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Cryptosporidium spp. in caged exotic psittacines from Brazil: Evaluation of diagnostic methods and molecular characterization

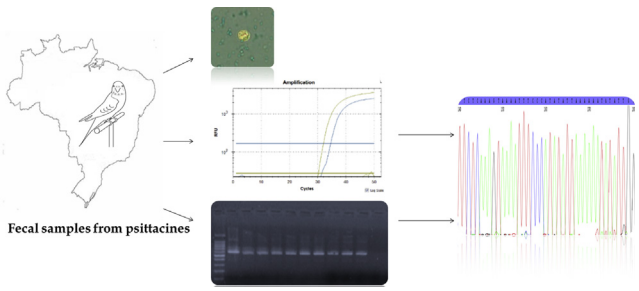
Elis Domingos Ferrari [a], Alex Akira Nakamura [a], Ana Rita Moraes Nardi [b], Bruna Nicoleti Santana [a], Vinicius da Silva Camargo [a], Walter Bertequini Nagata [a], Katia Denise Saraiva Bresciani [a], Marcelo Vasconcelos Meireles [a, *]

[a] Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba, Brazil – Clóvis Pestana St., 793 - Dona Amélia, 16050-680, Araçatuba, SP, Brazil
[b] Fundação Municipal de Ensino Superior, Bragança Paulista, Brazil - Estevão Diamant St., 210, Penha, 12929-590, Bragança Paulista, SP, Brazil

HIGHLIGHTS

- Prevalence of *Cryptosporidium* spp. in exotic psittacines from Brazil.
- Malachite green negative staining, *nested* PCR and duplex real-time PCR.
- The most prevalent species/genotype in parrots was avian genotype III.
- The best diagnostic method for gastric *Cryptosporidium* was duplex real-time PCR.
- *C. parvum* and *C. canis* have been found in fecal samples from psittacines.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to evaluate the prevalence of and diagnostic methods for *Cryptosporidium* spp. in caged adult exotic parrots from Southern and Southeastern Brazil. Oocysts were purified from fecal samples from 463 psittacines by centrifugal-flotation in Sheather's sugar solution. *Cryptosporidium* spp. were detected by malachite green negative staining and nested PCR targeting the 18S rRNA gene. *Cryptosporidium* species were identified by sequencing nested PCR amplicons. Samples were also tested by duplex real-time PCR targeting the 18S rRNA gene of *Cryptosporidium galli* and *Cryptosporidium* avian genotype III. The prevalence rates of *Cryptosporidium* spp. determined by microscopy and nested PCR were 3.0% (14/463) and 5.0% (23/463), respectively. The nested PCR/sequencing identified avian genotype III (1.7%; 8/463), *Cryptosporidium parvum* (0.9%; 4/463) and *Cryptosporidium canis* (0.2%; 1/463). Duplex real-time PCR was positive for gastric *Cryptosporidium* in 9.5% (44/463) of the samples. Among them, 1.9% (9/463) were positive for *C. galli*, 5.8% (27/463) were positive for avian genotype III and 1.7% (8/463) showed mixed infections with *C. galli* and avian genotype III. With regards to the positive detection of *Cryptosporidium* spp., there was no statistically significant difference between nested PCR and microscopic analysis ($p = .1237$), and a fair agreement existed between them (Kappa = 0.242). A statistically significant difference ($p < .0001$) and fair agreement (Kappa = 0.317) were obtained between nested PCR/sequencing and duplex real-time PCR for the detection of gastric *Cryptosporidium*. We determined that nested PCR and duplex real-time PCR are the best options for the detection of *Cryptosporidium* spp. and

* Corresponding author.
E-mail addresses: elisd.ferrari@yahoo.com.br (E.D. Ferrari), akiravt@gmail.com (A.A. Nakamura), nardi.vet@gmail.com (A.R.M. Nardi), brunanicoleti.ata@hotmail.com (B.N. Santana), viniciuscamargo.biologo@yahoo.com.br (V. da Silva Camargo), walter.bn@hotmail.com (W.B. Nagata), bresciani@fmva.unesp.br (K.D.S. Bresciani), marcelo@fmva.unesp.br (M.V. Meireles).

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gastric *Cryptosporidium*, respectively, and that avian genotype III is the most common *Cryptosporidium* genotype/species in psittacines.

1. Introduction

Cryptosporidium spp. are protists typically found in the gastrointestinal tract (Valigurová et al., 2008) that occasionally infect respiratory (Dhillon et al., 1981), biliary (Kovatch and White, 1972) and urinary tracts (Trampel et al., 2000), causing clinical and subclinical infections (Santín, 2013) in a wide range of vertebrate hosts.

In birds, the valid species are *Cryptosporidium meleagridis* (Slavin, 1955), *Cryptosporidium baileyi* (Current et al., 1986), *Cryptosporidium galli* (Pavlásek, 1999) and *Cryptosporidium avium* (Holubová 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, IV (Ng et al., 2006), and VI (Chelladurai et al., 2016); the black duck genotype (Morgan et al., 2001), the Eurasian woodcock genotype (Ryan et al., 2003), the goose genotypes I-IV (Jellison et al., 2004; Zhou et al., 2004) and the duck genotype (Zhou et al., 2004) infecting birds.

Avian species belonging to 16 orders have already been identified as hosts of *Cryptosporidium* (Nakamura and Meireles, 2015). In Psittaciformes, cryptosporidiosis manifests as an acute disease of the digestive and respiratory tracts. Clinical and necroscopic diagnosis of cryptosporidiosis is difficult since the clinical signs and macroscopic lesions related to cryptosporidiosis are not pathognomonic. Furthermore, *Cryptosporidium* infections in birds are often associated with infections by other pathogens (Ravich et al., 2014).

Infections in birds by gastric *Cryptosporidium* such as *C. galli* and avian genotype III are subclinical or associated with anorexia, weight loss and chronic vomiting (Makino et al., 2010; Ravich et al., 2014). *C. baileyi* infection is frequently related to respiratory clinical signs (Current et al., 1986), and *C. meleagridis* infection occurs in the gut, causing symptoms related to the intestinal tract (Sréter et al., 2000).

C. meleagridis is the only zoonotic species in birds (Nakamura and Meireles, 2015). In some countries, *C. meleagridis* infection in humans may present a frequency similar to or greater than *C. parvum* infection (Cama et al., 2003; Morgan et al., 2001). Studies using phylogenetic analyses suggest that *C. meleagridis* isolated from humans and birds are genetically related (Wang et al., 2014). Furthermore, the detection of *C. parvum* in bird feces has been increasingly frequent, with reports in several avian species (Nakamura and Meireles, 2015). Due to these facts, the zoonotic potential of avian cryptosporidiosis must be observed carefully, owing to the direct or indirect contact between humans and food or pet animals.

Microscopic analysis is an inexpensive, easy and rapid technique that allows visualization of *Cryptosporidium* oocysts (Elliot et al., 1999), while molecular methods, although expensive, detect even low amounts of DNA in stool samples and allow for the identification of parasite species (Ryan et al., 2014). The decision of whether to opt for any diagnostic method in epidemiological studies depends on the cost-benefit analysis for each animal species.

The objective of this study was to determine the prevalence of *Cryptosporidium* spp. in caged psittacines. Furthermore, we accomplished the comparison between diagnostic methods for the detection of *Cryptosporidium* spp. and gastric avian *Cryptosporidium* species and genotypes.

2. Material and methods

2.1. Study population and fecal sample collection

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 463 adult exotic captive psittacines belonging to 24 species (Table A1), randomly selected from 36 aviaries located in the Southern and Southeastern regions of Brazil during a bird exhibition at the 2015 Ornithological Championship of the Ornithological Federation of Brazil (FOB).

Samples were collected from the bottom of the cage at the time of the bird's reception at the championship to avoid cross-contamination. Each sample was collected using a disposable wooden spatula and then stored at 4 °C in 2 mL microtubes containing 0.9% sodium chloride solution.

2.2. Fecal sample purification and microscopic examination

The fecal samples were purified in a 2.0 mL microtube by centrifugal-flotation using Sheather's sugar flotation solution [454 g of table sugar, 355 mL of phosphate buffered saline (PBS) pH 7.4, 0.1% Tween 20]. The resulting sediment from 463 fecal samples was resuspended in 200 μL of PBS/0.01% Tween 20 and divided into two aliquots. One aliquot was diluted with 10% buffered formaldehyde for microscopic analysis intended to screen for oocysts and the determination of morphological and morphometric data, and the other aliquot was frozen at −20 °C for DNA extraction.

Cryptosporidium spp. oocyst screening and morphometric analysis were accomplished using malachite green negative staining (Elliot et al., 1999). Length and width of avian genotype III oocysts (n = 20) from PCR/sequencing and duplex real-time PCR positive fecal samples were measured under optical microscopy at 1000× magnification (Olympus BX 50).

2.3. Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from the oocysts using the QIAamp® DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's guidelines, except that the samples were incubated for 60 min in ASL buffer at 99 °C before DNA extraction. Nested PCR was performed for the amplification of an ~587 bp fragment of the 18S subunit gene of *Cryptosporidium* spp. (Ryan et al., 2003). Genomic DNA from *C. parvum* and ultrapure water were used as positive and negative controls, respectively. The amplified fragments were visualized by GelRed® stained gel electrophoresis (Biotium, Fremont, USA).

Duplex real-time PCR was performed in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) using primers and minor groove binding (MGB) probes specific for gastric cryptosporidiosis (Nakamura et al., 2014). *C. galli* and avian genotype III genomic DNA and ultrapure water were used as positive and negative controls, respectively.

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