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Identification of novel and zoonotic *Cryptosporidium* species in fish from Papua New Guinea

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ABSTRACT

There is still limited information on the distribution of *Cryptosporidium* species and genotypes in fish. The present study investigated the prevalence of *Cryptosporidium* species in cultured freshwater ($n = 132$), wild freshwater ($n = 206$) and wild marine ($n = 276$) fish in Papua New Guinea (PNG) by PCR screening at the 18S rRNA locus. A total of seven fish (2 cultured freshwater, 1 wild freshwater and 4 wild marine fish) were identified as positive for *Cryptosporidium*. Specifically, *Cryptosporidium* was found in four different host species (Nile tilapia, *Oreochromis niloticus*; silver barb, *Puntius gonionotus*; mackerel scad, *Decapterus maracellus* and oblong silver biddy, *Gerres oblongus*), giving an overall prevalence of 1.14% (95% CI: 0.3–2%, $n = 7/614$). Of the seven positive isolates, five were identified as *C. parvum* and two were a novel piscine genotype, which we have named piscine genotype 8. Piscine genotype 8 was identified in two marine oblong silver biddies and exhibited 4.3% genetic distance from piscine genotype 3 at the 18S locus. Further subtyping of *C. parvum* isolates at the 60 kDa glycoprotein (*gp60*) locus identified 3 *C. parvum* subtypes (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) all of which are zoonotic and a *C. hominis* subtype (IdA15G1). The zoonotic *Cryptosporidium* were identified in fish samples from all three groups; cultured and wild freshwater and wild marine fish. Detection of *Cryptosporidium* among aquaculture fingerlings warrants further research to gain a better understanding of the epidemiology of *Cryptosporidium* infection in cultured fish. The identification of zoonotic *Cryptosporidium* genotypes in fish from PNG has important public health implications and should be investigated further.

1. Introduction

The apicomplexan protozoan parasite *Cryptosporidium* infects a wide range of mammals, birds, reptiles and fish, primarily causing diarrhoea in mammals, diarrhoea and/or catarrhal respiratory signs in birds and gastritis in reptiles and possibly fish (O'Donoghue, 1995; Ryan, 2010). *Cryptosporidium* has been described in more than 17 species of both fresh and salt water fish with parasitic stages located deep within and on the surface of the stomach or intestinal epithelium (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012). In fish, *Cryptosporidium* can cause high morbidity with clinical signs including variable levels of emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased mortality (Murphy et al., 2009).

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Table 1
Species/genotypes of *Cryptosporidium* reported in fish in previous studies (*N* = the number of specimens in which each species/genotype of *Cryptosporidium* was identified).

Species/genotype	*N*	Fish host	References
C. molnari	>100	Gilthead sea bream and European seabass	Alvarez-Pellitero and Sitja-Bobadilla (2002), Palenzuela et al. (2010)
C. scophthalmi	49	Turbot	Alvarez-Pellitero and Sitja-Bobadilla (2002), Alvarez-Pellitero et al. (2004)
C. molnari-like	7	Butter bream, madder seaperch, bristle tooth tang, upsidedown catfish, wedgetailed blue tang and green chromas, golden algae eater	Zanguee et al. (2010)
Piscine genotype 1	2	Guppy and neon tetra	Ryan et al. (2004), Zanguee et al. (2010)
Piscine genotype 2	>5	Angelfish, neon tetra and Oscar fish	Murphy et al. (2009), Zanguee et al. (2010)
Piscine genotype 3	2	Sea mullet	Reid et al. (2010)
Piscine genotype 4	4	Golden algae eater, kupang damsel, Oscar fish and neon tetra	Zanguee et al. (2010), Morine et al. (2012)
Piscine genotype 5	3	Angelfish, butter bream and golden algae eater	Zanguee et al. (2010)
Piscine genotype 6	1	Guppy	Zanguee et al. (2010)
Piscine genotype 6-like	1	Gold gourami	Morine et al. (2012)
Piscine genotype 7	3	Red-eye tetra	Morine et al. (2012)
Rat genotype 3-like		Goldfish	Morine et al. (2012)
C. scrofarum	2	School whiting	Reid et al. (2010)
C. parvum	1	School whiting	Reid et al. (2010)
C. xiaoi	1	School whiting	Reid et al. (2010)

Currently the only recognised species infecting fish is *Cryptosporidium molnari*, which was identified in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labarx*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterised genetically in 2010 (Palenzuela et al., 2010). *C. molnari* primarily infects the epithelium of the stomach and seldom the intestine (Alvarez-Pellitero and Sitja-Bobadilla, 2002). In 2004, *C. scophthalmi* was described in turbot (*Psetta maxima*. syn. *Scopthalmus maximus*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004). However, no genetic sequences are available for *C. scophthalmi* and it can thus not be considered a valid species due to the high genetic heterogeneity and morphological similarity among *Cryptosporidium* species in fish. In addition to *C. molnari*, a total of 3 species and 10 genotypes have been characterised genetically in fish (Table 1).

Fish are an important part of the diet and a source of income especially for people living on the coast and along the rivers in Papua New Guinea (PNG). Freshwater fish farming began in the 1960s with the introduction of carp and trout species; however, difficulties due to lack of knowledge among farmers impeded farming progress and the spread of its nutritional and financial benefits to rural communities (Smith, 2007). Since 1995, the number of inland aquaculture operations has increased in PNG due to international programmes that involve the expansion of hatcheries, training of farmers and the introduction of new fish species (Smith, 2007). To date, very little is known about the prevalence and genotypes of *Cryptosporidium* in fish or other animals in PNG (Owen, 2005; Koinari et al., 2012, 2013). The present study represents a detailed investigation of the prevalence and genetic characterisation of *Cryptosporidium* in cultured freshwater, wild freshwater and wild marine fish sampled from a number of different locations throughout PNG. It is therefore the first comprehensive study describing the distribution of *Cryptosporidium* species in PNG.

2. Materials and methods

2.1. Sample collection

A total of 614 fish from cultured freshwater, wild freshwater and wild marine environments were collected in PNG between February and August 2011 (Fig. 1). Cultured fish ($n = 133$) included three species, which were collected from four smallholder fish ponds in Kundiawa, Asaro, Mumeng and Bathem (Table 2). Wild freshwater fish ($n = 205$) included six species and were collected from the Ramu and Sepik Rivers, while 276 wild marine fish consisting of 16 species were bought from local fishermen in Bilbil, Madang, Tavana and Pilapila (Table 2). On average, time lag between collection or purchasing and processing of the fish was up to 4 h. All sampling was conducted under Murdoch University Animal Ethics permit R2369/10.

The fish were weighed, measured (length and weight) and dissected. Sections of intestine and stomach were cut using a sterile scalpel blade for each fish, placed in 2 mL Eppendorf tubes and preserved in 70% ethanol for molecular screening. The remaining stomach and intestine were fixed in 10% buffered formalin for histological analysis. All samples were stored at 4 °C in PNG until sample collection was completed. The samples were then transported to Murdoch University, Perth, Australia and stored at 4 °C until analysis.

2.2. DNA isolation

The preserved intestines and stomachs were washed 5 times with water to remove ethanol and the epithelial layers were scraped using a sterile scalpel blade for each fish. DNA was extracted from 25 mg of intestinal and stomach scrapings using a PowerSoil® DNA Isolation Kit (MO BIO laboratories, Carlsbad, California, USA) according to the manufacturer's instructions and incorporating five