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Detection and characterization of *Cryptosporidium* species and genotypes in three chicken production systems in Brazil using different molecular diagnosis protocols

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ABSTRACT

The objective of this study was to determine the occurrence of *Cryptosporidium* spp. in domestic chickens raised in different chicken production systems in Brazil using three nested PCR protocols. The purification and concentration of oocysts present in 190 fecal samples from chickens raised in extensive, semi-intensive and intensive production systems were accomplished by centrifugal flotation in Sheather's solution and were followed by the extraction of genomic DNA. The detection and molecular characterization of *Cryptosporidium* species and genotypes were performed using three nested polymerase chain reaction (nested PCR) protocols targeting the 18S rRNA gene followed by sequencing of the amplified fragments. Subgenotyping of *C. meleagridis* was performed using a nested PCR reaction targeting the gp60 gene. Sample identified as *Cryptosporidium* sp. genetically similar to *Cryptosporidium xiaoi* and *Cryptosporidium bovis* by 18S rRNA gene sequencing were further analyzed by nested PCR targeting the actin gene and subsequent sequencing of the amplified fragment. Positive amplification for *Cryptosporidium* spp. was observed in 12.6% (24/190) of the samples, including *C. baileyi* (9.8%; 18/190), *C. meleagridis* (0.5%, 1/190), *C. parvum* (2.1%; 4/190) and *Cryptosporidium* sp. (0.5%; 1/190). Subgenotyping of *C. meleagridis* revealed the presence of the zoonotic subtype IIIgA23G3R1. Sequencing of the 18S rRNA gene and the actin gene fragments revealed a *Cryptosporidium* genotype in an extensive poultry system genetically related to *C. xiaoi* and *C. bovis*. There was no significant difference in the frequency of positive results obtained by the three nested PCR protocols ($p > 0.05$); additionally, the agreement obtained by Kappa index ranged from substantial (0.70) to almost perfect (0.9).

1. Introduction

Cryptosporidiosis is one of the most prevalent protozoan infections in birds, and it manifests as a respiratory or digestive disease (Ryan, 2010; Nakamura and Meireles, 2015). Among the 31 species of *Cryptosporidium* that infect several vertebrates, only four infect birds: *C. meleagridis*, *C. baileyi*, *C. galli* and *C. avium* (previously known as avian genotype V) (Holubová et al., 2016; Ryan et al., 2016).

In addition to these species, there are descriptions of the *Cryptosporidium* avian genotypes I (Ng et al., 2006), II (Santos et al., 2005; Meireles et al., 2006; Ng et al., 2006), III (Ng et al., 2006), IV (Ng et al., 2006), VI (Chelladurai et al., 2016), VII, VIII and IX (Helmy et al., 2017), the goose genotypes I–IV (Jellison et al., 2004; Zhou et al., 2004), the black duck genotype (Morgan et al., 2001) and the Eurasian woodcock genotype (Ryan et al., 2003) infecting birds.

The literature related to the occurrence of *Cryptosporidium* infection in avian species is scarce, most likely because the techniques used to diagnose cryptosporidiosis are not routinely adopted in avian pathology laboratories. Infections by *C. baileyi*, *C. galli,* and other avian *Cryptosporidium* species/genotypes are present in a wide variety of bird species; in contrast, *C. meleagridis* has a more restricted number of avian hosts (Sréter and Varga, 2000; Ng et al., 2006; Qi et al., 2011; Nakamura and Meireles, 2015). There is only one report of *C. parvum* infection associated with clinical signs in birds (Zylan et al., 2008).

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Table 1
Nested PCR protocols used for the detection of *Cryptosporidium* spp. in domestic chicken fecal samples.

Protocols		Primers 5′-3′ sequences	Amplified product (bp)	References
Protocol 1	PCR	GGAAGGGTTGTATTTATTAGATAAAG CTCATAAGGTGCTGAAGGAGTA	~ 848	Xiao et al. (1999) Jiang et al. (2005)
	Nested PCR	AAGCTCGTAGTTGGATTTCTG TAAGGTGCTGAAGGAGTAAGG	~ 425	Johnson et al. (1995)
Protocol 2	PCR	GACATATCATTCAAGTTTCTGACC CTGAAGGAGTAAGGAACAACC	~ 761	Ryan et al. (2003)
	Nested PCR	CCTATCAGCTTTAGACGGTAGG TCTAAGAATTTCACCTCTGACTG	~ 585	
Protocol 3	PCR	TTCTAGAGCTAATACATGCG CCCATTTCCTTCGAAACAGGA	~ 1318	Xiao et al. (1999, 2000)
	Nested PCR	GGAAGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACCTCCA	~ 835	

Most reports of cryptosporidiosis in domestic chickens are related to *C. baileyi*, which infects the respiratory tract, bursa of Fabricius and cloaca (Current et al., 1986), and less frequently to *C. meleagridis*, which is restricted to the small intestine (Sréter and Varga, 2000). *C. galli* and *C. avium*, as well as the avian genotypes of *Cryptosporidium*, are rarely described in domestic chickens (Nakamura and Meireles, 2015).

The importance of cryptosporidiosis in domestic chickens is unclear. Snyder et al. (1988) reported that the prevalence of antibodies to *Cryptosporidium* sp. in broiler chickens was 24% per flock in 18 flocks. Infection with *C. baileyi* in broiler chickens has been related to decreased weight gain, higher incidence of airsacculitis, increased mortality, and higher carcass condemnation rates in slaughterhouses (Gorham et al., 1987; Goodwin et al., 1996). *C. meleagridis* infection in domestic chickens is asymptomatic (Nakamura and Meireles, 2015).

In Brazil, there have been several reports of natural infection by *C. baileyi* in chickens (Meireles and Figueiredo, 1992; Huber et al., 2007). Using experimental infection with a Brazilian isolate of *C. baileyi*, Meireles et al. (1998a, b, 1999) found that domestic chickens developed clinical signs and macroscopic lesions related to the respiratory tract, as well as macroscopic lesions in the bursa of Fabricius.

C. meleagridis is a parasite of the intestinal epithelial cells in several species of birds, particularly turkeys (Slavin, 1955; Sréter and Varga, 2000), and it is the only avian species of *Cryptosporidium* with zoonotic potential (Chalmers and Giles, 2010). In some countries, the occurrence of *C. meleagridis* infection in humans is similar or superior to that of *C. parvum* infections (Xiao et al., 2001; Cama et al., 2003). In Brazil, *C. meleagridis* has been found in fecal samples from domestic chickens (Huber et al., 2007; Nakamura et al., 2009) and humans (Araújo et al., 2008).

Cryptosporidium species can be identified using molecular methods, such as polymerase chain reaction (PCR), followed by sequencing of the amplified fragments. The most commonly used protocol is nested PCR targeting the 18S rRNA gene, developed by Xiao et al. (1999, 2000). However, Mirhashemi et al. (2015) compared three nested PCR protocols using equine, bovine and ovine samples and found that the protocol developed by Ryan et al. (2003) was the most sensitive. There are no studies on the effectiveness of different molecular diagnostic techniques for the detection of *Cryptosporidium* spp. in avian fecal samples.

Also, there are few studies related to the prevalence of *Cryptosporidium* spp. in chickens from extensive, semi-intensive and intensive production systems (Nakamura and Meireles, 2015). In addition to presenting clinical disease when infected with *Cryptosporidium*, domestic chickens may be a source of infection of zoonotic species of this protozoan, such as *C. meleagridis* and *Cryptosporidium* species and genotypes adapted to mammals. The present work aimed to determine the occurrence of *Cryptosporidium* spp. in domestic chickens raised in different production systems and to perform molecular characterization of *Cryptosporidium* spp. using three nested PCR protocols and sequencing of the amplified fragments.

2. Material and methods

2.1. Fecal samples

This study was approved by the Animal Use Ethics Committee (CEUA) of the São Paulo State University (Unesp), School of Veterinary Medicine, Araçatuba, process FOA 2015-00572.

Fecal samples were obtained from domestic chickens (*Gallus gallus domesticus*) in 20 municipalities of the State of São Paulo, Brazil. According to availability, the samples were collected in 61 chicken farms with extensive (24), semi-intensive (8), intensive-layers (25), and intensive-broilers (4) production systems. Mixed types of chickens (broilers and layers) were raised in farms with extensive and semi-intensive production systems. Samples consisted of 190 fecal pools (each containing 10–20 g of feces) formed from the collection of up to 5–10 single fecal droppings per farm with extensive and semi-intensive production systems. In intensive production systems, one fecal pool was collected from up to four flocks.

In the extensive poultry production systems, it was not possible to identify the age of the birds at the time of collection. In the semi-intensive and intensive-layers production systems, the birds were all adults or were 8–16 weeks old, respectively. Broiler chickens from intensive production systems were 35–46 days old.

The samples consisted of freshly discarded feces collected using a disposable wooden spatula and were preserved in 2.5% potassium dichromate at 4 °C. The concentration and purification of oocysts were performed by centrifugal-flotation using Sheather's sugar flotation solution.

2.2. Molecular characterization

Genomic DNA from oocysts was extracted from purified fecal sediment (Boom et al., 1990; McLauchlin et al., 2000). Three nested PCR protocols targeting the 18S rRNA gene were used for the detection of *Cryptosporidium* spp. (Table 1). Samples that were identified as *C. meleagridis* and *Cryptosporidium* sp. by sequencing of the 18S rRNA amplicons were subjected to nested PCR targeting the gp60 gene (Stensvold et al., 2014) or the actin gene (Sulaiman et al., 2002), respectively. Genomic DNA from *C. parvum* and ultrapure water were used as positive and negative controls, respectively.

2.3. DNA sequence analysis

The nested PCR amplicons were purified using the Illustra ExoProStar 1-Step® (GE Healthcare Life Sciences) or the QIAquick®Gel Extraction Kit (Qiagen), following the manufacturer's guidelines. Sequencing was accomplished using the ABI Prism® Dye Terminator Cycling Sequence kit (Applied Biosystems) in an Automatic sequencer ABI 3730XL (Applied Biosystems).

DNA sequences were assembled with Codoncode Aligner version