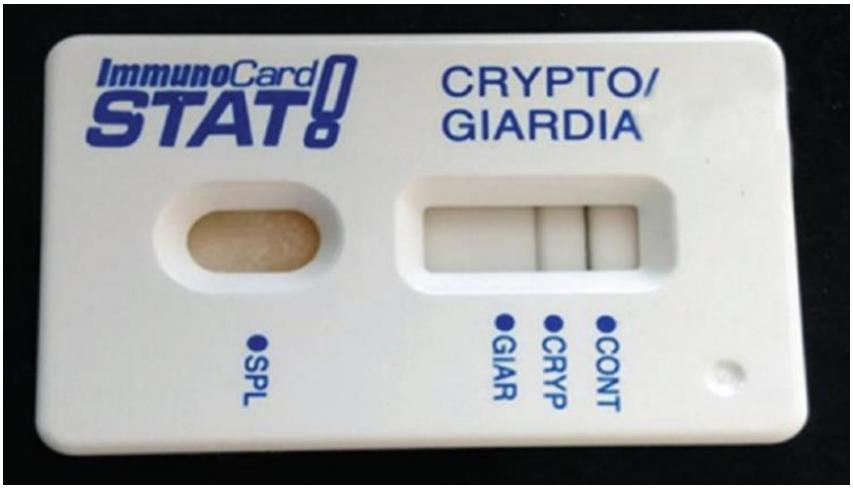

Figure 9. A commercial *Giardia* and *Cryptosporidium* antigen detection kit designed for rapid, patient-side results.

DNA-based techniques

With the advent of molecular diagnostics, it is now possible to detect the presence of very small amounts of DNA from a sample. The specific code of this DNA can then be sequenced, offering information of the specific genetic make-up of the isolate, which in turn allows us to draw conclusions about phylogeny, transmission pathways, epidemiology etc. These techniques are discussed in detail in the Section 10. Materials and Methods.

7.5 Clinical disease

The genera *Giardia*, *Cryptosporidium*, *Entamoeba*, and *Eimeria* are all primarily gastrointestinal parasites, and mainly cause disease associated with this organ system.

Giardiasis is generally a self-limiting illness, with symptoms including diarrhoea, abdominal cramps, weight loss and malabsorption. However, asymptomatic infection is also common (Feng and Xiao, 2011). Disease can be acute or chronic, and has been associated with a failure-to-thrive in children (Cacciò et al., 2005). Long-term sequelae have been reported, including nutritional deficiencies, growth stunting, and irritable bowel syndrome (Nakao et al., 2017; Einarsson et al., 2016). Interestingly, there are also some reports on the potential protective effect that *Giardia* may have against other enteric pathogens (Thompson, 2000).

Cryptosporidiosis has received particular interest within the medical field due to its clinical significance, lack of effective treatment, and recent extensive outbreaks associated with contaminated water sources. After its discovery as a cause of human disease in 1976, *Cryptosporidium* was recognised as a major cause of diarrhoea in immunocompromised people in the 1980s, particularly as the AIDS pandemic established, and by the 1990s it was known to be one of the major causes of childhood malnutrition and pre-mature death in developing nations (Checkley et al., 2015). The reason why *Cryptosporidium* remains resistant to antimicrobials, when other closely related taxa (*Toxoplasma*, *Eimeria*, *Plasmodium*) are sensitive, remains unknown, but may be due to its unique localisation within the host cell; the parasitophorous vacuole (Clark, 1999). Cryptosporidiosis also has a considerable impact on animals, particularly calves and lambs, within the livestock industry (Bouzid et al., 2013).

Cryptosporidiosis can present with a range of severities, based primarily on the host immune status, age, and nutritional status (Shirley et al., 2012). Infection is mainly associated with gastrointestinal symptoms, particularly diarrhoea (Mosier and Oberst, 2000). In children, cryptosporidiosis is associated with a long duration of diarrhoea, and causes high childhood morbidity and mortality in developing countries (Checkley et al., 2015). Cryptosporidiosis may also cause disease outside the GIT, including pancreatitis, cholecystitis, and infection within the renal and respiratory systems (Shirley et al., 2012).

Amoebiasis, or amoebic dysentery, is caused by *E. histolytica* in humans. Symptoms includes loose faeces, stomach pain, haematochezia, and fever; collectively called dysentery (CDC, 2016a; Fotedar et al., 2007; Urquhart et al., 1996). Less commonly, *E. histolytica* can spread beyond the intestinal tract and form abscesses in the liver, or even in the lungs and brain (CDC, 2016a). Despite this potential severity, it is estimated that 80 - 90 % of infections are asymptomatic (CDC, 2016a).

Coccidiosis is, almost exclusively, associated with the development of gastrointestinal symptoms including loose faeces, diarrhoea, maldigestion, haematochezia, poor weight gain, and other secondary bacterial infections due to mucosal disruption. Rarely, some animals are reported to be infected by a systemic form of coccidiosis, such as the short-beaked echidna (*Tachyglossus aculeatus*; Middleton, 2008; Dubey and Hartley, 1993). Pathogenicity varies markedly between different *Eimeria* spp., and clinical disease is dependent on the species involved, and the circumstances of infection, i.e., host age, immunity, level of exposure, etc. In domestic animals, the level of exposure is highly dependent on the animal husbandry such as stocking densities, sanitary conditions, and feeding strategies, all of which contribute to the level of faeco-oral contamination.

7.6 Epidemiology

Epidemiology is the study of the dynamics of a disease within a population; for parasites this refers to the transmission of the parasite between hosts, and how this transmission affects the dispersal of the parasite within and among host populations (Lymbery and Thompson, 2012). Understanding the epidemiology of parasites is important, both for public health as well as for the health of the animals they infect.

Traditionally, our understanding was based heavily on studying infection patterns within different hosts, primarily using parasite morphology to define a parasite species and thus elucidate suspected transmission pathways. However, the genera of *Giardia*, *Cryptosporidium*, and *Entamoeba* all contain morphologically identical variants that have separate pathogenicities, host ranges, and life cycles.

As a consequence, many wildlife populations were thought to be infected with the same parasite variants as those causing disease in humans or domestic animals, and, as such, were

labelled as disease reservoirs (Appelbee et al., 2005). Indeed, much of the information on infections in wild animals comes from prevalence studies focused on their potential to act as a reservoir of disease for humans or livestock. For instance, wild animals were thought to be the cause of early outbreaks of human giardiasis, leading to the disease being referred to as 'beaver fever'. A similar trend has been seen in *Cryptosporidium* in wildlife, with original reports of *C. parvum* in wildlife based on oocyst morphology, with later genetic studies showing these were indeed host-adapted species (Appelbee et al., 2005).

Our understanding of the taxonomy and epidemiology of many protozoa has been revolutionised by molecular typing tools. These have enabled the genetic characterisation of isolates, beyond describing simple morphology or time-consuming pathogenicity studies (Cacciò et al., 2005; Feng and Xiao, 2011). In many cases, these have shown wildlife not to be reservoirs of human disease, rather for wildlife species to be infected with their own genetically distinct, albeit morphologically identical, lineages (Appelbee et al., 2005). To date, molecular tools have not been used as extensively in the study of *Eimeria* spp., with the exception of those infecting domestic chickens. This is most likely due to the host specificity and morphological variation seen amongst *Eimeria* spp.

Giardia

Giardia spp. infect a range of host species from mammals to amphibians to birds (Table 1; Ryan and Caccio, 2013). In humans, it is known that there are around 200 million symptomatic cases of giardiasis in people from Asia, Africa and Latin America every year (Feng and Xiao, 2011). The prevalence of infection is lower in developed countries, 0.4 - 7.5 %, than in developing countries, 8 - 30 % (Feng and Xiao, 2011). In the developed world, it is often considered that the main burden is due to sporadic outbreaks, most of which are associated with contaminated water sources (Ryan and Caccio, 2013).

Within the *G. duodenalis* species complex, Assemblages A and B both infect humans, with local variations in their respective prevalences. Overall, there appears to be a higher prevalence of Assemblage B (58 %) than Assemblage A (37 %) in humans (Ryan and Caccio, 2013; Feng and Xiao, 2011). Although Assemblages other than A and B are occasionally found in humans, these results have been criticized as they are often based on the SSU rRNA gene alone, which has poor resolution in distinguishing Assemblages (Feng and Xiao, 2011). The usability of the SSU rRNA gene is debated, however, with others

claiming that it can reliably group isolates into their Assemblages (Wielinga and Thompson, 2007). Nevertheless, it can be safely assumed that infection in humans with Assemblage C to H only occurs under exceptional circumstances.

Giardia infection is common in a range of livestock including pigs, cattle, sheep, goats, deer, and other ruminants, as well as in various wildlife species (Feng and Xiao, 2011; Appelbee et al., 2005). Despite this, confirmed cases of zoonotic transmission appear to be rare, with weak evidence of zoonotic transmission of *Giardia* from dogs to humans, or livestock to humans (Thompson and Smith, 2011). Indeed, through quite extensive studies of the prevalence of human pathogenic *Giardia* Assemblages in domestic animals, it has become clear that the risk of humans being infected with *Giardia* from domestic animals is quite small, with most livestock and domestic pets infected with their host-adapted Assemblages (Feng and Xiao, 2011). In cattle, sheep, and pigs, the dominating genotype is Assemblage E, with only a small percentage (< 20 %) infected with zoonotic Assemblage A. Although there are insufficient data available to draw conclusions on similar risks with wildlife, preliminary data suggest that zoonotic *Giardia* assemblages may be more common in wildlife. For instance, in Europe, 74 % of 172 *Giardia* isolates from wild animals were Assemblages A and B (Sprong et al., 2009). Similar trends have been seen in marsupials in Australia (Thompson et al., 2008).

Cryptosporidium

Cryptosporidium spp. infect at least 79 species of animal, including a range of wildlife species (Table 2) (Mosier and Oberst, 2000; Appelbee et al., 2005). In contrast to *Giardia*, *Cryptosporidium* spp. found in wildlife are often host adapted, and thus do not pose a threat to public health (Zhou et al., 2004). Contrastingly, livestock, notably calves and lambs, have been linked as a common source of *C. parvum* in humans. *Cryptosporidium* spp., mainly *C. hominis* and *C. parvum*, have been responsible for large-scale waterborne epidemics in the developed world. It is, however, in the children of the developing world where *Cryptosporidium* has its greatest burden, with this pathogen amongst the top four causes of moderate-to-severe paediatric diarrhoea, and associated with increased mortality (Kotloff et al., 2013; Sow et al., 2016; Shirley et al., 2012; Checkley et al., 2015).

Early reports underestimated the prevalence of *Cryptosporidium* due to the difficulty in identifying oocysts using conventional light microscopy. Today, it is estimated that 15 - 25

% of children with diarrhoea are infected with *Cryptosporidium* (Checkley et al., 2015). Geographical variation in the prevalence of different *Cryptosporidium* spp. occurs at both the continental and regional level; *C. hominis* is more common in the Americas, Africa and Australia, whereas *C. parvum* is more common in Europe and the Middle East (Cacciò et al., 2005; Shirley et al., 2012).

Although a number of *Cryptosporidium* spp. infect wildlife species, the majority of these isolates do not appear to be capable of infecting humans (Table 2). Indeed, when it comes to zoonotic transmission, the most significant transmission route appears to be from livestock, particularly calves and lambs, which can excrete large quantities of *C. parvum* oocysts.

Entamoeba

Entamoeba histolytica is responsible for up to 100 000 human deaths annually, with infection most common in tropical regions where sanitary conditions are poor (Stanley Jr, 2003; WHO/PAHO/UNESCO, 1997; Fotedar et al., 2007; CDC, 2016a). Despite this, the epidemiology of *E. histolytica*, *E. dispar*, and *E. moshkovskii* remains uncertain, primarily since most existing data do not distinguish between the three species.

Our understanding is even more limited when it comes to the epidemiology of *Entamoeba* spp. other than *E. histolytica*. Some species are known to infect animals and humans, thus should be considered zoonotic; e.g., *E. dispar* infects both humans and NHPs, *E. polecki* infects humans and pigs (Clark et al., 2006). Although various *Entamoeba* spp., including *E. dispar*, *E. coli*, *E. chattoni*, *E. hartmanni*, and *E. nutteli*, are commonly found in the faeces of NHPs, their virulence in these hosts remains unknown (Feng et al., 2011; Feng et al., 2013). Experimental infections have demonstrated that rhesus macaques are susceptible to infection from *E. histolytica*, and that the resulting disease mimics human infection (Haq et al., 1985). However, the extent to which natural infections occur remains unclear. Further complicating the issue, is that whilst there are some reports of naturally occurring *E. histolytica* in captive NHPs, other studies have first suspected infection with *E. histolytica* in NHPs based on preliminary molecular results, only to conclude that these isolates are genetically distinct to those infecting humans following further molecular characterisation of the isolates (Tachibana et al., 2007; Takano et al., 2007).

Eimeria

The epidemiology of *Eimeria* spp. is highly dependent on the species in question. With over 1 000 species described, and likely many more that have not been described to date, a review of this genus is beyond the scope of this thesis. It is noteworthy, however, that infections with *Eimeria* are most common in younger animals, and where environmental conditions lead to extensive contamination of food and water with faeces. Also of importance, is that whilst the species of *Eimeria* described in domestic livestock and poultry are well described, those that infect closely related host species of wildlife remain largely unknown. Thus the extent of the potential for particular species of this otherwise host-specific genus, to transmit between domestic and wild animals, is not fully resolved. Although there have not been any records of such transmission, this is an area that receives little attention from the scientific community.

7.7 Emerging diseases at the wildlife–human–domestic animal interfaces

The barriers between wildlife, domestic animals, and humans appear to be becoming more porous, at least from a pathogen's perspective. Habitat fragmentation, urbanization, sharing of water sources, international travel, ecotourism, commercial bush-meat industry, logging, and climate change, have all increased the contact between previously remote wildlife populations and the global human / domestic animal populations. It may be argued that humans were more exposed to wildlife pathogens when living as hunter-gather societies. However, this was limited to a local scale, and pathogens did not have the ability to spread rapidly between larger wildlife / human / domestic animal populations. Indeed, it may be argued that in today's world there are no longer ecosystems void of anthropogenic influence. This potential currently exists, owing to higher densities of both humans and domestic animals, as well as the extensive and unprecedented movement and transport of humans and animals around the globe. These factors together, mean that the potential consequences of disease transmission are much greater.

The extent to which these different variables actually influence transmission of diseases between humans, their animals, and wildlife is unknown, and is probably affected by a range of factors associated with the pathogens, their hosts, and the environment. Understanding these transmission routes and their potential is usually considered within the discipline of "One Health", in which animal health, human health, and the environment are considered together rather than in isolation. The importance of the One Health approach has

been highlighted by recent disease epidemics such as West Nile Virus, avian influenza, Hendra virus, Ebola virus, and MERS-CoV (Appelbee et al., 2005). The benefits of a One Health approach to managing disease have been seen in the control and reduction of trichinellosis (Figure 10). Surveys of published literature state that over 70 % of emerging infectious diseases originate in wildlife (Jones et al., 2008). However, these have since been criticised as overstating the true risk of wildlife to public health (Kock, 2014).

When looking at emerging infectious diseases at the wildlife-human-domestic animal interface, it is first important to determine whether humans, livestock, and wildlife are susceptible to the same infectious agent. Here, it is not sufficient to consider only the genus, nor necessarily the species, but whether parasites that are genetically very similar can infect different host species; in some cases, maybe only infection studies can resolve this question. Once the question of host-specificity is resolved, then the next question is whether transmission pathways that enable hosts in different compartments to infect each other are established. The potential for transmission depends on a multitude of factors include population locations and densities, food / water sources, ranging patterns, etc. Although answering the first question indicates the potential for zoonotic transmission, it does not answer how often zoonotic transmission actually occurs.

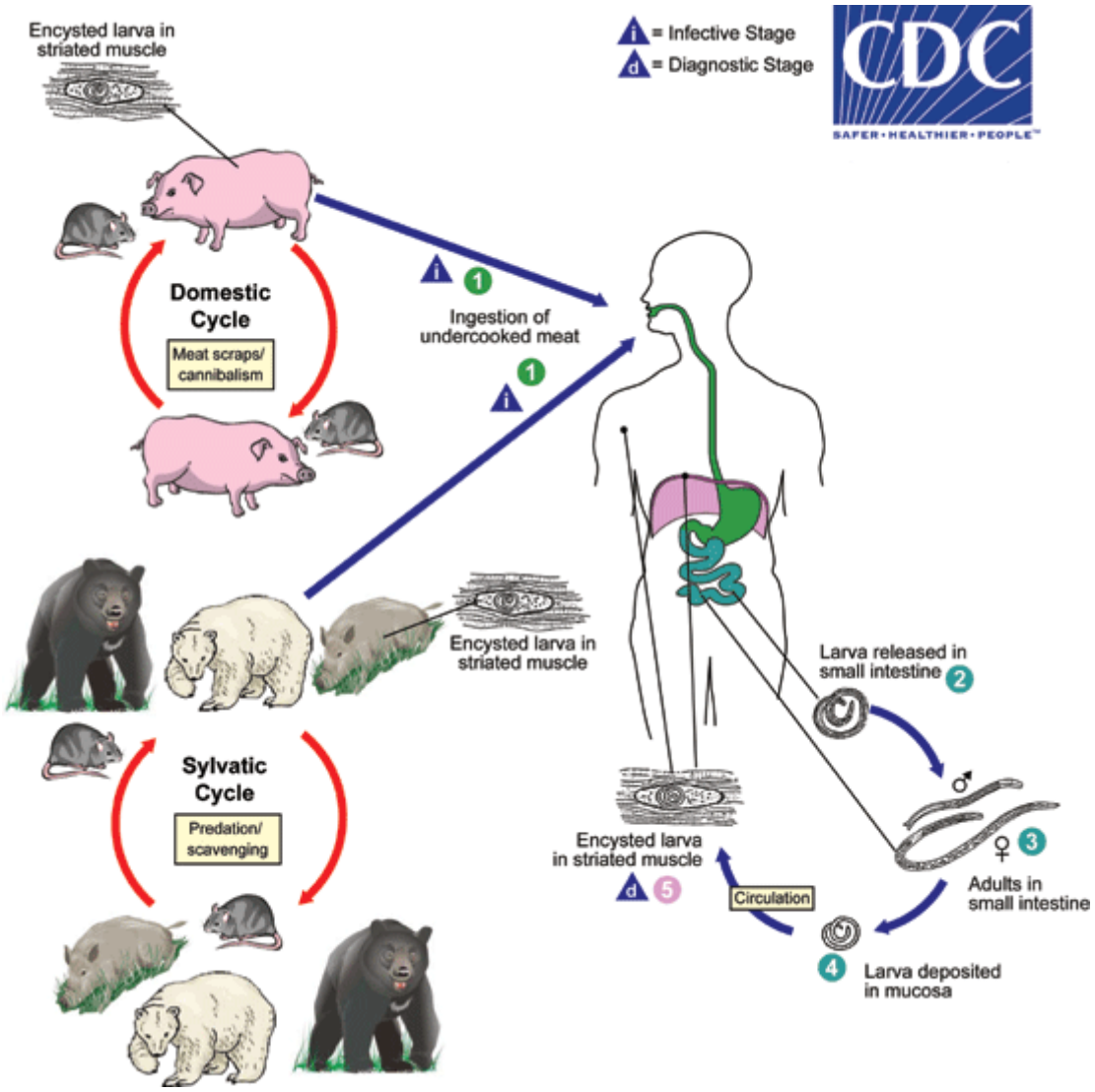

Figure 10. Life cycle of *Trichinella*, an example of a pathogen with a life cycle that involves wild animals, domestic animals, and humans (CDC, 2016d).

Regarding the transmission of pathogens between humans and wildlife, the risk is probably greatest for those wildlife species that have a close taxonomic relationship to humans e.g. NHPs, as pathogens may be more easily able to cross the species boundary. With respect to threats to domestic livestock or food safety, wildlife species that are closely related to livestock species, e.g. wild ungulates, may present the greatest risk. Also of importance, is studying wildlife that are closely related to domestic pets, such as wild canids. Their importance is seen in the epidemiology of echinococcosis, particularly *Echinococcus multilocularis*, where wild canids serve as a reservoir for disease that then spills over to domestic dogs, which may be more likely than foxes to transmit the disease to humans.

Giardia, *Cryptosporidium*, and *Entamoeba* are distributed throughout the world, causing substantial health risks where there is faecal contamination of food and water sources (Stanley Jr, 2003). Although all three protozoa may cause disease in the developed world, their real burden lies in developing regions. Developing nations often suffer from poverty, lack of hygiene, poor cooking facilities, free-roaming animals, high population densities, insufficient access to health care, and infrastructure inadequacies regarding water supply and sanitation, all of which facilitate infection. This not only makes the public susceptible to diseases from the animals with which they live, but also threatens the wild animals under anthropogenic influence. For instance, rhesus macaques (*Macaca mulatta*) are one of the most common primates in India, particularly in human-dominated habitats (Kumar et al., 2013). Indeed, in some Indian districts, the close contact between rhesus macaques and human activities means that they are regarded as a nuisance, particularly due to crop-raiding activities (Saraswat et al., 2015). Macaque species have already been implicated as wildlife reservoirs for zoonotic pathogens, such as Kyasanur Forest disease, a zoonotic tick-borne viral haemorrhagic fever (Singh and Gajadhar, 2014). Nevertheless, it is unclear whether there is transmission of intestinal protozoa between humans and urban monkeys, and, if so, how significant this is for public health and for the conservation of the macaques.

7.8 Disease: a major conservation concern

Wildlife species across the planet are under threat, with a wave of extinctions across all animal taxa (Ceballos et al., 2015). This is being driven by a range of factors including habitat loss, habitat fragmentation, hunting for sport / food / fur / pets, and disease. In general, disease is not the major force pushing species towards extinction. With this said, it is recognised that the threat of disease increases as a species moves towards extinction; i.e., as the population of a species declines, the role of disease in the survival of that species increases (Heard et al., 2013). Importantly, certain disease events can have catastrophic impacts on animal populations and thus conservation, as has been seen with chytridiomycosis in amphibians, white nose syndrome in bats, and facial tumour disease in Tasmanian devils (Alves et al., 2014; Pye et al., 2016; Kilpatrick et al., 2010).

7.9 Knowledge gaps

At the moment very little is understood about the epidemiology and impact of *Giardia, Cryptosporidium*, *Entamoeba*, or *Eimeria* in wildlife species. A number of species from within these genera have been identified in wildlife, leading to theories that they may act as reservoirs for human disease. However such transmission cycles have yet to be definitively demonstrated. This has led to a number of questions:

- Which wildlife species are infected with which species within these genera?

- What is the impact of these parasites on wildlife populations?

- Can wildlife act as a disease reservoir for human infections?

- Do humans or domestic animals pose a threat to the conservation of wildlife through the transmission of these protozoan parasites?

- Does the prevalence of infection in wildlife correlate with the extent of human contact or the types of human or livestock activities to which the wildlife species are exposed?

- Is there evidence of host-parasite co-evolution amongst non-host specific parasites; i.e., are wildlife species that are closely related to humans, such as NHPs, infected with parasites closely related to, or the same as, those infecting humans? Are similar relationships seen between the parasites in wild ungulates and domestic livestock, or wild canids and domestic dogs?

8. Aims of the Study

General Objective: To investigate the epidemiology of zoonotic intestinal protozoa, *G. duodenalis*, *Cryptosporidium* spp., and *Entamoeba* spp., in wildlife in order to provide a foundation for investigation of cross-transmission possibilities between host groups, and to examine the co-evolution of hosts with their parasites.

Specific objectives:

1. Review literature regarding epidemiology of *Giardia*, *Cryptosporidium*, *Entamoeba*, and *Eimeria* in wildlife species.

2. Investigate the prevalence of *Giardia*, *Cryptosporidium*, and *Entamoeba* in NHPs with varying degrees of human contact and use molecular analyses to determine whether anthropozoonotic or zoonotic transmission is likely to be occurring.

3. Perform a meta-analysis on the available sequence data on *Giardia* in NHPs and compare these with isolates from humans.

4. Investigate the prevalence of *Giardia* and *Cryptosporidium* in wild ungulates and livestock in Tanzania, and use molecular analyses to determine whether transmission is occurring between these groups.

5. Identify the species of *Eimeria* that infect wild ungulates in Tanzania, and compare the phylogenetic relationships of *Eimeria* spp. in wild ungulates with those infecting livestock to determine whether wild ungulate – domestic ungulate transmission may be occurring.

6. Investigate the prevalence of *Giardia* and *Cryptosporidium* in wild Swedish red foxes and use molecular analyses to determine whether zoonotic transmission is possible, or whether other transmission cycles, e.g., with domestic dogs, is occurring.

7. Investigate the prevalence of *Giardia* and *Cryptosporidium* in captive Norwegian reptiles, and determine whether there is a zoonotic potential.

9. Summary of the papers

Paper I:

Occurrence of *Giardia* and *Cryptosporidium* in captive chimpanzees (*Pan troglodytes*), mandrills (*Mandrillus sphinx*) and wild red colobus monkeys (*Procolobus kirkii*).

This study investigated the occurrence of *G. duodenalis* and *Cryptosporidium* spp. in primates and determined their zoonotic or anthropozoonotic potential. Direct immunofluorescence was used to identify *Giardia* and *Cryptosporidium* from faecal samples. PCR and DNA sequencing was performed on positive results. *Giardia* cysts were identified from 5.5 % (5 / 90) of captive chimpanzees and 0 % (0 / 11) of captive mandrills in the Republic of Congo; 0 % (0 / 10) of captive chimpanzees in Norway; and 0 % of faecal samples (n = 49) from wild Zanzibar red colobus monkeys. Two *Giardia* positive samples were also positive by PCR, and sequencing revealed identical isolates of Assemblage B. *Cryptosporidium* oocysts were not detected in any of the samples. In these primate groups, in which interactions with humans and human environments are quite substantial, *Giardia* and *Cryptosporidium* are rare pathogens. In chimpanzees, *Giardia* may have a zoonotic or anthropozoonotic potential.

Paper II:

Occurrence of *Giardia, Cryptosporidium*, and *Entamoeba* in wild rhesus macaques (*Macaca mulatta*) living in urban and semi-rural North-West India.

This study investigated the occurrence *G. duodenalis*, *Cryptosporidium* spp., and *Entamoeba* spp. in rhesus macaques (*Macaca mulatta*) in India. This provides preliminary information on the potential for transmission of these pathogens between macaques and humans. Faecal samples (n = 170) were collected from rhesus macaques from four districts of North-West India. Samples were analysed for *Giardia / Cryptosporidium* using a direct immunofluorescence after purification via immunomagnetic separation. Positive samples were characterised by sequencing PCR products. Occurrence of *Entamoeba* was first investigated by using a genus-specific PCR, and positive samples further investigated via species-specific PCRs for *E. coli*, *E. histolytica*, *E. dispar*, and *E. moshkovskii*. *Giardia*

cysts were found in 31 % of macaque faecal samples, with all isolates belonging to Assemblage B. *Cryptosporidium* oocysts were found in 1 sample, but this sample did not result in amplification by PCR. *Entamoeba* spp. were found in 79 % of samples, 49 % of which were positive for *E. coli*. Multiplex PCR for *E. histolytica*, *E. dispar*, and *E. moshkovskii* did not result in amplification in any of the samples. Thus, in 51 % of the samples positive at the genus-specific PCR, the *Entamoeba* species was not identified. This study provides baseline information on the potential for transmission of these zoonotic parasites at the wildlife-human interface.

Paper III

Phylogenetic analysis of *G. duodenalis* sequences in primates: evidence of potential zoonotic and anthropozoonotic transmission

This study used publicly available genotyping data to investigate the relatedness of human and NHP *Giardia* isolates in order to assess the potential for zoonotic transmission and evaluate the usefulness of the current taxonomic classification. Our final data set consisted of 165 isolates, 111 from NHP and 54 from humans. Sequence data consisted of the four commonly sequenced loci: SSU rRNA, *tpi*, *gdh*, and *bg*. Assemblages were well defined, but sub-Assemblages across Assemblage B were not resolved. Although sub-Assemblages AI and AII were resolved, the terms were not found to capture any useful molecular or host / deme properties. Nonhuman primate isolates were scattered among *Homo* isolates across Assemblage A and B, and were even found in Assemblage E. We evaluated the relative merit of the four genes for use in genotyping studies. The *tpi*, *gdh*, and *bg* genes gave relatively congruent tree topologies, but the SSU rRNA gene did not even resolve Assemblages consistently. Based on our results, there does not appear to be any molecular distinction between human and NHP *Giardia* isolates across these molecular markers. The risk for zoonotic and anthropozoonotic transmission of Assemblage A and B isolates must therefore be viewed as present, irrespective of sub-Assemblage classification. Future *Giardia* genotyping efforts should aim for multilocus or whole-genome approaches and, in particular, avoid using the SSU rRNA gene as the sole marker. However, due to the fact that the SSU rRNA gene is present in multiple copies, and thus may be more likely to give a positive result by PCR, in some samples it may the only PCR target that provides a positive result for indicating Assemblage.

Paper IV:

Low occurrence of *Giardia* and *Cryptosporidium* in domestic cattle and wild herbivores in and around Mikumi National Park, Tanzania.

This study investigated the prevalence of *Giardia* and *Cryptosporidium* at the wildlife-human-livestock interface around Mikumi National Park, Tanzania, in order to identify if transmission of these protozoa is occurring between different wild and domestic herbivores. Faecal samples were collected from wild herbivores (*n* = 110; African buffalo, eland, giraffe, impala, wildebeest and zebra) within Mikumi National Park, as well as from domestic cattle (*n* = 48) from two villages in the immediate proximity. *Giardia* and *Cryptosporidium* were detected via direct immunofluorescence after immunomagnetic separation, and positive samples further characterised by PCR. All faecal samples were negative for *Giardia* cysts. *Cryptosporidium* oocysts were found in 5 % (2 / 39) of African buffalo samples, whilst samples from all other taxa (domestic cattle, eland, giraffe, impala, blue wildebeest, and zebra) were negative. Neither of these two positive samples resulted in amplification by PCR. These results suggest either that the conditions around Mikumi National Park are not conducive to the spread of these two pathogens, or that these are largely naïve populations, and thus may be susceptible to the emergence of giardiasis or cryptosporidiosis in the future.

Paper V:

Five species of coccidia (Apicomplexa: Eimeriidae), including four new species, identified in the faeces of blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

This study identified and described the species of *Eimeria* infecting blue wildebeest (*Connochaetes taurinus*), and determined whether there was the potential for transmission to domestic livestock. During October 2013, 112 faecal samples were collected from wild blue wildebeest in Mikumi National Park, Tanzania, and examined for coccidians. Coccidia were present in 46 % of samples, with wildebeest shedding 60 to 18 000 OPG of faeces (median, 300; mean, 1 236). Five species, including 4 new species, were identified. Oocysts of *Eimeria gorgonis* from 18 % of samples were ellipsoidal, 23 × 18.4 µm, with a length/width (L / W) ratio of 1.3, oocyst wall 1-1.5 µm thick. Micropyle, oocyst residuum

and polar granule absent. Oocysts of *Eimeria donaldi* n. sp. from 34 % of samples were spherical to oblong, 13.4 × 12.3 µm, L / W ratio 1.1, oocyst wall 1 µm thick. Micropyle, oocyst residuum and polar granule absent. Oocysts of *Eimeria nyumbu* n. sp. were ellipsoidal, 30.8 × 22.1 µm, L / W 1.4, oocyst wall 2 µm thick. Large micropyle present, oocyst residuum and polar granule absent. Oocysts of *Eimeria burchelli* n. sp. in 16 % of samples were 34.8 × 24.4 µm, L / W 1.4, oocyst wall 2-2.5 µm thick, with a brown, lightly stippled outer layer. Micropyle present, oocyst residuum and polar granule absent. Oocysts of *Eimeria sokoine* n. sp. in 5 % of samples were 45.8 × 29 µm, L / W 1.6, oocyst wall 3-4 µm thick with a dark brown, very rough, stippled outer layer. Micropyle present, oocyst residuum and polar granule absent. There was no apparent cross transmission of coccidia found in blue wildebeest with those generally reported to infect domestic cattle.

Paper VI:

Occurrence of *Giardia* in Swedish red foxes (*Vulpes vulpes*)

The aim of this study was to investigate the occurrence of *G. duodenalis* in wild Swedish red foxes (*Vulpes vulpes*), with the aim of providing preliminary information on how this abundant predator may be involved in the transmission and epidemiology of *G. duodenalis*. Faecal samples (*n* = 104) were analysed for *G. duodenalis* using direct immunofluorescence. *Giardia duodenalis* cysts were found in 44 % (46 / 104) of samples, with foxes excreting 100 to 140 500 CPG of faeces (mean: 4 930; median: 600). Molecular analysis, using PCR with sequencing of PCR amplicons, was performed on fourteen samples, all containing over 2 000 CPG of faeces. Amplification only occurred in four samples at the *tpi* gene, sequencing of which revealed Assemblage B in all four samples. This study provides baseline information on the role of red foxes in the transmission dynamics of *G. duodenalis* in Sweden, and suggests that zoonotic or anthropozoonotic transmission may be possible.

10. Materials and Methods

10.1 Target wildlife populations

Disease transmission at the wildlife-livestock-human interface is a broad field, covering a wide range of parasites, geographical locations, and host species. This thesis focused on four broad groups of wild animals: NHPs, wild ungulates, wild canids, and reptiles. The first three of these target groups were chosen due to their close taxonomic relationship to humans, livestock, and domestic dogs, respectively. This choice was based on the principle that, in general, pathogens are more likely to transmit between host species that are closely related, and thus disease transmission may be more likely at these interfaces. Reptiles were studied due to the recent proposal to remove the ban on keeping reptiles as pets in Norway, something which would increase human-reptile contact. The populations studied are also representative of a broad range of geographical locations including Europe (Norway and Sweden), Africa (Republic of Congo and Tanzania), and Asia (India).

Nonhuman primates

1. Tchimpounga Chimpanzee Rehabilitation Centre (TCRC), the Republic of Congo.

Faecal samples (n = 269) were collected from captive chimpanzees (*Pan troglodytes*, n = 90) and mandrills (*Mandrillus sphinx*, n = 11). The majority (88 / 90) of chimpanzees and all mandrills were wild born and entered captivity as young orphans. The chimpanzees were housed in 5 permanent enclosures as well as various quarantine holding facilities, whilst mandrills were housed in 3 different enclosures. Infant and juvenile chimpanzees had daily direct physical contact with caregivers, whereas mandrills and adult chimpanzees had limited physical contact with carers.

2. Kristiansand Zoo, Norway.

Faecal samples (n = 29) were collected from captive chimpanzees (n = 10). All bar one chimpanzee was captive born, with the remaining chimpanzee having been in European zoos for over 20 years. All chimpanzees were housed together in a single enclosure, and had limited physical contact with carers.

3. Jozani Forest, Zanzibar, Tanzania.

Faecal samples (*n* = 58) were collected from wild Zanzibar red colobus monkeys (*Procolobus kirkii*) belonging to five separate troops. Direct physical contact between humans and the wild Zanzibar red colobus monkeys was minimal. Nevertheless, these troops were under anthropogenic influence through an ecotourism venture, where over one hundred tourists would enter Jozani Forest and come within 1 m of the monkeys on a daily basis.

4. North-west India.

Faecal samples (*n* = 170) were collected from free-living rhesus macaques (*Macaca mulatta*) in four non-overlapping locations in North-west India.

Troop 1: Located at Punjab University, Chandigarh. Monkeys move freely throughout the campus, spending large amounts of time feeding, defecating, and sleeping near areas used for preparation of human food. Estimated troop size, 300 animals.

Troop 2: Located at Jakhoo Temple, Himachal Pradesh. Primarily based around a forested hilltop temple, however also move freely into the surrounding city of Shimla. Estimated troop size, 200 animals.

Troop 3: Located around a small local temple in the municipality of Kurali, Punjab. This temple also owns a cattle-breeding facility where the troop spends much of its time. There is direct contact between the cows and the monkeys, with macaques eating grain provided to the cattle and picking up food from the ground that is contaminated with cattle faeces. Estimated troop size, 100 animals. In addition, faecal samples (*n* = 14) were collected from calves from the breeding facility in Kurali with which Troop 3 was in close contact.

Troop 4: Located on the outskirts of a semi-rural town Nada Sahib, Haryana. Co-exists with roughly 30 Tarai grey langurs (*Semnopuithecus hector*). Estimated troop size, 200 animals.

Wild and domestic herbivores, Mikumi, Tanzania

Freshly voided faeces were collected from wild African buffalo (*Syncerus caffer*; *n* = 39), eland (*Taurotragus oryx*; *n* = 8), giraffe (*Giraffa camelopardalis*; *n* = 12), impala (*Aepyceros melampus*; *n* = 21), blue wildebeest (*Connochaetes taurinus*; *n* = 21) and zebra (*Equus quagga*; *n* = 9) in Mikumi National Park, Tanzania (7°16'58.6"S, 37°7'03.1"E), during a one-week period in October 2013. In addition, freshly voided faeces were collected from domestic cattle (*Bos indicus*; *n* = 48) overnighting in two locations on the fringe of

Mikumi NP; Mikumi town (7°24'12.1"S, 36°58'56.0"E) and another settlement (7°03'55.6"S, 37°02'01.5"E) during the same period.

Wild red foxes (Vulpes vulpes), Sweden
Faecal samples (*n* = 104), originating from 16 Swedish counties, were collected opportunistically by hunters between August and December 2014, as part of a national *Echinococcus multilocularis* survey.

Captive reptiles, Norway
Faecal samples (*n* = 54) were collected from 24 species (Table 4) of clinically healthy reptiles from three anonymous zoos in Norway.

10.2 Collecting faeces in the field

Studying intestinal parasites in wild animals poses several challenges. Wildlife often live in hard-to-reach places, can be difficult to find and even more difficult to approach, and data on the health and history of these animals are usually lacking. Methods to collect samples from wildlife include utilising hunted specimens, non-invasive sample collection (i.e., picking up faeces from the ground), and directly collecting faeces from the rectum of animals that have been physically / chemically immobilised. All of these techniques have advantages and disadvantages, and the most appropriate technique depends on the study design, geographical location, host species, and target parasite.

Some important points to consider for each technique are:

- Hunted specimens: difficult to predict when / how many samples, represents a biased portion of the population, often cheap.
- Direct collection: dependent on invasive physical / chemical immobilisation, expensive, time consuming, more difficult to get approval.
- Non-invasive collection: difficult to link a sample to a specific individual or even host species, cheap.

The primary technique utilised throughout this thesis was non-invasive collection. Non-invasive collection required observation of the target species, and then collection of fresh, morphologically consistent stools from the area where they had been immediately prior to collection. For reptiles, this was easier since animals were confined to their terrariums.

Additionally, for the study on *Giardia* in Swedish red foxes, samples from a national Echinococcus survey were utilised, and originated from hunted specimens.

Table 4. Reptiles sampled for examination for *Cryptosporidium* and *Giardia*

Common name of Species	Scientific name of Species	Nr. of samples
Snakes		
Western hognose snake	*Heterodon nasicus*	3
Green tree python	*Morelia viridis*	1
California king snake	*Lampropeltis getula californiae*	1
Corn snake	*Pantherophis guttatus*	1
Ball python	*Python regius*	6
Pine snake	*Pituophis melanoleucus*	3
Burmese python	*Python bivittatus*	1
Indian rock python	*Python molurus*	2
Cave racer	*Othriophis taeniurus ridleyi*	2
Boa constrictor	*Boa constricto*	1
Common kingsnake	*Lampropeltis getula*	1
Carpet python	*Morelia spilota*	1
Lizards		
Bearded dragon	*Pogona vitticeps*	4
Madagascar giant day gecko	*Phelsuma kochi*	1
Green iguana	*Iguana*	11
Crested gecko	*Correlophus ciliates*	3
Blue tongued skink	*Tiliqua scincoides*	1
Leopard gecko	*Eublepharis macularius*	2
Ocellated lizard	*Timon lepidus*	2
Mossy New Caledonian gecko	*Mniarogekko chahoua*	1
Desert iguana	*Dipsosaurus dorsalis*	1
Chelonians		
Leopard tortoise	*Stigmochelys pardalis*	1
Pancake tortoise	*Malacochersus tornieri*	2
Hermann's tortoise	*Testudo hermanni*	1
Russian Tortoise	*Testudo horsfieldii*	1

10.3 Faecal preservation

Due to the time delay between sample collection and sample analysis, it was often necessary to preserve samples such that the parasites of interest remained intact and detectable. The ideal preservative is cheap, safe to work with, and preserves the morphology and DNA of the target parasite species. Unfortunately such a substance does not exist, or is prohibitively

expensive. The two preservatives used in these studies were formalin and potassium dichromate.

- Formalin (3.7 % w / v, also known as 10 % concentration of formaldehyde) is cheap and good at preserving morphology. However, is hazardous to work with, and causes cross-linking of the DNA helix, thus preventing molecular diagnostics.
- Potassium dichromate 2.5 % (w / v): cheap, allows future molecular diagnostics, allows coccidians to sporulate (Inoue et al., 2006), however is also hazardous to work with.

Other preservatives considered were 75 % ethanol (Jongwutiwes et al., 2002) and RNA-later. Interestingly, although 2.5 % potassium dichromate is considered the standard for preservation of *Cryptosporidium* oocysts at 4 °C, tap water has been shown in a single study to be equally effective (Chen et al., 2007).

Fox samples were sent to the National Veterinary Institute in Sweden and frozen, unpreserved, at –80 °C, then thawed and then re-frozen at –20 °C for 1 to 5 months, prior to being transported to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis. At NMBU, samples were stored at 4 °C.

10.4 Concentration techniques

After removal of preservative through washing, faecal concentration is a common technique that aims to remove the debris within the faecal pellet, making it easier to identify the parasites of interest via microscopy. In most instances this was performed by passing the homogenised sample through a fine sieve (125 µm mesh), allowing the parasite (oo)cysts to move through, and removing larger particulates.

Another concentration technique that can be used to separate parasites from the faecal pellet is flotation. This uses the difference in the density of the parasite egg / oocyst / cyst compared with the rest of the faecal debris. When the faecal pellet is mixed with a solution (e.g. sodium chloride, zinc sulphate, sucrose) that is less dense than most of the faecal debris but more dense than the parasite, then the parasite egg / oocyst / cyst will float to the top, whilst the rest of the debris sinks. This creates a plane to focus on or sample, and is very effective at concentrating parasite life stages. When combined with a McMaster chamber,

then this techniques allows a quantitative evaluation of the number of eggs / oocysts per gramme of faeces.

10.5 Oocyst sporulation

Most species of *Eimeria* are excreted in the faeces as unsporulated oocysts, whose morphology varies primarily only in size and wall thickness / texture. Through the process of sporulation, which requires oxygen and humidity, the oocyst develops a number of distinguishing morphological characteristics. Faeces were mixed with potassium dichromate 2.5 % (w / v) to facilitate sporulation of oocysts. This helps preserve oocyst viability whilst preventing bacterial growth that degrades oocysts.

10.6 Oocyst description

Descriptions of oocysts and sporocysts followed established guidelines (Wilber et al., 1998) as follows: oocyst length (L) and width (W), length to width ratio (L/W), oocyst wall (OW), micropyle (M), oocyst residuum (OR), polar granules (PG), sporocyst (SP) length (L), width (W), and length to width ratio (L/W), Stieda body (SB), sporocyst residuum (SR), sporozoite (SZ), refractile body (RB), and nucleus (N).

10.7 Immunomagnetic separation (IMS)

This technique utilizes magnetic beads that are coated in antibodies against the parasite surface antigens, in this case *Giardia* and *Cryptosporidium* oo(cyst) wall glycoproteins. When mixed with the faecal suspension, these antibodies bind to the antigens creating a magnetic bead-antibody-antigen ((oo)cyst) complex. Through the use of a magnet, these complexes can be separated from the remaining faecal suspension. The (oo)cyst is then removed from the magnetic bead through shear forces (vortexing) whilst in an acidic environment (hydrochloric acid) to prevent rebinding post-shaking. This has been shown to increase the sensitivity of detecting both *Giardia* and *Cryptosporidium* by PCR and IFAT (Coklin et al., 2011).

10.8 Immunofluorescent antibody testing (IFAT)

Immunofluorescence antibody testing uses antibodies that bind specifically to an antigen, in this case the same or similar surface wall glycoproteins on *Giardia* and *Cryptosporidium*

(oo)cysts as used for IMS. These antibodies have a pre-bound fluorescence molecule, allowing them to be observed under the right wavelength of fluorescence. For this project, a commercial test kit was used (Aqua-Glo, Waterborne Inc., New Orleans). Briefly, 5 to 10 µl of sample (either concentrated faecal pellet or product of IMS) was placed on a microscope slide, air dried, then fixed with methanol (96 %). It was then covered with the fluorescent antibody solution and incubated for 30 minutes at 37 °C, before the excess antibodies were removed. Ten microliters of a second stain, DAPI (4´, 6-diamidino-2-phenylindole), which binds non-selectively to DNA, was then added, and a coverslip placed over the sample. Stained samples were screened using a fluorescence microscope equipped with appropriate filters (for FITC and DAPI) and Normarksi optics.

10.9 DNA isolation

Disruption of the oocysts / cysts is often performed to ensure that the DNA is available for extraction. Techniques that can be used include freeze-thawing, boiling, bead-beating, and chemical lysis (Checkley et al., 2015).

For *Giardia* and *Cryptosporidium*, DNA was isolated using a QIAmp DNA mini kit (Qiagen GmbH) at NMBU. This was performed either directly on the faecal pellet, on the purified (oo)cysts after IMS, or after scraping oocysts off the IFAT slide as previously described (Robertson et al., 2009). The protocols followed the manufacturer instructions with slight modifications; cysts / oocysts were first mixed with 150 µl of TE buffer (100 mM Tris and 100 mM EDTA) and incubated at 90 °C / 100 °C (*Giardia* / *Cryptosporidium*) for 1 h before an overnight proteinase K lysis step at 56 °C and spin column purification. DNA was finally eluted in 30 µl of PCR-grade water and stored at 4 °C.

For *Entamoeba*, DNA was isolated using DNA was isolated using QIAamp Fast DNA Stool Mini Kit, with an incubation at 70 °C for 5 min, in accordance with the manufacturer's instructions.

10.10 Conventional polymerase chain reaction (PCR)

PCR is a technique that allows detection of very small quantities of parasite DNA within a sample. As it generates many copies of the target DNA, the result can then be sequenced, and this genetic code used to characterise an isolate and determine its taxonomy and

phylogenetic relationship to other isolates (Lymbery and Thompson, 2012). The PCR reaction occurs in a welled chamber consisting of: two primer sets, that bind specifically to the target parasites' DNA; RNAse-free water; bovine serum albumin (BSA), that stabilises the enzymes and reactions; DNA polymerase, which is the enzyme that creates the copies of DNA; and a mixture of DNA nucleotides. PCR products are visualised after separation across a 2 % agarose gel using electrophoresis, and can be compared with a DNA ladder to determine fragment lengths.

For *Cryptosporidium*, the SSU rRNA gene is a commonly used target in PCR, and can be used to distinguish between species, which can then be subtyped at the 60 kDA glycoprotein (*gp60*) gene (Checkley et al., 2015; Shirley et al., 2012). PCR protocols used in this study are given in Table 5 and 6.

For *Giardia*, four commonly targeted genes for molecular characterisation of an isolate were utilised in these studies; SSU rRNA, *bg*, *gdh*, and *tpi*. These vary in the rate of substitution per nucleotide, with 0.01, 0.03, 0.06 and 0.12 substitutions, respectively (Feng and Xiao, 2011). As such, their ability to distinguish isolates at different levels of genetic detail vary, with SSU rRNA useful to distinguish species, and the more heterogeneous, *tpi*¸ to type isolates at the sub-Assemblage level (Feng and Xiao, 2011).

10.11 Sanger sequencing

PCR products were sent to external laboratories where conventional Sanger DNA sequencing was performed. This process involves a similar system to the PCR described above, however some nucleotides have fluorescent labels and result in cessation of the amplification process. The solution is then passed through a reading chamber, with the order dependent on the length (size) of the amplified sequence, and the fluorescent labels are counted by machine. In this way, a chromatogram of the resulting fluorescent labels is created, representing the sequential nucleotides of the original PCR products, which can be read in the form of a DNA sequence.

These sequences were manually checked for consistency using the program Genious ®, and the resulting sequence compared against other reported sequences in the GenBank database.

Table 5. PCR conditions for amplification of *Giardia*.

Locus	bp	Primer	Cycle conditions		Ref.
Giardia					
Small Subunit (SSU) rRNA	292	1st amplification F: 5'-CATCCGGTCGATCCTGC-3' R: 5'-AGTCGAACCCTGATTCTCCGCCAGG-3'	96 °C, 5 min		(Hopkins et al., 1997) (Read et al., 2002)
			96 °C, 30 sec 59 °C, 40 sec 72 °C, 40 sec	40 x	
			72 °C, 7 min		
	175	2nd amplification F: 5'-GACGCTCTCCCCAAGGAC-3' R: 5'-CTGCGTCACGCTGCTCG-3'	96 °C, 5 min		
			96 °C, 30 sec 55 °C, 40 sec 72 °C, 30 sec	50 x	
			72 °C, 7 min		
Triosephosphate Isomerase (*tpi*)	605	1st amplification F: 5'-AAATYATGCCTGCTCGTCG-3' R: 5'-CAAACCTTYTCCGCAAACC-3'	95 °C, 10 min		(Sulaiman et al., 2003)
			94 °C, 45 sec 50 °C, 45 sec 72 °C, 60 sec	45 x	
			72 °C, 10 min		
	563	2nd amplification F: 5'-CCCTTCATCGGNGGTAACTT-3' R: 5'-GTGGCCACCACVCCCGTGCC-3'	95 °C, 10 min		
			94 °C, 45 sec 50 °C, 45 sec 72 °C, 60 sec	45 x	
			72 °C, 10 min		
Glutamate Dehydrogenase (*gdh*)	755	1st amplification: F: 5'-TTCCGTRTYCAGTACAACTC-3' R: 5'-ACCTCGTTCTGRGTGGCGCA-3'	94 °C, 2 min		(Caccio et al., 2008)
			94 °C, 30 sec 50 °C, 30 sec 72 °C, 60 sec	35 x	
			72 °C, 7 min		
	530	2nd amplification: F: 5'-ATGACYGAGCTYCAGAGGCACGT-3' R: 5'-GTGGCGCARGGCATGATGCA-3'	94 °C, 2 min		
			94 °C, 30 sec 50 °C, 30 sec 72 °C, 60 sec	35 x	
			72 °C, 7 min		
Glutamate Dehydrogenase (*gdh*)		1st amplification: F: 5'-TCAACGTYAAYCGYGGYTTCCGT-3' R: 5'-GTTRTCCTTGCACATCTCC-3'	94 °C, 15 min		(Read et al., 2004) (Robertson et al., 2006)
			94 °C, 45 sec 54 °C, 45 sec 72 °C, 45 sec	50 x	
			72 °C, 10 min		
Beta Giardin (*bg*)	753	1st amplification: F: 5'-AAGCCCGACGACCTCACCCGCAGTGC-3' R: 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'	95 °C, 15 min		(Caccio et al., 2002) (Lalle et al., 2005)
			94 °C, 30 sec 60 °C, 30 sec 72 °C, 60 sec	35 x	
			72 °C, 10 min		
	511	2nd amplification F: 5'-GAACGAGATCGAGGTCCG-3' R: 5'-CTCGACGAGCTTCGTGTT-3'	95 °C, 15 min		
			95 °C, 30 sec 53 °C, 30 sec 72 °C, 60 sec	40 x	
			72 °C, 10 min		
Cryptosporidium					
SSU rRNA	1 325	1st amplification: F: 5'-TTCTAGAGCTAATACATGCG-3'	94 °C, 3 min		(Xiao et al., 1999)
			94 °C, 45 sec 55 °C, 45 sec 72 °C, 60 sec	35 x	

		R: 5'-CCCTAATCCTTCGAAACAGGA-3'	72 °C, 7 min		
		2nd amplification: F: 5'-GAAGGGTTGTATTTATTAGATAAAG-3' R: 5'-AAGGAGTAAGGAACAACCTCCA-3'	94 °C, 3 min		
			94 °C, 45 sec 55 °C, 45 sec 72 °C, 60 sec	35 x	
			72 °C, 7 min		
Entamoeba					
SSU rRNA *Entamoeba* spp. (genus-specific)	550	F: 5'-GTTGATCCTGCCAGTATTATATG-3' R: 5'-CACTATTGGAGCTGGAATTAC-3'	95 °C, 15 min		(Verweij et al., 2003)
			95 °C, 30 sec 53 °C, 30 sec 72 °C, 60 sec	38 x	
			72 °C, 2 min		
SSU rRNA *E. histolytica* *E. moshkovskii* *E. dispar*	166 580 752	F: 5'-ATGCACGAGAGCGAAAGCAT-3' R: 5'-GATCTAGAAACAATGCTTCTCT-3' R: 5'-TGACCGGAGCCAGAGACAT-3' R: 5'-CACCACTTACTATCCCTACC-3'	94 °C, 5 min		(Hamzah et al., 2006)
			94 °C, 1 min 58 °C, 1 min 72 °C, 1 min	35 x	
			72 °C, 10 min		
18S rRNA *E. coli*	180	F: 5'-GAATGTCAAAGCTAATACTTGACG-3' R: 5'-GATTTCTACAATTCTCTTGGCATA-3'	94 °C, 5 min		(Tachibana et al., 2009)
			94 °C, 30 sec 56 °C, 30 sec 72 °C, 30 sec	40 x	
			72 °C, 5 min		

10.12 GenBank survey

Giardia duodenalis sequences obtained from isolates from NHPs were identified from the GenBank database by searching for "*Giardia*" and the different genera within the order Primates e.g. "*Giardia Macaca*", or "*Giardia Cheirogaleus*". In total, 76 searches were performed for 76 different NHP genera. Gene sequences that were shorter than 100 bp or longer than 1 100 bp were excluded. Only sequences from the SSU rRNA, *tpi*, *gdh*, and *bg* genes were included for analyses. Sequences from isolates described as mixed infections in the original research article were excluded (e.g., Isolate SQ694, Accession Number: FJ890962 and FJ890966). Isolates for which different Assemblages were apparently identified from sequences at different genetic loci, but where the original research article did not report mixed infection, were included (e.g., Isolate RC0458, Accession Number: GQ502964 and GQ502999).

Human-derived isolates were recruited gene-wise by searching on GenBank for "*Giardia Homo*". Sample isolates that did not clearly report sequences from at least three out of the following four genes were excluded: SSU, *tpi*, *gdh*, and *bg*. Sequences at different genes from the same isolates but that were submitted to GenBank under different names were not included. Gene sequences that were shorter than 100 bp or longer than 1100 bp were excluded. Finally, the reference sequences WB (human, Assemblage A), GS (human, Assemblage B), DH (human, Assemblage A2) and P15 (pig, Assemblage E) (Smith et al., 1982; Nash et al., 1985; Nash and Keister, 1985) were included to guide multiple sequence alignments and to contribute leaves with high-confidence Assemblage status in the phylogenetic tree.

10.13 Sequence alignment

(Performed by Ola Brynildsrud)

Sequences were parsed individually by gene, reverse complemented if necessary, and end repaired with ambiguous characters. Multiple sequence alignments were created individually for each gene using MAFFT v7.305b (Katoh and Standley, 2013), with the gap open penalty increased to 50 to prevent splitting mid-sequence; without the latter detail, the sequence alignments would sometimes favour alignment of non-homologous sites. Six samples for which only SSU information was available were excluded at this stage due to poor alignment. Sequence alignments were then concatenated into a single multi-gene alignment (hereafter referred to as the global alignment) of length 2 761 bp. Isolates that did not have at least 200 non-gap sites in the global alignment were excluded from further analysis.

10.14 Phylogenetic tree construction and annotation

(Performed by Ola Brynildsrud)

Sequence alignments were visually inspected in SeaView v4.4.2 (Gouy et al., 2010) and obvious misalignments were manually curated using Aliview 1.18 (Larsson, 2014). Maximum-likelihood phylogenetic trees (for individual gene and global alignments) were created using IQ-TREE (Nguyen et al., 2015; Trifinopoulos et al., 2016). We allowed for automatic model selection with free rate heterogeneity, and performed 1 000 iterations of the ultrafast bootstrap (Minh et al., 2013). In the global alignment, we allowed each locus partition to evolve under different, edge-unlinked substitution models (Chernomor et al.,

2016). The perturbation strength for nearest neighbour interchange (NNI) was set to 0.5, and was set to stop after 100 unsuccessful iterations. Trees were annotated using Interactive Tree Of Life (ITOL) (Letunic and Bork, 2016).

10.15 Testing for phylogenetic incongruence

(Performed by Ola Brynildsrud)

Tanglegrams were created to test for phylogenetic incongruence between the global alignment tree and individual loci trees using Dendroscope v3.4.0 (Huson et al., 2007). The congruency index were calculated by a web-based tool available at http://max2.ese.u-psud.fr/bases/upresa/pages/devienne/index.htm (de Vienne et al., 2007). The Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), as implemented in the *phangorn* package (Schliep, 2011) for the R statistical environment (Team, 2014), was performed to evaluate congruency between individual loci tree-character models and the global tree-character model.

10.16 Statistics

The prevalences of *Giardia*, *Cryptosporidium*, *Entamoeba*, and *Eimeria* were compared using contingency tables (Chi-squared test or Fisher's exact test). Excretion rates of *Eimeria* were compared by using the two-sample T-test.

11. Results and General Discussion

11.1 Prevalence of *Giardia* in NHPs

Examination of chimpanzee faecal samples from TCRC revealed the presence of *Giardia* cysts in 4.5 % (7 / 154) of samples (Table 7), with 5.5 % (5 / 90) of chimpanzees positive for *Giardia* in one or more sample. The proportion of chimpanzees at TCRC shedding *Giardia* cysts in one or more sample did not differ by sex (χ^2, P = 0.87), enclosure (χ^2, P = 0.23), faecal consistency (χ^2, P = 0.50) or age (χ^2, P = 0.70). Examination of rhesus macaque faecal samples revealed the presence of *Giardia* cysts in 31 % (53 / 170) of samples (Table 7). Macaques excreted 55 to 6 325 CPG of faeces (mean, 555; median, 165). There was a significant difference in the prevalence of *Giardia* cysts between Troops 1, 2, 3 and 4; 45 % (25 / 55), 20 % (9 / 55), 33 % (15 / 46) and 17 % (4 / 24), respectively ($p < 0.05$). All faecal samples from chimpanzees held at KZ (n = 29), mandrills at TCRC (n = 28) and wild Zanzibar red colobus (n = 58) were negative for *Giardia* (Table 7).

Table 7. Prevalence of *Giardia* in different nonhuman primate species

Host species	Country	Origin	No. samples	Prevalence (%)
Chimpanzee	Congo	Rehabilitation centre	154	4.5
Chimpanzee	Norway	Zoo	29	0
Mandrill	Congo	Rehabilitation centre	28	0
Red colobus	Tanzania	Wild	58	0
Rhesus macaque	India	Wild	170	31

The moderately high prevalence of *Giardia* in rhesus macaques found in this study was higher than reported for other macaque species, 2.4 – 9 %, where IFAT / PCR was used for diagnosis (Sricharern et al., 2016; Ye et al., 2013; Ye et al., 2012). As these studies also investigated macaque populations in close contact with humans, the difference in *Giardia* prevalence may be due to innate differences in the study populations, or, alternatively, due to different levels of contamination of food, water, or the environment where these population live. The study population in our study may have an increased exposure to *Giardia* due to its high prevalence amongst humans, domestic animals, and environmental water sources in India (Daniels et al., 2015; Laishram et al., 2012). *Giardia* infection has

been associated with human contact in other primate species (Gillespie and Chapman, 2008; Graczyk et al., 2002; Salzer et al., 2007).

Despite having high stocking densities, close human contact, and regular admission to the centre of sick young wild chimpanzees, the occurrence of *Giardia* amongst chimpanzees at TCRC was low. These results are consistent with previous reports of *Giardia* in chimpanzees (Huffman et al., 1997; Ashford et al., 2000; Muehlenbein, 2005; Gillespie et al., 2009; Petrzelkova et al., 2010; Sa et al., 2013). Given the prevalence of these parasites in humans in Sub-Saharan Africa is quite high (Mbae et al., 2013), this suggests that chimpanzees may not be suitable as long-term hosts of *Giardia*. Alternatively it may suggest that the biosecurity measures in place at TCRC (quarantine of new arrivals, treatment and isolation of sick individuals, enforced leave for sick staff and regular faecal parasitological examination), have been effective at preventing transmission of these parasites. This may also be responsible for the absence of *Giardia* in the mandrills at TCRC and captive chimpanzees at KZ.

For the remaining three primate groups (captive mandrills at TCRC, captive chimpanzees at KZ, and wild Zanzibar red colobus monkeys), the absence of *Giardia* infection indicates either that these animals are not being exposed, or are being exposed and are not susceptible to infection. Previously published data on *Giardia* in mandrills are limited to one study from a Belgian Zoo that reports detection of *Giardia* cysts in 7 % (5 / 75) of samples from 14 individuals (Levecke et al., 2007). This suggests that this host-species is susceptible to *Giardia*.

Our study represents the first investigation on the occurrence of *Giardia* in Zanzibar red colobus monkeys and therefore we have no comparative data. Research on another species of red colobus in Uganda reported low levels of *Giardia* associated with forest fragmentation (Gillespie and Chapman, 2008), thus it is possible that the sample size of this study was insufficient to detect very low prevalence levels. Alternatively, as Zanzibar red colobus monkeys are folivorous and drink from natural rainwater collection depots (Nowak, 2008), they may not be exposed to contaminated food or water despite their ground environment being contaminated with *Giardia* and *Cryptosporidium* from human and livestock waste.

Table 8. Prevalence of *Giardia* in nonhuman primates published since 2000.

Species	W/C	Country	Diagnostic test			No.	Prev. (%)	Reference
			LM	IFAT	PCR			
Colobus guereza	W	Uganda	x			495	0	(Gillespie et al., 2005b)
Pan troglodytes	W	Uganda	x			121	0	(Muehlenbein, 2005)
2 different spp.	W	Brazil	x			12	0	(de Carvalho Filho et al., 2006)
Pongo abelii	W	Indonesia	x			32	0	(Mul et al., 2007)
Colobus guereza	W	Uganda	x			200	0	(Gillespie and Chapman, 2008)
16 different spp.[1]	C	Malaysia	x			99	0	(Lim et al., 2008)
Lemur catta	W	Madagascar		x		99	0	(Villers et al., 2008)
Piliocolobus sp.	W	Uganda	x			1608	1	(Gillespie et al., 2005b)
Pongo abelii	C	Indonesia	x			73	1	(Mul et al., 2007)
Gorilla gorilla	W	Congo		x		67	1	(Gillespie et al., 2009)
34 different spp.	C	China	x			3349	1	(Li et al., 2017)
Gorilla gorilla	W	Uganda		x	x	100	2	(Graczyk et al., 2002)
Pan troglodytes	W	Tanzania	x			201	2	(Petrasova et al., 2010)
Gorilla gorilla	W	CAR			x	201	2	(Sak et al., 2013)
Macaca fasicularis	C	China			x	205	2	(Ye et al., 2014)
Piliocolobus sp.	W	Uganda	x			951	2	(Gillespie and Chapman, 2008)
Gorilla gorilla	W	Rwanda	x			70	3	(Sleeman et al., 2000)
3 different spp.	W	Uganda		x		115	3	(Salzer et al., 2007)
Pan troglodytes	W	Uganda	x			123	5	(Ashford et al., 2000)
Cercopithecus sp.	W	Uganda	x			157	6	(Gillespie et al., 2005a)
Pan troglodytes	W	Congo		x		67	6	(Gillespie et al., 2009)
9 different spp.	C	Italy	x	x	x	133	6	(Berrilli et al., 2011)
Pan troglodytes	W	Guinea-Bissau	x			102	6	(Sa et al., 2013)
34 different spp.	C	China			x	1882	6	(Li et al., 2017)
Macaca fasicularis	W	Thailand			x	200	7	(Sricharern et al., 2016)
Macaca mulatta	W	China			x	411	9	(Ye et al., 2012)
Lemur catta	C	Madagascar		x		50	10	(Villers et al., 2008)
Lemur spp.	W	Madagascar		x		38	11	(Rasambainarivo et al., 2013)
3 different spp.	W	Uganda			x	81	11	(Johnston et al., 2010)
Chlorocebus aothiops	W	Tanzania	x			111	14	(Petrasova et al., 2010)
Papio anubis	W	Uganda	x			56	16	(Ocaido et al., 2003)
Colobus guereza	W	Tanzania	x			49	19	(Petrasova et al., 2010)
Gorilla gorilla	W	Gabon		x		95	20	(Langhout et al., 2010)
12 different spp.	C	Croatia		x	x	32	28	(Beck et al., 2011a)
Alouatta pigra	W	Belize/Mexico		x	x	285	32	(Vitazkova and Wade, 2006)
31 different spp.	C	Belgium	x			910	41	(Levecke et al., 2007)
Alouatta caraya	W	Argentina		x		90	54	(Kowalewski et al., 2011)
Papio cynocephalus	W	Uganda		x		140	58	(Hope et al., 2004)
Macaca fasicularis	W	Bali				468	61	(Lane et al., 2011)
Colobus vellerosus	W	Ghana		x	x	109	69	(Teichroeb et al., 2009)
16 different spp.	C	Spain	x		x	20	70	(Martinez-Diaz et al., 2011)
Alouatta clamitans	C	Brazil			x	28	100	(Volotão et al., 2008)

W/C, wild / captive; LM, light microscopy; Prev., reported prevalence of *Giardia*.
[1], where multiple species were examined, only the number of species in the study is provided.

11.2 Molecular characterization of *Giardia* in NHPs

PCR resulted in successful DNA amplification at the *bg* gene in two of the positive samples from chimpanzees at TCRC, with sequencing of results revealing identical isolates of *G. duodenalis* Assemblage B (Table 9). A BLAST search showed that this isolate (GenBank Accession No. KM102527) had one SNP difference to *Giardia* isolates from humans in Bangladesh, China, India, and Sweden (GenBank Accession nos. KJ188088.1, JX994238.1, JF918494.1 and HM165218.1, respectively), and two SNP differences from an isolate from a ring-tailed lemur (GenBank Accession no. HQ616629.1) from a zoo in Spain.

Table 9. PCR results of faecal samples from captive chimpanzees at Tchimpounga Chimpanzee Rehabilitation Centre that were excreting *Giardia* cysts.

Sample	Number of cysts	DAPI[1]	*gdh*[2]	*bg*[3]	*tpi*-B[4]
25	10 per 200x field	-	+	+	+
64	50 per 200x field	-	+	+	+
107	1 on slide	+	-	-	-
121	1 on slide	+	-	-	-
136	2 on slide	+	-	-	-

[1] DAPI, 4´, 6-diamidino-2-phenylindole

[2] *gdh*, glutamate dehydrogenase (Caccio et al., 2008)

[3] *bg*, β-giardin (Lalle et al., 2005)

[4] *tpi*-B, triose phosphate isomerase Assemblage B-specific (Amar et al., 2002)

Of the twenty-six *Giardia* positive samples from rhesus macaques selected for molecular characterisation, positive results from PCR were obtained at one or more genes for 17 isolates, with the SSU rRNA loci being the most sensitive (Table 10). Amplification by PCR was significantly more likely ($p < 0.05$) if more than twenty DAPI-positive cysts were used for DNA isolation (80 %; 12 / 15), than if 10 or fewer DAPI-positive cysts were used (27 %; 3 / 11). No correlation between the total number of cysts and the likelihood of a sample being positive by PCR was observed.

Sequencing of PCR products from the macaques revealed Assemblage B in all samples. Sequences were submitted to GenBank and Accession numbers are provided (Table 10). Multiple alignment of consensus sequences at the *tpi*, *bg*, *gdh*, and SSU rRNA genes

showed that the *Giardia* excreted by the macaques were very similar to each other, (98-99 % identify), with differences primarily due to ambiguous nucleotides. Importantly, there was heterozygosity of alleles within the sequences corresponding to the reverse internal primer at the BG gene and the reverse internal primer at the SSU rRNA genes. BLAST results of macaque sequences at the *tpi*, *gdh*, and *bg* genes showed 99 % identity to *Giardia* isolates from humans, common marmosets, and a beaver. Two samples, 5 and 8 (Table 10), showed 100 % identity at the *bg* gene to a *Giardia* isolate from a sheep and human.

Macaques in China and Thailand have been reported to be infected with *G. duodenalis* of Assemblages A and B, as seen in other NHPs (Levecke et al., 2009; Sricharern et al., 2016; Ye et al., 2012). However, in this study macaques around Chandigarh were found to be infected with only Assemblage B. Although this indicates a zoonotic potential for *Giardia* infections in macaques in this location, the results should be interpreted with caution as most of the samples were only positive at one gene and it has been shown that some isolates may show a particular taxonomic grouping at one gene and a different grouping at another gene (Lebbad et al., 2010; Robertson et al., 2006). Furthermore, it is difficult to interpret the zoonotic potential of these isolates, as multi-locus typing data can reveal animal isolates to be distinct from human isolates, despite them appearing similar based on a single locus (Ryan and Caccio, 2013; Sprong et al., 2009). Despite close contact with cattle shedding Assemblage A and E cysts, these genotypes were not found in samples from macaques, suggesting that Assemblage B infection probably represents the detection of an actual infection, rather than a technical artefact.

Table 10. Results of PCR from *Giardia*-positive faecal samples from wild rhesus macaques with close human contact

#	Cysts[a]	DAPI[b]	*tpi*[c]	*gdh*[d]	*gdh*[e]	*bg*[f]	SSU rRNA[g]
1	950	800	-	B (KX787059)	B (KX787059)	B (KX787068)	B (KX787044)
2	1 150	600	-	-	-	-	B (KX787042)
3	200	150	-	-	B (KX787061)	-	B (KX787047)
4	130	70	-	-	-	-	Positive
5	190	60	-	B (KX787060)	-	B (KX787069)	-
6	110	50	-	-	-	-	B (KX787043)
7	110	50	-	-	-	B (KX787055)	B (KX787046)
8	80	50	-	-	-	B (KX787056)	B (KX787050)
9	50	40	-	-	-	-	B (KX787045)
10	70	20	-	-	-	-	B (KX787049)
11	40	20	-	-	-	-	-
12	30	20	-	-	-	-	Positive
13	30	20	B (KX787057)	-	-	-	-
14	20	20	-	-	-	-	Positive
15	20	20	B (KX787058)	-	-	-	B (KX787048)
16	160	10	-	-	-	-	-
17	80	10	-	-	-	-	Positive
18	40	10	-	-	-	-	-
19	320	0	-	-	-	-	Positive
20	240	0	-	-	-	-	-
21	170	0	-	-	-	-	-
22	160	0	-	-	-	-	-
23	130	0	-	-	-	-	-
24	130	0	-	-	-	-	-
25	130	0	-	-	-	-	-
26	110	0	-	-	-	-	Positive

-, PCR negative; Positive, amplification on PCR however no sequencing results; Assemblage (Accession number) provided where sequence of PCR products was obtained.

[a] Number of *Giardia* cysts used for DNA isolation

[b] Number of DAPI positive *Giardia* cysts used for DNA isolation

[c] Sulaiman et al., 2003

[d] Caccio et al., 2008

[e] Read et al., 2004; Robertson et al., 2006

[f] Lalle et al., 2005

[g] Hopkins et al., 1997; Read et al., 2002

11.3 Meta-analysis of *G. duodenalis* isolates in NHPs

Sequences from 111 isolates of *G. duodenalis* from NHP hosts from GenBank were included in our analyses. Nonhuman primates were found to be infected by *G. duodenalis* Assemblages A, B, and E in 26 %, 71 %, and 3 % of the isolates, respectively. Our phylogenetic analyses clearly distinguished between Assemblages A, B, and E, with Assemblages A and E more closely related to each other than to Assemblage B (Figure 11).

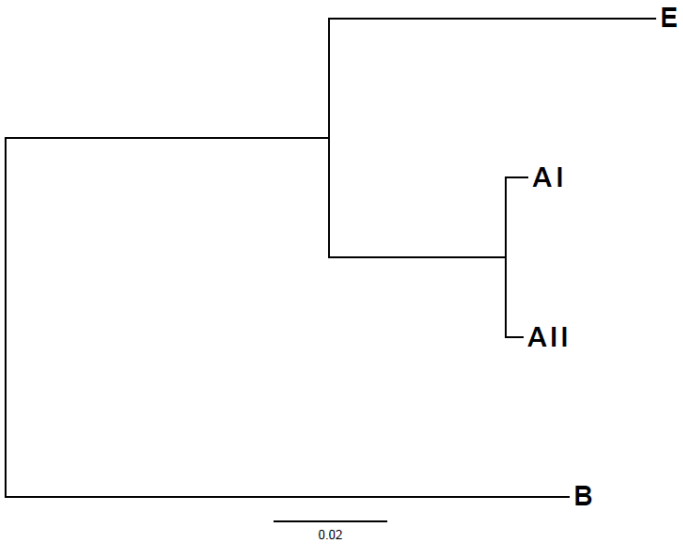

Figure 11. Phylogenetic overview of Assemblages, showing relative distance between Assemblages as well as sister taxa AI and AII.

Within Assemblage A (Figure 12), sub-Assemblages AI and AII were well resolved as monophyletic sister taxa, and the average distance between AI isolates and AII isolates was found to be in the order of 1/10 of the average distance between an Assemblage A and an Assemblage E isolate. However, the phylogenetic distances within these sub-Assemblages were much larger than the distance between them.

Within Assemblage B (Figure 13), a number of sub-Assemblage classifications are reported, but very few isolates have the same annotation. In fact, the only two with the same annotation are two Chinese BII isolates, one from *Macaca mulatta* and one from *Macaca fascicularis*, and these do not cluster together at all. From a molecular distance perspective, there does not appear to be any phylogenetic evidence, across either Assemblage A or B, for a simple sub-Assemblage classification system. Even reference isolates WB and DH,

representing AI and AII respectively, differ at only 18 sites across the full-length alignment of the four typing genes, averaging one nucleotide difference in every 150.

Visual inspection of the phylogenetic trees (Figures 12 and 13) suggests that there may be some degree of clustering based on geographical location and host species / group. However, this trend disappears when isolates from the same study are collapsed, suggesting that true geographic / host population structuring does not occur.

In order to evaluate the relative merit of each locus, the phylogenetic relationship inferred from individual loci was compared with that resulting from the global alignment of all four. The Shimodaira-Hasegawa (SH) test was performed, which tests whether input tree topologies are equally good explanations of the data, or alternatively if one tree is a better fit. In all cases, the global alignment tree represented the data better than the individual trees. (SH-test gave a probability of 1.0 to the global alignment tree and 0.0 to all individual gene trees). This strongly indicates that a multi-locus approach should be favoured when sequencing *Giardia*. We quantified this topological congruency by calculating the incongruency index (de Vienne et al., 2007). The worst congruence is seen for the SSU gene tree. With an Icong of 1.094, this tree is no more related to the global tree than would be expected by chance alone. The *tpi*, *gdh*, and *bg* gene trees are all congruent with the global tree, as measured by the incongruency index. Note, however, that the null hypothesis of this test is that the two trees are not more congruent than pairs of randomly generated trees, and, as such, is not a very strict test for the purpose of testing whether two topologies are similar.

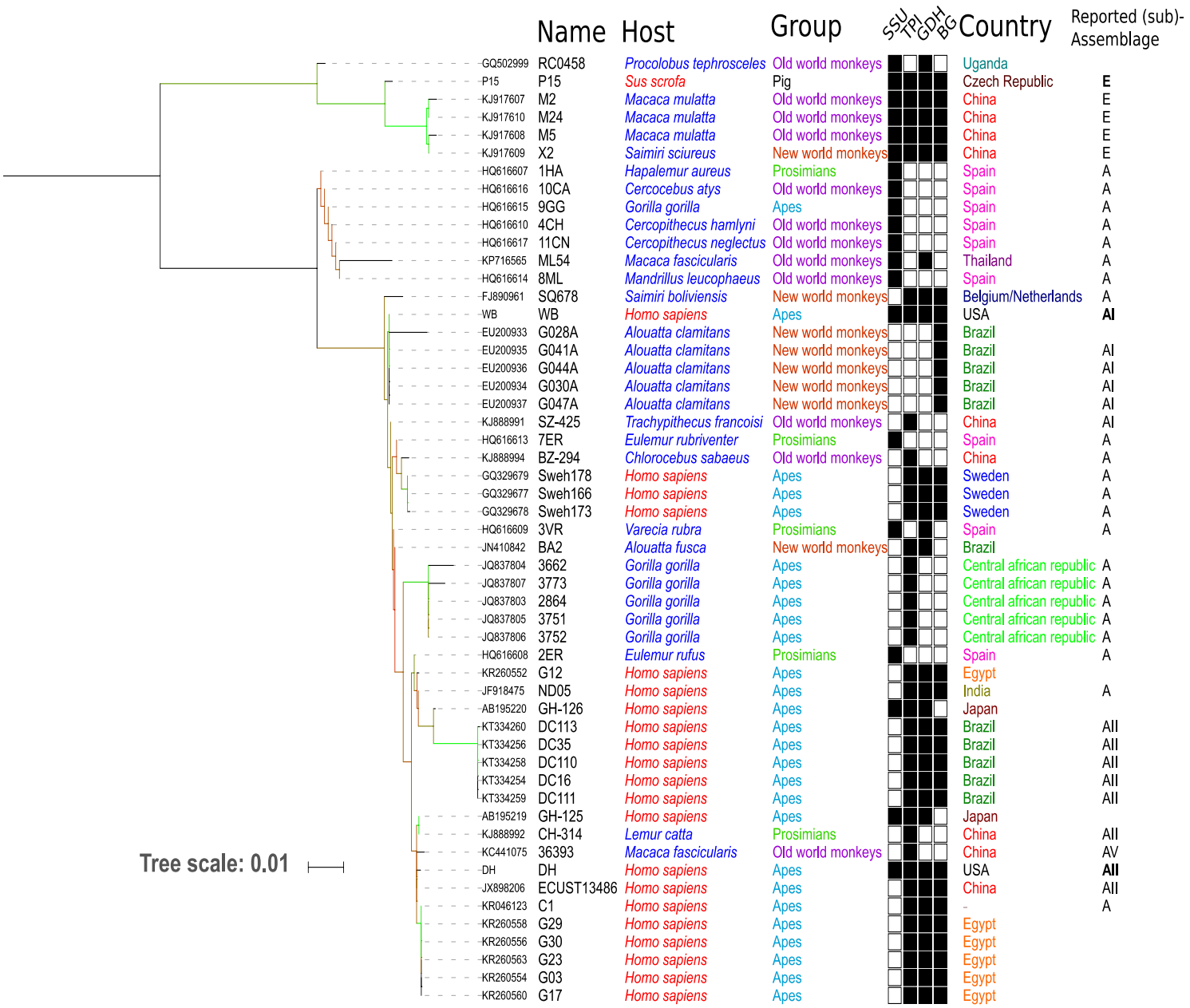

Figure 12. Phylogenetic tree of *Giardia duodenalis* Assemblages A and E. Branches are coloured according to the ultrafast bootstrap value, where green corresponds to higher and red to lower values. Leaves carry the first accession number in the concatenation of genes, except in the case of reference strains WB, DH and P15. Name = name of the sample (where available); Species = species from which *Giardia* isolate was sampled; Group = whether the host species was an ape (including *Homo*), old / new world monkey, or prosimian. Genes = indicator of gene sequence availability. Black indicates partial or complete availability. Country = country in which isolate was sampled. Reported Assemblage = the Assemblage (in some cases sub-Assemblage) that was reported in the associated GenBank file.

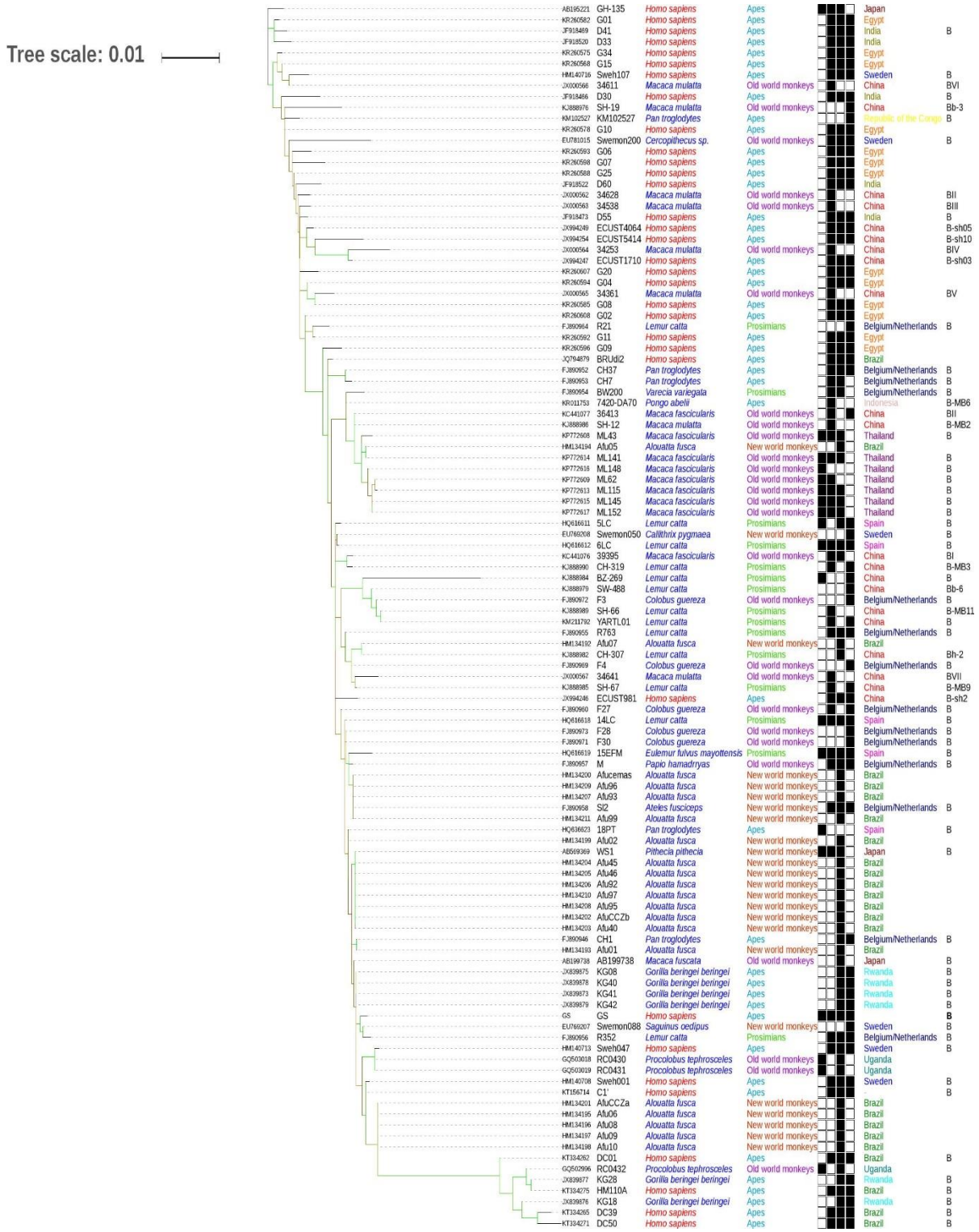

Figure 13. Phylogenetic tree of *Giardia duodenalis* Assemblage B. Branches are coloured according to the ultrafast bootstrap value, where green corresponds to higher and red to lower values. Leaves carry the first accession number in the concatenation of genes, except in the case of reference strain GS. Name = name of the sample (where available); Species = species from which *Giardia* isolate was sampled; Group = whether the host species was an ape (including *Homo*), old / new world monkey, or prosimian. Genes = indicator of gene sequence availability. Black indicates partial or complete availability. Country = country in which isolate was sampled. Reported Assemblage = the Assemblage (in some cases sub-Assemblage) that was reported in the associated GenBank file.

11.4 *Cryptosporidium* in NHPs

Examination of rhesus macaque faecal samples using immunofluorescent microscopy revealed the presence of *Cryptosporidium* oocysts in 1 of 170 samples, with this animal from Troop 3. This sample contained 50 OPG of faeces, all of which showed nuclear staining with DAPI, but was negative by PCR at the SSU rRNA gene.

Two faecal samples from adult male chimpanzees housed in separate enclosures at TCRC contained objects whose size, shape and fluorescence resembled *Cryptosporidium* spp. oocysts. However, due to the limited number of these objects, lack of DAPI staining, and negative PCR, identification of *Cryptosporidium* could not be confirmed.

All faecal samples from chimpanzees held at KZ (n = 29), mandrills at TCRC (n = 28), and wild Zanzibar red colobus (n = 58) were negative for *Cryptosporidium*.

Together, these results suggest that *Cryptosporidium* is not an important parasite in the host populations sampled. Indeed, the one confirmed positive sample contained few oocysts and was from the troop that had intimate contact with the calves shedding *Cryptosporidium* oocysts. Thus it is possible that this sample represents carriage, and not a true infection. *Cryptosporidium* may be more common in very young macaques that are likely under-represented in this study due to the sampling technique relying on stool morphology. Such reasoning does not apply for the chimpanzees at TCRC, where many of the sampled individuals were young and had close contact with humans.
Reported prevalence of *Cryptosporidium* vary markedly in NHPs (Table 11), and this variation may be based on host species and differing exposures in different environments.

Table 11. Reported prevalence of *Cryptosporidium* in nonhuman primates published since 2000.

Species	W/C	Country	Diagnostic test			No.	Prev. (%)	Reference
			AF	IFAT	PCR			
39 spp.[1]	C	Portugal	x		x		0	(Alves et al., 2005)
Pan troglodytes	W	Congo		x		67	0	(Gillespie et al., 2009)
Gorilla gorilla	W	Congo		x		67	0	(Gillespie et al., 2009)
Alouatta caraya	W	Argentina		x		90	0	(Kowalewski et al., 2011)
Gorilla gorilla	W	Gabon		x		11	0	(Langhout et al., 2010)
3 spp.	W	Uganda		x		35	0	(Salzer et al., 2007)
Lemur catta	W	Madagascar		x		99	0	(Villers et al., 2008)
Lemur catta	C	Madagascar		x		50	0	(Villers et al., 2008)
Macaca sp.	C	China			x	205	0.4	(Ye et al., 2014)
Gorilla gorilla	W	CAR			x	201	0.5	(Sak et al., 2013)
Macaque sp.	W	Thailand			x	200	1	(Sricharern et al., 2016)
3 spp.	W	Uganda		x		80	6	(Salzer et al., 2007)
Pan troglodytes	W	Tanzania	x			406	8.9	(Gonzalez-Moreno et al., 2013)
Lemur spp.	W	Madagascar		x		38	10.5	(Rasambainarivo et al., 2013)
Gorilla gorilla	W	Uganda	x	x		100	11	(Nizeyi et al., 1999)
Macaca mulatta	W	China			x	411	11	(Ye et al., 2012)
Papio anubis	W	Tanzania			x	47	11	(Parsons et al., 2015)
Papio anubis	W	Ethiopia	x			59	12	(Mengistu and Berhanu, 2004)
16 spp.	C	Malaysia	x			99	14	(Lim et al., 2008)
Gorilla gorilla	W	Gabon		x		84	19	(Langhout et al., 2010)
Pan troglodytes	W	Tanzania			x	84	19	(Parsons et al., 2015)
Trachypithecus sp.	W	Sri Lanka	x		 x	15 15	0 26	(Ekanayake et al., 2006)
Chlorocebus sp.	W	Ethiopia	x			41	29	(Mengistu and Berhanu, 2004)
Papio anubis	B	Kenya	x			63	30	(Muriuki et al., 1997b)
Papio anubis	W	Uganda		x		140	32	(Hope et al., 2004)
Macaca sinica	W	Sri Lanka	x		 x	89 89	29 44	(Ekanayake et al., 2006)
21 spp.	C	Spain	x			36	44	(Gomez et al., 2000)
Semnopithecus sp.	W	Sri Lanka	x		 x	21 21	38 48	(Ekanayake et al., 2006)
Propithecus sp.	C	USA	x	x		48	63	(Charles-Smith et al., 2010)
Chlorocebus sp.	B	Kenya	x			51	78	(Muriuki et al., 1997a)
2 spp.	W	Brazil	x			12	92	(de Carvalho Filho et al., 2006)

W/C, wild / captive, B = both wild and captive; AF, Acid-fast stain; Prev., reported prevalence of *Cryptosporidium*.

[1], where multiple species were examined, only the number of species in the study is provided.

11.5 *Entamoeba* in urban-living wild rhesus macaques

Examination of rhesus macaque faecal samples using a genus-specific conventional PCR revealed the presence of *Entamoeba* spp. in 79 % (132 / 168) of samples. There was no significant difference in the prevalence of *Entamoeba* spp. between Troops 1, 2, 3 and 4; 78 % (43 / 55), 69 % (31 / 45), 83 % (19 / 23), and 87 % (39 / 45), respectively ($p = 0.21$). Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii*, did not result in amplification in any of the samples (0 / 168). Species-specific PCR for *E. coli* resulted in amplification in 49 % (63 / 128) of samples positive at the genus-specific PCR. Thus, in the other 51 % (65 / 128), no species of *Entamoeba* was identified. There was a significant difference in the prevalence of *E. coli* between Troops 1, 2, 3 and 4; 26 % (11 / 42), 75 % (21 / 28), 56 % (10 / 18), and 45 % (21 / 39), respectively ($p < 0.01$).

The high prevalence of *Entamoeba* spp. in the macaques is consistent with results from studies in other closely related NHPs (Feng et al., 2011; Tachibana et al., 2009). *Entamoeba dispar* was not identified in this study, but has been detected in macaques from China and Nepal (Tachibana et al., 2013; Feng et al., 2013). Macaques were not infected with *E. histolytica* and *E. moshkovskii*, consistent with previous reports from other wild urban dwelling macaques (Tachibana et al., 2013; Feng et al., 2013). Since *E. histolytica*, *E. moshkovskii* and *E. dispar* are commonly reported in humans in India, this suggests that macaques are not a wildlife reservoir for these human pathogens, and that transmission from humans to macaques is not common among the macaque troops investigated (Nath et al., 2015; Parija and Khairnar, 2005; Parija et al., 2014).

Molecular identification of *Entamoeba* spp. in 520 samples from a range of captive NHP species revealed *E. hartmanni* (51.9 %), *E. polecki*-like (42.7 %), *Entamoeba histolytica* NHP variant (36 %), *E. coli* (21.5 %), *E. dispar* (2.4 %) and *E. moshkovskii* (1.9 %) , as well as unidentified *Entamoeba* spp. (18.9 %) (Levecke et al., 2010). *Entamoeba polecki* and *E. hartmanni* were not tested for in our study as they are not considered pathogenic to humans, however they may be responsible for the unidentified *Entamoeba* spp. observed. *Entamoeba hartmanni* has also been detected in wild NHPs (Jirků-Pomajbíková et al., 2016). The reason for different prevalences amongst the macaque troops is not clear and could be due to

a combination of various factors including diet, water sources, microbiome, genetics, and interactions with other humans or animals.

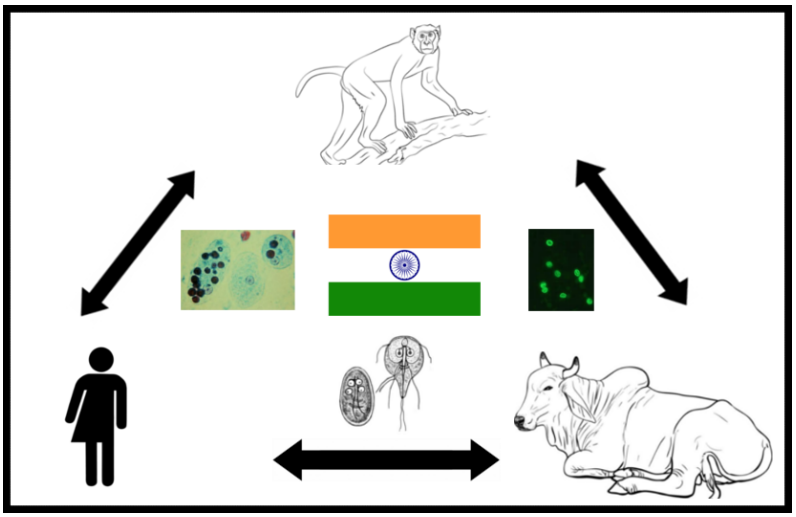

Figure 14. Representation of the One Health aspects of studying transmission of intestinal protozoa at the human-wildlife-domestic animal interface.

11.6 *Eimeria* in wild ungulates at the wildlife-livestock interface

Examination of faeces from wild blue wildebeest revealed coccidians in 46 % (52 / 112) of samples. The prevalence of *Eimeria* oocysts in faecal samples from juveniles was 92 % (11 / 12), which was significantly higher than the prevalence from adults 41 % (41 / 100) (Fisher's exact test, $p < 0.01$). No significant difference was observed in the prevalence of infection between the 3 different herds (Fisher's exact test, $p = 0.09$). Overall, wildebeest shed 60 to 18 000 OPG (median, 300; mean, 1 236) of faeces. The concentration of oocysts shed by juvenile animals, mean 4 002 (median, 700; range, 300 – 18 000) was significantly greater than shed by adult animals, mean 577 (median, 220; range, 60 – 3 500) when compared by mean log OPG (two-sample *t*-test, $P < 0.01$).

The overall prevalence of *Eimeria* observed in wild blue wildebeest faecal samples, 46 % (52 / 112), was similar to that of a previous report in this host-species. Few data are available about the prevalence of *Eimeria* spp. infections in wild artiodactyls, but there appears to be considerable species and seasonal variation (Pyziel and Demiaszkiewicz,

2013; Tomczuk et al., 2014; Turner and Getz, 2010). Overall, the concentration of oocysts in faeces was low (range 60 – 18 000; median, 300; mean, 1 236 OPG), and similar to that seen in domestic ruminants in Tanzania and other free ranging ungulates (Kusiluka et al., 1996; Singh et al., 2009; Turner and Getz, 2010). In this study, juvenile wildebeest were shedding a greater concentration of *Eimeria* spp. oocysts in the faeces, which is consistent with the infection patterns seen in livestock. At the infection levels observed in this study, the *Eimeria* spp. described probably act more as part of the commensal gastrointestinal flora. However, exposure of young, naïve, or immunosuppressed individuals, particularly at high stocking densities that facilitate faeco-oral transmission, may lead to disease.

In total, five different species of *Eimeria* were observed, with four of these being new species (i.e., not previously described).

Eimeria gorgonis (Figure 15; Prasad, 1960) was detected in 18 % (20 / 112) of faecal samples, with oocysts being ellipsoidal; L × W: 23 (19.9 - 28.0) × 18.4 (16 - 23); L / W ratio 1.3 (1.0 - 1.5); OW: 1-1.5 µm thick, UV autofluorescence, outer layer pale yellow and smooth; M, OR, PG: all absent. Four SP each with 2 SZ, SP shape: oval with a pointed tip; L × W: 12.2 (9.6 - 15.1) × 6 (4.5 - 8); L / W ratio 2.1 (1.6 - 2.4); smooth wall; SB: present; SR: loose granules to tightly packed rosette centrally located; SZ: large spherical posterior RB with strong UV autofluorescence, ~4 µm diameter.

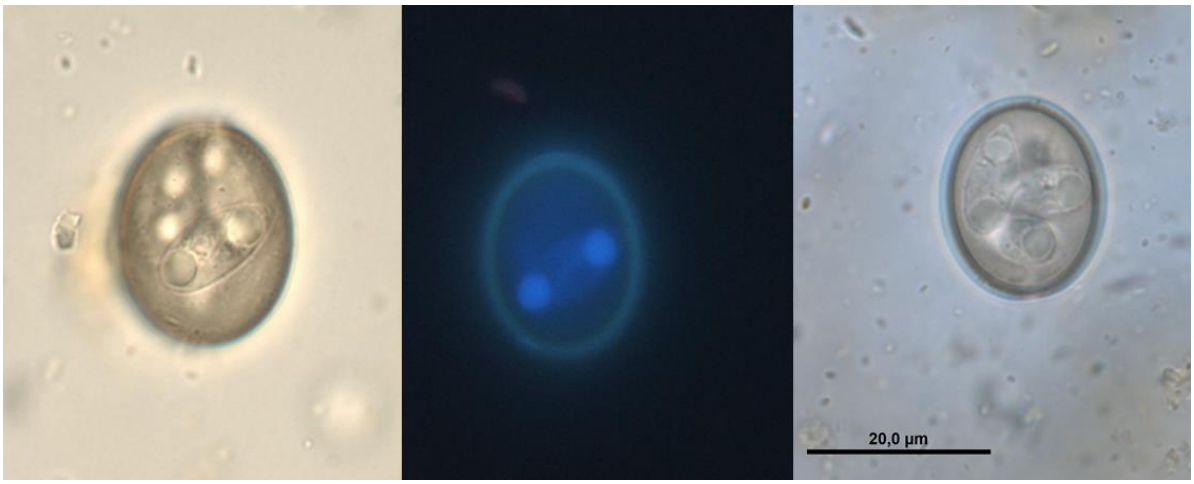

Figure 15. Photomicrographs of oocysts of *Eimeria gorgonis* from the faeces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria donaldi n. sp. (Figure 16) was detected in 34 % (38 / 112) of faecal samples, with oocysts being spherical to oblong; L × W: 13.4 (11 - 16) × 12.3 (10 - 15); L / W ratio 1.1 (1 - 1.4); OW: 1 µm thick, UV autofluorescence, colorless, and outer layer smooth; M, OR, PR: all absent. Four SP each with 2 SZ, SP shape: oval with a pointed tip; L × W: 6.7 (5 - 10) × 3.9 (2.7 - 3.9); L / W ratio: 1.7 (1.1 - 2.3); smooth thin wall; SR: variably present as tight rosette of small granules; SB: present; SZ: large posterior RB with centrally located spherical darker structure with UV autofluorescence, ~1 µm diameter; N: centrally located without UV autofluorescence.

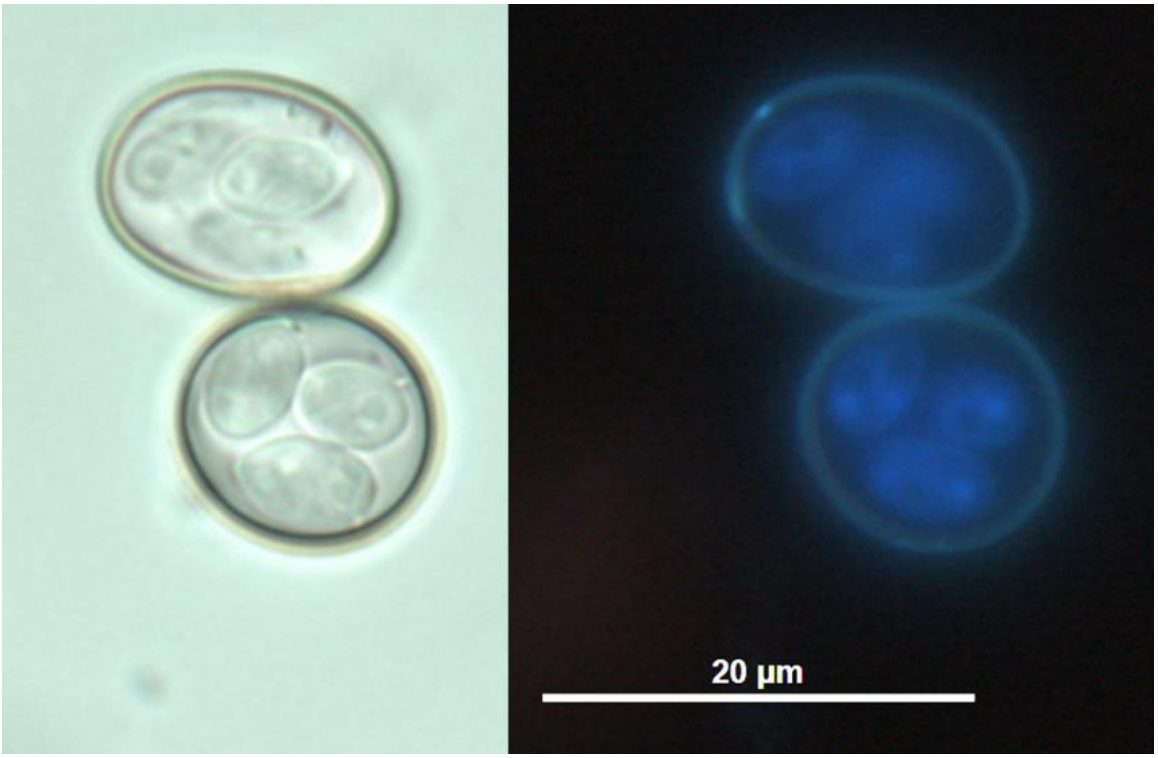

Figure 16. Photomicrographs of oocysts of *Eimeria donaldi* n. sp. from the faeces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria nyumbu n. sp. (Figure 17) was detected in 6 % (7 / 112) of faecal samples, with oocysts being ellipsoidal; L × W: 30.8 (27 - 33.5) × 22.1 (20 - 24.3); L / W ratio: 1.4 (1.2 - 1.6); OW: 2 µm thick, outer surface smooth with light brown color, inner layer with UV autofluorescence; M: present, large with domed shape; OR, PG: absent. Four SP each with 2 SZ, SP shape: oval with pointed tip; L × W: 14.8 (12 - 19) × 7.3 (5 - 9); L / W ratio: 2.1 (1.5 - 2.8); smooth wall; SB: present; SR: absent; SZ: large RB located centrally without UV autofluorescence.

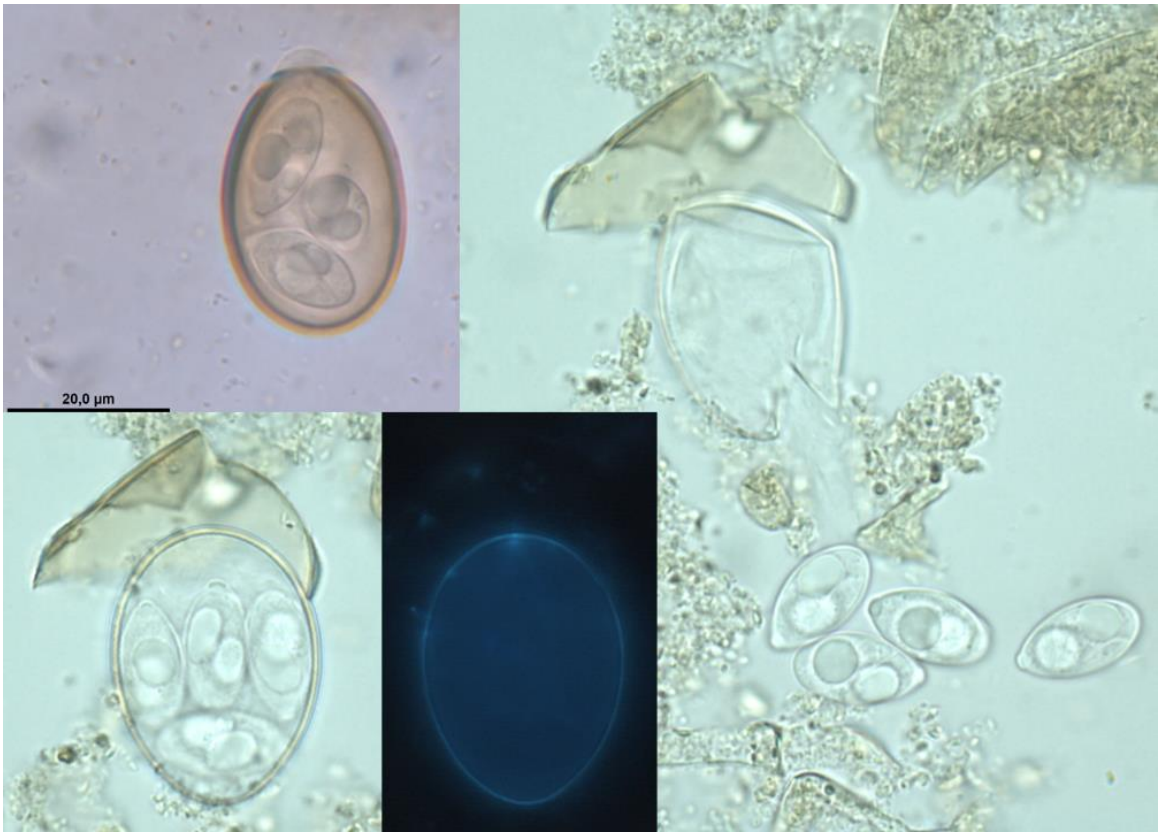

Figure 17. Photomicrographs of oocysts of *Eimeria nyumbu* n. sp. from the faeces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria burchelli n. sp. (Figure 18) was detected in 16 % (18 / 112) of faecal samples, with oocysts being ellipsoidal; L × W: 34.8 (30 - 39) × 24.4 (20 - 27.5); L / W ratio: 1.4 (1.2 - 1.8); OW: 2 - 2.5 µm thick, outer surface lightly stippled with a brown color, inner layer with weak UV autofluorescence; M: present; OR, PG: absent. Four SP each with 2 SZ, SP shape: oval with pointed tip; L × W: 16.8 (15 - 19) × 7.9 (6.2 - 9); L / W ratio: 2.1 (1.9 - 2.6); smooth wall; SB: present; SR: present as granules 0.5 – 1.5 µm scattered throughout; SZ: equally sized posterior RB and anterior RB present with weak UV autofluorescence.

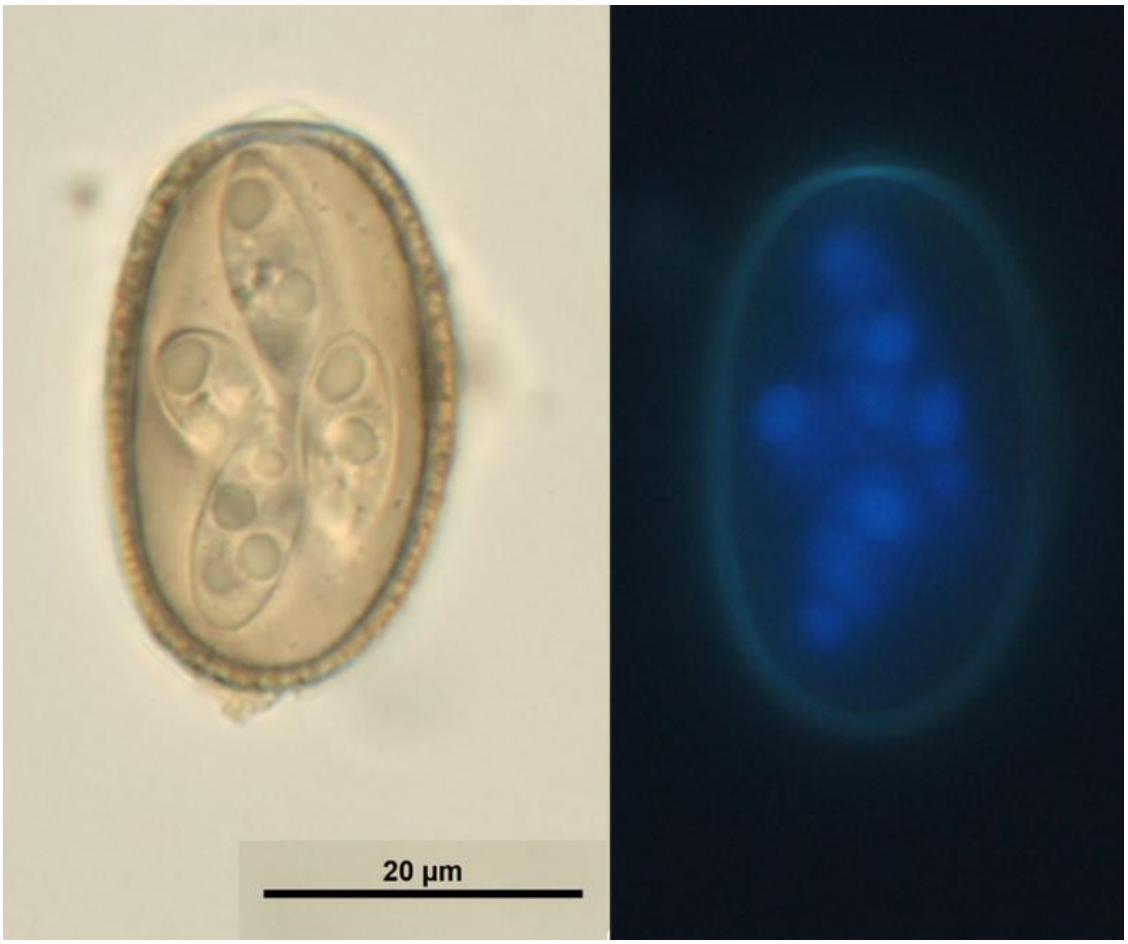

Figure 18. Photomicrographs of oocysts of *Eimeria burchelli* n. sp. from the faeces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria sokoine n. sp. (Figure 19) was detected in 5 % of the faecal samples, with oocysts being oval to ellipsoidal; L × W: 45.8 (39.8 - 52) × 29 (26.2 - 34.5); L / W ratio: 1.6 (1.3 - 1.9); OW: 3 - 4 µm thick, outer layer very rough, stippled and dark brown in color, inner layer with UV autofluorescence; M: present; OR, PG: absent. Four SP each with 2 SZ, SP shape: ellipsoidal to cylindrical with a pointed tip; L × W: 18.7 (15.6 - 21.1) × 8.3 (6.9 - 10.4); L / W ratio: 2.3 (1.7 - 2.8); smooth wall; SB: present; SR: present as granules 0.5 – 1.5 µm scattered throughout; SZ: large posterior RB and anterior RB of equal size, both with strong UV autofluorescence.

Figure 19. Photomicrographs of oocysts of *Eimeria sokoine* n. sp. from the faeces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania

In this study, four new species of *Eimeria* are described from blue wildebeest. Based on the oocyst morphology of the fifth species, it was concluded to be likely to be synonymous with the previously described *E. gorgonis* (Prasad, 1960). However, oocysts of *E. gorgonis* from the current study were found to be wider, lacking a polar granule, and had a less distinct sporocyst neck than originally reported. All 5 species have unique morphologies when compared with published *Eimeria* spp. oocysts from hosts of the subfamily Alcelaphinae. As such, none of these species are considered to be the same as those infecting domestic cattle, sheep, or goats. It therefore appears that cross-transmission of *Eimeria* between the domestic livestock and the wild wildebeest at Mikumi NP, Tanzania does not occur, which is consistent with the host-specificity described for other *Eimeria* spp.

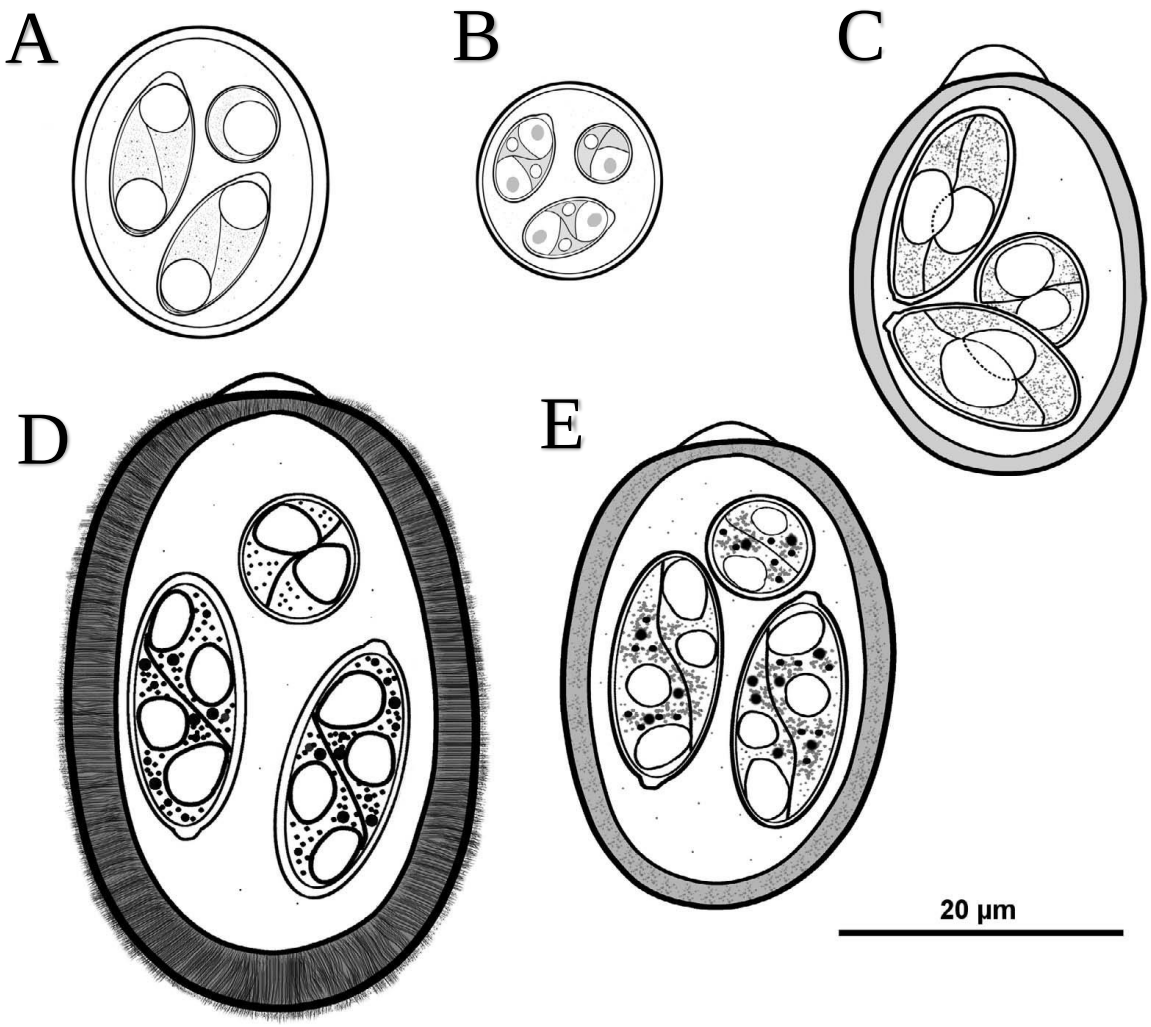

Figure 20. Composite line drawings of sporulated oocysts in the faeces of wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania. (**A**) *Eimeria gorgonis*. (**B**) *Eimeria donaldi* n. sp. (**C**) *Eimeria nyumbu* n. sp. (**D**) *Eimeria sokoine* n. sp. (**E**) *Eimeria burchelli* n. sp.

11.7 *Giardia* and *Cryptosporidium* in wild ungulates

Examination of faecal samples from African buffalo revealed *Cryptosporidium* oocysts in 5 % (2 / 39) of samples, whilst samples from all other taxa (domestic cattle, eland, giraffe, impala, blue wildebeest, and zebra) were negative for *Cryptosporidium* oocysts. The two *Cryptosporidium* positive samples contained three and one oocyst on the IFAT slide, which is equivalent to an excretion rate of 30 and 90 OPG of faeces, respectively. Nuclei in all four oocysts were observed by DAPI staining, but DNA amplification was not successful in either sample by PCR at the SSU rRNA gene. All faecal samples were negative for *Giardia* cysts.

Of the wildlife species examined in this study, *Cryptosporidium* has previously been reported from wildebeest (Morgan et al., 1999; Alves et al., 2005), impala (Abu Samra et al., 2011), zebra (Mtambo et al., 1997), and African buffalo (Hogan et al., 2014), but not from eland. However, only in the wildebeest has this infection been confirmed by molecular characterisation of the isolate. *Giardia* has been reported in African buffalo (Hogan et al., 2014), but not from wildebeest, zebra, impala, or eland. Prevalence rates of these pathogens based on mZN stain must be interpreted with care, as several studies have identified false positives when compared with PCR or IFAT (Szonyi et al., 2008; Chang'a et al., 2011). This may explain why the results of the present study are considerably different to those from a study from the same region using mZN 18 years earlier, which found *Cryptosporidium* in cattle (5.3 %, 26 / 486), African buffalo (22 %, 8 / 36), zebra (27 %, 7 / 25) and wildebeest (27 %, 7 / 26; Mtambo et al., 1997). This previous study relied upon mZN for initial detection, with a confirmatory ELISA test, for which specificity and sensitivity detail are lacking, so diagnostic variables may be the reason for this discrepancy. Other explanations include different sampling seasons (April vs October), changing pathogen dynamics over time, or deterioration of the parasite transmission stages during storage.

The current low prevalence of *Cryptosporidium*, and absence of *Giardia*, in domestic cattle and wild herbivores from Mikumi National Park suggests these are not important parasites in these populations at the time of sampling. This result was unexpected, given these protozoa are generally considered ubiquitous. Diagnosis of *Cryptosporidium* via IFAT is very sensitive and specific, particularly when combined with DAPI staining. However,

given the low oocyst counts in the African buffalo, it is possible that the two positive samples represented carriage, and not true infection. Unfortunately the two positive samples did not result in amplification by PCR, as sequencing PCR products would have identified the *Cryptosporidium* sp. present, and thus whether it is able to infect bovids. Thus, it would appear that, either the domestic cattle and wildlife studied are being exposed to *Cryptosporidium* and *Giardia*, but that the conditions are not conducive to spread, or that these are largely naïve populations. An earlier study from this region of Tanzania also did not detect *Cryptosporidium* infection in over 900 dairy calf samples (Chang'a et al., 2011); in this study the authors suggested that transmission of human cryptosporidiosis in this region may be largely anthropogenic, and that zoonotic infections in cattle may be rather limited. Furthermore, survival of both (oo)cysts is known to be optimal under cool, humid conditions, and the high ambient temperature and significant solar radiation in the Mikumi region may result in rapid inactivation of these transmission stages.

Nevertheless, changes in environmental conditions that facilitate the spread of infection, such as drought causing higher animal densities around water sources, increased rainfall supporting survival of (oo)cysts in the environment, or the introduction of these pathogens from infected cattle or people, all have the potential to allow these parasites to gain a foothold within these populations. This may have a significant effect on the health status of both the wildlife and the domestic cattle, thus influencing the livelihood of the already marginalised peoples in this region.

11.8 *Giardia* in wild Swedish red foxes

Examination of red fox faecal samples using IFAT for *Giardia* revealed the presence of *G. duodenalis* cysts in 44 % (46 / 104) of samples. Foxes excreted 100 to 140 500 CPG (mean, 4 930; median, 600) of faeces. In general, low numbers of cysts were shed, with only two samples containing over 10 000 CPG of faeces. All cysts examined directly by IFAT were negative by DAPI staining for the presence of nuclei.

Of the fourteen *G. duodenalis* positive samples selected for molecular characterisation, no amplification of DNA by PCR was seen at the *gdh*, SSU rRNA, or *bg* genes. Four samples

were positive at the *tpi* gene. Sequencing of these PCR products revealed Assemblage B in all four samples (Accession numbers; KY304077 - KY304080). All sequences were identical except for two ambiguous nucleotides in one isolate and a single SNP in another isolate. All samples had identical protein translations. BLAST comparison of nucleotide sequences revealed 100 % of the consensus region (498 bp) to be identical to *Giardia* isolates from a variety of sources e.g. rhesus macaque in China, water from the USA, and a human sample from Malaysia.

This study describes a high prevalence of low intensity infections of *G. duodenalis* in wild red foxes in Sweden, with only Assemblage B identified. *Giardia duodenalis* infection has previously been reported from a range of Swedish animals (Lebbad et al., 2010). However, this is the first report in Swedish red foxes and suggests that they may be important players in *G. duodenalis* epidemiology in this country. Infection prevalence was higher than reported prevalences in red foxes elsewhere in Europe; 2.8 % (10 / 217) in Romania, 4.5 % (3 / 66) in Croatia, 4.8 % in Norway (13 / 269), 7.3 % (9 / 123) in Bosnia and Herzegovina, and 19 % (4 / 21) in Poland (Beck et al., 2011b; Hamnes et al., 2007; Hodzic et al., 2014; Onac et al., 2015; Stojecki et al., 2015). Similar infection rates have been seen in other wild canids (Oates et al., 2012; Trout et al., 2007). The high prevalence found in the Swedish population may be due to innate differences in this population, e.g. associated with diet, proximity to farming or domestic animals, water sources, human contact, population densities etc. The low intensity of cyst shedding observed is important to consider when assessing zoonotic potential, as these animals will be less likely to lead to environmental contamination than animals with large excretion rates or higher faecal outputs.

In this study, PCR had limited success, similar to other studies trying to characterise *G. duodenalis* isolates from other canids (Sommer et al., 2015; Stojecki et al., 2015). The lack of DNA observed within the *G. duodenalis* cysts may indicate that the DNA was degraded or located free within the faeces matrix. If this is the case, then the processing steps designed to remove faecal debris, may have resulted in the loss of this DNA, thus causing false negatives. However, direct PCR on the faeces, with and without DNA fishing, was attempted on some samples (data not shown) and did not provide any further positive results.

Red foxes have previously been reported to be infected with *G. duodenalis* Assemblage A and B (Beck et al., 2011b; Hamnes et al., 2007; McCarthy et al., 2007; Onac et al., 2015), whereas only one study has reported the canine specific Assemblage D in two samples (Ng et al., 2011), and no reports of Assemblage C. In our study, Swedish red foxes were found to be infected with Assemblage B. In contrast, the vast majority of *Giardia* isolates from dogs are Assemblage C and D (Feng and Xiao, 2011), even in environments where Assemblage B is considered to predominate (Lebbad et al., 2008). It is therefore intriguing that Assemblage B is apparently common in red foxes, but rarely establishes in dogs, suggesting a considerably different host-parasite relationship between these two canids. Finding Assemblage B in Swedish red foxes may indicate that they act as a disease reservoir for zoonotic *G. duodenalis*. However, care must be taken when interpreting the zoonotic potential of these isolates based on a single gene locus, especially when taxonomic grouping can vary based on which genes are used for comparison (Lebbad et al., 2010).

11.9 *Giardia* and *Cryptosporidium* in captive Norwegian reptiles

Cryptosporidium oocysts were detected in 6 % (3 / 54) of the faecal samples. The three *Cryptosporidium* positive reptiles were a Western hognose snake, a California king snake, and a green iguana, which were shedding 5 145 OPG, 1 250 OPG, and 365 OPG of faeces, respectively. Nuclei were identified in oocysts by DAPI staining in samples from the Western hognose snake and California king snake, but not from the green iguana. *Giardia* cysts were not detected in any of the samples.

These results are consistent with reported prevalences of *Cryptosporidium* in reptiles; 3 % - 35 % (Rinaldi et al., 2012; Kuroki et al., 2008; da Silva et al., 2014; Díaz et al., 2013). The lack of *Giardia* in these samples is also consistent with previous finding (Dellarupe et al., 2016; Rinaldi et al., 2012). There are two recognised species of *Cryptosporidium* that cause cryptosporidiosis in reptiles; *C. serpentis*, primarily in snakes, and *C. varanii*, primarily in lizards (Table 2; Pavlasek and Ryan, 2008; Ryan et al., 2014b). Neither of these species is considered to have a zoonotic potential (Fayer, 2010; Ryan et al., 2014b). Several other species of *Cryptosporidium* have been detected in reptile faeces, including *C. parvum*, however these are thought to represent carriage from ingested prey, not true infection (Xiao

et al., 2004; Traversa et al., 2008). Indeed, *C. parvum* has been shown to be unable to establish infection in some reptile species (Graczyk et al., 1996). Thus, whilst the zoonotic potential of these isolates from Norwegian reptiles remains unknown, it is considered unlikely that they pose a significant zoonotic risk

12. Concluding remarks & future perspectives

Species across all taxa are being driven towards extinction, as humans try to squeeze the planet for every resource they can. Many environmentalists claim that the world is standing upon a precipice, and that if we continue to degrade nature's ecosystems, then we will have a huge down-turn in the services that they provide, and thereby, ultimately, a reduction in the quality of our own lives. It thus seems prudent to understand the health and disease of the animals with which we share the planet. The aim of this thesis was to try to explore a small portion of this enormous jigsaw. However, even with the small amount of data generated by the research described here, how we are going to use this information remains unclear. Is the focus of studies on wildlife parasitology purely from the stand-point of how animals may be harbouring parasites that can infect us, or are we also concerned with how the parasites that we ourselves might harbour may be infecting them when we move out into nature? And what are the consequences that this may have for wildlife health?

My research has tried to have a balanced perspective, looking at both the zoonotic potential of wildlife diseases, as well as the conservation importance that such shared diseases may have on wildlife populations. Integral to this is the understanding that we must stop referring to parasites by their genus alone (e.g., *Giardia*), but rather, come to realise that genetic groups within a genus, or even within a species, may all have their own independent epidemiologies. This highlights the critical role of molecular characterization in such studies, which allows a much more detailed understanding of the potential transmission pathways. However, it should not be overlooked that molecular analyses are sometimes difficult, due to few specimens, mixed infections that may provide confounding results, or degraded DNA that may prevent amplification. In such situations, we are obliged to draw inferences based on *a priori* knowledge and to try to design experiments that may yield more useful results.

Also of importance, and often overlooked, are the other factors that may impact pathogen spread, such as geographical range of host species and infection patterns, or the intensities of infections. For example, whilst both foxes and calves are able to contaminate a rural landscape with *G. duodenalis* Assemblage A cysts, the number of cysts being excreted by each host may differ greatly by many magnitudes, not only due to the intensity of infection,

but also due to the amount of faeces excreted. Furthermore, whereas calves are more likely to be geographically restrained by the farmer, the fox may range over a large area, and thus has the potential to spread parasites further afield. These host species may therefore have different, albeit both important, roles in the epidemiology of *Giardia*; the fox as a means of new strains being transmitted to a new region, and the domestic cow as a vehicle through which the parasite is able to contaminate the immediate environment. Other environmental factors, such as rivers or humans, may disseminate this contamination further.

One important question that should be addressed is the risk that ecotourism may have on wildlife populations through the spread / introduction of disease. Ecotourism is often seen as one of the key techniques that may be used to conserve wild animals in the future, encouraging tourists to visit pristine areas and thereby appreciate them. Nevertheless, we have already seen some potentially negative consequences such as changed behavior and migration patterns in whale sharks that are being fed, or increased *Giardia* infection in habituated NHPs. It is clear that ecotourism can help place a financial value on wildlife, thus motivating local communities to conserve them. However, how this benefit compares against the risks, including disease, which ecotourism has on the wildlife, is a question that needs to be considered. Is the 'shit' left by tourists visiting the mountain gorillas doing more damage than the economic benefit generated by their visit? Although answering this is beyond the scope of this thesis, it would seem wise that, until the risk of disease is known, operators of ecotourism ventures use the precautionary principle and ensure that visitors behave in a way that minimizes the chance of pathogen transmission.

Another important question generated by this thesis, and related to the last question, is whether *Cryptosporidium* and *Giardia* truly are rare / nonexistent pathogens in some wildlife populations. For instance, is the 'snap-shot' we captured of the herbivores of Mikumi National Park reflective of the epidemiology of these parasites in this region, and if so, does this situation represent larger areas of East Africa. If it does, then this poses a real threat for future disease emergence, since we know that closely related livestock can be severely affected by these pathogens, and that these herbivores can gather in very large numbers and high densities, something which facilities parasite transmission. Tourists to game reserves already expect showers and swimming pools, placing already constrained resources under further pressures. But tourists from areas where *C. parvum* and *G. duodenalis* are prevalent, may also bring with them these parasites, and potentially others,

providing the route by which the wild herbivores that they have come to East Africa to marvel, at can be infected.

Although the papers presented here have helped to answer some questions regarding which wildlife populations are infected by which parasites, they have not provided data on how these parasites impact those populations. This is the natural next step, and can best be achieved by large-scale longitudinal studies of wildlife. Countries that have been wise and wealthy enough to establish long-term monitoring of wildlife ecology and disease, have the benefit of knowing how and when these animals are affected. This then facilitates targeted, evidence-based management, which can help protect and conserve their wildlife. I would encourage all countries to have such a proactive disease surveillance programme for their wildlife, and to ensure that intestinal protozoa are amongst those pathogens towards which testing and control are focused.

13. References

Abu Samra, N, Jori, F, Samie, A & Thompson, P 2011. The prevalence of *Cryptosporidium* spp. oocysts in wild mammals in the Kruger National Park, South Africa. *Vet Parasitol,* 175**,** 155-159.

Alves, DM, Terribile, LC & Brito, D 2014. The potential impact of white-nose syndrome on the conservation status of north american bats. *PLoS One,* 9**,** e107395.

Alves, M, Xiao, L, Lemos, V, Zhou, L, Cama, V, Da Cunha, MB, Matos, O & Antunes, F 2005. Occurrence and molecular characterization of *Cryptosporidium* spp. in mammals and reptiles at the Lisbon Zoo. *Parasitol Res,* 97**,** 108-12.

Amar, CFL, Dear, PH, Pedraza-Diaz, S, Looker, N, Linnane, E & Mclauchlin, J 2002. Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. *J Clin Microbiol,* 40**,** 446-452.

Appelbee, AJ, Thompson, RCA & Olson, ME 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife - current status and future needs. *Trends Parasitol,* 21**,** 370-376.

Arisue, N & Hashimoto, T 2015. Phylogeny and evolution of apicoplasts and apicomplexan parasites. *Parasitol Int,* 64**,** 254-9.

Ashford, RW, Reid, GDF & Wrangham, RW 2000. Intestinal parasites of the chimpanzee *Pan troglodytes* in Kibale Forest, Uganda. *Ann Trop Med Parasit,* 94**,** 173-179.

Beck, R, Sprong, H, Bata, I, Lucinger, S, Pozio, E & Caccio, SM 2011a. Prevalence and molecular typing of *Giardia* spp. in captive mammals at the zoo of Zagreb, Croatia. *Vet Parasitol,* 175**,** 40-46.

Beck, R, Sprong, H, Lucinger, S, Pozio, E & Caccio, SM 2011b. A large survey of Croatian wild mammals for *Giardia duodenalis* reveals a low prevalence and limited zoonotic potential. *Vector-Borne Zoonot,* 11**,** 1049-1055.

Berrilli, F, Prisco, C, Friedrich, KG, Cerbo, PD, Cave, DD & Liberato, CD 2011. *Giardia duodenalis* assemblages and *Entamoeba* species infecting non-human primates in an

Italian zoological garden: zoonotic potential and management traits. *Parasites Vector,* 4**,** 199.

Bouzid, M, Hunter, PR, Chalmers, RM & Tyler, KM 2013. *Cryptosporidium* pathogenicity and virulence. *Clin Microbiol Rev,* 26**,** 115-34.

Caccio, SM, Beck, R, Lalle, M, Marinculic, A & Pozio, E 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *Int J Parasitol,* 38**,** 1523-1531.

Caccio, SM, De Giacomo, M & Pozio, E 2002. Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *Int J Parasitol,* 32**,** 1023-30.

Cacciò, SM, Thompson, RCA, Mclauchlin, J & Smith, HV 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends Parasitol,* 21**,** 430-437.

CDC. 2016a. *Amebiasis* [Online]. U.S. Department of Health & Human Services Available: https://www.cdc.gov/dpdx/amebiasis/index.html [Accessed 8th May 2017].

CDC. 2016b. *Cryptosporidiosis* [Online]. U.S. Department of Health & Human Services. Available: https://www.cdc.gov/dpdx/cryptosporidiosis/index.html [Accessed 8th May 2017].

CDC. 2016c. *Giardiasis* [Online]. U.S. Department of Health & Human Services. Available: https://www.cdc.gov/dpdx/giardiasis/index.html [Accessed 8th May 2017].

CDC. 2016d. *Trichinellosis* [Online]. U.S. Department of Health & Human Services. Available: https://www.cdc.gov/parasites/trichinellosis/biology.html [Accessed 18th May 2017].

Ceballos, G, Ehrlich, PR, Barnosky, AD, García, A, Pringle, RM & Palmer, TM 2015. Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Sci Adv,* 1**,** e1400253.

Chang'a, JS, Robertson, LJ, Mtambo, MMA, Mdegela, RH, Loken, T & Reksen, O 2011. Unexpected results from large-scale cryptosporidiosis screening study in calves in Tanzania. *Ann Trop Med Parasit,* 105**,** 513-519.

Charles-Smith, LE, Cowen, P & Schopler, R 2010. Environmental and physiological factors contributing to outbreaks of *Cryptosporidium* in Coquerel´s Sifaka (*Propithecus coquereli*) at the Duke Lemur Centre: 1999-2007. *J Zoo Wildlife Med,* 41**,** 438-444.

Checkley, W, White, AC, Jr., Jaganath, D, Arrowood, MJ, Chalmers, RM, Chen, XM, Fayer, R, Griffiths, JK, Guerrant, RL, Hedstrom, L, Huston, CD, Kotloff, KL, Kang, G, Mead, JR, Miller, M, Petri, WA, Jr., Priest, JW, Roos, DS, Striepen, B, Thompson, RC, Ward, HD, Van Voorhis, WA, Xiao, L, Zhu, G & Houpt, ER 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *Lancet Infect Dis,* 15**,** 85-94.

Chen, F, Huang, K, Qin, S, Zhao, Y & Pan, C 2007. Comparison of viability and infectivity of *Cryptosporidium parvum* oocysts stored in potassium dichromate solution and chlorinated tap water. *Vet Parasitol,* 150**,** 13-7.

Chernomor, O, Von Haeseler, A & Minh, BQ 2016. Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology***,** syw037.

Clark, CG, Kaffashian, F, Tawari, B, Windsor, JJ, Twigg-Flesner, A, Davies-Morel, MC, Blessmann, J, Ebert, F, Peschel, B, Le Van, A, Jackson, CJ, Macfarlane, L & Tannich, E 2006. New insights into the phylogeny of *Entamoeba* species provided by analysis of four new small-subunit rRNA genes. *Int J Syst Evol Microbiol,* 56**,** 2235-9.

Clark, DP 1999. New insights into human cryptosporidiosis. *Clin Microbiol Rev,* 12**,** 554-63.

Coklin, T, Farber, JM, Parrington, LJ, Bin Kingombe, CI, Ross, WH & Dixon, BR 2011. Immunomagnetic separation significantly improves the sensitivity of polymerase chain reaction in detecting *Giardia duodenalis* and *Cryptosporidium* spp. in dairy cattle. *J Vet Diagn Invest,* 23**,** 260-7.

Da Silva, DC, Paiva, PRSO, Nakamura, AA, Homem, CG, Souza, MSD, Grego, KF & Meireles, MV 2014. The detection of *Cryptosporidium serpentis* in snake fecal samples by real-time PCR. *Vet Parasitol,* 204**,** 134-138.

Daniels, ME, Shrivastava, A, Smith, WA, Sahu, P, Odagiri, M, Misra, PR, Panigrahi, P, Suar, M, Clasen, T & Jenkins, MW 2015. *Cryptosporidium* and *Giardia* in humans, domestic animals, and village water sources in rural India. *Am J Trop Med Hyg,* 93**,** 596-600.

De Carvalho Filho, PR, Cardozo, SV, Torres Ribeiro, C, De Medeiros, SM & Gomes Lopes, CW 2006. Intestinal protozoa in apprehended new world nonhuman primates. *Braz J Vet Res Anim Sci,* 43**,** 354-361.

De Vienne, DM, Giraud, T & Martin, OC 2007. A congruence index for testing topological similarity between trees. *Bioinformatics,* 23**,** 3119-3124.

Dellarupe, A, Unzaga, JM, Moré, G, Kienast, M, Larsen, A, Stiebel, C, Rambeaud, M & Venturini, MC 2016. *Cryptosporidium varanii* infection in leopard geckos (*Eublepharis macularius*) in Argentina. *Open Vet J,* 6**,** 98-101.

Díaz, P, Rota, S, Marchesi, B, López, C, Panadero, R, Fernández, G, Díez-Baños, P, Morrondo, P & Poglayen, G 2013. *Cryptosporidium* in pet snakes from Italy: Molecular characterization and zoonotic implications. *Vet Parasitol,* 197**,** 68-73.

Dubey, JP & Hartley, WJ 1993. Disseminated coccidiosis in short-beaked echindas (*Tachyglossus aculeatus*) from Australia. *J Vet Diag Invest,* 5**,** 483-488.

Dupont, HL, Chappell, CL, Sterling, CR, Okhuysen, PC, Rose, JB & Jakubowski, W 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N Engl J Med,* 332**,** 855-9.

Einarsson, E, Ma'ayeh, S & Svärd, SG 2016. An up-date on *Giardia* and giardiasis. *Curr Opin Microbiol,* 34**,** 47-52.

Ekanayake, DK, Arulkanthan, A, Horadagoda, NU, Sanjeevani, GKM, Kieft, R, Gunatilake, S & Dittus, WPJ 2006. Prevalence of *Cryptosporidium* and other enteric parasites

among wild non-human primates in Polonnaruwa, Sri Lanka. *Am J Trop Med Hyg,* 74**,** 322-329.

Fayer, R 2010. Taxonomy and species delimitation in *Cryptosporidium*. *Exp Parasitol,* 124**,** 90-97.

Feng, M, Cai, J, Min, X, Fu, Y, Xu, Q, Tachibana, H & Cheng, X 2013. Prevalence and genetic diversity of *Entamoeba* species infecting macaques in southwest China. *Parasitol Res,* 112**,** 1529-1536.

Feng, M, Yang, B, Yang, L, Fu, Y, Zhuang, Y, Liang, L, Xu, Q, Cheng, X & Tachibana, H 2011. High prevalence of *Entamoeba* infections in captive long-tailed macaques in China. *Parasitol Res,* 109**,** 1093-1097.

Feng, Y & Xiao, L 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. *Clin Microbiol Rev,* 24**,** 110-40.

Field, H, Schaaf, K, Kung, N, Simon, C, Waltisbuhl, D, Hobert, H, Moore, F, Middleton, D, Crook, A, Smith, G, Daniels, P, Glanville, R & Lovell, D 2010. Hendra virus outbreak with novel clinical features, Australia. *Emerg Infect Dis,* 16**,** 338-40.

Fotedar, R, Stark, D, Beebe, N, Marriott, D, Ellis, J & Harkness, J 2007. Laboratory diagnostic techniques for *Entamoeba* species. *Clin Microbiol Rev,* 20**,** 511-532.

Funk, S, Nishiura, H, Heesterbeek, H, Edmunds, WJ & Checchi, F 2013. Identifying transmission cycles at the human-animal interface: the role of animal reservoirs in maintaining gambiense human African trypanosomiasis. *PLoS Comput Biol,* 9**,** e1002855.

Gao, F, Bailes, E, Robertson, DL, Chen, Y, Rodenburg, CM, Michael, SF, Cummins, LB, Arthur, LO, Peeters, M, Shaw, GM, Sharp, PM & Hahn, BH 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature,* 397**,** 436-41.

Garcia, LS & Shimizu, RY 1997. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. *J Clin Microbiol,* 35**,** 1526-9.

Gillespie, TR & Chapman, CA 2008. Forest fragmentation, the decline of an endangered primate, and changes in host-parasite interactions relative to an unfragmented forest. *Am J Primatol,* 70**,** 222-230.

Gillespie, TR, Chapman, CA & Greiner, EC 2005a. Effects of logging on gastrointestinal parasite infections and infection risk in African primates. *J Appl Ecol,* 42**,** 699-707.

Gillespie, TR, Greiner, EC & Chapman, CA 2005b. Gastrointestinal parasites of the colobus monkeys of Uganda. *J Parasitol,* 91**,** 569-573.

Gillespie, TR, Morgan, D, Deutsch, JC, Kuhlenschmidt, MS, Salzer, JS, Cameron, K, Reed, T & Sanz, C 2009. A legacy of low-impact logging does not elevate prevalence of potentially pathogenic protozoa in free-ranging gorillas and chimpanzees in the Republic of Congo: logging and parasitism in African apes. *EcoHealth,* 6**,** 557-564.

Gomez, MS, Torres, J, Gracenea, M, Fernandez-Moran, J & Gonzalez-Moreno, O 2000. Further report on *Cryptosporidium* in Barcelona zoo mammals. *Parasitol Res,* 86**,** 318-323.

Gonzalez-Moreno, O, Hernandez-Aguilar, RA, Piel, AK, Stewart, FA, Gracenea, M & Moore, J 2013. Prevalence and climatic associated factors of *Cryptosporidium* sp infections in savanna chimpanzees from Ugalla, Western Tanzania. *Parasitol Res,* 112**,** 393-399.

Gouy, M, Guindon, S & Gascuel, O 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol biol evol,* 27**,** 221-224.

Graczyk, TK, Bosco-Nizeyi, J, Ssebide, B, Thompson, RCA, Read, C & Cranfield, MR 2002. Anthropozoonotic *Giardia duodenalis* genotype (assemblage) A infections in habitats of free-ranging human-habituated gorillas, Uganda. *J Parasitol,* 88**,** 905-909.

Graczyk, TK, Fayer, R & Cranfield, MR 1996. *Cryptosporidium parvum* is not transmissible to fish, amphibians, or reptiles. *J Parasitol,* 82**,** 748-751.

Hamnes, IS, Gjerde, BK, Forberg, T & Robertson, LJ 2007. Occurrence of *Giardia* and *Cryptosporidium* in Norwegian red foxes (*Vulpes vulpes*). *Vet Parasitol,* 143**,** 347-353.

Hamzah, Z, Petmitr, S, Mungthin, M, Leelayoova, S & Chavalitshewinkoon-Petmitr, P 2006. Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. *J Clin Microbiol,* 44**,** 3196-200.

Haq, A, Sharma, A, Ahmad, S, Khan, HM & Khan, N 1985. Experimental infection of rhesus monkeys with *Entamoeba histolytica* mimics human infection. *Lab Anim Sci,* 35**,** 481-4.

Heard, MJ, Smith, KF, Ripp, KJ, Berger, M, Chen, J, Dittmeier, J, Goter, M, Mcgarvey, ST & Ryan, E 2013. The threat of disease increases as species move toward extinction. *Conserv Biol,* 27**,** 1378-88.

Heimann, S. 2017. *Life cycle of coccidia (Eimeria spp.)* [Online]. Saxonet. Available: http://www.saxonet.de/coccidia/coccid02.htm [Accessed 11th May 2017].

Hodzic, A, Alic, A & Omeragic, J 2014. Occurrence of *Cryptosporidium* spp. and *Giardia duodenalis* in red foxes (*Vulpes vulpes*) in Bosnia and Herzegovina. *Maced Vet Rev,* 37**,** 189-192.

Hogan, JN, Miller, WA, Cranfield, MR, Ramer, J, Hassell, J, Noheri, JB, Conrad, PA & Gilardi, KVK 2014. *Giardia* in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda. *J Wildlife Dis,* 50**,** 21-30.

Hope, K, Goldsmith, ML & Graczyk, T 2004. Parasitic health of olive baboons in Bwindi Impenetrable National Park, Uganda. *Vet Parasitol,* 122**,** 165-170.

Hopkins, RM, Meloni, BP, Groth, DM, Wetherall, JD, Reynoldson, JA & Thompson, RC 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. *J Parasitol,* 83**,** 44-51.

Huffman, MA, Gotoh, S, Turner, LA, Hamai, M & Yoshida, K 1997. Seasonal trends in intestinal nematode infection and medicinal plant use among chimpanzees in the Mahale Mountains, Tanzania. *Primates,* 38**,** 111-125.

Hunter, PR & Thompson, RCA 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. *Int J Parasitol,* 35**,** 1181-1190.

Huson, DH, Richter, DC, Rausch, C, Dezulian, T, Franz, M & Rupp, R 2007. Dendroscope: An interactive viewer for large phylogenetic trees. *BMC Bioinformatics,* 8**,** 460.

Inoue, M, Uga, S, Oda, T, Rai, SK, Vesey, G & Hotta, H 2006. Changes of physical and biochemical properties of *Cryptosporidium* oocysts with various storage conditions. *Water Res,* 40**,** 881-6.

Jirků-Pomajbíková, K, Čepička, I, Kalousová, B, Jirků, M, Stewart, F, Levecke, B, Modrý, D, Piel, AK & Petrželková, KJ 2016. Molecular identification of *Entamoeba* species in savanna woodland chimpanzees (*Pan troglodytes schweinfurthii*). *Parasitology,* 143**,** 741-8.

Johnston, AR, Gillespie, TR, Rwego, IB, Mclachlan, TLT, Kent, AD & Goldberg, TL 2010. Molecular epidemiology of cross-species *Giardia duodenalis* transmission in Western Uganda. *PloS Negl Trop Dis,* 4**,** e683.

Johnston, SP, Ballard, MM, Beach, MJ, Causer, L & Wilkins, PP 2003. Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. *J Clin Microbiol,* 41**,** 623-6.

Jones, KE, Patel, NG, Levy, MA, Storeygard, A, Balk, D, Gittleman, JL & Daszak, P 2008. Global trends in emerging infectious diseases. *Nature,* 451**,** 990-3.

Jongwutiwes, S, Tiangtip, R, Yentakarm, S & Chantachum, N 2002. Simple method for long-term copro-preservation of *Cryptosporidium* oocysts for morphometric and molecular analysis. *Trop Med Int Health,* 7**,** 257-64.

Katoh, K & Standley, DM 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol Biol Evol,* 30**,** 772-780.

Kilpatrick, AM, Briggs, CJ & Daszak, P 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol Evol,* 25**,** 109-18.

Kock, R 2014. Drivers of disease emergence and spread: is wildlife to blame? *Onderstepoort J Vet Res,* 81**,** E1-4.

Kotloff, KL, Nataro, JP, Blackwelder, WC, Nasrin, D, Farag, TH, Panchalingam, S, Wu, Y, Sow, SO, Sur, D, Breiman, RF, Faruque, ASG, Zaidi, AKM, Saha, D, Alonso, PL, Tamboura, B, Sanogo, D, Onwuchekwa, U, Manna, B, Ramamurthy, T, Kanungo, S, Ochieng, JB, Omore, R, Oundo, JO, Hossain, A, Das, SK, Ahmed, S, Qureshi, S, Quadri, F, Adegbola, RA, Antonio, M, Hossain, MJ, Akinsola, A, Mandomando, I, Nhampossa, T, Acacio, S, Biswas, K, O'reilly, CE, Mintz, ED, Berkeley, LY, Muhsen, K, Sommerfelt, H, Robins-Browne, RM & Levine, MM 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet,* 382**,** 209-222.

Kowalewski, MM, Salzer, JS, Deutsch, JC, Rano, M, Kuhlenschmidt, MS & Gillespie, TR 2011. Black and gold howler monkeys (*Alouatta caraya*) as sentinels of ecosystem health: patterns of zoonotic protozoa infection relative to degree of human-primate contact. *Am J Primatol,* 73**,** 75-83.

Kumar, R, Sinha, A & Radhakrishna, S 2013. Comparative demography of two commensal macaques in India: implications for population status and conservation. *Folia Primatol (Basel),* 84**,** 384-93.

Kuroki, T, Izumiyama, S, Yagita, K, Une, Y, Hayashidani, H, Kuro-O, M, Mori, A, Moriguchi, H, Toriba, M, Ishibashi, T & Endo, T 2008. Occurrence of *Cryptosporidium* sp. in snakes in Japan. *Parasitol Res,* 103**,** 801-805.

Kusiluka, LJM, Kambarage, DM, Matthewman, RW, Harrison, LJS & Daborn, CJ 1996. Coccidiosis of small ruminants in Tanzania. *Small Rum Res,* 21**,** 127-131.

Laishram, S, Kang, G & Ajjampur, SS 2012. Giardiasis: a review on assemblage distribution and epidemiology in India. *Indian J Gastroenterol,* 31**,** 3-12.

Lalle, M, Pozio, E, Capelli, G, Bruschi, F, Crotti, D & Caccio, SM 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int J Parasitol,* 35**,** 207-13.

Lane, KE, Holley, C, Hollocher, H & Fuentes, A 2011. The anthropogenic environment lessens the intensity and prevalence of gastrointestinal parasites in Balinese long-tailed macaques (*Macaca fascicularis*). *Primates,* 52**,** 117-128.

Langhout, MVZ, Reed, P & Fox, M 2010. Validation of multiple diagnostic techniques to detect *Cryptosporidium* sp and *Giardia* sp in free-ranging western lowland gorillas (*Gorilla gorilla gorilla*) and observations on the prevalence of these protozoan infections in two populations in Gabon. *J Zoo Wildlife Med,* 41**,** 210-217.

Larsson, A 2014. AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics,* 30**,** 3276-3278.

Lebbad, M, Ankarklev, J, Tellez, A, Leiva, B, Andersson, JO & Svard, S 2008. Dominance of *Giardia* assemblage B in Leon, Nicaragua. *Acta Trop,* 106**,** 44-53.

Lebbad, M, Mattsson, JG, Christensson, B, Ljungstrom, B, Backhans, A, Andersson, JO & Svard, SG 2010. From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. *Vet Parasitol,* 168**,** 231-9.

Letunic, I & Bork, P 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucl Acid Res,* 44**,** W242-W245.

Levecke, B, Dorny, P, Geurden, T, Vercammen, F & Vercruysse, J 2007. Gastrointestinal protozoa in non-human primates of four zoological gardens in Belgium. *Vet Parasitol,* 148**,** 236-246.

Levecke, B, Dreesen, L, Dorny, P, Verweij, JJ, Vercammen, F, Casaert, S, Vercruysse, J & Geldhof, P 2010. Molecular identification of *Entamoeba* spp. in captive nonhuman primates. *J Clin Microbiol,* 48**,** 2988-90.

Levecke, B, Geldhof, P, Claerebout, E, Dorny, P, Vercammen, F, Caccio, SM, Vercruysse, J & Geurden, T 2009. Molecular characterisation of *Giardia duodenalis* in captive

non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. *Int J Parasitol,* 39**,** 1595-1601.

Li, J, Dong, H, Wang, R, Yu, F, Wu, Y, Chang, Y, Wang, C, Qi, M & Zhang, L 2017. An investigation of parasitic infections and review of molecular characterization of the intestinal protozoa in nonhuman primates in China from 2009 to 2015. *Int J Parasitol Parasites Wildl,* 6**,** 8-15.

Lim, YaL, Ngui, R, Shukri, J, Rohela, M & Naim, HRM 2008. Intestinal parasites in various animals at a zoo in Malaysia. *Vet Parasitol,* 157**,** 154-159.

Lips, KR 2016. Overview of chytrid emergence and impacts on amphibians. *Philos Trans R Soc Lond B Biol Sci,* 371.

Lymbery, AJ & Thompson, RC 2012. The molecular epidemiology of parasite infections: tools and applications. *Mol Biochem Parasitol,* 181**,** 102-16.

Martinez-Diaz, RA, Sansano-Maestre, J, Martinez-Herrero, MDC, Ponce-Gordo, F & Gomez-Munoz, MT 2011. Occurrence and genetic characterization of *Giardia duodenalis* from captive nonhuman primates by multi-locus sequence analysis. *Parasitol Res,* 109**,** 539-44.

Mbae, CK, Nokes, DJ, Mulinge, E, Nyambura, J, Waruru, A & Kariuki, S 2013. Intestinal parasitic infections in children presenting with diarrhoea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. *BMC Infect Dis,* 13.

Mccarthy, S, Ng, J, Gordon, C, Miller, R, Wyber, A & Ryan, UM 2007. Prevalence of *Cryptosporidium* and *Giardia* species in animals in irrigation catchments in the southwest of Australia. *Exp Parasitol,* 118**,** 596-9.

Mengistu, L & Berhanu, E 2004. Zoonotic intestinal parasites in *Papio anubis* (baboon) and *Cercopithecus aethiops* (vervet) from four localities in Ethiopia. *Acta Tropica,* 90**,** 231-236.

Middleton, D 2008. Echidnas. *In:* VOGELNEST, L. (ed.) *Medicine of Australian Mammals.* Collingwood: CSIRO Publishing.

Minh, BQ, Nguyen, MaT & Von Haeseler, A 2013. Ultrafast approximation for phylogenetic bootstrap. *Mol Biol Evol***,** mst024.

Morgan, KJ, Castro, I, Lopez-Villalobos, N, Pomroy, WE, Alley, MR, Gartrell, BD, Hunter, S & Howe, L 2014. Prevalence of and risk factors for coccidiosis in kiwi between 1977 and 2011. *N Z Vet J,* 62**,** 315-20.

Morgan, UM, Sturdee, AP, Singleton, G, Gomez, MS, Gracenea, M, Torres, J, Hamilton, SG, Woodside, DP & Thompson, RC 1999. The *Cryptosporidium* "mouse" genotype is conserved across geographic areas. *J Clin Microbiol,* 37**,** 1302-5.

Mosier, DA & Oberst, RD 2000. Cryptosporidiosis: A Global challenge. *Ann NY Acad Sci: Trop Vet Dis,* 102-111.

Mtambo, MMA, Sebatwale, JB, Kambarage, DM, Muhairwa, AP, Maeda, GE, Kusiluka, LJM & Kazwala, RR 1997. Prevalence of *Cryptosporidium* spp. oocysts in cattle and wildlife in Morogoro region, Tanzania. *Preven Vet Med,* 31**,** 185-190.

Muehlenbein, MP 2005. Parasitological analyses of the male chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *Am J Primatol,* 65**,** 167-179.

Mul, IF, Paembonan, W, Singleton, I, Wich, SA & Van Bolhuis, HG 2007. Intestinal parasites of free-ranging, semicaptive, and captive *Pongo abelii* in sumatra, indonesia. *Int J Primatol,* 28**,** 407-420.

Muriuki, SM, Farah, IO, Kagwiria, RM, Chai, DC, Njamunge, G, Suleman, M & Olobo, JO 1997a. The presence of *Cryptosporidium* oocysts in stools of clinically diarrhoeic and normal nonhuman primates in Kenya. *Vet Parasitol,* 72**,** 141-7.

Muriuki, SMK, Farah, IO, Kagwiria, RM, Chai, DC, Njamunge, G, Suleman, M & Olobo, JO 1997b. The presence of *Cryptosporidium* oocysts in stools of clinically diarrhoeic and normal nonhuman primates in Kenya. *Vet Parasitol,* 72**,** 141-147.

Nakao, JH, Collier, SA & Gargano, JW 2017. Giardiasis and subsequent irritable bowel syndrome: a longitudinal cohort study using health insurance data. *J Infect Dis,* 215**,** 798-805.

Nash, TE & Keister, DB 1985. Differences in excretory-secretory products and surface-antigens among 19 isolates of *Giardia*. *J Infect Dis,* 152**,** 1166-1171.

Nash, TE, Mccutchan, T, Keister, D, Dame, JB, Conrad, JD & Gillin, FD 1985. Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates bbtained from humans and animals. *J Infect Dis,* 152**,** 64-73.

Nath, J, Ghosh, SK, Singha, B & Paul, J 2015. Molecular epidemiology of amoebiasis: a cross-sectional study among North East Indian population. *PLoS Negl Trop Dis,* 9**,** e0004225.

Newman, C, Macdonald, DW & Anwar, MA 2001. Coccidiosis in the European badger, *Meles meles* in Wytham Woods: infection and consequences for growth and survival. *Parasitol,* 123**,** 133-142.

Ng, J, Yang, R, Whiffin, V, Cox, P & Ryan, U 2011. Identification of zoonotic *Cryptosporidium* and *Giardia* genotypes infecting animals in Sydney's water catchments. *Exp Parasitol,* 128**,** 138-144.

Nguyen, L-T, Schmidt, HA, Von Haeseler, A & Minh, BQ 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol,* 32**,** 268-274.

Nizeyi, JB, Mwebe, R, Nanteza, A, Cranfield, MR, Kalema, G & Graczyk, TK 1999. *Cryptosporidium* sp and *Giardia* sp infections in mountain gorillas (*Gorilla gorilla beringei*) of the Bwindi Impenetrable National Park, Uganda. *J Parasitol,* 85**,** 1084-1088.

Nowak, K 2008. Frequent water drinking by zanzibar red colobus (*Procolobus kirkii*) in a mangrove forest refuge. *Am J Primatol,* 70**,** 1081-1092.

Oates, SC, Miller, MA, Hardin, D, Conrad, PA, Melli, A, Jessup, DA, Dominik, C, Roug, A, Tinker, MT & Miller, WA 2012. Prevalence, Environmental Loading, and Molecular characterization of *Cryptosporidium* and *Giardia* isolates from domestic and wild animals along the central California coast. *Appl Environ Microbiol,* 78**,** 8762-8772.

Ocaido, M, Dranzoa, C & Cheli, P 2003. Gastrointestinal parasites of baboons (*Papio anubis*) interacting with humans in West Bugwe Forest Reserve, Uganda. *Afr J Ecol,* 41**,** 356-359.

Onac, D, Oltean, M, Mircean, V, Jarca, A & Cozma, V 2015. Occurence of *Giardia duodenalis* zoonotic assemblages in red foxes from Romania. *Sci Parasitol,* 16**,** 177-180.

Parija, SC & Khairnar, K 2005. *Entamoeba moshkovskii* and *Entamoeba dispar*-associated infections in pondicherry, India. *J Health Popul Nutr,* 23**,** 292-5.

Parija, SC, Mandal, J & Ponnambath, DK 2014. Laboratory methods of identification of *Entamoeba histolytica* and its differentiation from look-alike *Entamoeba* spp. *Trop Parasitol,* 4**,** 90-5.

Parsons, MB, Travis, D, Lonsdorf, EV, Lipende, I, Roellig, DM, Collins, A, Kamenya, S, Zhang, H, Xiao, L & Gillespie, TR 2015. Epidemiology and molecular characterization of *Cryptosporidium* spp. in humans, wild primates, and domesticated animals in the Greater Gombe Ecosystem, Tanzania. *PLoS Negl Trop Dis,* 9**,** e0003529.

Pavlasek, I & Ryan, U 2008. *Cryptosporidium varanii* takes precedence over *C. saurophilum*. *Exp Parasitol,* 118**,** 434-437.

Petrasova, J, Modry, D, Huffman, MA, Mapua, MI, Bobakova, L, Mazoch, V, Singh, J, Kaur, T & Petrzelkova, KJ 2010. Gastrointestinal parasites of indigenous and introduced primate species of Rubondo Island National Park, Tanzania. *Int J Primatol,* 31**,** 920-936.

Petrzelkova, KJ, Hasegawa, H, Appleton, CC, Huffman, MA, Archer, CE, Moscovice, LR, Mapua, MI, Singh, J & Kaur, T 2010. Gastrointestinal parasites of the chimpanzee population introduced onto Rubondo Island National Park, Tanzania. *Am J Primatol,* 72**,** 307-316.

Plutzer, J & Karanis, P 2009. Genetic polymorphism in *Cryptosporidium* species: An update. *Vet Parasitol,* 165**,** 187-199.

Prasad, H 1960. Studies on the coccidia of some mammals of the families Bovidae, Cervidae and Camelidae. *Z Parasitenkd,* 20**,** 390-400.

Pye, RJ, Woods, GM & Kreiss, A 2016. Devil facial tumor disease. *Vet Pathol,* 53**,** 726-36.

Pyziel, AM & Demiaszkiewicz, AW 2013. Coccidia (Apicomplexa: Eimeriidae) of elk (*Alces alces*) in Poland. *Parasitol Res,* 112**,** 2083-5.

Rasambainarivo, FT, Gillespie, TR, Wright, PC, Arsenault, J, Villeneuve, A & Lair, S 2013. Survey of *Giardia* and *Cryptosporidium* in lemurs from the Ranomafana National Park, Madagascar. *J Wildlife Dis,* 49**,** 741-743.

Read, C, Walters, J, Robertson, ID & Thompson, RC 2002. Correlation between genotype of *Giardia duodenalis* and diarrhoea. *Int J Parasitol,* 32**,** 229-31.

Read, CM, Monis, PT & Thompson, RCA 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infect Genet Evol* 4**,** 125-30.

Rhyan, JC & Spraker, TR 2010. Emergence of diseases from wildlife reservoirs. *Vet Pathol,* 47**,** 34-9.

Rinaldi, L, Capasso, M, Mihalca, AD, Cirillo, R, Cringoli, G & Cacciò, S 2012. Prevalence and molecular identification of *Cryptosporidium* isolates from pet lizards and snakes in Italy. *Parasite,* 19**,** 437-440.

Robertson, L, Gjerde, B, Hansen, EF & Stachurska-Hagen, T 2009. A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies. *J Water Health,* 7**,** 55-66.

Robertson, LJ, Hermansen, L, Gjerde, BK, Strand, E, Alvsvag, JO & Langeland, N 2006. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. *Appl Environ Microbiol,* 72**,** 2212-7.

Ryan, U & Caccio, SM 2013. Zoonotic potential of *Giardia*. *Int J Parasitol,* 43**,** 943-56.

Ryan, U, Fayer, R & Xiao, L 2014a. *Cryptosporidium* species in humans and animals: current understanding and research needs. *Parasitology,* 141**,** 1667-1685.

Ryan, U, Paparini, A & Oskam, C 2017. New technologies for detection of enteric parasites. *Trends Parasitol*.

Ryan, UNA, Fayer, R & Xiao, L 2014b. *Cryptosporidium* species in humans and animals: current understanding and research needs. *Parasitology,* 141**,** 1667-1685.

Sa, RM, Petrasova, J, Pomajbikova, K, Profousova, I, Petrzelkova, KJ, Sousa, C, Cable, J, Bruford, MW & Modry, D 2013. Gastrointestinal symbionts of chimpanzees in Cantanhez National Park, Guinea-Bissau with respect to habitat fragmentation. *Am J Primatol,* 75**,** 1032-1041.

Sak, B, Petrzelkova, KJ, Kvetonova, D, Mynarova, A, Shutt, KA, Pomajbikova, K, Kalousova, B, Modry, D, Benavides, J, Todd, A & Kvac, M 2013. Long-term monitoring of *Microsporidia, Cryptosporidium* and *Giardia* infections in Western lowland gorillas (*Gorilla gorilla gorilla*) at different stages of habituation in Dzanga Sangha Protected Areas, Central African Republic. *PLoS One,* 8**,** e71840.

Salb, AL, Barkema, HW, Elkin, BT, Thompson, RC, Whiteside, DP, Black, SR, Dubey, JP & Kutz, SJ 2008. Dogs as sources and sentinels of parasites in humans and wildlife, northern Canada. *Emerg Infect Dis,* 14**,** 60-3.

Salzer, JS, Rwego, IB, Goldberg, TL, Kuhlenschmidt, MS & Gillespie, TR 2007. *Giardia* sp and *Cryptosporidium* sp infections in primates in fragmented and undisturbed forest in western Uganda. *J Parasitol,* 93**,** 439-440.

Saraswat, R, Sinha, A & Radhakrishna, S 2015. A god becomes a pest? Human-rhesus macaque interactions in Himachal Pradesh, Northern India. *Eu J Wildlife Res,* 61**,** 435-443.

Savioli, L, Smith, H & Thompson, A 2006. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. *Trends Parasitol,* 22**,** 203-208.

Schliep, KP 2011. Phangorn: phylogenetic analysis in R. *Bioinformatics,* 27**,** 592-593.

Shimodaira, H & Hasegawa, M 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol biol evol,* 16**,** 1114-1116.

Shirley, DA, Moonah, SN & Kotloff, KL 2012. Burden of disease from cryptosporidiosis. *Curr Opin Infect Dis,* 25**,** 555-63.

Singh, BB & Gajadhar, AA 2014. Role of India's wildlife in the emergence and re-emergence of zoonotic pathogens, risk factors and public health implications. *Acta Trop,* 138**,** 67-77.

Singh, S, Shrivastav, AB & Sharma, RK 2009. The epidemiology of gastrointestinal parasitism and body condition in free-ranging herbivores. *J Threat Taxa,* 1**,** 535-537.

Sleeman, JM, Meader, LL, Mudakikwa, AB, Foster, JW & Patton, S 2000. Gastrointestinal parasites of mountain gorillas (*Gorilla gorilla beringei*) in the Parc National des Volcans, Rwanda. *J Zoo Wildlife Med,* 31**,** 322-328.

Smith, PD, Gillin, FD, Kaushal, NA & Nash, TE 1982. Antigenic analysis of *Giardia lamblia* from Afghanistan, Puerto Rico, Ecuador, and Oregon. *Infect Immun,* 36**,** 714-9.

Sommer, MF, Beck, R, Ionita, M, Stefanovska, J, Vasic, A, Zdravkovic, N, Hamel, D, Rehbein, S, Knaus, M, Mitrea, IL, Shukullari, E, Kirkova, Z, Rapti, D, Capari, B & Silaghi, C 2015. Multilocus sequence typing of canine *Giardia duodenalis* from South Eastern European countries. *Parasitol Res,* 114**,** 2165-74.

Sow, SO, Muhsen, K, Nasrin, D, Blackwelder, WC, Wu, Y, Farag, TH, Panchalingam, S, Sur, D, Zaidi, AK, Faruque, AS, Saha, D, Adegbola, R, Alonso, PL, Breiman, RF, Bassat, Q, Tamboura, B, Sanogo, D, Onwuchekwa, U, Manna, B, Ramamurthy, T, Kanungo, S, Ahmed, S, Qureshi, S, Quadri, F, Hossain, A, Das, SK, Antonio, M, Hossain, MJ, Mandomando, I, Nhampossa, T, Acacio, S, Omore, R, Oundo, JO, Ochieng, JB, Mintz, ED, O'reilly, CE, Berkeley, LY, Livio, S, Tennant, SM, Sommerfelt, H, Nataro, JP, Ziv-Baran, T, Robins-Browne, RM, Mishcherkin, V, Zhang, J, Liu, J, Houpt, ER, Kotloff, KL & Levine, MM 2016. The Burden of Cryptosporidium Diarrheal Disease among Children < 24 Months of Age in Moderate/High Mortality Regions of Sub-Saharan Africa and South Asia, Utilizing

Data from the Global Enteric Multicenter Study (GEMS). *PLoS Negl Trop Dis,* 10**,** e0004729.

Sprong, H, Caccio, SM, Van Der Giessen, JWB, Network, Z & Partners 2009. Identification of zoonotic genotypes of *Giardia duodenalis*. *Plos Negl Trop Dis,* 3**,** e558.

Sricharern, W, Inpankaew, T, Keawmongkol, S, Supanam, J, Stich, RW & Jittapalapong, S 2016. Molecular detection and prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. among long-tailed macaques (*Macaca fascicularis*) in Thailand. *Infect Genet Evol,* 40**,** 310-4.

Stanley Jr, SL 2003. Amoebiasis. *The Lancet,* 361**,** 1025-1034.

Stojecki, K, Sroka, J, Caccio, SM, Cencek, T, Dutkiewicz, J & Kusyk, P 2015. Prevalence and molecular typing of *Giardia duodenalis* in wildlife from eastern Poland. *Folia Parasitol (Praha),* 62.

Sulaiman, IM, Fayer, R, Bern, C, Gilman, RH, Trout, JM, Schantz, PM, Das, P, Lal, AA & Xiao, L 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. *Emerg Infect Dis,* 9**,** 1444-52.

Szonyi, B, Kang'ethe, EK, Mbae, CK, Kakundi, EM, Kamwati, SK & Mohammed, HO 2008. First report of *Cryptosporidium* deer-like genotype in Kenyan cattle. *Vet Parasitol,* 153**,** 172-5.

Tachibana, H, Yanagi, T, Akatsuka, A, Kobayash, S, Kanbara, H & Tsutsumi, V 2009. Isolation and characterization of a potentially virulent species *Entamoeba nuttalli* from captive Japanese macaques. *Parasitology,* 136**,** 1169-1177.

Tachibana, H, Yanagi, T, Lama, C, Pandey, K, Feng, M, Kobayashi, S & Sherchand, JB 2013. Prevalence of *Entamoeba nuttalli* infection in wild rhesus macaques in Nepal and characterization of the parasite isolates. *Parasitol Int,* 62**,** 230-5.

Tachibana, H, Yanagi, T, Pandey, K, Cheng, X-J, Kobayashi, S, Sherchand, JB & Kanbara, H 2007. An *Entamoeba* sp strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*. *Mol Biochem Parasitol,* 153**,** 107-114.

Takano, J, Narita, T, Tachibana, H, Terao, K & Fujimoto, K 2007. Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates. *Parasitol Res,* 101**,** 539-46.

Team, RC 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013.

Teichroeb, JA, Kutz, SJ, Parkar, U, Thompson, RCA & Sicotte, P 2009. Ecology of the gastrointestinal parasites of *Colobus vellerosus* at Boabeng-Fiema, Ghana: possible anthropozoonotic transmission. *Am J Physic Anthropol,* 140**,** 498-507.

Thompson, J, Yang, R, Power, M, Hufschmid, J, Beveridge, I, Reid, S, Ng, J, Armson, A & Ryan, U 2008. Identification of zoonotic *Giardia* genotypes in marsupials in Australia. *Exp Parasitol,* 120**,** 88-93.

Thompson, RCA 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. *Int Jr Parasitol,* 30**,** 1259-1267.

Thompson, RCA & Ash, A 2016. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. *Infect Genet Evol,* 40**,** 315-323.

Thompson, RCA, Lymbery, AJ & Smith, A 2010. Parasites, emerging disease and wildlife conservation. *Int J Parasitol,* 40**,** 1163-1170.

Thompson, RCA & Smith, A 2011. Zoonotic enteric protozoa. *Vet Parasitol,* 182**,** 70-8.

Tomczuk, K, Szczepaniak, K, Grzybek, M, Studzinska, M, Demkowska-Kutrzepa, M, Roczen-Karczmarz, M, Kostro, K & Krakowski, L 2014. Gastro-intestinal parasite prevention in wild roe deer in selected hunting areas in south-eastern Poland. *Medycyna Weterynaryjna* 70**,** 630-635.

Traversa, D, Iorio, R, Otranto, D, Modrý, D & Šlapeta, J 2008. *Cryptosporidium* from tortoises: Genetic characterisation, phylogeny and zoonotic implications. *Mol Cell Probes,* 22**,** 122-128.

Trifinopoulos, J, Nguyen, L-T, Von Haeseler, A & Minh, BQ 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research***,** gkw256.

Trout, JM, Santin, M & Fayer, R 2007. *Giardia* and *Cryptosporidium* species and genotypes in coyotes (*Canis latrans*). *J Zoo Wildl Med,* 37**,** 141-4.

Turner, WC & Getz, WM 2010. Seasonal and demographic factors influencing gastrointestinal parasitism in ungulates of Etosha National Park. *J Wildlife Dis,* 46**,** 1108-1119.

Urquhart, G, Armour, J, Dunn, A & Jennings, F 1996. *Veterinary Parasitology,* Blackwell Science.

Verweij, JJ, Laeijendecker, D, Brienen, EA, Van Lieshout, L & Polderman, AM 2003. Detection and identification of *Entamoeba* species in stool samples by a reverse line hybridization assay. *J Clin Microbiol,* 41**,** 5041-5.

Villers, LM, Jang, SS, Lent, CL, Lewin-Koh, S-C & Norosoarinaivo, JA 2008. Survey and comparison of major intestinal flora in captive and wild ring-tailed lemur (*Lemur catta*) populations. *Am J Primatol,* 70**,** 175-184.

Vitazkova, SK & Wade, SE 2006. Parasites of free-ranging black howler monkeys (*Alouatta pigra*) from Belize and Mexico. *Am J Primatol,* 68**,** 1089-97.

Volotão, ACC, Júnior, JCS, Grassini, C, Peralta, JM & Fernandes, O 2008. Genotyping of *Giardia duodenalis* from Southern brown howler monkeys (*Alouatta clamitans*) from Brazil. *Vet Parasitol,* 158**,** 133-137.

WHO/PAHO/UNESCO 1997. Expert consultation on Amoebiasis, Mexico City, Mexico 28-29 January. *Epidemiol Bull,* 18**,** 13-14.

Wielinga, CM & Thompson, RCA 2007. Comparative evaluation of *Giardia duodenalis* sequence data. *Parasitology,* 134**,** 1795-1821.

Wilber, RG, Duszynski, DW, Upton, SJ, Seville, RS & Corliss, JO 1998. A revision of the taxonomy and nomenclature of the *Eimeria* spp. (Apicomplexa: Eimeriidae) from the rodents in the tribe Marmotini (Sciuridae). *Syst Parasitol,* 39**,** 113-135.

Xiao, L, Ryan, UM, Graczyk, TK, Limor, J, Li, L, Kombert, M, Junge, R, Sulaiman, IM, Zhou, L, Arrowood, MJ, Koudela, B, Modrý, D & Lal, AA 2004. Genetic diversity of *Cryptosporidium* spp. in captive reptiles. *Appl Environ Microbiol,* 70**,** 891-899.

Xiao, LH, Escalante, L, Yang, CF, Sulaiman, I, Escalante, AA, Montali, RJ, Fayer, R & Lal, AA 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Appl Environ Microbiol,* 65**,** 1578-1583.

Ye, J, Xiao, L, Li, J, Huang, W, Amer, SE, Guo, Y, Roellig, D & Feng, Y 2013. Occurrence of human-pathogenic *Enterocytozoon bieneusi*, *Giardia duodenalis* and *Cryptosporidium* genotypes in laboratory macaques in Guangxi, China. *Parasitol Int,* 63**,** 132-7.

Ye, J, Xiao, L, Ma, J, Guo, M, Liu, L & Feng, Y 2012. Anthroponotic enteric parasites in monkeys in public park, China. *Emerg Infect Dis,* 18**,** 1640-1643.

Zhou, L, Fayer, R, Trout, JM, Ryan, UA, Schaefer, FW & Xiao, LH 2004. Genotypes of *Cryptosporidium* species infecting fur-bearing mammals differ from those of species infecting humans. *Appl Environ Microbiol,* 70**,** 7574-7577.

Paper 1

ORIGINAL ARTICLE

Occurrence of *Giardia* and *Cryptosporidium* in captive chimpanzees (*Pan troglodytes*), mandrills (*Mandrillus sphinx*) and wild Zanzibar red colobus monkeys (*Procolobus kirkii*)

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Abstract

Background The aim of this study was to investigate the occurrence of *Giardia duodenalis* and *Cryptosporidium* spp. in primates and determine their zoonotic or anthropozoonotic potential.
Methods Direct immunofluorescence was used to identify *Giardia* and *Cryptosporidium* from faecal samples. PCR and DNA sequencing was performed on positive results.
Results Giardia cysts were identified from 5.5% (5/90) of captive chimpanzees and 0% (0/11) of captive mandrills in the Republic of Congo; 0% (0/10) of captive chimpanzees in Norway; and 0% of faecal samples (n = 49) from wild Zanzibar red colobus monkeys. Two *Giardia* positive samples were also positive on PCR, and sequencing revealed identical isolates of Assemblage B. *Cryptosporidium* oocysts were not detected in any of the samples.
Conclusions In these primate groups, in which interactions with humans and human environments are quite substantial, *Giardia* and *Cryptosporidium* are rare pathogens. In chimpanzees, *Giardia* may have a zoonotic or anthropozoonotic potential.

Introduction

The transmission of pathogens between human and non-human primates (primates) is facilitated by their close taxonomic relationship, posing a threat to public health and wildlife conservation. This risk seems to be increasing as the barriers between wildlife, domestic animals and humans become more porous through habitat fragmentation, urbanisation, sharing of water sources, ecotourism and climate change [27]. Of particular concern are ubiquitous protozoan parasites *Giardia* and *Cryptosporidium*, which are able to infect humans, livestock and wild animals. Whilst it is recognised that *Giardia* and *Cryptosporidium* are major causes of human and livestock diarrhoeal disease, information on their significance and potential impact on wildlife populations is scanty [4, 26]. This is important because it has been shown that the occurrence of *Giardia* in some wild primate populations is linked to an anthropogenic effect [9].

Giardia duodenalis is a mammalian parasite, existing as a species complex comprised of at least seven distinct genetic groups, Assemblage A to F, with Assemblages A and B having zoonotic potential [25]. *Cryptosporidium* spp. are known to infect over 79 species of animals, which have received growing interest due to their clinical significance for immunocompromised people, human outbreaks associated with contaminated water sources as well as their economic significance in livestock [19].

Both these parasites can be transmitted directly, or via contaminated food or water [28].

The limited literature on the epidemiology of *Giardia* and *Cryptosporidium* in primates reports a wide range of prevalences amongst different populations. This may represent true differences in susceptibility and exposure between populations; however, due to variation in study design and the diagnostic tests used, it is difficult to compare results directly. To provide further information regarding the potential for zoonotic and anthropozoonotic spread of *Giardia* and *Cryptosporidium* between humans and primates, and the significance of these parasites in primate populations, we investigated the occurrence of these parasites in three primate species with varying degrees of human contact.

Materials and methods

Animals

This study was approved by the Jane Goodall Institute Animal Welfare Committee and was conducted in accordance with the Norwegian Animal Welfare Act. Faecal samples (n = 269) were collected from captive chimpanzees (*Pan troglodytes*, n = 90) and mandrills (*Mandrillus sphinx*, n = 11) housed at the Tchimpounga Chimpanzee Rehabilitation Centre (TCRC; Tchimpounga, the Republic of Congo); captive chimpanzees (n = 10) housed at Kristiansand Zoo (KZ; Kristiansand, Norway); and five wild Zanzibar red colobus (*Procolobus kirkii,* $n_{samples}$ = 58) monkeys troops from the Jozani Forest (Zanzibar, Tanzania) between June 2012 and March 2013. At TCRC, the majority (88/90) of chimpanzees and all mandrills were wild born and entered captivity as young orphans. The chimpanzees were housed in five permanent enclosures as well as various quarantine holding facilities, whilst mandrills were housed in three different enclosures. Infant and juvenile chimpanzees had daily direct physical contact with caregivers, whilst mandrills and adult chimpanzees had limited physical contact with carers. At KZ, nine of the 10 chimpanzees were captive born, with the remaining chimpanzee having been in European zoos for over 20 years. All chimpanzees at KZ were housed together in a single enclosure and had limited physical contact with carers. At both TCRC and KZ, caregivers were responsible for all food preparation for the chimpanzees and mandrills, as well as entering the enclosures on a daily basis for cleaning. Direct physical contact between humans and the wild Zanzibar red colobus monkeys was minimal; however, through an ecotourism venture, over 100 tourists would enter Jozani Forest and come within 1 m of the monkeys on a daily basis.

Sample collection and parasitological analysis

For all animals, samples were collected opportunistically when animals were observed to defecate. The sample was given a subjective consistency score from 1 (liquid diarrhoea) to 5 (dry hard stool). Faecal samples from chimpanzees at TCRC were mixed thoroughly with water, then a drop of the liquid portion was placed on a welled slide and allowed to air dry, and then fixed with ethanol (96%) and stored for a period of 1–4 months prior to analysis. Faecal samples from mandrills and red colobus were preserved in potassium dichromate 2.5% (w/v) at a ratio of 1:4 and processed within 4 months. Faecal samples from chimpanzees at KZ were kept unpreserved at <4°C and analysed within 7 days. Samples from the mandrills, red colobus and KZ chimpanzees were mixed thoroughly with a food blender, passed through a fine sieve and then centrifuged. Roughly 10 μl of the pellet was placed on a slide, air-dried and methanol-fixed. For all samples, *Giardia* cysts and *Cryptosporidium* oocysts were detected using a commercially available *Cryptosporidium*/*Giardia* direct fluorescent antibody (DFA; Aqua-Glo, Waterborne Inc., New Orleans) test, in accordance with manufacturer's instructions. Prior to being screened, all slides were also stained with 4′6 diamidino-2-phenylindole (DAPI), a non-specific fluorescent stain that binds to double-stranded DNA. Stained samples were screened using a fluorescence microscope equipped with appropriate filters (for FITC and DAPI) and Normarksi optics. Samples were initially screened at ×200, and possible findings examined more closely at ×400 and ×1000. Photographs were taken of positive or presumptive positive findings. Faecal samples were scored for the presence or absence of each parasite, and positive samples were given a semi-quantitative score by counting the number of oocysts or cysts per 200× microscope field. Score 1 = individual cysts/oocysts counted; score 2 = 1 to 20 per 200× field; score 3 = 21 to 100 per 200× field; and score 4 = over 100 per 200× field.

PCR and sequencing

When presumptive or confirmed *Giardia* cysts or *Cryptosporidium* oocysts were detected by DFA, cysts/oocysts were isolated from potassium dichromate-preserved samples by zinc sulphate floatation, or directly from slides by published methods [23]. DNA was isolated from cysts using QIAmp DNA mini kit (Qiagen GmbH, Hilden, Germany), including an overnight lysis step at 56°C and an initial 5 freeze-thaw cycles in liquid nitrogen. Nested PCR for *Giardia* positive samples was conducted on the glutamate dehydrogenase (*gdh*) gene,

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triose phosphate isomerase-B (*tpi-B*) and the β-giardin (*bg*) genes using published protocols, and PCR for *Cryptosporidium* positive samples was conducted on the Small Ribosomal Subunit (*SSU*) gene [3, 6, 14, 29]. In all cases, the primary PCR consisted of 8.3 μl PCR water, 1 μl forward primer, 1 μl reverse primer, 0.2 μl BSA (20 mg/l), 12.5 μl of HotStartTaqMaster and 2 μl of template DNA. For each PCR, positive and negative controls were included. PCR products were visualised by electrophoresis on 2% agarose gel with ethidium bromide staining and then sequenced by a commercial company (Eurofins Genomics, Ebersberg, Germany) in both directions. Sequences were assembled with DNA Baser (Version 3; Heracle BioSoft S.R.L., Pitesti, Romania) and compared with reference sequences in GenBank. New sequences were submitted to GenBank (Accession No. KM102527).

Statistical analysis

The prevalence of each parasite at each captive institution was calculated overall as well as by individual enclosure, age group (<5 year; 5–10 year; 10–15 year; 15–20 year; 20 + year), gender and faecal consistency. Animals were considered positive if one or more sample was positive on DFA testing. Chi-squared test was used to compare the prevalence of each parasite between different variables. A P-value <0.05 was considered significant.

Results

Direct immunofluorescent antibody tests

Examination of chimpanzee faecal samples from TCRC revealed the presence of *Giardia* cysts in 4.5% (7/154) of samples, with 5.5% (5/90) of chimpanzees testing positive for *Giardia* in one or more sample (Table 1). The proportion of chimpanzees at TCRC shedding *Giardia* cysts in one or more sample did not differ by sex (χ^2, $P = 0.87$), enclosure (χ^2, $P = 0.23$), faecal consistency (χ^2, $P = 0.50$) or age (χ^2, $P = 0.70$). One female chimpanzee (age: 1.5 years) had three consecutive clinically diarrhoeic stools containing moderate to high numbers of *Giardia* cysts. However, she had been tested five times over the preceding 21 days due to her diarrhoea, and all five of these samples were negative for *Giardia*.

Two faecal samples from adult male chimpanzees housed in separate enclosures at TCRC contained objects whose size, shape and fluorescence resembled *Cryptosporidium* spp. oocysts. However, due to the limited number of these objects, negative DAPI staining and negative PCR, identification of *Cryptosporidium* could not be confirmed.

All faecal samples from chimpanzees held at KZ (n = 29), mandrills at TCRC (n = 28) and wild Zanzibar red colobus (n = 58) were negative for both *Giardia* and *Cryptosporidium*.

PCR and DNA sequencing

Results for PCR are presented in Table 2. PCR products from samples 25 and 64 were sequenced and a 442 base-pair fragment from the *bg* gene was obtained. Both sequences were identical and belonged to *Giardia duodenalis* Assemblage B. A BLAST search showed that this isolate (GenBank Accession No. KM102527) had one SNP difference to *Giardia* isolates from humans in Bangladesh, China, India and Sweden (GenBank Accession nos. KJ188088.1, JX994238.1, JF918494.1 and HM165218.1, respectively), and two SNPs difference from an isolate from a ring-tailed lemur (GenBank Accession no. HQ616629.1) from a zoo in Spain.

Discussion

Our study describes a low occurrence of *Giardia* in captive chimpanzees in Congo, *Giardia* and *Cryptosporidium* occurrence below the level of detection in captive mandrills in Congo, captive chimpanzees in Norway

Table 1 Demographics of chimpanzees at Tchimpounga Chimpanzee Rehabilitation Centre for which *Giardia* cysts were identified in faecal samples

Sample	Gender	Age (years)	Enclosure	Consistency[1]	Giardia score[2]
25	Female	6	7	3	2
64, 83, 86[3]	Female	1.5	7	1	3
107	Male	19	2	2	1
121	Male	11	3	3	1
136	Male	10	3	4	1

[1]Consistency: 1 = liquid diarrhoea to 5 = dry hard stool.
[2]*Giardia* score: 1, individual cysts counted; 2, 1–20 cysts per 200× field; 3, 21–100 cysts per 200× field; and 4, over 100 cysts per 200× field.
[3]This chimpanzee had three consecutive faecal samples over 14 days which all were consistency 1 and score 3 for *Giardia* cysts.

Table 2 PCR results of faecal samples from captive chimpanzees at Tchimpounga Chimpanzee Rehabilitation Centre that were excreting *Giardia* cysts

Sample	Number of cysts	DAPI[1]	GDH[2]	BG[3]	TPI-B[4]
25	10 per 200× field	–	+	+	+
64	50 per 200× field	–	+	+	+
107	1 on slide	+	–	–	–
121	1 on slide	+	–	–	–
136	2 on slide	+	–	–	–

[1]DAPI, 4′,6-diamidine-2-phenylindole.
[2]GDH, glutamate dehydrogenase [6].
[3]TPI-B, triose phosphate isomerase Assemblage B-specific [3].
[4]BG, β-giardin [14].

and wild Zanzibar red colobus monkeys. Published prevalence data of *Giardia* or *Cryptosporidium* in chimpanzees vary considerably, with reports (where n > 10) of *Giardia* prevalences of 0%, 0.5%, 2%, 5%, 6% and 32%, and *Cryptosporidium* prevalences of 0%, 8.9% and 36% [20, 13, 22, 5, 24, 15, 10, 11, 16, respectively]. In only one of these studies [10] was DFA staining used, which is considered the gold standard for diagnosis of these parasites [1, 8]. This study investigated the occurrence of *Giardia* and *Cryptosporidium* amongst wild chimpanzees in the Republic of Congo (n = 67) and reported a prevalence of 6% for *Giardia* and 0% *Cryptosporidium*, which is consistent with the data in the present study. Similar results have also been obtained using DFA in mountain gorillas in Rwanda [12]. Interestingly, this latter study also reports the presence of '*Cryptosporidium*-like particles' – suggesting that objects with epitopes that cross-react with the anti-*Cryptosporidium* monoclonal antibody used in DFA may be excreted in low numbers in the faeces of non-human primates. It might be speculated that in some studies that report high *Cryptosporidium* prevalence, a misidentification may have occurred; this would not be a unique situation, as such misidentification has previously been proposed regarding *Cryptosporidium* excretion in cattle in sub-Saharan Africa [7]. Alternatively, these might indeed by *Cryptosporidium* oocysts, but the numbers are insufficient for successful molecular confirmation.

Despite having high stocking densities, close human contact and regular admission to the centre of sick young wild chimpanzees, the occurrence of *Giardia* and *Cryptosporidium* amongst chimpanzees at TCRC was low. Given the prevalence of these parasites in humans in sub-Saharan Africa is quite high, this suggests that the biosecurity measures in place at TCRC (quarantine of new arrivals, treatment and isolation of sick individuals, enforced leave of sick staff and regular faecal parasitological examination) have been effective at preventing transmission of these parasites [17, 18]. An alternative explanation could be that chimpanzees are not suitable as long-term hosts of *Giardia* and *Cryptosporidium*.

Of the five chimpanzees found to be excreting *Giardia* cysts, three were adults shedding very low numbers of cysts, whilst two were infants shedding moderate to high numbers of cysts. At the time of infection, these two infants were housed together and had extensive daily physical contact with their human caregivers. The sequencing results for the *Giardia* isolates from these chimpanzees indicates either horizontal transmission or common exposure. The sequence similarity (1 SNP difference) to human-derived isolates suggest that zoonotic and anthropozoonotic transmission is possible. Unfortunately, the human caregivers were not sampled, as this may have provided further insight into transmission routes.

For the remaining three primate groups (captive mandrills at TCRC, captive chimpanzees at KZ and wild Zanzibar red colobus monkeys), the lack of positive results indicates either that these animals are not being exposed, or are being exposed and are not susceptible to infection. Previously published data on *Giardia* and *Cryptosporidium* in mandrills are limited to one study from a Belgian Zoo that reports detection of *Giardia* cysts in 7% (5/75) of samples though did not investigate *Cryptosporidium*, and one report from a Portuguese zoo which found no *Cryptosporidium* though did not investigate *Giardia* [2, 15]. This indicats that mandrills can be susceptible to *Giardia*, and the results from TCRC along with results from other studies [5, 11, 13, 15, 16, 22, 24] indicates that chimpanzees can be susceptible to *Giardia* and may be susceptile to *Cryptosporidium* also. Our study represents the first investigation on the occurrence of *Giardia and Cryptosporidium* in Zanzibar red colobus monkeys, and therefore, we have no comparative data. Research on another species of red colobus in Uganda reported low levels of *Giardia* associated with forest fragmentation; thus, it is also possible that the sample size of this study was insufficient to detect very low prevalence levels [9]. Furthermore, wild Zanzibar colobus monkeys are folivorous and drink from natural rainwater collection depots, and thus, whilst their ground environment may be contaminated with *Giardia* and *Cryptosporidium* from human and livestock waste, they may not be exposed to contaminated food or water [21]. For the mandrills and captive chimpanzees at KZ, the absence of infection may be due to effective biosecurity measures in place and low levels of human contact.

This study investigated the prevalence of *Giardia* and *Cryptosporidium* in three primate species and found that these parasites are not common pathogens in the populations examined. Whilst *Giardia* isolates almost identical to those found in humans seem capable of establishing in chimpanzees, suggesting the potential for zoonotic and anthropozoonotic transmission, the low prevalence found in populations with extremely close human contact suggest that this is probably not a common occurrence. Investigation of the *Giardia* and *Cryptosporidium* status of the human contacts for each of these primate groups may have provided further indications of the potential for human transmission. However, this was not possible in this study. Furthermore, unless identification of identical isolates in humans and primates were found, ideally through a longitudinal study showing a time-related transmission event, then anthropozoonotic or zoonotic transmission remains speculation.

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References

1 Alles AJ, Waldron MA, Sierra LS, Mattia AR: Prospective comparison of direct immunofluorescence and conventional staining methods for detection of *Giardia* and *Cryptosporidium* in human fecal specimens. *J Clin Microbiol* 1995; **33**:1632–4.

2 Alves M, Xiao LH, Lemos V, Zhou L, Cama V, da Cunha MB, Matos O, Antunes F: Occurrence and molecular characterization of *Cryptosporidium* spp. in mammals and reptiles at the Lisbon Zoo. *Parasitol Res* 2005; **97**:108–112.

3 Amar CFL, Dear PH, Pedraza-Diaz S, Looker N, Linnane E, McLauchlin J: Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia* duodenalis in human feces. *J Clin Microbiol* 2002; **40**:446–52.

4 Appelbee AJ, Thompson RCA, Olson ME: *Giardia* and *Cryptosporidium* in mammalian wildlife – current status and future needs. *Trends Parasitol* 2005; **21**:370–6.

5 Ashford RW, Reid GDF, Wrangham RW: Intestinal parasites of the chimpanzee *Pan troglodytes* in Kibale Forest, Uganda. *Ann Trop Med Parasitol* 2000; **94**: 173–9.

6 Caccio SM, Beck R, Lalle M, Marinculic A, Pozio E: Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *Int J Parasitol* 2008; **38**:1523–31.

7 Chang'a JS, Robertson LJ, Mtambo MMA, Mdegela RH, Loken T, Reksen O: Unexpected results from large-scale cryptosporidiosis screening study in calves in Tanzania, Anna. *Trop Med Parasitol* 2011; **105**:513–9.

8 Geurden T, Berkvens D, Casaert S, Vercruysse J, Claerebout E: A Bayesian evaluation of three diagnostic assays for the detection of *Giardia duodenalis* in symptomatic and asymptomatic dogs. *Vet Parasitol* 2008; **157**:14–20.

9 Gillespie TR, Chapman CA: Forest fragmentation, the decline of an endangered primate, and changes in host-parasite interactions relative to an unfragmented forest. *Am J Primatol* 2008; **70**:222–30.

10 Gillespie TR, Morgan D, Deutsch JC, Kuhlenschmidt MS, Salzer JS, Cameron K, Reed T, Sanz C: A legacy of low-impact logging does not elevate prevalence of potentially pathogenic protozoa in free-ranging gorillas and chimpanzees in the Republic of Congo: logging and parasitism in African Apes. *EcoHealth* 2009; **6**:557–64.

11 Gonzalez-Moreno O, Hernandez-Aguilar RA, Piel AK, Stewart FA, Gracenea M, Moore J: Prevalence and climatic associated factors of *Cryptosporidium* sp. infections in savanna chimpanzees from Ugalla, Western Tanzania. *Parasitol Res* 2013; **112**:393–399.

12 Hogan JN, Miller WA, Cranfield MR, Ramer J, Hassell J, Noheri JB, Conrad PA, Gilardi KV: *Giardia* in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda. *J Wildl Dis* 2014; **50**:21–30.

13 Huffman MA, Gotoh S, Turner LA, Hamai M, Yoshida K: Seasonal trends in intestinal nematode infection and medicinal plant use among chimpanzees in the Mahale Mountains, Tanzania. *Primates* 1997; **38**:111–25.

14 Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D, Caccio SM: Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int J Parasitol* 2005; **35**:207–213.

15 Levecke B, Dorny P, Geurden T, Vercammen F, Vercruysse J:

Gastrointestinal protozoa in non--human primates of four zoological gardens in Belgium. *Vet Parasitol* 2007; **148**:236–46.
16 Lim YAL, Ngui R, Shukri J, Rohela M, Naim HRM: Intestinal parasites in various animals at a zoo in Malaysia. *Vet Parasitol* 2008; **157**:154–9.
17 Mbae CK, Nokes DJ, Mulinge E, Nyambura J, Waruru A, Kariuki S: Intestinal parasitic infections in children presenting with diarrhoea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. *BMC Infect Dis* 2013; **13**:243.
18 Mor SM, Tzipori S: Cryptosporidiosis in children in sub-Saharan Africa: a lingering challenge. *Clin Infect Dis* 2008; **47**:915–21.
19 Mosier DA, Oberst RD: Cryptosporidiosis: a global challenge. *Ann N Y Acad Sci* 2000; **916**: 102–11.
20 Muehlenbein MP: Parasitological analyses of the male chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *Am J Primatol* 2005; **65**:167–79.
21 Nowak K: Frequent water drinking by Zanzibar red colobus (*Procolobus kirkii*) in a mangrove forest refuge. *Am J Primatol* 2008; **70**:1081–92.
22 Petrasova J, Modry D, Huffman MA, Mapua MI, Bobakova L, Mazoch V, Singh J, Kaur T, Petrzelkova KJ: Gastrointestinal parasites of indigenous and introduced primate species of Rubondo Island National Park, Tanzania. *Int J Primatol* 2010; **31**:920–936.
23 Robertson L, Gjerde B, Hansen EF, Stachurska-Hagen T: A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies. *J Water Health* 2009; **7**:55–66.
24 Sa RM, Petrasova J, Pomajbikova K, Profousova I, Petrzelkova KJ, Sousa C, Cable J, Bruford MW, Modry D: Gastrointestinal symbionts of chimpanzees in Cantanhez National Park, guinea-bissau with respect to habitat fragmentation. *Am J Primatol* 2013; **75**:1032–41.
25 Sprong H, Caccio SM, van der Giessen JWB: Identification of zoonotic genotypes of *Giardia duodenalis*. *PLoS Negl Trop Dis* 2009; **3**:e558.
26 Thompson RCA, Palmer CS, O'Handley R: The public health and clinical significance of *Giardia* and *Cryptosporidium* in domestic animals. *Vet J* 2008; **177**:18–25.
27 Wolfe ND, Escalante AA, Karesh WB, Kilbourn A, Spielman A, Lal AA: Wild primate populations in emerging infectious disease research: the missing link? *Emerg Infect Dis* 1998; **4**:149–58.
28 Xiao L: Molecular epidemiology of cryptosporidiosis: an update. *Exp Parasitol* 2010; **124**:80–9.
29 Xiao LH, Escalante L, Yang CF, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA: Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Appl Environ Microbiol* 1999; **65**:1578–83.

Paper 2

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Invited article

Occurrence of *Giardia, Cryptosporidium*, and *Entamoeba* in wild rhesus macaques (*Macaca mulatta*) living in urban and semi-rural North-West India

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ABSTRACT

Giardia duodenalis, Cryptosporidium spp., and *Entamoeba* spp. are intestinal protozoa capable of infecting a range of host species, and are important causes of human morbidity and mortality. Understanding their epidemiology is important, both for public health and for the health of the animals they infect. This study investigated the occurrence of these protozoans in rhesus macaques (*Macaca mulatta*) in India, with the aim of providing preliminary information on the potential for transmission of these pathogens between macaques and humans. Faecal samples ($n = 170$) were collected from rhesus macaques from four districts of North-West India. Samples were analysed for *Giardia/Cryptosporidium* using a commercially available direct immunofluorescent antibody test after purification via immunomagnetic separation. Positive samples were characterised by sequencing of PCR products. Occurrence of *Entamoeba* was investigated first by using a genus-specific PCR, and positive samples further investigated via species-specific PCRs for *Entamoeba coli*, *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii*. *Giardia* cysts were found in 31% of macaque samples, with all isolates belonging to Assemblage B. *Cryptosporidium* oocysts were found in 1 sample, however this sample did not result in amplification by PCR. *Entamoeba* spp. were found in 79% of samples, 49% of which were positive for *E. coli*. Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii*, did not result in amplification in any of the samples. Thus in 51% of the samples positive at the genus specific PCR, the *Entamoeba* species was not identified. This study provides baseline information on the potential for transmission of these zoonotic parasites at the wildlife-human interface.

1. Introduction

Giardia duodenalis, Cryptosporidium spp., and *Entamoeba* spp. are intestinal protozoa capable of infecting a range of host species, and are important causes of human morbidity and mortality (Hunter and Thompson, 2005; Kotloff et al., 2013; Stanley Jr, 2003). *Cryptosporidium* spp., mainly *C. hominis* and *C. parvum,* have been responsible for large-scale waterborne epidemics in the developed world, and are amongst the top four causes of moderate-to-severe diarrhoea in young children in the developing world (Checkley et al., 2015; Kotloff et al., 2013; Shirley et al., 2012; Sow et al., 2016). Around 200 million people in Asia, Africa and Latin America are reported to have symptomatic giardiasis (Feng and Xiao, 2011). *Entamoeba histolytica*, the cause of amoebic colitis and amoebic liver disease, is responsible for up to 100 000 deaths annually (Stanley Jr, 2003).

Understanding the epidemiology of these parasites is important, both for public health as well as for the health of the animals they infect. This is made difficult by morphologically identical parasites sometimes having separate pathogenicity, host ranges and life cycles. Thus, molecular characterisation is required to elucidate transmission pathways. For instance, *Giardia duodenalis* is considered a species complex comprised of at least 8 distinct genetic

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groups (Assemblage A to H), with Assemblages A and B found both in humans and a range of animal species (Thompson and Smith, 2011).

Cryptosporidiosis, giardiasis and amoebiasis are all important diseases in India where poverty, lack of hygiene, free roaming animals, high population density, and infrastructure inadequacies regarding water supply and sanitation, facilitate infection (Kaur et al., 2002; Nath et al., 1999, 2015b). Rhesus macaques (*Macaca mulatta*) are one of the most common primates in India, particularly in human-dominated habitats (Kumar et al., 2013). Indeed, in some Indian districts, the close contact between rhesus macaques and human activities means that they are regarded as a nuisance, particularly due to crop raiding activities (Saraswat et al., 2015). Macaque species have been implicated as wildlife reservoirs for zoonotic pathogens such as Kyasanur forest disease, a zoonotic tick-borne viral haemorrhagic fever (Singh and Gajadhar, 2014). Nevertheless, it is unclear whether there is transmission of intestinal protozoans between humans and urban monkeys, and if so, how significant this is for public health and for the conservation of the macaques. This study investigated the occurrence of *Giardia duodenalis, Cryptosporidium* spp., and *Entamoeba* spp. in rhesus macaques in four districts of North-West India, with the aim of using molecular characterisation of isolates to provide preliminary information on the potential for transmission of these pathogens between macaques and humans.

2. Materials and methods

2.1. Animals

2.1.1. Rhesus macaques

Faecal samples ($n = 170$) were collected from free-living rhesus macaques in four non-overlapping locations in North-west India.

Troop 1: Located at Punjab University, Chandigarh. Monkeys move freely throughout the campus, spending large amounts of time feeding, defecating and sleeping near areas used for preparation of human food. Estimated troop size, 300 animals.
Troop 2: Located at Jakhoo Temple, Himachal Pradesh. Primarily based around a forested hilltop temple, however also move freely into the surrounding city of Shimla. Estimated troop size, 200 animals.
Troop 3: Located around a small local temple in the municipality of Kurali, Punjab. This temple also owns a cattle-breeding facility where the troop spends much of its time. There is direct contact between the cows and the moneys, with macaques eating grain provided to the cattle and picking food off the ground contaminated with cattle faeces. Estimated troop size, 100 animals.
Troop 4: Located on the outskirts of a semi-rural town Nada Sahib, Haryana. Co-exists with roughly 30 Tarai grey langurs (*Semnopuithecus hector*). Estimated troop size, 200 animals.

2.1.2. Domestic cattle (Bos indicus)

Faecal samples ($n = 14$) were collected from calves from the breeding facility in Kurali with which Troop 3 was in close contact.

2.2. Sample collection and preservation

Rhesus macaques faecal samples were collected non-invasively, and were identified by being morphologically consistent fresh stools located where these monkeys had been observed immediately preceding collection. Each stool sample was considered to be from a separate individual. Calf faecal samples were collected directly from the stool after the animal had been observed to defecate.

Approximately two grammes of faecal material, collected from the middle of the fecal mass, was placed in an 8 ml aliquot of 2.5% (w/v) potassium dichromate, mixed thoroughly, and transported to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis. One gram of faecal material was transported to the Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, and kept unpreserved at 4 °C for 2 weeks prior to DNA isolation.

2.3. Sample processing

At NMBU, the samples were washed twice with phosphate buffered saline, and then passed through a faecal parasite concentrator with a pore diameter 425 μm (Midi Parasep, Apacor, Berkshire, England) and centrifuged to create a pellet. *Giardia* cysts and *Cryptosporidium* oocysts were isolated using an in-house immunomagnetic separation method (IMS) using Dynabeads™ (GC-Combo, Life Technologies, Carlsbad, CA) as previously published (Robertson et al., 2006). Briefly, 10 μl anti-*Giardia* beads, 10 μl anti-*Cryptosporidium* beads, 100 μl SL buffer A and 100 μl SL Buffer B, were used to generate 55 μl of purified sample from approximately 200 mg of the faecal pellet. Five μl of the resulting purified sample was dried and methanol-fixed to welled slides for detection of *Giardia* cysts and *Cryptosporidium* oocysts using a commercially available *Cryptosporidium/Giardia* direct immunofluorescent antibody test (IFAT; Aqua-Glo, Waterborne Inc., New Orleans), in accordance with manufacturer's instructions. Prior to being screened, dried samples were also counterstained with 4′6 diamidino-2-phenylindole (DAPI), a non-specific fluorescent stain that binds to double-stranded DNA. Stained samples were screened using a fluorescence microscope equipped with appropriate filters (for FITC and DAPI) and Nomarksi optics. Samples were initially screened at ×200, and possible findings examined more closely at x 400 and x 1000. The total number and DAPI staining of cysts and oocysts on the slide was recorded. Due to the large number of *Giardia* positive samples, only those with either over 100 DAPI negative cysts, or over 20 DAPI positive cysts were included in molecular analyses. These criteria resulted in 26 *Giardia* positive samples being included. All *Cryptosporidium*-positive samples were included in molecular analysis.

2.4. DNA isolation

2.4.1. Entamoeba

At PGIMER, DNA was isolated using QIAamp® Fast DNA Stool Mini Kit, with an incubation at 70 °C for 5 min, in accordance with the manufacturer's instructions.

2.4.2. Giardia and Cryptosporidium

For *Giardia*/*Cryptosporidium*-positive samples, DNA was isolated using the remaining 50 μl of purified cysts/oocysts after IMS using the QIAmp DNA mini kit (Qiagen GmbH) at NMBU. The protocols followed the manufacturer instructions with slight modifications; cysts/oocysts were first mixed with 150 μl of TE buffer (100 mM Tris and 100 mM EDTA) and incubated at 90 °C/100 °C (*Giardia*/*Cryptosporidium*) for 1 h before an overnight proteinase K lysis step at 56 °C and spin column purification. DNA was finally eluted in 30 μl of PCR grade water and stored at 4 °C.

2.5. PCR and sequencing

In all cases, the primary PCR consisted of 8.3 μl PCR water, 1 μl forward and 1 μl reverse primer (at a final concentration of

0,4 mM), 0.2 μl BSA (20 mg/l), 12.5 μl of 2× HotStartTaqMaster and 2 μl of template DNA. For each PCR, positive and negative controls were included. PCR products were visualized by electrophoresis on 2% agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, CA). Target genes and PCR conditions are provided in Supplementary Table 1.

2.5.1. Giardia

Conventional PCR was performed on *Giardia* positive samples at the glutamate dehydrogenase (GDH), triosephosphate isomerase (TPI), β-giardin (BG) and small subunit rRNA (SSU rRNA) genes (Caccio et al., 2008; Hopkins et al., 1997; Lalle et al., 2005; Read et al., 2002, 2004; Robertson et al., 2006; Sulaiman et al., 2003). Positive samples were purified using a High Pure PCR Product Purification Kit (Roche, Oslo, Norway) and sent to a commercial company (GATC Biotech, Germany) for sequencing in both directions. Sequences from both directions were assembled and manually corrected by analysis of the chromatograms using the program Geneious™.

2.5.2. Cryptosporidium

Conventional PCR was performed on *Cryptosporidium* positive samples at the SSU rRNA gene (Xiao et al., 1999).

2.5.3. Entamoeba

An *Entamoeba* genus-specific conventional PCR was performed on rhesus macaque samples as previously published (Verweij et al., 2003). Two samples were not analysed due to insufficient faecal material for DNA isolation. A single round multiplex PCR targeting the SSU rRNA gene, and that identifies *E. histolytica*, *E. dispar* and *E. moshkovskii*, was performed on all samples (Hamzah et al., 2006). For samples that tested positive on the genus-specific PCR, a species-specific PCR for *E. coli* was performed as previously described (Tachibana et al., 2009). Four *Entamoeba* genus-specific positive samples were not tested for *E. coli* due to laboratory error.

2.6. Statistics

Prevalence of *Giardia*, *Cryptosporidium* and *Entamoeba* were compared for the four different macaque troops using the Chi-squared test. Proportion of samples that resulted in amplification by PCR was compared using Fischers exact test.

3. Results

3.1. Prevalence of Giardia cysts shed by wild rhesus macaques

Examination of rhesus macaque faecal samples using immunofluorescent microscopy revealed the presence of *Giardia* cysts in 31% (53/170) of samples. Macaques excreted 55 to 6325 cysts per gramme faeces (mean, 555; median, 165). There was a significant difference in the prevalence of *Giardia* cysts between Troops 1, 2, 3 and 4; 45% (25/55), 20% (9/55), 33% (15/46) and 17% (4/24), respectively ($p < 0.05$).

3.2. Giardia genotyping

Of the twenty-six *Giardia* positive samples selected for molecular characterisation, seventeen tested positive at one or more gene, with the SSU rRNA loci being the most sensitive (Table 1).

Table 1
Results of PCR from *Giardia* positive faecal samples from wild rhesus macaques (*Macaca mulatta*) with close human contact.

#	Cysts[a]	DAPI[b]	TPI[c]	GDH[d]	GDH[e]	BG[f]	SSU[g]
1	950	800	–	B (KX787059)	B (KX787059)	B (KX787068)	B (KX787044)
2	1150	600	–	–	–	–	B (KX787042)
3	200	150	–	–	B (KX787061)	–	B (KX787047)
4	130	70	–	–	–	–	Positive
5	190	60	–	B (KX787060)	–	B (KX787069)	–
6	110	50	–	–	–	–	B (KX787043)
7	110	50	–	–	–	B (KX787055)	B (KX787046)
8	80	50	–	–	–	B (KX787056)	B (KX787050)
9	50	40	–	–	–	–	B (KX787045)
10	70	20	–	–	–	–	B (KX787049)
11	40	20	–	–	–	–	–
12	30	20	–	–	–	–	Positive
13	30	20	B (KX787057)	–	–	–	–
14	20	20	–	–	–	–	Positive
15	20	20	B (KX787058)	–	–	–	B (KX787048)
16	160	10	–	–	–	–	–
17	80	10	–	–	–	–	Positive
18	40	10	–	–	–	–	–
19	320	0	–	–	–	–	Positive
20	240	0	–	–	–	–	–
21	170	0	–	–	–	–	–
22	160	0	–	–	–	–	–
23	130	0	–	–	–	–	–
24	130	0	–	–	–	–	–
25	130	0	–	–	–	–	–
26	110	0	–	–	–	–	Positive

TPI, triosephosphate isomerase; GDH, glutamate dehydrogenase; BG, beta giardin; SSU, small subunit rRNA; -, PCR negative; Positive, amplification on PCR however no sequencing results; Assemblage (Accession number) provided where sequence of PCR products was obtained.

[a] Number of *Giardia* cysts used for DNA isolation.
[b] Number of DAPI positive *Giardia* cysts used for DNA isolation.
[c] Sulaiman et al. (2003).
[d] Caccio et al. (2008).
[e] Read et al. (2004) & Robertson et al. (2006).
[f] Lalle et al. (2005).
[g] Hopkins et al. (1997) & Read et al. (2002).

Amplification by PCR was more likely if more than twenty DAPI-positive cysts were used for DNA isolation, 80% (12/15), than if 10 or less DAPI positive cysts were used, 27% (3/11) ($p < 0.05$). There was no observed correlation observed between the total number of cysts and the likelihood of a sample being positive by PCR.

Sequencing of PCR products revealed Assemblage B in all samples. Sequences were submitted to GenBank and Accession numbers are provided (Table 1). Multiple alignment of consensus sequences at the TPI, GDH, BG and SSU rRNA genes showed *Giardia* excreted by macaques to be very similar to each other, 98–99%, with differences primarily due to ambiguous nucleotides. Importantly, there was heterozygosity of alleles within the sequences corresponding to the reverse internal primer at the BG gene and the reverse internal primer at the SSU rRNA genes. BLAST results of macaque sequences at the TPI, GDH and BG genes showed 99% identity to *Giardia* isolates from humans, common marmosets and a beaver. Two samples, 5 and 8 (Table 1), showed 100% identity at the BG gene to a *Giardia* isolate from a sheep and human.

3.3. Prevalence of Cryptosporidium spp. oocysts shed by wild rhesus macaques

Examination of rhesus macaque faecal samples using immunofluorescent microscopy revealed the presence of *Cryptosporidium* oocysts in 1 of 170 samples, with this animal from Troop 3. This sample contained 50 oocysts per gramme of faeces, all of which stained positively with DAPI, however was negative by PCR at the SSU rRNA gene.

3.4. Entamoeba coli and unknown Entamoeba spp. in wild rhesus macaques

Examination of rhesus macaque faecal samples using a genus-specific conventional PCR revealed the presence of *Entamoeba* spp. in 79% (132/168) of samples. There was no significant difference in the prevalence of *Entamoeba* spp. between Troops 1, 2, 3 and 4; 78% (43/55), 69% (31/45), 83% (19/23) and 87% (39/45) respectively ($p = 0.21$).

Multiplex PCR for *E. histolytica*, *E. dispar* and *E. moshkovskii*, did not result in amplification in any of the samples (0/168). Species-specific PCR for *E. coli* resulted in amplification in 49% (63/128) of samples positive at the genus-specific PCR. Thus, in the other 51% (65/128), no species of *Entamoeba* was identified. There was a significant difference in the prevalence of *E. coli* between Troops 1, 2, 3 and 4; 26% (11/42), 75% (21/28), 56% (10/18) and 45% (21/39), respectively ($p < 0.01$).

3.5. Giardia and Cryptosporidium in calves living in association with wild rhesus macaques (Troop 3)

Examination of faeces from domestic calves living together with Troop 3, revealed *Giardia* spp. cysts in 64% (9/14) of samples. Calves excreted 55 to 19 250 cysts per gramme faeces (mean, 4746; median, 302). Five positive samples were analysed further by PCR, and all five tested positive at one or more loci. Sequencing of PCR products revealed Assemblage A (KX787052, KX787054) in two calves, Assemblage A1 (KX787067) in one calf, Assemblage E (KX787051, KX787063, KX787065) in one calf, and a mixed infection of Assemblage A1 (KX787062, KX787053) and E (KX787064, KX787066) in one calf.

Cryptosporidium spp. oocysts were detected in 36% (5/14) of samples. Calves were excreting 100 to 5000 oocysts per gramme faeces (mean; 1480, median; 700). PCR at the SSU rRNA gene was negative for all 5 samples.

4. Discussion

This study describes a very high prevalence of *Entamoeba* spp., a moderate prevalence of *Giardia duodenalis* Assemblage B, and a very low prevalence of *Cryptosporidium* spp. in wild rhesus macaques in India, some of which have relatively close contact with humans and domestic animals.

The high prevalence of *Entamoeba* spp. in the macaques is consistent with results from studies in other closely related nonhuman primates (Feng et al., 2011; Feng and Xiao, 2011; Tachibana et al., 2009). *E. dispar* was not identified in this study, but has been detected in macaques from China and Nepal (Feng et al., 2013; Tachibana et al., 2013). Macaques were not infected with *E. histolytica* and *E. moshkovskii*, consistent with previous reports from other wild urban dwelling macaques (Feng et al., 2013; Tachibana et al., 2013). Since *E. histolytica*, *E. moshkovskii* and *E. dispar* are commonly reported in humans in India, this suggests that macaques are not a wildlife reservoir for these human pathogens, and that transmission from humans to macaques is not common among the macaque troops investigated (Nath et al., 2015a; Parija and Khairnar, 2005: Parija et al., 2014). Molecular identification of *Entamoeba* spp. in 520 samples from a range of captive nonhuman primate species revealed *E. hartmanni* (51.9%), *E. polecki*-like (42.7%), *Entamoeba histolytica* nonhuman primate variant (36%), *E. coli* (21.5%), *E. dispar* (2.4%) and *E. moshkovskii* (1.9%), as well as unidentified *Entamoeba* spp (18.9%). *E. polecki* and *E. hartmanni* were not tested for in our study as they are not considered pathogenic to humans, however they may be responsible for the unidentified *Entamoeba* spp observed. The reason for different prevalences amongst the macaque troops is not clear and could be due to a combination of various factors including diet, water sources, microbiome, genetics, and interactions with other humans or animals.

The moderately high prevalence of *Giardia* in rhesus macaques in this study is higher than reported for other macaque species, 2.4–9%, where IFA/PCR was used for diagnosis (Sricharern et al., 2016; Ye et al., 2012, 2014). As these studies also investigated macaque populations in close contact with humans, the difference in *Giardia* prevalence may be due to innate differences in the study populations, or, alternatively, due to different levels of food, water or environmental contamination where these population live. The study population in our study may have an increased exposure to *Giardia* due to its high prevalence amongst humans, domestic animals, and environmental water sources in India (Daniels et al., 2015; Laishram et al., 2012). *Giardia* infection has been associated with human contact in other primate species (Gillespie and Chapman, 2008; Graczyk et al., 2002; Salzer et al., 2007).

Macaques in China and Thailand have been reported to be infected with *G. duodenalis* Assemblage A and B, as seen in other nonhuman primates (Levecke et al., 2009; Sricharern et al., 2016; Ye et al., 2012), while in this study macaques around Chandigarh were only found to be infected with Assemblage B. Although this indicates a zoonotic potential for *Giardia* infections in macaques, the results should be interpreted with caution as most of the samples were only positive at one gene and it has been show that some isolates show certain taxonomic grouping at one gene and a different grouping at another gene (Lebbad et al., 2010; Robertson et al., 2006). Furthermore, it is difficult to interpret the zoonotic potential of these isolates, as multi-locus typing data can reveal animal isolates to be distinct from human isolates, despite them appearing similar based on a single locus (Ryan and Caccio, 2013; Sprong et al., 2009). Despite close contact with cattle shedding Assemblage A and E cysts, these genotypes were not found in samples from macaques.

There was only a single macaque faecal sample that was positive

for *Cryptosporidium*, suggesting that this protozoan in not an important parasite in rhesus macaques in this region of India. Since this positive sample contained few oocysts and was from the troop that had intimate contact with the calves shedding *Cryptosporidium* oocysts, it is possible that this sample represents carriage, and not a true infection. *Cryptosporidium* may be more common in very young macaques that are likely under-represented in this study due to the sampling technique relying on stool morphology.

In this study, using IFA as the gold standard, then PCR at different gene loci had the following sensitivities; SSU rRNA (58%), BG (15%), GDH (12%) and TPI (8%). Overall sensitivity of PCR, using all loci, was 65% (17/26) in macaques, and 100% (6/6) in the calves. PCR sensitivity may have been limited by the low number of DAPI positive cysts available for DNA isolation. Alternatively, the allelic sequence heterozygosity observed at the primer binding sites would suggest that the primers used in this study are not optimal for the *Giardia* isolates found in the study population. Similar limitations of PCR have been observed in genotyping canine *Giardia* isolates (Sommer et al., 2015). Not surprisingly, positive DAPI staining of cysts, indicating the presence of nuclear DNA, was associated with increased likelihood of a positive PCR result.

5. Conclusion

Entamoeba coli, unknown *Entamoeba* spp. and *G. duodenalis* Assemblage B were common in urban dwelling rhesus macaques around Chandigarh, India. *Cryptosporidium* spp., *E. histolytica* and *E. moshkovskii* do not appear to be important pathogens in this population. Further molecular investigation is needed to firmly establish the zoonotic potential of *Giardia* infections in macaques.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2016.12.002.

References

Caccio, S.M., Beck, R., Lalle, M., Marinculic, A., Pozio, E., 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int. J. Parasitol. 38, 1523–1531.

Checkley, W., White Jr., A.C., Jaganath, D., Arrowood, M.J., Chalmers, R.M., Chen, X.M., et al., 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. Lancet Infect. Dis. 15, 85–94.

Daniels, M.E., Shrivastava, A., Smith, W.A., Sahu, P., Odagiri, M., Misra, P.R., et al., 2015. *Cryptosporidium* and *Giardia* in humans, domestic animals, and village water sources in rural India. Am. J. Trop. Med. Hyg. 93, 596–600.

Feng, M., Cai, J., Min, X., Fu, Y., Xu, Q., Tachibana, H., et al., 2013. Prevalence and genetic diversity of *Entamoeba* species infecting macaques in southwest China. Parasitol. Res. 112, 1529–1536.

Feng, M., Yang, B., Yang, L., Fu, Y., Zhuang, Y., Liang, L., et al., 2011. High prevalence of *Entamoeba* infections in captive long-tailed macaques in China. Parasitol. Res. 109, 1093–1097.

Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin. Microbiol. Rev. 24, 110–140.

Gillespie, T.R., Chapman, C.A., 2008. Forest fragmentation, the decline of an endangered primate, and changes in host-parasite interactions relative to an unfragmented forest. Am. J. Primatol. 70, 222–230.

Graczyk, T.K., Bosco-Nizeyi, J., Ssebide, B., Thompson, R.C.A., Read, C., Cranfield, M.R., 2002. Anthropozoonotic *Giardia duodenalis* genotype (assemblage) A infections in habitats of free-ranging human-habituated gorillas, Uganda. J. Parasitol. 88, 905–909.

Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., Chavalitshewinkoon-Petmitr, P., 2006. Differential detection of *Entamoeba histolytica, Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. J. Clin. Microbiol. 44, 3196–3200.

Hopkins, R.M., Meloni, B.P., Groth, D.M., Wetherall, J.D., Reynoldson, J.A., Thompson, R.C., 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. J. Parasitol. 83, 44–51.

Hunter, P.R., Thompson, R.C.A., 2005. The zoonotic transmission of *Giardia* and *Cryptosporidium*. Int. J. Parasitol. 35, 1181–1190.

Kaur, R., Rawat, D., Kakkar, M., Uppal, B., Sharma, V.K., 2002. Intestinal parasites in children with diarrhea in Delhi, India. Southeast Asian J. Trop. Med. Public Health 33, 725–729.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., et al., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382, 209–222.

Kumar, R., Sinha, A., Radhakrishna, S., 2013. Comparative demography of two commensal macaques in India: implications for population status and conservation. Folia Primatol. (Basel) 84, 384–393.

Laishram, S., Kang, G., Ajjampur, S.S., 2012. Giardiasis: a review on assemblage distribution and epidemiology in India. Indian J. Gastroenterol. 31, 3–12.

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Caccio, S.M., 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35, 207–213.

Lebbad, M., Mattsson, J.G., Christensson, B., Ljungström, B., Backhans, A., Andersson, J.O., et al., 2010. From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. Vet. Parasitol. 168, 231–239.

Levecke, B., Geldhof, P., Claerebout, E., Dorny, P., Vercammen, F., Caccio, S.M., et al., 2009. Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. Int. J. Parasitol. 39, 1595–1601.

Nath, G., Choudhury, A., Shukla, B.N., Singh, T.B., Reddy, D.C., 1999. Significance of *Cryptosporidium* in acute diarrhoea in North-Eastern India. J. Med. Microbiol. 48, 523–526.

Nath, J., Ghosh, S.K., Singha, B., Paul, J., 2015a. Molecular epidemiology of amoebiasis: a cross-sectional study among North East Indian population. PLoS Negl. Trop. Dis. 9, e0004225.

Nath, J., Hussain, G., Singha, B., Paul, J., Ghosh, S.K., 2015b. Burden of major diarrheagenic protozoan parasitic co-infection among amoebic dysentery cases from North East India: a case report. Parasitology 142, 1318–1325.

Parija, S.C., Mandal, J., Ponnambath, D.K., et al., 2014. Laboratory methods of identification of *Entamoeba histolytica* and its differentiation from look-alike *Entamoeba* spp. Trop. Parasitol. 4, 90–95.

Parija, S.C., Khairnar, K., 2005. *Entamoeba moshkovskii* and *Entamoeba dispar*-associated infections in pondicherry. India. J. Health Popul. Nutr. 23, 292–295.

Read, C., Walters, J., Robertson, I.D., Thompson, R.C., 2002. Correlation between genotype of *Giardia duodenalis* and diarrhoea. Int. J. Parasitol. 32, 229–231.

Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4, 125–130.

Robertson, L.J., Hermansen, L., Gjerde, B.K., Strand, E., Alvsvag, J.O., Langeland, N., 2006. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl. Environ. Microbiol. 72, 2212–2217.

Ryan, U., Caccio, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956.

Salzer, J.S., Rwego, I.B., Goldberg, T.L., Kuhlenschmidt, M.S., Gillespie, T.R., 2007. *Giardia* sp and *Cryptosporidium* sp infections in primates in fragmented and undisturbed forest in western Uganda. J. Parasitol. 93, 439–440.

Saraswat, R., Sinha, A., Radhakrishna, S., 2015. A god becomes a pest? Human-rhesus macaque interactions in Himachal Pradesh, northern India. Eur. J. Wildl. Res. 61, 435–443.

Shirley, D.A., Moonah, S.N., Kotloff, K.L., 2012. Burden of disease from cryptosporidiosis. Curr. Opin. Infect. Dis. 25, 555–563.

Singh, B.B., Gajadhar, A.A., 2014. Role of India's wildlife in the emergence and re-emergence of zoonotic pathogens, risk factors and public health implications. Acta Trop. 138, 67–77.

Sommer, M.F., Beck, R., Ionita, M., Stefanovska, J., Vasic, A., Zdravkovic, N., et al., 2015. Multilocus sequence typing of canine *Giardia duodenalis* from South Eastern European countries. Parasitol. Res. 114, 2165–2174.

Sow, S.O., Muhsen, K., Nasrin, D., Blackwelder, W.C., Wu, Y., Farag, T.H., et al., 2016. The burden of *Cryptosporidium* diarrheal disease among children < 24 months of age in moderate/high mortality regions of Sub-Saharan Africa and South Asia, utilizing data from the Global Enteric Multicenter Study (GEMS). PLoS Negl. Trop. Dis. 10, e0004729.

Sprong, H., Caccio, S.M., van der Giessen, J.W.B., 2009. Identification of zoonotic genotypes of *Giardia duodenalis*. PLoS Negl. Trop. Dis. 3, e558.

Sricharern, W., Inpankaew, T., Keawmongkol, S., Supanam, J., Stich, R.W., Jittapalapong, S., 2016. Molecular detection and prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. among long-tailed macaques (*Macaca*

fascicularis) in Thailand. Infect. Genet. Evol. 40, 310–314.
Stanley Jr., S.L., 2003. Amoebiasis. Lancet 361, 1025–1034.
Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., et al., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452.
Tachibana, H., Yanagi, T., Akatsuka, A., Kobayash, S., Kanbara, H., Tsutsumi, V., 2009. Isolation and characterization of a potentially virulent species *Entamoeba nuttalli* from captive Japanese macaques. Parasitology 136, 1169–1177.
Tachibana, H., Yanagi, T., Lama, C., Pandey, K., Feng, M., Kobayashi, S., et al., 2013. Prevalence of *Entamoeba nuttalli* infection in wild rhesus macaques in Nepal and characterization of the parasite isolates. Parasitol. Int. 62, 230–235.
Thompson, R.C.A., Smith, A., 2011. Zoonotic enteric protozoa. Vet. Parasitol. 182, 70–78.
Verweij, J.J., Laeijendecker, D., Brienen, E.A., van Lieshout, L., Polderman, A.M., 2003. Detection and identification of *Entamoeba* species in stool samples by a reverse line hybridization assay. J. Clin. Microbiol. 41, 5041–5045.
Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., et al., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578–1583.
Ye, J., Xiao, L., Li, J., Huang, W., Amer, S.E., Guo, Y., et al., 2014. Occurrence of human-pathogenic *Enterocytozoon bieneusi, Giardia duodenalis* and *Cryptosporidium* genotypes in laboratory macaques in Guangxi, China. Parasitol. Int. 63, 132–137.
Ye, J., Xiao, L., Ma, J., Guo, M., Liu, L., Feng, Y., 2012. Anthroponotic enteric parasites in monkeys in Public Park, China. Emerg. Infect. Dis. 18, 1640–1643.

Paper 3

Phylogenetic analysis of *Giardia duodenalis* sequences in primates: evidence of potential zoonotic and anthropozoonotic transmission

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Abstract (Max 350 words)

Background: *Giardia duodenalis*, a protozoan parasite, colonizes the gastrointestinal tract of a wide range of hosts. Genotypically, it is classified into eight different Assemblages and beyond that into more ill-defined sub-Assemblages. Isolates belonging to Assemblages A and B can be found in both humans and non-human primates (NHP), but the zoonotic and anthropozoonotic potential is unknown. The aim of this article was to use publicly available genotyping data to investigate the relatedness of human and NHP *Giardia* isolates in order to evaluate the potential for zoonotic transmission and evaluate the usefulness of the current taxonomic classification.

Results: Our final data set consisted of 165 isolates, 111 from NHP and 54 from humans. Sequence data consisted of the four commonly sequenced loci: SSU, *tpi*, *gdh*, and *bg*. Assemblages were well defined, but sub-Assemblages across Assemblage B were not resolved. Although sub-Assemblages AI and AII were resolved, the terms were not found to capture any useful molecular or host/deme properties. NHP isolates were scattered among human isolates across Assemblages A and B, and were even found in Assemblage E. We evaluated the relative merit of the four genes for use in genotyping studies. The *tpi*, *gdh* and *bg* genes gave relatively congruent tree topologies, but the SSU gene did not even resolve Assemblages correctly.

Conclusions: There does not appear to be any molecular distinction between human and NHP *Giardia* isolates across these molecular markers. The risk for zoonotic and anthropozoonotic transmission of Assemblage A and B isolates must therefore be viewed as present, irrespective of sub-Assemblage classification. Future genotyping efforts of *Giardia* should aim for multilocus or whole-genome approaches and, in particular, avoid sequencing of the SSU gene as the sole marker.

Keywords (3-10): disease, protozoa, nonhuman primate, wildlife, monkey, ape, *Giardia*, genotyping, phylogeny

Background

Giardia duodenalis is an intestinal protozoan parasite capable of causing gastrointestinal disease in a range of vertebrate hosts. Transmission occurs via the faecal-oral route, either through direct ingestion, or through contaminated food and water. Giardiasis is a significant cause of human morbidity globally, affecting over 200 million people in Asia, Africa, and Latin America, as well as being responsible for large-scale human waterborne outbreaks in the developed world [1, 2]. Consequences of infection vary from subclinical carriage, to gastrointestinal symptoms, to chronic growth stunting [3]. Prolonged symptoms and / or post-infectious sequelae may occur, and treatment may be problematic [3]. Although *G. duodenalis* has been reported to infect a wide range of wildlife species, little information is available on the clinical significance of infection on the individual, nor whether it may impact wildlife conservation.

Giardia duodenalis is considered to exist as a species complex that is comprised of at least eight distinct genetic groups, referred to as Assemblages A to H, based on their protein and DNA polymorphisms. It has been suggested that the differences between some of these genetic groups are sufficient to consider them unique species [4, 5]. This article, however, uses the more widely accepted terminology of Assemblages [2]. Assemblages A and B both have wide host ranges, infecting humans and a range of animal species, and are therefore considered to have zoonotic potential. In contrast, Assemblages C to H are considered to be more host specific, infecting predominantly canids (C and D), some ungulates (E), felids (F), rodents (G), and pinnipeds (H) [2]. In surveys that attempt to determine the prevalences of different *G. duodenalis* Assemblages in particular host populations, isolates are often assigned to one or other of these Assemblages based only on the sequences derived from a single gene locus, or part thereof, representing a very small fragment of that isolate's genome. Some isolates may also be assigned to different Assemblages depending on which gene is sequenced [6]. As more sequence data become available, some researchers are now describing isolates according to intra-Assemblage genetic groups, with certain of these sub-Assemblages apparently showing unique infection patterns for particular hosts e.g. Assemblage AIII not infecting humans [6]. In the

light of the low typing resolution using single loci, this indicates that multi-locus genotyping, or, preferentially, whole genome sequencing [7] may be better suited when attempting to investigate host-*Giardia* relationships or to reach conclusions on potential transmission pathways.

The primates are a diverse order of mammals found throughout the world, to which we, *Homo sapiens*, belong. Nonhuman primate (NHP) species are under pressure as the forces of expanding human populations, deforestation, habitat fragmentation and the commercial bush meat industry threaten their conservation. The close taxonomic relationship between humans and NHPs facilitates the transmission of pathogens between them, and the forces listed increase the chances of transmission events occurring by drive the overlap between habitats, resulting in the exposure of humans and NHP to each other's pathogens. In our opinion, understanding the potential zoonotic and anthropozoonotic spread of different pathogens between humans and NHPs is imperative for public health, as well as for NHP conservation.

The aim of the study described here was to use the available genetic sequences from *G. duodenalis* isolates from NHPs to determine whether there is evidence for host–specificity within certain taxonomic groups. Additionally, by comparing *G. duodenalis* isolates from NHPs and humans, we aim to identify whether there is molecular evidence for zoonotic or anthropozoonotic transmission. Finally, we aimed to test the four most commonly sequenced gene loci for their relative ability to resolve phylogenetic relationships, and whether the sub-Assemblage classification is supported by the available molecular data.

Methods

GenBank survey

Giardia duodenalis sequences obtained from isolates from NHP were identified from the GenBank database by searching for "Giardia" and the different genera within the order Primates e.g. "Giardia Macaca", or "Giardia Cheirogaleus". In total, 76 searches were performed for 76 different NHP genera. Gene sequences that were shorter than 100 bp or longer than 1100 bp were excluded. Only

sequences from the small subunit rRNA (SSU), triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*), and beta-giardin (*bg*) genes were included for analysis. Sequences from isolates described as mixed infections in the original research article were excluded (e.g., Isolate SQ694, Accession Number: FJ890962 and FJ890966). Isolates for which different Assemblages were apparently identified from sequences at different genetic loci, but where the original research article did not report mixed infection, were included (e.g., Isolate RC0458, Accession Number: GQ502964 and GQ502999).

Human-derived isolates were recruited gene-wise by searching on GenBank for "Giardia Homo". Sample isolates that did not report sequences from at least three out of the following four genes were excluded: SSU, *tpi*, *gdh*, and *bg*. Gene sequences that were shorter than 100 bp or longer than 1100 bp were excluded. Finally, the full-length gene sequences of reference sequences WB (human, Assemblage A), GS (human, Assemblage B), DH (human, Assemblage A2) and P15 (pig, Assemblage E) [8-10], for which complete and draft whole genomes are available from www.giardiadb.org, were included to guide multiple sequence alignments and to contribute leaves with high-confidence Assemblage status in the phylogenetic tree. A full list of isolates with accession numbers can be found in Table 1.

Sequence alignment

Sequences were parsed individually by gene, reverse complemented if necessary, and *in silico* end-repaired with ambiguous characters. Multiple sequence alignments were created individually for each gene using MAFFT v7.305b [11], with the gap open penalty increased to 50 to prevent splitting mid-sequence; without the latter detail, the sequence alignments would sometimes favour alignment of non-homologous sites. Six samples for which only SSU information was available were excluded at this stage due to poor alignment. Sequence alignments were then concatenated into a single multi-gene alignment (hereafter referred to as the global alignment) of length 2761 bp. Isolates that did not have at least 200 non-gap sites in the global alignment were excluded from further analysis.

Phylogenetic tree construction and annotation

Sequence alignments were visually inspected in SeaView v4.4.2 [12] and obvious misalignments were manually curated using Aliview 1.18 [13]. Maximum-likelihood phylogenetic trees (for individual gene and global alignments) were created using IQ-TREE. [14, 15]. We allowed for automatic model selection with free rate heterogeneity, and performed 1000 iterations of the ultrafast bootstrap [16]. In the global alignment, we allowed each locus partition to evolve under different, edge-unlinked substitution models [17]. The perturbation strength for nearest neighbour interchange (NNI) was set to 0.5, and was set to stop after 100 unsuccessful iterations. Trees were annotated using Interactive Tree Of Life (ITOL), using metadata from the Genbank files and published papers (if applicable) [18].

Testing for phylogenetic incongruence between individual gene trees and global trees

Tanglegrams were created between the global alignment tree and individual loci trees using Dendroscope v3.4.0 [19]. The congruency index were calculated by a web-based tool available at http://max2.ese.u-psud.fr/bases/upresa/pages/devienne/index.htm [20].

Results

Sequences from 111 isolates of *G. duodenalis* from NHP hosts from GenBank were included in our analyses. Nonhuman primates were infected by *G. duodenalis* Assemblages A, B, and E in 24 % (26/111), 72 % (79/111), and 4 % (5/111) of the isolates, respectively. Our phylogenetic analyses clearly distinguished Assemblages A, B and E, with Assemblages A and E more closely related to each other than to Assemblage B (Fig 1).

Within Assemblage A (Fig. 2), sub-Assemblages AI and AII were well resolved as monophyletic sister taxa, and the average distance between AI isolates and AII isolates was found to be in the order of 1/10 of the average distance between an Assemblage A and an Assemblage E isolate. However, the phylogenetic distances within these sub-Assemblages were much larger than the distance between them. Within Assemblage B (Fig. 3), a number of sub-Assemblage classifications are reported, but

very few isolates have the same annotation. In fact, the only two with the same annotation are two Chinese BII isolates, one from *Macaca mulatta* and one from *Macaca fascicularis*, and these do not cluster together at all. From a molecular distance perspective, there does not appear to be any phylogenetic evidence, across either Assemblage A or B, for a simple sub-Assemblage classification system. Even reference isolates WB and DH, representing AI and AII respectively, differ at only 18 sites across the full-length alignment of the four typing genes (0, 3, 13 and 2 differences in SSU, *tpi*, *gdh* and *bg*, respectively), averaging one nucleotide difference in every 150.

Visual inspection of the phylogenetic trees (Figs. 2 and 3) suggests that there may be some degree of clustering based on geographical location and host species / group. However, this trend disappears when isolates from the same study are collapsed, suggesting that true geographic / host population structuring does not occur.

Congruency between gene trees and the global tree

In order to evaluate the relative merit of each locus sequenced, we compared the phylogenetic relationship inferred from individual loci to that resulting from the global alignment of all four using tanglegrams, as can be seen in supplementary figures S1-S4. We also quantified this topological congruency by calculating the incongruency index [20]. These results strongly favour a multi-locus approach when characterising *Giardia* isolates based on sequence data. The lowest congruence is seen for the SSU gene tree. With an Icong of 1.094, this tree is no more related to the global tree than would be expected by chance alone (*i.e.* more than 5 % of randomly generated tree topologies are more closely related to the global tree than the SSU tree). The *tpi*, *gdh*, and *bg* gene trees are all congruent with the global tree, as measured by the incongruency index (See Table 2). Note, however, that the null hypothesis of this test is that the two trees are not more congruent than pairs of randomly generated trees, and as such is not a very strict test for testing whether two topologies are similar. It is not clear if the slightly different topologies between the *tpi*, *gdh* and *bg* trees are a result of differing evolutionary histories or the differences are due to random error.

Discussion

Giardia Assemblages in nonhuman primates

Phylogenetic clustering of Assemblages A, B, and E was similar to that reported previously [2, 21]. The close clustering of A and E, compared with B and E, is supported by comparative studies of whole genomes from Assemblage A, B, and E reference isolates, for which a higher degree of nucleotide and protein identity between A and E (90 / 87 %), compared with B and E (81 / 77 %), has been demonstrated [22].

Assemblages A and B were the most common Assemblages in NHPs, representing 97 % of all isolates. This is similar to the situation in humans where the global prevalence of Assemblage A and B together is 96 % (39 % Assemblage A and 57% Assemblage B) [2]. The difference between the distribution of Assemblage A and B among human and NHP isolates may be that NHP isolates frequently represent the results of non-selective surveys, whereas studies of human *Giardia* isolates often, but not exclusively, select for symptomatic infections. However, the evidence for differences in the virulence between Assemblages A and B is somewhat conflicting [23, 24].

Assemblage E is generally considered to infect only ungulates, however Assemblage E infection in humans [25, 26] and also in NHP [27, 28] has recently been reported. The Assemblage E isolates included in our analyses, excluding the reference isolate, represent 4 different isolates sequenced at all 4 gene loci, and were collected directly from the rectum of each NHP host, suggesting contamination or misidentification are unlikely [28]. However, they are all derived from a single study from a single geographical location, and therefore may indicate a particular set of circumstances. Although whole genome sequencing studies have identified Assemblage-specific and isolate-specific genes [22], indicating host adaptation, these data suggest that this Assemblage may be less host-specific than previously reported, and if host-specific sub-Assemblages exist within E these are probably difficult to distinguish at the four most commonly sequenced molecular markers..

Host-parasite co-evolution

The lack of phylogenetic clustering of *G. duodenalis* isolates from NHPs compared with isolates from humans suggests that there is no hard genetic evidence that isolates from these two host groups differ

from each other. Although there probably are locally adapted biotypes in vicariate populations, the present article provides supportive evidence for a general zoonotic / anthropozoonotic potential of these isolates. This is logical, as, from a phylogenetic perspective, humans are no more distinct from other primates than other NHP are from each other. For example, in terms of evolutionary distance, lemurs and macaques are much more distinct than macaques and humans [29].

Likewise, no genetic clustering was observed for *Giardia* isolates from different groups of NHP hosts when compared at the suborder / superfamily level; apes, old world monkeys, new world monkeys, prosimians. This suggests that interspecies transmission between these hosts is possible, and that if host adaptation has occurred since the primate host lineages separated it has since been washed out by subsequent gene flow. Of course, it should be emphasised that although these molecular data indicate the potential for zoonotic / anthropozoonotic transmission, zoonotic risk is impacted by a range of non-pathogen-related factors, including both human/animal, environment and the interaction between these, such as for example host ranges, individual host susceptibility under various climates, and shared food/water resources [30].

Mixed infections

It seems likely that the literature underestimates the frequency of mixed *Giardia* infections, as most molecular data from *Giardia* isolates are based on the use of Assemblage non-specific primers. These favour the amplification of the more prevalent / dominant Assemblage, and minor or underlying infections with another Assemblage may not be detected [31]. Mixed infections have been shown to be common in a range of host species, including NHP [6, 32]. This indicates the importance of a multi-locus sequencing approach and the use of Assemblage-specific PCRs in prevalence studies, or alternatively whole-genome sequencing with subsequent deconvolution of the mixed infections.

Sub-Assemblage grouping

Nomenclature and taxonomy within *G. duodenalis* remain debated issues within the literature. Rules or guidelines on the establishment of new sub-Assemblages do not exist. Many researchers apparently report the Assemblage of an isolate that they have sequenced based on the results of a simple BLAST

search, and report their isolate as being of the same Assemblage as the most similar isolate in the database. Although this is perhaps acceptable for identical sequences, clearly, if there is a 1% genetic difference in each consecutively reported (sub-) Assemblage, or an Assemblage identity is based on a very short sequence length, then this database can rapidly contain isolates with considerable genetic variability that are labelled as the same (sub-) Assemblage. We suggest that a more robust system would use comparisons with internationally recognised reference strains for each (sub-) Assemblage, with isolates classified according to their similarity to these reference strains.

Sub-Assemblage groups AI / AII are reported as being more robust across commonly examined gene loci [21]. Sequences in our analyses grouped according to their reported sub-Assemblage, AI or AII. However, clusters within these sub-Assemblages had greater genetic distances between them than occur between the AI and AII groups, casting further doubt on the validity of this classification system. The Assemblage A cluster (Accession Numbers: JQ837803 to JQ837807) obtained from *Giardia* from *Gorilla gorilla* in the Central African Republic could, for example, represent a putative sub-Assemblage, but since these only had sequences from the *gdh* gene, the actual distance to other sub-Assemblages is uncertain and might be over- or underestimated. However, the same cannot be said for the cluster (Accession Numbers: KT334254, KT334256, KT334258, KT334259, KT334260) of Brazilian AII *Giardia* isolates from *Homo sapiens*, for which sequences from *tpi*, *gdh* and *bg* are available, which are quite distinct from other AII isolates. If molecular distance is used to define sub-Assemblages, then these isolates should clearly belong in their own sub-Assemblage. Obfuscating the picture even further, one Chinese *Macaca fascicularis* isolate (Accession Number: KC441075), reportedly of sub-Assemblage AV, clustered relatively close to AI isolates, including reference isolate WB. It seems, in fact, as if sub-Assemblage classification corresponds weakly, or not at all, to molecular distance across these commonly used typing genes.

Assemblage B sequences from NHPs showed a larger degree of genetic variability than Assemblage A or E. This is consistent with previous findings, and has been attributed to the greater degree of genetic variability within Assemblage B in general [6]. Indeed, allelic sequence heterozygosity even occurs from single Assemblage B trophozoites [33], creating ambiguous nucleotides within the

generated sequences. This has led to a wide range of reported sub-Assemblages within Assemblage B, highlighting flaws in some of the proposed nomenclature [34] . We find no genetic clustering that would support a simple sub-Assemblage classification system within Assemblage B, and advise against creating further sub-Assemblage groups, particularly when based on minor genetic differences. The inadequacy of criteria for naming sub-Assemblages and the problem of creating sub-Assemblages based solely on a few nucleotide polymorphisms has previously been noted [1]. This illustrates the futility of believing that relevant typing information may be gained from short sequences.

Methodological considerations

Although large amounts of missing data can distort phylogenetic relationships, the phylogenetic trees described here are moderately robust, even if the exact branching order in short clades is in some cases unclear, and some branches have relatively low (<50%) bootstrap support. In particular, samples with only short stretches of SSU sequences are vulnerable to a bootstrap approach, since the probability of sampling a segregating site becomes very low. Note, however, that in general large data gaps perturbs, but does not downright preclude, phylogenetic inference, [35] and the detrimental effects of missing data from multi-locus sequence alignments can be mitigated by partitioned analysis [36].

Our results clearly favour a multi-locus approach to *Giardia* typing. In particular, the SSU gene alone performed poorly in recreating the presumed phylogenetic relationship of the samples. In fact SSU by itself did not even group isolates correctly by Assemblage. It is unknown whether this is due to low phylogenetic resolution from the SSU gene (due to a low number of segregating sites and possible homoplasies), or if the SSU gene in this data set has a different evolutionary history from that of the *tpi*, *gdh* and *bg* genes. Since the SSU tree did not group isolates according to their annotated Assemblage, this latter possibility would presumably point to some type of non-vertical inheritance, such as hybridization, recombination, gene duplication or loss or horizontal gene transfer. In this respect, it is perhaps worth mentioning that some authors have discussed the concept of sexual

recombination in trophozoites [37]. The usefulness of SSU is made even worse by the fact that it is a multi-copy gene, and it is not clear whether copies are necessarily identical, nor if sequencing results represent a single copy or a mixture.

Conclusion

The available data on *Giardia* in NHPs shows no molecular evidence for host-specificity within different groups of NHPs in the four commonly sequenced genes SSU, *tpi*, *gdh* or *bg*. Furthermore, there is no evidence that *Giardia* isolates from humans are distinct from *Giardia* isolates from NHPs. Thus *Giardia* that infect primates, whether human or not, should be considered to have zoonotic and anthropozoonotic potential. Future genotyping efforts of *Giardia* should favour a multi-locus or whole-genome approach. In particular, sequencing of the SSU gene as the only marker should be discouraged.

List of abbreviations

bg = beta-giardin

gdh = glutamate dehydrogenase

NHP = Non-human primate

SSU = small subunit rRNA

tpi = triose phosphate isomerase

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data was downloaded through GenBank and is available through accession numbers as shown in Table 1.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived study: JJD, OB, LJR, KRT. Collected data: JJD, KRT. Performed analysis: OB. Wrote manuscript: JJD, OB, LJR, KRT. All authors have read and approved the final version of this manuscript.

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Literature cited

1. Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N: **Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004**. *Appl Environ Microbiol* 2006, **72**(3):2212-2217.
2. Feng Y, Xiao L: **Zoonotic potential and molecular epidemiology of Giardia species and giardiasis**. *Clin Microbiol Rev* 2011, **24**(1):110-140.
3. Robertson LJ, Hanevik K, Escobedo AA, Mørch K, Langeland N: **Giardiasis--why do the symptoms sometimes never stop?** *Trends Parasitol* 2010, **26**(2):75-82.
4. Ryan U, Caccio SM: **Zoonotic potential of Giardia**. *Int J Parasitol* 2013, **43**(12-13):943-956.
5. Thompson RC, Ash A: **Molecular epidemiology of Giardia and Cryptosporidium infections**. *Infect Genet Evol* 2016, **40**:315-323.
6. Sprong H, Caccio SM, van der Giessen JWB, Network Z, Partners: **Identification of Zoonotic Genotypes of Giardia duodenalis**. *Plos Neglected Tropical Diseases* 2009, **3**(12):e558.
7. Ankarklev J, Franzen O, Peirasmaki D, Jerlstrom-Hultqvist J, Lebbad M, Andersson J, Andersson B, Svard SG: **Comparative genomic analyses of freshly isolated Giardia intestinalis assemblage A isolates**. *BMC Genomics* 2015, **16**:697.
8. Smith PD, Gillin FD, Kaushal NA, Nash TE: **Antigenic analysis of Giardia lamblia from Afghanistan, Puerto Rico, Ecuador, and Oregon**. *Infect Immun* 1982, **36**(2):714-719.
9. Nash TE, Mccutchan T, Keister D, Dame JB, Conrad JD, Gillin FD: **Restriction-Endonuclease Analysis of DNA from 15 Giardia Isolates Obtained from Humans and Animals**. *Journal of Infectious Diseases* 1985, **152**(1):64-73.

10. Nash TE, Keister DB: **Differences in Excretory-Secretory Products and Surface-Antigens among 19 Isolates of Giardia**. *Journal of Infectious Diseases* 1985, **152**(6):1166-1171.
11. Katoh K, Standley DM: **MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability**. *Molecular Biology and Evolution* 2013, **30**(4):772-780.
12. Gouy M, Guindon S, Gascuel O: **SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building**. *Molecular biology and evolution* 2010, **27**(2):221-224.
13. Larsson A: **AliView: a fast and lightweight alignment viewer and editor for large datasets**. *Bioinformatics* 2014, **30**(22):3276-3278.
14. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ: **IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies**. *Molecular biology and evolution* 2015, **32**(1):268-274.
15. Trifinopoulos J, Nguyen L-T, von Haeseler A, Minh BQ: **W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis**. *Nucleic acids research* 2016:gkw256.
16. Minh BQ, Nguyen MAT, von Haeseler A: **Ultrafast approximation for phylogenetic bootstrap**. *Molecular biology and evolution* 2013:mst024.
17. Chernomor O, von Haeseler A, Minh BQ: **Terrace aware data structure for phylogenomic inference from supermatrices**. *Systematic biology* 2016:syw037.
18. Letunic I, Bork P: **Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees**. *Nucleic acids research* 2016, **44**(W1):W242-W245.
19. Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R: **Dendroscope: An interactive viewer for large phylogenetic trees**. *BMC bioinformatics* 2007, **8**(1):460.
20. de Vienne DM, Giraud T, Martin OC: **A congruence index for testing topological similarity between trees**. *Bioinformatics* 2007, **23**(23):3119-3124.
21. Wielinga CM, Thompson RCA: **Comparative evaluation of Giardia duodenalis sequence data**. *Parasitology* 2007, **134**:1795-1821.
22. Jerlstrom-Hultqvist J, Franzen O, Ankarklev J, Xu F, Nohynkova E, Andersson JO, Svard SG, Andersson B: **Genome analysis and comparative genomics of a Giardia intestinalis assemblage E isolate**. *BMC Genomics* 2010, **11**:543.
23. Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, Svard SG: **Multilocus Genotyping of Human Giardia Isolates Suggests Limited Zoonotic Transmission and Association between Assemblage B and Flatulence in Children**. *Plos Neglected Tropical Diseases* 2011, **5**(8).
24. Haque R, Mondal D, Karim A, Molla IH, Rahim A, Faruque AS, Ahmad N, Kirkpatrick BD, Houpt E, Snider C *et al*: **Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh**. *Clin Infect Dis* 2009, **48**(9):1191-1197.
25. Abdel-Moein KA, Saeed H: **The zoonotic potential of Giardia intestinalis assemblage E in rural settings**. *Parasitol Res* 2016, **115**(8):3197-3202.
26. Fantinatti M, Bello AR, Fernandes O, Da-Cruz AM: **Identification of Giardia lamblia Assemblage E in Humans Points to a New Anthropozoonotic Cycle**. *J Infect Dis* 2016, **214**(8):1256-1259.
27. Johnston AR, Gillespie TR, Rwego IB, McLachlan TLT, Kent AD, Goldberg TL: **Molecular Epidemiology of Cross-Species Giardia duodenalis Transmission in Western Uganda**. *PLoS Negl Trop Dis* 2010, **4**(5):e683.
28. Du SZ, Zhao GH, Shao JF, Fang YQ, Tian GR, Zhang LX, Wang RJ, Wang HY, Qi M, Yu SK: **Cryptosporidium spp., Giardia intestinalis, and Enterocytozoon bieneusi in Captive Non-Human Primates in Qinling Mountains**. *The Korean journal of parasitology* 2015, **53**(4):395-402.

29. Perelman P, Johnson WE, Roos C, Seuánez HN, Horvath JE, Moreira MA, Kessing B, Pontius J, Roelke M, Rumpler Y: **A molecular phylogeny of living primates**. *PLoS Genet* 2011, **7**(3):e1001342.
30. Lal A, Hales S, French N, Baker MG: **Seasonality in human zoonotic enteric diseases: a systematic review**. *PLoS One* 2012, **7**(4):e31883.
31. Benere E, Geurden T, Robertson L, Van Assche T, Cos P, Maes L: **Infectivity of Giardia duodenalis Assemblages A and E for the gerbil and axenisation of duodenal trophozoites**. *Parasitol Int* 2010, **59**(4):634-637.
32. Levecke B, Geldhof P, Claerebout E, Dorny P, Vercammen F, Caccio SM, Vercruysse J, Geurden T: **Molecular characterisation of Giardia duodenalis in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms**. *Int J Parasitol* 2009, **39**(14):1595-1601.
33. Ankarklev J, Svard SG, Lebbad M: **Allelic sequence heterozygosity in single Giardia parasites**. *BMC Microbiol* 2012, **12**:65.
34. Monis PT, Andrews RH, Mayrhofer G, Ey PL: **Genetic diversity within the morphological species Giardia intestinalis and its relationship to host origin**. *Infect Genet Evol* 2003, **3**(1):29-38.
35. Roure B, Baurain D, Philippe H: **Impact of missing data on phylogenies inferred from empirical phylogenomic data sets**. *Molecular biology and evolution* 2013, **30**(1):197-214.
36. Wiens JJ, Morrill MC: **Missing data in phylogenetic analysis: reconciling results from simulations and empirical data**. *Systematic Biology* 2011:syr025.
37. Ankarklev J, Jerlström-Hultqvist J, Ringqvist E, Troell K, Svärd SG: **Behind the smile: cell biology and disease mechanisms of Giardia species**. *Nature Reviews Microbiology* 2010, **8**(6):413-422.
38. Sak B, Petrzelkova KJ, Kvetonova D, Mynarova A, Shutt KA, Pomajbikova K, Kalousova B, Modry D, Benavides J, Todd A *et al*: **Long-term monitoring of microsporidia, Cryptosporidium and Giardia infections in western Lowland Gorillas (Gorilla gorilla gorilla) at different stages of habituation in Dzanga Sangha Protected Areas, Central African Republic**. *PLoS One* 2013, **8**(8):e71840.
39. Ye J, Xiao L, Li J, Huang W, Amer SE, Guo Y, Roellig D, Feng Y: **Occurrence of human-pathogenic Enterocytozoon bieneusi, Giardia duodenalis and Cryptosporidium genotypes in laboratory macaques in Guangxi, China**. *Parasitology international* 2014, **63**(1):132-137.
40. Martinez-Diaz RA, Sansano-Maestre J, Martinez-Herrero Mdel C, Ponce-Gordo F, Gomez-Munoz MT: **Occurrence and genetic characterization of Giardia duodenalis from captive nonhuman primates by multi-locus sequence analysis**. *Parasitology research* 2011, **109**(3):539-544.
41. Ye J, Xiao L, Ma J, Guo M, Liu L, Feng Y: **Anthroponotic enteric parasites in monkeys in public park, China**. *Emerging infectious diseases* 2012, **18**(10):1640-1643.
42. Mynarova A, Foitova I, Kvac M, Kvetonova D, Rost M, Morrogh-Bernard H, Nurcahyo W, Nguyen C, Supriyadi S, Sak B: **Prevalence of Cryptosporidium spp., Enterocytozoon bieneusi, Encephalitozoon spp. and Giardia intestinalis in Wild, Semi-Wild and Captive Orangutans (Pongo abelii and Pongo pygmaeus) on Sumatra and Borneo, Indonesia**. *PLoS One* 2016, **11**(3):e0152771.
43. Itagaki T, Kinoshita S, Aoki M, Itoh N, Saeki H, Sato N, Uetsuki J, Izumiyama S, Yagita K, Endo T: **Genotyping of Giardia intestinalis from domestic and wild animals in Japan using glutamete dehydrogenase gene sequencing**. *Veterinary parasitology* 2005, **133**(4):283-287.
44. Soares RM, de Souza SL, Silveira LH, Funada MR, Richtzenhain LJ, Gennari SM: **Genotyping of potentially zoonotic Giardia duodenalis from exotic and wild animals kept in captivity in Brazil**. *Veterinary parasitology* 2011, **180**(3-4):344-348.
45. Levecke B, Geldhof P, Claerebout E, Dorny P, Vercammen F, Caccio SM, Vercruysse J, Geurden T: **Molecular characterisation of Giardiaduodenalis in captive non-human**

primates reveals mixed assemblage A and B infections and novel polymorphisms. *International journal for parasitology* 2009, **39**(14):1595-1601.

46. Karim MR, Wang R, Yu F, Li T, Dong H, Li D, Zhang L, Li J, Jian F, Zhang S *et al*: **Multi-locus analysis of Giardia duodenalis from nonhuman primates kept in zoos in China: geographical segregation and host-adaptation of assemblage B isolates**. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2015, **30**:82-88.

47. Aguiar JM, Silva SO, Santos VA, Rossa GA, Taniwaki SA, Gregori F, Keid LB, Soares RM: **Multilocus amplification of genomic DNA from single cysts of Giardia duodenalis separated using micromanipulation technique**. *Experimental parasitology* 2015, **157**:84-87.

48. Wang L, Xiao L, Duan L, Ye J, Guo Y, Guo M, Liu L, Feng Y: **Concurrent infections of Giardia duodenalis, Enterocytozoon bieneusi, and Clostridium difficile in children during a cryptosporidiosis outbreak in a pediatric hospital in China**. *PLoS neglected tropical diseases* 2013, **7**(9):e2437.

49. Fahmy HM, El-Serougi AO, El Deeb HK, Hussein HM, Abou-Seri HM, Klotz C, Aebischer T, El Sayed Khalifa Mohamed K: **Giardia duodenalis assemblages in Egyptian children with diarrhea**. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2015, **34**(8):1573-1581.

50. Volotao AC, Junior JC, Grassini C, Peralta JM, Fernandes O: **Genotyping of Giardia duodenalis from Southern Brown Howler Monkeys (Alouatta clamitans) from Brazil**. *Veterinary parasitology* 2008, **158**(1-2):133-137.

51. Abe N, Kimata I, Tokoro M: **Genotyping of Giardia isolates from humans in Japan using the small subunit ribosomal RNA and glutamate dehydrogenase gene sequences**. *Japanese journal of infectious diseases* 2005, **58**(1):57-58.

52. Oliveira-Arbex AP, David EB, Oliveira-Sequeira TC, Bittencourt GN, Guimaraes S: **Genotyping of Giardia duodenalis isolates in asymptomatic children attending daycare centre: evidence of high risk for anthroponotic transmission**. *Epidemiology and infection* 2016, **144**(7):1418-1428.

53. Hogan JN, Miller WA, Cranfield MR, Ramer J, Hassell J, Noheri JB, Conrad PA, Gilardi KV: **Giardia in mountain gorillas (Gorilla beringei beringei), forest buffalo (Syncerus caffer), and domestic cattle in Volcanoes National Park, Rwanda**. *Journal of wildlife diseases* 2014, **50**(1):21-30.

54. Debenham JJ, Atencia R, Midtgaard F, Robertson LJ: **Occurrence of Giardia and Cryptosporidium in captive chimpanzees (Pan troglodytes), mandrills (Mandrillus sphinx) and wild Zanzibar red colobus monkeys (Procolobus kirkii)**. *Journal of medical primatology* 2015, **44**(2):60-65.

55. Sricharern W, Inpankaew T, Keawmongkol S, Supanam J, Stich RW, Jittapalapong S: **Molecular detection and prevalence of Giardia duodenalis and Cryptosporidium spp. among long-tailed macaques (Macaca fascicularis) in Thailand**. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2016, **40**:310-314.

56. Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, Svard SG: **Multilocus genotyping of human Giardia isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children**. *PLoS neglected tropical diseases* 2011, **5**(8):e1262.

57. Lebbad M, Mattsson JG, Christensson B, Ljungstrom B, Backhans A, Andersson JO, Svard SG: **From mouse to moose: multilocus genotyping of Giardia isolates from various animal species**. *Veterinary parasitology* 2010, **168**(3-4):231-239.

58. Suzuki J, Murata R, Kobayashi S, Sadamasu K, Kai A, Takeuchi T: **Risk of human infection with Giardia duodenalis from cats in Japan and genotyping of the isolates to assess the route of infection in cats**. *Parasitology* 2011, **138**(4):493-500.

59. Felsenstein J: **Evolutionary trees from DNA sequences: a maximum likelihood approach**. *Journal of molecular evolution* 1981, **17**(6):368-376.
60. Kimura M: **A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences**. *Journal of molecular evolution* 1980, **16**(2):111-120.
61. Soubrier J, Steel M, Lee MS, Der Sarkissian C, Guindon S, Ho SY, Cooper A: **The influence of rate heterogeneity among sites on the time dependence of molecular rates**. *Molecular biology and evolution* 2012, **29**(11):3345-3358.
62. Tamura K, Nei M: **Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees**. *Molecular biology and evolution* 1993, **10**(3):512-526.

Tables

Table 1 – Isolates included in the current study

Isolate	Host	Country	*SSU*	*tpi*	*gdh*	*bg*	Ref
3662	*Gorilla gorilla*	Central African Republic		JQ837804			[38]
3751	*Gorilla gorilla*	Central African Republic		JQ837805			[38]
3752	*Gorilla gorilla*	Central African Republic		JQ837806			[38]
3773	*Gorilla gorilla*	Central African Republic		JQ837807			
36413	*Macaca fascicularis*	China		KC441077		KC441079	[39]
10CA	*Cercocebus atys*	Spain	HQ61661				[40]
11CN	*Cercopithecus neglectus*	Spain	HQ61661				[40]
14LC	*Lemur catta*	Spain	HQ61661	HQ616626	HQ616623	HQ616630	[40]
15EFM	*Eulemur fulvus mayottensis*	Spain	HQ61661	HQ616627	HQ616624	HQ616631	[40]
18PT	*Pan troglodytes*	Spain	HQ63662				[40]
1HA	*Hapalemur aureus*	Spain	HQ61660				[40]
2864	*Gorilla gorilla*	Central African Republic		JQ837803			[38]
2ER	*Eulemur rufus*	Spain	HQ61660				[40]
34361	*Macaca mulatta*	China		JX000565			[41]
34524	*Macaca mulatta*	China		JX000564			[41]
34538	*Macaca mulatta*	China		JX000563			[41]
34611	*Macaca mulatta*	China		JX000566			[41]
34628	*Macaca mulatta*	China		JX000562			[41]
34641	*Macaca mulatta*	China		JX000567			[41]
36393	*Macaca fascicularis*	China		KC441075			[39]
39395	*Macaca fascicularis*	China		KC441076		KC441078	[39]
3VR	*Varecia rubra*	Spain	HQ61660		HQ616620		[40]
4CH	*Cercopithecus hamlyni*	Spain	HQ61661				[40]
5LC	*Lemur catta*	Spain	HQ61661		HQ616621	HQ616628	[40]
6LC	*Lemur catta*	Spain	HQ61661	HQ616625	HQ616622	HQ616629	[40]
7420-DA70	*Pongo abelli*	Indonesia		KR011753			[42]
7ER	*Eulemur rubriventer*	Spain	HQ61661				[40]
8ML	*Mandrillus leucophaeus*	Spain	HQ61661				[40]
9GG	*Gorilla gorilla*	Spain	HQ61661				[40]
AB199738	*Macaca fuscata*	Japan			AB199738		[43]
Afu01	*Alouatta fusca*	Brazil			HM134193		[44]
Afu02	*Alouatta fusca*	Brazil			HM134199		[44]
Afu05	*Alouatta fusca*	Brazil			HM134194		[44]
Afu06	*Alouatta fusca*	Brazil			HM134195		[44]
Afu07	*Alouatta fusca*	Brazil			HM134192		[44]
Afu08	*Alouatta fusca*	Brazil			HM134196		[44]
Afu09	*Alouatta fusca*	Brazil			HM134197		[44]
Afu10	*Alouatta fusca*	Brazil			HM134198		[44]
Afu40	*Alouatta fusca*	Brazil			HM134203		[44]
Afu45	*Alouatta fusca*	Brazil			HM134204		[44]
Afu46	*Alouatta fusca*	Brazil			HM134205		[44]
Afu92	*Alouatta fusca*	Brazil			HM134206		[44]
Afu93	*Alouatta fusca*	Brazil			HM134207		[44]
Afu95	*Alouatta fusca*	Brazil			HM134208		[44]
Afu96	*Alouatta fusca*	Brazil			HM134209		[44]
Afu97	*Alouatta fusca*	Brazil			HM134210		[44]
Afu99	*Alouatta fusca*	Brazil			HM134211		[44]
AfuCCZa	*Alouatta fusca*	Brazil			HM134201		[44]
AfuCCZb	*Alouatta fusca*	Brazil			HM134202		[44]
Afucemas	*Alouatta fusca*	Brazil			HM134200		[44]
BA1	*Alouatta caraya*	Brazil		JN410841	JN172997		N/A
BA2	*Alouatta fusca*	Brazil		JN410842	JN172998		N/A
BA3	*Alouatta caraya*	Brazil		JN410843	JN172999		N/A
BRUdi2	*Homo sapiens*	Brazil		JQ794879	JQ794888	JX033115	N/A

BW200	*Varecia variegata*	Belgium/Netherlands		FJ890954	FJ890949		[45]
BZ-269	*Lemur catta*	China	KJ888984			KJ888977	[46]
BZ-294	*Chlorocebus sabaeus*	China		KJ888994			[46]
C1	*Homo sapiens*	-		KR046123	KR046111	KR046109	[47]
C1'	*Homo sapiens*	-		KT156714	KT156713	KT156712	[47]
CH1	*Pan troglodytes*	Belgium/Netherlands			FJ890946	FJ890976	[45]
CH-307	*Lemur catta*	China			KJ888982		[46]
CH-314	*Lemur catta*	China		KJ888992			[46]
CH-319	*Lemur catta*	China		KJ888990		KJ888975	[46]
CH37	*Pan troglodytes*	Belgium/Netherlands		FJ890952	FJ890945	FJ890963	[45]
CH7	*Pan troglodytes*	Belgium/Netherlands		FJ890953	FJ890950		[45]
D30	*Homo sapiens*	India		JF918466	JF918511	JF918485	N/A
D33	*Homo sapiens*	India		JF918520	JF918439	JF918487	N/A
D41	*Homo sapiens*	India		JF918469	JF918442	JF918493	N/A
D55	*Homo sapiens*	India		JF918473	JF918445	JF918499	N/A
D60	*Homo sapiens*	India		JF918522	JF918448	JF918500	N/A
DC01	*Homo sapiens*	Brazil		KT334262	KT334245	KT334287	N/A
DC110	*Homo sapiens*	Brazil		KT334258	KT334238	KT334283	N/A
DC111	*Homo sapiens*	Brazil		KT334259	KT334239	KT334284	N/A
DC113	*Homo sapiens*	Brazil		KT334260	KT334240	KT334285	N/A
DC16	*Homo sapiens*	Brazil		KT334254	KT334235	KT334279	N/A
DC35	*Homo sapiens*	Brazil		KT334256	KT334236	KT334282	N/A
DC39	*Homo sapiens*	Brazil		KT334265	KT334248	KT334293	N/A
DC50	*Homo sapiens*	Brazil		KT334271	KT334251	KT334291	N/A
DH	*Homo sapiens*	USA					Giardiadb
ECUST13486	*Homo sapiens*	China		JX898206	JX898211	JX898209	N/A
ECUST1710	*Homo sapiens*	China		JX994247	JX994232	JX994238	[48]
ECUST4064	*Homo sapiens*	China		JX994249	JX994234	JX994239	[48]
ECUST5414	*Homo sapiens*	China		JX994254	JX994236	JX994243	[48]
ECUST981	*Homo sapiens*	China		JX994246	JX994231	JX994240	[48]
F19	*Colobus guereza*	Belgium/Netherlands		FJ890959	FJ890942	FJ890974	[45]
F27	*Colobus guereza*	Belgium/Netherlands		FJ890960		FJ890970	[45]
F28	*Colobus guereza*	Belgium/Netherlands				FJ890973	[45]
F3	*Colobus guereza*	Belgium/Netherlands				FJ890972	[45]
F30	*Colobus guereza*	Belgium/Netherlands				FJ890971	[45]
F4	*Colobus guereza*	Belgium/Netherlands				FJ890969	[45]
G01	*Homo sapiens*	Egypt		KR260582	KR260512	KR260668	[49]
G02	*Homo sapiens*	Egypt		KR260608	KR260533	KR260663	[49]
G028A	*Alouatta clamitans*	Brazil				EU200933	[50]
G03	*Homo sapiens*	Egypt		KR260554	KR260554	KR260624	[49]
G030A	*Alouatta clamitans*	Brazil				EU200934	[50]
G04	*Homo sapiens*	Egypt		KR260594	KR260549	KR260675	[49]
G041A	*Alouatta clamitans*	Brazil				EU200935	[50]
G044A	*Alouatta clamitans*	Brazil				EU200936	[50]
G047A	*Alouatta clamitans*	Brazil				EU200937	[50]
G06	*Homo sapiens*	Egypt		KR260593	KR260545	KR260666	[49]
G07	*Homo sapiens*	Egypt		KR260598	KR260507	KR260669	[49]
G08	*Homo sapiens*	Egypt		KR260585	KR260526	KR260673	[49]
G09	*Homo sapiens*	Egypt		KR260596	KR260521	KR260648	[49]
G10	*Homo sapiens*	Egypt		KR260578	KR260548	KR260639	[49]
G11	*Homo sapiens*	Egypt		KR260592	KR260550	KR260647	[49]
G12	*Homo sapiens*	Egypt		KR260552	KR260492	KR260630	[49]
G15	*Homo sapiens*	Egypt		KR260568	KR260534	KR260656	[49]
G17	*Homo sapiens*	Egypt		KR260560	KR260495	KR260618	[49]
G20	*Homo sapiens*	Egypt		KR260607	KR260514	KR260654	[49]
G23	*Homo sapiens*	Egypt		KR260563	KR260482	KR260623	[49]
G25	*Homo sapiens*	Egypt		KR260588	KR260543	KR260664	[49]
G29	*Homo sapiens*	Egypt		KR260558	KR260493	KR260626	[49]
G30	*Homo sapiens*	Egypt		KR260556	KR260489	KR260627	[49]
G34	*Homo sapiens*	Egypt		KR260575	KR260537	KR260658	[49]
GH-125	*Homo sapiens*	Japan	AB19521	AB516350	AB195222		[51]

GH-126	*Homo sapiens*	Japan	AB19522	AB516351	AB195223		[51]
GH-135	*Homo sapiens*	Japan	AB19522	AB516532	AB195224		[51]
GS	*Homo sapiens*	USA					Giardiadb
HM110A	*Homo sapiens*	Brazil		KT334275	KT334243	KT334292	[52]
KG08	*Gorilla beringei beringei*	Rwanda			JX839875	JX839880	[53]
KG18	*Gorilla beringei beringei*	Rwanda			JX839876	JX839881	[53]
KG28	*Gorilla beringei beringei*	Rwanda			JX839877	JX839882	[53]
KG40	*Gorilla beringei beringei*	Rwanda			JX839878	JX839883	[53]
KG41	*Gorilla beringei beringei*	Rwanda			JX839873	JX839884	[53]
KG42	*Gorilla beringei beringei*	Rwanda			JX839879	JX839885	[53]
KM102527	*Pan troglodytes*	Republic of the Congo				KM10252	[54]
M	*Papio hamadrryas*	Belgium/Netherlands		FJ890957	FJ890943	FJ890968	[45]
M2	*Macaca mulatta*	China	KJ917607	KJ917619	KJ917615	KJ917611	[28]
M24	*Macaca mulatta*	China	KJ917610	KJ917621	KJ917617	KJ917613	[28]
M5	*Macaca mulatta*	China	KJ917608	KJ917620	KJ917616	KJ917612	[28]
ML115	*Macaca fascicularis*	Thailand	KP772613	KP772626	KP772619		[55]
ML141	*Macaca fascicularis*	Thailand	KP772614	KP772627	KP772620		[55]
ML145	*Macaca fascicularis*	Thailand	KP772615	KP772628	KP772621		[55]
ML148	*Macaca fascicularis*	Thailand	KP772616				[55]
ML152	*Macaca fascicularis*	Thailand	KP772617	KP772629	KP772622		[55]
ML43	*Macaca fascicularis*	Thailand	KP772608	KP772624	KP772618		[55]
ML54	*Macaca fascicularis*	Thailand	KP716565		KP772623		[55]
ML62	*Macaca fascicularis*	Thailand	KP772609	KP772625			[55]
ND05	*Homo sapiens*	India		JF918475	JF918517	JF918502	N/A
P15	*Sus scrofa*	Czech Republic					Giardiadb
R21	*Lemur catta*	Belgium/Netherlands				FJ890964	[45]
R352	*Lemur catta*	Belgium/Netherlands		FJ890956	FJ890948	FJ890977	[45]
R763	*Lemur catta*	Belgium/Netherlands		FJ890955	FJ890947	FJ890967	[45]
RC0430	*Procolobus tephrosceles*	Uganda	GQ50301		GQ502965		[27]
RC0431	*Procolobus tephrosceles*	Uganda	GQ50301		GQ502966		[27]
RC0432	*Procolobus tephrosceles*	Uganda	GQ50299		GQ502967		[27]
RC0458	*Procolobus tephrosceles*	Uganda	GQ50299		GQ502964		[27]
SH-12	*Macaca mulatta*	China		KJ888986			[46]
SH-19	*Macaca mulatta*	China				KJ888976	[46]
SH-66	*Macaca mulatta*	China		KJ888989			[46]
SH-67	*Lemur catta*	China		KJ888985		KJ888980	[46]
SI2	*Ateles fusciceps*	Belgium/Netherlands		FJ890958	FJ890944	FJ890965	[45]
SQ678	*Saimiri boliviensis*	Belgium/Netherlands		FJ890961	FJ890951	FJ890978	[45]
SQ694	*Saimiri boliviensis*	Belgium/Netherlands		FJ890962		FJ890966	[45]
SW-488	*Lemur catta*	China				KJ888979	[46]
Sweh001	*Homo sapiens*	Sweden		HM14070	HM13688	HM16520	[56]
Sweh047	*Homo sapiens*	Sweden		HM14071	HM13689	HM16521	[56]
Sweh107	*Homo sapiens*	Sweden		HM14071	HM13689	HM16522	[56]
Sweh166	*Homo sapiens*	Sweden		GQ329677	GQ329674	GQ329671	[56]
Sweh173	*Homo sapiens*	Sweden		GQ329678	GQ329675	GQ329672	[56]
Sweh178	*Homo sapiens*	Sweden		GQ329679	GQ329676	GQ329673	[56]
Swemon050	*Callithrix pygmaea*	Sweden				EU769208	[57]
Swemon088	*Saguinus oedipus*	Sweden				EU769207	[57]
Swemon200	*Cercopithecus sp.*	Sweden		EU781015	EU769226	EU769211	[57]
SZ-425	*Trachypithecus francoisi*	China		KJ888991			[46]
WB	*Homo sapiens*	USA					Giardiadb
WS1	*Pithecia pithecia*	Japan	AB56936	AB569405	AB569387		[58]
X2	*Saimiri sciureus*	China	KJ917609	KJ917622	KJ917618	KJ917614	[28]
YARTL01	*Lemur catta*	China		KM21179		KM21179	N/A

N/A = Data not published outside of GenBank. Giardiadb = Downloaded directly through Giardiadb.

Table 2 – Tree substitution models and congruency/topology tests.

Data	Subst. mod.	Δ ln L	Icong	Icong *p*
Global	Partitioned*	0		
SSU	F81 + I [59]	733.38	1.09	0.30
tpi	K80 + 2R [60, 61]	292.86	2.48	2.5E-15
gdh	TN + 2R [62]	875.70	2.30	6.0E-14
bg	TN + 2R	605.46	1.90	1.1E-8

Subst. mod. = DNA substitution model. Δ ln L = Difference in log-likelihood between best (global) model and current. Icong = Index of congruence, as described in Vienne et al. Tested pairwise between current and best (global) model. Icong p = Icong associated p-value (low means more congruent than by chance). * = In global model, each partition could have its own substitution model (equal to that of constituent partitions below).

Figure legends

Fig 1 – Phylogenetic overview of Assemblages, showing relative distance between Assemblages as well as sister taxa AI and AII.

Fig 2 – Phylogenetic tree of *Giardia duodenalis* Assemblages A and E. Branches are coloured according to the ultrafast bootstrap value, where green corresponds to higher and red to lower values. Leaves carry the first accession number in the concatenation of genes, except in the case of reference strains WB, DH and P15. Name = Name of the sample (where available); Species = Species from which *Giardia* isolate was sampled; Group = Whether the host species was an ape (including *Homo*), old / new world monkey, or prosimian. Genes = Indicator of gene sequence availability. Black indicates partial or complete availability. Country = Country in which isolate was sampled. Reported Assemblage = The Assemblage (in some cases sub-Assemblage) that was reported in the associated GenBank file.

Fig 3 – Phylogenetic tree of *Giardia duodenalis* Assemblage B. Branches are coloured according to the ultrafast bootstrap value, where green corresponds to higher and red to lower values. Leaves carry the first accession number in the concatenation of genes, except in the case of reference strain GS. Name = Name of the sample (where available); Species = Species from which *Giardia* isolate was sampled; Group = Whether the host species was an ape (including *Homo*), old / new world monkey, or prosimian. Genes = Indicator of gene sequence availability. Black indicates partial or complete

availability. Country = Country in which isolate was sampled. Reported Assemblage = The Assemblage (in some cases sub-Assemblage) that was reported in the associated GenBank file.

Figure S1 – Tanglegram of phylogenetic tree from global alignment versus SSU only. The SSU tree was not congruent with the global alignment tree, and grouped strains from different Assemblages together. It is unknown whether this is due to poor phylogenetic resolution from the SSU locus due to a low number of segregating sites, or if the evolutionary history of the SSU gene in these strains have been subject to horizontal inheritance such as hybridization or recombination. Taxa are coloured according to Assemblage: Red = Assemblage E. Blue = Assemblage A. Green = Assemblage B.

Figure S2 – Tanglegram of phylogenetic tree from global alignment versus *tpi* only. The *tpi* tree is relatively congruent with the global alignment tree. Some polytomies have been collapsed and for readability not all strain names are shown, however all tangle edges are included.

Figure S3 – Tanglegram of phylogenetic tree from global alignment versus *gdh* only. The *gdh* tree is relatively congruent with the global alignment tree. Some polytomies have been collapsed and for readability not all strain names are shown, however all tangle edges are included.

Figure S2 – Tanglegram of phylogenetic tree from global alignment versus *tpi* only. The *tpi* tree is relatively congruent with the global alignment tree. Some polytomies have been collapsed and for readability not all strain names are shown, however all tangle edges are included.

Figure 1

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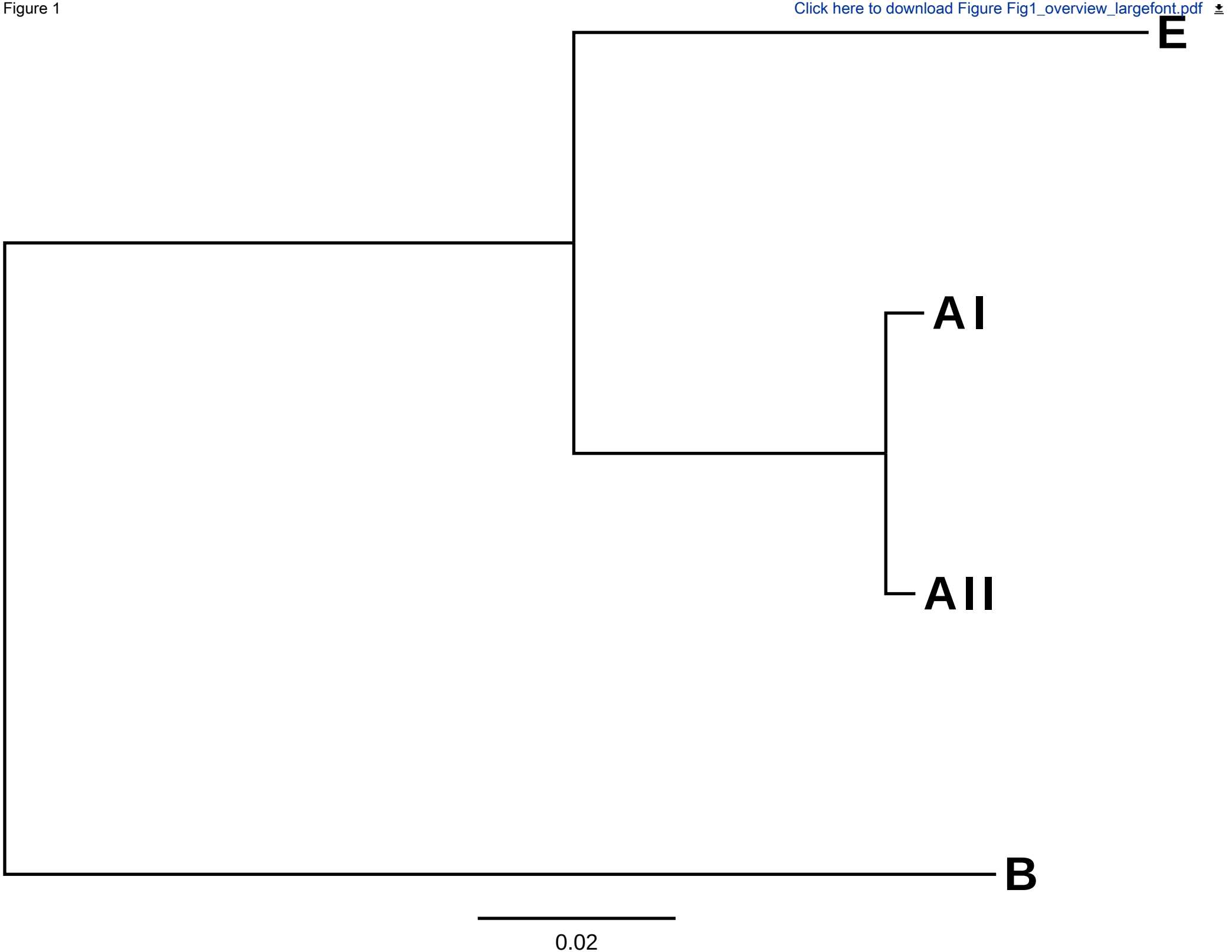


Figure 2

Click here to download Figure A_and_E_allbranchesintact.pdf

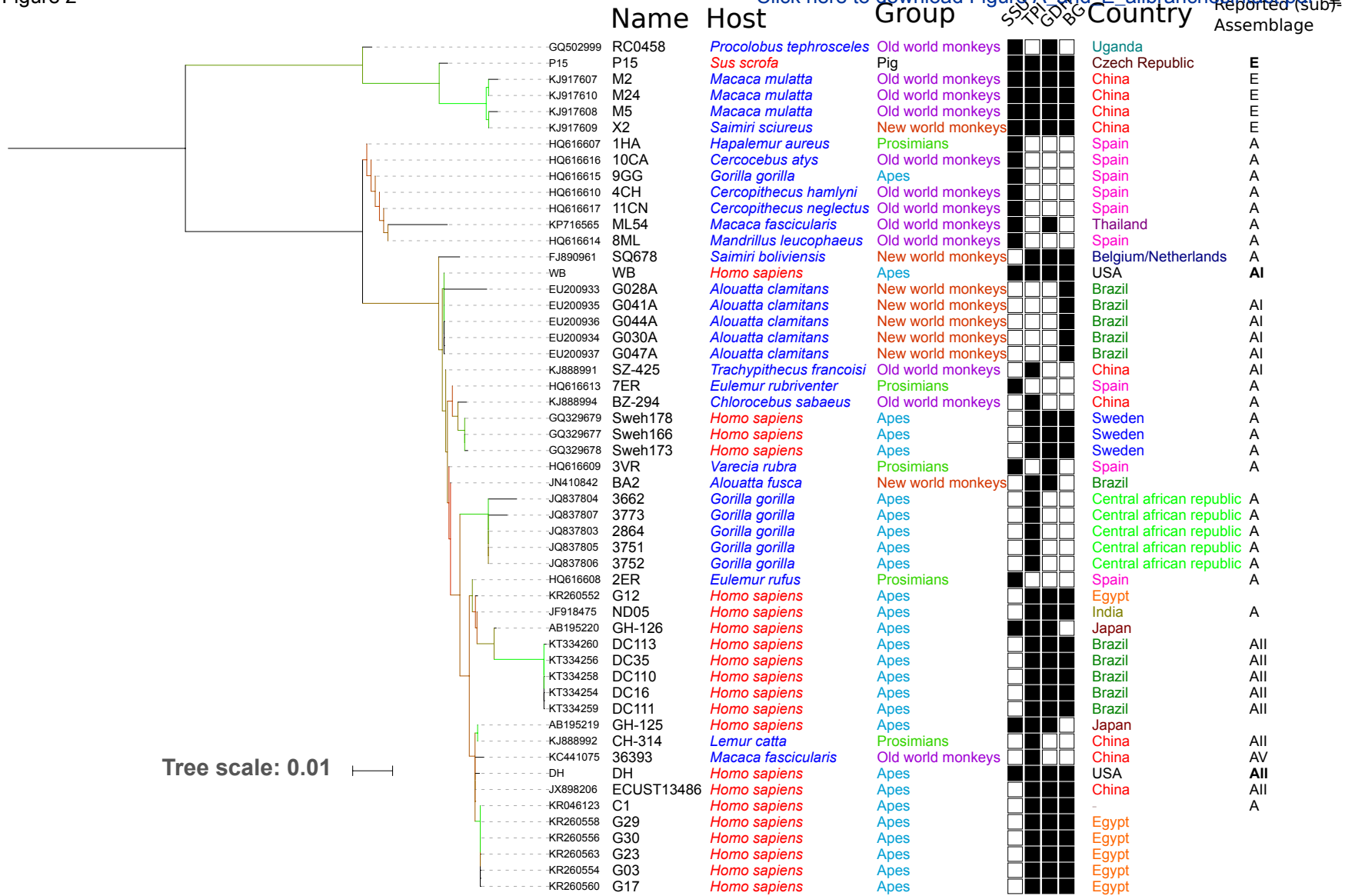
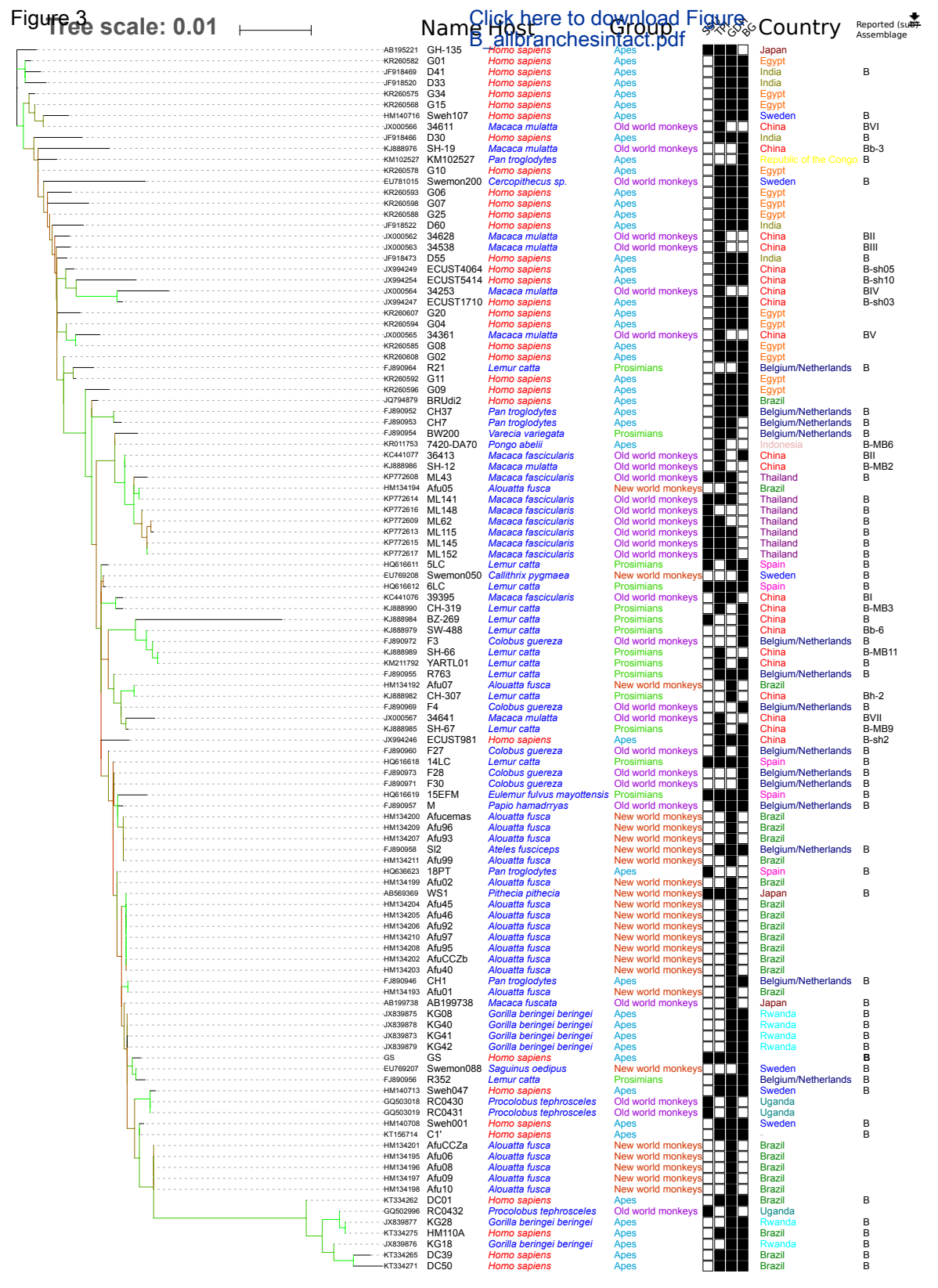


Figure 3


Tree scale: 0.01
Click here to download Figure B_allbranchesintact.pdf
Name
Host
Group
SRT
GD
BG
Country
Reported (sub)
Assemblage
AB195221 GH-135 Homo sapiens Apes Japan
KR260582 G01 Homo sapiens Apes Egypt
JF918469 D41 Homo sapiens Apes India B
JF918520 D33 Homo sapiens Apes India
KR260575 G34 Homo sapiens Apes Egypt
KR260568 G15 Homo sapiens Apes Egypt
HM140716 Sweh107 Homo sapiens Apes Sweden B
JX000566 34611 Macaca mulatta Old world monkeys China BVI
JF918466 D30 Homo sapiens Apes India B
KJ888976 SH-19 Macaca mulatta Old world monkeys China Bb-3
KM102527 KM102527 Pan troglodytes Apes Republic of the Congo B
KR260578 G10 Homo sapiens Apes Egypt
EU781015 Swemon200 Cercopithecus sp. Old world monkeys Sweden B
KR260593 G06 Homo sapiens Apes Egypt
KR260598 G07 Homo sapiens Apes Egypt
KR260588 G25 Homo sapiens Apes Egypt
JF918522 D60 Homo sapiens Apes India
JX000562 34628 Macaca mulatta Old world monkeys China BII
JX000563 34538 Macaca mulatta Old world monkeys China BIII
JF918473 D55 Homo sapiens Apes India B
JX994249 ECUST4064 Homo sapiens Apes China B-sh05
JX994254 ECUST5414 Homo sapiens Apes China B-sh10
JX000564 34253 Macaca mulatta Old world monkeys China BIV
JX994247 ECUST1710 Homo sapiens Apes China B-sh03
KR260607 G20 Homo sapiens Apes Egypt
KR260594 G04 Homo sapiens Apes Egypt
JX000565 34361 Macaca mulatta Old world monkeys China BV
KR260585 G08 Homo sapiens Apes Egypt
KR260608 G02 Homo sapiens Apes Egypt
FJ890964 R21 Lemur catta Prosimians Belgium/Netherlands B
KR260592 G11 Homo sapiens Apes Egypt
KR260596 G09 Homo sapiens Apes Egypt
JQ794879 BRUdi2 Homo sapiens Apes Brazil
FJ890952 CH37 Pan troglodytes Apes Belgium/Netherlands B
FJ890953 CH7 Pan troglodytes Apes Belgium/Netherlands B
FJ890954 BW200 Varecia variegata Prosimians Belgium/Netherlands B
KR011753 7420-DA70 Pongo abelii Apes Indonesia B-MB6
KC441077 36413 Macaca fascicularis Old world monkeys China BII
KJ888986 SH-12 Macaca mulatta Old world monkeys China B-MB2
KP772608 ML43 Macaca fascicularis Old world monkeys Thailand B
HM134194 Afu05 Alouatta fusca New world monkeys Brazil
KP772614 ML141 Macaca fascicularis Old world monkeys Thailand B
KP772616 ML148 Macaca fascicularis Old world monkeys Thailand B
KP772609 ML62 Macaca fascicularis Old world monkeys Thailand B
KP772613 ML115 Macaca fascicularis Old world monkeys Thailand B
KP772615 ML145 Macaca fascicularis Old world monkeys Thailand B
KP772617 ML152 Macaca fascicularis Old world monkeys Thailand B
HQ616611 5LC Lemur catta Prosimians Spain B
EU769208 Swemon050 Callithrix pygmaea New world monkeys Sweden B
HQ616612 6LC Lemur catta Prosimians Spain B
KC441076 39395 Macaca fascicularis Old world monkeys China BI
KJ888990 CH-319 Lemur catta Prosimians China B-MB3
KJ888984 BZ-269 Lemur catta Prosimians China B
KJ888979 SW-488 Lemur catta Prosimians China Bb-6
FJ890972 F3 Colobus guereza Old world monkeys Belgium/Netherlands B
KJ888989 SH-66 Lemur catta Prosimians China B-MB11
KM211792 YARTL01 Lemur catta Prosimians China B
FJ890955 R763 Lemur catta Prosimians Belgium/Netherlands B
HM134192 Afu07 Alouatta fusca New world monkeys Brazil
KJ888982 CH-307 Lemur catta Prosimians China Bh-2
FJ890969 F4 Colobus guereza Old world monkeys Belgium/Netherlands B
JX000567 34641 Macaca mulatta Old world monkeys China BVII
KJ888985 SH-67 Lemur catta Prosimians China B-MB9
JX994246 ECUST981 Homo sapiens Apes China B-sh2
FJ890960 F27 Colobus guereza Old world monkeys Belgium/Netherlands B
HQ616618 14LC Lemur catta Prosimians Spain B
FJ890973 F28 Colobus guereza Old world monkeys Belgium/Netherlands B
FJ890971 F30 Colobus guereza Old world monkeys Belgium/Netherlands B
HQ616619 15EFM Eulemur fulvus mayottensis Prosimians Spain B
FJ890957 M Papio hamadrryas Old world monkeys Belgium/Netherlands B
HM134200 Afucemas Alouatta fusca New world monkeys Brazil
HM134209 Afu96 Alouatta fusca New world monkeys Brazil
HM134207 Afu93 Alouatta fusca New world monkeys Brazil
FJ890958 SI2 Ateles fusciceps New world monkeys Belgium/Netherlands B
HM134211 Afu99 Alouatta fusca New world monkeys Brazil
HQ636623 18PT Pan troglodytes Apes Spain B
HM134199 Afu02 Alouatta fusca New world monkeys Brazil
AB569369 WS1 Pithecia pithecia New world monkeys Japan B
HM134204 Afu45 Alouatta fusca New world monkeys Brazil
HM134205 Afu46 Alouatta fusca New world monkeys Brazil
HM134206 Afu92 Alouatta fusca New world monkeys Brazil
HM134210 Afu97 Alouatta fusca New world monkeys Brazil
HM134208 Afu95 Alouatta fusca New world monkeys Brazil
HM134202 AfuCCZb Alouatta fusca New world monkeys Brazil
HM134203 Afu40 Alouatta fusca New world monkeys Brazil
FJ890946 CH1 Pan troglodytes Apes Belgium/Netherlands B
HM134193 Afu01 Alouatta fusca New world monkeys Brazil
AB199738 AB199738 Macaca fuscata Old world monkeys Japan B
JX839875 KG08 Gorilla beringei beringei Apes Rwanda B
JX839878 KG40 Gorilla beringei beringei Apes Rwanda B
JX839873 KG41 Gorilla beringei beringei Apes Rwanda B
JX839879 KG42 Gorilla beringei beringei Apes Rwanda B
GS GS Homo sapiens Apes B
EU769207 Swemon088 Saguinus oedipus New world monkeys Sweden B
FJ890956 R352 Lemur catta Prosimians Belgium/Netherlands B
HM140713 Sweh047 Homo sapiens Apes Sweden B
GQ503018 RC0430 Procolobus tephrosceles Old world monkeys Uganda
GQ503019 RC0431 Procolobus tephrosceles Old world monkeys Uganda
HM140708 Sweh001 Homo sapiens Apes Sweden B
KT156714 C1' Homo sapiens Apes B
HM134201 AfuCCZa Alouatta fusca New world monkeys Brazil
HM134195 Afu06 Alouatta fusca New world monkeys Brazil
HM134196 Afu08 Alouatta fusca New world monkeys Brazil
HM134197 Afu09 Alouatta fusca New world monkeys Brazil
HM134198 Afu10 Alouatta fusca New world monkeys Brazil
KT334262 DC01 Homo sapiens Apes Brazil B
GQ502996 RC0432 Procolobus tephrosceles Old world monkeys Uganda
JX839877 KG28 Gorilla beringei beringei Apes Rwanda B
KT334275 HM110A Homo sapiens Apes Brazil B
JX839876 KG18 Gorilla beringei beringei Apes Rwanda B
KT334265 DC39 Homo sapiens Apes Brazil B
KT334271 DC50 Homo sapiens Apes Brazil B

Supplementary fig S1

Supplementary fig S2

Supplementary fig S3

Supplementary fig S4

Paper 4

RHH:-SHORT COMMUNICATIONS

Low occurrence of *Giardia* and *Cryptosporidium* in domestic cattle and wild herbivores in and around Mikumi National Park, Tanzania

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Word count: 2037

ABSTRACT: The interface between humans, domestic animals and wildlife is changing, and as a result, it is becoming more porous to the spread of disease, something that is important to both public health and wildlife conservation. Two pathogens that have the potential to transmit between taxonomic groups are the ubiquitous protozoa *Giardia* and *Cryptosporidium*, both of which are important causes of human and livestock morbidity. The aim of this study was to investigate the prevalence of these protozoa at the wildlife-human-livestock interface around Mikumi National Park, Tanzania, in order to identify if disease transmission is occurring. Fecal samples were collected from wild herbivores (*n*=110; African buffalo, eland, giraffe, impala, wildebeest and zebra), as well as from domestic cattle (*n*=48) from two surrounding villages. *Giardia* and *Cryptosporidium* were detected via immunofluorescence antibody testing after immunomagnetic separation, and positive samples further characterised by PCR. All fecal samples were negative for *Giardia* cysts. *Cryptosporidium* oocysts were found in 5 % (2 / 39) of African buffalo samples, whilst samples from all other taxa (domestic cattle, eland, giraffe, impala, blue wildebeest, and zebra) were negative. Neither of these two positive samples resulted in amplification by PCR. These results suggest either that the conditions around Mikumi National Park are not conductive to the spread of these two pathogens, or that these are largely naïve populations, and thus may be susceptible to the emergence of giardiasis or cryptosporidiosis in the future.

Key words: zoonoses, diseases, one health, pathogen, protozoa

1. INTRODUCTION

The interface between humans, domestic animals and wildlife is changing. Drivers of this change include expanding human populations, alterations in land use, sharing of food and water sources, increased international mobility of humans and animals, commercialisation of the bushmeat industry, climate change, and intensification of animal production systems (Kock 2014). As this interface becomes more porous, the potential for transmission of infections between wildlife and humans/domestic animals increases, resulting in a spectrum of emerging infectious diseases (Jones et al., 2008). This can have consequences for human health and wildlife conservation, as evidenced by rabies, plague, AIDS, tularaemia and tuberculosis (Gao et al., 1999, Rhyan and Spraker, 2010). Interestingly, zoonotic transmission often utilizes a domesticated animal "bridge" e.g. dogs for echinococcosis or livestock for African trypanosomiasis (Salb et al., 2008, Funk et al., 2013).

Two pathogens that have documented abilities to transmit between different host species are ubiquitous protozoa *Giardia* and *Cryptosporidium*. Both these pathogens are known to cause significant economic losses within the livestock industry, mainly through causing high morbidity, albeit usually low mortality, in young animals (Thompson et al., 2008). *Giardia* and *Cryptosporidium* have been reported to infect a range of wildlife species (Appelbee et al., 2005), however their significance in these wild populations remains unknown.

Mikumi National Park (NP), covering over 3 000 km^2, lies in Eastern Tanzania, consisting of mixed habitats of primarily open grasslands and woodland. Although cattle grazing in Mikumi NP is strictly prohibited, inadvertent grazing by pastoralists in search of food and water does occur. Additionally, wildlife often crosses the border, and both poaching and crop raiding are common in the areas outside the NP. This creates possible contact points, either

direct or indirect, between these livestock and the wildlife. We investigated the prevalence of *Giardia* and *Cryptosporidium* in domestic cattle from just outside Mikumi NP, and from a number of species of wild ungulates and zebra from within the Park, with the aim of identifying the presence or potential for transmission of these pathogens at the wildlife-livestock-human interface.

2. MATERIALS AND METHODS

2.1 Study area and animals

Freshly voided feces were collected from African buffalo (*Syncerus caffer*; *n*=39), eland (*Taurotragus oryx*; *n*=8), giraffe (*Giraffa camelopardalis*; *n*=12), impala (*Aepyceros melampus*; *n*=21), wild blue wildebeest (*Connochaetes taurinus*; *n*=21) and zebra (*Equus quagga*; *n*=9) in Mikumi National Park, Tanzania (7°16'58.6"S, 37°7'03.1"E), during a one-week period in October 2013. In addition, freshly voided feces were collected from domestic cattle (*Bos indicus*; *n*=48) overnighting in in two locations on the fringe of Mikumi NP; Mikumi town (7°24'12.1"S, 36°58'56.0"E, *n*=16) and another unnamed settlement (7°03'55.6"S, 37°02'01.5"E) during the same period.

2.2 Sample collection

Fecal samples were collected non-invasively, and were identified by being fresh morphologically consistent stools located where the animal had been observed immediately prior to collection. Each stool was considered to be from a separate individual. As fecal morphology was used to determine host species, no diarrhoeic stools were collected.

2.3 Parasitological analysis

Approximately two grams of fecal material, either entire fecal pellets or from within the fecal mass, was placed into an 8 ml aliquot of 2.5 % (w/v) potassium dichromate, mixed thoroughly, and transported to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis for *Giardia* and *Cryptosporidium*. In October and November 2016, samples were washed twice with water followed by sieving and centrifugation. *Giardia* cysts and *Cryptosporidium* oocysts were isolated using an in-house immunomagnetic separation method (IMS) using Dynabeads™ (GC-Combo, Life Technologies, Carlsbad, CA) as previously published (Robertson et al., 2006). Briefly, 10 µl anti-*Giardia* beads, 10 µl anti-*Cryptosporidium* beads, 100 µl SL buffer A and 100 µl SL Buffer B, were used to generate 55 µl of purified sample from approximately 200 mg of the fecal pellet. Five µl of the resulting purified sample was air-dried and methanol-fixed to welled slides for detection of *Giardia* cysts and *Cryptosporidium* oocysts using a commercially available *Cryptosporidium/Giardia* direct immunofluorescent antibody test (IFAT; Aqua-Glo, Waterborne Inc., New Orleans), in accordance with manufacturer's instructions. Prior to being screened, dried samples were also stained with 4'6 diamidino-2-phenylindole (DAPI), a non-specific fluorescent stain that binds to double-stranded DNA. DAPI staining was used to assist in parasite identification and whether or not the cysts/oocysts contained nuclei and were therefore suitable for molecular analyses. Stained samples were screened using a fluorescence microscope equipped with appropriate filters (for FITC and DAPI) and Nomarski optics. Samples were initially screened at x 200, and possible findings examined more closely at x 400 and x 1000. The total number of cysts and oocysts on the slide and the DAPI staining characteristics were recorded.

2.4 DNA isolation

For *Cryptosporidium*-positive samples, DNA was isolated using the remaining 50 µl of purified oocysts after IMS using the QIAamp DNA mini kit (Qiagen GmbH) at NMBU. The protocol followed the manufacturer instructions with slight modifications to sample preparation; oocysts were first mixed with 150 µl of TE buffer (100 mM Tris and 100 mM EDTA) and incubated at 100 °C for 1 hour before an overnight proteinase K lysis step at 56 °C and spin column purification. DNA was finally eluted in 30 µl of PCR grade water and stored at 4 °C.

2.5 PCR

Conventional PCR was performed on *Cryptosporidium*-positive samples at the SSU rRNA gene as previously described (Xiao et al., 1999). PCR consisted of 8.3 µl PCR water, 1µl forward and 1 µl reverse primer (at a final concentration of 0.4 mM), 0.2 µl BSA (20mg/l), 12.5 µl of 2x HotStartTaq Master, and 2 µl of template DNA. For each PCR, positive controls and negative controls were included. PCR products were visualized by electrophoresis on 2 % agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, CA).

3. RESULTS

All fecal samples were negative for *Giardia* cysts. Examination of fecal samples from African buffalo revealed *Cryptosporidium* oocysts in 5% (2/39) of samples, whilst samples from all other taxa (domestic cattle, eland, giraffe, impala, blue wildebeest, and zebra) were negative for *Cryptosporidium* oocysts. The two *Cryptosporidium* positive samples contained 3 and 1 oocyst on the IFAT slide, which is equivalent to an excretion rate of 30 and 90 oocysts per gram of feces, respectively. All 4 oocysts observed were positive for DAPI staining, but DNA amplification was not obtained in either sample by PCR at the SSU rRNA gene.

149

150 **4. DISCUSSION**

151 Of the wildlife species examined in this study, *Cryptosporidium* has previously been detected
152 from wildebeest (Morgan et al., 1999, Alves et al., 2005), impala (Abu Samra et al., 2011),
153 zebra (Mtambo et al., 1997), and African buffalo (Hogan et al., 2014), but not from eland.
154 *Giardia* has been reported in African buffalo (Hogan et al., 2014), but not from wildebeest,
155 zebra, impala or eland. Prevalence rates of these pathogens based on modified Ziehl-Neelsen
156 (mZN) stain must be interpreted with care, as several studies have identified false positives
157 when compared with PCR or IFAT (Szonyi et al., 2008, Chang'a et al., 2011). This may
158 explain why the results of the present study are considerably different to those from a study
159 from the same region using mZN 18 years earlier, which found *Cryptosporidium* in cattle
160 (5.3%, 26/486), African buffalo (22% 8/36), zebra (27%, 7/25) and wildebeest (27%, 7 /26)
161 (Mtambo et al., 1997). This study relied upon mZN for initial detection, with a confirmatory
162 ELISA test, for which specificity and sensitivity detail are lacking, so diagnostic variables
163 may be the reason for this discrepancy. Other explanations include different sampling
164 seasons (April vs October), changing pathogen dynamics over time, or deterioration of
165 pathogens through storage.

166

167 The current low prevalence of *Cryptosporidium*, and absence of *Giardia*, in domestic cattle
168 and wild herbivores from Mikumi National Park suggests these are not important parasites in
169 these populations at the time of sampling. This result was unexpected, given these protozoa
170 are generally considered ubiquitous. Diagnosis of *Cryptosporidium* via IFAT is very sensitive
171 and specific, particularly when combined with DAPI staining. However, given the low oocyst
172 counts in the African buffalo, it is possible that the two positive samples represented carriage,
173 and not true infection. Unfortunately the two positive samples did not result in amplification

by PCR, as sequencing PCR products would have identified the *Cryptosporidium* sp. present, and thus whether it is able to infect bovids. Thus, it would appear that either the domestic cattle and wildlife studied are being exposed to *Cryptosporidium* and *Giardia*, but that the conditions are not conducive to spread, or that these are largely naïve populations. An earlier study from this region of Tanzania also did not detect *Cryptosporidium* infection in over 900 dairy calf samples (Chang'a et al., 2011); in this study the authors suggested that transmission of human cryptosporidiosis in this region may be largely anthropogenic, and that zoonotic infections in cattle may be rather limited. Furthermore, survival of both cysts and oocysts is known to be optimal under cool, damp conditions, and the high ambient temperature and significant solar radiation may result in rapid inactivation of these transmission stages. Nevertheless, changes in environmental conditions that facilitate the spread of infection, such as drought causing higher animal densities around water sources, increased rainfall supporting survival of oocysts/cysts in the environment, or the introduction of these pathogens from infected cattle or people, all have the potential to allow these parasites to gain a foothold within these populations. This may have a significant effect on the health status of both the wildlife and the domestic cattle, thus influencing the livelihood of the already marginalised peoples in this region.

ACKNOWLEDGEMENTS

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REFERENCES

Abu Samra, N., Jori, F., Samie, A., Thompson, P., 2011. The prevalence of *Cryptosporidium* spp. oocysts in wild mammals in the Kruger National Park, South Africa. Vet. Parasitol. 175, 155-159.

Alves, M., Xiao, L., Lemos, V., Zhou, L., Cama, V., da Cunha, M.B., Matos, O., Antunes, F., 2005. Occurrence and molecular characterization of *Cryptosporidium* spp. in mammals and reptiles at the Lisbon Zoo. Parasitol. Res. 97, 108-112.

Appelbee, A.J., Thompson, R.C.A., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife - current status and future needs. Trends Parasitol. 21, 370-376.

Chang'a, J.S., Robertson, L.J., Mtambo, M.M.A., Mdegela, R.H., Loken, T., Reksen, O., 2011. Unexpected results from large-scale cryptosporidiosis screening study in calves in Tanzania. Anna. Trop. Med. Parasit. 105, 513-519.

Funk, S., Nishiura, H., Heesterbeek, H., Edmunds, W.J., Checchi, F., 2013. Identifying transmission cycles at the human-animal interface: the role of animal reservoirs in maintaining gambiense human african trypanosomiasis. PLoS Comput. Biol. 9, e1002855.

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., Sharp, P.M., Hahn, B.H., 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. Nature 397, 436-441.

Hogan, J.N., Miller, W.A., Cranfield, M.R., Ramer, J., Hassell, J., Noheri, J.B., Conrad, P.A., Gilardi, K.V., 2014. *Giardia* in mountain gorillas (*Gorilla beringei beringei*), forest buffalo (*Syncerus caffer*), and domestic cattle in Volcanoes National Park, Rwanda. J. Wildl. Dis. 50, 21-30.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451, 990-993.

Kock, R., 2014. Drivers of disease emergence and spread: is wildlife to blame? Onderstepoort J. Vet. Res. 81, E1-4.

Morgan, U.M., Sturdee, A.P., Singleton, G., Gomez, M.S., Gracenea, M., Torres, J., Hamilton, S.G., Woodside, D.P., Thompson, R.C., 1999. The *Cryptosporidium* "mouse" genotype is conserved across geographic areas. J. Clin. Microbiol. 37, 1302-1305.

226 Mtambo, M.M.A., Sebatwale, J.B., Kambarage, D.M., Muhairwa, A.P., Maeda, G.E., Kusiluka,
227 L.J.M., Kazwala, R.R., 1997. Prevalence of *Cryptosporidium* spp. oocysts in cattle and wildlife in
228 Morogoro region, Tanzania. Prevent. Vet. Med. 31, 185-190.
229 Rhyan, J.C., Spraker, T.R., 2010. Emergence of diseases from wildlife reservoirs. Vet. Pathol. 47, 34-
230 39.
231 Robertson, L.J., Hermansen, L., Gjerde, B.K., Strand, E., Alvsvag, L.N., 2006. Application of
232 genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn
233 and winter 2004. Appl. Environ. Microb. 72, 2212-2217.
234 Salb, A.L., Barkema, H.W., Elkin, B.T., Thompson, R.C., Whiteside, D.P., Black, S.R., Dubey, J.P.,
235 Kutz, S.J., 2008. Dogs as sources and sentinels of parasites in humans and wildlife, northern Canada.
236 Emerg. Infect. Dis. 14, 60-63.
237 Szonyi, B., Kang'ethe, E.K., Mbae, C.K., Kakundi, E.M., Kamwati, S.K., Mohammed, H.O., 2008.
238 First report of *Cryptosporidium* deer-like genotype in Kenyan cattle. Vet. Parasitol. 153, 172-175.
239 Thompson, R.C.A., Palmer, C.S., O'Handley, R., 2008. The public health and clinical significance of
240 *Giardia* and *Cryptosporidium* in domestic animals. Vet. J. 177, 18-25.
241 Xiao, L.H., Escalante, L., Yang, C.F., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal,
242 A.A., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA
243 gene locus. Appl. Environ. Microb. 65, 1578-1583.

Paper 5

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FIVE SPECIES OF COCCIDIA (APICOMPLEXA: EIMERIIDAE), INCLUDING FOUR NEW SPECIES, IDENTIFIED IN THE FECES OF BLUE WILDEBEEST (*CONNOCHAETES TAURINUS*) IN MIKUMI NATIONAL PARK, TANZANIA

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ABSTRACT: During October 2013, 112 fecal samples were collected from wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania, and examined for coccidians. Coccidia were present in 46% of samples, with wildebeest shedding 60 to 18,000 oocysts per gram feces (median, 300; mean, 1,236). Five species, including 4 new species, were identified. Oocysts of *Eimeria gorgonis* from 18% of samples were ellipsoidal, 23 × 18.4 μm, with a length/width (L/W) ratio of 1.3, oocyst wall 1–1.5 μm thick. Micropyle, oocyst residuum, and polar granule absent. Oocysts of *Eimeria donaldi* n. sp. from 34% of samples were spherical to oblong, 13.4 × 12.3 μm, L/W ratio 1.1, oocyst wall 1 μm thick. Micropyle, oocyst residuum, and polar granule absent. Oocysts of *Eimeria nyumbu* n. sp. were ellipsoidal, 30.8 × 22.1 μm, L/W 1.4, oocyst wall 2 μm thick. Large micropyle present, oocyst residuum and polar granule absent. Oocysts of *Eimeria burchelli* n. sp. in 16% of samples were 34.8 × 24.4 μm, L/W 1.4, oocyst wall 2–2.5 μm thick, with a brown, lightly stippled outer layer. Micropyle present, oocyst residuum and polar granule absent. Oocysts of *Eimeria sokoine* n. sp. in 5% of samples were 45.8 × 29 μm, L/W 1.6, oocyst wall 3–4 μm thick with a dark brown, very rough, stippled outer layer. Micropyle present, oocyst residuum and polar granule absent. There was no apparent cross transmission of coccidia found in blue wildebeest with those generally reported to infect domestic cattle.

The blue wildebeest (*Connochaetes taurinus*) is found in Southern and Eastern Africa, inhabiting areas of short-grass plains, open bushland, and woodland (IUCN SSC Antelope Specialist Group, 2008). It is closely related to the black wildebeest (*Connochaetes gnou*), found only in Southern Africa, both belonging to the family Bovidae, subfamily Alcelaphinae.

Tourism significantly contributes to economic growth and development in Tanzania, contributing over US $570 million in 1998, and the cornerstone to tourism is the country's wildlife and natural resources. Despite this, there is limited information available on the potential transmission of pathogens from humans or livestock to wildlife. Coccidiosis is a worldwide disease that results in annual losses of over US $2 billion in the cattle and poultry industries (Wunderlich et al., 2014). While coccidian infections are usually host-specific, wildebeest are closely related to domestic ruminants, and it remains unclear if they may share some of these pathogens (Matthee and Davis, 2001).

One species of *Eimeria* has been described in blue wildebeest, *Eimeria gorgonis* (Prasad, 1960), and 2 species described in black wildebeest; *Eimeria ellipsoidalis* (Prasad, 1960) and *Eimeria gnui* (Alyousif and Al-Shawa, 1998). There are no reports on the pathogenicity of these species. The present study aimed to determine the prevalence and intensity of infection of *Eimeria* spp. infecting wild blue wildebeest in Tanzania.

MATERIALS AND METHODS

Animals

Freshly voided feces ($n = 112$) were collected from wild blue wildebeest in Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E), during a 1-wk period in October 2013. Three regions across the park were sampled. All animals were assessed from a distance by a veterinarian, and no overtly sick animals were observed.

Sample collection and parasitological examination

All fecal samples were collected non-invasively and were identified as belonging to blue wildebeest by being morphologically consistent fresh stools located where blue wildebeest had been observed immediately preceding collection. Relative stool diameter was used as a proxy indicator of whether it had been excreted from an adult or juvenile wildebeest, with adults pellets over 2 cm. On this basis, of the 112 samples, 12 were considered to have been excreted by juveniles and 100 by adults. Owing to the reliance on fecal morphology for host age determination, no diarrheic stools were collected. Each stool sample was considered to be from a separate individual. Four grams of fecal material, collected from the middle of the fecal mass, was equally divided and placed in 8 ml dual aliquots of 2.5% (w/v) potassium dichromate and 10% formalin, mixed thoroughly, and transported to the Parasitology Department, Norwegian University of Life Sciences for analysis.

Routine fecal examination was performed by flotation in Sheather's sugar solution (specific gravity = 1.28) after samples had been washed twice with phosphate buffered saline, vortexed for 2 min, and then passed through a fecal parasite concentrator with a pore diameter 425 μm (Midi Parasep, Apacor, Berkshire, England) and centrifuged again to create a pellet. An estimate of oocysts per gram (OPG) was calculated by adding 2 drops of Sheather's solution to 0.17 g of the resulting pellet on a microscope slide and then adding a large cover slip (24 × 50 mm) and counting the total number of oocysts on 1/3 of the slide. Sporulation was determined by comparing oocysts found in formalin and dichromate samples. Sporulated oocysts were measured using a calibrated ocular micrometer using bright-field microscopy×100 oil objective on a DM2700 M microscope (Leica Microsystems, Lysaker, Norway) and are reported in micrometers (μm) as means followed by the ranges in parentheses. Oocysts were observed for autofluorescence under ultraviolet (UV) filter (excitation, 360 nm; emission, 460 nm). Photographs were taken using a DFC450 camera (Leica Microsystems). Descriptions of oocysts and sporocysts follow guidelines of Wilber et al. (1998) as follows: oocyst length (L) and width (W), length to width ratio (L/W), oocyst wall (OW), micropyle (M), oocyst residuum (OR), polar granules (PG), sporocyst (SP) length (L), width (W), and length to width ratio (L/W), Stieda body (SB), sporocyst residuum (SR), sporozoite (SZ), refractile body (RB), and nucleus (N).

Haplotypes, paratypes, and phototypes of each species were stored at the Natural History Museum at the University of Oslo, Norway.

Statistical analysis

The proportion of positive fecal samples and the summary statistics of oocyst counts were calculated overall and also by individual herd and age

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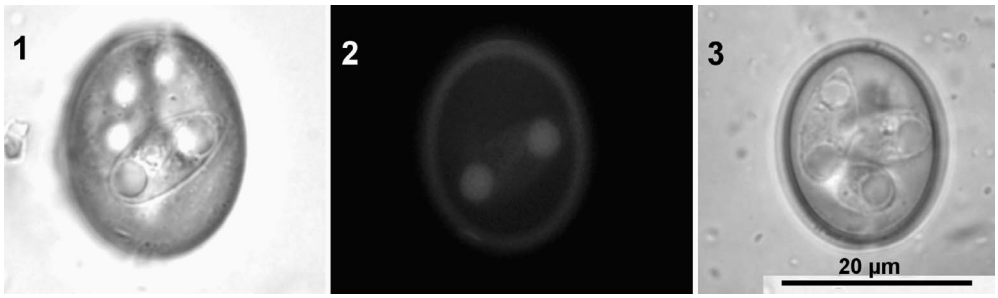


FIGURES 1–3. Photomicrographs of oocysts of *Eimeria gorgonis* from the feces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

group and compared by Fisher's exact test. Two-sample *t*-tests were used to compare the mean log OPG between juvenile and adult animals.

DESCRIPTION

Eimeria gorgonis (Prasad, 1960)

(Figs. 1–3, 15)

Description of sporulated oocyst: Oocyst (n = 72) shape, ellipsoidal; L × W, 23 (19.9–28.0) × 18.4 (16–23); L/W ratio, 1.3 (1.0–1.5); OW, 1–1.5 µm thick, UV autofluorescence, outer layer pale yellow and smooth; M, OR, PG, all absent.

Description of sporocyst and sporozoites: Four SP each with 2 SZ, SP (n = 51) shape, oval with a pointed tip; L × W, 12.2 (9.6–15.1) × 6 (4.5–8); L/W ratio, 2.1 (1.6–2.4); smooth wall; SB, present; SR, loose granules to tightly packed rosette centrally located; SZ, large spherical posterior RB with strong UV autofluorescence, ~4 µm diameter.

Taxonomic summary

Type host: Blue wildebeest, *C. taurinus* (Burchell, 1823) (Artiodactyla: Bovidae).

Type locality: Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E).

Prevalence: In 20/112 (18%).

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Site of infection: Unknown, oocysts recovered from feces.

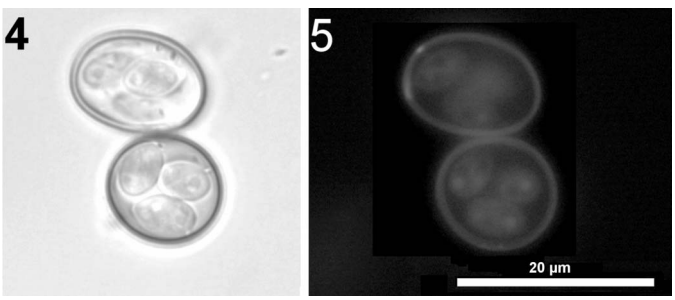
Endogenous stages: Unknown.

Cross transmission: None to date.

Materials deposited: Holotype (NHMO-Prot00015) and several paratypes (NHMO-Prot00016, NHMO-Prot00017, NHMO-Prot00019, NHMO-Prot00020, NHMO-Prot00021) are deposited together with photographs of the symbiotype host at the Natural History Museum, University of Oslo, Norway.

Remarks

Eimeria gorgonis can be distinguished from the other *Eimeria* spp. in blue wildebeest based on oocyst size, L/W ratio, and the lack of a micropyle. It is distinguished from *E. ellipsoidalis* in black wildebeest (*C. gnou*) by its greater width and thus smaller L/W ratio. While oocysts of *E. gorgonis* in this study were found to be slightly wider and with a less pronounced Stieda body and sporocyst neck than the composite drawings provided in the original description (Prasad, 1960), these morphological discrepancies were considered insufficient to distinguish a new species.



FIGURES 4, 5. Photomicrographs of an oocyst of *Eimeria donaldi* n. sp. from the feces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria donaldi n. sp.

(Figs. 4, 5, 16)

Description of sporulated oocyst: Oocyst (n = 125) shape, spherical to oblong; L × W, 13.4 (11–16) × 12.3 (10–15); L/W ratio, 1.1 (1–1.4); OW, 1 µm thick, autofluorescent under UV light, colorless, and outer layer smooth; M, OR, PR, all absent.

Description of sporocyst and sporozoites: Four SP each with 2 SZ, SP (n = 125) shape, oval with a pointed tip; L × W, 6.7 (5–10) × 3.9 (2.7–3.9); L/W ratio, 1.7 (1.1–2.3); smooth thin wall; SR, variably present as tight rosette of small granules; SB, present; SZ, large posterior RB with centrally located spherical darker structure with UV autofluorescence, ~1 µm diameter; N, centrally located without UV autofluorescence.

Taxonomic summary

Type host: Blue wildebeest, *C. taurinus* (Burchell, 1823) (Artiodactyla: Bovidae).

Type locality: Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E).

Prevalence: In 38/112 (34%).

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Site of infection: Unknown, oocysts recovered from feces.

Endogenous stages: Unknown.

Cross transmission: None to date.

Materials deposited: Holotype (NHMO-Prot00022) and several paratypes (NHMO-Prot00023, NHMO-Prot00024, NHMO-Prot00025, NHMO-Prot00026) are deposited together with photographs of the symbiotype host at the Natural History Museum, University of Oslo, Norway.

Etymology: The specific epithet is given in honor of Donald W. Duszynski for his work on *Eimeria*.

Remarks

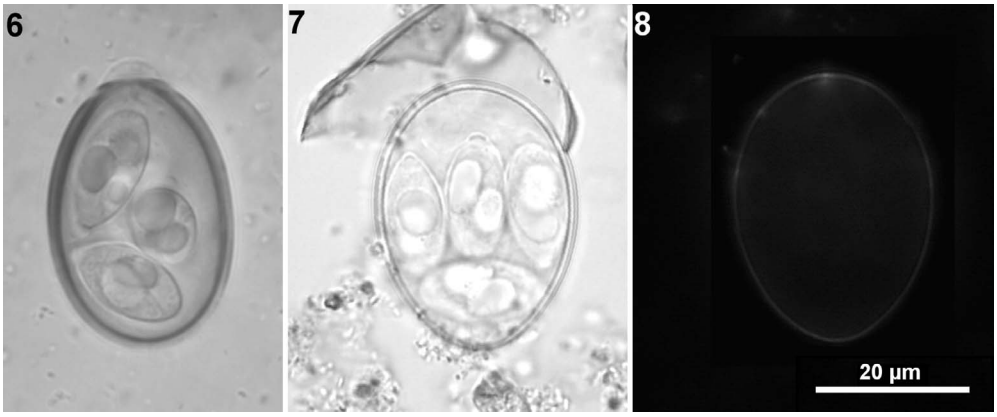
Eimeria donaldi n. sp. is the smallest *Eimeria* spp. that was identified in this population of wildebeest and is most easily distinguished based on the oocyst size and shape, as well as the smaller L/W ratio of sporocysts.

Eimeria nyumbu n. sp.

(Figs. 6–8, 17)

Description of sporulated oocyst: Oocyst (n = 57) shape, ellipsoidal; L × W, 30.8 (27–33.5) × 22.1 (20–24.3); L/W ratio, 1.4 (1.2–1.6); OW, 2 µm thick, outer surface smooth with light brown color, inner layer with UV autofluorescence; M, present, large with domed shape; OR, PG, absent.

Description of sporocyst and sporozoites: Four SP each with 2 SZ, SP (n = 60) shape, oval with pointed tip; L × W, 14.8 (12–19) × 7.3 (5–9); L/W



FIGURES 6–8. Photomicrographs of oocysts of *Eimeria nyumbu* n. sp. from the feces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

ratio, 2.1 (1.5–2.8); smooth wall; SB, present; SR, absent; SZ, large RB located centrally without UV autofluorescence.

Taxonomic summary

Type host: Blue wildebeest, *C. taurinus* (Burchell, 1823) (Artiodactyla: Bovidae).

Type locality: Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E).

Prevalence: In 7/112 (6%).

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Site of infection: Unknown, oocysts recovered from feces.

Endogenous stages: Unknown.

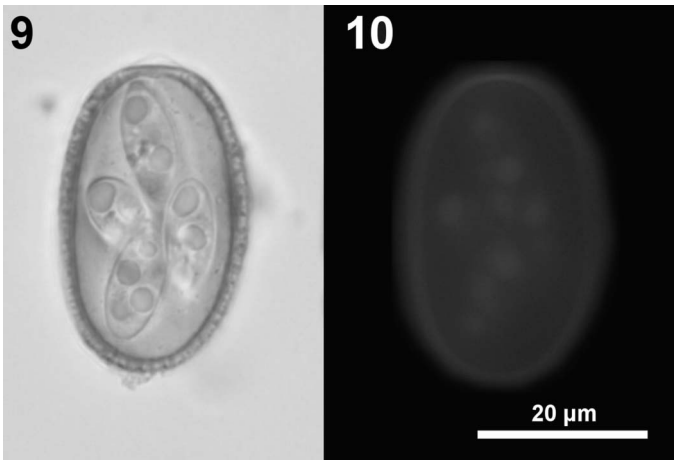
Cross transmission: None to date.

Materials deposited: Holotype (NHMO-Prot00027) and several paratypes (NHMO-Prot00028, NHMO-Prot00029, NHMO-Prot00030, NHMO-Prot00031) are deposited together with photographs of the symbiotype host at the Natural History Museum, University of Oslo, Norway.

Etymology: The specific epithet is derived from the Swahili word nyumbu, meaning wildebeest or hartebeest.

Remarks

Eimeria nyumbu n. sp. oocysts are best distinguished by the smooth outer layer of the oocyst wall, large extruding micropyle, and the large sporozoite refractile bodies located relatively centrally within the sporocyst. There is a slight overlap in oocyst size with *Eimeria burchelli*.



FIGURES 9, 10. Photomicrographs of an oocyst of *Eimeria burchelli* n. sp. from the feces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Eimeria burchelli n. sp.

(Figs. 9, 10, 19)

Description of sporulated oocyst: Oocyst (n = 46) shape, ellipsoidal; L × W, 34.8 (30–39) × 24.4 (20–27.5); L/W ratio, 1.4 (1.2–1.8); OW, 2–2.5 µm thick, outer surface lightly stippled with a brown color, inner layer with weak UV autofluorescence; M, present; OR, PG, absent.

Description of sporocyst and sporozoites: Four SP each with 2 SZ, SP (n = 22) shape, oval with pointed tip; L × W, 16.8 (15–19) × 7.9 (6.2–9); L/W ratio, 2.1 (1.9–2.6); smooth wall; SB, present; SR, present as granules 0.5–1.5 µm scattered throughout; SZ, equally sized posterior RB and anterior RB present with weak UV autofluorescence.

Taxonomic summary

Type host: Blue wildebeest, *C. taurinus* (Burchell, 1823) (Artiodactyla: Bovidae).

Type locality: Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E).

Prevalence: In 18/112 (16%).

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Site of infection: Unknown, oocysts recovered from feces.

Endogenous stages: Unknown.

Cross transmission: None to date.

Materials deposited: Holotype (NHMO-Prot00032) and several paratypes (NHMO-Prot00033, NHMO-Prot00034, NHMO-Prot00035) are deposited together with photographs of the symbiotype host at the Natural History Museum, University of Oslo, Norway.

Etymology: The specific epithet is given in honor of William John Burchell (1781–1863), the English explorer who first described the type host in 1823.

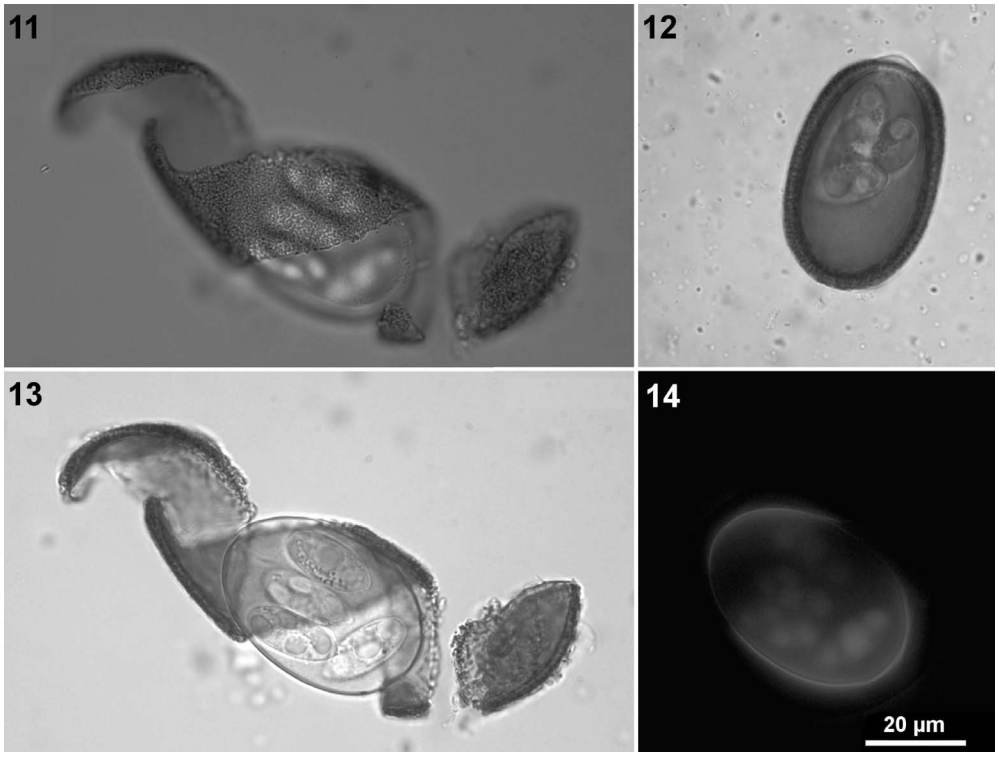
Remarks

Eimeria burchelli n. sp. oocysts are best distinguished by their shape and size, the thick brown wall, with mildly rough or stippled outer layer, and by having 2 refractile bodies per sporozoite. The oocysts overlap slightly in size with *E. nyumbu* oocysts.

Eimeria sokoine n. sp.

(Figs. 11–14, 18)

Description of sporulated oocyst: Oocyst (n = 90) shape, oval to ellipsoidal; L × W, 45.8 (39.8–52) × 29 (26.2–34.5); L/W ratio, 1.6 (1.3–1.9); OW, 3–4 µm thick, outer layer very rough, stippled and dark brown in color, inner layer with uneven UV autofluorescence; M, present; OR, PG, absent.



FIGURES 11–14. Photomicrographs of oocysts of *Eimeria sokoine* n. sp. from the feces of a wild blue wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania.

Description of sporocyst and sporozoites: Four SP each with 2 SZ; SP (n = 89) shape, ellipsoidal to cylindrical with a pointed tip; L × W, 18.7 (15.6–21.1) × 8.3 (6.9–10.4); L/W ratio, 2.3 (1.7–2.8); smooth wall; SB, present; SR, present as granules 0.5–1.5 µm scattered throughout; SZ, large posterior RB and anterior RB of equal size, both with strong UV autofluorescence.

Taxonomic summary

Type host: Blue wildebeest, *C. taurinus* (Burchell, 1823) (Artiodactyla: Bovidae).

Type locality: Mikumi National Park, Tanzania (7°16′58.6″S, 37°7′03.1″E).

Prevalence: In 6/112 (5%).

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Site of infection: Unknown, oocysts recovered from feces.

Endogenous stages: Unknown.

Cross transmission: None to date.

Materials deposited: Holotype (NHMO-Prot00036) and 2 paratypes (NHMO-Prot00037, NHMO-Prot00038) are deposited together with photographs of the symbiotype host at the Natural History Museum, University of Oslo, Norway.

Etymology: The specific epithet is given in honor of Edward Moringe Sokoine for his work in establishing the Sokoine University of Agriculture (SUA) in Morogoro, Tanzania.

Remarks

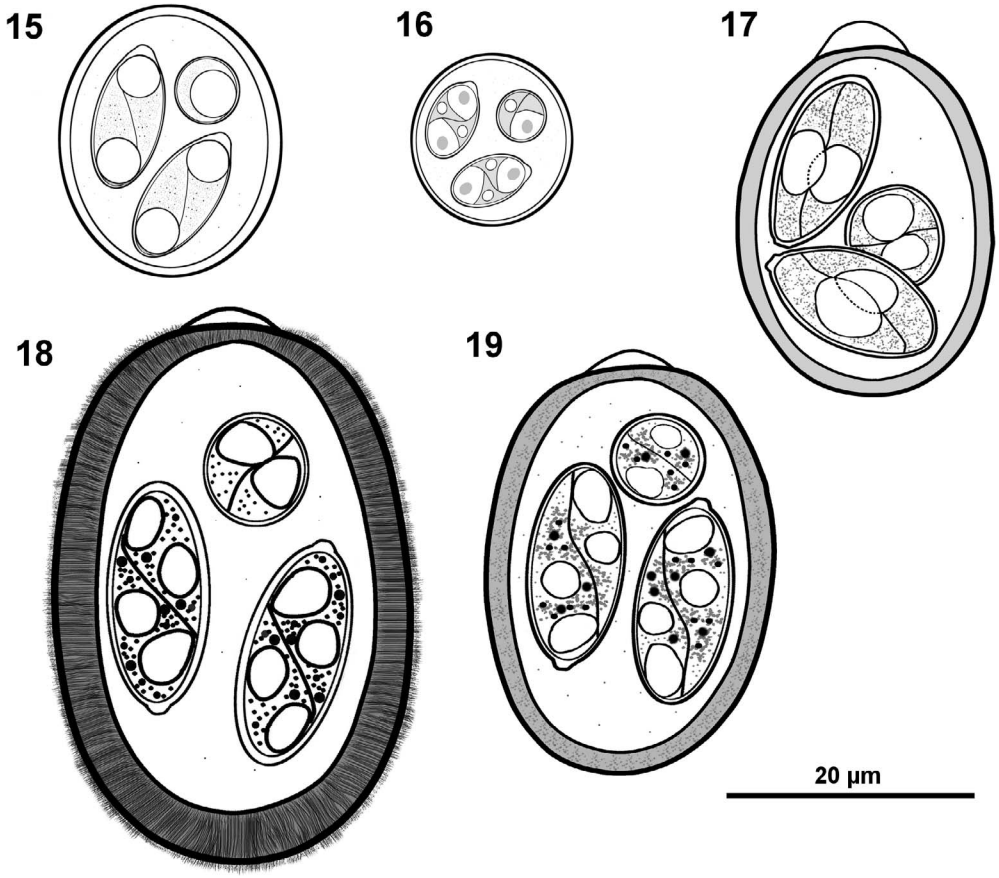
Eimeria sokoine n. sp. is the largest of the *Eimeria* spp. infecting this population of wildebeest. The oocysts can easily be distinguished by their size; by their dark brown, thick, and very rough outer wall; and by the 2 refractile bodies per sporozoite. Oocyst size overlaps with *E. gnui* (Alyousif and Al-Shawa, 1998) from black wildebeest, however *E. sokoine* has a thicker, more heavily pitted outer oocyst wall, a less tapered oocyst width toward the poles, and the anterior and posterior sporozoite refractile bodies are equal in size.

Low numbers of Eimeria spp. oocysts are shed by wild blue wildebeest, with a higher prevalence and intensity seen in juvenile compared with adult animals: *Eimeria* spp. oocysts were detected in 46% (52/112) of the fecal samples. Of 52 positive samples, 33 (63%) had mixed infections. The prevalence of *Eimeria* oocysts in fecal samples from juveniles was 92% (11/12), which was significantly higher than the prevalence from adults, 41% (41/100) (Fisher's exact test, $P < 0.01$). No significant difference was observed in the prevalence of infection between the 3 different herds (Fisher's exact test, $P = 0.09$). Overall, wildebeest shed 60 to 18,000 OPG (median, 300; mean, 1,236). The concentration of oocysts shed by juvenile animals, mean 4,002 (median, 700; range, 300–18,000), was significantly greater than shed by adult animals, mean 577 (median, 220; range, 60–3,500), when compared by mean log OPG (2-sample *t*-test, $P < 0.01$).

DISCUSSION

Despite the close taxonomic relationship of wildebeest and domestic cattle, and the importance coccidiosis plays in the cattle industry, the coccidian fauna of wildebeest have remained relatively unstudied. In this study, 4 new species of *Eimeria* are described from blue wildebeest. A fifth oocyst morphology was observed and concluded to be likely to be synonymous with the previously described *E. gorgonis* (Prasad, 1960). However, oocysts of *E. gorgonis* from the current study were found to be wider, lacking a polar granule, and had a less distinct sporocyst neck than originally reported. All 5 species have unique morphologies when compared with published *Eimeria* spp. oocysts from hosts of the subfamily Alcelaphinae.

Oocysts were detected in 46% (52/112) of wild blue wildebeest samples, which is consistent with previous reports (Turner and



FIGURES 15–19. Composite line drawings of sporulated oocysts from the feces of wild blue wildebeests (*Connochaetes taurinus*) in Mikumi National Park, Tanzania. (**15**) *Eimeria gorgonis*. (**16**) *Eimeria donaldi* n. sp. (**17**) *Eimeria nyumbu* n. sp. (**18**) *Eimeria sokoine* n. sp. (**19**) *Eimeria burchelli* n. sp.

Getz, 2010). Little information is available about the prevalence of *Eimeria* spp. infections in wild artiodactyls, but there appears to be considerable species and seasonal variation (Turner and Getz, 2010; Pyziel and Demiaszkiewicz, 2013; Tomczuk et al., 2014). Overall, the concentration of oocysts in feces was low (range 60–18,000; median 300; mean 1,236 OPG) and similar to that seen in domestic ruminants in Tanzania and other free ranging herbivores (Kusiluka et al., 1996; Singh et al., 2009; Turner and Getz, 2010). In this study, juvenile wildebeest were shedding a greater concentration of *Eimeria* spp. oocysts in the feces, as has been previously reported (Turner and Getz, 2010). At the infection levels observed in this study, the *Eimeria* spp. described probably act more as part of the commensal gastrointestinal flora. However, exposure of young, naive, or immunosuppressed individuals, particularly at high stocking density that facilitates feco–oral transmission, may lead to disease.

We suggest that the information presented here indicates that cross transmission of coccidian species between domestic cattle and sympatric wildebeest is unlikely to occur and does not seem to present a threat to either group. However, cross transmission of less host-specific pathogens between wildlife and domestic animals may still be of relevance and importance to the pastoral communities and/or to Tanzanian wildlife on which the national economy is so reliant.

ACKNOWLEDGMENTS

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LITERATURE CITED

ALYOUSIF, M. S., AND Y. R. AL-SHAWA. 1998. *Eimeria gnui* sp. n. (Apicomplexa: Eimeriidae) from white-tailed gnu, *Connochaetes gnu*. Acta Protozoologica **37:** 53–55.

INTERNATIONAL UNION FOR CONSERVATION OF NATURE, SPECIES SURVIVAL COMMISSION (IUCN SSC) ANTELOPE SPECIALIST GROUP. 2008. *Connochaetes taurinus*. The IUCN Red list of threatened species. Version 2015.1. Available at: www.iucnredlist.org. Accessed 2 June 2015.

KUSILUKA, L. J. M., D. M. KAMBARAGE, R. W. MATTHEWMAN, L. J. S. HARRISON, AND C. J. DABORN. 1996. Coccidiosis of small ruminants in Tanzania. Small Ruminant Research **21:** 127–131.

MATTHEE, C. A., AND S. K. DAVIS. 2001. Molecular insights into the evolution of the family Bovidae: A nuclear DNA perspective. Molecular Biology and Evolution **18:** 1220–1230.

PRASAD, H. 1960. Studies on the coccidia of some mammals of the families Bovidae, Cervidae and Camelidae. Zeitschrift für Parasitenkunde **20:** 390–400.

Pyziel, A. M., and A. W. Demiaszkiewicz. 2013. Coccidia (Apicomplexa: Eimeriidae) of elk (*Alces alces*) in Poland. Parasitology Research **112:** 2083–2085.

Singh, S., A. B. Shrivastav, and R. K. Sharma. 2009. The epidemiology of gastrointestinal parasitism and body condition in free-ranging herbivores. Journal of Threatened Taxa **1:** 535–537.

Tomczuk, K., K. Szczepaniak, M. Grzybek, M. Studzinska, M. Demkowska-Kutrzepa, M. Roczen-Karczmarz, K. Kostro, and L. Krakowski. 2014. Gastro-intestinal parasite prevention in wild roe deer in selected hunting areas in south-eastern Poland. Medycyna Weterynaryjna-Veterinary Medicine—Science and Practice **70:** 630–635.

Turner, W. C., and W. M. Getz. 2010. Seasonal and demographic factors influencing gastrointestinal parasitism in ungulates of Etosha National Park. Journal of Wildlife Diseases **46:** 1108–1119.

Wilber, P. G., D. W. Duszynski, S. J. Upton, R. S. Seville, and J. O. Corliss. 1998. A revision of the taxonomy and nomenclature of the *Eimeria* spp. (Apicomplexa: Eimeriidae) from the rodents in the tribe Marmotini (Sciuridae). Systematic Parasitology **39:** 113–135.

Wunderlich, F., S. Al-Quraishy, H. Steinbrenner, H. Sies, and M. A. Dkhil. 2014. Towards identifying novel anti-*Eimeria* agents: Trace elements, vitamins, and plant-based natural products. Parasitology Research **113:** 3547–3556.

Paper 6

Short Communications

Occurrence of *Giardia* in Swedish red foxes (*Vulpes vulpes*)

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Running head: *Giardia* in Swedish red foxes

Word count: 1941

Total Figure: 0

Total Tables: 0

Abstract (Max 300 words)

Giardia duodenalis is an intestinal protozoa capable of causing gastrointestinal disease in a range of vertebrate hosts. It is transmitted via the fecal-oral route. Understanding the epidemiology of *G. duodenalis* in animals is important, both for public health and for the health of the animals it infects. This study investigated the occurrence of *G. duodenalis* in wild Swedish red foxes (*Vulpes vulpes*), with the aim of providing preliminary information on how this abundant predator may be involved in the transmission and epidemiology of *G. duodenalis*. Fecal samples (n = 104) were analysed for *G. duodenalis* using a commercially available direct immunofluorescent antibody test. *Giardia duodenalis* cysts were found in 44 % (46 / 104) of samples, with foxes excreting 100 to 140 500 cysts per gram feces (mean, 4930; median, 600). Molecular analysis, using PCR with sequencing of PCR amplicons, was performed on fourteen samples, all containing over 2000 cysts per gram feces. Amplification only occurred in 4 samples at the *tpi* gene, all of which belonged to Assemblage B. This study provides baseline information on the role of red foxes in the transmission dynamics of *G. duodenalis* in Sweden.

Keywords: anthropozoonotic, carnivore, disease, parasite, protozoa, transmission, zoonotic

Giardia duodenalis is an intestinal protozoan parasite capable of causing gastrointestinal disease in a range of vertebrate hosts. It is transmitted via the fecal-oral route. *G. duodenalis* annually causes clinical disease in over 200 million people in Asia, Africa, and Latin America, as well as being responsible for large-scale human waterborne outbreaks in the developed world, including in Scandinavia (Robertson et al. 2006, Feng and Xiao 2011). Of the eight different *G. duodenalis* assemblages (A-H), the two that are considered to have zoonotic potential (A and B) have been found to be infective to a wide range of wildlife, including red foxes (Appelbee et al. 2005, Hamnes et al. 2007).

There is a lack of knowledge on the role of wildlife in transmission dynamics, and whether some wildlife species may be disease reservoirs for human infection with zoonotic *G. duodenalis* assemblages, or vice versa. In order to investigate the role of wildlife in the epidemiology of *G. duodenalis*, prevalence data in species living at the wildlife-human-domestic animal interface must be determined and isolates characterized at the molecular level to identify possible transmission pathways.

The red fox (*Vulpes vulpes*) has the widest geographical range of any member of the order Carnivora, (Hoffmann and Sillero-Zubiri 2016). Being an opportunistic omnivore that is often found at high densities in urban areas, the red fox is exposed to a range of pathogens through its diet, and may act as a reservoir of pathogens between humans, domestic animals and other wildlife species. Few studies have investigated the molecular epidemiology of *G. duodenalis* in red fox populations, and in order to reduce the knowledge gap, this study investigated the occurrence of *G. duodenalis* in wild Swedish red foxes.

Fecal samples (n = 104) were collected opportunistically by hunters between August and December 2014, as part of a national *Echinococcus multilocularis* survey. Samples were sent to the National Veterinary Institute in Sweden and frozen unpreserved at -80 °C, then thawed and then re-frozen at –

20 °C for 1 to 5 months, prior to being transported to the Parasitology Department, Norwegian University of Life Sciences (NMBU) for analysis. At NMBU, samples were stored at 4 °C.

Detection and quantification of *G. duodenalis* was by standard immunofluorescent antibody test (IFAT) with FITC-labelled monoclonal antibody (Aqua-Glo, Waterborne Inc., New Orleans, USA) and 4′,6-diamidino-2-phenyl indole (DAPI) staining on 10 µL sub-samples that had been prepared by re-suspending 3 g of feces in water followed by sieving and centrifugation, before air-drying and methanol fixing the sub-samples to welled slides. The stained samples were examined by fluorescence microscopy (DM2700M, Leica Microsystems, Lysaker, Norway) at X200 and X400 magnification. The total number of cysts on each slide were counted, and used to estimate the number of cysts per gram feces. The theoretical limit of detection of this method is 100 cysts per gram feces (CPG).

DNA isolation was performed directly from the fecal pellet on fourteen samples with over 2000 CPG using QIAmp DNA mini kit (Qiagen GmbH). The protocol followed the manufacturer's instructions with slight modifications; 0.1 g of fecal pellet was first mixed with 150 µl of TE buffer (100 mM Tris and 100 mM EDTA), incubated at 90 °C for 1 hour and an overnight proteinase K lysis step at 56 °C before spin column purification. DNA was eluted in 30 µl of PCR grade water, and stored at 4 °C.

Conventional PCR was performed on *G. duodenalis* positive samples at the small subunit rRNA (SSU), glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), and β-giardin (*bg*) genes (Hopkins et al. 1997, Sulaiman et al. 2003, Read et al. 2004, Lalle et al. 2005). In all cases, PCR consisted of 8.3 µl PCR water, 1µl forward and 1 µl reverse primer (at a final concentration of 0.4 mM), 0.2 µl BSA (20 mg/l), 12.5 µl of 2x HotStartTaqMaster and 2 µl of template DNA. PCR products were visualized by electrophoresis on 2 % agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, CA).

Positive samples were purified using a High Pure PCR Product Purification Kit (Roche, Oslo, Norway) and sequenced in both directions (GATC Biotech, Germany). Sequences were analysed using the program Geneious™.

Examination of red fox fecal samples using immunofluorescent microscopy revealed the presence of *G. duodenalis* cysts in 44 % (46/104) of samples. Foxes excreted 100 to 140 500 CPG (mean, 4930; median, 600). In general, low numbers of cysts were shed, with only two samples containing over 10 000 CPG. All cysts examined directly by IFAT were negative by DAPI for the presence of nuclei.

Of the fourteen *G. duodenalis* positive samples selected for molecular characterisation, no amplification of DNA by PCR was seen at the *gdh*, SSU, or *bg* genes. Four samples were positive at the *tpi* gene. Sequencing of these PCR products revealed Assemblage B in all four samples (Accession numbers; KY304077-KY304080). All sequences were identical except for two ambiguous nucleotides in one isolate and a single SNP in another isolate. All samples had identical protein translations. BLAST comparison of nucleotide sequences revealed 100 % of the consensus region (498 bp) to be identical to *Giardia* isolates from a variety of sources e.g. rhesus macaque in China, water from the USA, and a human sample from Malaysia.

This study describes a high prevalence of low intensity infections of *G. duodenalis* in wild red foxes in Sweden, with only Assemblage B identified. *G. duodenalis* infection has previously been reported from a range of Swedish animals (Lebbad et al. 2010), however this is the first report in Swedish red foxes and suggests that they may be important players in *G. duodenalis* epidemiology in this country. Infection prevalence was higher than reported prevalences in red foxes elsewhere in Europe; 2.8% (10/217) in Romania, 4.5 % (3/66) in Croatia, 4.8 % in Norway (13/269), 7.3 % (9/123) in Bosnia and Herzegovina, and 19 % (4/21) in Poland (Hamnes et al. 2007, Beck et al. 2011, Hodzic et al. 2014, Onac et al. 2015, Stojecki et al. 2015). Similar infection rates have been seen in other wild canids (Trout et al. 2006, Oates et al. 2012). The high prevalence found in the Swedish population may be due to innate differences in this population, e.g. associated with diet, proximity to farming or domestic

137 animals, water sources, human contact, population densities etc. The low intensity of cyst shedding
138 observed is important to consider when assessing zoonotic potential, as these animals will be less
139 likely to lead to environmental contamination than animals with large excretion rates or higher fecal
140 outputs.
141
142 In this study, PCR had limited success, similar to other studies trying to characterise *G. duodenalis*
143 isolates from other canids (Sommer et al. 2015, Stojecki et al. 2015). The lack of DNA observed
144 within the *G. duodenalis* cysts may indicate that the DNA was degraded or located free within the
145 feces matrix. If this is the case, then the processing steps designed to remove fecal debris, may have
146 resulted in the loss of this DNA, thus causing false negatives.
147
148 Red foxes have previously been reported to be infected with *G. duodenalis* Assemblage A and B
149 (Hamnes et al. 2007, McCarthy et al. 2008, Beck et al. 2011, Onac et al. 2015), whereas only one
150 study has reported the canine specific Assemblage D in two samples (Ng et al. 2011), and no reports
151 of Assemblage C. In our study, Swedish red foxes were found to be infected with Assemblage B. In
152 contrast, the vast majority of *Giardia* isolates from dogs are Assemblage C and D (Feng and Xiao
153 2011), even in environments where Assemblage B is considered to predominate (Lebbad et al. 2008).
154 It is therefore intriguing that Assemblage B is apparently common in red foxes, but rarely establishes
155 in dogs, suggesting a considerably different host-parasite relationship between these two canids.
156 Finding Assemblage B in Swedish red foxes may indicate that they act as a disease reservoir for
157 zoonotic *G. duodenalis*. However, care must be taken when interpreting the zoonotic potential of
158 these isolates based on a single gene locus, especially when taxonomic grouping can vary based on
159 which genes are used for comparison (Lebbad et al. 2010).
160
161
162 The stay of HL at the Parasitology Lab at NMBU was financed through the Erasmus
163 Program. Collection of fox samples were funded by Swedish Board of Agriculture.

Literature cited

Appelbee AJ, Thompson RCA, Olson ME. 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife - current status and future needs. *Trends Parasitol* 21(8):370-376.

Beck R, Sprong H, Lucinger S, Pozio E, Caccio SM. 2011. A large survey of croatian wild mammals for *Giardia duodenalis* reveals a low prevalence and limited zoonotic potential. *Vector-Borne Zoonot* 11(8):1049-1055.

Feng Y, Xiao L. 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. *Clin Microbiol Rev* 24(1):110-140.

Hamnes IS, Gjerde BK, Forberg T, Robertson LJ. 2007. Occurrence of *Giardia* and *Cryptosporidium* in Norwegian red foxes (*Vulpes vulpes*). *Vet Parasitol* 143(3-4):347-353.

Hodzic A, Alic A, Omeragic J. 2014. Occurrence of *Cryptosporidium* spp. and *Giardia duodenalis* in red foxes (*Vulpes vulpes*) in Bosnia and Herzegovina. *Mac Vet Rev* 37(2):189-192.

Hoffmann M, Sillero-Zubiri C: *Vulpes vulpes*. In: The IUCN Red List of Threatened Species 2016. IUCN.

Hopkins RM, Meloni BP, Groth DM, Wetherall JD, Reynoldson JA, Thompson RC. 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. *J Parasitol* 83(1):44-51.

Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D, Caccio SM. 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int J Parasitol* 35(2):207-213.

Lebbad M, Ankarklev J, Tellez A, Leiva B, Andersson JO, Svard S. 2008. Dominance of *Giardia* assemblage B in Leon, Nicaragua. *Acta Trop* 106(1):44-53.

Lebbad M, Mattsson JG, Christensson B, Ljungstrom B, Backhans A, Andersson JO, Svard SG. 2010. From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. *Vet Parasitol* 168(3-4):231-239.

McCarthy S, Ng J, Gordon C, Miller R, Wyber A, Ryan UM. 2008. Prevalence of *Cryptosporidium* and *Giardia* species in animals in irrigation catchments in the southwest of Australia. *Exp Parasitol* 118(4):596-599.

192 Ng J, Yang R, Whiffin V, Cox P, Ryan U. 2011. Identification of zoonotic *Cryptosporidium* and
193 *Giardia* genotypes infecting animals in Sydney's water catchments. *Exp Parasitol*
194 128(2):138-144.
195 Oates SC, Miller MA, Hardin D, Conrad PA, Melli A, Jessup DA, Dominik C, Roug A, Tinker MT,
196 Miller WA. 2012. Prevalence, environmental loading, and molecular characterization of
197 *Cryptosporidium* and *Giardia* isolates from domestic and wild animals along the central
198 California coast. *Appl Environ Microbiol* 78(24):8762-8772.
199 Onac, D, Oltean M, Mircean V, Jarca, A, Cozma, V. 2015. Occurence of *Giardia duodenalis* zoonotic
200 assemblages in red foxes from Romania. *Sci Parsitol* 16(4):177-180.
201 Read CM, Monis PT, Thompson RCA. 2004. Discrimination of all genotypes of *Giardia duodenalis*
202 at the glutamate dehydrogenase locus using PCR-RFLP. *Infect Genet Evol* 4(2):125-130.
203 Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N. 2006. Application of
204 genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during
205 autumn and winter 2004. *Appl Environ Microbiol* 72(3):2212-2217.
206 Sommer MF, Beck R, Ionita M, Stefanovska J, Vasic A, Zdravkovic N, Hamel D, Rehbein S, Knaus
207 M, Mitrea IL, Shukullari E, Kirkova Z, Rapti D, Capari B, Silaghi C. 2015. Multilocus
208 sequence typing of canine *Giardia duodenalis* from South Eastern European countries.
209 *Parasitol Res* 114(6):2165-2174.
210 Stojecki K, Sroka J, Caccio SM, Cencek T, Dutkiewicz J, Kusyk P. 2015. Prevalence and molecular
211 typing of *Giardia duodenalis* in wildlife from eastern Poland. *Folia Parasitol* 62:042.
212 Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA, Xiao L. 2003.
213 Triosephosphate isomerase gene characterization and potential zoonotic transmission of
214 *Giardia duodenalis*. *Emerg Infect Dis* 9(11):1444-1452.
215 Trout JM, Santin M, Fayer R. 2006. *Giardia* and *Cryptosporidium* species and genotypes in coyotes
216 (*Canis latrans*). *J Zoo Wildl Med* 37(2):141-144.
217
218
219