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Prevalence and Molecular Analysis of *Cryptosporidium* Spp. Isolated From Wild and Domestic Birds

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Abstract: The purpose of this studywas: Initially to revealed the distribution of *Cryptosporidium* spp. in wild and domestic birds in Al- Qadisiya province in Iraq, secondly to determinate genotypic characterization and phylogenetic analyze based on 18S rRNAsequences of *Cryptosporidium* which obtained from these birds. A total of 236 fecal specimens from six types of birds were screened microscopically to look for *Cryptosporidium* oocysts using modified Ziehl–Neelsen stain. Cryptosporidium spp. in 32 microscopy-positive specimens were analyzed genetically through DNA extraction after that DNA was amplified and processed with the small-subunit 18S rRNA gene by using ordinary PCR and then producedDNA wasanalyze to determinate their sequences. Results showed *Cryptosporidium* oocysts were observed in 137(58.1%) specimens of birds' feces. They was 54.5% of the turkey, 57.5% of the domestic chickens, 53.8% of the broiler chickens, 62.5% of the common ducks, 76.7% of the quails and 26.7% of the feral pigeon. Sequencing andfurther phylogenetic analyses identified *Cryptosporidiumparvum* ()in all birds in our study, *Cryptosporidium-baileyi*observed in domestic and broiler chickens, quail and feral pigeon ducks, *Cryptosporidium–meleagridis*isolated from turkey and quail, finally the species *Cryptosporidium-galli*only recorded from domestic chickens.Because of a high infection percentages with cryptosporidiosis in domestic and wild birds species. So that we suggesting that our birdsin the present study would be considered as biological transporters of *Cryptosporidium* spp. contributing to environmentaldissemination with pathogens which infect human and other animals.

Key words: *Cryptosporidium* Sp. · Domestic Birds · 18srrna of *Cryptosporidium*

INTRODUCTION

Cryptosporidium spp. are one of the most prevalent protozoan pathogen which have a wide range of varietyhosts like: mammalian, reptilian, amphibian and fish, beside of all species of birds [1]. Cryptosporidiosis, a disease caused by *Cryptosporidium* spp., has been reported in over 170 host species [2, 3] and in more than 30 avian species worldwide [4].

In birds there are three main *Cryptosporidium* species that have been described namely *C. baileyi, C. galli* and *C. meleagridis* [5, 6]. Only *C. meleagridis*, which infects turkeys and parrots, is a known threat to humans [7, 8]. While, *C. baileyi*is likely the most common avian *Cryptosporidium* species because its ability to infect many birds like: domestic and caged chickens, turkeys, geese, ducks, feral pigeon, lovebirds, budgerigars, cockatiels, quails and ostriches [6], finaly, *C.galli*, which discovered recently, infects several hosts such as finches, domestic chickens and pine grosbeaks [9]. In 1929, Tyzzerwas recordedthe first description of *Cryptosporidium* infection in the ceca of chickens [4]. Later, a report in 1955 described structurally similar parasites in turkeys and these parasites were named *C. meleagridis* [9]. In 1986, an organism from chickens was isolated, described and gave the name *C. baileyi* [10]. Currently, there are 16 species of *Cryptosporidium* that have been identified which have a different morphologies and hosts [11, 12]. Several researches refers to infection with another species of *Cryptosporidium* in birds in addition tothethree species mentioned previously. The most important of these species is *C. parvum*which have foundnatural infection with it in different wild and pet birds [4, 13].

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Avian cryptosporidiosis can manifest as respiratory form Dhillon *et al.* [14] and intestinal form Adejinmi and Oke [15]. In some cases, cryptosporidiosis might even manifest as a renal form, which can be fatal [16], also Trampel *et al.* [17] were described cryptosporidiosis in laying hens in tubule urinary and ureterand they named as"Urinary tract cryptosporidiosis" the stages of evolutionary parasite seen on the apical surface of epithelial cells of the collected tubules urinary and ureters.

The traditional methods of diagnosis of *cryptosporidium* spp. was confirmed through findingoocysts in the feces [18]. Recently, molecular diagnosis was followed to investigate a new species and genotypes of *cryptosporidium*in different host [19, 20].

Because of the local studieson Cryptosporidiosis in birds in Iraq were rarely and its risk on human health, economically as caused loss of livestock and therole of birds in the dissemination of infection with this parasite, our study aimed to investigate the prevalence of cryptosporidiosis in fecal samples of wild and domestic birds in different regions in Al-Qadisiya province, Iraq and molecular analysis of*cryptosporidium* species which isolated from birds and definethe relationship among species genetically according to 18S rRNA gene.

MATERIALS AND METHODS

Collection of Samples and Examination: A total of 236 birds were collected from different regions of Al-Qadisiya province between May 2013 to June 2014, Birds included six species which are: 22 Turkey (*Meleagrisgallopova*), 60 Quail (*Coturnixcoturnix*), 40domestic chicken (*Gallus gallusdomesticus*), 52broiler chicken (*Gallus gallus*), 32 commenduck (*Anasplatyrhynchos*) and 30 Feral pigeon (*Columba livia*). Fresh fecal samples which took from birds were examined by used hot modified Zeihl-Nelseen stain [18].

The samples were examined for *Cryptosporidium* oocysts by light microscopy at x400 magnification. Then the oocysts had been concentrated by Sheather's sugar flotation technique as described byWebster *et al.* [21]. Cryptosporidium-positive samples were stored in 2.5% potassium dichromate and kept at 4°C until DNA extraction.

DNA Extraction: *Cryptosporidium*oocysts were isolated from positive fecal samples using discontinuous density sucrose gradient centrifugation. Genomic DNA was extracted from the purified oocysts using a Stool Genomic DNA extraction Kit (Bioneer Company, South Korea) in accordance with the manufacturer's instructions and kept at ¯20°C until detected by the PCR method.

Molecular Analysis: A total of 32 Cryptosporidium-positive samples from studied birds were characterized genetically. Cryptosporidium spp. were genotyped by amplifying an 830bp of the small subunit 18S rRNA gene by direct PCR [9]. these samples from different birds were analyzed to identified DNA sequencingby using AB DNA sequencing system [22]. The acquired sequences were submitted to a BLAST search to initially define the species/genotypes and to confirm the high similarity and homology with other known sequences of Cryptosporidium spp. in NCBI-GenBank. Sequences of the partial 18S rRNA gene of *C.parvum*, *C. meleagridis C. baileyi* and *C. galli* which isolated from studied birds were compared with analogous species which deposited in NCBI-GenBank under accession numbers L1699.1, eu827314.1, AY954884.1 and HM116388.1 respectively.

Cryptosporidium Phylogeneticanalysis: All sequences were multiple-aligned and analyzedby using MEGA6 phylogenetic tree program (http://www.megasoftware.net). And then a neighbor-joining or phylogenetic tree was built.

Statistical Analysis: Data generated were analysed on the computerstatistical package SSPSusing Chi-square test. Differences expressed as significant at $P < 0.05$ [23].

RESULTS

Results revealed through examination of 236 samples from wild and domestic birds belonging to six species of birds under study that the percentage of the overall infection was 58.1%.The results in Table 1. Showed the prevalence of Cryptosporidiosis according to species of birds which revealed the highest infection percentage was in quails(*Coturnixcoturnix*) which reached to 76.7% and the least in feralpigeon (*Columba livia*) which reached to 26.7%.

Our study recorded four species belong to *Cryptosporidium* be responsible for intestinal cryptosporidiosis in birds which are: *C. parvum, C. meleagridis, C. baileyi* and *C. galli*. In addition to their high occurrence, oocysts were also observed in large numbers in fecal samples collected from the same bird.

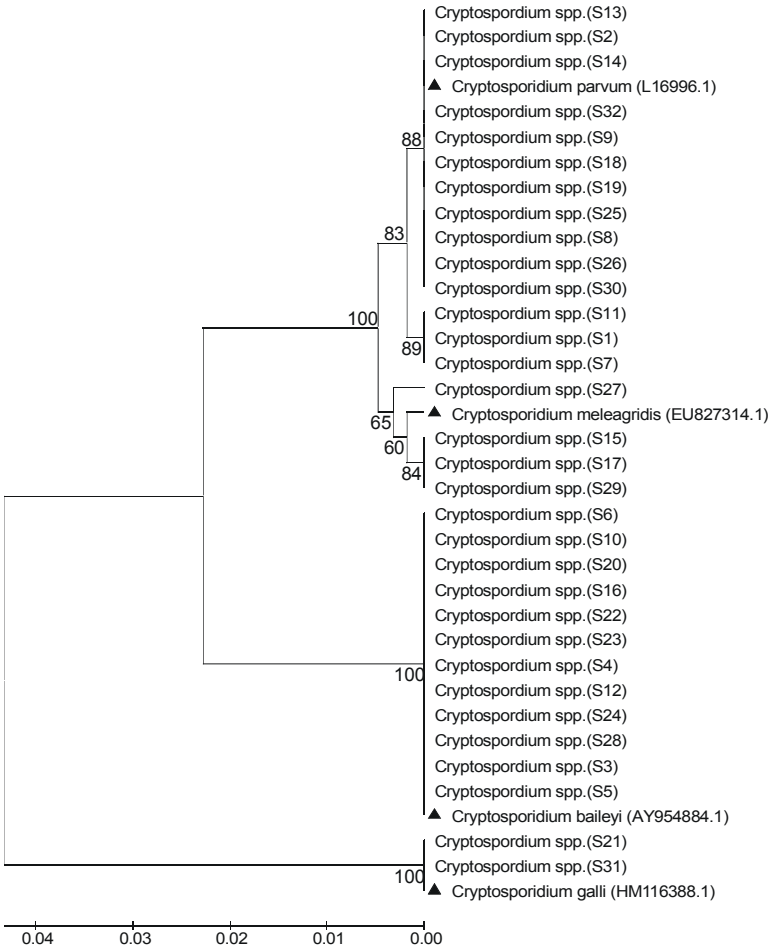

Fig. 1: Phylogenetic tree of *Cryptosporidium* spp. isolated from wild and domestic birds. The numbers on tree branches represent bootstrap values for neighbor-joining and the numbers near specimens represent the obtained samples from birds under study(1, 7, 15, 17 from Turkey; 2, 9, 11, 14, from Common duck; 3, 4, 5, 6, 16, 19, 21, 30, 31, 32 from Domestic chicken; 8, 13, 22, 27, 29 from Quail; 10, 23, 24, 25 from Broiler chicken; 18, 20, 26, 28 from Feral pigeon)

Table 1: The prevalence of Cryptosporidiosis according to species of birds

Species of birds	No. examined	No. positive	Positive %
Meleagrisgallopova	22	12	54.5
Gallus gallusdomesticus	40	23	57.5
Gallus gallus	52	28	53.8
Anasplatyrhynchos	32	20	62.5
Coturnixcoturnix	60	46	76.7
Columba livia	30	8	26.7
Total No.	236	137	58.1

The molecular analysis of *Cryptosporidium* species was done through extracting DNA amplified with 18SrRNA by used PCR technique. Results of electrophoresis revealed that DNA bands were 830 bp. and their sequences were determinate by sent DNA product to Bioneer company in South Korea.

Results of DNA sequences by used NCBI-BLAST showed that locally species of parasite, which isolate in this study, give a high identical ratio that was between 99%-100% compared with the global analogous species that recorded in NCBI Genbank for the same gene.

The phylogenic tree of parasite species was drown by used MEGA6 program, the results of neighboring tree revealed two major branches: First branch include specimens of *C. galli* while the second branch include the other three species (*C. parvu, C. meleagridis, C. baileyi*). Also genetic tree showed presence of two difference strains of *C. parvum* can infected the birds with neighboring ratio between them reached to 83% and the same thing was found for *C. meleagridis* but the neighboring ratio was 65% (Fig. 1). Sequencing and phylogenetic analyses identified *C. parvum*in all birds in

our study, *C. baileyi*observed in domestic and broiler chickens, quail and feral pigeon ducks, *C. meleagridis* isolated from turkey and quail, finally the species *C. galli* only recorded from domestic chickens.

DISCUSSION

The local birds in our study showed a higher prevalencerate of *Cryptosporidium*oocysts (58.1%) as compared withother concerned studies, the present study agree with previous researches [5, 15, 24, 25]. Statistical analysis showed no significant differences in infection ratios among turkeys, domestic and broiler chicken and common duck while a significant difference found in quail and feral pigeon compared with the other bird species under study. This variation in the percentages attributed to the different areas and environments which samples were collected from it, as well as the different among studied birds in their sensitivity and resistance to infect with oocysts of parasite, its age [13, 26] and bird management may also contribute to high infection rates such as the following methods of feeding, it may be opening breeding type (Free in the fields) as: domestic chickens, turkeys, common duck in agricultural areas [15, 27], or follow the closed breeding method (Caged inside poultry home), as in the case of broiler chicken and sometimes the presence of small rodents in the pet shops, which could be infected with *C. parvum*, probably could have caused the spread of oocysts in their home [27].

We know that birds play very important role as disseminators of many pathogens [28], *Cryptosporidium* are one of them, our study recorded two important species *C. parvum* and *C. meleagridis* which have ability to infect human and variation mammals [8, 29], so that the detection of Cryptosporidium oocysts inwild and domestic birds is very significant because of theirmovement from one source to another and contact withsporulatedoocysts by man and livestock lead to disseminate cryptosporidiosis infection beside of environmental contamination with viableoocysts [4, 28]. A huge outbreaksof cryptosporidiosis have been associated withdrinking water in Milwaukee in1994 in which about 403, 000 people wereinfected [26]. Therefore birds may be considered as a biological transporter of*Cryptosporidium*spp. that could contaminate rivers and sea or drinking water [24] thusinfection transport to human or other animals.

Four species of *Cryptosporidium* were diagnosed from the studied birds which are:*C.parvum, C. meleagridis, C. baileyi* and *C. galli*. Our results agreeing with many researches which recorded infection with Cryptosporidium spp. in different birds [4, 6, 15, 19, 20].

On the other hand, *C. parvum* was isolated from all sex species of studied birds feces, so this refer to ability to infect awide range of hosts [4, 12]. The presence of *C. baileyi* was observed in domestic and broiler chickens, quail and feral pigeon, this species is usually found as a parasite in many variety of birds hosts. *C. baileyi* is probably the most common avian Cryptosporidium spp. it reported in more than 17 other avian hosts [4, 11, 30]. While *C. meleagridis* just isolated from turkey and quail and *C. galli* diagnosed from domestic chickens only, these may be belong to its weak host specificity, exposure to infection sources, age of birdsand immunity of birds [26].

Molecular analysis of Cryptosporidium spp. isolated from birds depending on 18SrRNA revealed distinguish among species clearly, this gene used as a distinction gene, many studies confirmedthis fact [6, 12, 31]. According to molecular characterization and phenotypic differences the genus*C.galli*considerable a distinct species compared with other speciesbased on three genetic sites which are 18Sr RNA, HSP70 and actin locus [20, 32]. Among the species/genotypes, phylogenetic tree showed a big genetic similarity between *C. parvum* and *C. meleagridis* with neighbor-joining reached to 99%-100%. Many researches agreed with our results [28, 33]. *C. meleagridis*was the third most common Cryptosporidium parasite in human [12], *C. meleagridis* have a possible to transfer from infected birds to human [24, 29]. Through phylogenetic tree we observed two genotypes belong to *C. parvum* and *C. meleagridis* can infect birds with neighbor-joining reached to 83% and 65% respectively.

Finally, our study established the presence of four species belong to genus Cryptosporidium caused cryptosporidiosis in birds and these birds represent as a biological transporters for Cryptosporidium oocysts to human, livestock, poultry and other birds.

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