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The Importance of Poultry in Environmental Dissemination of *Cryptosporidium* spp.

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Abstract: The purpose of this study was to genotypically characterize and phylogenetically analyze the 18S rDNA sequences of *Cryptosporidium* obtained from poultry in Brazil. Fecal samples were obtained from ducks, chicks and quails sold in popular markets. DNA was isolated from *Cryptosporidium* positive feces, and 18S subunit rDNA was amplified and processed using nested-polymerase chain reaction (PCR). To identify the protozoan species, the PCR amplicons were used for restriction fragment length polymorphism (RFLP) and sequencing analyses. The presence of *Cryptosporidium* oocysts was observed in 76% of the ducks, 55% of the quails and 86% of the chicks. Sequencing and further phylogenetic analyses identified *C. parvum* in quails and chicks and *C. baileyi* in ducks. Although *C. baileyi* is usually a parasitic species found in birds, *C. parvum*, due to its weak host specificity, is the species most frequently diagnosed in various domestic and wild animal species, especially mammals. Thus, suggesting that the poultry observed in the present study would be considered as mechanical transporters of *C. parvum*, contributing to protozoa environmental dissemination.

Keywords: *Cryptosporidium*, poultry, occurrence, molecular diagnosis.

INTRODUCTION

Cryptosporidiosis is a parasitic disease prevalent in wild, domestic and captive birds from several parts of the world [1]. It has been reported in more than 30 species of birds from several orders such as Anseriformes, Chadriformes, Galliformes, Passeriformes, Psittaciformes and Struthiniformes [2-7].

Currently, three species of *Cryptosporidium* are considered to be bird parasites: *Cryptosporidium baileyi* [8-10] and until the moment there are 10 avian genotypes described [11]: avian genotype I-IV, black duck genotype, Eurasian Woodcock genotype and goose genotype I-IV [3, 7, 12-14]. In the future, these genotypes, combined with additional biological information, may be redescribed as species [15].

Studies have reported that in addition to the species and genotypes capable of infecting birds, there are other species and genotypes that are considered to be specific for their determined host, which would increase the number of hosts involved in the epidemiology of aviary cryptosporidiosis [12, 16]. Moreover, the low host specificity constitutes a risk factor for humans due to the zoonotic potential of some species, thus birds should be considered as a reservoir for human infection due to the possible mechanical transmission of *C. parvum* [13, 17-20].

In Brazil, some studies were conducted to evaluate *Cryptosporidium* infection in birds. The study of the occurrence of *Cryptosporidium* species in wild birds apprehended by environmental control agencies in São Paulo State, and demonstrated infection by *C. galli, C. baileyi* and the Avian genotype II [21]. In a study of exotic bird species [19], was diagnosed *C. parvum* infecting cockatiels; *C. baileyi* in a black vulture and a saffron finch; *C. galli* in canaries, a cockatiel, and lesser seed-finches; *Cryptosporidium* avian genotype I in a canary and an Indian peafowl; *Cryptosporidium* avian genotype II in ostriches and *Cryptosporidium* avian genotype III in a cockatiel and a peach-faced lovebird. Previous studies described *C. baileyi* infecting ducks and quails [22] and *C. meleagridis* infecting chicks [19, 22]. In exotic birds [23] described the infection with *C. parvum* in Bengalese finch and avian genotype III in Cockatiel and Java sparrow.

The aim of this study was to identify the species of *Cryptosporidium* in poultry sold in public markets in the state of Rio de Janeiro, Brazil.

MATERIALS AND METHODS

A total of 180 animals were purchased from public markets located in the city of Rio de Janeiro, Brazil. The markets were huge constructions with hundreds of different small stores, selling a great diversity of products, varying from food to electronics. Animal stores were near to some food (vegetables or meat) selling establishments or restaurants. The space for human circulation was usually very small and the contact between visitors, shoppers and animals was frequent.

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Generally, the animals were housed in piled up cages with overpopulation. Different kinds of animals were commercialized in the pet shops inside the markets, such as young poultry, little mammals (as puppies), guinea pigs, hamsters and rabbits. Besides the previously described animals, other mammals, such as young goats, could be observed being sold in some of those stores.

Fecal Sample Collection and Processing

A total of 180 birds were used in this study, which were acquired from two different markets (A and B). Thirty birds of each species were purchased from market A and the same amount was acquired from market B. The animals were divided into three groups, one for each animal species, and were composed of 60 chicks (*Gallus gallus domesticus*), 60 quails (*Coturnix coturnix japonica*) or 60 ducks (*Anas platyrhynchos domesticus*). The ducks and chicks studied were less than 30-days-old, and the quails were more than 30-days-old.

All the poultry were maintained in individual cages located in the Protozoology laboratory, until there was at least one fecal sample of each animal that could be submitted to coproparasitological analyses. The birds diagnosed as positive for *Cryptosporidium* spp. were maintained in the laboratory until the oocysts were no longer detected in their fecal samples for three consecutive analyses.

Coproparasitological Examinations

Coproparasitological examinations were carried out where fecal samples were weighed (4 g) and homogenized with 14 mL distilled water and filtered through plastic disposable sieves with an overlay of gauze. The filtered fecal material was then transferred to 15 ml conical test tubes and centrifuged at 402 x *g* for 10 minutes, and the supernatant was discarded. The precipitate was suspended in a saturated sugar solution (specific gravity = 1.3), homogenized and centrifuged at 402.48 xg for five minutes. The tube was then filled with a sugar solution and covered with a slide cover slip resting for three minutes. The cover slip was then mounted on a glass slide and examined under a microscope with and without phase contrast, using objectives at 40x and 100x magnification [24].

Molecular Analysis

Genomic DNA extractions were based on the protocols previously described in the literature [22, 23]. Nested-PCR was conducted in two amplification steps [25]. During the first step (i.e., the primary PCR), the following primers were used: 18 SF: 5`- TTCTAGAGCTAATACATGCG-3` (Forward) and 18 SR: 5`- CCCATTTCC TTCGAAACAGG A-3` (Reverse) (expected amplicon size: 1,325 bp). During the second step (i.e. secondary PCR), the following primers were used: 18 SNF: 5`- GGAAGGGTTGTATTTATTAG ATAAAG-3` (Forward) and 18 SNR 5`- AAGG AGTAA GGAACAACCTCCA-3` (Reverse) (expected amplicon size: 819 to 865 bp, depending on the species).   DNA extracted from oocysts obtained from calf feces was used as a positive control for DNA amplification during the primary and secondary PCR. Each positive sample was processed in duplicate, one for amplification of DNA for RFLP and another for sequencing.

The amplicon obtained in the nested-PCR was submitted to RFLP using two enzymes: *SspI* (Invitrogen®) and *VspI* (Fermentas®), following manufacturer's protocols. Digestion products electrophoresis was performed in 3% agarose gel along with a 1Kb plus DNA ladder (Invitrogen) molecular weight marker. The gel image was visualized and photographed using an image capture system (Bio-Rad®). The program Quantity One (Bio-Rad®) was used to calculate the molecular weight of the obtained products. All the restriction patterns obtained were compared to those already reported in the literature [26].

The second Nested-PCR products for each sample were purified using the GFX Kit from GE healthcare®, following the manufacturer's instructions. The purified amplicons were sent to the PDTIS/FIOCRUZ platform for quantification and sequencing using an automated 48-capilar 3730 sequencer, following laboratory protocols. Sequences were aligned and analyzed using MEGA version 4.1 and Bioedit softwares to obtain consensus sequences. The sequences were then used in a BLAST search (http://www.ncbi.mln-nih.goc/Genbank/ index.html) to determine their identity and to find homologous and similar sequences for each group of *Cryptosporidium* deposited in Genbank. Phylogenetic analyses were performed using the software MEGA version 4.1. Bootstrapping with 1000 replicates was used as a phylogenetic test, and a consensus tree was obtained using the neighbor-joining method, using the Kimura 2-parameter model. The sequences obtained were deposited in GenBank using the SeqIn computational program receiving accession numbers GU082384, GU082386, GU082387, GU082390 and GU082391.

Comparative phylogenetic analyses were carried out between the consensus sequences and the five first sequences listed during BLAST analysis. The following sequences deposited in GenBank were used: *C. parvum*: GU971622, GU971621, GU971620, GU971623, HQ009805, EF175936, EU553557, DQ833278, EU553550 and DQ656354, *C. baileyi*: GU816042, GU816039, GU816040, GU816041, GU816043. The following sequences deposited in GenBank were also used: *C. meleagridis*: EU827312, HM116384 and HM116383; avian genotype I: GQ227479; avian genotype II: DQ002931; avian genotype III: HM116386; avian genotype IV: DQ650344; Eurasian woodcock genotype: AY273769; duck genotype: AY504514; goose genotype I: AY120912; goose genotype II: AY504512; goose genotype III: AY324638; goose genotype IV: EF060289, *C. galli*: AY168848.

RESULTS

From the three species studied, 52 (86,6%) chicks (*Gallus gallus domesticus*), 33 (55%) quails (*Coturnix coturnix japonica*) and 46 (76,6%) ducks (*Anas platyrhynchos domesticus*) were diagnosed as positive to *Cryptosporidium* spp.

In addition to their high occurrence, oocysts were also observed in large numbers in fecal samples (> 30 oocysts/field) collected from the same bird.

Morphometric measurements of the *Cryptosporidium* isolates present in feces of quails, chicks and ducks were conducted and are shown in Table **1**.

Table 1. Morphometric Means, Restriction Fragment Length Patterns and Sequencing Results of the *Cryptosporidium* Isolates Obtained from Poultry with Corresponding GenBank Accession Numbers

Poultry	Species of Birds	Average Oocyst Size	Enzyme *Ssp*I	Enzyme *Vsp*I	Genbank Access Numbers and Sequence Size	Blast Results
Chicks I and II	*Gallus gallus domesticus*	5.8 x 4.6 µm (n=100)	465, 265, 100	651, 100	GU082390 (613 bp), GU082391 (613 bp)	*C. parvum*
Quails I and II	*Coturnix coturnix japonica*	6.1 x 4.8 µm (n=50)	465, 268, 102	655, 101	GU082384 (543 bp), GU082386 (600 bp)	*C. parvum*
Duck I	*Anas platyrhinchus domesticus*	6.0 x 4,5 µm (n=50)	648, 106* 469, 264*	650, 101	GU082387 (532 bp)	*C. baileyi*

*Cutting pattern was inconclusive to define species.
n = number of measured oocysts.
bp = base pair.

Results from the DNA amplification reactions from secondary PCR showed bands with amplicons varying in length from 850 to 871 bp. The results of restriction fragment length patterns and sequencing of the *Cryptosporidium* isolates are shown in Table **1**. Species identification of *Cryptosporidium* obtained in the present study was not possible through the observation of the fragments obtained with the use of restriction enzyme. Due to the lack of data to diagnose the species, the samples were sequenced using the Nested-PCR primers in both directions. All the 15 samples used in the RFLP were sequenced but only five of them presented good quality sequences.

When the sequences of the present study were submitted to BLAST search, the *C. parvum* species isolated from quails and chicks showed 99% sequence similarity with *C. parvum* sequences from human origin (GU971622, GU971621, GU971620 and GU971623), bovine origin (HQ009805 and EF175936), reptile origin (EU553557 and EU553550) and swine origin (DQ833278).

The *Cryptosporidium* sequence from duck shared high similarity, 99%, with GenBank sequences from *C. baileyi* obtained from birds (GU816042, GU816039, GU816040, GU816041, and GU816043).

During phylogenetic analysis, the isolates obtained in this study grouped with previously described species as confirmed by the topology of the phylogenetic tree generated using the neighbor-joining method. The isolates from the chicks and quails grouped with *C. parvum* species, and the one duck isolate grouped with *C. baileyi* species.

Phylogenetic tree of the *Cryptosporidium* spp. isolated from poultry and those deposited in GenBank. Bootstrap 1000, Kimura 2 Parameter (Fig. **1**). The phylogenetic tree suggests that the samples quail I and II; chick I and II are more related to *C. parvum* and the sample duck I presents more similarities to *C. baileyi*.

DISCUSSION

Worldwide, birds are considered as disseminators of many pathogens, being the genera *Cryptosporidium* one of the most important because it is diagnosed in a wide variety of them [1], where some of this coccidia species may have zoonotic potential [27].
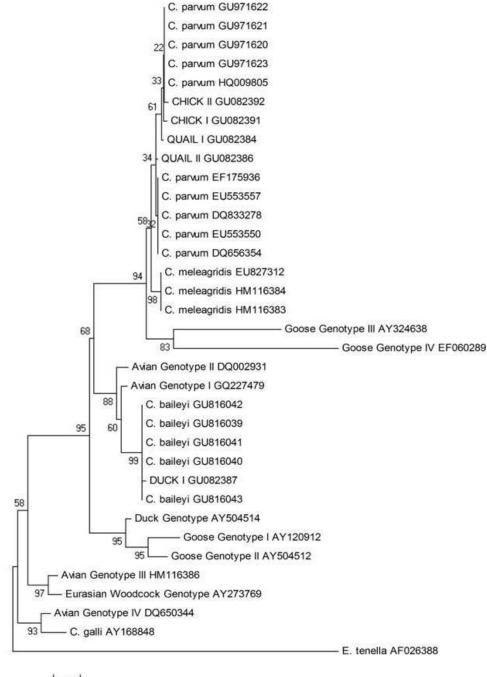
Despite the importance of species characterization related to the epidemiology of avian cryptosporidiosis, there are few studies that have tried to identify this protozoan in poultry. Among those, there are reports of *Cryptosporidium* spp. in ducks [2, 17, 22, 28], chicks [22, 28, 29] and quails [12, 22].

The high rate of infection for domestic birds and the large number of oocysts found have been previously demonstrated in the literature. Bird management may also contribute to high infection rates. Other study [3] verified that low infection rates in birds could be related to efficient management of the aviaries observed. However, in this study, the sanitary conditions were characterized as poor due to the lack of periodical cleaning of the cages, overpopulation and different species of birds in the same cage. Besides that, the presence of small mammals in the pet shops, which could be infected with *C. parvum*, probably could have caused the spread of oocysts in the surroundings, favoring the ingestion by birds that might act as an environment disperser. Similar situation was described in Malaysia [20], suggesting that a huge variety of birds may be considered as mechanical transporters of *C. parvum*, favoring the infection to human and other mammals.

On the other hand, some studies have concluded that the high prevalence of parasitic infection is influenced by avian age. Because all the purchased birds, with exception of quails, were less than one month of age, their immune system could exhibit a low efficiency [30, 31].

The high quantity of oocysts found in the feces of the birds (> 30 oocysts/field) allowed statistical morphometric analysis, however, due to morphometric similarities between the species, this technique did not allow for species identification. It was possible to make species identifications by combining these results with those obtained by molecular biology techniques.

Two species of *Cryptosporidium* were diagnosed from the studied birds, the first was *C. baileyi,* observed in the feces of an infected duck and the second was *C. parvum,* which was diagnosed from quails and chicks feces. The presence of *C. baileyi* was originally described in commercial broiler chickens and is usually found as a parasite in a large variety of birds hosts, including chicks, ducks, and quails [3, 4, 8, 9, 12, 13, 22, 28, 32] agreeing with the results reported in this research. The presence of oocysts of *C. parvum* in feces of some birds observed in the present study agree with previous researches, where the authors suggested that the birds would be acting as mechanical

Fig. (1). Phylogenetic tree of the Brazilian *Cryptosporidium* species isolated from domestic birds and species already deposited in GenBank. The numbers represent bootstrap values for neighbor-joining. The samples obtained in this study and presented in the phylogenetic tree: Quail I – GU082384; Quail II – GU082386; Duck I – GU082387; Chick I – GU082391; Chick II– GU082392.

vectors, shedding oocysts in the environment, even if at a low rate [18, 19, 33].

The diagnosis of *Cryptosporidium* in domestic chickens, ducks and quails is relevant because these animals are purchased and sold while young in order to maintain a small subsistence breeding program for egg and meat production within households. The introduction of animals shedding the infectious form of *Cryptosporidium* (which is easily disseminated on the environment) into a household is considered to be a risk factor of *Cryptosporidium* infection to other animals and humans.

An additional aspect that should be taken into consideration is the place where the birds were being sold. At these locations, in addition to other species of birds, there

were small mammals that were kept in shelved cages, which allowed contact between species. Therefore, all the animals being sold were exposed to this environment, which increased their chances of being infected. These unhealthy conditions would probably put uninfected small mammals and birds at risk of being infected from positive birds of the same or different species.

The markets where these birds were purchased were very crowded with people looking to buy low-cost food products sold "*in natura,*" such as fruits, vegetables, grains, etc. However, it was noticeable that in this environment, the close proximity of the stores favored a possible dissemination of the infectious form of *Cryptosporidium*.

The commercialization of very young birds is attractive to the young children that are commonly present in this environment. We frequently observed children petting ducks, chicks and quails. The ducks, chicks and quails could be purchased for these children and kept as pets inside households, becoming a risk for infection to humans of all ages.

The results obtained in the present study suggest that birds may be considered as mechanical transporters of *C. parvum* oocysts, besides that, the poultry studied were infected by *C. baileyi* which is cause of severe pathogenicity in these hosts.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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