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Research paper

First description of *Cryptosporidium parvum* in carrier pigeons (*Columba livia*)

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ABSTRACT

The carrier pigeon and the domestic pigeon are different breeds of the species *Columba livia*. Carrier pigeons are used for recreational activities such as bird contests and exhibitions. Due to the close contact with humans, these birds may potentially represent a public health risk, since they can host and disseminate zoonotic parasites, such as those belonging to the genus *Cryptosporidium* (phylum Apicomplexa). The purpose of this work was the detection by microscopic and molecular techniques of *Cryptosporidium* spp. oocysts in fecal samples of carrier pigeons, and subsequently to sequence the 18S ribosomal RNA marker of positive samples to identify the species. A total of 100 fecal samples were collected individually in two pigeon breeding facilities from Formiga and Araçatuba, cities located in Minas Gerais state and São Paulo state, Brazil, respectively. The age of the birds ranged from one to 12 years; 56 were females and 44 males. Fecal smears were stained with negative malachite green, whereas the molecular characterization was based on the sequence of a ~800 bp fragment of the 18S rRNA gene. Microscopic examination of fecal smears revealed 4% (4/100) oocyst positivity. On the other hand, 7% (7/100) of positivity were found using nested PCR. Three samples were 99% to 100% similar to *Cryptosporidium parvum* 18S rDNA type A (Genbank AH006572) and the other three samples had 99% to 100% similarity to *C. parvum* 18S rDNA type B (Genbank AF308600). To our knowledge, this is the first report of *C. parvum* oocysts in carrier pigeons.

1. Introduction

Pigeons belong to the genus *Columba*, which has more than 50 species found worldwide. These birds present a wide variation of plumage color, body size and habitats (Haro et al., 2005). The carrier pigeons belong to the same species as domestic pigeons found in urban environments, but they belong to a different breed; they have larger bodies and a more pronounced caruncle at the base of the beak.

During World War I and World War II, these birds were widely used for sending messages, as an alternative communication means. Nowadays, carrier pigeons are mostly used in competitions called pigeon races.

Birds belonging to the genus *Columba* may represent a public health concern, since they can potentially disseminate zoonotic pathogens and serve as reservoir of many parasitic diseases (Cooper, 1984; Kaminjolo et al., 1988; Piasecki, 2006; Lallo et al., 2012). Infections of domestic pigeons with helminths and coccidia have been reported in several regions of the world, such as in Turkey (Özkul and Aydin, 1994), Spain (Abreu-Acosta et al., 2009; Cano-Terriza et al., 2015), India (Gupta et al., 2011) and Iran (Radfar et al., 2012). In addition, infection with *Cryptosporidium* has also been found in these birds (Abreu-Acosta et al., 2009; Meng et al., 2011; Radfar et al., 2012; Li et al., 2015).

Cryptosporidiosis acutely affects the respiratory or digestive tract of birds, and has been reported in more than 30 host species of various orders, such as Anseriformes, Charadriiformes, Columbiformes, Galliformes, Passeriformes, Psittaciformes and Struthioniformes, in

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South and North America, Europe, Africa, Asia and Oceania (Nakamura and Meireles, 2015). Few studies have reported the occurrence of this protozoan in domestic pigeons and none have specifically focused on carrier pigeons.

To assess the potential of carrier pigeons to harbor *Cryptosporidium* parasites, this study examined fecal samples of carrier pigeons for the presence of *Cryptosporidium* spp.

2. Material and methods

2.1. Ethics committee approval

The study was approved by the Animal Use Ethics Committee (CEUA) of the São Paulo State University (Unesp), School of Veterinary Medicine, Araçatuba, process number FOA 2015-00327.

2.2. Experimental design

A total of 100 carrier pigeons, 44 males and 56 females, of different age raised by two pigeon breeders, one located in the city of Araçatuba, São Paulo state, and the other in the city of Formiga, Minas Gerais state, Brazil, were examined. This samples size was determined by the number of unique birds that could be identified. Fecal samples were collected with disposable wooden spatulas from the bottom of the cages, and transferred in duplicate to 2 mL microtubes until purification and analysis.

2.3. Processing of fecal samples

Initially, fecal samples were homogenized in modified Sheather's solution prepared with phosphate buffered saline (PBS) and 1% Tween 20, and centrifuged at 800 × *g* for 10 min. The supernatant was divided into two portions and both were transferred to new tubes. Each sample was washed in a solution of PBS with 0.1% Tween 20 followed by a second wash with PBS/0.01% Tween 20.Samples were then centrifuged at 2000 × *g* for 10 min, resulting in two pellets for each sample; to one of them 10% formaldehyde was added and used for microscopy; the other pellet was frozen at −20 °C for DNA extraction (Elliot et al., 1999).

Microscopic analysis for *Cryptosporidium* oocyst detection was performed using the malachite green negative staining technique (Elliot et al., 1999). DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen), according to the manufacturer's instructions.

Nested PCR (nPCR) was used to amplify an ~800 bp fragment of the *Cryptosporidium* 18S rRNA gene as described (Xiao et al., 2000). As positive and negative controls *Cryptosporidium serpentis* genomic DNA and ultrapure water, respectively, were used instead of sample DNA. The amplified fragments were electrophoresed in 1.5% agarose, stained with GelRed® (Biotium) and visualized on an UV transilluminator.

2.4. Sequencing

Amplicons were purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen) and sequenced using an ABI Prism Dye® Terminator Cycling Sequence kit (Applied Biosystems) on an ABI 3730XL (Applied Biosystems) automated sequencer. Amplicons were sequenced in both directions with primers used for the secondary PCR. Codoncode Aligner Software version 4.0.1 (CodonCode Corporation Dedham®, MA, USA) was used to determine the consensus sequence. Consensus sequences were aligned to homologous sequences downloaded from GenBank with Clustal W (Thompson et al., 1997) and BioEdit Sequence Alignment Editor (Hall, 1999).

2.5. Nucleotide sequence accession number

The nucleotide sequences generated in this study were deposited in GenBank under accession numbers KY514062 to KY514066.

2.6. Statistical analysis

Data analysis consisted of descriptive statistics and inferential analysis (Fisher's exact test) to verify the association between the presence and absence of this pathogen with each of the studied variables. The variable database was created with Microsoft Office Excel 2010, and statistics were considered significant when $p < 0.05$.

3. Results

Microscopic analysis of malachite green negative stained fecal smears from 100 samples revealed oocysts in four (4%) fecal samples of pigeons (three males and one female) that were morphologically similar to *Cryptosporidium* spp. oocysts. One of the samples originated from Formiga and three from Araçatuba. On the other hand, using nPCR with the 18S rRNA primers, seven (7%) positive amplifications (four females and three males) for *Cryptosporidium* spp. were obtained; four samples were from Formiga and three from Araçatuba. All samples positive by microscopy were also PCR positive.

According to inferential statistics, no association between PCR positivity and location, gender and age ($p > 0.05$) was found. The proportion of positive results by PCR and microscopy were not significantly different ($p > 0.05$).

Sequencing of 18S rRNA nPCR generated six good quality sequences. One sequence could not be read, possibly due to a mixed amplicon template. The sequences from the six samples were similar to *Cryptosporidium parvum.* Three samples were 99–100% identical to *C. parvum* 18S rDNA type A, one of them had a A to G nucleotide substitution at positions 500 and 667 (Genbank AH006572). The other three samples were 99%–100% identical to *C. parvum* 18S rDNA type B (Genbank AF308600), one of them showing a T to C nucleotide substitution at position 253 and another a T to C nucleotide substitution at position 580.

4. Discussion

This study reports on the detection of, *Cryptosporidium* spp. in carrier pigeons by two techniques. The analysis of 18S rRNA sequences is consistent with the presence of *C. parvum*. This finding was unexpected since birds are typically infected with other *Cryptosporidium* species, primarily *Cryptosporidium meleagridis* and *Cryptosporidium baileyi* and represents the first report of *C. parvum* in *C. livia*. *C. parvum* is known to have zoonotic potential and identification in birds is rare (Nakamura and Meireles, 2015). The literature on cryptosporidiosis in domestic birds is insufficient, but birds infected with *C. meleagridis* (Meng et al., 2011; Li et al., 2015), *C. baileyi* (Li et al., 2015) and *Cryptosporidium hominis* (Abreu-Acosta et al., 2009) have been reported from China and Spain, respectively.

C. parvum has been occasionally reported from aquatic birds such as canada geese (*Branta canadensis*), mallard ducks (*Anas platyrhynchos*) (Graczyk et al., 1998), mandarin ducks (*Aix galericulata*), common mergansers (*Mergus merganser*) and mute swans (*Cygnus olor*) (Majewska et al., 2008). It has also been detected in birds of prey, such as the Eurasian sparrowhawk (*Accipiter nisus*), common buzzard (*Buteo buteo*), black kite (*Milvus migrans*) and European honey buzzard (*Pernis apivorus*), as well as in birds of the Ciconiidae family, such as the white stork (*Ciconia ciconia*) (Majewska et al., 2008) and members of the Corvidade family, such as Eurasian magpie (*Pica pica*) (Reboredo-Fernández et al., 2015), rook (*Corvus frugilegus*) (Majewska et al., 2008) and carrion crow (*Corvus corone*) (Majewska et al., 2008; Reboredo-Fernández et al., 2015).

Most of the reports of land birds mentioned above were based on the analysis of fecal samples from wild birds using PCR. Because *C. parvum* has been identified in feces of birds of prey and waterfowl, the parasite

may be present both in the environment (Reboredo-Fernández et al., 2015) and in animals of other trophic levels. The PCR detection of *Cryptosporidium* DNA also leaves open the possibility that birds passively carry oocysts that were ingested with prey or water (Abreu-Acosta et al., 2009).

One of the characteristics of pigeons is the capacity to fly long distances. In Brazil, some of these birds have been reported to fly up to 900 km in a single championship day. Depending on the physical condition pigeons may stop to drink and eat and may come into contact with animals of any trophic levels, become infected, and upon returning may spread *Cryptosporidium* in pigeonry. Thus, infected pigeons could passively carry oocysts without becoming infected.

Carrier pigeons have some peculiar behaviour, differentiating them from urban pigeons commonly seen in streets and squares. Transmission of *C. parvum* can occur through interaction with other animals, such as wild birds, present in the surroundings of the pigeonry. Another possibility to be considered is the infection that occurs at the time of competition or training, when the birds are transported together in a contaminated environment.

As *C. parvum* is common in humans, a possible source of infection of domestic birds could be their owners, who often have daily contact with their birds. Similarly, there is also a risk of the owners becoming infected due to contact with oocysts eliminated by the birds. The potential for pigeons to contribute to the dissemination of zoonotic agents such as *Cryptosporidium* parasites remains to be investigated.

5. Conclusion

We detected *Cryptosporidium* spp. in carrier pigeon's fecal samples by microscopic technique and be PCR. This observation indicates that domestic birds may play a role in the transmission of *Cryptosporidium* parasites, and *C. parvum* in particular. The prevention of this disease in pigeonry should be considered, since pigeon races are popular in Brazil and in other countries.

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