

REVIEW ARTICLE

Cryptosporidium species in humans and animals: current understanding and research needsUNA RYAN^{1*}, RONALD FAYER² and LIHUA XIAO³¹ School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, 6150 Australia² Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705, USA³ Centers for Disease Control and Prevention, Atlanta, Georgia 30333, USA

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SUMMARY

Cryptosporidium is increasingly recognized as one of the major causes of moderate to severe diarrhoea in developing countries. With treatment options limited, control relies on knowledge of the biology and transmission of the members of the genus responsible for disease. Currently, 26 species are recognized as valid on the basis of morphological, biological and molecular data. Of the nearly 20 *Cryptosporidium* species and genotypes that have been reported in humans, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for the majority of infections. Livestock, particularly cattle, are one of the most important reservoirs of zoonotic infections. Domesticated and wild animals can each be infected with several *Cryptosporidium* species or genotypes that have only a narrow host range and therefore have no major public health significance. Recent advances in next-generation sequencing techniques will significantly improve our understanding of the taxonomy and transmission of *Cryptosporidium* species, and the investigation of outbreaks and monitoring of emerging and virulent subtypes. Important research gaps remain including a lack of subtyping tools for many *Cryptosporidium* species of public and veterinary health importance, and poor understanding of the genetic determinants of host specificity of *Cryptosporidium* species and impact of climate change on the transmission of *Cryptosporidium*.

Key words: *Cryptosporidium*, taxonomy, epidemiology, species, genotype, subtype.

INTRODUCTION

Cryptosporidiosis is a highly prevalent and extremely widespread disease documented by over 1000 reports in humans in 95 countries on all continents except Antarctica (Fayer *et al.* 1997). Considering that cryptosporidiosis is primarily spread by ingestion of contaminated water, was ranked fifth among the 24 most important food-borne parasites in a global ranking by a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) expert committee in 2012 (<http://www.who.int/foodsafety/micro/jemra/meetings/sep12/en/>), and can be spread by close proximity to infected humans and animals, the importance of this genus in human and animal health has long been underestimated. Of approximately 8 million worldwide annual deaths of children under 5 years of age, diarrhoea is associated with 10·5% (Liu *et al.* 2012). An epidemiological study of over 22 000 infants and children in Africa and Asia found that *Cryptosporidium* was one of the four pathogens responsible for most of the severe diarrhoea (Kotloff *et al.* 2013) and was considered the second greatest cause of diarrhoea and death in children after rotavirus (Striepen, 2013). In developing countries

cryptosporidiosis is reported to account for 20% of all cases of diarrhoea in children (Mosier and Oberst, 2000) and, depending on location, at some point in life the percentage of affected persons in a population was estimated to range from 20–90% (Dillingham *et al.* 2002). In the USA, there are approximately 748 000 cases of cryptosporidiosis annually and hospitalizations from cryptosporidiosis cost an estimated \$45·8 million (Scallan *et al.* 2011). Unfortunately, cryptosporidiosis data are lacking for 11 of the 30 European Union and EEA/EFTA countries and data are underreported for those that did report; in 2007, only 6253 cases, all confirmed, were reported (Anonymous, 2010).

In general, three factors contribute significantly to the success of *Cryptosporidium* as a parasite. Large numbers of oocysts are excreted into the environment by infected individuals. Oocysts are environmentally hardy and can survive for many months in temperate and moist conditions. And infection can be initiated by a very small number of oocysts; theoretically a single oocyst could cause infection in a susceptible person. In human volunteer studies a median infectious dose for *Cryptosporidium hominis* ranged from 10 to 83 oocysts and for *Cryptosporidium parvum* from below 10 to over 1000 oocysts can initiate infection (Okhuysen *et al.* 1999; Chappell *et al.* 2006).

The lack of widespread prophylactic and therapeutic treatment options for cryptosporidiosis in

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humans and animals also has permitted a high prevalence and widespread distribution of the parasite. Although hundreds of drugs have been tested for prophylaxis and treatment of cryptosporidiosis in animals and humans (Fayer *et al.* 1997), only one has been approved by the US Food and Drug Administration (FDA). Nitazoxanide (Alinia®) liquid has been indicated for treatment of diarrhoea caused by *Giardia* and *Cryptosporidium* in patients one year and older and Alinia tablets have been approved for patients 12 years and older. However, Alinia has not been found to be better than a placebo for treatment of cryptosporidiosis in HIV-infected or immunodeficient patients (<http://www.romark.com/alinia-product-information>). FAO's executive summary of the State of Food Insecurity in the World (<http://www.fao.org/docrep/018/i3458e/i3458e.pdf>) indicates there are 842 million chronically malnourished persons worldwide, a factor that also significantly contributes to impaired immunity. Furthermore, the epidemiology of diarrhoea in children is thought to overlap with that of pneumonia, perhaps owing to shared risk factors such as insufficient nutrition, suboptimal breastfeeding and zinc deficiency (Walker *et al.* 2013). Rotavirus, a major cause of diarrhoea in children, is vaccine-preventable, but there is no vaccine for cryptosporidiosis and few published reports of attempts to develop immunoprophylaxis-based modalities (McDonald, 2011).

Because oocysts of *Cryptosporidium* species from humans and animals are ubiquitous in the environment, cryptosporidiosis can be acquired through multiple routes (reviewed by Robertson and Fayer, 2013). Transmission of oocysts is by the faecal–oral route, either directly or indirectly. For humans, direct transmission can be from one person to others primarily from poor hygiene among household members and attendees in day-care centres, nursing facilities, or other institutions, or from animals to persons such as farm-workers, pet owners, veterinarians and farm visitors. Most indirect transmission is from contaminated drinking water or recreational water (especially from faecal accidents in swimming pools). Contaminated food can be a source of transmission, starting at production sites where crops can be contaminated from contact with manure, from contaminated irrigation water, or from the hands of agricultural workers. Food can be contaminated at any point during distribution and preparation by food handlers, washwater, preparation surfaces, equipment or utensils. Although the primary foods implicated in transmission are raw fruits and vegetables, transmission associated with unpasteurized milk and apple cider, raw meat and sauce has also been documented. Although transmission has been reported from contaminated soil virtually nothing is known of the prevalence of infections from camping or gardening activities.

With cryptosporidiosis so widespread and prevalent, and with prophylaxis and therapeutic treatment options so limited, the ability to prevent and control disease appears best served by sanitation. The availability of clean water and toilets, fastidious handling of food and a clear understanding of the sources of *Cryptosporidium* provide a basis for prevention of transmission. This last topic, prevention, encompasses epidemiology that requires knowledge of the biology and taxonomy of the members of the genus responsible for disease. Because the oocysts of many species are indistinguishable from one another, molecular methods are essential for identification of the species, genotype and subtype of *Cryptosporidium* to specifically identify the organism responsible for infection and the source and routes of transmission (Xiao and Feng, 2008).

STANDARDS FOR TAXONOMY OF *CRYPTOSPORIDIUM* SPP.

Clearly described species with stable scientific names are basic and essential for understanding epidemiology. Principals for naming and stability are provided by the International Code of Zoological Nomenclature (ICZN). For names published after 1930 Article 13 of the ICZN states that 'when describing a new nominal taxon, an author should make clear his or her purpose to differentiate the taxon by including with it a diagnosis, that is to say, a summary of the characters that differentiate the new nominal taxon from related or similar taxa'. The lack of a description of the morphology and other unique features renders the name a nomen nudum, i.e. a naked name, without support, making it non-valid. In such cases the name is available for use at a later time, if desired. Article 13 and recent descriptions that have adopted the guidelines of Xiao *et al.* (2004, 2007) have been applied for accepting or rejecting those species names provided in Tables 1 and 2. The guidelines include the need to: provide morphometric data on oocysts; provide genetic characterization; demonstrate natural, and when feasible, experimental host specificity; and comply with ICZN rules. Those species that meet the guidelines appear in Table 1 and those that do not appear in Table 2 with reasons for making them non-valid species. In addition to these established *Cryptosporidium* spp., there are also over 40 genotypes of unknown species status. Like most *Cryptosporidium* species, these genotypes appear largely host-adapted (Feng, 2010; Ryan and Power, 2012). Some of them are likely to be named new *Cryptosporidium* species when biological data become available.

MOLECULAR TYPING TOOLS

After DNA has been extracted from oocysts obtained from environmental, food or biological specimens,

Table 1. Valid species of *Cryptosporidium*. Many of these earlier species were originally described based on morphological criteria but have subsequently been validated using molecular data

Species name	Author(s)	Type hosts	Major host	Reports in humans
<i>C. muris</i>	Tyzzer (1907, 1910)	Tame mice	Rodents	Numerous reports (cf. Feng <i>et al.</i> 2011b)
<i>C. zvairi</i>	Vetterling <i>et al.</i> (1971)	<i>Cavia porcellus</i> (Guinea pig)	Guinea pigs	None reported
<i>C. felis</i>	Iseki (1979)	<i>Felis catus</i> (Cat)		Many reports (cf. Lucio-Forster <i>et al.</i> 2010)
<i>C. serpentis</i>	Levine (1980)	<i>Elaphe guttata</i> , <i>E. subocularis</i> , <i>Sanzinia madagascarensis</i> (Snakes)	Snakes and lizards	None reported
<i>C. meleagridis</i>	Slavin (1955)	<i>Meleagris gallopavo</i> (Turkey)	Birds and humans	Commonly reported in humans
<i>C. parvum</i>	Re: Upton and Current (1985) Tyzzer (1912) ^a	<i>Bos taurus</i> (Cattle)	Ruminants	Commonly reported in humans
<i>C. baileyi</i>	Current <i>et al.</i> (1986)	<i>Gallus gallus</i> (Chicken)	Birds	None reported
<i>C. varanii</i>	Pavlašek <i>et al.</i> (1995)	<i>Varanus prasinus</i> (Emerald Monitor)	Lizards	None reported
<i>C. andersoni</i>	Lindsay <i>et al.</i> (2000)	<i>Bos taurus</i> (Cattle)	Cattle	Leoni <i>et al.</i> (2006); Morse <i>et al.</i> (2007); Waldron <i>et al.</i> (2011); Agholi <i>et al.</i> (2013); Liu <i>et al.</i> (2014)
<i>C. canis</i>	Fayer <i>et al.</i> (2001)	<i>Canis familiaris</i> (Dog)	Dogs	Many reports (cf. Lucio-Forster <i>et al.</i> 2010)
<i>C. molnari</i>	Alvarez-Pellitero and Sitja-Bobadilla (2002)	<i>Sparus aurata</i> and <i>Dicentrarchus labrax</i> (Fish)	Fish	None reported
<i>C. hominis</i>	Morgan-Ryan <i>et al.</i> (2002)	<i>Homo sapiens</i> (Human)	Humans	Most common species in humans
<i>C. galli</i>	Re: Ryan <i>et al.</i> (2003c) Pavlašek (1999) ^a	Spermestidae, Frangillidae, <i>Gallus gallus</i> , <i>Tetrao urogallus</i> , <i>Pinicola enucleator</i> (Birds)	Birds	None reported
<i>C. suis</i>	Ryan <i>et al.</i> (2004)	<i>Sus scrofa</i> (Pig)	Pigs	Xiao <i>et al.</i> (2002a); Leoni <i>et al.</i> (2006); Cama <i>et al.</i> (2007); Wang <i>et al.</i> (2013)
<i>C. bovis</i>	Fayer <i>et al.</i> (2005)	<i>Bos taurus</i> (Cattle)	Cattle	Khan <i>et al.</i> (2010); Ng <i>et al.</i> (2012); Helmy <i>et al.</i> (2013)
<i>C. fayeri</i>	Ryan <i>et al.</i> (2008)	<i>Macropus rufus</i> (Kangaroo)	Marsupials	Waldron <i>et al.</i> (2010)
<i>C. fragile</i>	Jirku <i>et al.</i> (2008)	<i>Duttaphrynus melanostictus</i> (Toad)	Toads	None reported
<i>C. macropodum</i>	Power and Ryan (2008)	<i>Macropus giganteus</i> (Kangaroo)	Marsupial	None reported
<i>C. ryanae</i>	Fayer <i>et al.</i> (2008)	<i>Bos taurus</i> (Cattle)	Cattle	None reported
<i>C. xiaoi</i>	Fayer <i>et al.</i> (2010)	<i>Ovis aries</i> (Sheep)	Sheep and goats	Adamu <i>et al.</i> (2014)
<i>C. ubiquitum</i>	Fayer <i>et al.</i> (2010)	<i>Bos taurus</i> (Cattle)	Ruminants, rodents, primates	Commonly reported (cf. Fayer <i>et al.</i> 2010; Elwin <i>et al.</i> 2012a)
<i>C. cuniculus</i>	Re: Robinson <i>et al.</i> (2010) Inman and Takeuchi (1979) ^a	<i>Oryctolagus cuniculus</i> (European rabbit)	Rabbits	Chalmers <i>et al.</i> (2009b); Anon (2010); Molloy <i>et al.</i> (2010); Chalmers <i>et al.</i> (2011a)
<i>C. tyzzeri</i>	Re: Ren <i>et al.</i> (2012) Tyzzer (1912) (<i>C. parvum</i>) ^a	<i>Mus musculus</i> (Mouse)	Rodents	Raskova <i>et al.</i> (2013)
<i>C. viatorum</i>	Elwin <i>et al.</i> (2012b)	<i>Homo sapiens</i> (Human)	Humans	Elwin <i>et al.</i> (2012b); Insulander <i>et al.</i> (2013)
<i>C. scrofarum</i>	Kváč <i>et al.</i> (2013b)	<i>Sus scrofa</i> (Pig)	Pigs	Kváč <i>et al.</i> (2009a); Kváč <i>et al.</i> (2009b)
<i>C. erinacei</i>	Kváč <i>et al.</i> (2014b)	European hedgehog (<i>Erinaceus europaeus</i>)	Hedgehogs and horses	Kváč <i>et al.</i> (2014a)

Re, redescription.

^a Initial description.

Table 2. Non-valid species names associated with *Cryptosporidium*

Name	Author	Host	Reason for non-validity
<i>C. crotali</i>	Triffit (1925)	<i>Crotalis confluentus</i> (Rattlesnake)	Large oocysts (11 by 12.5 µm) are likely <i>Sarcocystis</i>
<i>C. vulpis</i>	Wetzel (1938)	<i>Vulpes vulpes</i> (Fox)	Large oocysts (8 by 13.5 µm) are likely <i>Sarcocystis</i>
<i>C. baikalika</i>	Matschoulsky (1947)	<i>Scolopax</i> sp. (Woodcock)	Gregarine oocyst mistaken for <i>Cryptosporidium</i>
<i>C. ctenosauris</i>	Duszynski (1969)	<i>Ctenosaura similis</i> (Lizard)	Large oocysts (11.5 by 21.7 µm) are likely <i>Sarcocystis</i>
<i>C. lampropeltis</i>	Anderson <i>et al.</i> (1968)	<i>Lampropeltis calligaster</i> (King snake)	Large oocysts (8–9 by 10–12 µm) are likely <i>Sarcocystis</i>
<i>C. amievae</i>	Arcay-de-Peraza and Bastardo-de-San Jose (1969)	<i>Ameiva ameiva</i> (Lizard)	No oocyst measurements; no useful taxonomic data
<i>C. agni</i>	Barker and Carbonnell (1974)	<i>Ovis aries</i> (Sheep)	No oocyst measurements; no useful taxonomic data
<i>C. anserinum</i>	Proctor and Kemp (1974)	<i>Anser anser</i> (Goose)	No oocyst measurements; no useful taxonomic data
<i>C. rhesi</i>	Levine (1980)	<i>Macaca mulatta</i> (Rhesus monkey)	Name provided for enteric stages reported by others; no oocyst measurements; no useful taxonomic data
<i>C. garnhami</i>	Bird (1981)	<i>Homo sapiens</i> (Human)	No oocyst measurements; no useful taxonomic data
<i>C. natorum</i>	Hoover <i>et al.</i> (1981)	<i>Naso lateratus</i> (Fish)	One fish; one possible oocyst measured; insufficient data
<i>C. enteriditis</i>	Payne <i>et al.</i> (1983)	<i>Homo sapiens</i> (Human)	No oocyst measurements; no useful taxonomic data
<i>C. villithecum</i>	Paperna <i>et al.</i> 1986	Cichlid fish	Name proposed in a meeting abstract; data never published; later amended to <i>Piscicryptosporidium cichlidis</i> new genus, new species
<i>C. curyi</i>	Ogassawara <i>et al.</i> (1986)	<i>Felis catis</i> (Cat)	Very large oocysts (25–34.5 by 31.4–37.7 µm)
<i>C. saurophilum</i>	Koudela and Modry (1998)	<i>Eumeces schneideri</i> (Skink)	Identical to <i>C. varanii</i> that was named earlier
<i>C. scophthalmi</i>	Alvarez-Pellitero <i>et al.</i> (2004)	<i>Scophthalmus maximus</i> (Fish)	Lacking molecular data, cannot differentiate from other fish <i>Cryptosporidium</i>
<i>C. pestis</i>	Slapeta (2006)	<i>Bos taurus</i> (Cattle)	Does not follow ICZN rule of priority: Article 23.9.1.2
<i>C. ducismarci</i>	Traversa (2010)	<i>Testuda marginata</i> (Tortoise)	Lacks biological data and formal taxonomic description

different types of molecular diagnostic tools have been used in the identification of *Cryptosporidium* spp. These tools can be categorized into genotyping, subtyping, multilocus typing/population genetics, and comparative genomics depending on the approaches and usages.

Genotyping

Many small subunit (SSU) rRNA-based tools have been developed for the identification of *Cryptosporidium* species in humans, animals and water samples. In particular, a PCR-RFLP tool that targets an ~830-bp fragment of the gene and uses SspI and VspI restrictions for genotyping (Xiao *et al.* 1999) is commonly used in the differentiation of *Cryptosporidium* species in humans, animals and environmental samples. For the analysis of specimens from ruminants, the method has been modified by using SspI and MboII in the RFLP analysis (Feng *et al.* 2007). Another format commonly used for genotyping is DNA sequencing of PCR products (Koinari *et al.* 2013). In recent years, qPCR assays using fluorescent probes and melting curve analysis are increasingly used in *Cryptosporidium* genotyping (Jothikumar *et al.* 2008; Hadfield *et al.* 2011; Burnet *et al.* 2012; Lalonde *et al.* 2013; Mary *et al.* 2013; Staggs *et al.* 2013; Yang *et al.* 2013). Although the range of detection or differentiation for *Cryptosporidium* species is in general narrower, these newer methods are simpler to use and less prone to PCR contamination. The widespread use of the SSU rRNA gene in *Cryptosporidium* genotyping is largely due to the multi-copy nature of the gene and presence of semi-conserved and hyper-variable regions, which facilitate the design of genus-species primers. In many *Cryptosporidium* species and genotypes, minor intra-isolate sequence variations are present among different copies of the SSU rRNA gene. Thus, new genotypes should not be named based on one or two nucleotide substitutions or insertions/deletions in the gene (Abeywardena *et al.* 2014).

PCR tools based on other genes in general only amplify DNA of *C. parvum*, *C. hominis*, *Cryptosporidium meleagridis* and species/genotypes closely related to them. Thus, studies that have used these tools have usually showed lower *Cryptosporidium* species diversity (Abd El Kader *et al.* 2012; Berrilli *et al.* 2012). These tools have limited usefulness in genotyping *Cryptosporidium* spp. of animals because of their narrow specificity. They, nevertheless, can be used in the identification of mixed infections with *C. hominis* or *C. parvum* in humans in developing countries that have been infected with rare *Cryptosporidium* species based on PCR analysis of the SSU rRNA gene (Cama *et al.* 2006). A few other markers such as the 90 kDa heat shock protein and A135 genes have been used in the development of genus-specific PCR-RFLP tools for genotyping

Cryptosporidium (Feng *et al.* 2009; Tosini *et al.* 2010). Recently, a genus-specific *Cryptosporidium* qPCR based on the actin gene has been developed. Although it currently can only be used in screening of *Cryptosporidium* spp., genotyping can be done subsequently using species-specific qPCR (Yang *et al.* 2014).

Subtyping

Subtyping tools have been used extensively in studies of the transmission of *C. hominis* in humans, *C. parvum* in humans and ruminants, and a few other related *Cryptosporidium* species such as *C. meleagridis* and *Cryptosporidium ubiquitum* in both humans and animals (Xiao, 2010; Li *et al.* 2014). One of the most common subtyping tools is the DNA sequence analysis of the 60 kDa glycoprotein (gp60, also known as gp40/15) gene. Most of the genetic heterogeneity in this gene is the variation in the number of a tri-nucleotide repeat (TCA, TCG or TCT) in the 5' end (gp40) of the coding region, although extensive sequence polymorphism is also present in the rest of the gene. The latter is used in defining subtype families within a species, whereas the former is used in identifying subtypes within a subtype family. It should be kept in mind that commonly used gp60 PCR primers do not amplify DNA of *C. ubiquitum*, *Cryptosporidium felis*, *Cryptosporidium canis* and other species distant from *C. parvum* and *C. hominis* (Feng *et al.* 2011a; Li *et al.* 2014).

An established subtype nomenclature is used in identifying gp60 subtype family. A subtype name starts with the species and subtype family designation (Ia, Ib, Id, Ie, If, etc. for *C. hominis*; IIa, IIb, IIc, IIId, etc. for *C. parvum*; IIIa, IIIb, IIIc, IIIId, etc. for *C. meleagridis*; see Table 3 for subtype families of other *Cryptosporidium* species) followed by the number of TCA (represented by the letter A), TCG (represented by the letter G) or TCT (represented by the letter T) repeats (Sulaiman *et al.* 2005; Xiao, 2010; Feng *et al.* 2011a). Thus, the name IeA11G3T3 indicates that parasite belongs to *C. hominis* subtype family Ie and has 11 copies of the TCA repeat, three copies of the TCG repeat and three copies of the TCT repeat in the trinucleotide repeat region of the gene.

In the gp60 gene of a few subtype families, variations in copy numbers of other repeat sequences (designed as R at the end of the subtype name) are also present in the gp40 region. Thus, in the *C. parvum* IIa subtype family, some subtypes have two or three copies of the ACATCA sequence right after the trinucleotide repeats, which are represented by 'R2' or R3 (R1 for most subtypes). Likewise, within the *C. hominis* subtype family Ia, subtypes are further differentiated by the copy number of a 15-bp repetitive sequence 5'-AA/GGACGGTGGTAAGG-3' (the last copy is 13-bp: AAA/GACGGTGAAGG)

Table 3. Major gp60 subtype families of *Cryptosporidium* spp. and representative sequences

Species	Subtype family	Dominant trinucleotide repeat	Other repeat (R) ^a	GenBank accession no. ^b	
<i>C. hominis</i>	Ia	TCA	AAGACGGTGGTAAGG	AF164502 (IaA23R4)	
	Ib	TCA, TCG, TCT	–	AY262031 (IbA10G2), DQ665688 (IbA9G3)	
	Id	TCA, TCG	–	DQ665692 (IdA16)	
	Ie	TCA, TCG, TCT	–	AY738184 (IeA11G3T3)	
	If	TCA, TCG	AAGAAGGCAAAGAAG	AF440638 (IfA19G1R5), FJ153244 (IfA22G1R4)	
	Ig	TCA	–	EF208067 (IgA24)	
	Ih	TCA, TCG	–	FJ971716 (IhA14G1)	
	Ii	TCA	–	HM234173 (IiA17)	
<i>C. parvum</i>	Ij	TCA	–	JF681174 (IjA14)	
	IIa	TCA, TCG	ACATCA	AY262034 (IIaA15G2R1), DQ192501 (IIaA15G2R2)	
	IIb	TCA	–	AF402285 (IIbA14)	
	IIc	TCA, TCG	–	AF164491 (IIcA5G3a), AF164501 (IIcA5G3b), EU095267 (IIcA5G3c), AF440636 (IIcA5G3d), HM234172 (IIcA5G3e), HM234171 (IIcA5G3f), AJ973154 (IIcA5G3h), AM947935 (IIcA5G3i), GQ259136 (IIcA5G3j), JF802123 (IicA5G3k)	
	IId	TCA, TCG	–	AY738194 (IIdA18G1)	
	IIe	TCA, TCG	–	AY382675 (IIeA12G1)	
	IIf	TCA	–	AY738188 (IIfA6)	
	IIg	TCA	–	AY873780 (IIgA9)	
	IIh	TCA, TCG	–	AY873781 (IIhA7G4)	
	IIi	TCA	–	AY873782 (IIiA10)	
	IIk	TCA	–	AB237137 (IIkA14)	
	III	TCA	–	AM937006 (IIIA18)	
	IIm	TCA, TCG	–	AY700401 (IImA7G1)	
	IIn	TCA	–	FJ897787 (IInA8)	
	IIo	TCA, TCG	–	JN867335 (IIoA16G1)	
	<i>C. meleagridis</i>	IIIa	TCA, TCG	–	AF401499 (IIIaA24G3)
		IIIb	TCA, TCG	–	AB539720 (IIIbA20G1)
IIIc		TCA	–	AF401497 (IIIcA6)	
IIId		TCA	–	DQ067570 (IIIdA6)	
IIIe		TCA, TCG	–	AB539721 (IIIeA20G1)	
IIIf		TCA, TCG	–	EU164813 (IIIfA16G2)	
IIIg		TCA, TCG	–	JX878614 (IIIgA19G5)	
<i>C. fayeri</i>	IVa	TCA, TCG, TCT	–	FJ490060 (IVaA11G3T1)	
	IVb	TCA, TCG, TCT	–	FJ490087 (IVbA9G1T1)	
	IVc	TCA, TCG, TCT	–	FJ490069 (IVcA8G1T1)	
	IVd	TCA, TCG, TCT	–	FJ490058 (IVdA7G1T1)	
	IVe	TCA, TCG, TCT	–	FJ490071 (IVeA7G1T1)	
	IVf	TCA, TCG, TCT	–	FJ490076 (IVfA12G1T1)	
Opossum genotype	XIa	TCA, TCG, TCT	–	HM234181 (XIaA4G1T1)	

<i>C. cuniculus</i>	Va	TCA	-	FJ262730 (VaA18)
	Vb	TCA	-	FJ262734 (VbA29)
Horse genotype	Vla	TCA, TCG	-	FJ435960 (VlaA11G3), DQ648547 (IIjA15G4)
	Vlb			FJ435961 (VlbA13)
<i>C. wrairi</i>	VIIa	TCA, TCT	-	GQ121020 (VIIaA17T1)
Ferret genotype	VIIIa	TCA, TCG	-	GQ121029 (VIIIaA5G2)
<i>C. fyszeri</i>	IXa	TCA	-	GQ121030 (IXaA6R3), AY378188 (IIFA6R2)
	IXb	TCA	-	HM234177 (IXbA6)
Mink genotype	Xa	TCA, TCG	-	HM234174 (XaA5G1)
Opossum genotype I	XIa	TCA, TCG, TCT	-	HM234181 (XIaA4G1T1)
<i>C. ubiquitum</i> ^b	XIIa	-	-	JX412915
	XIIb	-	-	JX412926
	XIIc	-	-	JX412925
	XIId	-	-	JX412922
	XIIe	-	-	KC204983
	XIIIf	-	-	KC204984
<i>C. erinacei</i>	XIIIa	TCA	ACATCA	KF055453 (XIIIaA20R10)

^a Consensus repeat; minor sequence variation exists among repeats.

^b The gp60 gene of *C. ubiquitum* subtypes has no trinucleotide repeats, thus cannot be named at the subtype level using the established nomenclature.

in the gp40 region of the gene. Thus, the name IaA28R4 indicates that parasite belongs to *C. hominis* subtype family Ia and has 28 copies of the TCA repeat in the trinucleotide region and four copies of the 13–15 bp repeats downstream. Variations in other repeats also occur in other less common *C. hominis* subtype families such as If. In some subtype families such as *C. parvum* subtype family IIc, there is no sequence difference in the trinucleotide region (all IIc subtypes have five copies of TCA and three copies of TCG repeats: IIcA5G3). Instead, subtypes differ from each other in the nucleotide sequence of the 3' region (mostly gp15) of the gene. Subtypes within this subtype families are differentiated by alphabetical extensions, with the original gp60 sequence for the subtype family (GenBank accession number AF164491) assigned as IIcA5G3a (Table 3). Representative sequences of some common subtype families are shown in Table 3.

An advantage of using gp60 for subtyping is the potential association between subtype families and phenotypes of *C. parvum* and *C. hominis*. This could be due to the biological importance of the gp60 protein, which is located on the surface of apical region of invasive stages of the parasite, and is one of the dominant targets for neutralizing antibody responses in humans (O'Connor *et al.* 2007). Some of the *C. parvum* subtype families, such as IIa and IIc, are found in both humans and ruminants, responsible for zoonotic cryptosporidiosis (Xiao, 2010). In areas with both IIa and IIc, such as Spain, IIa subtypes preferentially infect calves whereas IIc subtypes preferentially infect lambs and goat kids (Diaz *et al.* 2010a; Quilez *et al.* 2013). Some other *C. parvum* subtype families, especially IIc, have so far only been found in humans (Xiao and Feng, 2008; Widmer and Lee, 2010). Host adaptation has recently also been identified in *C. ubiquitum* based on sequence analysis of its gp60 gene (Li *et al.* 2014). There are also significant differences in clinical presentations and virulence among some common *C. hominis* or *C. parvum* subtype families in cryptosporidiosis-endemic areas (Cama *et al.* 2007, 2008; Del Chierico *et al.* 2011; Feng *et al.* 2012). Some gp60 subtypes of *C. hominis* and *C. parvum*, such as IaA10G2 of *C. hominis* and IIaA15G2R1 of *C. parvum*, are widely distributed in the world probably due to their biological fitness (Budú-Amoako *et al.* 2012a; Feng *et al.* 2013; Li *et al.* 2013).

Multilocus typing and population genetics

The whole genome sequencing (WGS) of *C. parvum*, *C. hominis* and *Cryptosporidium muris* has allowed the identification of microsatellite and minisatellite sequences in *Cryptosporidium* genomes and other targets that are highly polymorphic between *C. parvum*

and *C. hominis* (Robinson and Chalmers, 2012; Li *et al.* 2013). They are frequently used in either multi-locus fragment typing (MLFT) or multilocus sequence typing (MLST) to increase the subtyping resolution offered by gp60 sequence analysis (Quilez *et al.* 2011, 2013; Diaz *et al.* 2012; Herges *et al.* 2012; De Waele *et al.* 2013; Feng *et al.* 2013, 2014). Recently, a MLST tool has been developed for subtyping *C. muris* and *Cryptosporidium andersoni* (Feng *et al.* 2011b). In addition to subtyping, MLFT and MLST data can be analysed for linkage disequilibrium and recombination rates with various population genetic tools such as DnaSP (<http://www.ub.es/dnasp/>), LIAN (<http://pubmlst.org/per/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=lian&referer=pubmlst.org>) and Arlequin 3.1 (<http://cmpg.unibe.ch/software/arlequin3/>). These data can also be analysed for population differentiation using the Bayesian analysis tool STRUCTURE (<http://pritch.bsd.uchicago.edu/structure.html>) and network analysis software eBURST (<http://eburst.mlst.net/>). Any population differentiation can be confirmed by the calculation of the Wright's fixation index (F_{ST}). These tools have been used widely in population genetics studies and geographic tracking of *C. parvum*, *C. hominis*, *C. muris* and *C. andersoni* (Quilez *et al.* 2011, 2013; Herges *et al.* 2012; Diaz *et al.* 2012; Wang *et al.* 2012; De Waele *et al.* 2013; Feng *et al.* 2013, 2014; Zhao *et al.* 2013). Some of these studies have shown a panmictic population structure with frequent recombination in *C. parvum* (Herges *et al.* 2012; De Waele *et al.* 2013), whereas others have demonstrated the existence of a flexible reproductive strategy (co-occurrence of panmictic, clonal or epidemic structure) in this species (Tanriverdi *et al.* 2008; Drumo *et al.* 2012). Similarly, some genetic studies conducted on *C. hominis* identified largely a clonal population structure (Mallon *et al.* 2003; Gatei *et al.* 2007; Li *et al.* 2013; Feng *et al.* 2014), whereas others showed the common occurrence of genetic recombination in *C. hominis* in developing countries (Widmer and Sullivan, 2012). In areas with an overall clonal population structure of *C. hominis*, genetic recombination has been shown to be a driving force for the emergence of virulent subtypes such as Iba10G2 and IaA28R4 (Li *et al.* 2013; Feng *et al.* 2014). Genetic recombination appears also to play an important role in the emergence of the virulent *C. parvum* subtype IIaA15G2R1 (Feng *et al.* 2013).

Comparative genomics

The WGS of *C. parvum*, *C. hominis* and *C. muris* and recent advances in next-generation sequencing (NGS) techniques have made the comparative genomic analysis of *Cryptosporidium* spp. possible. It is expected that comparative genomics of *Cryptosporidium* spp. will significantly improve our

understanding of the transmission of cryptosporidiosis, especially in the investigation of outbreaks and monitoring of emerging and virulent subtypes (Widmer and Sullivan, 2012). Thus far, WGS analysis of *Cryptosporidium* spp. using NGS has only been done on one isolate each of the *C. parvum* anthroponotic subtype family IIc (Widmer *et al.* 2012) and *C. ubiquitum* (Li *et al.* 2014), although efforts are underway to sequence more isolates of *Cryptosporidium* species of public health and veterinary importance. NGS analysis of HSP70 and gp60 PCR products from two *C. parvum* isolates showed the presence of two HSP70 and 10 gp60 subtype in these isolates in spite of the initial detection of one subtype per locus using the traditional Sanger sequencing (Grinberg *et al.* 2013). If verified, this finding of high intra-isolate heterogeneity would have important implications in our understanding of the epidemiology and population genetics of *Cryptosporidium* spp.

CRYPTOSPORIDIUM SPECIES IN HUMANS

Currently, nearly 20 *Cryptosporidium* species and genotypes have been reported in humans, including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *Cryptosporidium cuniculus*, *C. ubiquitum*, *Cryptosporidium viatorum*, *C. muris*, *Cryptosporidium suis*, *Cryptosporidium fayeri*, *C. andersoni*, *Cryptosporidium bovis*, *Cryptosporidium scrofarum*, *Cryptosporidium tyzzeri*, *Cryptosporidium erinacei* and *Cryptosporidium* horse, skunk and chipmunk I genotypes (Xiao, 2010; Waldron *et al.* 2011; Elwin *et al.* 2012a; Ng *et al.* 2012; Kváč *et al.* 2013a; Raskova *et al.* 2013; Liu *et al.* 2014). Humans are most frequently infected with *C. hominis* and *C. parvum*. Other species, such as *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum* and *C. viatorum* are less common. The remaining *Cryptosporidium* species and genotypes have been found in only a few human cases (Xiao, 2010; Elwin *et al.* 2012a). These *Cryptosporidium* spp. infect both immunocompetent and immunocompromised persons.

The distribution of these species in humans is different among geographic areas and socioeconomic conditions. In European countries and New Zealand, both *C. hominis* and *C. parvum* are commonly detected in humans. In contrast, *C. parvum* is the dominant species in humans in Middle Eastern countries, whereas *C. hominis* is the dominant species in other industrialized nations and developing countries (Xiao and Feng, 2008; Xiao, 2010; Nazemalhosseini-Mojarad *et al.* 2012). Likewise, human infections with *C. canis* and *C. felis* are reported in studies conducted in developing countries, *C. ubiquitum* mostly in industrialized nations and *C. cuniculus* mostly in the UK. Differences also exist in the distribution of *C. parvum* and *C. hominis* between urban and rural

areas, with the former more commonly detected in rural and the latter in urban areas (Learmonth *et al.* 2004; Llorente *et al.* 2007; Zintl *et al.* 2009; Chalmers *et al.* 2011a). This difference in *Cryptosporidium* species distribution is probably the results of differences in infection sources and transmission routes (Xiao, 2010).

There are also temporal and age-associated variations in the disease burdens between *C. parvum* and *C. hominis* (Chalmers *et al.* 2009a, 2011a). Like earlier observations in the UK and New Zealand, *C. hominis* was more prevalent in autumn, and *C. parvum* was more prevalent in spring in some more recent studies conducted in Canada, Ireland and the Netherlands (Wielinga *et al.* 2008; Zintl *et al.* 2009; Budu-Amoako *et al.* 2012b). In the Netherlands, *C. hominis* was more commonly found in children and *C. parvum* more in adults (Wielinga *et al.* 2008). In the UK, *C. hominis* was more prevalent in infants less than one year, females aged 15–44 years and international travellers, and there has been a decline in *C. parvum* cases since 2001 (Chalmers *et al.* 2009a, 2011a). In some studies, rare *Cryptosporidium* species were more commonly detected in immunocompromised persons than immunocompetent persons (ANOFEL *Cryptosporidium* National Network, 2010), while in others, there were no significant differences in the distribution of *Cryptosporidium* species between children and HIV-positive persons (Cama *et al.* 2007, 2008).

Cryptosporidium parvum and *C. hominis* are responsible for most cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* (Xiao, 2010). This is even the case for the UK, where *C. parvum* and *C. hominis* are both common in the general population. Recently, there was one drinking water associated cryptosporidiosis outbreak caused by *C. cuniculus* (Chalmers *et al.* 2009b). An outbreak of *C. meleagridis* also occurred in a high school dormitory in Japan, although the infection source for the outbreak was not clear (Asano *et al.* 2006).

Subtyping studies based on gp60 have shown that many *C. parvum* infections in humans are not results of zoonotic transmission (Xiao, 2010). Among several *C. parvum* subtype families identified, IIa and IIc are the two most common families in humans. In developing countries, most *C. parvum* infections in children and HIV-positive persons are caused by the subtype family IIc, with IIa largely absent, indicating that anthroponotic transmission of *C. parvum* is common in these areas. In contrast, both IIa and IIc subtype families are seen in humans in developed countries (Xiao and Feng, 2008; Xiao, 2010), whereas IID is the dominant *C. parvum* subtype family in humans in Middle Eastern countries (Nazemalhosseini-Mojarad *et al.* 2012). In a recent study conducted in Sweden, all IIc infections were acquired when travelling in developing countries,

whereas almost all IIa and IID infections were acquired locally or in other European countries (Insulander *et al.* 2013). As expected infections with subtype family IIa in the UK are frequently associated with farm visits (Chalmers *et al.* 2011b).

CRYPTOSPORIDIUM SPECIES IN ANIMALS

Cryptosporidium in cattle, sheep and goats

Molecular studies have identified a wide range of *Cryptosporidium* species and genotypes in animals, many of which are not commonly found in humans. Livestock however, particularly cattle and sheep, are important reservoirs for *C. parvum*. In case-control studies, contact with cattle was implicated as a risk factor for human cryptosporidiosis in the USA, UK, Ireland and Australia (Robertson *et al.* 2002; Goh *et al.* 2004; Hunter *et al.* 2004; Roy *et al.* 2004).

Studies worldwide suggest that cattle are infected with four major *Cryptosporidium* species: *C. parvum*, *C. bovis*, *C. andersoni* and *C. ryanae* (Xiao and Feng, 2008; Xiao, 2010). Studies of dairy cattle in industrialized nations have shown a dominance of *C. parvum*, especially its IIa subtypes in pre-weaned calves (Xiao, 2010; Amer *et al.* 2013). Subtype IIaA15G2R1 is especially common and is overwhelmingly the dominant subtype in most areas (Xiao, 2010). However, the *C. parvum* IID subtype has been found in low frequencies in cattle in European countries. Intensive farming practices may facilitate the persistent transmission of *C. parvum* IIa subtypes on most dairy farms in industrialized nations (Xiao, 2010; Amer *et al.* 2013; Santin, 2013).

In developing countries *C. bovis* is the dominant species in pre-weaned calves, in addition to *C. parvum*, *C. ryanae* and *C. andersoni* (Silverlås *et al.* 2010; Meireles *et al.* 2011; Muhid *et al.* 2011; Wang *et al.* 2011a; Budu-Amoako *et al.* 2012b; Venu *et al.* 2012; Amer *et al.* 2013; Silva *et al.* 2013; Silverlås and Blanco-Penedo, 2013; Zhang *et al.* 2013a). A small number of these studies subtyped *C. parvum* (Amer *et al.* 2010; Imre *et al.* 2011; Meireles *et al.* 2011; Muhid *et al.* 2011; Wang *et al.* 2011a; Silva *et al.* 2013), with *C. parvum* IID subtypes identified as the dominant *C. parvum* in China and Malaysia (Muhid *et al.* 2011; Wang *et al.* 2011a; Zhang *et al.* 2013a) and both IIa and IID dominant in Egypt (Amer *et al.* 2010; 2013). Thus, the prevalence and consequently the potential for transmission of *Cryptosporidium* infecting dairy cattle differ in developing countries *vs* industrialized nations at both species and subtype levels.

Cryptosporidium bovis (Fayer *et al.* 2005) primarily infects young post-weaned cattle and has a wide geographic distribution (Feng *et al.* 2007). To date there have been only three reports of humans infected with *C. bovis*: a dairy farm worker in India, a farm

hand in Australia, and a 5-year-old village boy with animal contact in Egypt; the infection was asymptomatic in the first two cases and symptomatic in the third case (Khan *et al.* 2010; Ng *et al.* 2012; Helmy *et al.* 2013). There have been a few reports of *C. andersoni* in humans in the UK, Australia, Iran and Malawi (Leoni *et al.* 2006; Morse *et al.* 2007; Waldron *et al.* 2011; Agholi *et al.* 2013). A recent study in China identified *C. andersoni* in 34/252 diarrhoea patients in Shanghai (Liu *et al.* 2014), but this together with the observation of common occurrence of *Giardia duodenalis* assemblage C in these patients needs confirmation by other studies. One study in New Zealand identified *C. hominis* (subtype IbA10G2) in cattle (Abeywardena *et al.* 2012).

Globally, the prevalence of *Cryptosporidium* spp. in sheep can vary drastically from <5 to >70% (Robertson, 2009). At least eight *Cryptosporidium* species have been identified in sheep faeces including *C. parvum*, *C. hominis*, *C. andersoni*, *C. suis*, *Cryptosporidium xiaoi*, *C. fayeri*, *C. ubiquitum* and *C. scrofarum*, with *C. xiaoi*, *C. ubiquitum* and *C. parvum* most prevalent (Ryan *et al.* 2005; Santín *et al.* 2007; Fayer and Santín, 2009; Giles *et al.* 2009; Yang *et al.* 2009, 2014; Robertson, 2009; Díaz *et al.* 2010a; Wang *et al.* 2010a; Fiuza *et al.* 2011a; Sweeny *et al.* 2011; Cacciò *et al.* 2013; Connelly *et al.* 2013; Koinari *et al.* 2014). There are geographic differences in the distribution of the three dominant species in sheep; *C. parvum* is the dominant species in Europe, *C. xiaoi* is the dominant species in Australia, whereas *C. ubiquitum* appears to dominate in the Americas and Asia (see Table 2 in Ye *et al.* 2013). As with cattle, there are probably age-associated differences in the distribution of *Cryptosporidium* spp. in sheep (Sweeny *et al.* 2011; Ye *et al.* 2013), although the picture remains unclear due to a lack of longitudinal studies. In one longitudinal study in China, periparturient shedding of oocysts in ewes was identified as the source of *C. xiaoi* infection in newborn lambs (Ye *et al.* 2013).

In locations with common occurrence of *C. parvum* in sheep, the subtype IIaA15G2R1 is also dominant, although IId subtypes are also commonly reported (Xiao, 2010; Yang *et al.* 2014). The IIaA15G2R1 subtype was reported in three lambs linked to a human infection in the UK (Chalmers *et al.* 2005). Recently, sheep have also been implicated as a potential source for human *C. ubiquitum* infections in the UK by gp60 subtyping (Li *et al.* 2014). Furthermore, two studies reported the occurrence of the *C. hominis* IbA10G2 subtype in lambs in the UK (Giles *et al.* 2009; Connelly *et al.* 2013).

Although fewer epidemiological studies have examined *Cryptosporidium* spp. in goats, *C. parvum*, *C. hominis*, *C. ubiquitum* and *C. xiaoi* have also been identified in goats (Park *et al.* 2006; Goma *et al.* 2007; Geurden *et al.* 2008; Quílez *et al.* 2008; Giles *et al.* 2009; Robertson, 2009; Diaz *et al.* 2010b; Fayer *et al.*

2010; Koinari *et al.* 2014). The *C. parvum* IId subtype predominates in goats (Xiao, 2010).

Cryptosporidium in pigs

The main *Cryptosporidium* species identified in pigs worldwide are *C. suis* and *C. scrofarum* (formally pig genotype II), although *C. muris*, *C. tyzzeri* and *C. parvum* have been reported (Ryan *et al.* 2003a; Xiao *et al.* 2006; Zintl *et al.* 2007; Johnson *et al.* 2008; Kváč *et al.* 2009a, 2013b; Jeníková *et al.* 2010; Jenkins *et al.* 2010; Sevá *et al.* 2010; Xiao, 2010; Wang *et al.* 2010b; Budu-Amoako *et al.* 2011; Chen *et al.* 2011; Farzan *et al.* 2011; Fiuza *et al.* 2011b; Yin *et al.* 2011; Němejc *et al.* 2013; Yui *et al.* 2014). As in dairy cattle, there is also an age-associated distribution of *C. suis* and *C. scrofarum* in pigs, with the former more commonly seen in pre-weaned piglets and the latter more commonly seen in older pigs (Němejc *et al.* 2013; Zhang *et al.* 2013b; Yui *et al.* 2014). *Cryptosporidium suis* has been reported in humans (Xiao *et al.* 2002a; Leoni *et al.* 2006; Cama *et al.* 2007; Wang *et al.* 2013) and has been frequently recovered from water samples (Feng *et al.* 2011c). *Cryptosporidium scrofarum* has also been reported in an immunocompetent human (Kváč *et al.* 2013b).

Cryptosporidium parvum has been reported at least six times in pigs: (1) in four 19-day-old pre-weaned piglets with diarrhoea from an indoor farm in Western Australia (Morgan *et al.* 1999a); (2) in asymptomatic sows from intensive commercial pig production units in Ireland (Zintl *et al.* 2007); (3) in two piglets from Prince Edward Island, Canada (Budu-Amoako *et al.* 2011); (4) in piglets in Ontario, where it was the most prevalent species detected (55.4%) (Farzan *et al.* 2011); (5) in pig lagoons in the USA (Jenkins *et al.* 2010); and (6) in one pig isolate from the Czech Republic (IIa A16G1R1b) (Němejc *et al.* 2013). This suggests that pigs may play a potential role in the transmission of zoonotic *Cryptosporidium*. However, further research is required to understand the prevalence of *Cryptosporidium* species in wild pigs. Recently, *C. suis* and *C. scrofarum* have also been detected in wild boars in the Czech Republic (Němejc *et al.* 2012) and Spain (García-Precedo *et al.* 2013a). The latter also reported the occurrence of *C. parvum* subtypes IIaA16G2R1 and IIaA13G1R1, two common subtypes in humans in the country.

Cryptosporidium in cats and dogs

Genotyping studies of *Cryptosporidium* oocysts in faeces of dogs and cats have demonstrated that most infections in these animals are caused by *C. canis* and *C. felis*, respectively. *Cryptosporidium muris* and *C. parvum* have also occasionally been reported in dogs and cats (cf. Lucio-Forster *et al.* 2010). *Cryptosporidium muris* has a wide host range and has also been identified in a few humans in developing

countries (Gatei *et al.* 2006; Muthusamy *et al.* 2006; Palmer *et al.* 2008). *Cryptosporidium felis* has a much more restricted host range and, using molecular techniques, has been confirmed to infect cats, immunocompetent and immunocompromised humans and a cow (Bornay-Llinares *et al.* 1999; Lucio-Forster *et al.* 2010). Similarly, using molecular techniques, *C. canis* has been confirmed to infect dogs, foxes, wolves and immunocompetent and immunocompromised humans (Lucio-Forster *et al.* 2010). In children in developing countries, *C. felis* and *C. canis* are responsible for as much as 3.3 and 4.4%, respectively, of overall cryptosporidiosis cases (Lucio-Forster *et al.* 2010). However, most human cases of cryptosporidiosis, worldwide, are associated with *C. hominis* and *C. parvum* (Xiao, 2010) and therefore *C. muris*, *C. canis* and *C. felis* are of low zoonotic risk to humans.

Cryptosporidium in wild mammals

Cryptosporidium cuniculus (previously rabbit genotype) was first identified genetically in rabbits from the Czech Republic (Ryan *et al.* 2003b), then formally re-described as a species, based on biological and genetic data in 2010 (Robinson *et al.* 2010). *Cryptosporidium cuniculus* was initially thought to be host-specific until the discovery that *C. cuniculus* was linked to a human cryptosporidiosis outbreak in the UK (Chalmers *et al.* 2009b), which raised considerable awareness about the importance of investigating rabbits as a source of *Cryptosporidium* transmissible to humans. Subsequently, a study reported that *C. cuniculus* was the third most commonly identified *Cryptosporidium* species in patients with diarrhoea in the UK (Chalmers *et al.* 2011a). It has also been identified in a few human patients in France, South Australia and Nigeria (Anon, 2010; unpublished; Molloy *et al.* 2010).

Few studies have characterized *Cryptosporidium* spp. in wild deer, with *C. ubiquitum*, *C. bovis*, *C. ryanae*, the deer genotype and *C. parvum* and a *C. hominis*-like genotype reported (cf. Cinque *et al.* 2008; Amer *et al.* 2009; Feng, 2010; Ng *et al.* 2011; Nolan *et al.* 2013; García-Preedo *et al.* 2013b). In captive cervids *C. parvum* and *C. ubiquitum* predominated (cf. Feng, 2010).

House mice are commonly infected with *C. muris* and *C. tyzzeri* (formerly mouse genotype I), and occasionally with the mouse genotype II (Morgan *et al.* 1999b; Foo *et al.* 2007; Silva *et al.* 2013). There have been numerous reports of *C. muris* in humans (cf. Feng *et al.* 2011b) and a co-infection of *C. tyzzeri* and *C. parvum* in a young woman (Raskova *et al.* 2013). Confirmed *C. parvum* infections have been reported in relatively few rodents (Morgan *et al.* 1999b; Lv *et al.* 2009; Feng, 2010). Recently *C. erinacei* (formerly the hedgehog genotype) has been described

biologically and genetically from the European hedgehog (*Erinaceus europaeus*) (Kváč *et al.* 2014b). It has previously been described in horses and an immunocompetent human (Kváč *et al.* 2014a).

Of the few genotyping studies conducted with foxes, the *Cryptosporidium* fox genotype, *C. canis* fox subtype (a variant of *C. canis*) and a *Cryptosporidium macropodum*-like genotype have been reported (Xiao *et al.* 2002b; Ng *et al.* 2011; Ruecker *et al.* 2012, 2013; Nolan *et al.* 2013).

Cryptosporidium fayeri (previously marsupial genotype I) and *C. macropodum* (previously marsupial genotype II) infect marsupials (Ryan *et al.* 2008; Power and Ryan, 2008; Power, 2010; Ryan and Power, 2012). There has been one report of *C. fayeri* in a 29-year-old immunocompetent woman who suffered prolonged gastrointestinal illness in Sydney (Waldron *et al.* 2010). Identical gp60 subtypes were found in marsupials in the area, indicating potential zoonotic transmission (Waldron *et al.* 2010).

Cryptosporidium in birds

Currently only three avian *Cryptosporidium* spp. are recognized in birds: *C. meleagridis*, *Cryptosporidium baileyi* and *Cryptosporidium galli* (Table 1). *Cryptosporidium meleagridis* has a wide host range and is the third most prevalent species infecting humans (Leoni *et al.* 2006; Xiao, 2010; Elwin *et al.* 2012a). The ability of *C. meleagridis* to infect humans and other mammals, and its close phylogenetic relationship to *C. parvum* and *C. hominis*, has led to the suggestion that mammals actually were the original hosts, and that the species has later adapted to birds (Xiao *et al.* 2002b, 2004). Recently, sequence analysis of the SSU gene and HSP70 loci has been used to provide evidence of zoonotic transmission of *C. meleagridis* from chickens to a human on a Swedish farm (Silverlås *et al.* 2012). In addition to the three recognized species of *Cryptosporidium*, 11 genotypes: the avian genotypes I–V, the black duck genotype, the Eurasian woodcock genotype and goose genotypes I–IV have been reported (Ryan and Xiao, 2014), none of which are considered human pathogens. In addition, *C. hominis*, *C. parvum*, *Cryptosporidium serpentis*, *C. muris*, *C. andersoni* and muskrat genotype I have also been identified in a small number of birds, most of which were probably the results of accidental ingestion of oocysts by these organisms (cf. Ryan, 2010; Ryan and Xiao, 2014).

Cryptosporidium in marine mammals, reptiles, amphibians and fish

Very little is known about the prevalence and genetic diversity of species of *Cryptosporidium* in marine environments and the role that marine animals play in transmission of these parasites to humans. Molecular research on marine mammals has identified

C. muris, seal genotypes 1–4 and a genotype similar to the skunk genotype in seals (Santín *et al.* 2005; Rengifo-Herrera *et al.* 2011; Bass *et al.* 2012; Rengifo-Herrera *et al.* 2013).

Cryptosporidium serpentis (Levine, 1980) and *Cryptosporidium varanii* (syn. *Cryptosporidium saurophilum*) Pavlásek *et al.* 1995; Pavlasek and Ryan, 2008) are the only valid species in reptiles. Several genotypes including tortoise genotype I and snake genotypes I and II have also been identified (cf. Ryan and Xiao, 2014), which have not been reported in humans. A new intestinal species, *Cryptosporidium ducismarci* has been reported in several species of tortoises, snakes and lizards (Traversa, 2010). Because only molecular data are presented, this species has to be regarded as a nomen nudum, pending the support of morphological and biological data. Relatively little is known about *Cryptosporidium* in amphibians and currently the only accepted species is *Cryptosporidium fragile*, which was described from the stomach of naturally infected black-spined toads (*Duttaphrynus melanostictus*) from the Malay peninsula (Jirku *et al.* 2008).

Cryptosporidium has been described in both fresh and marine water piscine species, with *Cryptosporidium molnari* (Alvarez-Pellitero and Sitja-Bobadilla, 2002), the only currently recognized species. *Cryptosporidium scophthalmi* was described in 2004 in turbot (*Psetta maxima*, syn. *Scophthalmus maximus*) (Alvarez-Pellitero *et al.* 2004), but is considered a nomen nudum until genetic data are provided, as several morphologically identical species can be found in one fish species. A total of 13 additional species/genotypes have been identified in fish using molecular tools; piscine genotypes 1–8, rat genotype III, *C. parvum*, *C. hominis*, *C. xiaoi* and *C. scrofarum* (cf. Koinari *et al.* 2013; Ryan and Xiao, 2014). The very low prevalence of *C. parvum* and *C. hominis* in fish (<1%) suggests that fish are not an important reservoir for human infection.

The taxonomic status of *Cryptosporidium* in fish presents a problem based on the location of the oocyst in the basal part of the stomach epithelium and the retained residual parasitophorous sac with the retained remnants of the attachment organelle, features not found in species of *Cryptosporidium* from homoeothermic hosts. Recognition of these features prompted Paperna and Vilenkin (1996) to propose the new genus *Piscicryptosporidium* for two new species – *Piscicryptosporidium reichenbachklinkei* and *Piscicryptosporidium cichlidis* from cichlid fishes of the genus *Oreochromis*. Other forms of divergence however have been accepted for *Cryptosporidium* species found in hosts within the same major host taxon, such as the large oocysts of *Cryptosporidium* species that develop in the stomach *vs* the small oocysts of species that develop in the small intestine. Genetic data also suggest that *Cryptosporidium* in fish are genetically very distinct (Palenzuela *et al.* 2010;

Koinari *et al.* 2013), however additional genetic and biological data are required to better understand the taxonomic status of *Piscicryptosporidium*.

RESEARCH GAPS AND PERSPECTIVES

Despite the considerable progress in the last 20 years on the taxonomy and molecular epidemiology of *Cryptosporidium*, significant research gaps remain. Differences still exist on the interpretation of ICZN rules and opinions on validity of some *Cryptosporidium* species (Slapeta, 2013). Morphological and biological data are not yet available for some common *Cryptosporidium* genotypes with public health and veterinary importance, such as the horse and skunk genotypes. Taxonomic and molecular epidemiological studies on *Cryptosporidium* spp. in wildlife, especially those in watershed are still scarce. Resolutions to those issues should greatly improve our understanding of the species structure and transmission of *Cryptosporidium* in humans and animals.

Cryptosporidium was the aetiological agent in 60.3% (120) of the waterborne protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010, (Baldursson and Karanis, 2011). Yet the public health significance of various zoonotic *Cryptosporidium* species detected in animals in water catchments and in drinking water remains unclear. For example, do different species of *Cryptosporidium* have different sensitivities (inactivation rates) to drinking water treatments and environmental conditions such as temperature, UV and solar inactivation? Are there differences in infectious dose for different zoonotic *Cryptosporidium* species? There is also a need to confirm if molecular detection of zoonotic *Cryptosporidium* species in wildlife is commonly associated with actual infections or mechanical transmission. *Cryptosporidium cuniculus* is the only species besides *C. hominis* and *C. parvum* known to be associated with a waterborne outbreak of human cryptosporidiosis, yet little is known about the prevalence and oocyst shedding rates of *C. cuniculus* in rabbits. Measuring the infectivity of different *Cryptosporidium* species under different climatic conditions is also crucial for accurate risk assessment of public health implications, particularly as more extreme precipitation is predicted globally (IPCC, 2013).

Studies on the transmission of *Cryptosporidium* in humans and domesticated animals are currently hampered by the lack of suitable subtyping tools for *Cryptosporidium* species that are genetically distant from *C. parvum* and *C. hominis*. The ability to subtype all major *Cryptosporidium* species at the gp60 is also important for understanding the transmission dynamics, particularly as recent research suggests that gp60 plays an active and essential role in the life cycle of the parasite and that genetic variation at this locus might be essential for the parasite's long-term

success (Abal-Fabeiro *et al.* 2013; Feng *et al.* 2013, 2014; Li *et al.* 2014). Currently only *C. parvum*, *C. hominis*, *C. meleagridis*, *C. tyzzeri*, *C. cuniculus*, *C. fayeri* and *C. ubiquitum* can be subtyped at this locus (Chalmers *et al.* 2009b; Lv *et al.* 2009; Power *et al.* 2009; Xiao, 2010; Feng *et al.* 2011a; Kváč *et al.* 2013c; Li *et al.* 2014). The most recent study, which used NGS to identify and develop a gp60-based typing tool for *C. ubiquitum*, revealed that the gp60 gene of *C. ubiquitum* has extensive sequence differences from the gp60 gene of other *Cryptosporidium* spp. (Li *et al.* 2014). These findings highlight the need for WGS analysis of diverse *Cryptosporidium* species to develop subtyping tools for other common *Cryptosporidium* species in humans and domestic animals. Extensive WGS of *Cryptosporidium* spp. will likely lead to improved understanding of virulence factors in *C. parvum* and *C. hominis* and the genetic basis for host specificity and human infectivity of various *Cryptosporidium* species. This in turn will promote the development of vaccines and new therapies to help control the spread of *Cryptosporidium* (Striepen, 2013).

CONCLUSIONS

As molecular tools improve and data accumulate, our understanding of the role of zoonotic transmission in epidemiology and clinical manifestations is becoming clearer. In the future, these tools and advanced techniques developed from WGS of diverse *Cryptosporidium* species will be essential in understanding intricate associations between wildlife, domestic animals and humans in the context of health and climate changes to enable management of the zoonotic risk of *Cryptosporidium*.

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