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Molecular diagnosis and genetic diversity of *Cryptosporidium* spp. in exotic birds of southwest of Iran

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Abstract. *Cryptosporidium* parasites can infect a wide range of vertebrate hosts including reptiles, mammals, and birds. Due to the zoonotic nature of cryptosporidiosis and its close contact with exotic birds and humans, the present study aimed to determine the prevalence and genetic diversity of *Cryptosporidium* spp. in exotic birds of southwest of Iran, by the staining and molecular methods. In the present research, 369 stool specimens were randomly collected from exotic birds and stained by modified acid-fast stain using Ziehl-Neelsen method. The slides were examined using light microscopy at a magnification of 100X. Then, the extracted DNA was amplified using the PCR method. Finally, all genotypes and positive samples from PCR assay were sequenced by Bioneer Company (Daejeon, South Korea). Among 369 stool specimens, 25 and 27 cases were found to be positive for *Cryptosporidium* spp. by the modified Ziehl-Neelsen staining and the PCR methods, respectively. Based on the genotyping, *C. avian* genotype III and *C. meleagridis* were detected in 25 and 2 stool samples, respectively. The results revealed a relatively high prevalence of *Cryptosporidium* spp. in exotic birds in the southwest of Iran. Due to the zoonotic nature of *C. meleagridis*, these exotic birds can be a significant source of cryptosporidiosis. It is important that high-risk people, including immune-deficient patients, receive correct information about the risk of indirect and direct contact with infected exotic birds.

INTRODUCTION

Cryptosporidium protozoans are significant coccidian parasites that infect a wide range of vertebrate hosts including reptiles, mammals, and birds (Morgan *et al*., 2000; O'Donghue, 1995). *Cryptosporidium* species are important pathogens that can cause diarrhea in children, especially in developing countries (Senlling *et al.*, 2007). The largest outbreak of the infection has been observed in children aged 6-12 months (Mor & Tzipori, 2008; Perch *et al*., 2001). Although Cryptosporidiosis is self-limiting in immunocompetent hosts and often asymptomatic, it may be life-threatening in immune-deficient patients such as people with severe malnutrition or acquired immunodeficiency syndrome (AIDS).

Cryptosporidium parasite infects epithelial cells in the respiratory and gastrointestinal tracts of infected hosts. It is established that humans are susceptible to cryptosporidiosis that infect animals (Huber *et al*., 2007; Gomes *et al*., 2012). More than 90.0% of the human cryptosporidiosis are caused by *C. parvum* and *C. hominis*. In addition, other species that infect humans include *C. muris*, *C. canis*, *C. meleagridis*, *C. andersoni*, *C. felis*, and *C. suis* (Xiao, 2010).

Cryptosporidium species have been reported in several bird's species, including turkeys, quails, peacocks, domesticated

chickens, geese, ducks, pheasants, as well as a wide range of captive and wild birds (Morgan *et al*., 2000; Fayer *et al*., 1997; Meng *et al*., 2011). In domestic and wild birds, cryptosporidiosis is often related to infections by *C. meleagridis*, *C. galli*, and *C. baileyi*. The hosts of *C. galli* parasite include birds of Fringillidae family, Spermicide and domestic chickens (Huber *et al*., 2007; Da Paixo *et al*., 2011). *Cryptosporidium* genotypes are defined in birds that consist of the avian genotypes (I–V), the goose (*Branta canadensis*) genotypes (I–IV), the Eurasian woodcock (*Scolopax rusticola*) genotype, and the black duck (*Anas rubripes*) genotype (Morgan *et al*., 2001; Nakamura *et al*., 2009). Since, in Iran, the distribution of *Cryptosporidium* genotypes/species in exotic birds is still unclear and due to the zoonotic nature of cryptosporidiosis and its close contact with exotic birds and humans, the present study aimed to determine the prevalence and genetic diversity of *Cryptosporidium* spp. in exotic birds of southwest of Iran, by the staining and molecular methods.

MATERIAL AND METHODS

Sample collection

From March 2015 to January 2016, 369 fresh stool specimens were collected mostly from the cages with a bird. It is important to note that the birds were separated into single cages for each individual bird and then their fecal samples were collected. Feces were collected from several pet shops in the cities of Ahvaz, Abadan and Khorramshahr, Khuzestan province, southwest of Iran. 150 samples were collected from the city of Ahvaz, 119 from Abadan, and 100 from Khorramshahr respectively. All the fecal samples collected were labeled and transferred to the Department of Parasitology, Ahvaz Jundishapur University of Medical Sciences. Part of the specimen collected was used for the staining method and the rest of the feces were mixed with twice the volume of 2.5% potassium dichromate and kept at 4°C (Meng *et al*., 2011).

Staining method

All stool samples were stained by the modified acid-fast stain using Ziehl-Neelsen method. Following this, all stained slides were microscopically examined for the identification of *Cryptosporidium* oocytes at a magnification of 100X.

Extraction of DNA and molecular detection

The DNA was extracted by the DNA stool kit (Bioneer) and the extracted DNA was kept at -20°C. This kit consisted of spin column that absorbed the parasite DNA by the column and after washing it twice with special buffers, the purified DNA was obtained (Tavalla *et al*., 2017). The extracted DNA was analyzed by the PCR method. Primers used by the PCR method included the forward primer (Cry F; 5' CTG ACC TAT CAG CTT TAG A 3'), and the reverse primer (Cry R; 5' GCT GAA GGA GTA AGG AAC A 3') (Meamar *et al*., 2017) for amplification of a fragment of SSU rRNA (with the length of 749 bp). The primers were purchased from Bioneer Company and stored at -20°C. The PCR method was performed in 30 cycles including the stages of initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min (Ria *et al*., 2008).

Sequencing

The positive samples were sequenced by Bioneer Company (Daejeon, South Korea). Afterwards, the specified sequence was compared with the sequence of the registered isolates available in the GeneBank library (NCBI) and the homology between them was evaluated by BLAST software (Tavalla *et al*., 2017). Finally, the phylogenetic tree was drawn using the MEGA (version 7) software and Neighbor-Joining method.

RESULTS

Fig. 1 shows positive *Cryptosporidium* spp. were found in 25 exotic birds using the modified Ziehl-Neelsen staining method, and all the diagnosis were confirmed by the PCR method. Table 1 shows the results of the molecular analysis and the genotyping of stool samples obtained from exotic birds in the southwest of Iran. Accordingly, 7.2% of the specimens positive for *Cryptosporidium* spp. were detected using the PCR method. Based on the genotyping, *Cryptosporidium avian genotype III* and *C. meleagridis* were detected in 6.8% and 0.5% respectively. Fig. 2 shows the phylogenetic analysis of SSU rRNA sequences of *Cryptosporidium* spp. isolates recovered from exotic birds from different localities.

DISCUSSION

In this study, 369 exotic birds from 7 families were examined by PCR and Ziehl-Neelsen methods for *Cryptosporidium* spp. As seen in Table 1, 7.3% and 6.8% of specimens were diagnosed to be positive for *Cryptosporidium* spp. by PCR and Ziehl-Neelsen methods respectively. After sequencing, 25 cases of *C. avian III* and 2 cases of *C. meleagridis* were identified. The highest rate of Cryptosporidiosis infection was found in parrots (*Psittacus erithacus*) (22%), followed by finches (*Taeniopvgia gutata)* (10.6%), cockatiels (*Nymphicus holandicus*) (10%) and lovebirds (*Agapornis rosecolis)* (6.7%). The lowest infection rate was related to bulbuls (*White-eared bulbul*) (1.8%). In mynas (*Acridotheres tristris*) and larks (*Bimaculated lark*), no infection was found. This research is consistent with some other similar studies on the prevalence of *Cryptosporidium*. Gomes *et al*. (2012) evaluated the *Cryptosporidium* species in exotic commercial birds in popular markets, pet shops, and commercial aviaries located in Rio de Janeiro, Brazil. They showed that among 103 stool samples, 7 (6.8%) of them showed positive *Cryptosporidium* oocysts. Furthermore, Qi *et al*. (2011) evaluated *Cryptosporidium* species/genotypes in pet birds in Henan, China. They showed that among 434 fecal samples obtained from the

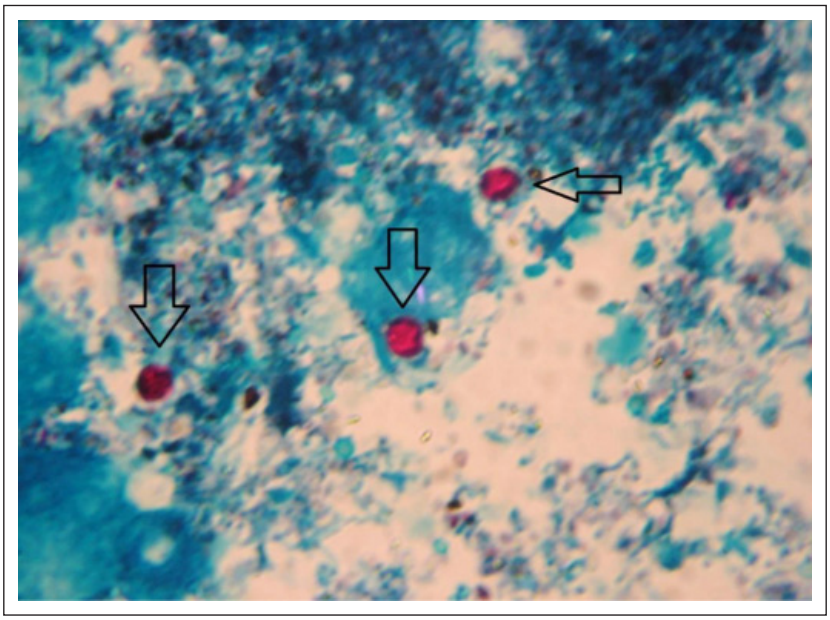

Fig. 1. *Cryptosporidium* spp. in the stool sample of exotic birds stained by the modified Ziehl-Neelsen staining and examined microscopically at a magnification of 100X.

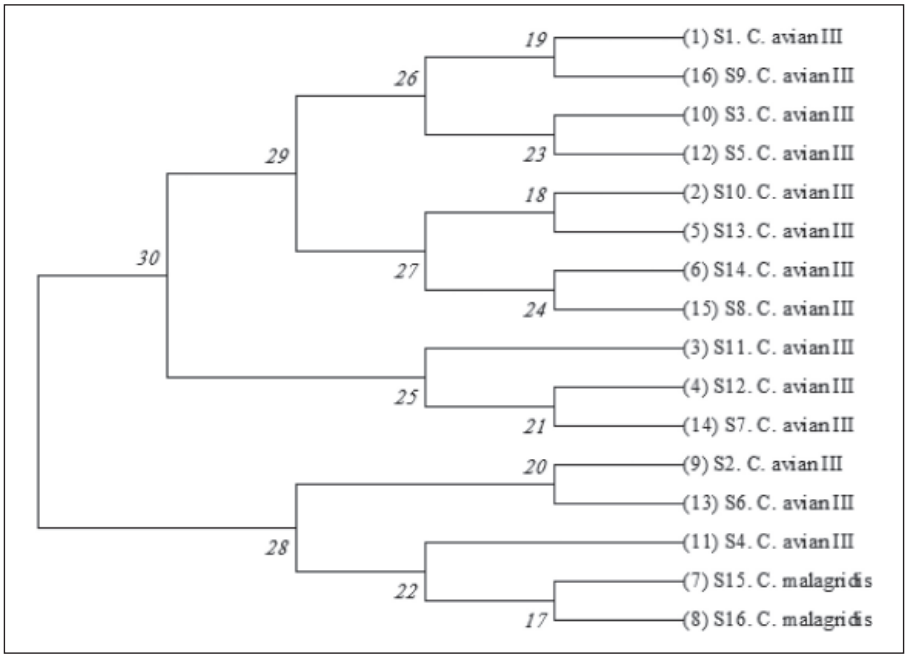

Fig. 2. The phylogenetic analysis of SSU rRNA sequences of *Cryptosporidium* spp. isolates recovered from exotic birds in the cities of Ahvaz, Abadan and Khorramshahr, Khuzestan province, Southwest of Iran. The phylogenetic tree was drawn using the MEGA (version 7) software and Neighbor-Joining method.

Table 1. The results of Ziehl-Neelsen and the molecular analysis of stool samples obtained from exotic birds in the cities of Ahvaz, Abadan and Khorramshahr, Khuzestan province, Southwest of Iran

Exotic Birds	Number	Positive in staining method	Positive in PCR method	DNA Sequencing	
				C. avian III	*C. meleagridis*
Canary (*Serinus canaria*)	90	3 (3.3%)	3 (3.3%)	3 (3.3%)	0 (0%)
Cockatiel (*Nymphicus hollandicus*)	40	3 (7.5%)	4 (10%)	3 (7.5%)	1 (2.5%)
Grey parrot *(psittacus erithacus*)	50	10 (20.0%)	11 (22.0%)	10 (22.0%)	0 (0%)
Finch *(Taeniopygia gutata)*	47	5 (10.6%)	5 (10.6%)	4 (8.5%)	1 (2.1%)
Bulbul *White-eared Bulbul*	55	0 (0%)	1 (1.8%)	1 (1.8%)	0 (0%)
Lovebird (*Agapornis roseicolis*)	45	3 (6.7%)	3 (6.7%)	3 (6.7%)	0 (0%)
Lark (*Bimaculated lark*)	12	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Myna (*Acridotheres tristis*)	30	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	369	6.8%	7.3%	25 (6.8%)	2 (0.5%)

birds, 8.1% (35/434) of them were positive for *Cryptosporidium* parasite. *Cryptosporidium* species/genotypes were observed in different types of birds. Nakamura *et al.* (2009) showed that of 966 stool specimens obtained from captive birds in Brazil, 47 (4.86%) cases were identified with *Cryptosporidium*. Da Paixo (2011) reported 6.6% *Cryptosporidium* in pet birds.

In the present study, 11 out of 50 parrots (*Psittacus erithacus*) were diagnosed with *C. avian genotype III*. This result is consistent with those of Da Paixo (2011).

Four cases of *Cryptosporidium* infection were found in 40 cockatiels (*Nymphicus holandicus*), of which one of them was *C. meleagridis* and 3 were *C. avian genotype III*. Qi (2011) and Gomes (2011) diagnosed *C. avian genotype III* infections in cockatiels (*Nymphicus holandicus*) while in the present study, not only *C. avian genotype III* was reported but also *C. meleagridis*. It is worth mentioning that Nakamura *et al.* (2009) identified *C. parvum* and *C. galli* in cockatiels (*Nymphicus holandicus*) as well.

Among 44 finches (*Taeniopvgia gutata)* 5 cases were found to be positive by PCR methods, of which 4 were *C. avian III* and 1 was *C. meleagridis*. It should be noted that the different species of finches showed a variety of genotypes. For example, Qi (2011) reported *C. bailey* in saffron nches (*Sicalis aveola*), Antunes (2008) found *C. galli* in zebra finches (*Taeniopvgia gutata)* and Nakamura *et al.* (2009) reported *C. galli* in lesser seed-nches (*Oryzoborus angolensis*) and *C. baillyei* in saffron nches (*Sicalis aveola*) but Gomes (2011) found *C. parvum* in Bengalese finches (*Lonchura striata domestica*). This was the first report in Brazil that detected *C. parvum* in Bengalese finches.

The other species of birds studied were canaries (*Serinus canaria*). Accordingly, 90 cases were examined by PCR and Ziehl-Neelsen methods, in which 3 cases were diagnosed with *C. avian III*. In comparison with the present study, Nakamura *et al.* (2009) isolate two different genotypes including *C. galli* and *C. avian I* in the same type of bird. *C. galli* was also observed in the study of Antunes (2008). It is worth pointing out that in a study conducted by Gomes (2011) an unnamed genotype of *Cryptosporidium*, genetically like *C. parvum*, was identified in canary.

An examination performed on 55 bulbuls (white-eared bulbul) by PCR and Ziehl-Neelsen methods, diagnosed 1 positive case of *Cryptosporidium avian III*.

The last two species, 30 mynas (*Acridotheres tristis*) and 12 larks (*Bimaculated lark*) showed no positive case of *Cryptosporidium* by PCR and Ziehl-Neelsen methods. Moreover, Qi (2011) identified *C. baillyei* in 4 mynas (*Acridotheres tristis*) and Dapixo (2011) diagnosed *C. galli* in 1 lark (*Bimaculated lark*).

As we discussed above, different types of exotic birds show various infection with *Cryptosporidium* genotype, which makes it dynamically challenging to report. What makes it more difficult is that there are still some unknown genotypes of *Cryptosporidium* that needs more comprehensive study worldwide (Gomes, 2011).

CONCLUSIONS

The results of present research revealed a high prevalence of *Cryptosporidium* spp. in exotic birds in the southwest of Iran. In this study, the results of both PCR and Ziehl-Neelsen staining methods were similar. Among selected specimens, 27 and 25 samples showed positive *Cryptosporidium*. The results of Qi square analysis inidcated that these two methods were not statistically different from each other. After sequencing and specifying the species, most of the *Cryptosporidium* genotypes were *C. avian III* (6.8%), which followed by *C. meleagridis* (0.5%). however, there has been no report of infection with *C. avian III* in human in previous studies so far. Due to the zoonotic nature of *C. meleagridis*, these exotic birds can be a significant source of cryptosporidiosis. It is important that high-risk people, including immune-deficient patients, receive correct information about the risk of direct and indirect contact with infected exotic birds.

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Compliance with ethical standards
Statement of animal rights
All animal experimental procedures were approved by the Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Conflict of interest
The authors declare no conflict of interests.

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