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Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil☆

Maria Júlia Rodrigues da Cunha[a,b,c], Márcia Cristina Cury[b], Monica Santín[a,*]

[a] Environmental Microbial and Food Safety Laboratory, Agricultural Research Service, United States Department of Agriculture, Building 173, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

[b] Universidade Federal de Uberlândia, Instituto de Ciências Biomédicas, Laboratório de Parasitologia, Av. Pará, 1720, Campus Umuarama, 38400-902 Uberlândia, Minas Gerais, Brazil

[c] CAPES Foundation, Ministry of Education of Brazil Caixa, Postal 250, 70040-020 Brasília, DF, Brazil

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ABSTRACT

Cryptosporidiosis is an important zoonotic disease caused by *Cryptosporidium*. Infections in birds are mainly caused by *C. meleagridis*, *C. baileyi*, and *C. galli*. *C. meleagridis* is the third most common cause of cryptosporidiosis in humans and the only *Cryptosporidium* species known to infect both birds and mammals. One hundred and fifty-five fecal specimens from different poultry species (chicken, turkey, ostrich, helmeted guinea fowl, quail, pheasant, and emu) were collected at local markets in the state of Minas Gerais, Brazil. Twenty-three (14.8%) birds (20 chickens, 2 quails, and 1 turkey) were found *Cryptosporidium*-positive. This constitutes the first report of *Cryptosporidium* in turkeys from Brazil. Nucleotide sequence analysis identified *C. meleagridis* in chickens (15), a turkey (1), and a quail (1), *C. baileyi* in chickens (4) and a quail (1), and a mixed infection *C. meleagridis*/*C. baileyi* in a chicken (1). This is the first report of *C. meleagridis* in turkeys and quails from Brazil. Using the *gp60* gene, three subtype families were identified, IIIa, IIIb and IIIg. Within subtype family IIIg, four subtypes were identified in chickens, two novel (IIIgA25G3R1 and IIIgA21G3R1) and two previously reported (IIIgA22G3R1 and IIIgA24G2R1). Within subtype family IIIb two subtypes were identified, IIIbA24G1R1 in a chicken and IIIbA23G1R1 in a quail. A novel subtype in the family IIIa was identified (IIIaA22G3R1) in a turkey. The finding of *C. meleagridis* subtypes previously identified in humans (IIIgA22G3R1, IIIbA24G1R1 and IIIbA23G1R1) indicates that they can be potentially zoonotic. Further subtyping studies that clarify genetic diversity of *C. meleagridis* are required to better understand host specificity, source of infection, and transmission dynamics of *C. meleagridis*.

1. Introduction

Cryptosporidiosis is a zoonotic disease recognized as a significant cause of diarrhea in both immunocompetent and immunocompromised worldwide (Chalmers and Davies, 2010; Kotloff et al., 2013). *Cryptosporidium* spp. infects a wide range of vertebrate host including humans, domestic, and wild animals (Ryan et al., 2014). There are approximately 30 species of *Cryptosporidium* that can infect fishes, amphibians, reptiles, birds, and mammals (Xiao and Ryan, 2015). Humans are most frequently infected with *C. hominis* and *C. parvum* (Xiao, 2010). However, *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum* and other *Cryptosporidium* genotypes that infect birds and mammals are also found in humans although the extent of zoonotic transmission and geographical differences in the distribution of these species are still unclear (Baroudi et al., 2013; Wang et al., 2014a; Li et al., 2016).

Cryptosporidium is an important parasite in poultry reported from all continents except Antarctica (Nakamura and Meireles, 2015; Wang et al., 2014b). Infections in birds are mainly caused by four species, *C. baileyi*, *C. galli*, *C. meleagridis*, and *C. avium* (Nakamura and Meireles, 2015; Holubová et al., 2016). In addition, there are other genotypes that also infect birds including avian genotypes I-IV and VI, goose genotypes I-IV, black duck genotype, and the Eurasian woodcock genotype, which are genetically different from the above species based on molecular analysis (Abe and Makino, 2010; Wang et al., 2014b; Chelladurai et al., 2016). *C. baileyi*, *C. meleagridis*, and *C. avium* are found in the small and large intestine and the bursa, but only *C. baileyi* is also found in respiratory tissues such as the conjunctiva, sinuses, and trachea (Sréter and Varga, 2000; Holubová et al., 2016). In contrast, *C. galli* has only been found in the proventriculus (Sréter and Varga, 2000). Infections with *C. meleagridis* and *C. galli* have been associated with enteritis, diarrhoea, and mortality (Santín, 2013) while infections with *C. baileyi* are mainly associated with respiratory symptomatology

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* Corresponding author.
E-mail address: monica.santin-duran@ars.usda.gov (M. Santín).

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linked to high morbidity and mortality (Lindsay and Blagburn, 1990; Sréter et al., 1995). On the other hand, birds infected with *C. avium* do not show clinical signs and no pathology is associated with the infection (Holubová et al., 2016).

C. meleagridis is the only *Cryptosporidium* species known to infect both birds and mammals (Ryan, 2010; Chappell et al., 2011) and is the third most common cause of cryptosporidiosis in immunocompromised and immunocompetent humans, indicating the epidemiological importance of avian hosts as reservoirs of this parasite (Baroudi et al., 2013; Nakamura and Meireles, 2015). The identification of *C. meleagridis* oocysts in drinking and recreational water indicates that water can be a potential vehicle for transmission of this parasite to both humans and animals (Plutzer et al., 2008; Koloren et al., 2013). In Sweden, there was a probable case of human *C. meleagridis* infection on a farm *via* direct contact with infected chickens (Silverlås et al., 2012). Therefore, humans can acquire cryptosporidiosis through direct contact with infected persons or animals and consumption of water or food contaminated with oocysts (Xiao and Ryan, 2015).

The diagnosis of cryptosporidiosis is based on microscopy, immunological methods, or molecular methods (Nakamura and Meireles, 2015). Molecular methods are of great importance due to the accuracy in the identification and characterization of species, genotypes, and subtypes of *Cryptosporidium* that is crucial for the assessment of public health importance and the identification of contamination sources of oocysts. The small subunit (SSU) rRNA gene is the most commonly gene used for determining *Cryptosporidium* species and genotypes (Ryan et al., 2014) whereas molecular subtyping employs the 60-kDa glycoprotein (*gp60*) gene (Stensvold et al., 2014; Xiao, 2010). Currently, there is little information about genetic diversity within *C. meleagridis* except that there are 6 subtype families (IIa to IIIe, and IIIg) with apparently low host specificity given that they have been found most of them in both birds and humans. (Stensvold et al., 2014). Although poultry infected with *C. meleagridis* may represent a public health concern given their close contact to humans and that they can potentially disseminate *Cryptosporidium* oocysts and act as reservoir for this parasite, there are few studies that have used molecular characterization of *Cryptosporidium* in poultry. Therefore, the present study was conducted to determine the occurrence and genetic diversity of *Cryptosporidium* species in several poultry species in Brazil and to determine the subtypes of *C. meleagridis*.

2. Materials and methods

2.1. Samples

A total of 155 fecal samples from different poultry species including chicken (n = 130), turkey (n = 1), ostrich (n = 2), helmeted guinea fowl (n = 5), quail (n = 5), pheseant (n = 10), and emu (n = 2), were obtained from October 2013 to September 2014 at 17 local markets in Uberlândia and Belo Horizonte in the state of Minas Gerais, Brazil (Table 1). Specimens from ostriches were collected at the farm which owner had also birds at Market 2 in Uberlândia. The birds ranged in age from 1 month to one year. They were raised in small farms for egg or meat consumption or to trade them at the local markets. On the farms, birds were raised free-range with frequent contact with humans and other animals. At the market, birds were kept separately in individual cages, which were sanitized every day. Food consisted of corn grain or ration in containers. All birds appeared in good health and no diarrhea was observed when samples were collected. Fresh fecal samples were collected from cages, or from the ground for ostriches, with care to collect only the portion that did not have direct contact with the cage to avoid contamination. Specimens were placed into sterile polystyrene tubes and transferred in isothermal boxes to the Parasitology Laboratory of Federal University of Uberlândia (UFU) and held at −20 °C until DNA extraction. The study was approved by the Ethics Committee in Animal Research of Federal University of Uberlândia (CEUA-UFU)".

2.2. DNA extraction

DNA was extracted directly from feces using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction with minor modifications. Modifications included the addition of 0.3 g of zirconia beads (Stratech Scientific, Luton, U.K.) to 0.2 g of feces and 1.4 ml of lysis buffer (McLauchlin et al., 1999); then, the mixture was heated at 95 °C for 5 min followed by vigorous shaking (2 rounds of 15 min) to facilitate the oocyst rupture. The nucleic acid was eluted in 150 µl of AE buffer to increase the quantity of DNA recovered. DNA was frozen and stored at −20 °C.

2.3. PCR, sequencing, and cloning

To detect *Cryptosporidium* species, a nested PCR protocol was used to amplify *ca.* 800-bp fragment of SSU rRNA gene, as previous described (Xiao et al., 1999). To subtype *C. meleagridis*, a nested PCR protocol was performed to amplify *ca.* 900-bp fragment of the *gp60* gene (Stensvold et al., 2014). Negative and positive controls were included in all PCR sets. Secondary PCR products were detected and visualized by electrophoresis in the QIAxcel Advanced system (Qiagen, Valencia, California).

All positive PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT) (USB Corporation; Cleveland, Ohio) and sequenced in both directions using the same PCR primers used in the secondary PCR in 10 µl reactions using BigDye 3.1v Chemistries and an ABI 3130 sequencer analyzer (Applied Biosystems, Foster City, California). Nucleotide sequences obtained in this study were aligned, examined, and compared with reference sequences downloaded from GenBank using SeqMan™ (DNAstar Inc., Madison, Wisconsin). The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers MH062741-MH062752.

When mixed infection within a specimen was suspected from the sequence traces, the PCR products were cloned using the TOPO TA cloning kit (Invitrogen Corp, Carlsbad, California) and transformants were selected from each specimen and screened by PCR, and sequenced in both directions using M13 forward (5′-GTA AAA CGA CGG CCA G-3′).

and reverse (5´-CAG GAA ACA GCT ATG AC-3′) primers. Up to ten clones from each specimen were sequenced.

3. Results

Of the 155 bird specimens examined 23 (14.8%) were PCR-positive for *Cryptosporidium* with positive birds at 9 of the 17 markets included in the study (Table 1). Prevalence at markets ranged from 0% (markets 3, 4, 6, 9, 11, 12, 13 and 16) to 33.3% (markets 5 and 10). *Cryptosporidium* was identified in three poultry species, chicken, quail, and turkey, with a prevalence of 15.4% (20/130), 40% (2/5), and 100% (1/1), respectively.

All PCR positive specimens were successfully sequenced and the analysis nucleotide sequences of SSU rRNA revealed the presence of two *Cryptosporidium* species, *C. baileyi* and *C. meleagridis* (Table 1). *C. meleagridis* was the most prevalent species and was identified in four chickens, one turkey and one quail from Uberlândia and in 11 chickens from Belo Horizonte. *C. baileyi* was identified in three chickens and one quail from Uberlândia and one chicken from Belo Horizonte (Table 1). A mixed infection was suspected after visual inspection of the nucleotide sequence traces from a specimen obtained from a chicken from Uberlândia. Cloning of the PCR product of this specimen confirmed the presence of *C. meleagridis* and *C. baileyi* (Table 1).

All 17 *C. meleagridis*-positive specimens generated the expected *gp60* PCR product. However, only 12 of the 17 were successfully sequenced (Table 2). Nucleotide sequence analysis revealed that the subtypes belonged to three subtype families of *C. meleagridis* (IIIa, IIIb, and IIIg). The most common subtype family was IIIg and it was identified in two