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A review of the current status of *Cryptosporidium* in fish

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Review Article

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Abstract

Species of the genus *Cryptosporidium* (phylum Apicomplexa) infect the epithelium of the gastrointestinal tract of several vertebrate hosts, including humans and domestic and wild animals. In the past 20 years, several studies have focused on *Cryptosporidium* in fish. To date, a total of four piscine-host-specific species (*Cryptosporidium molnari*, *Cryptosporidium huwi*, *Cryptosporidium bollandi* and *Cryptosporidium abrahamseni*), nine piscine genotypes and more than 29 unnamed genotypes have been described in fish hosts. In addition, *Cryptosporidium* species and genotypes typical of other groups of vertebrates have also been identified. This review summarizes the history, biology, pathology and clinical manifestations, as well as the transmission, prevalence and molecular epidemiology of *Cryptosporidium* in wild, cultured and ornamental fish from both marine and freshwater environments. Finally, the potential role of piscine hosts as a reservoir of zoonotic *Cryptosporidium* species is also discussed.

Introduction

Species of the genus *Cryptosporidium* (phylum Apicomplexa) are protozoan parasites that infect the epithelium of the gastrointestinal tract of several vertebrate hosts, including humans and domestic and wild animals. A total of 44 *Cryptosporidium* species that infect fish, amphibians, reptiles, birds and mammals are currently recognized. In addition, more than 70 genotypes have been described in different hosts (Ryan *et al.*, 2014; Chalmers *et al.*, 2018; Holubová *et al.*, 2020; Ježková *et al.*, 2021; Zahedi *et al.*, 2021). *Cryptosporidium* infection, which is transmitted by the fecal–oral route, can be acquired both directly, through contact with infected hosts, and indirectly, through the ingestion of food- and water-contaminated with oocysts (the infective form of the parasite) (Cacciò and Putignani, 2014; Gerace *et al.*, 2019). Although infection is asymptomatic in some immunocompetent hosts, watery diarrhoea, abdominal pain and vomiting are the most common symptoms of cryptosporidiosis, which can be severe or even fatal in immunocompromised patients (Shrivastava *et al.*, 2017). Detection of oocysts, antigens and/or nucleic acids of the parasite in stool samples is the most frequently used method for diagnosing this infection (Gerace *et al.*, 2019).

Outbreaks of cryptosporidiosis linked to drinking and recreational waters are common due to the ubiquity of the parasite, the low-infectious dose and the resistance of the oocysts to environmental pressures (temperature, desiccation and humidity conditions) and to conventional water disinfection treatments (mainly chlorination and ozonization) (King and Monis, 2007; Carneiro Santos *et al.*, 2020). *Cryptosporidium* is, thus, one of the most frequently detected pathogens in waterborne outbreaks of parasitic aetiology in developed countries, being involved in 63% of the reported outbreaks between 2011 and 2016 (Efstratiou *et al.*, 2017). Moreover, in a study on the global burden of foodborne diseases, it was estimated that *Cryptosporidium* was responsible for 8.6 million cases of illness and 3759 deaths in 2010 (World Health Organization, 2015). Food can be contaminated with *Cryptosporidium* oocysts at all points of food chain (ranging from primary production in agriculture and aquaculture to the transformation, distribution and sale of food) *via* direct contact with fecal material from infected hosts or indirectly *via* infected food handlers (Zahedi and Ryan, 2020). In developing countries, *Cryptosporidium*, along with Rotavirus and *Shigella*, are the three pathogens that most commonly cause diarrhoeal disease in children under 2 years old, with *Cryptosporidium* being responsible for 30–50% of childhood mortality in these countries (Sow *et al.*, 2016; Kotloff *et al.*, 2017).

In farmed ruminants, *Cryptosporidium* is also recognized as one of the main enteropathogens involved in the aetiology of neonatal diarrhoea syndrome, which causes significant economic losses on farms, owing to the associated high morbidity and mortality and the delayed growth of animals (De Graaf *et al.*, 1999; Meganck *et al.*, 2015). Furthermore, *Cryptosporidium* has been reported to be widely distributed in wildlife, which may act as both an important reservoir and source of infection (Zahedi *et al.*, 2016).

Brief historical review of *Cryptosporidium* in fish

Cryptosporidium parasites were first described by Clarke (1895) as 'swarm-spores' in the gastric epithelium of laboratory mice (*Mus musculus*). Tyzzer (1907) subsequently established the

genus *Cryptosporidium* to refer to this protozoan parasite infecting the same host. However, it was not until seven decades later that the first study on *Cryptosporidium* in fish was published, in which Hoover *et al.* (1981) described *Cryptosporidium nasoris* (syn. *Cryptosporidium nasorum*), a species found in the intestine of the tropical marine fish naso tang (*Naso lituratus*). Nevertheless, this species is currently considered as *nomen nudum* for the following reasons: (1) only developmental stages of the parasite on the microvillus surface of intestinal epithelial cells have been described by light and electron microscopy; (2) no measurements of viable oocysts have been provided; (3) no other taxonomically useful diagnostic features have been presented and (4) the lack of deposited museum specimens and molecular studies prevent completion of the description. Under these circumstances, it is impossible to differentiate between *C. nasorum* and other *Cryptosporidium* spp. (Ryan *et al.*, 2004, 2014; Xiao *et al.*, 2004; Fayer, 2010; Ryan, 2010).

After the first description, different histological studies detected developmental stages compatible with *Cryptosporidium* in the stomach and/or intestine of several fish species: carp (*Cyprinus carpio*; prevalence = 14.3%) (Pavlásek, 1983); cichlid fish (*Oreochromis* spp.; prevalence = 58.8%) (Landsberg and Paperna, 1986); barramundi (*Lates calcarifer*; prevalence not provided) (Glazebrook and Campbell, 1987); black Nile catfish (*Bagrus bayad*; prevalence = 10.0%), North African catfish (*Clarias lazera*; prevalence = 20.0%), Nile tilapia (*Tilapia nilotica* syn. *Oreochromis niloticus*; prevalence = 30.0%) (Hefnawy, 1989); brown trout (*Salmo trutta*; prevalence = 38.9%) (Rush *et al.*, 1990); red drum (*Sciaenops ocellatus*; prevalence = 21.7%) (Camus and López, 1996) and catfish (*Plecostomus* sp.; prevalence = 100%) (Muench and White, 1997).

Moreover, the results of ultrastructural studies led Paperna and Vilenkin (1996) to propose a new genus, *Piscicryptosporidium*, for species of this parasite infecting fish, on the basis of the following features: (1) piscine *Cryptosporidium* spp. are found in the stomachs of the hosts; (2) the species differs from all other known *Cryptosporidium* species in that the parasitic parasitophorous vacuole has microvilli on the surface and (3) the sporulated oocysts gradually penetrate the basal part of the gut epithelium or lamina propria rather than being released into the gut lumen (Paperna and Vilenkin, 1996). The organisms found in the stomachs of the gourami (*Trichogaster leeri*) and cichlid fish (*Oreochromis* spp.) were designated as *Piscicryptosporidium reinchenbachklinkei* and *Piscicryptosporidium cichlidis*, respectively. The validity of the genus remains to be determined and the two *Piscicryptosporidium* species may be considered *nomina nuda* because some of these features have been described in mammalian *Cryptosporidium* (i.e. gastric location) (Valigurová *et al.*, 2008) and there are no molecular studies to support this proposal.

In the current century, a new species, *Cryptosporidium molnari*, identified by Álvarez-Pellitero and Sitjá-Bobadilla (2002), was found in the stomach of farmed marine fish, specifically gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Álvarez-Pellitero *et al.* (2004) subsequently described the species *Cryptosporidium scophthalmi* after detecting developmental stages of this protozoon in the intestine of cultured turbot (*Scophthalmus maximus* syn. *Psetta maxima*). In the same year, Ryan *et al.* (2004) reported the results of histological, genetic and phylogenetic studies of a *C. molnari*-like isolate from the stomach of the ornamental fish guppy (*Poecilia reticulata*), which was designated as *Cryptosporidium* piscine genotype 1 in the absence of molecular data for *C. molnari*. In a later study, Palenzuela *et al.* (2010) carried out the molecular characterization of the *C. molnari* isolates, and Ryan *et al.* (2015) subsequently elevated the piscine genotype 1 to species level and named it *Cryptosporidium huwi* on the basis of genetic and morphological differences relative to other gastric and intestinal species of *Cryptosporidium* in these hosts. Although Costa and Saraiva (2015*a*, 2015*b*) described *C. scophthalmi*-like sequences in turbot, no genetic information on the original isolate of *C. scophthalmi* is yet available. Therefore, this species may be considered as not valid, owing to the observed high level of genetic heterogeneity and oocyst morphological similarity between *Cryptosporidium* species (Fayer, 2010; Ryan *et al.*, 2014, 2015). The species names *Cryptosporidium bollandi*, for piscine genotype 2 found in angelfish (*Pterophyllum scalare*) and Oscar fish (*Astronotus ocellatus*), and *Cryptosporidium abrahamseni*, for piscine genotype 7 found in red-eye tetra (*Moenkhausia sanctaefilomenae*), have been recently established (see Table 1) (Bolland *et al.*, 2020; Zahedi *et al.*, 2021).

The use of molecular techniques has also enabled the identification of a total of nine piscine *Cryptosporidium* genotypes (3–6, 8–10 and marine 1–2 genotypes), five different *C. molnari*-like genotypes and more than 29 unnamed novel genotypes in both freshwater and marine fish (Reid *et al.*, 2010; Zanguee *et al.*, 2010; Koinari *et al.*, 2013; Yang *et al.*, 2015, 2016; Couso-Pérez *et al.*, 2018, 2019; Certad *et al.*, 2019, 2020). The species and genotypes of *Cryptosporidium* currently recognized in fish are listed in Table 1.

Molecular studies have revealed the considerable genetic distance between piscine *Cryptosporidium* and remaining species of the genus infecting other host classes. Phylogenetic analysis of piscine-derived *Cryptosporidium* species/genotypes showed that the piscine clade has a basal position relative to all other *Cryptosporidium* species, which form two main broad branches: intestinal and gastric species. This suggests that piscine species may be the evolutionary ancestors of *Cryptosporidium* species infecting other host classes (Figs 1 and 2) (Palenzuela *et al.*, 2010; Reid *et al.*, 2010; Koinari *et al.*, 2013; Certad *et al.*, 2015, 2019, 2020; Ryan *et al.*, 2015; Couso-Pérez *et al.*, 2018, 2019; Bolland *et al.*, 2020; Zahedi *et al.*, 2021).

Moreover, several species and genotypes of *Cryptosporidium* typical of other hosts such as *Cryptosporidium parvum* (major host: livestock; Tyzzer, 1912), *Cryptosporidium hominis* (major host: humans; Morgan-Ryan *et al.*, 2002), *Cryptosporidium xiaoi* (major host: sheep; Fayer and Santín, 2009), *Cryptosporidium scrofarum* (major host: pig; Kváč *et al.*, 2013) and rat genotype 3 have been identified in fish (Table 2) (Reid *et al.*, 2010; Gibson-Kueh *et al.*, 2011; Morine *et al.*, 2012; Koinari *et al.*, 2013; Certad *et al.*, 2015, 2019; Palermo, 2016; Couso-Pérez *et al.*, 2018, 2019; Shahbazi *et al.*, 2020). Without ruling out the possibility that fish only act as mechanical carriers of these *Cryptosporidium* spp., the role of fish as potential hosts of zoonotic *Cryptosporidium* spp. will be discussed later.

Life cycle of piscine *Cryptosporidium*

Considering the different developmental stages of *Cryptosporidium* observed in ultrastructural studies (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004), the life cycle of *Cryptosporidium* spp. in fish can be assumed to involve the following different stages: (1) excystation and release of sporozoites; (2) schizogony or merogony; (3) gamogony; (4) zygote formation; (5) oocyst wall formation and (6) sporulation (Fig. 3). However, there are some notable differences in the life cycles of *Cryptosporidium* species in fish and in mammals, which we will highlight below. The cycle begins when a sporulated oocyst is ingested by a susceptible fish host. The oocyst undergoes excystation, releasing the sporozoites, which then reach the apical surface of the cells of the gastrointestinal epithelium through gliding motility. The host cell envelops the sporozoite inside a vacuolar space formed by

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Table 1. *Cryptosporidium* species and genotypes currently recognized in piscine hosts

Species/genotype	Fish host	Origin	Habitat	*P* (%)	GenBank	Reference
Cryptosporidium molnari Oocyst size (mean ± s.d.; μm): 4.7 ± 0.5 × 4.5 ± 0.5 Location: stomach	*Chromis viridis*	O	M	15.4	HM989832*	Zanguee *et al.* (2010)
	Ctenochaetus tominiensis	O	M	100	HM989832*	Zanguee *et al.* (2010)
	Dicentrarchus labrax	C	M	4.6–57.9	ND	Álvarez-Pellitero and Sitjà-Bobadilla (2002); Sitjà-Bobadilla *et al.* (2005)
	Exos lucius	W	FW	40.0	KP939352	Certad *et al.* (2015)
	Monodactylus argenteus	O	M	33.3	HM989832	Zanguee *et al.* (2010)
	Pseudanthias dispar	O	M	50.0	HM989832*	Zanguee *et al.* (2010)
	Sparus aurata[a]	C	M	6.5–100	HM243547	Álvarez-Pellitero and Sitjà-Bobadilla (2002); Sitjà-Bobadilla *et al.* (2005); Palenzuela *et al.* (2010)
C. molnari-like	*Amphiprion percula*	O	M	9.1	KR610356*	Yang *et al.* (2015)
	Astronotus ocellatus	O	FW	10.0	KR610356*	Yang *et al.* (2015)
	Carassius auratus	O	FW	2.7	KR610356*	Yang *et al.* (2015)
	Centropyge eibli	O	M	100	KR610356*	Yang *et al.* (2015)
	Chrysiptera hemicyanea	O	M	33.3	KR610356*	Yang *et al.* (2015)
	Crossocheilus aymonieri	O	FW	20.0	HM989836	Zanguee *et al.* (2010)
	Cyprinus carpio	O	FW	–	KX033348	Yang *et al.* (2016)
	Maccullochella peelii	C	FW	95.4	HQ585890	Barugahare *et al.* (2011)
	Opistognathus aurifrons	O	M	100	KR610356*	Yang *et al.* (2015)
	Paracanthurus hepatus	O	M	100	HM989832*/ KR610356*	Zanguee *et al.* (2010); Yang *et al.* (2015)
	Poecilia reticulata	O	FW	0.9	KR610356*	Yang *et al.* (2015)
	P. dispar	O	M	33.3	KR610356	Yang *et al.* (2015)
	Pterophyllum altum	O	FW	20.0	KR610356*	Yang *et al.* (2015)
	Synodontis nigriventris	O	FW	50.0	HM989832*/ KR610356*	Zanguee *et al.* (2010); Yang *et al.* (2015)
Cryptosporidium huwi Oocyst size (mean ± s.d.; μm): 4.6 ± 0.3 × 4.4 ± 0.4 Location: stomach	*Paracheirodon innesi*	O	FW	3.5–50.0	HM989835	Zanguee *et al.* (2010); Yang *et al.* (2015); Bolland *et al.* (2020)
	P. reticulata[a]	O	FW	1.9	AY524773	Ryan *et al.* (2014); Yang *et al.* (2015)
	Puntigrus tetrazona	O	FW	4.5	ND	Yang *et al.* (2015)
Cryptosporidium bollandi Oocyst size (mean ± s.d.; μm): 3.1 ± 0.5 × 2.8 ± 0.4 Location: stomach	*A. ocellatus*[a]	O	FW	5.0–75.0	MT169961	Zanguee *et al.* (2010); Yang *et al.* (2015); Bolland *et al.* (2020)
	Mugil cephalus	W	M	0.5	KR610347*	Yang *et al.* (2015)
	P. innesi	O	FW	50.0	ND	Zanguee *et al.* (2010)
	Pterophyllum scalare[a]	C	FW	–	FJ769050	Murphy *et al.* (2009)
Cryptosporidium abrahamseni Oocyst size (mean ± s.d.; μm): 3.8 ± 0.2 × 3.2 ± 0.2 Location: intestine	*Moenkhausia sanctaefilomenae*[a]	O	FW	27.3–62.5	MW075511	Morine *et al.* (2012); Bolland *et al.* (2020); Zahedi *et al.* (2021)
	P. innesi	O	FW	–	KR610354	Yang *et al.* (2015)
C. abrahamseni-like	*P. innesi*	O	FW	–	KR610355	Yang *et al.* (2015)
Genotype 3	*M. cephalus*	W	M	0.5–1.8	GQ925452/ KR610348	Reid *et al.* (2010); Yang *et al.* (2015)
Genotype 3-like	*C. auratus*	O	FW	0.9	ND	Yang *et al.* (2015)
Genotype 4	*Apteronotus albifrons*	O	FW	25.0–50.0	KR610346*	Yang *et al.* (2015); Bolland *et al.* (2020)
	A. ocellatus	O	FW	25.0	HM989833*	Zanguee *et al.* (2010)
	C. hemicyanea	O	M	100	HM989833*/ KR610346	Zanguee *et al.* (2010); Yang *et al.* (2015)
	C. aymonieri	O	FW	20.0	HM989833	Zanguee *et al.* (2010)
	P. innesi	O	FW	25.0	JQ995771	Morine *et al.* (2012)
	Pelvicachromis pulcher	O	FW	100	KR610346*	Yang *et al.* (2015)

(*Continued*)

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Table 1. (*Continued.*)

Species/genotype	Fish host	Origin	Habitat	*P* (%)	GenBank	Reference
Genotype 5	*A. albifrons*	O	FW	25.0	KR610344*	Yang *et al.* (2015)
	C. auratus	O	FW	2.7	KR610344*	Yang *et al.* (2015)
	C. aymonieri	O	FW	20.0	HM989837	Zanguee *et al.* (2010)
	M. argenteus	O	M	33.3	HM989834*/ KR610344	Zanguee *et al.* (2010); Yang *et al.* (2015)
	M. cephalus	W	M	0.5	KR610344*	Yang *et al.* (2015)
	P. hepatus	O	M	100	KR610344*	Yang *et al.* (2015)
	P. reticulata	O	FW	0.9	KR610344*	Yang *et al.* (2015)
	P. scalare	O	FW	25.0	HM989834/ KR610344*	Zanguee *et al.* (2010); Yang *et al.* (2015)
	P. altum	O	FW	20.0	KR610344*	Yang *et al.* (2015)
	Xiphophorus maculatus	O	FW	11.1	KR610344*	Yang *et al.* (2015)
Genotype 5-like	*A. ocellatus*	O	FW	5.0	KR610345	Yang *et al.* (2015)
Genotype 6	*P. reticulata*	O	FW	2.3	HM991857	Zanguee *et al.* (2010)
	Trichogaster trichopterus	O	FW	33.3	JQ995776	Morine *et al.* (2012)
Genotype 8	*Gerres oblongus*	W	M	3.6	KC807985	Koinari *et al.* (2013)
Genotype 9	*Oncorhynchus mykiss*	C	FW	0.6	MG951477	Couso-Pérez *et al.* (2018)
Genotype 10	*Salmo trutta*	W	FW	0.2	MH074869	Couso-Pérez *et al.* (2019)
Marine genotype 1	*Merlangius merlangus*	W	M	0.8	MK236539	Certad *et al.* (2019, 2020)
	Merluccius merluccius	W	M	0.7	ND	Certad *et al.* (2019, 2020)
	Molva dypterygia	W	M	2.9	MK236541	Certad *et al.* (2019, 2020)
	Molva molva	W	M	10.9	ND	Certad *et al.* (2019, 2020)
	Pollachius virens	W	M	14.4	MK236538	Certad *et al.* (2019, 2020)
Marine genotype 2	*Scomber scombrus*	W	M	0.9	MK236544	Certad *et al.* (2019, 2020)

S.D., standard deviation; C, cultured; FW, freshwater; M, marine; O, ornamental; W, wild; *P*, prevalence rate; ND, no data.
[a]Primary host species.
*GenBank accession number identical to the original sequence (no asterisk).

invagination of the cytoplasmic membrane; this parasitophorous vacuole has an intracellular, but extra-cytoplasmic location, in which the subsequent developmental stages take place (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004). The first remarkable difference observed in fish relative to mammal hosts occurs at the level of the membrane of the parasitophorous vacuole. Ultrastructural studies have shown that in piscine hosts the membrane of the parasitophorous vacuole has regularly spaced microvilli containing a dense, finely granular substance. In addition, the zone of attachment between the host cell and the parasite is also very different. In fish, rather than being continuous with the interlamellar layer of the membrane of the parasitophorous vacuole, the zone of attachment appears to consist of two electron-dense bands, where the inner band is connected to two plates by osmophilic points. The feeder organelle is composed of multiple parallel folds with vesiculate widening at their endings, being longer and more numerous in piscine *Cryptosporidium* than in other members of the genus (Fig. 4) (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996; Muench and White, 1997; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004; Gabor *et al.*, 2011).

The sporozoite differentiates into a trophozoite with a large nucleus. The nucleus undergoes division, through a process called schizogony or merogony, to produce two types of multinucleated meronts (Álvarez-Pellitero and Sitjà-Bobadilla, 2002). Type I meronts have eight nuclei and produce eight type I merozoites (Paperna and Vilenkin, 1996), which infect adjacent cells where they again divide asexually, producing new type I or type II meronts. Type II meronts mature to produce four type II merozoites (Álvarez-Pellitero and Sitjà-Bobadilla, 2002), which probably invade other cells and undergo sexual reproduction by gamogony, to produce microgamonts and macrogamonts. As a consequence of the nuclear division of the microgamont, aflagellate microgametes are generated (up to 12 microgametes were observed by Álvarez-Pellitero and Sitjà-Bobadilla, 2002). These forms leave the parasitophorous vacuole to fertilize the differentiated macrogamont or macrogamete. After fertilization, the zygote formed is internalized and undergoes sporogony to produce sporulated oocysts containing four naked sporozoites (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004). Sporulation deep within the epithelium appears to be characteristic of the piscine clade (Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Ryan *et al.*, 2004, 2015; Bolland *et al.*, 2020; Zahedi *et al.*, 2021). Thus, in *Cryptosporidium* species that infect fish, unlike in mammal hosts, the oocysts are found within a vacuolar space located deep within the cytoplasm in the epithelial cells (Paperna and Vilenkin, 1996; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Ryan *et al.*, 2004, 2015; Palenzuela *et al.*, 2010; Bolland *et al.*, 2020; Zahedi *et al.*, 2021). In addition, more than one oocyst can appear in clusters in the same infected cell, along with aggregates of cellular residues and necrotic substances (Fig. 5). The infected cells gradually

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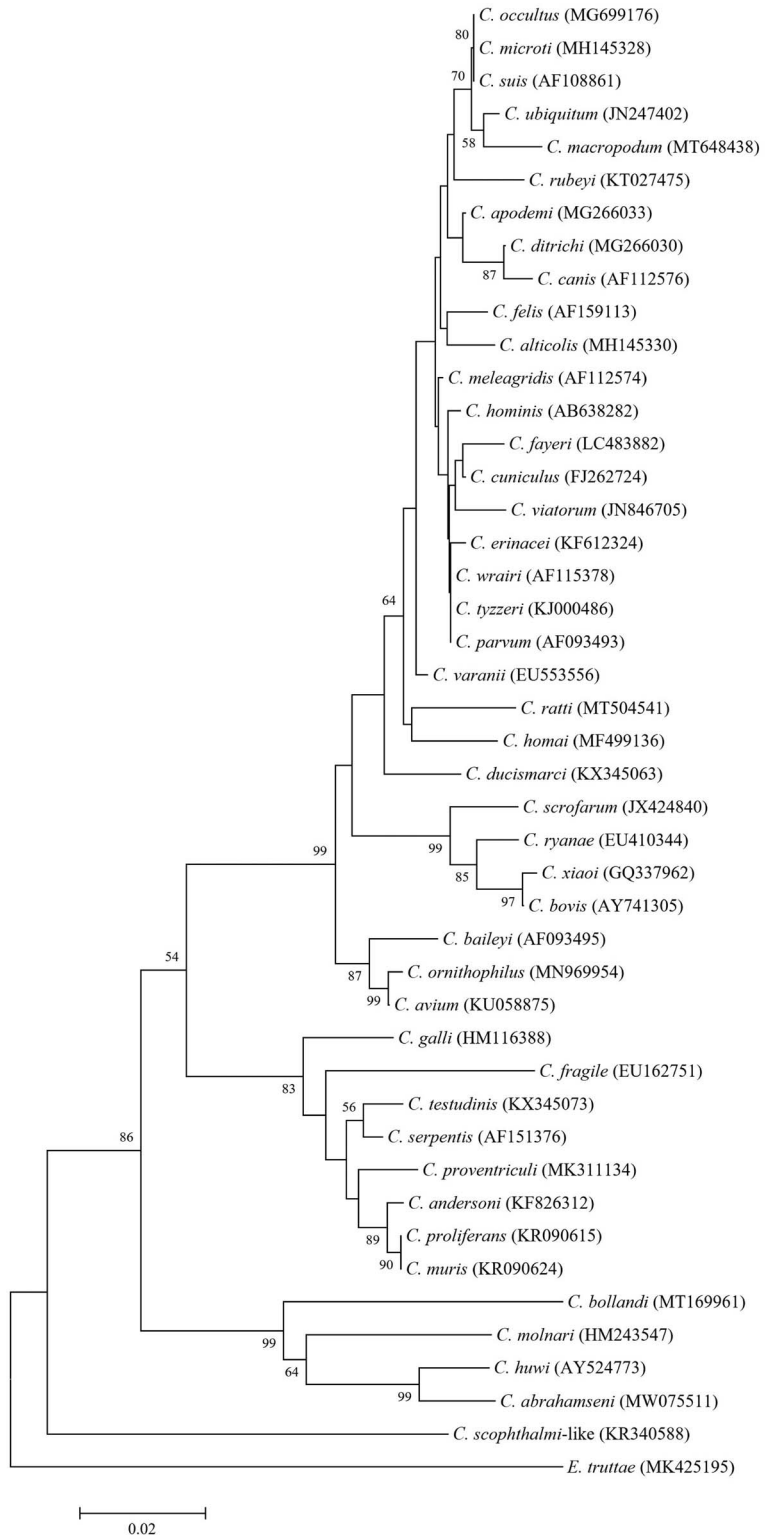

Fig. 1. Phylogenetic relationships in the genus *Cryptosporidium* inferred by neighbour-joining analysis of the small subunit ribosomal RNA (18S rRNA) gene on the basis of genetic distances calculated by the Tamura 3-parameter model (gamma distributed with five rate categories) using MEGA X software (Kumar *et al.*, 2018). The tree was generated using a total of 433 positions in the final dataset. The percentages of replicate trees in which associated taxa clustered together in the bootstrap test (10 000 replicates) are shown at the internal nodes for distance (>50%). Accession numbers are given in parentheses.

degenerate before finally disintegrating, leaving the oocysts or clusters of oocysts in the intercellular spaces or allowing these to escape to the lumen (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996; Álvarez-Pellitero and Sitjà-Bobadilla, 2002).

Most of the sporulated oocysts eliminated with the feces and capable of infecting other animals have a thick (trilamellar) wall. However, as occurs in mammals, some oocysts have a thin (bilamellar) envelope that is easily broken when the oocysts are released into the gastrointestinal lumen, and free sporozoites can then infect adjacent cells, reinitiating a cycle of endogenous autoinfection (Fig. 3) (Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004).

Cryptosporidium species that infect fish have been detected in different sections of the gastrointestinal tract. Thus, histological observations have revealed developmental stages of *C. molnari* lining the stomach epithelium, whereas zygotes and fully sporulated oocysts ($4.7 \pm 0.5\,\mu m \times 4.5 \pm 0.5\,\mu m$), sometimes in groups, have been located in the basal portion of the epithelial cell (Álvarez-Pellitero and Sitjà-Bobadilla, 2002). Parasitic stages of *C. huwi* have been found dispersed on the apical surface of the stomach mucosa, and groups of zygotes and oocysts ($4.6 \pm 0.3\,\mu m \times 4.4 \pm 0.4\,\mu m$) have also been located deep within the gastric epithelium (Ryan *et al.*, 2015). Similarly, trophozoites, meronts and gamonts of *C. bollandi* have been detected in large numbers

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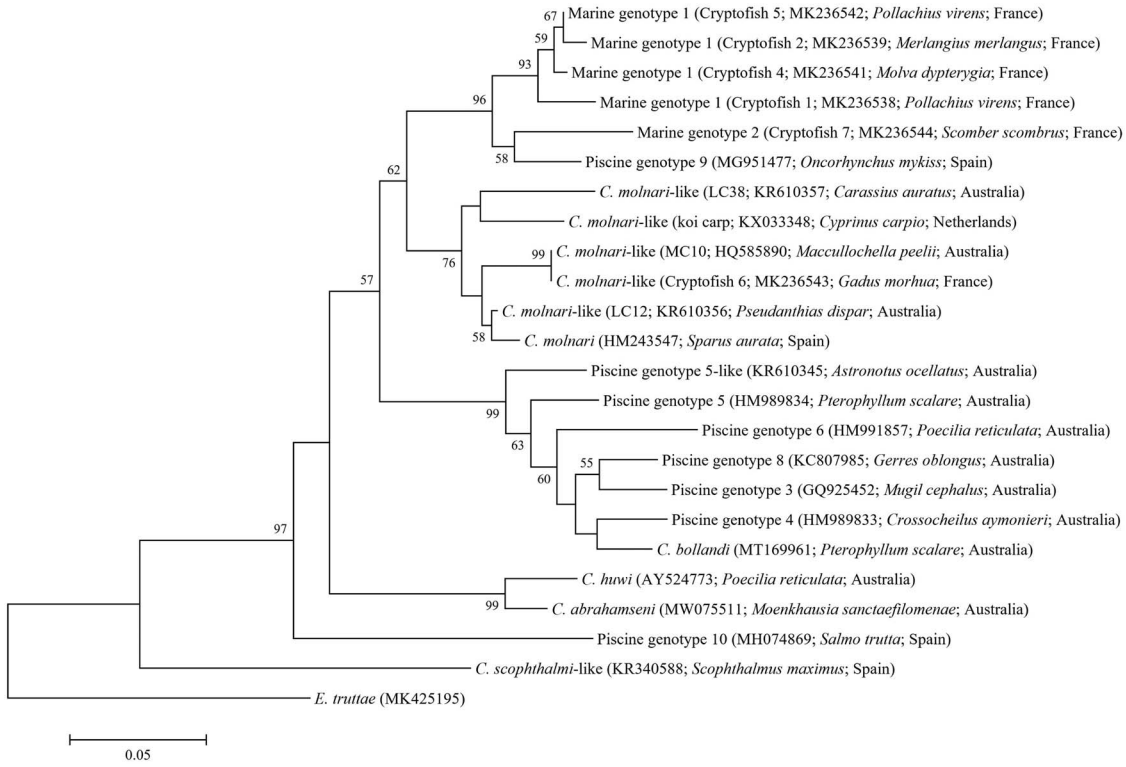

Fig. 2. Phylogenetic relationships of piscine *Cryptosporidium* species and genotypes inferred by neighbour-joining analysis of the small subunit ribosomal RNA (18S rRNA) gene on the basis of genetic distances calculated by the Tamura 3-parameter model (gamma distributed with five rate categories) using MEGA X software (Kumar *et al.*, 2018). The tree was generated using a total of 251 positions in the final dataset. The percentage numbers of replicate trees in which associated taxa clustered together in the bootstrap test (10 000 replicates) are shown at the internal nodes for distance (>50%). Accession numbers, host species and geographical regions are shown in parentheses.

attached to the gastric mucosa, with zygotes and oocysts ($3.1 \pm 0.5\,\mu m \times 2.8 \pm 0.4\,\mu m$) also located deep within the epithelium (Bolland *et al.*, 2020). By contrast, histological analysis has revealed meronts and micro- and macrogamonts of *C. scophthalmi* in an extracytoplasmic position in the intestine, whereas oocysts ($4.4 \pm 0.3\,\mu m \times 3.9 \pm 0.4\,\mu m$) have been observed deeply embedded within the epithelium (Álvarez-Pellitero *et al.*, 2004). More recently, Zahedi *et al.* (2021) observed the existence of a large number of *C. abrahamseni* oocysts ($3.8 \pm 0.2\,\mu m \times 3.2 \pm 0.2\,\mu m$) and clusters of parasites also located deep within the epithelium of the small intestine.

Piscine cryptosporidiosis

Transmission

Water is an excellent vehicle for the dissemination of pathogenic organisms, which are transmitted *via* the fecal–oral route. *Cryptosporidium* is one of the infectious agents most frequently detected in water and has been reported in different types of water worldwide (river, recreational, drinking and wastewater) (Omarova *et al.*, 2018; Vermeulen *et al.*, 2019). This waterborne protozoan parasite can occur in surface waters due to contamination with fecal matter of human or animal origin (both domestic and wild animals). The oocysts can spread to water bodies directly or indirectly *via* run-off from contaminated land surfaces with livestock manure (Lu *et al.*, 2011; Ahmed *et al.*, 2013; Sidhu *et al.*, 2013). In addition, the aquatic environment can become contaminated by effluents from wastewater treatment plants or from insufficient or deficient sewage treatment systems (Ahmed *et al.*, 2010; Schneeberger *et al.*, 2015; Vermeulen *et al.*, 2019).

Regarding fish farm facilities, the presence of *Cryptosporidium* may be associated with water quality. As previously reported by Sitjà-Bobadilla *et al.* (2005) and Álvarez-Pellitero *et al.* (2009), transmission occurs through the farm water supply (i.e. rivers and seas), and the use of filtration and ultraviolet irradiation in hatcheries and nurseries is not sufficient to prevent the entry of infective oocysts. Once the parasite is present inside the facilities, recirculation systems can contribute to dispersing and concentrating oocysts in the water. In addition, transmission of the parasite may be facilitated by the stress conditions that occur in aquaculture systems, in which fish cohabit in dense groups, and by cannibalism, a common phenomenon in piscine communities, which may also play a role in transmission (Sitjà-Bobadilla and Álvarez-Pellitero, 2003; Sitjà-Bobadilla *et al.*, 2005).

Cryptosporidium oocysts have been found in other eukaryotic organisms living in the aquatic environment, and their participation in the transmission of this protozoan parasite cannot be ruled out. Several rotifers, species of the genera *Epiphanes* and *Euchlanis*, and some free-living amoebas, such as *Acanthamoeba* spp., can retain *C. parvum* oocysts when they coexist in contaminated waters (Fayer *et al.*, 2000; Gómez-Couso *et al.*, 2007). Similarly, the brine shrimp *Artemia franciscana* can act as a carrier in the transmission of *Cryptosporidium* infection in cultured fish when it is used as a live diet (Méndez-Hermida *et al.*, 2006, 2007). Reboredo-Fernández *et al.* (2014) demonstrated the presence of *Cryptosporidium* oocysts in benthic macroinvertebrates in Galician rivers (NW Spain) and concluded that these organisms could contribute to the transmission of the parasite through the aquatic food chain.

Prevalence

Wild fish

So far, only seven studies have reported the presence of *Cryptosporidium* in both marine and freshwater wild fish.

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Table 2. Mammalian *Cryptosporidium* species and genotypes detected in fish

Species/genotype	Fish host	Origin	Habitat	*P* (%)	GenBank	Reference
Cryptosporidium hominis	*C. auratus*	O	FW	2.0–4.6	AF222998	Palermo (2016); Shahbazi *et al.* (2020)
Cryptosporidium parvum	*C. auratus*	O	FW	0.9	ND	Palermo (2016)
	Clupea harengus	W	M	0.9	ND	Certad *et al.* (2019)
	Coregonus lavaretus	W	FW	45.5	KP939343	Certad *et al.* (2015)
	Decapterus macarellus	W	M	6.9	ND	Koinari *et al.* (2013)
	Engraulis encrasicolus	W	M	0.7	ND	Certad *et al.* (2019)
	E. lucius	W	FW	20.0	KP939338	Certad *et al.* (2015)
	Gadus morhua	W	M	0.8	ND	Certad *et al.* (2019)
	Lates calcarifer	C	FW	20.0	JF285332	Gibson-Kueh *et al.* (2011)
	M. dypterygia	W	M	1.4	ND	Certad *et al.* (2019)
	O. mykiss	C	FW	1.9	MG951476	Couso-Pérez *et al.* (2018)
	Oreochromis niloticus	C	FW	2.4	ND	Koinari *et al.* (2013)
	Perca fluviatilis	W	FW	33.3	KP939346	Certad *et al.* (2015)
	Puntius gonionotus	W	FW	1.9	ND	Koinari *et al.* (2013)
	Rutilus rutilus	W	FW	100	KP939351	Certad *et al.* (2015)
	S. trutta	W	FW	7.7	MH074866	Couso-Pérez *et al.* (2019)
	Salvelinus alpinus	W	FW	66.7	KP939333	Certad *et al.* (2015)
	Sardina pilchardus	W	M	1.3	ND	Certad *et al.* (2019)
	Scomber japonicus	W	M	6.5	ND	Certad *et al.* (2019)
	S. scombrus	W	M	1.5	ND	Certad *et al.* (2019)
	Sillago vittata	W	M	1.8	ND	Reid *et al.* (2010)
Cryptosporidium scrofarum	*S. vittata*	W	M	3.6	ND	Reid *et al.* (2010)
Cryptosporidium xiaoi	*S. vittata*	W	M	1.8	ND	Reid *et al.* (2010)
Rat genotype 3	*C. auratus*	O	FW	5.3	JQ995772	Morine *et al.* (2012)

C, cultured; FW, freshwater; M, marine; O, ornamental; W, wild; *P*, prevalence rate; ND, no data.

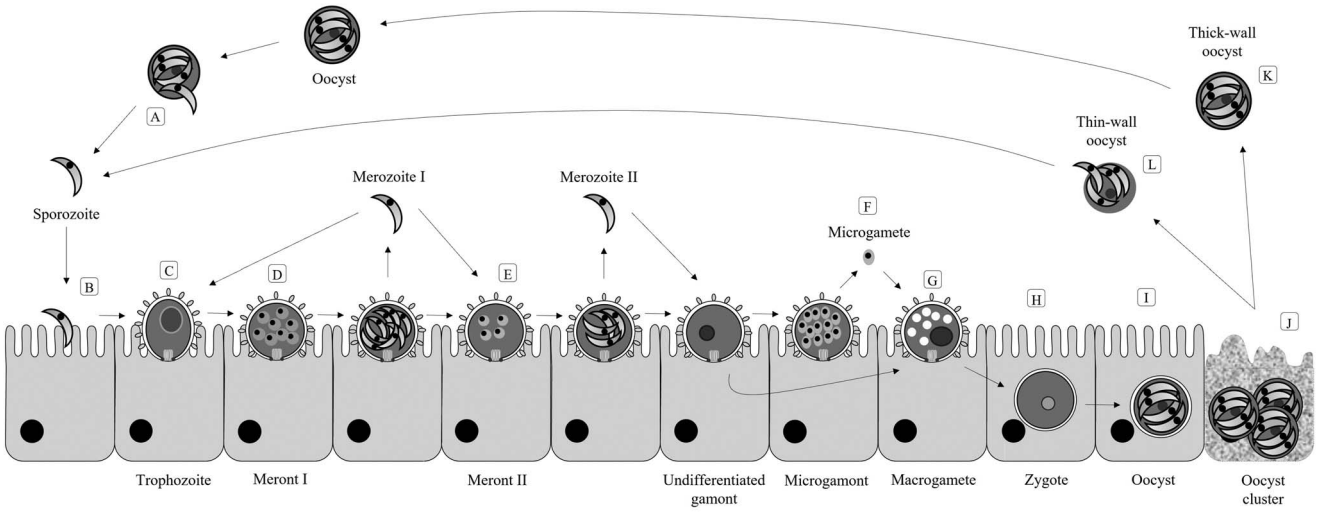

Fig. 3. Hypothetical life cycle of piscine *Cryptosporidium*. The sporozoites (A) reach the apical surface of the epithelial cells (B). The sporozoite is enveloped inside a parasitophorous vacuole (PV), the membrane of which has regularly spaced microvilli. Within the PV, the sporozoite is differentiated into a trophozoite (C), which undergoes nuclear division through merogony, producing a type I meront (D). Type I merozoites infect adjacent cells and new type I or type II meronts (E) are produced. Type II merozoites invade other cells and undergo gamogony, forming microgamonts and macrogamonts, which produce microgametes (F) and macrogametes (G). After fertilization, a zygote is formed (H), generating (by sporogony) sporulated oocysts containing four naked sporozoites (I). These oocysts are found within a vacuolar space located deep within the cytoplasm in the epithelial cells, and more than one oocyst can appear in clusters in the same infected cell (J). Thick-walled oocysts are released with the feces of the host and can infect other susceptible hosts (K). Some oocysts have a thin wall that is easily broken, thus enabling the cycle to be reinitiated by endogenous autoinfection (L).

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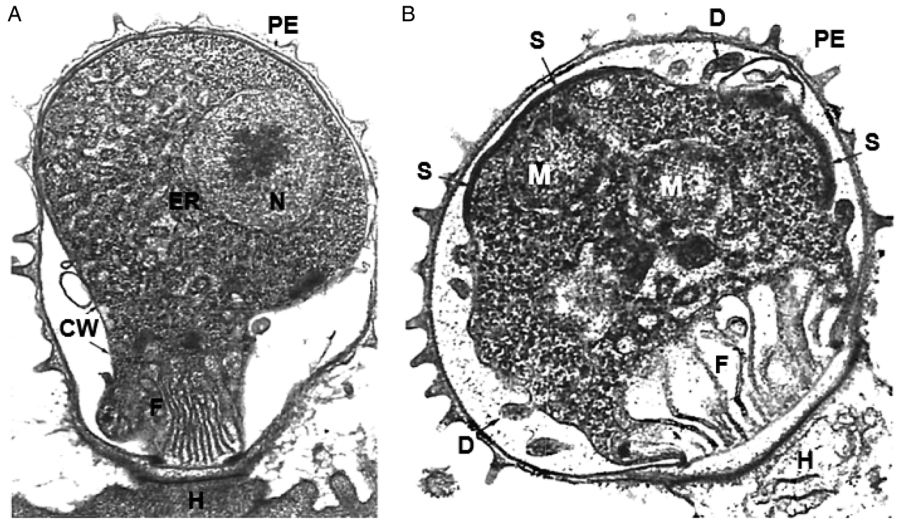

Fig. 4. Histological sections of the stomach of *Oreochromis aureus* × *Oreochromis niloticus* naturally infected with *Cryptosporidium* sp. showing (A) trophozoite and (B) early differentiating meront under transmission electron microscopy (29 300×). PE, parasitophorous membrane; ER, rough endoplasmic reticulum; N, nucleus; CW, parasite plasmalemma; F, feeder organelle; H, host cell; M, differentiating merozoite (Landsberg and Paperna, 1986) (© Inter-Research).

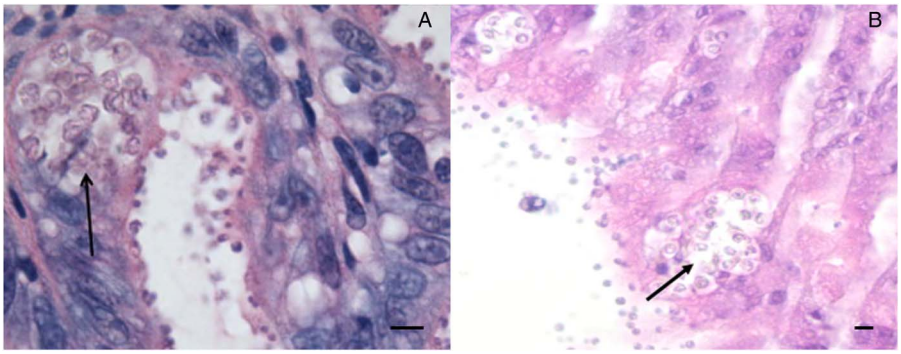

Fig. 5. Histological sections of the stomach of (A) guppy (*Poecilia reticulata*) and (B) angelfish (*Pterophyllum scalare*) stained with haematoxylin–eosin, showing large numbers of *Cryptosporidium* life cycle stages along the lining of the gastric mucosa with clusters of oocysts located deep within the epithelium (arrows). Scale bar = 10 μm (Ryan *et al.*, 2015; Bolland *et al.*, 2020) (© Elsevier).

In marine fish, molecular characterization of *Cryptosporidium* isolates from 20 different species collected on the coasts of Western Australia and Papua New Guinea led to the identification of *C. bollandi* and piscine genotypes 3 and 5 in sea mullet (*Mugil cephalus*) (0.5–33.3%) and piscine genotype 8 in silver biddy (*Gerres oblongus*) (3.6%) as well as *Cryptosporidium* species typical of other hosts, specifically *C. parvum* (1.8–6.9%), *C. xiaoi* (1.8%) and *C. scrofarum* (3.6%) (see Tables 1 and 2) (Reid *et al.*, 2010; Koinari *et al.*, 2013; Yang *et al.*, 2015; Bolland *et al.*, 2020). A recent study on *Cryptosporidium* was carried out in commercially important edible fish across marine areas around France collected in two surveys (Certad *et al.*, 2019). The study reported, by molecular analysis, overall prevalences of *Cryptosporidium* of 2.3 and 3.2% in the first and second surveys, respectively, identifying *C. parvum* (0.7–6.5%) (see Table 2) and also seven new piscine genotypes that exhibit genetic distances from *C. molnari* of between 0.5 and 12.5%. Thus, according to the terminology used by the authors, Cryptofish 1 genotype was found in saithe (*Pollachius virens*) (12.5%) and in blue ling (*Molva dypterygia*) (1.4%); Cryptofish 2 genotype was detected in ling (*Molva molva*) (10.9%) and in whiting (*Merlangius merlangus*) (0.8%); Cryptofish 3 and Cryptofish 4 genotypes were identified in ling (*M. molva*) (4.3%) and in blue ling (*M. dypterygia*) (1.4%), respectively; Cryptofish 5 genotype was found in saithe (*P. virens*) (6.3%) and in hake (*Merluccius merluccius*) (0.7%); finally, Cryptofish 6 and Cryptofish 7 genotypes were identified in cod (*Gadus morhua*) (0.8%) and in mackerel (*Scomber scombrus*) (0.9%), respectively (Certad *et al.*, 2019). Subsequent phylogenetic analysis at the 18S rDNA and actin loci led to the designation of these genotypes as novel marine genotype 1 (Cryptofish 1, 2, 4 and 5) and marine genotype 2 (Cryptofish 7) (see Table 1) (Certad *et al.*, 2020).

A lower diversity of *Cryptosporidium* species/genotypes (including *C. molnari*, piscine genotype 10 and *C. parvum*), although with higher overall prevalences (40.0%; 0.2 and 20.0–100%, respectively), has been identified by molecular methods in freshwater fish from lake Leman (France), Papua New Guinea and Galicia (NW Spain) (see Tables 1 and 2) (Koinari *et al.*, 2013; Certad *et al.*, 2015; Couso-Pérez *et al.*, 2019). Moreover, *Cryptosporidium* sp. was detected by flotation technique and enzyme-linked immunosorbent assay in alewife (*Alosa pseudoharengus*) (50.0%) in the New York State (USA) (Ziegler *et al.*, 2007).

Cultured fish

Data on *Cryptosporidium* in farmed fish are also very scarce, with information available for only three marine and six freshwater species (see Tables 1 and 2).

Álvarez-Pellitero and Sitjà-Bobadilla (2002) carried out the first study on gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) (both marine species), reporting prevalences of *C. molnari* in the range of 6.5–25.4 and 4.6–12.0%, respectively. Sitjà-Bobadilla *et al.* (2005) later conducted an epidemiological study on these same hosts, observing that the prevalence of *C. molnari* differed in specimens from hatcheries and in those from ongrowing tanks. Thus, prevalences of 11.6–100 and 11.1–50.0% were detected in the smallest specimens of gilthead sea bream and European sea bass, respectively, whereas those corresponding to fish from the ongrowing tanks were in the range of 3.6–55.0% for gilthead sea bream to 57.9% for European sea bass (Table 2). In another cultured species, the turbot *P. maxima*, the prevalence rate of *C. scophthalmi* was in the range of 15.0–100%, with the higher values corresponding to the juvenile specimens, observing a decrease in the prevalence as the size of the fish increased (Álvarez-Pellitero *et al.*, 2004).

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Regarding freshwater species, the presence of *Cryptosporidium* was investigated in angelfish (*P. scalare*) by Murphy *et al.* (2009), who described piscine genotype 2, currently recognized as *C. bollandi* (Bolland *et al.*, 2020). Sequences similar to *C. molnari* have been identified in stomach samples from Murray cod (*Maccullochella peelii*) (95.4%) (Barugahare *et al.*, 2011). Molecular characterization of the *Cryptosporidium* isolates from Asian sea bass (*L. calcarifer*) and Nile tilapia (*O. niloticus*) enabled identification of *C. parvum* in these species (20.0 and 2.4%, respectively) (Gibson-Kueh *et al.*, 2011; Koinari *et al.*, 2013) (see Tables 1 and 2). Moreover, after histological examination, *Cryptosporidium*-like organisms were observed in the apical border of gastric and enteric epithelial sections of intensively reared barramundi (*L. calcarifer*) (prevalence of 92.5%) (Gabor *et al.*, 2011). More recently, Couso-Pérez *et al.* (2018) conducted a study on *Cryptosporidium* in farmed rainbow trout (*Oncorhynchus mykiss*), reporting an overall prevalence of 9.2% by immunofluorescence microscopy, although a higher rate was observed in the smallest fish (14.2%), and identifying *C. parvum* (1.9%) and piscine genotype 9 (0.6%) (see Tables 1 and 2).

Ornamental fish

Most studies on piscine *Cryptosporidium* have involved ornamental fish, including about 70 species collected from various aquariums and pet shops in Perth (Western Australia) (Zanguee *et al.*, 2010; Morine *et al.*, 2012; Yang *et al.*, 2015, 2016; Palermo, 2016; Bolland *et al.*, 2020; Zahedi *et al.*, 2021) and in different cities in Iran (Nematollahi *et al.*, 2016; Shahbazi *et al.*, 2020). *Cryptosporidium* was detected by molecular methods in a total of 12 and 16 species of marine and freshwater ornamental fish, respectively, with prevalences in the range of 9.1–100% in marine fish and 0.9–100% in freshwater species (Zanguee *et al.*, 2010; Morine *et al.*, 2012; Yang *et al.*, 2015, 2016; Palermo, 2016; Bolland *et al.*, 2020; Shahbazi *et al.*, 2020; Zahedi *et al.*, 2021). Molecular characterization of the isolates enabled identification of *C. molnari* (15.4–100%), *C. molnari*-like (0.9–100%), *C. huwi* (1.9–50.0%), *C. bollandi* (0.5–75.0%) and *C. abrahamseni* (27.3–62.5%) (see Table 1). Moreover, several authors have characterized a total of three genotypes of *Cryptosporidium*, specifically piscine genotypes 4 (20.0–100%), 5 (0.5–100%) and 6 (2.3–33.3%), as well as piscine genotype 3-like (0.9%), initially described in the sea mullet (*M. cephalus*) (see Table 1) (Zanguee *et al.*, 2010; Morine *et al.*, 2012; Yang *et al.*, 2015, 2016; Bolland *et al.*, 2020; Zahedi *et al.*, 2021). In the goldfish (*Carassius auratus*), *C. hominis* (4.6%), *C. parvum* (0.9%) and rat genotype 3 (5.3%) were detected in addition to *C. molnari*-like (2.7%) and piscine genotypes 3-like and 5 (0.9 and 2.7%, respectively) (see Tables 1 and 2) (Morine *et al.*, 2012; Palermo, 2016). Although Shahbazi *et al.* (2020) reported the presence of *C. parvum* in *C. auratus*, the sequence analysis revealed 99% similarity with the sequence corresponding to accession number AF222998, which is actually *C. hominis* (2.0%) (see Table 2). Furthermore, in histological studies on parasites in freshwater ornamental fish collected in aquarium fish shops in Iran, *Cryptosporidium* spp. were detected by histology in the 16% of examined fish, specifically in sailfin molly (*Poecilia latipinna*), Siamese fighting fish (*Betta splendens*), gourami (*T. leeri*), rosy barb (*Puntius conchonius*), platy fish (*Xiphophorus maculatus*), angelfish (*P. scalare*), electric yellow (*Labidochromis caeruleus*), goldfish (*C. auratus*), Oscar fish (*A. ocellatus*) and slender rainbow (*Melanotaenia gracilis*) (Nematollahi *et al.*, 2016).

Pathology and clinical manifestations

Although several authors have reported high morbidity and mortality rates, mainly in juvenile fish specimens, the pathology of cryptosporidiosis in fish has not been extensively studied (Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Ryan *et al.*, 2004; Yang *et al.*, 2016). However, infection by *Cryptosporidium* is known to be influenced by the following: (1) the species/genotype considered; (2) the host fish species; (3) the age/size of the fish and (4) co-infections with other pathogens (Sitjà-Bobadilla *et al.*, 2005, 2006).

In most cases of *Cryptosporidium* infection, no clinical signs of disease are observed, although several authors have described clinical manifestations such as emaciation, atrophy of skeletal muscle, flattening of the abdomen, low growth rate, anorexia, listlessness, whitish feces, abdominal swelling and ascites (Hoover *et al.*, 1981; Gratzek, 1993; Camus and López, 1996; Muench and White, 1997; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004; Ryan *et al.*, 2004; Murphy *et al.*, 2009; Gabor *et al.*, 2011; Nematollahi *et al.*, 2016).

Some studies have demonstrated the existence of histopathological damage induced by the accumulation of oocysts of *C. molnari* and *C. scophthalmi* (Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004). The presence of large vacuoles disturbing the usual architecture of the mucosa, followed by massive necrosis of the epithelial cells and the consequent cellular detachment in specimens with high intensities of infection have been reported (Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004). Furthermore, a strong inflammatory response and intense leucocyte infiltration have also been observed (Álvarez-Pellitero *et al.*, 2004; Yang *et al.*, 2016).

Fish as potential hosts of zoonotic *Cryptosporidium* species

The use of molecular subtyping tools has led to a better understanding of the transmission dynamics of *Cryptosporidium* infections in humans and animals. The most widely used marker is a hypervariable portion of the 60-kDa glycoprotein (GP60) gene, sequencing of which enables identification of human-specific, animal-specific and zoonotic subtypes. Regarding *Cryptosporidium* in fish, the GP60 gene has been amplified in very few studies. Thus, *C. hominis* subtype IdA15G1 was detected in wild marine fish, specifically in mackerel scad (*Decapterus macarellus*) from Papua New Guinea (Koinari *et al.*, 2013), and subtype IbA10G2 was identified in a freshwater goldfish (*C. auratus*) from a farm in Western Australia (Palermo, 2016). Other studies have identified different *C. parvum* subtypes in both freshwater and marine fish, including subtypes IIaA13G1R1, IIaA14G2R1, IIaA15G2R1, IIaA16G2R1, IIaA17G2R1, IIaA18G3R1 and IIaA19G4R1 (see Table 3) (Reid *et al.*, 2010; Koinari *et al.*, 2013; Certad *et al.*, 2015, 2019; Couso-Pérez *et al.*, 2019). However, to date there are no reports on the identification of piscine *Cryptosporidium* species/genotypes in human hosts (Ryan *et al.*, 2021).

Detection of zoonotic *Cryptosporidium* subtypes in fish raises the following questions: Are fish actually infected by *Cryptosporidium* spp. that infect mammals or are they simply carriers of this protozoan parasite? If so, would this be important from a public health point of view? Detection of zoonotic *Cryptosporidium* species/genotypes in fish collected from the environment does not necessarily indicate real infection, because fish can act as mechanical carriers, as low prevalences and parasite loads are usually detected. Thus, experimental cross-transmission and/or histological studies are needed to confirm the role of fish as competent hosts for zoonotic *Cryptosporidium* species. Several authors have considered whether *Cryptosporidium* of mammalian origin can infect lower vertebrate hosts. Graczyk *et al.* (1996) inoculated (by gastric intubation) some fish, amphibians and reptiles with *C. parvum* (AUCP-1 strain) oocysts obtained from the feces of experimentally infected Holstein calves, which were infectious to neonatal BALB/c mice. Nevertheless, histological sections

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Table 3. Subtypes of *C. parvum* identified in piscine hosts by analysis of the GP60 gene

Subtype	Fish host	Origin	Habitat	GenBank	Reference
IIaA14G2R1	*O. niloticus*	C	FW	ND	Koinari *et al.* (2013)
IIaA15G2R1	*C. lavaretus*	W	FW	KP939340	Certad *et al.* (2015)
	D. macarellus	W	M	ND	Koinari *et al.* (2013)
	P. fluviatilis	W	FW	KP939345	Certad *et al.* (2015)
	S. trutta	W	FW	MH107845	Couso-Pérez *et al.* (2019)
	S. alpinus	W	FW	KP939335	Certad *et al.* (2015)
IIaA16G2R1	*P. fluviatilis*	W	FW	KP939348	Certad *et al.* (2015)
IIaA17G2R1	*C. lavaretus*	W	FW	KP939341	Certad *et al.* (2015)
	E. lucius	W	FW	KP939338	Certad *et al.* (2015)
	R. rutilus	W	FW	KP939350	Certad *et al.* (2015)
	S. alpinus	W	FW	KP939334	Certad *et al.* (2015)
IIaA18G3R1	*S. vittata*	W	M	ND	Reid *et al.* (2010)
	S. trutta	W	FW	MH107846	Couso-Pérez *et al.* (2019)
IIaA19G4R1	*O. niloticus*	C	FW	ND	Koinari *et al.* (2013)
	P. gonionotus	W	FW	ND	Koinari *et al.* (2013)

C, cultured; FW, freshwater; M, marine; O, ornamental; W, wild; ND, no data.

from gastrointestinal tissues were negative for developmental stages of *Cryptosporidium*. These authors consider that *C. parvum* does not infect fish, but that under some circumstances such as after the ingestion of *C. parvum*-infected prey, lower vertebrates may disseminate oocysts in the environment by acting as mechanical carriers.

However, other authors consider that *C. parvum* can infect piscine hosts. Thus, Freire-Santos *et al.* (1998) experimentally infected rainbow trout (*O. mykiss*) with *C. parvum* oocysts collected from a naturally infected Friesian-Holstein neonatal calf, after evaluating the viability and infectivity of the oocysts in a suckling murine model. During the histological examination, no life-cycle stages of *Cryptosporidium* were observed in any part of the apical border of the digestive tract sections. However, large numbers of 5–7 μm spherical structures compatible with *Cryptosporidium* developmental stages were found located deep within the epithelial tissue of pyloric caeca sections. Moreover, an indirect fluorescent antibody test with immunoglobulin (Ig) M and IgG anti-*Cryptosporidium* antibodies revealed fluorescence reactivity in these structures, the number of which increased remarkably when the specimens were subjected to stress conditions.

Similarly, Couso-Pérez *et al.* (2016) experimentally infected 25 young specimens of cultured turbot (*P. maxima*), of weight 20–40 g, with *C. parvum* oocysts (subtype IIaA16G3R1) obtained from a naturally infected neonatal calf. The fish were maintained for 2 h under stress conditions in a tank contaminated with 25×10^6 purified *C. parvum* oocysts and then placed in another tank with clean sea water. The application of a direct immunofluorescence method with monoclonal anti-*Cryptosporidium* antibodies led to microscopic detection of *C. parvum* oocysts in the intestine and the pyloric caeca from fish collected 7 and 10 days post-exposure, respectively. Considering that *C. parvum* requires between 48 and 72 h to complete its life cycle in a suitable host and that the emptying time of turbot gastrointestinal tract is approximately 24 h, the authors suggested that *C. parvum* infection in young cultured turbot is possible, although infection must be confirmed by further histological studies (Couso-Pérez *et al.*, 2016). Moreover, in response to high mortality rates detected in different fish farm systems in Galicia (NW Spain), the presence of *Cryptosporidium* in pyloric caeca and intestinal homogenates from cultured turbot (*P. maxima*) was investigated, revealing the presence of *Cryptosporidium* sp. oocysts in 21 of 29 (72.4%) juvenile specimens examined, with a mean intensity of 14.6 oocysts/turbot. Subsequent molecular characterization identified the hypertransmissible subtype IIaA15G2R1 of *C. parvum* (unpublished results).

Recent epidemiological studies have suggested that *C. parvum* can complete its life cycle and multiply in piscine hosts. Thus, histological analysis of gastric and intestine sections from *C. parvum* polymerase chain reaction positive freshwater and marine fish carried out by Certad *et al.* (2015, 2019) revealed the presence of round bodies resembling developmental stages of *Cryptosporidium* in an apical position within the cells. As a consequence of these observations, the authors suggested that *C. parvum* was actually infecting fish, rather than being passively transmitted. Couso-Pérez *et al.* (2018, 2019) subjected gastrointestinal samples of brown and rainbow trout (*S. trutta* and *O. mykiss*, respectively) to a homogenization treatment using a bar homogenizer and applied a monoclonal anti-*Cryptosporidium* antibody test, detecting clusters of oocysts in the pyloric caeca of both fish species. Molecular analysis revealed that oocysts belonged to the species *C. parvum*, specifically subtypes IIaA15G2R1 and IIaA18G3R1. These findings suggest the existence of real infections, because fully sporulated oocysts, which can appear in clusters in the same infected cell, are only found within the vacuolar space located deep in the mucosal epithelium in piscine hosts (Landsberg and Paperna, 1986; Álvarez-Pellitero and Sitjà-Bobadilla, 2002; Álvarez-Pellitero *et al.*, 2004; Ryan *et al.*, 2004, 2015; Bolland *et al.*, 2020; Zahedi *et al.*, 2021). The homogenization treatment to which the samples were subjected would have ruptured the epithelial cells, thereby releasing oocysts, individually or in clusters. Unfortunately, histological analysis of the samples was not possible as the tissue had been deteriorated by autolysis (Couso-Pérez *et al.*, 2018, 2019).

Identification of zoonotic *C. parvum* subtypes in piscine hosts suggests that fish are a potential source of infection in humans, with an associated risk to public health. Anglers and food handlers could be infected directly while eviscerating or preparing the captured specimens or indirectly *via* contact with contaminated surfaces or fomites during the storage of the fish. The only study

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that has quantified the risk of infection by *Cryptosporidium* was carried out by Roberts *et al.* (2007) and was based on fish and hand wash samples taken from urban anglers in Baltimore (USA). Using the United States Environmental Protection Agency's dose–response model, these authors determined that the mean probability of acquiring *Cryptosporidium* infection in anglers was 41.0% (although it could reach 100%) on the basis of positive fish samples. A significantly higher mean probability of infection (91.0%) was estimated on the basis of positive hand wash samples. When all data (positive fish and hand wash samples) were considered and, depending on host factors, such as immune status and pre-existing illnesses, it was estimated that on average 1–8 out of 10 anglers could become infected by *Cryptosporidium* (Roberts *et al.*, 2007). Thus, edible fish would extend the range of foodstuffs involved in the transmission of cryptosporidiosis, the aetiological agent of which is responsible for 8.6 million cases of foodborne illness annually worldwide (Ryan *et al.*, 2018; Moratal *et al.*, 2020; Zahedi and Ryan, 2020).

Conclusions

Research on *Cryptosporidium* in piscine hosts has increased in recent years, reaffirming the ubiquitous nature of this protozoan parasite, which has been detected in a large number of free-living, cultured and ornamental fish species worldwide, from both marine and freshwater environments. Future studies will increase the range of piscine hosts, and novel *Cryptosporidium* species and genotypes will be proposed. Therefore, the taxonomy and evolutionary relationships in the genus must be clarified to enable consensus to be reached regarding the nomenclature used to designate new piscine species/genotypes. Whole genome sequencing of *Cryptosporidium* species/genotypes from fish is also required to assist the taxonomic clarification. Moreover, experimental cross-transmission and/or histological studies are needed to confirm the role of fish as competent hosts for zoonotic *Cryptosporidium* species as very few such studies have been conducted to date.

The existence of *Cryptosporidium* infections in cultured fish may have a significant economic impact on the aquaculture industry due to the morbidity and mortality rates reported in some fish species. The implementation of control programmes may be necessary to remove the *Cryptosporidium* oocysts from aquaculture facilities, where fish cohabit in dense groups and are subjected to other stress factors that can enhance transmission of this parasite.

Finally, the identification of zoonotic *Cryptosporidium* species in edible fish extends the range of foodstuffs potentially involved in the transmission of cryptosporidiosis, representing a risk to public health, although further risk assessment studies are required to confirm this possibility.

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