Molecular identification of *Cryptosporidium parvum* from avian hosts

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SUMMARY

Cryptosporidium species are protozoan parasites that infect humans and a wide variety of animals. This study was aimed at identifying *Cryptosporidium* species and genotypes isolated from avian hosts. A total of 90 samples from 37 different species of birds were collected throughout a 3-month period from April 2008 to June 2008 in the National Zoo of Kuala Lumpur, Malaysia. Prior to molecular characterization, all samples were screened for *Cryptosporidium* using a modified Ziehl-Neelsen staining technique. Subsequently samples were analysed with nested-PCR targeting the partial SSU rRNA gene. Amplicons were sequenced in both directions and used for phylogenetic analysis using Neighbour-Joining and Maximum Parsimony methods. Although 9 (10%) samples were positive for *Cryptosporidium* via microscopy, 8 (8·9%) produced amplicons using nested PCR. Phylogenetic trees identified all the isolates as *Cryptosporidium parvum*. Although *C. parvum* has not been reported to cause infection in birds, and the role of birds in this study was postulated mainly as mechanical transporters, these present findings highlight the significant public health risk posed by birds that harbour the zoonotic species of *Cryptosporidium*.

Key words: birds, *Cryptosporidium parvum*, zoo, Malaysia.

INTRODUCTION

Cryptosporidium species are protozoan parasites that infect humans and a wide variety of animals. Human cryptosporidiosis has been recognized as the most common cause of protozoal diarrhoea worldwide leading to significant morbidity and mortality both in developing and industrialized nations (Marshall *et al*. 1997; Clark, 1999). Furthermore, *Cryptosporidium* has caused multiple waterborne outbreaks in developed and developing countries (Insulander *et al*. 2005) with the largest *Cryptosporidium* waterborne outbreak reported in Milwaukee, USA, affecting 403 000 persons with more than 100 fatal cases (MacKenzie *et al*. 1994).

Cryptosporidium has recently been classified into 23 valid species (Fayer, 2010; Fayer *et al*. 2010; Traversa, 2010) and nearly 61 genotypes have been reported (Plutzer and Karanis, 2009). Of these, 1 species was reported from amphibians, 2 from reptiles, 3 from birds and 12 were reported from mammals (Fayer, 2010). Of the *Cryptosporidium* species infecting mammals, *C. parvum* and *C. hominis* are the most common species responsible for human infections. Although *C. felis* (Matos *et al*. 2004), *C. muris* (Katsumata *et al*. 2000), and *C. canis* (Cama *et al*. 2003) have also been reported to infect humans, they are less common.

Avian cryptosporidiosis has been reported from more than 30 avian species and is usually caused by 3 *Cryptosporidium* species and 10 *Cryptosporidium* genotypes (Fayer, 2010). The recognized avian species are *C. meleagridis* (Slavin, 1955), *C. baileyi* (Current *et al*. 1986) and *C. galli* (Pavlásek, 1999). Avian genotypes include avian genotypes I – IV that were identified from various avian hosts (Ryan, 2010) such as Eurasian woodcock genotype (Ryan *et al*. 2003), duck genotype from black duck (Morgan *et al*. 2001) and goose genotypes 1 – IV from Canada geese (Jellison *et al*. 2004).

The risk of zoonotic transmission arises from *C. meleagridis* which is an emerging human pathogen and has been considered the third most common *Cryptosporidium* species infecting humans (Xiao and Fayer, 2008). The role of *C. parvum* infection among birds is not fully understood and even the reported cases from wild birds were attributed to mechanical transmission rather than an established infection (Dieter *et al*. 2001; Zhou *et al*. 2004; Majewska *et al*. 2009). In Malaysia, no information on *Cryptosporidium* species and genotypes infecting avian hosts is currently available. Thus, the present study was carried out to determine the species and genotypes of *Cryptosporidium* found in birds at the National Zoo, Kuala Lumpur and whether these could be potential reservoirs for human infections.

MATERIALS AND METHODS

Source of samples

Ninety faecal samples were collected from 37 species of birds from different locations (i.e. the aviary, bird

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house, breeding area and the lake) in the National Zoo for a period of 3 months (i.e. April to June 2008). Samples were collected early in the morning in clean plastic containers and transported to the laboratory. The faecal samples were fixed with 2·5% potassium dichromate before being stored in a cold room at 4 °C for further analysis.

Microscopic examination

The faecal samples were concentrated to obtain a better yield of *Cryptosporidium* oocysts with the formalin-ether concentration technique. Using a pipette, a drop of the sediment was placed on a glass slide and evenly spread and stained with Ziehl-Neelsen stain. Briefly the slide was dried and stained with carbol fuchsin for 10 min, destained using acid alcohol for 10–15 s and stained with malachite green for 3 min. Stained smears were examined under 1000 × magnification using a light microscope.

Polymerase chain reaction

DNA was extracted from approximately 1 g of faecal samples using QIAmp DNA stool mini kit according to the manufacturer's instructions (QIAgen) and stored at −20 °C until use. Nested-PCR was used to amplify a partial, polymorphic region of 18S rRNA, according to the method described by Nichols *et al.* (2003). Primary PCR amplified a 655 to 667 bp fragment, depending on the species of *Cryptosporidium* or *C. parvum* genotype, using the forward primer N-DIAGF2 (5′-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3′) and the reverse primer N-DIAGR2 (5′-CCT TCC TAT GTC TGG ACC TGG TGA GT-3′), while the secondary PCR amplified a 435 bp fragment using the forward primer CPB-DIAGF (5′-AAG CTC GTA GTT GGA TTT CTG-3′) and the reverse primer CPB-DIAGR (5′-TAA GGT GCT GAA GGA GTA AGG-3′) that were previously developed by Johnson *et al.* (1995).

Both primary and secondary PCR were conducted in a 50 μl reaction mixture containing 200 μM of each dNTP (Fermentas, Ontario, Canada), 0·2 μM of each of the primers CPB-DIAGF2/R2 (Research Biolab, Singapore), 400 μg/ml BSA (New England Biolabs, Ipswich, MA, USA), 3·5 mM $MgCl_2$ (Fermentas, Ontario, Canada), 2·5 U *Taq* polymerase (New England Biolabs, Ipswich, MA, USA), and 1X ThermoPol PCR buffer (New England Biolabs, Ipswich, MA, USA). Two μl of DNA template was used in the primary PCR whereas 5 μl of the first PCR product was used as template in the secondary PCR. DNA template extracted from oocysts of *C. parvum* H3 isolate, was used as positive control. The cycling condition was as follows; hot start at 95 °C for 5 min, followed by 35 cycles of denaturing for 30 s at 94 °C, annealing for 1 min at 68 °C and extension for 30 s at 72 °C, followed by a final extension at 72 °C for 10 min. The secondary PCR had a similar cycling condition except that the annealing temperature was 60 °C instead of 68 °C. The PCR products were electrophoresed on 2% agarose gels at 100 V.

DNA sequencing and phylogenetic analysis

The DNA was purified using the QIAquick Gel Extraction Kit (QIAgen, Germany) according to manufacturer's instructions. Cycle sequencing was carried out using the ABI PRISM® BigDye™ terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions, and amplified products were analysed using the ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, USA). DNA sequencing was carried out in both directions. Forward and reverse sequences were edited and assembled, and the consensus sequence was derived for each sample using BioEdit (www.mbio.ncsu.edu). Multiple sequence alignment was performed using MEGA4 (www.megasoftware.net). To root the trees, *Eimeria tenella* (GenBank Accession no. AF026388) sequence was retrieved as it appears to be the closest sister taxon to the ingroup of the present study. Neighbour-joining (NJ) and maximum parsimony (MP) analyses were performed in MEGA4. NJ analyses were performed with distances calculated with the Kimura 2-parameter (Kimura, 1980). Unweighted parsimony analyses were performed using the Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000). To evaluate the support for inferred topologies, bootstrapping (Felsenstein, 1985) was done with 1000 replicates. Homology search was carried out using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997).

RESULTS

A total of 90 bird faecal samples were collected and 9 (10%) out of these were positive for *Cryptosporidium* spp. microscopically. *Cryptosporidium* oocysts were identified as pink to red spheres with a size of approximately 4–6 μm. Nine samples from 8 avian species that were found to be positive with *Cryptosporidium* are shown in Table 1. However, the numbers of oocysts in each positive faecal sample were not counted.

Microscopic results were in concordance with nested PCR results except in 1 case where, microscopically, the sample was positive but negative by nested PCR. Amplicons from all PCR positive samples were successfully sequenced except 1 sample. Seven sequences from this study (Table 1) and 13 reference sequences from GenBank, representing avian *Cryptosporidium* species; *C. meleagridis*,

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Table 1. List of bird species positive for *Cryptosporidium* oocysts using Ziehl-Neelson stain at the National Zoo

(A, Aviary; BH, Bird house; BA, Breeding area); A, Lake.)

ID	Common Name	Species	Location	No. of samples	No. positive/species/ Genbank Accession number
71	Black Swan	*Cygnus atratus*	A	2	1/*C. parvum*/HM059832
15	Crestless Fireback Pheasant	*Lophura erthrophthalma*	BH, A	5	2/*C. parvum*/HM059827#
20	Fischer's Lovebird	*Agopornis fisheri*	BH, A	2	1/*C. parvum*/HM059829
17	Golden Pheasant	*Chrysolophus pictus*	A	3	1/*C. parvum*/HM059828
5	Great Argus Pheasant	*Argusianus argus*	A	4	1/*Cryptosporidium* spp*
31	Great Curassow	*Crax rubra*	A	2	1/*C. parvum*/HM059830
37	Pink Backed Pelican	*Pelecanus rufescens*	A	1	1/*C. parvum*/HM059831
13	Wrinkled Hornbill	*Aceros corrugatus*	A	3	1/*C. parvum*/HM059826

* *Cryptosporidium* species was not identified due to failure in DNA sequencing.
\# Two samples were microscopically positive but only one was confirmed using PCR.

C. baileyi, C. galli and avian genotypes I–IV were multiple aligned and used for constructing the phylogenetic trees. The Neighbour-Joining method (Fig. 1) grouped all the isolates from this study together with *C. parvum* in 1 cluster (83% bootstrap support). Maximum Parsimony was concordant in topology with NJ tree placing the 7 isolates in 1 clade with *C. parvum* (72% bootstrap) (Fig. 1). Similarity search using BLAST showed that isolates 20 and 31 were identical while isolates 13, 15, 17, 37 and 71 were 99% similar to *C. parvum* (GenBank Accession number FJ984565) which was isolated from avian hosts in Hungary (Plutzer and Tomor, 2009).

DISCUSSION

The current study carried out at the National Zoo, Kuala Lumpur showed evidence that birds on display in the zoo have the potential to be sources of dissemination of *Cryptosporidium* in the environment. Two previous studies have been carried out to determine the prevalence of cryptosporidiosis in the birds in the same zoo. The two studies indicated that the prevalence of *Cryptosporidium* was rated at 6% (Rohela *et al*. 2005) and 3·4% (Mahat, 2006) compared to 10% in this study. However, the two previous studies did not conduct molecular characterization of the *Cryptosporidium* species recovered from infected birds.

In this present study, phylogenetic analysis using Maximum Parsimony and Neighbour-Joining methods identified all the 7 isolates as *C. parvum*. This finding was confirmed by BLAST search in which the 7 isolates were almost identical to *C. parvum* which was isolated from the faeces of wild aquatic birds (mallard, *Anas platyrhynchos* and coot, *Fulica atra*), in Hungary (Plutzer and Tomor, 2009). Previous studies have detected *C. parvum* in the faeces of Canada geese in the United States (Zhou *et al*. 2004; Graczyk *et al*. 1998; Kassa *et al*. 2004). In Western Poland, of 499 faecal specimens collected

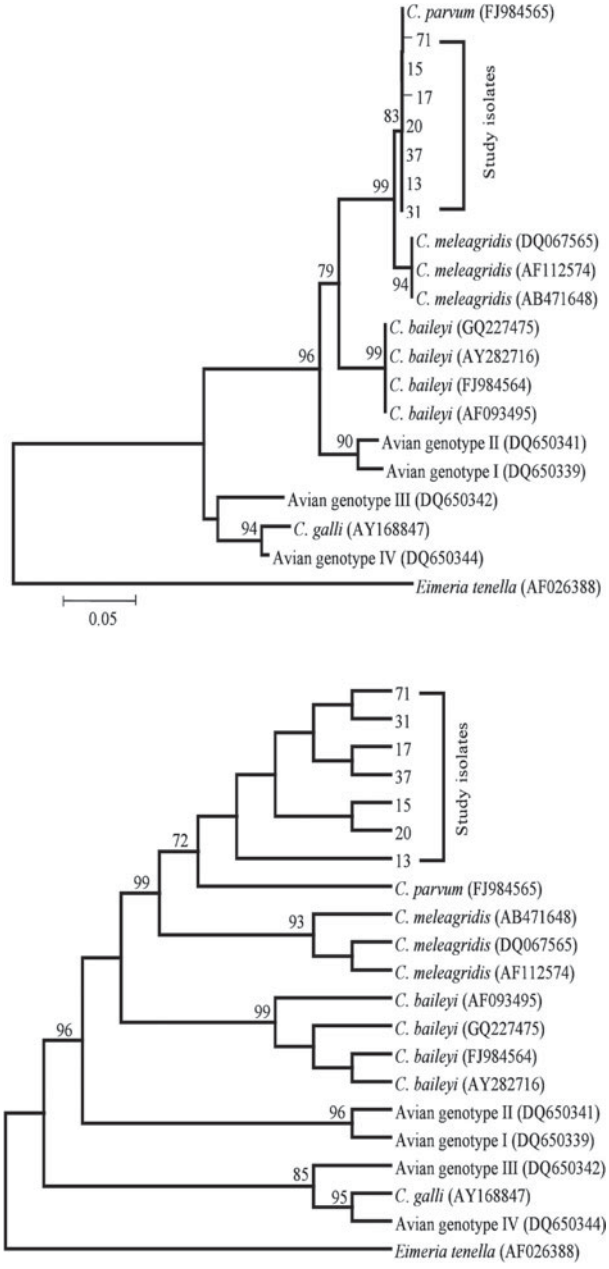

Fig. 1. Phylogenetic tree for *Cryptosporidium* spp.

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from birds, *C. parvum* was identified in the faeces of 1 mandarin duck (*Aix galericulata*), 2 common mergansers (*Mergus merganser*), 4 mute swans (*Cygnus olor*), 3 white storks (*Ciconia ciconia*), 6 carrion crow (*Corvus corone*) and 3 rooks (*Corvus frugilegus*) (Majewska *et al*. 2009).

Since studies that attempted to infect birds with *C. parvum* from mammals had inconsistent results with mild to moderate and unsuccessful establishment of the infection, detection of *C. parvum* in the faeces of birds has been thought to be the result of mechanical transmission rather than established infection (Lindsay *et al*. 1987; Palkovic and Marousek, 1989; Sreter and Varga, 2000; Darabus and Olariu, 2003). However, it was reported that *C. parvum* oocysts retain viability and infectivity following passage through the avian hosts (Graczyk *et al*. 1997). In addition, previous studies showed that the concentration of *C. parvum* oocysts was $3{\cdot}7\times10^{6}$/g in the faeces of Canada geese (Graczyk *et al*. 1998) and $4{\cdot}8\times10^{2}$/g in the faeces of ducks (Kuhn *et al*. 2002).

A prediction model was developed to correlate between bird abundance, faeces input and faecal indicator bacteria (FIB) in aquatic environments (Kirschner *et al*. 2004). Application of this model showed that approximately $9{\cdot}3\times10^{6}$ infectious *C. parvum* oocysts could be introduced into the water due to a single visitation of an average-sized waterfowl flock (Graczyk *et al*. 2008). Thus, birds may still play a significant role in environmental contamination with the human infectious *C. parvum*. It must be noted that *C. parvum* oocysts are highly infectious, with an ID_{50} ranging from 9 to 2980 oocysts based on the type of isolates (Okhuysen *et al*. 1999, 2002; Messner *et al*. 2001). In the study area of the present investigation it is important to note that the birds at the lake area are free to fly out of the National Zoo to the Putrajaya Lake which is about 35 km away. Therefore there is the possibility that if these birds are infected they may contaminate another water body. However, this postulation needs further study to confirm its significance since the present investigation was limited by the fact that oocysts were not counted.

In conclusion, the results of the present study showed a high prevalence of *C. parvum* among Zoo birds. Although *C. parvum* has not been determined to cause infection in birds, and the role of birds was postulated to be mainly as mechanical transporters, the present findings highlight the significant public health risk posed by birds that harbour the zoonotic species of *Cryptosporidium*. In addition, even though this study found no avian species or genotypes in zoo birds, it cannot be concluded that birds in Malaysia are free of avian *Cryptosporidium* species or genotypes. Therefore, further studies are warranted in order to have a better understanding of the epidemiology of cryptosporidiosis of birds in Malaysia.

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