Infection, Genetics and Evolution 40 (2016) 315–323

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular epidemiology of *Giardia* and *Cryptosporidium* infections

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ARTICLE INFO

Article history:
Received 31 May 2015
Received in revised form 23 September 2015
Accepted 24 September 2015
Available online 11 October 2015

Keywords:
Giardia
Cryptosporidium
Molecular epidemiology
Detection
Taxonomy
Phylogeny

ABSTRACT

Giardia and *Cryptosporidium* are ubiquitous enteric protozoan pathogens of vertebrates. Although recognised as the aetiological agents of disease in humans and domestic animals for many years, fundamental questions concerning their ecology have been unresolved. Molecular tools have helped to better understand their genetic diversity and in so doing have helped to resolve questions about their transmission patterns and associated impacts on public health. However, the value of molecular tools is often complicated by questions concerning their applications, interpretation of results and terminology. Taxonomic issues have, until recently, made it difficult to determine the epidemiology of infections with both *Giardia* and *Cryptosporidium*. Similarly, improved understanding of their respective phylogenetic relationships has helped to resolve questions about zoonotic potential and distribution in wildlife. In the case of *Cryptosporidium*, imaging technologies have complemented phylogenetic studies in demonstrating the parasite's affinities with gregarine protozoa and have further supported its extracellular developmental capability and potential role as an environmental pathogen.

1. Introduction

Giardia and *Cryptosporidium* are ubiquitous protozoan parasites of the small intestine and stomach of vertebrates (Checkley et al., 2014; Fletcher et al., 2012; Thompson, 2011;). Their host range is broad and diverse, including all vertebrate groups. They have direct life cycles comprising an environmentally resistant infective stage, cyst or oocyst, which initiates infection following ingestion. The cyst and oocyst (Fig. 1) play essential roles in the plasticity of transmission routes available to both parasites and are the stages most frequently used in molecular epidemiological studies (Fletcher et al., 2012; Thompson, 2003, 2004), although trophozoites are expelled in the faeces in acute infections.

Asexual multiplication is the dominant means of proliferation in the gut and both *Giardia* and *Cryptosporidium* have clonal population structures (Tibayrenc and Ayala, 2014). Sexual reproduction is not a feature in the life cycle of *Giardia* and no mechanisms of genetic exchange have been identified. However, epidemiological evidence indicates that occasional bouts of genetic exchange may occur, particularly in circumstances where the frequency of transmission is high (Caccio and Sprong, 2010; Thompson and Monis, 2012). A sexual phase of gametogony does occur in the life cycle of *Cryptosporidium*, as in other apicomplexans.

Asexual multiplication allows rapid multiplication in the gut leading to acute, often asymptomatic infection, although chronic infections can occur. Clinically, the most significant impact of *Giardia* and *Cryptosporidium* is in the very young, particularly children and domestic animals (Checkley et al., 2014; FAO, 2014). Their importance as parasites of children in the developing world and disadvantaged communities has resulted in both giardiasis and cryptosporidiosis being considered neglected diseases (Hotez et al., 2015; Savioli et al., 2006). As such, *Giardia* and *Cryptosporidium* are common in areas that support the transmission of other parasites, particularly enteric protozoa and soil-transmitted helminths (Lymbery and Thompson, 2011). Thus *Giardia* and *Cryptosporidium* are rarely present as mono-infections in developing countries and the resultant polyparasitic scenarios exacerbate the clinical impact of individual parasites, and complicate diagnosis, treatment and control (Thompson and Smith, 2011; Thompson, 2015).

With both parasites, the host plays an important role in the clinical impact of infections and expression of disease. With *Giardia*, the nutritional status of the host is very important, particularly in young children with poor nutrition who may suffer failure to thrive (FAO, 2014; Thompson, 2015). In individuals with a compromised or deficient immune system, *Cryptosporidium* infections persist leading to intractable diarrhoea and potentially death (Checkley et al., 2014).

Drug treatment is inadequate for infections with both parasites, and does not provide a reliable strategy for control (Checkley et al., 2014; Fletcher et al. 2012; Leitsch, n.a). The few available drugs to treat *Giardia* require multiple doses and in the case of the most widely used drugs, the nitroimidazoles, there is often poor patient compliance, toxicity issues and adverse effects on the normal gut microflora (Thompson, 2011). There are no curative drugs to treat infections with *Cryptosporidium* (Checkley et al., 2014; Thompson et al., 2005).

In terms of control, there are different priorities in developed and developing countries. In the former, the need is for effective treatment for individuals, and the prevention of food and waterborne transmission. The latter is a significant issue for water utilities and the relevant

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http://dx.doi.org/10.1016/j.meegid.2015.09.028

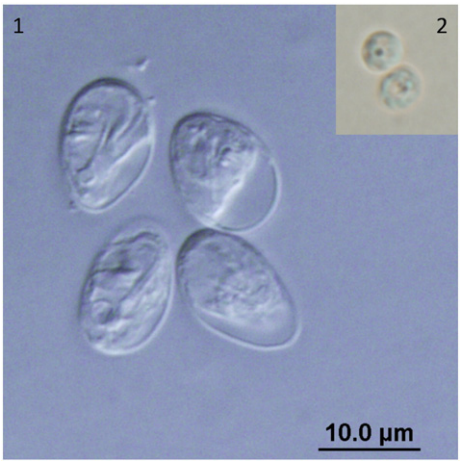

Fig. 1. Light microscopy images of *Giardia canis* cysts (1) from the faeces of a dog, and oocysts of *Cryptosporidium pestis* (2) from the faeces of an experimentally infected mouse.

authorities in terms of both economic and legislative aspects. In the developing world, the need is to lessen the burden of disease in those most at risk of infection, particularly children (Hotez et al. 2015).

The taxonomy of both *Giardia* and *Cryptosporidium* has been controversial since the early 20th century, and remains so today. This has principally been due to the broad host range of both parasites, and a paucity of reliable morphological features on which to define species. These problems have been resolved to some extent with the application of molecular tools. However, the most valuable contribution of these tools has been a better understanding of the epidemiology of infections with both parasites.

2. Molecular detection

The development and use of molecular tools to detect species of *Giardia* and *Cryptosporidium* has undergone great expansion in recent times and as a result so has the understanding of host ranges and transmission dynamics of these two protozoan parasites. Indeed, these morphologically indistinguishable parasites have been found to consist of numerous additional species solely through the use of molecular tools (Hopkins et al., 1997; Lalle et al., 2007; Monis et al., 1998; Reid et al., 2010; Ryan et al., 2003; Xiao et al., 1999; Yang et al., 2011).

Commonly targeted genes used for characterising species of *Giardia* include the small subunit ribosomal DNA (SSU-rDNA), the closely situated internal transcriber regions (ITS1-2), the *Giardia* specific ß-giardin, the triosephosphate isomerase (TPI) and the glutamate dehydrogenase (GDH) genes (Caccio et al., 2002; Hopkins et al., 1997; Lalle et al., 2005; Read et al., 2004; Sulaiman et al., 2003) (Table 1). The SSU-rDNA was one of the first genes commonly used for genotyping *Giardia* and gave rise to the realisation that *Giardia duodenalis* contained several assemblages (A–G) (Andrews et al., 1989; Hopkins et al., 1997; Monis et al., 1999) and more recently have been assigned species names according to host specificity (Monis et al., 2009; Thompson and Monis, 2004) (Table 2). Additional research using multiple genes in various combinations has consolidated this understanding and through the identification of intra-specific genetic variation has also highlighted the existence of sub-genotypes, particularly within *G. duodenalis* (Assemblage A) and *Giardia enterica* (Assemblage B) (Adam et al., 2013; Caccio et al., 2008; Sprong et al., 2009; Weilinga and Thompson, 2007; Wielinga et al., 2015). The significance of these sub-genotypes has gained importance as the question of zoonotic transmission continues to be unravelled.

As seen with *Giardia* the most commonly targeted gene used for characterising species of *Cryptosporidium* is the SSU-rDNA (Xiao, 2010) and has largely been responsible for the proliferation of new species and host ranges identified (Slapeta, 2013; Xiao and Fayer, 2008). A major research area has been concerned with those species commonly infecting humans (*Cryptosporidium hominis*, *Cryptosporidium pestis*; Fig. 3) and understanding the possible transmission routes from the environment and co-habiting animals such as companion animals and livestock (Fayer et al., 2000; Hunter and Thompson, 2005). Invariably this requires genotyping at additional genes which commonly include the 70 kDA heat-shock protein (HSP70), the *Cryptosporidium* oocyst wall protein (COWP) and the internal transcriber region 1 (ITS-1)

Table 1
Commonly targeted genes for the molecular characterisation of *Giardia* and *Cryptosporidium* species.

Gene/locus	Gene copy number	Reliable differentiation of species and sub-genotyping	Reported use and benefits of specific genes
Giardia sp.			
SSU-rDNA	Multiple	Species information	Commonly used
			Often provides greatest amplification success
ITS1-5.8S-ITS2	Multiple	Species information	Recently reintroduced to the literature
		Some sub-genotypic information obtained	Good amplification success
TPI	Single	Species information	Commonly used
		Sub-genotypic information	Variable amplification success
		Species specific primers designed	Useful for suspected mixed infection
ß-giardin	Single	Species information	Commonly used
		Sub-genotypic information	Variable amplification success
			Specific to *Giardia*
GDH	Single	Species information	Commonly used
		Sub-genotypic information	Variable amplification success
ef1-α	Single	Species information	Not commonly used
		Sub-genotypic information	Variable amplification success
Cryptosporidium sp.			
SSU-rDNA	Multiple	Species information	Commonly used
		Genotype information	Often provides greatest amplification success
ITS-1	Multiple	Species information	Not commonly used
		Genotype information	Good amplification success
HSP70	Single	Species information	Commonly used
		Genotype information	Good amplification success
GP60	Single	Species information	Commonly used
		Sub-genotypic information	Variable amplification success
COWP	Single	Species information	Commonly used
		Genotype information	Variable amplification success

Information obtained from cited publications in this review.

Table 2
Species of *Giardia* in mammals.

Species	Assemblage	Host(s)
G. duodenalis	A	Humans and other primates and a wide range of mammals
G. enterica	B	Humans and other primates, dogs, cats, and some species of wild animals
G. canis	C/D	Dogs and other canids
G. bovis	E	Cattle and other hoofed animals
G. cati	F	Cats
G. simondi	G	Rats
G. muris	–	Rodents
G. microti	–	Microtine voles and muskrats

Adapted from Thompson and Monis, 2012

(Geurden et al., 2009; Jex et al., 2008a; Leoni et al., 2006; Ryan et al., 2008; Xiao et al., 2001; Zhou et al., 2007). For greater detail on possible transmission routes intra-specific genotyping is required whereby the 60 kDA glycoprotein (GP60) gene is commonly used (Alves et al., 2003; Xiao and Fayer, 2008) (Table 1).

For both *Cryptosporidium* and *Giardia* identifying intraspecific variation has been central to understanding transmission dynamics of the zoonotic species of both parasites. With respect to *Giardia* this has provided both illuminating and confusing data. Numerous studies have been published describing sub-genotypic information for the zoonotic species *G. duodenalis and G. enterica* (Bonhomme et al., 2011; Caccio et al., 2008; Lalle et al., 2005, 2007; Lebbad et al., 2010; Pallant et al., 2015) and whilst consensus has largely been achieved for *G. duodenalis, G. enterica* is still somewhat of an enigma (Lebbad et al., 2010). Genetically *G. enterica* is highly variable and investigations continually reveal 'new' sub-genotypes which are provisionally given an alpha/numerical name. The variability detected at the various genes and subsequent nomenclature has lead to difficulties for comparative studies (Bonhomme et al., 2011; Pallant et al., 2015; Thompson and Monis, 2012). Reasons suggested for the observed high sequence variability include allelic sequence heterogeneity, genetic recombination through cryptic sex and mixed infections (Birky, 2010; Lalle et al., 2005; Lebbad et al., 2010; Teodorovic et al., 2007) but for the moment definitive answers remain elusive.

Whilst the benefit of molecular tools as a means to identify cryptic species is unquestionable it is still not clear if amplification by PCR is a dependable detection tool for screening purposes. In the case of detecting *Giardia* traditional microscopy and immunoflourescent (IMF) techniques appear to be more sensitive than amplification by PCR (Table 3). Some investigations have reported similar and sometimes greater detection rates with PCR (Cacciò and Ryan, 2008; Santín et al., 2007) strengthening the presumption that PCR is more sensitive and consequently studies discounting the use of microscopy techniques entirely have been conducted (Nolan et al., 2010; Vermeulen et al. 2015). However, there are a large proportion of published studies which report greater detection rates using microscopy/IMF and of the varying PCR amplification success attained across the different genes targeted (Table 3). In some cases, the reported amplification rate has been greater than the subsequent sequencing of amplicons (Fava et al., 2013; Johansen et al., 2014; Sommer et al., 2015; Vermeulen et al., in press). Reasons for this can include messy product giving unreadable sequences or amplification of non-specific product such as bacteria; the latter being of more concern as this indicates the reporting of false positives if sequencing is not conducted to discount this.

As a multi-copy gene the SSU-rDNA has often been found to achieve the greatest amplification success, whilst the other commonly used single-copy genes achieved a much less and variable amplification rate (Table 3). The need for sub-genotype information drives the use of the less successful single-copy genes as these provide more discriminating genetic variation, as opposed to the SSU-rDNA which does not (Beck et al., 2012; Caccio et al., 2008; Lalle et al., 2005; Lebbad et al., 2010; Sulaiman et al., 2003). Given the stated successful amplification of multi-copy genes, of potential is the ITS1-5.8 s-ITS2 region, which recently has been shown to provide reasonable amplification success and also much greater genetic variation than that obtained targeting the SSU-rDNA (Beck et al., 2011a; Beck et al., 2011b; Caccio et al., 2010; Veronesi et al., 2012). The full potential of this region for sub-genotyping however is not clear, particularly for *G. enterica*, as the intra-specific variation observed has not been matched with genotypic information obtained at the other sub-genotyping genes such as ß-giardin and GDH (Caccio et al., 2010).

The variation in reported success with PCR protocols can be dependent on numerous factors such as DNA extraction methods, PCR inhibitors and whether multi-copy or single-copy genes are being targeted, all of which influence the successful amplification of DNA (Elwin et al., 2014). However the disparity in detection rates would suggest that until molecular tools can consistently achieve similar detection levels as those obtained with microscopy/IMF, prevalence studies investigating *Giardia* in host populations should be conducted utilizing both methodologies in tandem. This would ensure a greater confidence

Table 3
Reported detection rates of *Giardia* using both microscopy/IMF and molecular techniques obtained from published research. The amplification success achieved at each gene is given as a percentage of the samples which had tested positive with microscopy/IMF. *Numerous studies could not be included in this table due to insufficient detail provided to allow for comparison.

Host species	Positive by microscopy/IMF	SSU-rDNA	ITS	TPI	ß-g	GDH	Reference
Zoo animal	27	85%	70%	75%	41%	30%	Beck et al. (2011a)
Dogs	96	–	58%	64%	54%	48%	Beck et al. (2012))
Wild animals	26	88%	62%	34%	–	–	Beck et al. (2011b)
Dogs and cats	133	66%	–	–	31%	16%	Mcdowal et al. (2011)
Dogs and cats	190	92%	–	–	42%	13%	Pallant et al. (2015)
Humans	84	–	–	70%	33%	45%	Huey et al. (2013)
Livestock	59	–	–	34%	–	40%	Fava et al. (2013)
Dogs	196	83%	–	–	21%	–	Johansen et al. (2014)
Dogs and cats	100	90%	–	–	34%	58%	Dado et al. (2012)
Dogs	52	69%	–	–	69%	–	Paz Silva et al. (2012)
Domestic animals	202	–	–	19%	85%	91%	Scorza et al. (2012)
Humans	29	83%	–	55%	–	–	Traub et al. (2004)
Dogs	20	70%	–	35%	–	–	Traub et al. (2004)
Dogs	60	91%	–	–	–	33%	Leonhard et al. (2007)
Buffalo	15	–	–	–	–	53%	Caccio et al. (2007)
Chinchilla	41	–	75%	–	–	–	Veronesi et al. (2012)
Dogs	133	82%	31%	1.5%	12%	11%	Sommer et al. (2015)

level in ascertaining the true prevalence whilst also determining the composition of *Giardia* species present within the sampled population.

3. Giardia

3.1. Taxonomy

The taxonomy of *Giardia* has largely been resolved with the advent of molecular tools which have shown that the observations of early taxonomists in the field were correct (Thompson and Monis, 2011, 2012). A series of host-adapted species are now recognised as well as two species with low host specificity and demonstrated zoonotic potential (Table 2). It has been proposed that the original species names be used for the different species which is gaining acceptance and are used here. From an epidemiological point of view, most attention has focussed on the zoonotic species which have been shown to exhibit intraspecific genetic variability but the taxonomic significance of this has yet to be resolved. This is exacerbated to some extent by a confusing and contrasting nomenclature associated with the different loci that are used (Caccio et al., 2008, Pallant et al., 2015; Sprong et al., 2009 and see above).

3.2. Cycles of transmission

Giardia is maintained in a series of independent host adapted cycles of transmission, as well as cycles involving transmission of the two zoonotic species (Fig. 2). The frequency of zoonotic transmission has been a question that has dominated discussions about the epidemiology of *Giardia* infections for decades. The application of molecular tools has largely confirmed the belief that *Giardia* is zoonotic but the epidemiology of zoonotic infections remains a subject of some controversy (Fletcher et al., 2012; Thompson 2011).

Particular attention has focussed on the relationship between *Giardia* infections in humans and companion animals, principally dogs and cats. *Giardia* is common in domestic dogs and cats throughout the world, and is often the most common enteric parasite (Ballweber et al., 2010; Barutzki and Schaper 2011; Covacin, et al. 2011; Palmer et al. 2008a). Numerous studies have demonstrated that populations of dogs and cats are often infected with zoonotic species of *Giardia* and thus represent potential sources of zoonotic infection in people (Palmer et al. 2008b; Covacin et al. 2011; Pallant et al., 2015; Table 4). However, such studies provide no evidence that zoonotic transmission is actually occurring. It is also difficult to interpret and compare published data on *Giardia* infections in dogs and cats because of differences in the diagnostic tests used, the populations of animals surveyed with respect to age, breed and basis of selection, i.e. random or clinical condition (Table 4). In addition, and most importantly, protocols for genotypic characterisation vary considerably between studies making it very difficult to compare results due to differences in the type and number of loci used, methods of DNA extraction and PCR conditions (Table 3).

Some genetic sub structuring/intraspecific variation has been reported in both zoonotic species, *G. duodenalis* and *G. enterica* (Caccio et al., 2008; Sprong et al., 2009; Pallant et al., 2015; Wielinga and Thompson, 2007; Wielinga et al., 2015). The epidemiological significance of this in terms of host specificity is not clear. More isolates of *Giardia* from the same hosts in different geographical areas need to be characterised using the same loci in order to determine the host range of intraspecific variants (sub-assemblages/genotypes).

The most valuable approach to better understand the molecular epidemiology of zoonotic infections with *Giardia* is to study transmission at a local level where the frequency of transmission is high. A number of such studies in defined endemic foci have provided convincing evidence of zoonotic transmission involving dogs, livestock, lower

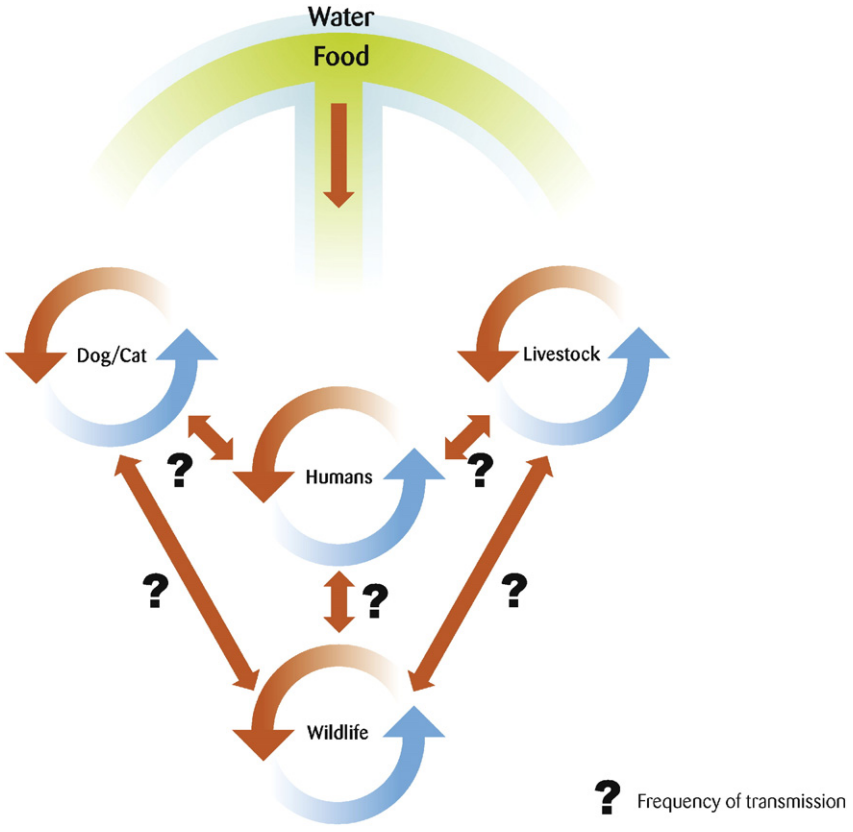

Fig. 2. Major cycles of transmission of *Giardia* species (see Table 2). Some species are host specific and cycle between their respective hosts (blue) whereas others have low host specificity and capable of zoonotic transmission (Orange). Frequency of transmission between host species is largely unknown, and is exacerbated by transmission via water and food.

Table 4
Selected reports of *Giardia* genotyping in dogs and cats.

Location	Type	Dogs			Cats			Reference
		n	% Positive	Genotype (%)	n	% Positive	Genotype (%)	
Italy	Urban	253	16–25	C/D (54/45)	156	22–37	A (83); D (17)	Zanzani et al. (2014)
Sardinia	Mixed	655	26	C/D (49/36); A[a] (5)	–	–	–	Pipia et al. (2014)
Germany	Vet clinics	30	100	A + mixed (91);	–	–	–	Leonhard et al. (2007)
	Shelters	30	100	C/D(9) A + mixed (81); C/D (19)	–	–	–	
Spain	Shelters	81	63	C/D of 4 genotyped	–	–	–	Ortuño et al. (2014)
	Hunting	88	15					
USA	Vet clinics	–	–	–	250	7	A (35)*; F (65)	Vasilopulos et al. (2007)
USA	Vet clinics	128	100	C/D (30); A/B[a] (70); + mixed	–	–	–	Covacin et al. (2010)
Australia	Vet clinics	810	6	C/D (100); + 1mixed	572	1	F (100)	Palmer et al. (2008a, 2008b)
	Shelters	590	14	A C/D (100)	491	3	F (100)	
China	Police & farm	205	13	A (12)[a]; C (1)	–	–	–	Li et al. (2013)
Canada	Shelters	62	6	C/D (100)	–	–	–	Uehlinger et al. (2013)
	Vet clinics	78	38	C/D (54); A (12);	–	–	–	
	Pet shops	69	39	mixed (12) C/D (82); A(6); mixed (12)	–	–	–	
Spain	Urban	604	16	A or B (89); C/D, E (11); + mixed	144	4	A + F (25)	Dado et al. (2012)
Canada	Urban	118	100	C/D (1%B)	15	87	A(100) or B (1)	Mcdowal et al. (2011)
USA	Urban	183	100	C/D (93); A or mixed (7)	13	100	F(54), rest C/D or A	Scorza et al. (2012)
USA	Shelters	672	29	C/D (100); A mixed (1)	–	–	–	Johansen et al. (2014)
Australia	Urban	130	100	A,B,C/D[b, a] mixed	60	100	A,F,D[b, a] mixed	Pallant et al.(2015)

[a] Sub structuring also identified.
[b] Proportions varied with locus used.

primates and humans (Traub et al., 2004, Inpankaew et al., 2007, 2014, Salb et al. 2008, Teichroeb et al., 2009, Johnston et al., 2010). These studies have also demonstrated that 'reverse zoonotic transmission' (zooanthroponotic) is an important factor that must be considered in understanding the epidemiology of infections with *Giardia*, particularly in wildlife (Thompson, 2013).

Wildlife is commonly infected with *Giardia* (Table 5). Unlike *Cryptosporidium* which has numerous species and genotypes adapted to particular species of wildlife with little evidence of any clinical impact reflecting well-balanced host parasite relationships, this is not the case with *Giardia*. Three species of *Giardia* have been described from rodents, *Giardia simondi* (rats), *Giardia muris* (mice), and *Giardia microti* (microtine rodents) which are all genetically distinct (reviewed in Thompson and Monis, 2004). A novel genotype of *Giardia* has been described in an Australian marsupial, a bandicoot known as the quenda (*Isoodon obesulus*), which on the basis of genetic characteristics would appear to represent a distinct species that on present evidence appears to be host specific to quenda (Adams et al., 2004; Thompson et al., 2010b). However, these and other wildlife species are also susceptible to infection with zoonotic species of *Giardia* (Table 5). Indeed, the majority of reported *Giardia* infections in wildlife are with the so-called zoonotic species and are considered to reflect accidental infections in naïve hosts (Table 5). *Giardia* serves as the archetypal example of a zoonotic parasite that is principally found in wildlife as a result of human activities (Thompson, 2013). In all cases, epidemiological evidence supports humans, or cohabiting livestock, as the source of infection through environmental contamination, either directly or indirectly via domestic animal hosts (Thompson et al., 2010a; Thompson, 2013). The impact of these zoonotic species of *Giardia* on wildlife is not known, but *Giardia* has been reported in several species of non-human primates in Africa

Table 5
Giardia in free-ranging wildlife.

Host species	Location	Species/genotype	Reference
Marsupials	Australia	Novel; *G. duodenalis* (A1); *G. enterica* (BIV); *G. bovis*	Thompson et al. (2010a, 2010b); Vermeulen et al. (2015)
Mice	Boullanger & Macquarie Islands — Australia	Novel; *G. duodenalis*; *G. canis*	Moro et al. (2003)
Beavers	Canada	*G. duodenalis* *G. enterica*	Prystajecky et al. (2015)
Coyotes	Canada	*G. duodenalis*	Thompson et al. (2009)
Coyotes	California	*G. duodenalis; G. enterica; G. canis*	Oates et al. (2012)
Painted dogs	Africa	*G. duodenalis; G. enterica*; *G. canis*	Ash et al. (2010)
Seals	Canada	*G. duodenalis*; *G. enterica*	Dixon et al. (2008); Appelbee et al. (2010)
Seals	Australia	*G. duodenalis*; *G. enterica*	Delport et al. (2014)
Dolphins	Spain	*G. duodenalis* (A1 & A2) *G. enterica*	Reboredo-Fernández et al. (2014)
Reindeer	Finland	*G. duodenalis*	Unpublished
Reindeer & moose	Norway	*G. duodenalis*	Robertson et al. (2007)
Red & roe deer	Poland	*G. duodenalis* (AI & AIII); *G. enterica*	Solarczyk et al. (2012)
Moose & Deer	Sweden	*G. duodenalis, G. bovis*	Lebbad et al. (2010)
Muskox	Canadian Arctic	*G. duodenalis* (AI) *G. enterica* (BIV)	Kutz et al. (2008)
Muskox	Norway	*G. duodenalis*	Davidson et al. (2014).
Wolves	Canada	*G. duodenalis*; *G. enterica*	Bryan et al. (2012)
Gorillas	Rwanda	*G. enterica*	Hogan et al. (2014)
Gorillas	Central African Republic	*G. duodenalis* (AII)	Sak et al. (2013)
Colobus monkeys	Ghana	*G. enterica*	Teichroeb et al. (2009)
Colobus monkeys	Uganda	*G. enterica* (BIV); *G. bovis*	Johnston et al. (2010)

and is considered to be a cause of morbidity (Johnston et al., 2010; Teichroeb et al., 2009).

3.3. Polyparasitism

Mixed infections with different species and intraspecific variants (sub-assemblages/genotypes) of *Giardia* occur frequently, particularly in non-human domestic hosts (Pallant et al., 2015; Thompson and Smith, 2011; Upjohn et al., 2010). Mixed infections appear to be particularly common in companion animals, dogs and to a lesser extent cats (Pallant et al., 2015), raising questions about how a diversity of species and intraspecific variants are acquired, and why they persist when competitive interactions would be expected to lead to the dominance of particular species. Most published data has been obtained from individual domestic dogs and cats (Covacin et al. 2011; Pallant et al., 2015; Palmer et al. 2008b). Mixed infections in these animals would suggest exposure to highly contaminated environments and/or frequent contact with other dogs and cats. This is supported by the fact that mixed infections are less common in cats that tend to be more often restricted to inside the house (Pallant et al., 2015). However, the persistence of mixed infections may suggest that the frequency of transmission between domestic dogs and cats is less than in community and kennel/cattery situations where available data suggests that competitive exclusion may be the reason why particular species of *Giardia* are dominant (reviewed in Thompson, 2011).

4. Cryptosporidium

4.1. Taxonomy

Unlike the more conservative approach that has been taken with *Giardia* towards the recognition of species, with *Cryptosporidium* there has been a proliferation of new host records complemented by novel genotypic characterisation (Ryan et al., 2014; Slapeta 2013). This has formed the basis for many new species descriptions, to which biological and epidemiological data has yet to be supplemented. The taxonomy of *Cryptosporidium* has gone through periods where a multitude of species were recognised followed by rationalisation and reduction to what we have now with over 30 named species, proposed principally on the basis of molecular characterisation. However, the population structure of *Cryptosporidium* is uncertain and requires further study (Tibayrenc and Ayala, 2014) and once determined will provide some stability to the species taxonomy of *Cryptosporidium*. In this respect, recent advances in nucleic-based approaches for the diagnosis and analysis of genetic diversity in species of *Cryptosporidium* (Jex et al., 2008b) represent a significant step towards an improved understanding of epidemiology and population structure (Beck et al. 2009).

4.2. Cycles of transmission

The question of zoonotic transmission is more clear-cut with *Cryptosporidium* than with *Giardia*. Two species, *C. hominis* and *C. pestis* are responsible for the majority of human infections but only the latter species is zoonotic, with cattle as its principal host (Fig. 3). Other species, and genotypes, have been reported in humans but only occasionally (Slapeta, 2013) and susceptibility to infection with other host adapted species and genotypes is largely governed by the immune status of the host (Slapeta, 2013).

The most important application of molecular tools in the epidemiology of *Cryptosporidium* infections has been in determining the source of infection in outbreak situations and risk factors of public health significance (Hunter and Thompson, 2005). This is because the oocysts of enteric *Cryptosporidium* species are not distinguishable morphologically, and molecular characterisation provides the only way to identify species or genotypes and thus the likely host origin of contaminating oocysts. Such tools are also widely used for routine surveillance by water utilities. However, apart from environmental detection, molecular epidemiological studies on *Cryptosporidium* infections are considered to be still in their infancy (Beck et al., 2009). The molecular epidemiological potential of such studies has been demonstrated by Mallon et al. (2003) and Peng et al. (2003), who have provided evidence that the population structure of *Cryptosporidium parvum* (= *C. pestis*, Slapeta, 2011) and *C. hominis* is more complex than previously suggested, but more genome information from different *Cryptosporidium* species and sub-types is needed in order to resolve these issues.

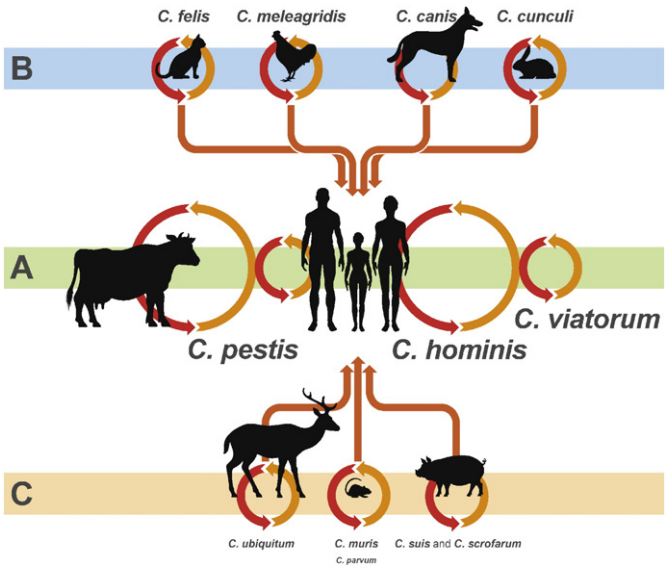

Fig. 3. Species and genotypes of *Cryptosporidium* with zoonotic potential. Frequency of human infection is indicated by the font size given to the species/genotype names, and the colour bands: green most frequently found in humans with *C. pestis* and *C. viatorum* less commonly cycling from human to human; blue and orange much less frequent and usually infect humans with impaired immune systems. The new terminology with respect to *C. pestis* is used here based on the proposed ICZN Code-based change and readers are referred to Slapeta, 2011, 2013 for discussion of this.

4.3. Phylogenetic relationships

From a phylogenetic perspective, molecular tools have proved valuable in questioning *Cryptosporidium*'s affinities with the coccidia. It was known for some time that *Cryptosporidium* lacks key morphological characteristics of coccidians and is insensitive to anti-coccidial drugs (O'Donoghue, 1995; Fayer et al. 1997). These suspicions were reinforced when SSU-rDNA sequencing demonstrated that *Cryptosporidium* is more closely related to gregarines (Barta and Thompson, 2006; Carreno et al., 1999; Leander and Ramey, 2006; Templeton et al. 2010). Most recently, Cavalier-Smith (2014) has undertaken a revision of gregarine higher classification, and the evolutionary diversification of sporozoa on the basis of gregarine site-heterogeneous SSU-rDNA trees. This has firmly placed *Cryptosporidium* with the gregarines, demonstrating that some 'eugregarines' and all 'neogregarines' are closely related to *Cryptosporidium*. A new subclass, the Orthogregarinia was established for gregarines most closely related to *Cryptosporidium*, which has been placed in its own subclass Cryptogregaria (Cavalier-Smith, 2014). This subclass is defined as comprising epicellular parasites of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast.

Biologically, *Cryptosporidium* shares many features in common with gregarines, including an extracytoplasmic location, connection to the host cell via a myzocytosis-like feeding mechanism and other structural similarities (Aldeyarbi and Karanis, 2015; Barta and Thompson, 2006; Borowski et al., 2008, 2010; Clode et al., in press). *Cryptosporidium* has

also been shown to have the capacity to multiply both intracellularly and extracellularly again reflecting the fact that *Cryptosporidium* is closely related to gregarine protozoa (Hijjawi et al. 2004; Karanis et al. 2008, Koh et al., 2013, 2014; Rosales et al. 2005), which can also multiply by either means. Most recently, *Cryptosporidium* has been shown to survive, multiply and develop in biofilms salvaging nutrients from their environment (Koh et al., 2014). Without molecular evidence of *Cryptosporidium*'s affinities with gregarine protozoa it is unlikely that studies on its developmental plasticity would have been undertaken to the extent that they have thus losing valuable information on the parasite's free-living potential and capacity for environmental persistence.

5. Concluding comments

There is no doubt that molecular tools have progressed our understanding of *Giardia* and *Cryptosporidium* and the infections they cause. However, as many questions have been answered they have been replaced by many more, along with continuing controversial issues. Surprisingly, nomenclature remains a problem with both parasites and seems to reflect a continuing lack of consensus between workers in the field. This hinders understanding of transmission patterns in endemic areas and can only be resolved with more discussion and healthy dialogue and a realisation that correct scientific names are required for effective communication.

With *Giardia*, molecular epidemiological studies require the use of multiple loci but again there needs to be some standardisation as to how the nomenclature that has 'evolved' with proponents of one locus relate to the nomenclature used for another locus. With *Cryptosporidium*, molecular epidemiological investigations should be directed to achieving a better understanding of the population genetics rather than documenting diversity and naming species. The fact that *Cryptosporidium* is a gregarine is likely to reflect an enormous diversity and host range and we have probably only sampled the surface to date. Most importantly in terms of *Cryptosporidium*'s gregarine characteristics are the implications with respect to water safety and public health, given the plasticity in terms of host and environmental development (Clode et al., in press). We need to overcome the entrenched dogma and realise we are dealing with an environmental pathogen.

Acknowledgements

We thank Aileen Elliot for the excellent images and Mark Preston of Murdoch Design for his skill in preparing the illustrations.

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