Are molecular tools clarifying or confusing our understanding of the public health threat from zoonotic enteric protozoa in wildlife?

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Abstract

Emerging infectious diseases are frequently zoonotic, often originating in wildlife, but enteric protozoa are considered relatively minor contributors. Opinions regarding whether pathogenic enteric protozoa may be transmitted between wildlife and humans have been shaped by our investigation tools, and has led to oscillations regarding whether particular species are zoonotic or have host-adapted life cycles.

When the only approach for identifying enteric protozoa was morphology, it was assumed that many enteric protozoa colonized multiple hosts and were probably zoonotic. When molecular tools revealed genetic differences in morphologically identical species colonizing humans and other animals, host specificity seemed more likely. Parasites from animals found to be genetically identical - at the few genes investigated - to morphologically indistinguishable parasites from human hosts, were described as having zoonotic potential. More discriminatory molecular tools have now sub-divided some protozoa again. Meanwhile, some infection events indicate that, circumstances permitting, some "host-specific" protozoa, can actually infect various hosts. These repeated changes in our understanding are linked intrinsically to the investigative tools available.

Here we review how molecular tools have assisted, or sometimes confused, our understanding of the public health threat from nine enteric protozoa and example wildlife hosts (*Balantoides coli* - wild boar; *Blastocystis* sp. - wild rodents; *Cryptosporidium* spp. - wild fish; *Encephalitozoon* spp. - wild birds; *Entamoeba* spp. - non-human primates; *Enterocytozoon bieneusi* - wild cervids; *Giardia duodenalis* - red foxes; *Sarcocystis nesbitti* - snakes; *Toxoplasma gondii* - bobcats).

Molecular tools have provided evidence that some enteric protozoa in wildlife may infect humans, but due to limited discriminatory power, often only the zoonotic potential of the parasite is indicated. Molecular analyses, which should be as discriminatory as possible, are one, but not the only, component of the toolbox for investigating potential public health impacts from pathogenic enteric protozoa in wildlife.

1. Zoonotic enteric protozoa in wildlife as agents of "emerging" infectious diseases in humans

The potential for wildlife to be a source of infectious diseases in humans was brought into focus in a landmark paper published in 2008, in which the authors estimated that emerging infectious disease (EID) events occurring between 1940 and 2004 were dominated by zoonoses (60.3%), the majority of which (71.8%) originated in wildlife (Jones et al., 2008). These figures, or approximations thereof, have been widely quoted since. The authors of the original article report that the majority of the EID events included in their calculations involve bacteria or rickettsiae (54.3%), but note that in making these estimates they classified every individual drug-resistant microbial strain as a separate pathogen. Although the importance of antimicrobial resistance to global health should be emphasized, this classification may have resulted in the contribution of other pathogen types (virus, protozoa, helminths, etc.) being underestimated; the authors calculated the percentages of EID events caused by other pathogen types to be 25.4% for viral or prion pathogens, 10.7% for protozoa, 6.3% for fungi, and 3.3% for helminths (Jones et al., 2008).

Although wildlife parasitology is of importance in its own right, particularly in consideration of such elements as loss of biodiversity, conservation issues, alterations in land use, impacts of climate change, and the role of invasive species (Thompson and Polley, 2014), it is clear that much of the research in wildlife parasitology is driven by determining whether or not parasitic infections in a wildlife population may serve as a reservoir of diseases that may affect domestic animals or humans (Appelbee et al., 2005). A clear and early example of this was the investigation of beavers for *Giardia* infection, following an outbreak of waterborne giardiasis in Washington State, USA in 1976, in which *Giardia* cysts were detected in the raw water and storage reservoirs (Dykes et al., 1980). Three beavers trapped in the watershed area were infected with *Giardia*, implicating them as a potential source of the outbreak. However, the lack of morphological differences between genetic variants means that, at that time, it was not possible to determine whether the *Giardia* in the beavers was of the same species as in the patients, or the extent of genetic similarity or difference between the *Giardia* from the beavers and the infected people. If molecular tools had been available then, it may have been possible to exclude the beavers as the source of the *Giardia* contaminating the water supply – but what if molecular tools had shown a similar genotype? Would this have indicated that the beavers were

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the "guilty party", or would it have simply indicated that humans and some animals in this area were infected with similar genotypes of *Giardia*? And what of disease potential? Although the infected humans in this 1976 outbreak were obviously symptomatic, it is not evident that the beavers themselves were suffering from clinical disease. Two of the three beavers were dead-trapped, but the third one was live-trapped, and sent, alive, from Camas, Washington to Fort Collins, Colorado for infection studies in beagles (Dykes et al., 1980); given that no comment is made regarding signs in the beaver, it has to be assumed that none were observed.

In the database provided as supplementary information to the article considering EIDs in humans (Jones et al., 2008), of the 335 EID events noted to have occurred, 36 were designated as being due to protozoa. Table 1 is extracted from this reference, and gives an overview of the protozoal EID events considered; of the 35 protozoa listed (the nematode *Angiostrongylus cantonensis* is incorrectly described as a protozoa), a substantial proportion (19/35, 54%) are non-enteric, vectorborne parasites. Of the remaining 16, seven are enteric in some hosts; for a further six, all of which are microsporidia, it is currently unknown whether they may be enteric in some hosts. Of the remaining three, two are not enteric (residing in the urogenital tract), and one (a free-living, opportunistic amoeba) is probably not enteric. Of those that are categorized here as enteric or that their enteric potential for all hosts is unknown (n=13, highlighted in Table 1), only two, both microsporidia in the genus *Encephalitozoon*, are classified as being pathogens of wildlife origin (red font in Table 1).

2. Which enteric protozoan pathogens are zoonotic and what are their wildlife hosts? What information on public health importance has been obtained using molecular tools?

Despite the data from Jones et al. (2008) indicating that enteric protozoan parasites in wildlife are of relatively low public health relevance, we have chosen here to re-visit this topic. In particular, we have focussed upon the greater information obtained in the past decade by the use of molecular tools, and consider whether this extra information has assisted or confused us in determining the extent to which wildlife may act as a reservoir for enteric protozoa that may pose a threat to public health.

The first molecular tools used to address this question involved the use of antibodies and isoenzyme analysis. Currently, however, the most common approaches involve amplification and sequencing of one or more genomic DNA targets, selected to be either more conserved or more variable, depending on the focus of the investigation, followed by the use of phylogenetic analyses, including sequence polymorphism analyses, to determine relationships between the sequences obtained. It is these approaches and their results that are used predominantly in the following parasite-host specific sections.

One of the difficulties that we have in discussing this topic is the terminology. For example, throughout this manuscript we use the term "protozoa" to encompass the group of single-celled eukaryotic parasites under consideration, despite "protozoa" having no real taxonomic meaning. The term "protista" could have been used instead, but, again, the terminology is not founded on phylogenetic relationships, and the debate regarding how microorganisms, particularly eukaryotes, should be most appropriately classified has been a source of debate for centuries (Scamardella, 1999). Here we have chosen to use the term "protozoa" for convenience and to enable simpler comparison with other relevant articles, although some of the parasites covered are no longer considered to fit properly within this terminology. For example, although microsporidia are currently considered to be more related to fungi than other protozoan parasites (in the clade Opisthosporidia), we have chosen to include them here in line with Jones et al., (2008). We also include *Blastocystis* (which is not mentioned by Jones et al. (2008)), although this organism is now known to belong to the Stramenopiles, a group of organisms that includes, among others, brown algae, diatoms, and oomycetes.

Another terminology issue concerns what we actually mean when we refer to a pathogen as zoonotic. For example, if a pathogen that usually infects only animals is reported on just a single occasion in low numbers from a highly immunocompromised human patient, perhaps as an incidental finding, should it then be considered zoonotic? For the purposes of this document, we have described protozoans in such instances as that as being "potentially zoonotic"; an example of this could be *Cryptosporidium suis*. The adjective "zoonotic" is only used when we have clear evidence that the protozoa will readily establish in both humans and animals. The concept of zoonanthroponosis, which refers to diseases that are primarily infections of humans, but that

have the potential to be naturally transmitted to animals, is also of relevance – but not a major theme of this manuscript.

It is clear that enteric protozoan infections, in which, generally, robust transmission stages are excreted in the faeces, have the ability to contaminate the environment. These may be ingested by another possible host, be that wildlife, domestic animals, or humans, potentially resulting in infection, and possibly disease. However, the extent to which this spillover between groups of potential host species actually occurs is not necessarily clear. Not only does the likelihood of cross-infection between potential host groups depend on the ability of the parasite itself to infect the different hosts, but it also depends on factors relevant to the host (immunity, age, foraging or grazing habits, etc.), and also to the environment where the defecation occurs - whether survival and onwards movement of the transmission stage is favoured, and the likelihood that both host groups use the same environment. It is these interactions between the environment and the health status of people, their domestic animals, and wildlife that together form the basis of the One Health concept.

The likelihood of transmission of an enteric protozoan parasite to a person thus depends not only on whether the parasite has zoonotic potential, but also on a range environmental factors and aspects of the wildlife hosts' activity, mode of existence, and behaviour. Therefore, rather than list the potential wildlife hosts for each of the relevant protozoan pathogenic parasites considered here, a particular wildlife host has been selected per parasite. An overview of the parasite-wildlife host pairs considered is provided in Table 2. Brief information for each parasite is provided in the table, along with the rationale for the selection of the host species under consideration; that is, particular characteristics of the host that are relevant to transmission of enteric protozoa to people. The protozoan-wildlife hosts are then described in focused vignettes that indicate the current extent of our knowledge regarding zoonotic transmission and public health threat, with particular emphasis on the role of molecular methods in aiding our understanding of the threat to public health encompassed by the particular parasite in that specific wildlife host.

2.1 *Balantioides coli* (*Balantidium coli*) in wild boar

Paramecium coli, described by Malsten (1857) in human samples, was renamed *Balantidium coli* by Stein (1863) after describing it from pigs. Over the subsequent years, several *Balantidium* species were described from different wild and domestic mammals and birds based on morphological differences or the host species (Neiva et al., 1914; McDonald, 1922; Hegner, 1934). Alexeieff (1931) proposed transferring *B. coli* to a new genus, *Balantioides*, but, until recently, when Pomajbíková et al. (2013) proposed a new genus, *Neobalantidium*, to accommodate species from mammals, this was not taken into consideration; Chystyakova et al. (2014) consider *Neobalantidium* to be a junior synonym of *Balantioides*. As not all authors follow this taxonomic change, different names are used in scientific articles and in genetic databases: veterinarians and researchers not specialized in taxonomic discussions continue to use *Balantidium coli*, whereas specialists have used *Neobalantidium coli* and more recently, *Balantioides coli*. This gradual correction in naming of the parasite may be a source of confusion.

In a very detailed morphological study, McDonald (1922) proposed that pigs could be infected by two species, *B. coli* (which could also infect humans) and *B. suis* (pig-specific). However, there was some controversy about the validity of the second species and finally it was generally accepted as a synonym of *B. coli* (Awakian, 1937; Levine, 1961, 1985). Nevertheless, even in some recent papers (e.g., Schuster and Ramirez-Avila, 2008; Supriadi et al., 2012; Petrova et al., 2017), *B. suis* is still used as a name for the species found in pigs. Curiously, although pigs and wild boar are the same species, findings from wild boar are reported as *B. coli* or *Balantidium* sp., but not *B. suis* (e.g., Solaymani-Mohammadi et al., 2004; Mundim et al., 2004; Navarro-González et al., 2013; Yaghoobi et al., 2016).

Balantioides coli is commonly found in both pigs and wild boar (with prevalences ranging up to 100% in domestic pigs, and up to 70% in wild boar; Ponce-Gordo and Jirků-Pomajbíková 2018). Soon after the first description of this parasite, the epidemiological importance of pigs for human infections was noted. Pigs are considered the main reservoir and people living in close contact with them are at greatest risk of becoming infected with this parasite (Ponce-Gordo and Jirků-Pomajbíková, 2018). The epidemiological importance of wild boar is unknown; however, when infected animals congregate in the catchment area of public drinking water sources, then the risk

of transmission of their parasites to humans via contaminated water is likely to increase (Hampton et al., 2006). Transmission to humans via contamination of the environment is also likely to be associated with the apparently recent and increasing tendency for wild boar to invade urban areas, mainly searching for feed (Cahill et al., 2012). In Muslim countries, where pig farming is forbidden, wild boar are considered the main reservoirs of *B. coli* by some authors, but others consider other domestic mammals (camels, donkeys, sheep and goats) as the reservoirs of greatest importance to human health (Ponce-Gordo and Jirků-Pomajbíková, 2018). If parasite identification in human infections is based only on trophozoite and/or cyst morphology, it is not possible to identify the origin of the infections, and thus the most likely transmission routes remain unknown.

Genetic studies on *B. coli* started around 15 years ago, and currently the only genetic data available are for the nuclear small subunit rRNA gene (SSU-rDNA) and the internal transcribed spacer (ITS1-5.8S rDNA-ITS2) regions; *B. coli* does not possess mitochondria, and data for other genes have not been published to date. Despite only two ribosomal genes being currently available for comparative work, their analysis is useful for taxonomic studies and interesting results have been obtained. The comparison of SSU-rDNA sequences is a valid tool for taxon differentiation at the genus level or above, and sometimes also at the species level; however, for differentiating between closely related subtypes, analysis of the ITS region, and especially the ITS2 fragment, is considered the best option (Yao et al., 2010; Gou et al., 2012; Han et al., 2013).

The first *B. coli* sequence published was the SSU-rDNA from a gorilla isolate (Strüder-Kypke et al., 2006), but the first comparison of *B. coli* DNA isolated from different hosts (pig and ostrich) was made by Ponce-Gordo et al. (2008) by analysing sequences from the SSU-rDNA and ITS regions. In that study, pooled cysts were analysed; SSU-rDNA sequences showed some unresolved ambiguities common to all cyst isolates, and ITS sequences indicated two different sequence variants. To determine the importance of this ITS polymorphism (which could have represented two different species, but with low host specificity), a detailed analysis of individual cysts was made (Ponce-Gordo et al., 2011) and three important results were obtained: (1) the two ITS sequence variants previously identified had clear differences in both the ITS1 and ITS2 regions. (2) Both variants were found within single parasite cells, indicating that the

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polymorphism was present within one single species. (3) The same ITS sequences of both variants occurred in isolates from human, gorilla, pig and ostrich, indicating that the same species (*B. coli*) was found in all of them. Pomajbíková et al. (2013) and da Silva Barbosa et al. (2017) have also found no significant differences between *B. coli* DNA isolated from non-human primates (NHPs) and wild boar, or NHPs and pigs, respectively. Thus, these genetic data support the presence of a single zoonotic species, *B. coli*, that infects homeothermic vertebrates and can be transmitted between wild fauna (mainly wild boar), domestic animals (mainly pigs), and humans.

2.2 *Blastocystis* sp. in wild rodents

Blastocystis sp. has been found in most animal species investigated to date. Its pathogenicity is controversial (Stensvold and Clark, 2016a), although more recent articles describe it as being part of the microbiome, and influencing, or influenced by, the composition of bacterial communities (Nieves-Ramirez et al., 2018; Tito et al., 2018). It is clear that subtyping of *Blastocystis* isolates is critical for evaluating the relationship between the parasite, gut microbiota profile, and host health. It was long assumed that one species of *Blastocystis*, *Blastocystis hominis*, infected humans, and different species of *Blastocystis* infected other animals. However, genetic analyses have demonstrated that there is no single *Blastocystis* entity that infects only humans; many (but not all) of the subtypes (ST) identified in animals also infect humans, and currently *Blastocystis* tends to be identified by the genus name and the ST number.

There are only a few epidemiological studies that investigate the transmission of *Blastocystis* from rodents, and these mainly focus on trapping and euthanizing the hosts to collect their caeca (Seifollahi et al., 2016; Yoshikawa et al., 2016b; Katsumata et al., 2018), followed by detection using a smear examined by light microscopy or incubation of the sample in Jones' medium (Katsumata et al., 2018) or agar slant (Yoshikawa et al., 2016b) for three to five days, before microscopy examination of stained or unstained samples. Although these approaches continue to be used (Katsumata et al., 2018), they are being replaced by molecular techniques, not only because *Blastocystis* can be easily confused with other microorganisms (e.g., *Entamoeba*) – and thus determining the prevalence of *Blastocystis* by microscopy or culture methods is likely to result in an inaccurate estimate (Wawrzyniak et al., 2013) - but also because the culture methods

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may select for particular subtypes (Roberts et al., 2011). PCR and qPCR methods that partially or completely amplify the SSU-rDNA are sufficiently sensitive for both identifying and subtyping *Blastocystis* (Roberts et al., 2011; Wawrzyniak et al., 2013; Stensvold and Clark, 2016b).

Using a combination of these techniques, in both restricted (Betts et al., 2018; Farah Haziqah et al., 2018) and wider (Cian et al., 2017) sampling regions, has demonstrated that subtypes ST1, ST2, ST4, ST5, ST10, and ST17 predominate in rodents, and, in some cases, mixed infections (of two STs) have been identified (Cian et al., 2017; Betts et al., 2018; Farah Haziqah et al., 2018). Of these subtypes, ST1, ST2, ST4, and ST5 have also been found in humans (and thus have zoonotic potential), whereas ST10 and ST17 have not, to date, been identified in humans, being exclusively found in animals. In human infections, ST3 and ST4 often predominate, but this may vary between locations and circumstances.

Although the different rodent studies using molecular tools have used similar approaches, each used a different set of primers to target the same (5'-end) region of the SSU-rDNA and the (phylogenetic) analyses of the amplified and sequenced regions tend to be inconsistent, sometimes due to the short length of the amplified fragment. For subtyping studies to provide meaningful results, phylogenetic trees should include all 17 subtypes, and be rooted and constructed using both maximum likelihood and Bayesian inference methods (Betts et al., 2018); this has not been the case in all studies.

This means that although some articles have reported on *Blastocystis* in rodent populations, it is difficult to investigate transmission dynamics, and further studies are important to elucidate the circulation of different *Blastocystis* subtypes within rodent populations. For example, Betts et al. (2018) identified *Blastocystis* infections in both wild and captive water voles (*Arvicola amphibius*), but the subtypes differed, with ST4 dominant in the wild voles and ST1, which was not identified in wild water voles, also present in captive voles. When wild water voles were brought into captivity, ST1 started circulating within this population. How this happened is difficult to determine, although it is tempting to speculate that this could reflect a microbiota-associated effect, related to life in captivity (Betts et al., 2018).

Current molecular methods for the investigation of *Blastocystis* subtypes in rodents and other wildlife, especially those described in Betts et al. (2018), match those used for investigating *Blastocystis* subtypes in humans (Alfellani et al., 2013; Yoshikawa et al., 2016a). The potential

for zoonotic transmission is indicated by the same ST being found in rodents and humans. However, further studies are needed to determine the extent to which this occurs, whether the single locus evaluation is sufficient to determine genetic identity, and, overall, whether this similarity in *Blastocystis* subtypes in humans and rodents is of public health significance.

2.3 *Cryptosporidium* spp. in wild fish

Wild fish represent a source of *Cryptosporidium* infection for humans. This may be via: (1) consumption of raw or undercooked fish flesh that has been contaminated with oocysts, and (2) consumption of water contaminated with oocysts shed in fish faeces. Despite these two potential routes for transmission, relatively few molecular studies have been conducted on *Cryptosporidium* in fish and the majority of these have been on farmed or aquarium fish (Murphy et al., 2009; Zanguee et al., 2010; Barugahare et al., 2011; Gibson-Kueh et al., 2011; Morine et al., 2012; Ryan et al., 2015; Yang et al., 2015; Palermo, 2016; Yang et al., 2016; Paparini et al., 2017; Couso-Pérez et al., 2018), with only a handful of studies on wild fish or, particularly, wild fish commonly consumed by people (Alvarez-Pellitero and Sitja-Bobadilla, 2002, Palenzuela et al., 2010; Reid et al., 2010; Certad et al., 2015) (Table 3).

Currently, three species of *Cryptosporidium* have been described from fish that are not found in other hosts. These are: (1) *Cryptosporidium molnari*, which was originally described in wild gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Alvarez-Pellitero and Sitjà-Bobadilla, 2002) and was characterized genetically some years later (Palenzuela et al., 2010); (2) *Cryptosporidium scophthalmi*, described in wild turbot (*Psetta maxima* syn. *Scophthalmus maximus*) (Alvarez-Pellitero et al., 2004), and a *C. scophthalmi*-like strain characterized genetically in 2015 (GenBank accession numbers: KR340588 and KR340589), and (3) *Cryptosporidium huwi* (previously piscine genotype 1) in a captive guppy (*Poecilia reticulata*) (Ryan et al., 2015).

Molecular characterisation has also identified piscine genotypes 2-9, two different *C. molnari*-like genotypes, more than 8 un-named novel genotypes, *C. parvum*, *C. hominis*, *C. xiaoi*, *C. scrofarum*, and rat genotype III in fish (Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Barugahare et al., 2011; Morine et al., 2012; Koinari et al., 2013; Certad et al., 2015; Ryan

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et al., 2015; Yang et al., 2015; Yang et al., 2016; Palermo, 2016; Couso-Pérez et al., 2018). Both *C. scrofarum* and *C. xiaoi* have been identified in western school whiting (*Sillago vittata*) (Reid et al., 2010) and species could be of some public health importance; *C. scrofarum* has been reported in several cases of human cryptosporidiosis (Kváč et al., 2009; Xiao, 2010), and *C. xiaoi* has been reported in two patients in Ethiopia (Adamu et al., 2014). *Cryptosporidium hominis* was identified in wild mackerel scad (*Decapterus macarellus*) in Papua New Guinea (Koinari et al., 2013) and more recently in farmed goldfish (*Carassius auratus*) in Australia (Palermo, 2016). *Cryptosporidium parvum*, the species most associated with zoonotic infection, was identified in western school whiting (Reid et al., 2010) and in goldfish (Palermo, 2016) in Australia, in wild-caught mackerel scad and silver barb (*Puntius gonionotus*) from Papua New Guinea and in cultured Nile tilapia (*Oreochromis niloticus*) (Koinari et al., 2013). In Lake Geneva, France, *C. parvum* was detected in Arctic char (*Salvelinus alpinus*), European whitefish (*Coregonus lavaretus*), European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) (Certad et al., 2015). In the latter study, *C. parvum* was identified at a high prevalence in freshwater fish (13/15, 87%) and *C. parvum* developmental stages were detected in fish intestines, suggesting that this was infection, rather than simply carriage (Certad et al., 2015).

Only three studies have conducted glycoprotein-60 (*gp60*) subtyping on *Cryptosporidium* DNA isolated from wild fish samples, although this tool has become relatively standard for identifying subtypes in potential reservoir species for human infection: (1) *C. parvum* IIaA18G3R1 was identified in western school whiting from Australia (Reid et al., 2010), (2) *C. hominis* IdA15G1 in mackerel scad and *C. parvum* IIaA15G2R1 and IIaA19G4R1 subtypes in mackerel scad and silver barb respectively from Papua New Guinea, and (3) in the study in France, *C. parvum* subtypes IIaA15G2R1, IIaA16G2R1, and IIaA17G2R1 were reported (Certad et al., 2015). These *C. parvum* subtypes commonly infect both livestock and humans (Xiao, 2010). In the study in Papua New Guinea, the *C. hominis* detected in the fish could have come from spillback from the human population due to poor sanitation infrastructure, but as parasites were not observed by histology, it was not possible to determine whether this was an actual infection or carriage.

The results presented here show that use of molecular tools has been instrumental in determining the zoonotic potential of *Cryptosporidium* detected in wild fish. They have demonstrated that

although wild fish are infected with apparently host-specific species and genotypes of *Cryptosporidium* (*C. molnari*, *C. scophthalmi*, and piscine genotype 3), there is also the potential that human-infectious species (*C. hominis*, *C. parvum*, *C. scrofarum*, and *C. xiaoi*) may be identified in samples from wild fish. For *C. parvum*, at least, the investigations represented true infections, indicating propagation. However, these studies are preliminary and scattered, and further investigations to support these initial findings, preferably with infection studies, are essential in order to better understand the likelihood of wild fish representing a public health risk for transmission of *Cryptosporidium*.

2.4 *Encephalitozoon* spp. in wild birds

Birds are often infected by microsporidian parasites in the genus *Encephalitozoon* (Hinney et al., 2016; Sak et al., 2010). *Encephalitozoon* spp. have been identified in a wide variety of avian hosts, including in the Orders Anseriformes, Apodiformes, Ciconiiformes, Columbiformes, Falconiformes, Gruiformes, Passeriformes, Podicipediformes, Struthioniformes, and Suliformes, and also in many countries (Hinney et al., 2016). *Encephalitozoon intestinalis*, which is the most prevalent *Encephalitozoon* species in humans and also infects various mammalian species (e.g. livestock, dogs, and NHPs), has been reported only sporadically from birds (Pirestani et al., 2013; Galvan-Diaz et al., 2014; Tavalla et al., 2018), whereas *E. hellem*, which is considered bird-specific, and, to a lesser extent, *E. cuniculi*, which is considered mammal-specific, have been reported frequently from birds (Hinney et al., 2016).

Most human infections are thought to result from faecal-oral transmission of spores. *Encephalitozoon* spores have been detected in various water sources (irrigation water, recreational water, drinking water, and wastewater) and food (Dowd et al., 1998; Fournier et al., 2000; Decraene et al., 2012; Kváč et al., 2016). In addition, spores can be aerosolized from disturbed excrement and could be inhaled by hosts as airborne particles (Graczyk et al., 2008). As *Encephalitozoon* spp. from birds have been shown to be capable of infecting people, both immunocompetent and immunocompromised (Didier and Weiss, 2011), there is a need for better understanding of the role of birds as a reservoir of human microsporidiosis (Table 4).

Genotyping has proven useful for high-throughput sample screening and, despite a limited number of molecular markers, has revealed some important details about heterogeneity among and within *Encephalitozoon* species. Most studies have targeted the ITS region of ribosomal RNA genes (ITS, Fig. 1A) (Hinney et al. 2016). Less common markers include the polar tube protein (PTP; Fig. 1B), SSU-rDNA, intergenic spacers of ribosomal genes, (IGS) IGS-TH and IGS-HZ, and the spore wall protein (SWP) gene (Mathis et al., 1999; Xiao et al., 2001a,b; Haro et al., 2003). Sequence analysis of these markers has distinguished four *E. cuniculi* genotypes (I, II, III, and IV) and seven *E. hellem* genotypes (1A, 1B, 1C, 2A, 2B, 2C, and 2D) (Mathis et al., 1999; Xiao et al., 2001; Haro et al., 2003; Galván et al. 2013; Table 4). *Encephalitozoon cuniculi* genotypes I, II and III have also been differentiated by fragment size analysis of the Spo11 gene (Selman et al., 2013).

Although several studies have been conducted, genetic heterogeneity in the ITS and the PTP genes has not yet been observed in *E. intestinalis* (Didier et al., 1996; del Aguila et al., 1998; Rinder et al., 1999; Sobottka et al., 1999; Liguory et al., 2000; Xiao et al., 2001b). Genotypic variation of *E. intestinalis*, genotypes I and II, has been demonstrated by Galván et al. (2013) at the M2A, M3 M5, M7A, and M8 loci, but this approach has not been applied to broader surveys. As almost all *Encephalitozoon* genotypes can be distinguished by sequencing at the ITS and PTP loci, these are most commonly used as they enable comparison of results among studies, and these loci can be recommended for routine investigations.

A major constraint to investigations of whether birds are a relevant source of human *Encephalitozoon* infection is that spore shedding is generally intermittent and therefore difficult to detect; the limit of detection may also be reached at lower levels of shedding. Extensive sampling is therefore required to determine whether an animal is infected; for example, Sak et al. (2010) screened nine budgerigars (*Melopsittacus undulatus*) naturally infected with *E. hellem* genotypes 1A and 2C (previously known as genotype 3) and *E. cuniculi* genotype III for spore shedding. Although the cumulative prevalence of *Encephalitozoon* spp. was 100%, daily prevalence ranged from 0 to 67%, with a mean of 27%. Such intermittent shedding of spores has also been reported for infections in humans, rodents, and horses (Sak et al., 2011a, 2017; Wagnerová et al., 2013), suggesting that, without repeated sampling, the prevalence of *Encephalitozoon* spp. in host populations could be underestimated.

A second major limitation to investigations of whether birds are a relevant source of human *Encephalitozoon* infection is due to the broad host range, which may limit the value of genotyping for identifying sources of human infection and means that molecular results must be supported by traditional epidemiological investigations. For example, *E. intestinalis* and all *E. cuniculi* genotypes infect several mammals and birds (Hinney et al., 2016). In contrast, *E. hellem* almost exclusively infects birds, and genotypes show no differences in host specificity. Nevertheless, genotyping should be an integral component of epidemiological surveillance and genetic data should be interpreted together with the case report and not separately. As an example, Haro et al. (2003, 2005) demonstrated that *E. hellem* genotype 1A from human immunodeficiency virus-positive patients was apparently the identical genotype to that of *E. hellem* identified in urban pigeons in a park (using a PCR amplifying a gene fragment of 208 bp, including the ITS region), and, based on this, suggested that these birds could be considered a potential source of human infection. However, direct evidence of human infection through contact with pigeons was lacking. Kašičková et al. (2009) and Sak et al. (2011b) later demonstrated that this genotype infected other avian and mammalian hosts, and therefore, in the absence of traditional epidemiological associations, pigeons were not necessarily the source of the infection. Use of further genetic markers may strengthen or weaken an association, but traditional epidemiological investigations cannot usually be replaced by molecular techniques at this time for confirming zoonotic transmission routes.

2.5 Pathogenic *Entamoeba* spp. in non-human primates (NHPs)

In the early 1900s, many species of *Entamoeba* were described in NHPs. By light microscopy, these were, for the most part, indistinguishable from the species found in humans. In his great work of 1919, "The Amoebae Living in Man", Dobell concluded on p. 133 that "it is by no means impossible that the amoebae are really identical" to *E. histolytica* and *E. coli* (the only *Entamoeba* species he recognized as colonising the human gut), and that "there is as yet no proof that monkeys harbour *Entamoebae* in any way different from those of man". Subsequently, based on his own life-cycle studies and experimental infection results, he concluded that the species were indeed the same.

This understanding of *Entamoeba* species in NHPs persisted unchallenged for many decades. Implicit in this view is that NHPs are a potential reservoir for *E. histolytica* and therefore for an important disease of humans. Supporting his conclusion were reports of dysentery (e.g., Eichhorn and Gallagher, 1916) and liver abscesses (e.g., Castellani, 1908) in NHPs that, based on both morphology and histology, were apparently caused by *E. histolytica*.

Molecular tools arrived with the use of antibodies and isoenzyme analyses in the 1970s. Almost immediately both approaches identified that there were two distinct groups within *E. histolytica*, one linked to cases of disease (named pathogenic zymodemes) and one linked to asymptomatic infections (non-pathogenic zymodemes). Application of isoenzyme analyses to isolates from captive (e.g., Smith and Meerovitch, 1985) and wild (Jackson et al., 1990) NHPs initially indicated that all their *E. histolytica* belonged to non-pathogenic zymodemes. The accumulation of DNA data – restriction fragment length polymorphisms, Southern blots, gene sequences - and other information, eventually led to non-pathogenic zymodemes being reclassified as a distinct species of *Entamoeba*, *E. dispar* (Diamond and Clark, 1993); it was concluded that NHPs mostly harboured *E. dispar*.

Thus, use of molecular tools initially led to a switch from viewing NHPs as a potential reservoir of *E. histolytica* to viewing them as carrying primarily non-pathogenic *Entamoeba* species. The use of DNA-based tools for detection and differentiation of *Entamoeba* species spread rapidly, initially with the use of PCR alone and subsequently with PCR combined with DNA amplicon sequencing. The PCR target was primarily SSU-rDNA, but the divergence between *E. histolytica* and *E. dispar* is such that many genes are suitable targets for species-specific PCR tests, if desired.

One of the applications of these tools was screening of primates imported for medical research in order to identify those carrying pathogens, including *E. histolytica*. This led to the discovery that *E. histolytica* was not the (only) pathogenic *Entamoeba* in NHPs (Suzuki et al., 2007; Tachibana et al., 2007; Takano et al., 2007). Initially described as a variant of *E. histolytica*, this *Entamoeba* species is now generally referred to as *E. nuttalli* – the name originally given to the species responsible for liver abscesses in macaques by Castellani in 1908. Pathogenic in both primates and rodent models, *E. nuttalli* is closely related to, but definitely distinct from, *E. histolytica*. However, most tools that had been developed at that time to differentiate *E. histolytica* from *E.*

dispar did not differentiate *E. histolytica* from *E. nuttalli*. The SSU-rDNA sequences of *E. histolytica* and *E. nuttalli* differ by less than 1%, compared with over 2% divergence between *E. histolytica* and *E. dispar*, and, as a result, many previously designed PCR primers annealed to sequences that were identical in *E. histolytica* and *E. nuttalli*. Species-specific primers for *E. nuttalli* SSU-rDNA were developed quickly (Tachibana et al., 2007). Gene sequences, such as those encoding chitinase and the serine-rich protein (Takano et al., 2007; Tachibana et al., 2007), and isoenzymes (Suzuki et al., 2007; Tachibana et al., 2007) also differentiate between *E. histolytica* and *E. nuttalli*, as do the tRNA-linked non-coding short-tandem-repeats that can be used for genotyping of all three species (e.g., Guan et al., 2016; Feng et al., 2018). In no sequence datasets do the two species overlap.

It seemed likely, therefore, that reports of *E. histolytica* carriage and disease in NHPs attributed to *E. histolytica* were actually due to *E. nuttalli*. In addition, because isoenzyme analyses can differentiate between *E. histolytica* and *E. nuttalli*, the absence of the *E. nuttalli* 'variant' pattern among the thousands of human samples that were analysed in the 1980s (Sargeaunt, 1989) suggested that humans are not hosts for *E. nuttalli*. Thus, a host specificity seemed clear; *E. histolytica* was infective to humans and *E. nuttalli* infected NHPs – neither species was zoonotic.

This simple view did not last long; some true *E. histolytica* infections were reported in NHPs (Verweij et al., 2003; Rivera et al., 2010) and then infection of a zookeeper with *E. nuttalli* was reported, indicating that humans could be susceptible to colonisation if exposed (Levecke et al., 2015). No cases of invasive disease in humans attributed to *E. nuttalli* have been reported to date. Most recently, using high throughput sequencing, populations of humans and wild gorillas living in a nature reserve in Cameroon were both found to be carrying *E. histolytica* and *E. nuttalli* and *E. dispar* (Vlčková et al., 2018). The patterns of transmission in this location remain unclear, but this finding does demonstrate unequivocally that humans and great apes can be colonised by both *E. histolytica* and *E. nuttalli*.

Over time, our perception of the potential for NHPs to be reservoirs for *E. histolytica* has shifted several times, from microscopy indicating that NHPs were reservoirs of *E. histolytica*, to isoenzymes indicating that they were not, to NHPs carrying a pathogen - but not *E. histolytica* - to the current view, which is, we suspect, "it depends". Where contact exists between NHPs and humans, the potential for *E. histolytica* to be transmitted between the two host groups is real.

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However, the circumstances in Cameroon are not likely to be common and even if, technically, NHPs can be a reservoir for human infection with *E. histolytica*, it seems unlikely that they are a major source of the parasites responsible for invasive amoebiasis in humans. Molecular tools may assist in clarifying the species of *Entamoeba* present in NHPs, but as yet they cannot fully clarify the public health threat that they pose.

2.6 *Enterocytozoon bieneusi* in wild cervids

Among the 1300 –1500 formally described species (within 187 genera) of microsporidia (Wang et al., 2018), 14 species infect humans, and, of these, *Enterocytozoon bieneusi* is the most common and reported to be responsible for more than 90% of human cases of microsporidiosis (Matos et al., 2012).

Although light microscopy of stained clinical smears is commonly used for diagnosis of microsporidia infections in humans and animals (Zhao et al., 2017), the small spore size and lack of definitive staining characteristics mean that detection of *E. bieneusi* by this technique is difficult (Li et al., 2016). As genotype identification cannot be assessed by microscopy, many of the investigations of *E. bieneusi* infections in cervids (and other hosts) use molecular tools. Distinguishing between different genotypes of *E. bieneusi* to date has usually been based on analysis of the ITS region, amplified by nested PCR. The occurrence of both single-nucleotide polymorphisms (SNPs) and short polymorphic insertions or deletions in these sequences is usually used to identify *E. bieneusi* ITS genotypes (Buckholt et al., 2002), and based on nucleotide divergence within the ITS gene fragment, over 300 *E. bieneusi* genotypes have been defined in humans and animals (Wang et al., 2018).

Two pairs of primers (EBITS3 and EBITS4, and EBITS1 and EBITS2.4) have mostly been used to amplify a 390-base pair (bp) fragment of the rDNA (containing 76 bp of the 3 ′-end of SSU-rDNA, 243 bp of the ITS, and 71 bp of 5′-region of the large subunit (LSU)-rDNA) for studies on *E. bieneusi* in cervids (Buckholt et al., 2002). However, another study used the alternative primers (MSP-1 and MSP-2B, and MSP-3 and MSP-4B) to amplify a 535 bp sequence, which also includes the 243 bp ITS gene sequence (Zhang et al., 2018).

Based on ITS sequence identification, an average prevalence of *E. bieneusi* in cervids of 18.4% (415/2251) has been reported from the combined results of 15 studies, the majority of which have been conducted in China and have included at least 8 different cervid species (Table 5). Among the studies conducted, genotype BEB6 predominates, having been identified in 40.3% (170/422) of the known genotypes and in 10 of the studies (Table 5).

In such studies, zoonotic potential is assessed by phylogenetic analysis of the ITS sequences, with those isolates of *E. bieneusi* that cluster with known zoonotic isolates being considered to be potentially zoonotic (Figure 2). Overall, 62 different *E. bieneusi* genotypes have been identified in cervids using ITS sequence-polymorphism analysis (Table 5); of these, around 44% (27/62) clustered into the group with zoonotic potential, thereby raising public health concerns (Santín and Fayer, 2009). Although a genotype (BEB6) that is not in the zoonotic potential group appears to predominate in cervids, the second most prevalent genotype SC03 (9.2%; 39/422), reported from two studies, clusters in the zoonotic potential group. However, most of the genotypes (54.8%; 34/62) were only identified in a single specimen.

Multilocus sequence typing (MLST) analysis, with high-resolution and targeting three microsatellites (MS1, MS3 and MS7) and one minisatellite (MS4), has been widely used for investigating multilocus genotypes (MLG) of *E. bieneusi* in humans and animals (Feng et al., 2011; Wang et al., 2018). For cervids, this MLST approach has been used in studies including *E. bieneusi* DNA isolated from samples from red deer, hog deer, sika deer, and musk deer (Li et al., 2016; Song et al., 2018). In one study, strains of the same ITS genotype (BEB6) were identified as including two different MLGs (Li et al., 2016), and in another study DNA of the potentially zoonotic ITS genotype, SC03, was reported to include two different microsatellite MS3 types (Song et al., 2018). The significance of the higher resolution of the MLST tool regarding the zoonotic potential of *E. bieneusi* in cervids is yet to be clarified. However, these data suggest that use of a single genetic locus to determine whether two isolates are similar enough to be considered identical may be insufficient, and may result in strains being incorrectly classified as indicating a particular transmission source, whereas additional genetic information may provide a more nuanced and accurate picture. Thus, although molecular tools have indicated the potential for *Enterocytozoon* spp. from cervids to pose a threat to public health, as tools become more discriminatory, our current understanding may require revision.

2.7 *Giardia duodenalis* in red foxes

Giardia duodenalis, often described as being a ubiquitous protozoan of major global public health significance, is commonly found in a range of host species, including humans, domestic animals, and wildlife (Feng and Xiao, 2011). However, although considerable molecular data on *G. duodenalis* isolated from samples from various host species have been accumulated, interpreting these data, regarding whether they represent zoonotic potential, is not entirely straightforward; even nomenclature remains controversial, with some authors referring to genetic clusters as Assemblages A to H (with some sub-assemblages within these larger groups), whereas others report the assemblages as representing distinct species (Feng and Xiao, 2011; Thompson and Ash, 2016). Indeed, the lack of consistent systems for characterising and naming strains from different host species has led to some authors claiming "new" sub-assemblage groups, even when this is based on only a few minor SNPs compared with a previously reported genotype, and without any other defining epidemiological traits (Ye et al., 2012).

Nevertheless, it is clear that different assemblages exhibit different patterns of infection; both Assemblages A and B have wide host ranges, infecting humans and a variety of animal species, whereas Assemblages C to H are considered to be more host specific, infecting predominantly canids (C and D), bovids and suids (E), felids (F), rodents (G), and pinnipeds (H) (Feng and Xiao, 2011). Based on this nomenclature and division, it is common to refer to *Giardia* cysts belonging to Assemblages A or B as having zoonotic potential. However, this is not clear-cut, as some sub-Assemblage groups apparently are not zoonotic (e.g., AIII; Sprong et al., 2009), and there are an increasing number of reports of assemblages other than A and B found in humans (Cacciò et al., 2018).

Various characteristics of the red fox (see Table 2), mean that this species is of particular interest as a reservoir of infections of importance to public health. Nevertheless, although red foxes have been commonly reported to excrete *Giardia* cysts, the role that they play, if any, in the zoonotic transmission of *G. duodenalis* is unclear (Onac et al., 2015; Debenham et al., 2017; Mateo et al., 2017). Dogs are in the same family (Canidae) as foxes but have a much closer relationship and contact with humans, and thus a greater potential to share pathogens. However, dogs tend not to

be considered an important source of zoonotic transmission, being largely infected with canid-specific Assemblages C and D (Ballweber et al., 2010).

Molecular studies seeking to investigate the potential role of red foxes as a reservoir of *G. duodenalis* of public health importance, have primarily focused on using conventional PCR to amplify sequences within various genetic loci (including SSU-rDNA and ITS1 and ITS2, and targets in glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), and β-giardin (*bg*) genes) to determine the assemblages most commonly occurring (Hamnes et al., 2007; McCarthy et al., 2007; Beck et al., 2011; Onac et al., 2015; Debenham et al., 2017; Mateo et al., 2017). For sub-assemblage genotyping, SSU-rDNA has insufficient genetic variation, thus *tpi*, *bg*, and *gdh*, and, to a lesser extent, ITS1 and ITS2 genetic targets are used (Stojecki et al., 2015; Debenham et al., 2017; Mateo et al., 2017).

In general, however, amplification of these sequences of *G. duodenalis* from red fox samples has a poor success rate, compared with *G. duodenalis* from humans and other animals, such as ruminants. Among those studies in which PCR was performed on samples already confirmed to contain *Giardia* cysts by immunofluorescent antibody microscopy (IFA), poor amplification success was observed across all five of the common gene loci (Table 6). In *G. duodenalis* isolated from samples from other host species, SSU-rDNA appears to provides the greatest amplification success, probably at least in part due to it being found in multiple gene copies, and is therefore often used for detection (Thompson and Ash, 2016). However, in these studies on *Giardia* from fox samples, even at this locus, good amplification was usually not observed. Similar limitations of PCR have been reported by Onac et al. (2015), who obtained positive amplification in 10 out of 217 fox samples at the *bg* gene, but only three of these gave positive results for *G. duodenalis* when sequenced, and by Mateo et al. (2017) who obtained DNA amplification in seven out of 87 fox samples at the SSU-rDNA, but none of these resulted in amplification by qPCR at the *gdh* gene.

Due to their close relationship with domestic dogs, it may be expected that red foxes would be primarily infected with *G. duodenalis* Assemblages C or D (reported to be specific to canid hosts; Thompson and Ash, 2016), and the most frequently detected assemblages in dogs (Ballweber et al., 2010). However, this does not appear to be the case, with the majority of *Giardia* DNA isolated from red fox samples, and for which characterisation has been successful,

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being found to be Assemblage A or B (Hamnes et al., 2007; McCarthy et al., 2007; Beck et al., 2011; Onac et al., 2015). Additionally, Ng et al. (2011) amplified *Giardia* SSU-rDNA in 32 % (6/19) of fox samples, and sequence results revealed the presence of Assemblage D in only two samples, but A and/or E in four samples. Given that foxes coexist with domestic dogs infected with Assemblages C and D, and are known to eat rodents, as well as both wild and domestic ungulates, these findings are both unexpected and interesting and may suggest that foxes play a greater role in the zoonotic transmission of this parasite than dogs. However, most of these results are based on sequences obtained from amplification of a single gene locus, and thus should be interpreted with caution.

Despite some results having been obtained, the generally poor success of these molecular tools in investigating *Giardia* isolated from the red fox samples (and, to some extent, from other canids also) is interesting when compared with the much better results obtained using the same tools on *Giardia* collected from human or domestic livestock samples. One reason for this poor amplification success could be due to collection variables; samples collected from wildlife often require extended storage and this could impact cyst or DNA integrity. In addition, the sample populations are usually non-selected apparently healthy foxes, rather than symptomatic individuals suffering from giardiasis, which is the case in many human studies and sometimes for studies in domestic livestock. However, given similar limitations of PCR are seen in other canids then a more host-specific explanation should be considered; for example, the faecal matrix of canids may contain inhibitors that are not accounted for using traditional DNA extraction or PCR amplification techniques (Stojecki et al., 2015; Sommer et al., 2015).

Overall, although molecular tools have suggested that red foxes should not be dismissed as potential reservoirs of *Giardia* of public health relevance, the apparently poor sensitivity of conventional PCR tools for amplifying gene sequences from *Giardia* DNA obtained from red foxes has greatly limited our ability to reach a meaningful conclusion. The most widely used molecular tools for *Giardia* investigations remain of limited value for both detection and characterisation of *Giardia* from red foxes. Until these issues are addressed and resolved, our understanding of the role of this widespread predator as a reservoir of zoonotic *G. duodenalis* will remain limited.

2.8 *Sarcocystis nesbitti* in snakes

Between the years 1993 and 2014, several outbreaks of extra-intestinal or invasive muscle sarcocystosis occurred among travellers returning from central Malaysia and Malaysian islands. Until then, reports on muscle sarcocystosis in humans had been relatively rare, although an unexplained accumulation of reports from South-East Asia, especially from Malaysia, had been noticed (Beaver et al., 1979; Kan and Pathmanathan, 1991; Wong and Pathmanathan, 1992; Fayer et al., 2015). Differences in tissue cyst morphology between cases had indicated that various *Sarcocystis* species were involved and probably humans were aberrant, dead-end hosts (Beaver et al., 1979; Fayer et al., 2015). Most of the case descriptions from Malaysia were incidental biopsy findings, with the exception of an outbreak affecting seven members of a 15-man U.S. military team, for which a positive muscle biopsy was also reported in one of the cases (Arness et al., 1999).

The most recent outbreaks were large, involving more than 100 persons, and initiated a more through diagnostic investigation and follow-up of patients (Von Sonnenburg et al., 2012; Abubakar et al., 2013; Esposito et al., 2014; Italiano et al., 2014; Tappe et al., 2014). One of the characteristics observed in affected humans was a biphasic course of the disease starting about two weeks after return from Malaysia, often with fever, frequently myalgia, fatigue, and headache, and, less often, arthralgia. After a period of remission, a second phase started around six weeks after return, this time myalgia was the dominating symptom, followed by fever and fatigue and less often arthralgia and headache (Esposito et al., 2014).

Biopsies were taken from the patients involved in the more recent outbreaks on the Malaysian islands of Tioman and Pankor, but sarcocysts were identified by histology in only a few patients (Abubakar et al., 2013; Esposito et al., 2014; Italiano et al., 2014). However, using PCR primers targeting part of the SSU-rDNA locus and originally developed to amplify *Sarcocystis* DNA from ruminants, amplicons were obtained. Sequencing revealed them to be identical to an SSU-rDNA sequence obtained from sarcocysts from a crab-eating macaque (*Macaca fascicularis*) in China that had been suspected to be infected with *Sarcocystis nesbitti* (Yang et al., 2005; Tian et al., 2012). *Sarcocystis nesbitti* had first been described from a rhesus macaque (*Macaca mulatta*) from Northern India (Mandour, 1969). As material from this initial description was not available for morphological and molecular confirmation that Mandour (1969) and Yang et al. (2005) were

actually describing the same species, the *Sarcocystis* found in these infected patients were referred to as *S. nesbitti*-like by others (Dubey, 2015).

Phylogenetic analyses (Tian et al., 2012; Abubakar et al., 2013) of sequences from *S. nesbitti*-like parasites from *M. fascicularis* grouped this species close to *Sarcocystis singaporensis* (Jäkel et al., 2001) and *Sarcocystis atheridis* (Šlapeta et al., 2003), both of which infect snakes. Based on this and further phylogenetic analyses, it appeared very likely that sporocysts derived from the faeces of infected snakes may have been the source of the Malaysian outbreaks of extra-intestinal sarcocystosis (Lau et al., 2013, 2014), and that people may have become infected by oral uptake of these sporocysts in, for example, contaminated water or on fresh produce consumed raw (Lau et al., 2014; Fayer et al., 2015). Other reptiles, such as monitors, have also been discussed as potential definitive hosts and the source of the human infections (Tappe et al., 2013). *Sarcocystis nesbitti*-like sequences were also identified among DNA isolated from faecal samples from wild reticulated python (*Braghammerus reticulatus*) and monocled cobra (*Naja kaouthia*) captured in Malaysia (Lau et al., 2013) and in sediment samples from tank and river water collected at Tioman Island, Malaysia (Shahari et al., 2016). The suggested life cycle at that time included monkeys (*Macaca mulatta*, *Macaca fascicularis*, *Cercocebus atys*, and *Papio papio*) as natural intermediate hosts with snakes (cobra and python) as definitive hosts (Lau et al., 2013, 2014). Humans were regarded as aberrant, dead-end intermediate hosts (Dubey, 2015); their susceptibility may result from the close phylogenetic relationship with NHPs, the natural intermediate hosts.

Although molecular tools assisted diagnosis of this sarcocystosis outbreak, their diagnostic sensitivity seems to be low. The first signs of invasive sarcocystosis in people were generally non-specific (fever, headache, myalgia) and similar to those seen during other infectious diseases, including parasitoses such as toxoplasmosis and trichinellosis. Whether circulating parasites or parasite DNA could be detected in blood of early-stage patients is unknown, and more sensitive diagnostic methods are necessary. During the second phase of invasive sarcocystosis, patients developed myositis but obtaining positive biopsies nevertheless remains challenging. Sarcocysts were only observed in 6/14 patients in the Malaysian outbreak cohort, despite intensive searches including examination of more than 60 sections from a single muscle biopsy (Esposito et al., 2014). It has been suggested that the chance of a positive finding may

increase should biopsy material be collected from sites where magnetic resonance imaging suggests myositis (Italiano et al., 2014) or sites where muscles are swollen, painful, or show signs of inflammation (Fayer et al., 2015).

Despite molecular tools having been key in clarification of the outbreaks of sarcocystosis in Malaysia, the full identity and life cycle of *S. nesbitti* is still a matter of discussion. A recent publication reported a finding of *S. nesbitti*-like SSU-rDNA in an Australian scrub python (*Simalia amethistina*, sampled at the Cape York Peninsula, Queensland); as NHPs, which were regarded as the natural intermediate hosts of *S. nesbitti* are not found in Australia, such a finding is unexpected (Wassermann et al., 2017). Australian scrub pythons prey preferentially on birds and mammals, including rodents and wallabies, and therefore it was hypothesized that *S. nesbitti* might actually have a snake-rodent life-cycle, with primates and humans as aberrant or – due to low intermediate host-specificity – alternative hosts (Wassermann et al., 2017). Thus, it is possible that the true natural intermediate hosts of the *S. nesbitti*-like parasites that caused the outbreaks in Malaysia may not yet have been identified.

Another complication is that the suitability of the SSU-rDNA locus to differentiate between *Sarcocystis* spp. is questionable (Gjerde, 2013; Poulsen and Stensvold, 2014; Dubey, 2015), and some *Sarcocystis* species that are phylogenetically closely related, are actually biologically distinct from each other (e.g., *Sarcocystis neurona*, *Sarcocystis falcatula*; Dubey, 2015). Thus, although the *Sarcocystis* species in NHPs, snakes, and humans have very similar SSU-rDNA sequences, it is possible that they belong to different species, with their own distinct life cycles and host ranges.

Based on research on *Sarcocystis* species in domestic and wild ruminants, the ITS1 locus and parts of the LSU-rDNA gene may also be valuable targets for further characterizations (Gjerde, 2016). Furthermore, sequencing the partial mitochondrial cytochrome c oxidase subunit I gene (*cox1*) may offer a useful possibility for distinguishing between even closely related *Sarcocystis* species (Gjerde, 2013, 2016). However, *cox1*-based tools may be of limited suitability for investigations of crude clinical or environmental samples in which parasite DNA is not enriched. Although the analytical sensitivity is not yet characterized, it is likely to be much lower than those targeting the SSU-rDNA locus. The ideal molecular targets for resolving the *S. nesbitti* question would be species-specific targets including highly repetitive gene elements, similar to

those developed for *Toxoplasma gondii* (Burg et al., 1989; Homan et al., 2000). However, with the limited availability of reference material, the identification of such repetitive elements currently seems out of reach.

In conclusion, although SSU-rDNA sequencing was extremely helpful in identifying potential sources and routes of transmission, and obviously aided in the establishment of interventions to reduce the likelihood of further human infections, the heading in one of the early reviews is valid still "Unsolved mysteries: The source of infection …" (Tappe et al., 2013).

2.9 *Toxoplasma gondii* in bobcats

Wild felids are important in the sylvatic cycle of *Toxoplasma gondii*. In a study of *T. gondii* infections in feral domestic and wild felids in California, USA, *T. gondii* DNA was detected in 49 of 166 feral cats (*Felis catus*), 10 of 73 mountain lions (*Puma concolor*), and 11 of 27 bobcats (*Lynx rufus*) (VanWormer et al., 2014). The researchers concluded that wild cats that come in contact with feral and domestic cycles may introduce atypical genotypes to domestic cats and thereby facilitate the transmission of potentially more virulent genotypes to humans, domestic animals and wildlife (VanWormer et al., 2014). In that study, bobcats, with > 40% seroprevalence, seemed to be more likely to be seropositive, and thus, presumably, more exposed to *T. gondii* infection than the other wild or feral cats investigated. This subject is of more than simply academic interest, because atypical genotypes circulating in wildlife have been found recently in humans in the USA (Pomares et al., 2018). In addition, atypical genotypes were identified in cases of fatal human toxoplasmosis in French Guiana (Carme et al., 2009).

In North America, the bobcat is a common wild felid and could play an important role in the transmission of *T. gondii*. There are millions of bobcats in the USA (Roberts and Crimmins, 2010) and their *T. gondii* seroprevalence tends to be high. It was shown previously that 71% (15/21) of bobcats from northern California (Riemann et al., 1975), 52% (30/58) from the US and Mexico (Kikuchi et al., 2004), 83% (109/131) from Pennsylvania (Mucker et al., 2006), and 100% (35/35) from Mississippi (Verma et al., 2017), were seropositive for *T. gondii*. These data are not dissimilar to those of domestic cats in the USA, ranging from 34-100% depending on cat type, age, and habitat (Dubey and Jones, 2008); felids existing entirely on a prey diet are more

likely to be infected than domestic cats that are fed only canned or dried food. Given the high infection rates, bobcats are probably responsible for a very high (> 65%) *T. gondii* infection rate in deer in USA. In 2004, unfrozen samples (blood, heart, tongue, and faeces) were collected from 35 free ranging wild bobcats from Mississippi, USA. *Toxoplasma gondii* antibodies were detected in serum by the modified agglutination test (1 ≥:200) in all 35 bobcats. Hearts from all bobcats were bioassayed in mice and viable *T. gondii* was isolated from 21; these strains were further propagated in cell culture. Additionally, DNA was extracted from digests of tongues and hearts of all 35 bobcats; *T. gondii* DNA was detected in tissues of all 35 bobcats. Genetic characterization of DNA from cell culture-derived isolates was performed by multiplex PCR using 10 PCR-RFLP markers. Results showed that ToxoDB genotype #5 predominated (in 18 isolates) with a few other genotypes (#24 in two isolates, and #2 in one isolate). The genotype #5 (also known as clonal type 12) is a major genotype in wildlife in North America (Dubey et al., 2011; Khan et al, 2011). From the above 35 bobcats, PCR-DNA sequencing at two polymorphic marker loci, GRA6 and GRA7, detected mixed strains co-infecting the tissues of bobcats; most possessing Type II ToxoDB genotype #1 or #3 alleles at GRA7 versus Type X (type 12, ToxoDB genotype #5) alleles at GRA6 (Verma et al., 2017). These results suggest that individual bobcats have been exposed to more than one parasite strain during their life time. Together with high seroprevalence, the data suggest that these bobcats were exposed and contributed to a high level of contamination of their environment with *T. gondii*.

Genetic typing of *T. gondii* strains has evolved since the seminal study of Howe and Sibley (1995) who first used six multilocus PCR-RFLP markers to study genetic diversity. This method is simple, cost-effective, and, as no special equipment is needed, it is cheaper than MLST typing. Later, a multiplex multilocus PCR-RFLP method was developed using 10 markers (Su et al., 2010). This method improved resolution in distinguishing different *T. gondii* strains, and as it is a nested PCR, it is more sensitive. This effort generated an integrated database to reveal global *T. gondii* diversity (Su et al., 2012), distribution of genotypes world-wide (Shwab et al., 2014), and partition of genotypes into a spatial gradient and animal species in North America (Jiang et al., 2018). Surprisingly, the phylogenetic network generated from the PCR-RFLP data is similar to that obtained from whole genome sequence data (Lorenzi et al., 2016).

There is very little variation in SSU-rDNA of *T. gondii*, so it is not a useful marker for genotyping (strain identification). However, due to the high copy number of this gene, it is an excellent marker for identification of *T. gondii* infection. Currently 10 PCR-RFLP markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, are used for genotyping. Among these markers, c22-8, c29-2, and L358 are non-coding polymorphic DNA sequences, Apico is an intron sequence in the apicoplast, and the others are polymorphic genes.

Toxoplasma gondii genotype data from humans is very limited due to the limited access to human tissue for isolation and characterization of the parasite. Indeed, > 90% of existing genotype data on *T. gondii* is derived from animal sources. However, the accepted view is that humans can be infected by all sub-types. With regards to the interplay with bobcats, human infection may arise indirectly from bradyzoites in undercooked meat from domestic or game animals that have themselves been infected via oocysts originating from the faeces of bobcats, or directly from oocysts in contaminated water, soil, or fresh produce consumed raw. It should also be noted that bobcat meat is exported for human consumption, and may also be eaten by hunters.

The virulence of different *T. gondii* strains is also of relevance. Experimental studies have confirmed that some genotypes are lethal to mice, whereas others are non-lethal, and that the virulence phenotype is linked to polymorphic virulence genes ROP18 and ROP5 (Shwab et al., 2016). Although epidemiological data are clear that some *T. gondii* genotypes are more virulent to humans than others, we do not have sufficient information at present to know whether particular genotypes associated with particular wildlife species are more or less likely to be virulent in humans. This is clearly an area that could be usefully explored using the molecular tools available.

3. Conclusions

The number of wildlife species that may be hosts to enteric protozoan parasites of potential zoonotic importance is huge. However, regardless of whether a particular protozoan can infect both humans and a particular wildlife species, the public health importance is determined not only by that possibility, but also by the likelihood of transmission occurring, along with the

severity of disease from any infections that may arise. Rather than consider each enteric protozoan of potential zoonotic importance and consider how molecular tools have contributed to our understanding of different wildlife species as reservoir hosts, we have decided to use selected parasite-host pairs to illustrate some of the progress and frustrations in this field.

Although these different example vignettes make it clear that there is no simple answer, it is also obvious that the use of molecular tools for different protozoa in different wildlife hosts has enabled improved understanding of the complexity of the situation. These examples also highlight the challenges of applying molecular tools to unravel this complexity and to gain a better understanding of the extent to which enteric protozoa in wildlife may present a threat to public health. For each of our selected parasite-wildlife host pairs, we summarize in Table 7 three of the major advances and challenges associated with the use of molecular tools to investigate zoonotic potential.

Among the commonalities in the advances, are the increasing accumulation of data and sequences and an improved understanding of the phylogeny, interrelationships, and differences between species (although a related challenge for all is that some of the main sequence data resources are not curated, and contain errors). Among the commonalities of the challenges are that the DNA from protozoan transmission stages in faecal samples or environmental samples is not always easily accessible for amplification, and may be in small quantities or be of low quality. This encourages researchers to use multicopy genes, such as SSU-rDNA, as targets for amplification. However, as these are well conserved, they do not necessarily provide the necessary resolution to determine whether two very similar sequences are actually derived from the same genotype or even the same species. As molecular tools become more sophisticated, enabling easier and faster comparison of greater stretches of the genome, up to whole genome sequencing, we may find that different strains of similar parasites that we previously combined in the same sub-type due to being very similar or even identical at one genetic locus, are actually very different at other loci. The extent and meaning of these similarities and differences will require careful interpretation; even strains that appear different may still utilize the same spectrum of hosts.

In conclusion, molecular tools have provided us with more information about the possibility that several enteric protozoa from wildlife may infect humans, resulting in cases or outbreaks of

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diseases occurring that have not necessarily been included in important articles on this subject. The usefulness of these tools to indicate plausible transmission sources and routes for specific cases or outbreaks has been well proven. Nevertheless, this is not the end of the story, and molecular tools often only indicate the possibility that a particular protozoan parasite may be zoonotic, not that it is. Nor do these tools necessarily indicate the likelihood of transmission occurring or the impact, both of which are relevant when assessing a threat. Well-designed traditional epidemiological studies, experimental studies to confirm reservoir hosts, and identification of markers for virulence are all important approaches that should not be overlooked for ensuring that the results obtained using molecular tools are useful and of relevance when considering potential public health impacts.

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Figure legends

Figure 1: Phylogenetic relationships among genotypes of *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon intestinalis* based on: **A)** partial sequences of internal transcribed spacer gene (ITS), and **B)** partial sequences of polar tube protein gene (PTP). Numbers at the nodes represent the bootstrap values gaining more than 50% support based on 1000 replications. Phylogenetic trees were inferred by the Neighbor-Joining method with the A) Jukes-Cantor and B) Kimura 2-parameter models in MEGA6 software.

Figure 2: Phylogenetic relationships of the *Enterocytozoon bieneusi* genotypes identified in cervids. The phylogenetic tree was inferred with a neighbour-joining analysis of the *E. bieneusi* ITS sequences, based on distances calculated with the Kimura two-parameter model. Bootstrap values > 50 % from 1,000 replicates are shown on the nodes. The *E. bieneusi*-ITS genotypes detected in cervids (more than one isolate) are shown, and those isolates that clustered into the same clade as those considered to be zoonotic are considered to be in Group 1, the "potentially zoonotic group".

Table 1: The 35 protozoa (and one incorrectly classified helminth) responsible for the EID events included in the database, as extracted from Supplementary Table 1 (Jones et al., 2008)

	Location[1]	Driver[2] and Wildlife association (0, N, W, U)[3]	Classification (enteric or otherwise)[4]
Angiostrongylus cantonensis	Taiwan	International travel & commerce (W)	Incorrect classification as protozoan – *A. cantonensis* is a nematode
Babesia divergens	Eastern Europe	Land use changes (N)	Vectorborne (not enteric)
Babesia microti	US	Land use changes (W)	Vectorborne (not enteric)
Babesia microti-like	Croatia	Human susceptibility to infection (W)	Vectorborne (not enteric)
Babesia microti–like WA1-type	US	Human susceptibility to infection (W)	Vectorborne (not enteric)
Balamuthia mandrillaris	US	Human susceptibility to infection (W)	Free-living amoeba (probably not enteric)
Brachiola vesicularum	**Czech Republic**	**Human susceptibility to infection (0)**	**Microsporidian (enteric association unknown)**
Cryptosporidium parvum	**US**	**Land use changes (N)**	**Coccidian-like (enteric)**
Cyclospora cayetanensis	**Papua New Guinea**	**Human susceptibility to infection (U)**	**Coccidian (enteric - limited zoonotic potential for this species)**
Encephalitozoon cuniculi	**Switzerland**	**Human susceptibility to infection (W)**	**Microsporidian (maybe enteric in some hosts)**
Encephalitozoon hellem	**US**	**Human susceptibility to infection (W)**	**Microsporidian (maybe enteric in some hosts)**
Encephalitozoon intestinalis	**US**	**Human susceptibility to infection (0)**	**Microsporidian (enteric)**
Enterocytozoon bieneusi	**Haiti**	**Human susceptibility to infection (N)**	**Microsporidian (enteric)**
Giardia duodenalis	**US**	**Human demographics & behavior (0)**	**Flagellate (enteric)**
Isospora (Cystoisospora) belli	**US**	**Human susceptibility to infection (0)**	**Coccidian (enteric)**
Leishmania donovani	Germany	Human susceptibility to infection (W)	Vectorborne (not enteric)
Leishmania infantum	Southern Europe	Land use changes (W)	Vectorborne (not enteric)
Leishmania tropica	Saudi Arabia	War and famine (W)	Vectorborne (not enteric)
Nosema connori	**Japan**	**Human susceptibility to infection (0)**	**Microsporidian (enteric association unknown)**
Plasmodium falciparum chloroquine-resistant	Venezuela	Antimicrobial agent use (0)	Vectorborne (not enteric)
P. falciparum mefloquine-resistant	Thailand	Antimicrobial agent use (0)	Vectorborne (not enteric)
P. falciparum multiple drug-resistant	Thailand	Land use changes (0)	Vectorborne (not enteric)
P. falciparum proguanil-resistant	Malaysia	Antimicrobial agent use (0)	Vectorborne (not enteric)

P. falciparum quinine-resistant	Thailand	Antimicrobial agent use (0)	Vectorborne (not enteric)
P. falciparum sulfadoxine-pyrimethamine-resistant	Thailand	Antimicrobial agent use (0)	Vectorborne (not enteric)
Plasmodium vivax	India	Breakdown of public health measures (0)	Vectorborne (not enteric)
P. vivax chloroquine-resistant	Papua New Guinea	War & famine (0)	Vectorborne (not enteric)
P. vivax proguanil-resistant	Malaysia	Antimicrobial agent use (0)	Vectorborne (not enteric)
Toxoplasma gondii	**Various**	**Human susceptibility to infection (N)**	**Coccidian (enteric in definitive felid host)**
Trachipleistophora anthropophthera	**Czech Republic**	**Human susceptibility to infection (0)**	**Microsporidian (enteric association unknown)**
Trachipleistophora hominis	**Australia**	**Human susceptibility to infection (0)**	**Microsporidian (enteric association unknown)**
Trichomonas vaginalis	US	Human susceptibility to infection (0)	Flagellate (not enteric)
Trichomonas vaginalis metronidazole-resistant	Austria	Antimicrobial agent use (0)	Flagellate (not enteric)
Trypanosoma brucei gambiense	Democratic Republic of Congo	War & famine (W)	Vectorborne (not enteric)
Trypanosoma brucei rhodesiensis	Sudan	Breakdown of public health measures (W)	Vectorborne (not enteric)
Trypanosoma cruzi	Brazil	Land use changes (W)	Vectorborne (not enteric)

1: The location column is based on the location designations used in Table 1 of the Supplementary Material of Jones et al. (2008), in the penultimate column of that table headed "Location". When a single location is given this is copied verbatim for the country or region heading, regardless of political affiliation. However, more specific pinpointing provided in the original table (such as city, county, or even institution) are not included in the current table. Furthermore, where several countries were noted we have grouped them if feasible (e.g., Eastern Europe for *Babesia microti*, rather than the various countries listed)

2: The drivers written in this column have been copied verbatim from the "Driver" column used in Table 1 of the Supplementary Material of Jones et al. (2008).

3: The wildlife association designations used here are based on those used in Table 1 of the Supplementary Material of Jones et al. (2008), specifically, the column in the original table described as "Zoonotic type". Derived directly from the definitions used for that table (although with a different coding system), with the variable definitions copied verbatim from those provided by Jones et al. 2008, 0 means non-zoonotic (disease emerged via human to human transmission); N means non-wildlife (zoonotic EID event caused by a pathogen with no known wildlife origin); W means wildlife (zoonotic EID event caused by a pathogen with a wildlife origin); and U means unspecified (zoonotic EID event caused by a pathogen with an unknown origin).

4. The classifications used in this column are <u>not</u> derived directly from Table 1 of the Supplementary Material of Jones et al. (2008). Instead, for the purposes of the current article, a very brief description indicates whether each protozoa could be considered as enteric.

Table 2: Overview of enteric protozoa (including Microsporidia and *Blastocystis*) and a potential wildlife host species selected for review in this article, including the reasons for their inclusion.

Enteric protozoan parasite	Brief description	Wildlife (definitive) host[a]	Host characteristics of relevance to transmission of enteric protozoa from wildlife hosts to people
Balantioides coli (*Balantidium coli*)	Ciliate: relatively large trophozoites inhabit the colon and caecum of the host. Both asexual and sexual replication. Transmission via cysts in environment.	**Wild boar**	*Sus scrofa*: most widespread species of wild pig. Distribution from Western Europe to the Far East and Southeast Asia. Introduced populations on all continents except Antarctica. High reproductive rate. Suitable habitats include mixed landscape (agricultural fields and forest). Likely to contaminate environments where humans work, have leisure activities, and produce food.
Blastocystis sp.	Stramenopiles: exist in several different morphological forms – vacuolar, granular, and amoeboid - that inhabit the intestine. Transmission via cysts in environment.	**Rodents**	Very diversified mammalian order, living in huge numbers on all continents except Antarctica. Inhabit a wide variety of terrestrial habitats, including man-made environments. Many species are considered pests. Likely to contaminate environments where humans live and produce food.
Cryptosporidium spp.	Apicomplexan: numerous (>30) species. Sporozoites invade epithelial cells. Epicellular location. Both asexual and sexual replication. Transmission via oocysts in environment.	**Wild fish**	Some wild fish species represent not only a food source for humans, but may also inhabit waterways used as drinking water sources or for recreation. Defecation into water favours survival for parasite transmission stages, but also may enable wide dissemination.
Encephalitozoon spp.	Microsporidian: host cells infected via an extruded polar tubule that injects infective sporoplasm. Multiplication within cells by merogony and schizogony. Spores released by cell bursting; transmission stage in environment.	**Wild birds**	With birds living and breeding in nearly all terrestrial habitats and all continents, and some migrating over vast distances, birds provide a mechanism for dissemination of transmission stages of enteric protozoa into all environments, including urban and agricultural, depending on bird species.
Pathogenic *Entamoeba* spp.	Amoebozoan: trophozoites inhabit large intestine and multiply by binary fission. Transmission via cysts in environment.	**Non-human primates (NHP)**	The close taxonomic relationship between humans and NHPs facilitates transmission of pathogens between them. Drivers such as urbanization, habitat fragmentation, deforestation, tourism, increase the likelihood of overlap between habitats.

Enterocytozoon bieneusi	Microsporidian: infects intestinal epithelial cells via injection of sporoplasm through a polar tubule. Multiplication within cells by merogony and schizogony. Transmission via spores in environment.	**Wild cervids**	Deer are widely distributed, and indigenous species are found in all continents, except Australia and Antarctica. Some live in sizeable populations. Depending on species, cervids occupy different biomes, from tundra to tropical forest, but mostly inhabit mixed habitats. Adjacent croplands benefit several species, and enable contamination of environments where human food is produced. Further human interaction as many species are important game animals.
Giardia duodenalis	Flagellate (Order, Diplomonadida): trophozoites inhabit small intestine and replicate by binary fission. Transmission via cysts in environment.	**Red foxes**	*Vulpes vulpes*: widely distributed member of the Carnivora. Occurs throughout the northern hemisphere, adapting rapidly to new environments and food sources. Very successful at adapting to and colonizing urban environments.
Sarcocystis nesbitti	Apicomplexan with two host (prey, predator) life cycle. Sexual reproduction in intestine of definitive host. Asexual reproduction in sarcocysts in muscle of intermediate host. Oocysts shed in faeces and may lyse, releasing two sporocysts; transmission stages in environment. Snakes are indicated as definitive hosts for *S. nesbitti*.	**Snakes**	Although snakes do not possess specific characteristics that are relevant for transmission of enteric protozoa to people, they are the wildlife species that has been associated with transmission of *S. nesbitti* to humans.
Toxoplasma gondii	Apicomplexan with two host (prey, predator) life cycle. Felids are definitive host. Asexual reproduction in bradyzoites in muscle of intermediate host. Sexual reproduction in intestine of definitive host. Oocysts transmission stage in environment.	**Bobcat**	*Lynx rufus*: although not possessing specific characteristics relevant for transmission of enteric protozoa to people, felids are the only definitive hosts of *Toxoplasma*. Bobcats range from southern Canada to central Mexico, including most of the USA. Living near agricultural areas, prey population are a main driver for distribution, unrestricted by human populations, provided a suitable habitat is available.

a: The wildlife (definitive) hosts listed here are not the only wildlife hosts for the parasite under consideration, but those selected for consideration for the purpose of this article.

Table 3: *Cryptosporidium* species and genotypes reported in wild fish using molecular tools.

Species	Host	Marine/ Freshwater	Site of Infection	Overall % Prevalence	GenBank accession number (SSU-rDNA)*	gp60 subtype**	Reference
C. molnari	Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*),	Marine	Stomach (and intestine)	5-25	HM243548, HM243550, HQ585890	-	Alvarez-Pellitero and Sitja-Bobadilla, 2002, Palenzuela et al., 2010
C. molnari	Northern pike (*Esox lucius*)	Freshwater	-	40	KP939354	-	Certad et al., 2015
C. scophthalmi	Turbot (*Scophthalmus maximus*)	Marine	Intestine	100	KR340588	-	Alvarez-Pellitero et al, 2004
Cryptosporidium Piscine genotype 3	Mullet (*Mugil cephalus*)	Marine	Intestine	2	GQ925452	-	Reid et al., 2010
C. scrofarum	School whiting (*Sillago vittata*)	Marine	-	4	-	-	Reid et al., 2010
C. xiaoi	School whiting (*Sillago vittata*)	Marine	-	2	-	-	Reid et al., 2010
C. parvum	School whiting (*Sillago vittata*)	Marine	Intestine	2	-	IIaA18G3R1	Reid et al., 2010
C. hominis	Mackerel scad (*Decapterus macarellus*)	Marine	-	3	-	IdA15G1	Koinari et al., 2013
C. parvum	Mackerel scad (*Decapterus macarellus*)	Marine	-	7	-	IIaA15G2R1	Koinari et al., 2013
C. parvum	Silver barb (*Puntius gonionotus*)	Freshwater	-	2	-	IIaA19G4R1	Koinari et al., 2013
C. parvum	Arctic char (*Salvelinus alpinus*), European whitefish (*Coregonus lavaretus*), European perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*)	Freshwater	Intestine	33-100	KP939335, KP939337, KP939351	IIaA15G2R1, IIaA16G2R1, IIaA17G2R1	Certad et al., 2015

* in most cases either only one isolate was identified or only one or no representative sequence was submitted to GenBank

** corresponding *gp60* subtype identified in samples that were positive for either *C. hominis* or *C. parvum* at the SSU locus

Table 4. Zoonotic potential of *Encephalitozoon intestinalis*, *E. cuniculi* and *E. hellem* and their genotyping at various gene targets (Galván et al., 2013; Mathies et al., 1999; Xiao et al., 2001).

Species	ITS[1]	SSU[2]	Genotype PTP[3]	SWP[4]-1	IGS[5]-TH	IGS[5]-HZ	Zoonotic potential
E. intestinalis	-[6]	-	-	NA[7]	NA	NA	Yes
E. cuniculi	I	-	I	Ia, Ib	NA	NA	Yes
	II	-	II	II	NA	NA	Yes
	III	-	III	IIIa, IIIb	NA	NA	Yes
	IV	-	NA	NA	NA	NA	Yes
E. hellem	1A	1A	1A	NA	1	1	Yes
	1A	1A	1B	NA	2	1	Yes
	1A	1C	1C	NA	2	2	Yes
	2	NA	2A	NA	NA	NA	No
	2	2B	2B	NA	NA	NA	Yes
	3	NA	2C	NA	3	NA	No
	3	NA	2D	NA	NA	NA	Yes

[1]the internal transcribed spacer (ITS) of the rRNA; [2]the small subunit rRNA (SSU); [3]the polar tube protein (PTP); [4]the spore wall protein 1 (SWP-1); [5]the intergenic spacers of the ribosomal genes; [6]not differentiated into genotypes; [7]NA: sequence of the gene is not available

Table 5. The presence and distributions of different genotypes of *Enterocytozoon bieneusi* in cervids

Species	Collection sites	% prevalence (positive/total)	ITS Genotype (no.)	Potentially zoonotic genotypes	Reference
Sika deer (*Cervus nippon*)[1]	China: Heilongjiang and Jilin	33 (28/86)	BEB6 (20), HLJD-I (1), HLJD-II (1), HLJD-III (1), HLJD-IV (1), HLJD-V (4)	HLJD-II, HLJD-III	Zhao et al., 2014
Red deer (*Cervus elaphus*)[2]	China: Heilongjiang	20 (1/5)	HLJD-V (1)	--	Zhao et al., 2014
Pere David's deer (*Elaphurus davidianus*)[3]	China: Henan	34 (16/47)	Type IV (4), EbpC (4), EbpA (4), BEB6 (2), COS-I (1), COS-II (1)	type IV, EbpC, EbpA	Zhang et al., 2015
Wild reindeers (*Rangifer tarandus*)[3]	China: Northeast forest region of Great Hinggan Mountains	17 (21/125)	Peru6 (6), CHN-RD1 (12), CHN-RD2 (1), CHN-RD3 (1), CHN-RD4 (1)	Peru6, CHN-RD1, CHN-RD2, CHN-RD3, CHN-RD4	Liu et al., 2015
Sika deer (*Cervus nippon*)[1]	China: Jilin	7 (23/326)	J (11), BEB6 (4), EbpC (1), KIN-1 (1), CHN-DC1 (1), JLD-1 (2), JLD-2 (2), JLD-3 (1)	CHN-DC-1, KIN-1, EbpC, JLD-2, JLD-3	Zhang et al., 2016
Hog deer (*Axis porcinus*)[2]	China: Sichuan	75 (3/4)	BEB6 (2), CHS9 (1)	--	Li et al., 2016
Red deer (*Cervus elaphus*)[2]	China: Sichuan	25 (1/4)	BEB6 (1)	--	Li et al., 2016
Sika deer (*Cervus nippon*)[2]	China: Sichuan	50 (2/4)	BEB6 (1), SC03 (1)	SC03	Li et al., 2016
Red deer (*Cervus elaphus*)[3]	China: Heilongjiang and Jilin	8 (8/104)	BEB6 (7), HLJD-VI (1)	HLJD-VI	Zhao et al., 2017
Siberian roe deer (*Capreolus pygargus*)[3]	China: Heilongjiang and Jilin	11 (2/18)	BEB6 (2)	--	Zhao et al., 2017

Species	Location	Positive (n/N)	Genotypes	Potentially zoonotic genotypes	Reference
Sika deer (*Cervus nippon*)[1]	China: Henan and Jilin	36 (215/599)	BEB6 (129), HLJD-I (18), EbpC (3), HLJD-IV (2), COS-I (1), EbpA (1), D (1), JLD-I (7), JLD-II (5), HND-I (4), JLD-III (2), HND-II (1), JLD-IV (3), JLD-V (2), JLD-VI (5), HND-III (1), JLD-VII (1), JLD-VIII (16), JLD-IX (1), JLD-X (1), HND-IV (1), JLD-XI (2), JLD-XII (1), JLD-XIV (7)	D, HND-I, EbpC, HND-II, JLD-I, JLD-II, JLD-III, JLD-IV, JLD-V, JLD-VI, EbpA	Huang et al., 2017
Red deer (*Cervus elaphus*)[1]	China: Henan and Jilin	38 (6/16)	BEB6 (2), JLD-IV (3), JLD-XIII (1)	JLD-IV	Huang et al., 2017
Musk deer (*Moschus berezovskii*)[4]	China: Sichuan	17 (38/223)	SC03 (38)	SC03	Song et al., 2018
White-tailed deer (*Odocoileus virginianus*)[5]	USA: Maryland	33 (26/80)	WL4 (11), I (7), J (1), LW1 (1), DeerEb1-DeerEb13 (one each)	--	Santin and Fayer, 2015
Wild deer[5]	Australia: Melbourne	4 (25/610)*	D (3), J (1), Type IV (1), MWC_d1 (19), MWC_d2 (1)	D, Type IV, MWC_d1, MWC_d2	Zhang et al., 2018
Total	**China, USA, Australia**	**18% (415/2251)**	BEB6 (170), **SC03 (39)**, **MWC_d1 (19)**, HLJD-I (19), JLD-VIII (16), J (13), **CHN-RD1 (12)**, WL4 (11), **EbpC (8)**, I (7), JLD-XIV (7), **JLD-I (7)**, **JLD-IV (6)**, **Peru6 (6)**, **JLD-II (5)**, JLD-VI (5), HLJD-V (5), **Type IV (5)**, EbpA (5), **D (4)**, **HND-I (4)**, HLJD-IV (3), JLD-1 (2), **JLD-2 (2)**, JLD-III (2), JLD-XI (2), **JLD-V (2)**, COS-I (2), COS-II (1), **KIN-1 (1)**, **HLJD-II (1)**, **HLJD-III (1)**, **HLJD-VI (1)**, **CHN-DC1 (1)**, **CHN-RD2 (1)**, CHN-RD3 (1), **CHN-RD4 (1)**, **JLD-3 (1)**, JLD-VII (1), JLD-IX (1), JLD-X (1), JLD-XII (1), JLD-XIII (1), CHS9 (1), **HND-II (1)**, HND-III (1), HND-IV (1), LW1 (1), **MWC_d2 (1)**, DeerEb1-DeerEb13 (one each)	Potentially zoonotic genotypes are in bold font:	

* Mixed infections

[1] Farmed deer; [2] Zoo deer; [3] Forest farmed; [4] Deer breeding centre; [5] Wild deer

Table 6: Amplification of *Giardia duodenalis* DNA by PCR at different gene loci for isolates from red fox samples already confirmed to contain *Giardia* cysts by immunofluorescent antibody microscopy.

Country	% prevalence by IFA[1] (number positive / samples examined)	Amplification at genes targeted for PCR (successful amplification/isolates tested)					Assemblages reported	Reference
		SSU-rDNA[2]	BG[3]	TPI[4]	GDH[5]	ITS1 & ITS2[6]		
Croatia	5 (3/66)	1/3	NA[7]	0/3	NA[7]	0/3	A	Beck et al., 2011
Norway	5 (13/269)	NA[7]	1/3	NA[7]	7/12	NA[7]	A and B	Hamnes et al., 2007
Poland	19 (4/21)	NA[7]	0/21[8]	NA[7]	NA[7]	NA[7]	-	Stojecki et al., 2015
Sweden	45 (46/104)	0/14	0/14	4/14	0/14	NA[7]	B	Debenham et al., 2017

[1] immunofluorescent antibody microscopy; [2] small subunit rDNA; [3] β-giardin; [4] triosephosphate isomerase; [5] glutamate dehydrogenase; [6] internal transcribed spacer loci 1 and 2; [7] not attempted; [8] PCR was performed on all samples, not only those positive by IFA.

Table 7: Overview of advances and challenges associated with the use of molecular tools for understanding the public health threat from enteric protozoan parasites in specific wildlife host species.

Enteric protozoan parasite	Wildlife host	Use of molecular tools for understanding the public health threat from enteric protozoa in wildlife	
		Main advances made	**Challenges and remaining hurdles**
***Balantioides coli* (*Balantidium coli*)**	**Wild boar**	1. Resolution of the taxonomic position of the genus. 2. Evidence that there is only a single species. 3. Confirmation that the species is zoonotic.	1. Changes in taxonomical status over time means that the current genus name still not used widely. 2. Published genetic data currently not extensive for comparative studies; currently only a couple of genetic markers have been used. 3. Within-cyst sequence polymorphisms may create confusion. 4. The importance of wild boar (and other wildlife species) as a reservoir for human infection remains unresolved despite improved knowledge of zoonotic potential.
***Blastocystis* sp.**	**Rodents**	1. Greater understanding of the phylogeny of the genus. 2. Improved understanding of subtypes (STs) and their host specificity. 3. ST discrimination based on SSU-rDNA.	1. The interaction between different STs and the host microbiome is not yet understood. 2. Phylogenetic studies sometimes inconsistent; GenBank sequences that are not *Blastocystis*. 3. The importance of rodents (and other wildlife species) as a reservoir for human infection remains unresolved despite knowledge of zoonotic potential for some STs.
***Cryptosporidium* spp.**	**Wild fish**	1. Greater understanding of the phylogeny of the genus. 2. Considerable data accrued on *Cryptosporidium* species (and some on subtypes) found in some fish species, although most studies on wild or aquarium fish. 3. Understanding that wild fish may harbour both zoonotic species and fish-specific species.	1. Differentiation between true infections of fish and carriage not always clear. 2. Many of the studies are ad hoc and preliminary, without epidemiology-based planning. 3. The importance of wild fish (and other wildlife species) as a reservoir for human infection remains unresolved, despite knowledge of zoonotic potential for some *Cryptosporidium* species.
***Encephalitozoon* spp.**	**Wild birds**	1. Greater understanding of the heterogeneity within and between species. 2. Considerable data accrued on	1. Spore shedding intermittent, and therefore extensive sampling required. 2. Broad host range can limit the value of genotyping for determining zoonotic potential.

Organism	Host		
		Encephalitozoon species and subtypes at different genes, enabling comparisons. 3. Genetic loci recommended for routine investigations	3. The importance of wild birds (and other wildlife species) as a reservoir for human infection remains unresolved, despite knowledge of zoonotic potential for some species and genotypes.
Pathogenic *Entamoeba* spp.	**Non-human primates (NHPs)**	1. Understanding that different species of morphologically identical *Entamoeba*, with different pathogenicity, infect humans. 2. Identification of *Entamoeba nuttalli* in NHPs. 3. Realisation that, should suitable circumstances arise, humans and NHPs may be infected by the same *Entamoeba* species.	1. Although SSU-rDNA sequence divergence between *E. histolytica* and *E. dispar* is over 2%, it is below 1% for *E. histolytica* and *E. nuttalli*, so primer design is crucial. 2. Predictive markers for invasiveness are yet to be identified. 3. The importance of NHPs as a reservoir for human infection remains unresolved, despite an improved understanding of zoonotic potential for some species.
Enterocytozoon bieneusi	**Wild cervids**	1. Greater understanding of the heterogeneity within the *E. bieneusi* species. 2. Phylogenetic analysis can be used to determine potentially zoonotic isolates. 3. Cervids may harbour zoonotic genotypes.	1. Identical subtypes at one gene may differ when multilocus genotyping is applied. 2. The significance of results from higher resolution tools is yet to be understood, and more discriminatory tools may alter our understanding. 3. The importance of cervids as a reservoir for human infection remains unresolved, despite an improved understanding of zoonotic potential for some isolates.
Giardia duodenalis	**Red foxes**	1. Understanding that foxes may harbour zoonotic assemblages. 2. Dogs and red foxes appear not to harbour the same assemblages. 3. Understanding that PCR may not provide most sensitive identification method for some isolates or isolates in some matrices.	1. Amplification of DNA from *Giardia* isolates in fox faeces often unsuccessful. 2. Low resolution from SSU-rDNA. 3. The importance of red foxes as a reservoir for human infection remains unresolved, despite an improved understanding of the zoonotic potential for some isolates.
Sarcocystis nesbitti	**Snakes**	1. Phylogenetic analyses used to indicate potential definitive hosts. 2. Potential lifecycles indicated by detection of particular sequences. 3. Identification of potential	1. Lack of accessible material hinders development of appropriate tools, with high sensitivity and sufficient resolution. 2. Natural intermediate hosts still not necessarily identified. 3. Although snakes may be a reservoir for human infection, their importance in transmission seems to be unclear; the severity of

Toxoplasma gondii	**Bobcat**	transmission routes enabling planning of appropriate interventions. 1. Greater understanding of the heterogeneity within the *T. gondii* species. 2. Several useful PCR-RFLP markers have been identified. 3. Global database developed from PCR-RFLP markers supported by whole genome sequencing data.	infection means that even infrequent infections or outbreaks may be considered of public health importance. 1. Lack of accessible material from human infections means that our knowledge of the strains of greatest public health significance is limited. 2. Identification of markers for pathogenicity will assist in determining those strains of greatest virulence. 3. Although bobcats are a reservoir for human infection, their importance remains unresolved; information on the virulence of isolates associated with bobcats would be useful.

Highlights

- Wildlife is a potential reservoir of disease agents that may infect humans
- The public health threat from enteric protozoa in wildlife is poorly understood
- Molecular tools may help in understanding this threat, but may also confuse
- We use nine enteric protozoa-wildlife host examples to review the current position
- Data accumulate, but more discriminatory tools and other approaches are important

A

87 — *E. cuniculi* genotype III [EU001242]

73 — *E. cuniculi* genotype IV [HM045511]

100 — *E. cuniculi* genotype I [GU198946]

E. cuniculi genotype II [GU213880]

E. intestinalis [JF792394]

E. hellem genotype 1A [AF338367]

99 — *E. hellem* genotype 2A [AF110327]

99 — *E. hellem* genoype 2B [AF338368]

52 — *E. hellem* genotype 2C [AF110328]

50

0.02

E. romaleae [FJ026013]

B

E. hellem genotype 1B [AY024342]

E. hellem genotype 1C [AF338362]

E. hellem genotype 1A [AF338361]

83 — *E. hellem* genotype 2B [AF338363]

E. hellem genotype 2C [AY171240]

E. intestinalis [AJ880382]

76 — *E. cuniculi* genotype III [AF310679]

84 — *E. cuniculi* genotype II [AF310678]

88 — *E. cuniculi* genotype I [AF310677]

1.0

Nosema ceranae [KC520248]

KR815515 JLD-2 Sika deer
MF496204 MWC d1 Sambar deer
AY371281 Peru6 Reindeer
KX383628 HND-I Sika deer
67 | KP057598 Type-IV Pere David s deer
KX383625 JLD-I Sika deer
KX383627 JLD-II Sika deer
KX383624 D Sika deer
57 | KX383629 JLD-III Sika deer
KX383623 EbpA Sika deer
KR632538 CHN-RD1 Reindeer
KX383632 JLD-IV Red deer
KX383635 JLD-VI Sika deer
KX383620 Ebpc Sika deer
KU852479 SC03 Sika deer
KX383633 JLD-V Sika deer

Group 1
potentially zoonotic group

KX383621 HLJD-IV Sika deer
72 | KX383643 JLD-XI Sika deer
99 | KX383618 HLJD-I Sika deer
73 | KX383647 JLD-XIV Sika deer
65 | KR815518 JLD-1 Sika deer
70 | KX383614 BEB6 Sika deer
92 | KJ556988 HLJD-V Deer
62 | KX383622 COS-I Sika deer

Group 2

89

99

KR815512 J Sika deer
96 | KX383638 JLD-VIII Sika deer
KJ867479 I White tailed deer

86

KJ867476 WL4 White-tailed deer | Group 3

62

56 | Group 7
76 | Group 8
92 Group 9

100 | Group 5

87 | Group 4
100 | Group 6
59

AF059610 Dog PtEbIX

0.05