OCCURRENCE OF *CRYPTOSPORIDIUM* OOCYSTS IN WRINKLED HORNBILL AND OTHER BIRDS IN THE KUALA LUMPUR NATIONAL ZOO

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Abstract. The occurrence of a coccidian parasite, *Cryptosporidium*, among birds in the Kuala Lumpur National Zoo was investigated in this study. A hundred bird fecal samples were taken from various locations of the zoo. Fecal smears prepared using direct smear and formalin ethyl acetate concentration technique were stained with modified Ziehl-Neelsen stain. Samples positive for *Cryptosporidium* with Ziehl-Neelsen stain were later confirmed using the immunofluorescence technique and viewed under the epifluorescence microscope. Six species of bird feces were confirmed positive with *Cryptosporidium* oocysts. They included Wrinkled Hornbill (*Aceros corrugatus*), Great Argus Pheasant (*Argusianus argus*), Black Swan (*Cygnus atratus*), Swan Goose (*Anser cygnoides*), Marabou Stork (*Leptoptilos crumeniferus*), and Moluccan Cockatoo (*Cacatua moluccencis*). These birds were located in the aviary and lake, with the Moluccan Cockatoo routinely used as a show bird. Results obtained in this study indicated that animal sanctuaries like zoos and bird parks are important sources of *Cryptosporidium* infection to humans, especially children and other animals.

INTRODUCTION

Cryptosporidium from birds was first described in 1929 by Tyzzer in the ceca of chickens. In 1955, Slavin reported a structurally similar parasite in turkeys and named it *C. meleagridis* (Slavin, 1955). It has been reported that cryptosporidiosis is one of the most prevalent parasitic infections in domesticated, caged, and wild birds. Cryptosporidiosis has been detected in more than 30 bird species belonging to orders Anseriformes, Charadriiformes, Columbiformes, Galliformes, Passeriformes, Psittaciformes and Struthiniformes (Goodwin *et al*, 1981; Tsai *et al*, 1983; Glisson *et al*, 1984; Lindsay *et al*, 1991).

In birds, cryptosporidiosis manifests itself mainly in two clinical forms, which are respiratory (Mason and Hartley 1980; Dhillon *et al*, 1994) and intestinal (Doster *et al*, 1979; Goodwin and Krabill, 1989; Lindsay *et al*, 1990) diseases. Studies have shown that it may also manifest as renal disease and may even be fatal (Hoerr *et al*, 1986; Latimer *et al*, 1992). In respiratory cryptosporidiosis in birds, the parasite can infect the nasal turbinates, nasopharynx, sinuses, larynx, trachea, lungs, air sacs, and conjunctiva (Sréter and Varga 1999). Intestinal cryptosporidiosis, on the other hand, demonstrates *Cryptosporidium* in salivary and esophageal glands, proventriculus, small intestine, cecum, colon, cloaca and bursa of Fabricius (Xiao *et al*, 2004).

Although studies have indicated that *Cryptosporidium* infections in avian species are probably not a zoonotic threat to humans and cross transmission studies have shown that *Cryptosporidium* is host specific (Lindsay *et al*, 1986; O'Donoghue *et al*, 1987; Graczyk and Cranfield, 1998), some studies have proven that birds may act as mechanical vectors and may shed oocysts which are especially significant in waterborne-cryptosporidiosis (Howe *et al*, 2002). Three avian *Cryptosporidium* species have been named *C. meleagridis*, *C. baileyi* and *C. galli* (Graczyk *et al*, 1996a; Awad-el-Kariem *et al*, 1997) and out of these, only *C. meleagridis*, which infects turkeys and parrots, is a known threat to human beings. Nevertheless, *C. baileyi* is probably the most common avian *Cryptosporidium* species because it is able to infect chickens, turkeys, ducks, cockatiels, quails and ostriches, whereas *C.galli*, the latest addition to the family, infects hosts such as finches, domestic chickens, capercaille and pine grosbeaks (Xiao *et al*, 2004).

Studies done in many countries have indicated the presence of *Cryptosporidium* in various bird species, and have implicated the epidemiological and epizootiological role of birds in the spread of this parasite. In Malaysia, studies on *Cryptosporidium* in animals have been limited to cattle and rodents (Lim and Ahmad 2001; Sabri *et al*, 2004). Currently no study has attempted to ascertain the occurrence of *Cryptosporidium* in birds, especially those in the zoo and pet birds. The aim of the present study is to

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determine the occurrence of *Cryptosporidium* species in feces of birds in Kuala Lumpur National Zoo. Based on the results of this study, further studies and actions can be taken to determine the significance between the presence of this particular parasite and the possibility of zoonotic infection, especially among zoo workers and visitors. This could then determine whether animal sanctuaries like zoos and bird parks can be sources of *Cryptosporidium* infection to humans.

MATERIALS AND METHODS

The Kuala Lumpur National Zoo, located 13 km northeast of Kuala Lumpur, was selected as the study site because it is the largest zoo in the country and the birds are caged and segregated according to species. A total of 100 fresh bird fecal samples were collected and kept in fecal containers labeled according to bird species and location. Feces were collected carefully to avoid contamination with soil. Collection of feces and identification of bird species from their respective locations were carried out in the morning with the assistance of the zookeepers. The fecal samples were brought to the laboratory and kept in the cold room at 4^{o}C before processing.

Direct smear and formalin ethyl acetate concentration techniques were employed to prepare the fecal smears. Both types of smears were then stained using Ziehl-Neelsen (acid-fast) stain. Slides were examined using oil immersion under 400x. *Cryptosporidium* oocysts appeared as bright rose-pink spheres (5±1 μm) on a pale green background. All slides positive for *Cryptosporidium* using the Ziehl-Neelsen staining method were recorded.

To confirm the findings, a more specific and sensitive method using immunofluorescence was employed. For this assay, the fecal specimens had to be concentrated using the formalin ethyl acetate concentration technique. The sediment in formalin was vortexed to ensure a homogenized suspension. Samples of 50 μl were then applied onto a teflon coated welled slide. The slide was then air-dried. One drop of methanol was added for fixation, followed by 25 μl of fluorescein isothiocynate (FITC)-labeled anti-*Cryptosporidium* monoclonal antibody (mAb) (Waterborne Inc, New Orleans, Los Angelas, USA). The slide was then incubated in a humid chamber for 35 minutes at 37^{o}C and the stain was aspirated. A drop of phosphate buffered saline (PBS) was added to wash any excess stain. This was repeated twice followed by staining with 25 μl of $4x10^{-4}$ mg/ml 4´, 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical, Missouri, USA). After 2 minutes, the stain was aspirated before adding 50 μl of distilled water. The distilled water was subsequently aspirated and the slide was then mounted with a drop of mounting medium before placing a glass cover slip on it. Slides were viewed under a 400x epifluorescence microscope (Olympus BX52, Japan) with a wide band excitation color separation filter (exciter 460-490 nm, barrier 550 nm) to detect FITC stain, a wide band UV filter (exciter 330-335 nm, barrier 420 nm) for DAPI stain and Nomarski-DIC optics to observe the internal structures. Positive control slides containing oocysts and negative control slides were included in the analysis.

Oocysts which were stained with FITC-mAb exhibited bright apple green fluorescence, typically concentrated on the periphery of the oocysts when observed under the epifluorescence microscope. *Cryptosporidium* oocysts are spherical in shape with a diameter ranging from 4 to 6 μm (Anonymous, 1990). To further confirm the presence of oocysts, putative oocysts were also examined under the UV filter to determine the presence of four sky-blue sporozoite nuclei (highlighted by the fluorochrome DAPI). Nomarski-differential interference contrast (DIC) optics were used to view internal structures for confirmation.

RESULTS

A total of 100 fecal specimens were collected from 56 species of birds (Table 1) located at 6 different locations (Table 2). By using the Ziehl-Neelsen staining technique, 28 specimens (28%) were positive for *Cryptosporidium* (Table 1). *Cryptosporidium* oocysts were identified as bright rose-pink spheres (5 ± 1 μm) on a pale green background. The oocyst load was very minimal since most smears revealed approximately 5 oocysts per slide. Besides oocysts of *Cryptosporidium*, the smears also contained large numbers of bacteria and yeasts, which had more uniform dull red color as compared to *Cryptosporidium* oocysts.

Of the above 28 positive specimens, only 6 (21.4%) were confirmed positive with immunofluorescence technique. With FITC-labeled mAb, the periphery of the oocysts exhibited bright apple green fluorescence whereas with DAPI, the nuclei of the oocysts appeared sky-blue in color.

The 6 specimens positive for *Cryptosporidium* were detected in species such as the Great Argus Pheasant, the Black Swan and the Wrinkled Hornbill from the aviary, the Swan Goose and the Marabou

Stork from the lake and the Moluccan Cockatoo (Salmon-crested Cockatoo), which is used for the animal show (Tables 1 and 2). Only 14.3% (1 out of 7) of the specimens taken from the Great Argus Pheasant was positive for *Cryptosporidium* oocysts, while 50% (1 out of 2) of the specimens taken from the Wrinkled Hornbill, Swan Goose and Moluccan Cockatoo and 100% (1 out of 1) of the specimens taken from Marabou Stork and Black Swan were positive for *Cryptosporidium* oocysts (Table 1).

Locations free from *Cryptosporidium* were the birdhouse, the breeding area and the Children's World (Table 2). The lake in the zoo had the highest percentage (28.6%) of bird fecal specimens being positive. Almost 8% of the feces from the aviary and 8.3% from the animals in the show were positive for *Cryptosporidium* oocysts.

DISCUSSION

The image of *Cryptosporidium* has changed from that of a relatively mild parasite to one that could be lethal in the immunocompromized host, most notably, AIDS (Acquired Immune Deficiency Syndrome) patients. Waterborne outbreaks caused by *Cryptosporidium* have also caused great public concern in the international community (Sterling, 1990; Widmer *et al*, 1996; Smith and Rose, 1998). The most outstanding of these was the largest *Cryptosporidium* outbreak, which occurred in the early spring of 1993 in Milwaukee, Wisconsin, USA. An estimated of 1.5 million people were exposed to *Cryptosporidium* contamination in the public water supply. Out of this total, 403,000 became ill and 104 died (most were immunocompromized) (MacKenzie *et al*, 1994).

Besides its impact on humans and waterborne outbreaks, *Cryptosporidium* has also been detected in many animal reservoirs: rats, beavers, cattle, dogs, cats, sheep, and birds, indicating a broad host range (Tzipori and Campbell, 1981). The widespread distribution of cryptosporidiosis in a variety of domestic and wild animals and household pets indicates the potential of zoonotic transmission (Anderson *et al*, 1982; O'Donoghue, 1995).

This present study is the first in Malaysia to report the occurrence of *Cryptosporidium* in birds. Out of 100 fecal specimens examined from 56 species of birds, *Cryptosporidium* oocysts were detected in species such as the Wrinkled Hornbill (*Aceros corrugatus*), Great Argus Pheasant (*Argusianus argus*), Black Swan (*Cygnus atratus*), Swan Goose (*Anser cygnoides*), Marabou Stork (*Leptoptilos crumeniferus*) and Moluccan Cockatoo (*Cacatua moluccencis*). This indicates that birds in our National Zoo do pose a threat to zookeepers and the public alike in serving as carriers of *Cryptosporidium*. The fact that birds which shed oocysts in their stools did not show any clinical symptoms suggests two possibilities: (1) either the birds were only minimally infected, thus explaining the small parasite load or (2) that they retained infective *Cryptosporidium* oocysts after intestinal passage and hence may serve as mechanical vectors for the parasite (Graczyk *et al*, 1996b, 1998).

Detection of cryptosporidiosis in cockatoos is not new. In 1992, Latimer and colleagues diagnosed cryptosporidiosis in 4 cockatoos with psittacin beak and feather disease. All of the birds had intermittent to protracted diarrhea before death (Latimer *et al*, 1992). In a study on the cryptosporidial infections in zoo and pet birds, Lindsay *et al* (1991) identified infections in cockatiels, white-lored euphonies, bronze mannikin finches and Australian diamond firetailed finches. The *Cryptosporidium* species responsible for causing proventritricular infection in the zoo and pet birds was said to be different from *C. meleagridis* and *C. baileyi*. In Taiwan, avian cryptosporidiosis has been found in chickens, ducklings and canaries (Zhonghua *et al*, 1983).

With regards to the occurrence of *Cryptosporidium* in birds according to the different locations in the zoo, we discovered that birds from the lake, aviary and those involved in the animal show had cryptosporidiosis. Birds located at the lake are free to take flight to any location, thus facilitating the transmission of cryptosporidiosis. It is vital then to ascertain whether water from the lake empties into a river within the zoo compound, because this could contaminate the environment and increase the risk of waterborne transmission. Water samples could be taken from the river to confirm the presence or absence of *Cryptosporidium* oocysts.

Parasitic waterborne transmission caused by contamination with infected animal feces in water has been documented. In 1980, it was reported that 128 children were infected while swimming in a pool in Washington. The source of the outbreak was traced to 3 *Giardia*-infected beavers which contaminated the pool water (Dykes *et al*, 1980). The aviary and the animal show are popular locations in the zoo among visitors, therefore the chances of transmitting *Cryptosporidium* oocysts to humans is higher. It is fortunate that birds from the Children's World were free from *Cryptosporidium* because children are allowed to handle the birds in this location.

One unique species of bird confirmed to be positive

Table 1
Fecal specimens positive for *Cryptosporidium* oocysts using Ziehl-Neelsen staining technique and immunofluorescence technique.

No.	Common name	Species	No. of specimens taken	No. positive with Ziehl-Neelsen	No. positive with immuno-fluorescence
1.	Blue and Gold Macaw	*Ara ararauna*	3	1	-
2.	Green-winged Macaw	*Ara chloroptera*	3	1	-
3.	Hill Myna	*Gracula religiosa*	1	1	-
4.	Storm's Stork	*Ciconia stormi*	1	1	-
5.	Milky Stork	*Mycteria cinerea*	2	-	-
6.	Painted Stork	*Mycteria leucocephala*	1	-	-
7.	Marabou Stork	*Leptoptilos crumeniferus*	1	1	1
8.	Shikra/Bird of prey	*Accipiter badius*	1	-	-
9.	Wreathed Hornbill	*Aceros undulates*	3	-	-
10.	Greater Hornbill	*Buceros bicornis*	5	1	-
11.	Bushy-crested Hornbill	*Anorrhinus galeritus*	1	-	-
12.	Wrinkled Hornbill	*Aceros corrugatus*	2	1	1
13.	African Pied Hornbill	*Tockus fasciatus*	1	-	-
14.	Black Hornbill	*Anthracoceros malayanus*	1	1	-
15.	Rhinoceros Hornbill	*Buceros rhinoceros*	4	1	-
16.	African Grey Parrot	*Psittacus erithacus*	1	-	-
17.	Eclectus Parrot	*Eclectus roratus*	1	1	-
18.	Great Argus Pheasant	*Argusianus argus*	7	2	1
19.	Malayan Peacock-Pheasant	*Polyplectron malacense*	4	-	-
20.	Mountain Peacock- Pheasant	*Polyplectron inopinatum*	3	2	-
21.	Crestless Fireback Pheasant	*Lophura erythrophthalma*	2	-	-
22.	Crested Fireback Pheasant	*Lophura ignita*	2	1	-
23.	Common Peafowl	*Pavo cristatus*	2	1	-
24.	Helmeted Guineafowl	*Numida meleagris*	1	-	-
25.	Brahminy Kite	*Haliastur indus*	2	-	-
26.	Black Kite	*Milvus migrans*	1	-	-
27.	Buffy Fish Owl	*Ketupa ketupu*	2	-	-
28.	Barred Eagle-Owl	*Bubo sumatranus*	1	-	-
29.	Barn Owl	*Tyto alba*	1	-	-
30.	Hadada Ibis	*Bostrychia hagedash*	1	-	-
31.	Black-headed Ibis	*Threskiornis melanocephalus*	1	-	-
32.	Lovebird	*Agapornis fischeri*	1	-	-
33.	Emu	*Dromaius novaehollandiae*	3	3	-
34.	Greater Flamingo	*Phoenicopterus ruber*	1	-	-
35.	Pink-backed Pelican	*Pelecanus rufescens*	2	1	-
36.	White Bellied Sea Eagle	*Haliaetus leucogaster*	1	-	-
37.	Common Raven	*Corvus corax*	2	-	-
38.	Bulbul	*Pycnonotus*	2	2	-
39.	Pigeon	*Columba*	3	-	-
40.	Common Crowned Pigeon	*Goura cristata*	1	-	-
41.	Nicobar Pigeon	*Caloenas nicobarica*	3	-	-
42.	Great Currasow	*Crax rubra*	1	-	-
43.	Silver Pheasant	*Lophura nycthemera*	1	-	-
44.	Golden Pheasant	*Chrysolophus pictus*	1	1	-
45.	Whitewinged Magpie	*Urocissa whiteheadi*	1	-	-

Table 1 Continued

No.	Common name	Species	No. of specimens taken	No. positive with Ziehl-Neelsen	No. positive with immuno-fluorescence
46.	Black Swan	*Cygnus atratus*	1	1	1
47.	Swan Goose	*Anser cygnoides*	2	1	1
48.	Mandarin Duck	*Aix galericulata*	1	-	-
49.	Domestic Duck	*Anas domesticus*	2	-	-
50.	Goffins Cockatoo	*Cacatua goffini*	1	-	-
51.	Greater Sulfur Crested Cockatoo	*Cacatua galerita*	1	1	-
52.	Moluccan Cockatoo/Salmon-crested Cockatoo	*Cacatua moluccencis*	2	2	1
53.	White Cockatoo	*Cacatua alba*	1	-	-
54.	Lesser Sulphur Crested Cockatoo	*Cacatua sulphurea*	2	-	-
55.	Little Corella	*Cacatua sanguinea*	1	-	-
56.	Red Junglefowl	*Gallus gallus*	1	-	-
	Total		100	28	6

Table 2
Percentage of bird fecal specimens positive for *Cryptosporidium* oocysts using the immunofluorescence technique according to location.

Location	Total no. of fecal specimen collected	No. of fecal specimens positive using immunofluorescence technique	Percentage positve
Birdhouse	26	0	0
Breeding area	9	0	0
Aviary	40	3	7.5
Lake	7	2	28.6
Animal Show	12	1	8.3
Children's World	6	0	0

with *Cryptosporidium* oocysts was the Wrinkled Hornbill (*Aceros corrugatus*) from the aviary. Previous studies have never revealed any findings in such species and as a consequence, this fact may be a starting point for further studies to be done on *Cryptosporidium* in this species. The other five species of birds positive in this study have also been identified with *Cryptosporidium* in other studies. They were the Great Argus Pheasant (*Argusianus argus*), Black Swan (*Cygnus atratus*), Swan Goose (*Anser cygnoides*), Marabou Stork (*Leptoptilos crumeniferus*) and the Moluccan Cockatoo/Salmon-crested Cockatoo (*Cacatua moluccencis*) (Doster *et al*, 1979; Tsai *et al*, 1983; Una *et al*, 2001). The presence of *Cryptosporidium* in birds in the zoo indicates that animal sanctuaries like zoos and bird parks are important potential sources of *Cryptosporidium* infection to humans, especially children and other animals.

The great difference in recovery between modified Ziehl-Neelsen technique and immunofluorescence technique is due to low specificity of the modified Ziehl-Neelsen technique (Weber *et al*, 1991; Webster *et al*, 1996). Results from the modified Ziehl-Neelsen technique had many false positive *Cryptosporidium* oocyst-like organisms. On the other hand, examination of feces by immunofluorescence is more specific (McLauchlin *et al*, 1987; Grimason *et al*, 1994).

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