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

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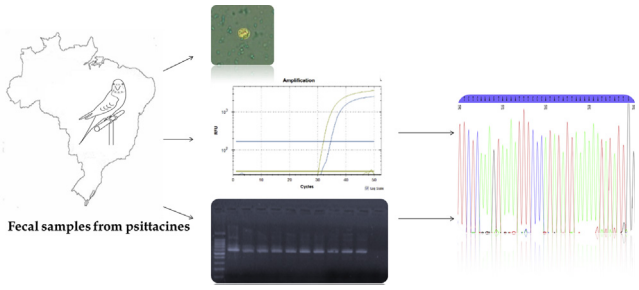
HIGHLIGHTS

- 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

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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.

2.4. Molecular cloning

The amplicons from 14 samples that were positive by duplex real-time PCR and negative by microscopy and nested PCR were purified using the PureLink™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, USA) and cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, USA), following the manufacturer's guidelines.

2.5. DNA sequence analysis

The nested PCR amplicons and plasmids from molecular cloning were purified using Illustra ExoProStar 1-Step® (GE Healthcare Life Sciences, Little Chalfont, England), following the manufacturer's guidelines. Sequencing was accomplished using the ABI Prism® Dye Terminator Cycling Sequence kit (Applied Biosystems, Foster City, USA) in the automated sequencer, ABI 3730XL (Applied Biosystems, Foster City, USA). DNA sequences were assembled with CodonCode Aligner version 4.0.1 software (CodonCode Corporation, Dedham, USA). The consensus sequences were aligned with homologous sequences published in GenBank using Clustal W (Thompson et al., 1997) and the BioEdit Sequence Alignment Editor (Hall, 1999).

Nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MF462153 to MF462158.

2.6. Statistical analysis

The data analysis consisted of descriptive statistics, frequency analysis (McNemar test) to verify the paired proportions and the Kappa correlation coefficient to evaluate the agreement between the methods. The database was created in Microsoft Office Excel (2010), and statistical results were considered significant when $p < .05$.

3. Results and discussion

The rates of results positive for *Cryptosporidium* spp. obtained by microscopy and nested PCR were 3.0% (14/463) and 5.0% (23/463), respectively. The sequencing of nested PCR amplicons allowed the identification of avian genotype III (1.7%; 8/463), *C. parvum* (0.9%; 4/463) and *Cryptosporidium canis* (0.2%; 1/463). All the sequences had 100% genetic similarity with sequences previously published in GenBank (GQ227480, AH006572, AF308600 and AF112576).

The use of formalin for the preservation of oocysts was aimed at preventing excystation (Widmer et al., 2007) and oocyst disintegration (Jirků et al., 2008) during the storage of oocysts from gastric *Cryptosporidium*, as observed in our laboratory with *C. serpentis* oocysts preserved in potassium dichromate (unpublished results). The mean size of avian genotype III oocysts was described elsewhere as 6.7 × 5.2 μm (Makino et al., 2010), which is similar to the 6.4 × 5.0 μm (n = 20) mean size we found for oocysts from samples positive for avian genotype III. Although structural variation is expected over time during the storage of oocysts in several fixatives, Duszynski and Gardner (1991) found that *Eimeria* oocysts preserved in 10% buffered formalin maintained structural integrity for at least 115 days.

Microscopic staining techniques are widely used for *Cryptosporidium* spp. diagnosis because they have low costs and are easy to perform (Elliot et al., 1999). Several samples that were classified as negative by microscopy tested positive by nested PCR and duplex real-time PCR (Table 1). Oocyst screening by microscopy showed that all positive samples had a low number of oocysts per slide (Fig. 1). Since infections with gastric species of *Cryptosporidium* in birds, as a rule, have a low number of oocysts in fecal samples (Nakamura et al., 2009), false-negative results may occur when microscopy is used for *Cryptosporidium* detection in birds.

Two samples that were considered positive by microscopy tested negative by nested PCR and duplex real-time PCR. Numerous factors may decrease the sensitivity of PCR and generate false-negative results, such as a low number of oocysts in feces of birds infected by gastric *Cryptosporidium* species (Nakamura et al., 2009) associated with PCR inhibitors coextracted with DNA (Schrader et al., 2012). The rates of positive results for *Cryptosporidium* spp. obtained by nested PCR were similar to those described in the literature in which psittacines were included in the studied populations (Nakamura et al., 2009, 2014; Qi et al., 2011).

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 (Table 1).

Duplex real-time PCR aims to quickly and specifically detect the gastric species/genotypes of *Cryptosporidium* that are the most frequent causes of cryptosporidiosis in Passeriformes and Psittaciformes (Nakamura et al., 2014). The amplification of fragments of smaller sizes and the use of MGB probes increases the sensitivity and the specificity of real-time PCR, respectively (Chakravorty et al.,

Table 1
Results of three diagnostic techniques for *Cryptosporidium* spp. in fecal samples of parrots.

Malachite green negative staining (*Cryptosporidium* spp.)	Nested PCR		Duplex Real-Time PCR		No. samples (%)
	Cryptosporidium spp.	Amplicon sequencing (No. samples)	*C. galli*	Avian genotype III	
–	–	–	+	–	8 (1.7)
–	+	ns[a](1)	+	–	1 (0.2)
+	–	–	+	+	1 (0.2)
–	–	–	+	+	7 (1.5)
–	–	–	–	+	13 (2.8)
–	+	Avian genotype III (3)	–	+	3 (0.7)
+	+	Avian genotype III (5)	–	+	5 (1.1)
+	–	–	–	+	6 (1.3)
+	–	–	–	–	2 (0.4)
–	+	C. *canis* (1) C. *parvum* (4) ns (9)	–	–	14 (3.0)
–	–	–	–	–	403 (87.0)
					463 (100)

[a] Samples not sequenced.

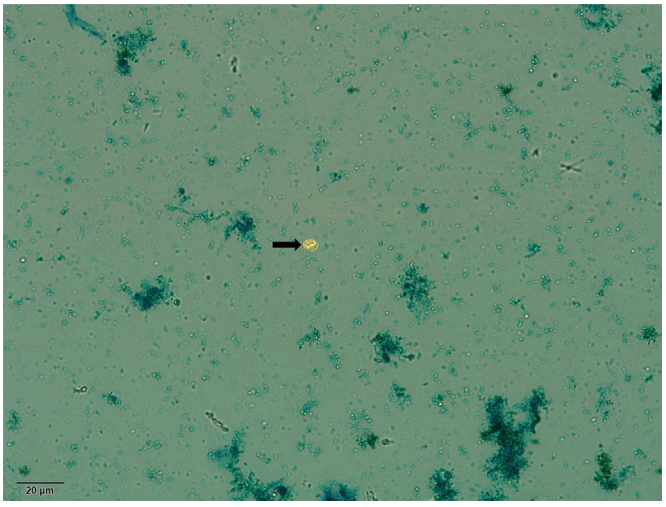

Fig. 1. *Cryptosporidium* avian genotype III oocyst (arrow). Fecal sample from a *Nymphicus hollandicus* (MG1 aviary – Table A.2) using malachite green negative staining.

2006). Nine samples that were positive by microscopy were negative by nested PCR but positive by duplex real-time PCR. All samples classified as avian genotype III by nested PCR/sequencing also tested positive by duplex real-time PCR. We detected mixed infection in eight samples using duplex real-time PCR. The identification of mixed infections by gastric species is another advantage of duplex real-time PCR since mixed infections are not easily identified by nested PCR/sequencing.

Positivity for gastric *Cryptosporidium* obtained by duplex real-time PCR was lower than that described by Nakamura et al. (2014), who used fecal samples from birds originating from either captivity or the wild and housed in individual cages closely placed side by side. Samples from this study were collected at the time of each bird's reception at the facility to avoid cross-contamination. Additionally, the birds originated from a championship, so it is assumed that the owners have exhibited birds with better physical status and, consequently, better health status, therefore reducing the chance of clinical or subclinical infections and oocyst shedding.

The amplicons from 12 duplex real-time PCRs were successfully cloned and sequenced. The cloned amplicons were 100% similar to sequences from avian genotype III (GenBank GQ227480) or *C. galli* (GenBank GU734645 and KT352999) (Table A.2). Molecular cloning was performed to confirm the specificity of duplex real-time PCR. Nakamura et al. (2014) have described high specificity for both primers and probes to detect avian genotype III and *C. galli*.

Avian genotype VI was described in the proventriculus of red-winged blackbirds (*Agelaius phoeniceus*) (Chelladurai et al., 2016). Even though we have not detected avian genotype VI in 12 samples submitted for cloning and sequencing, we cannot ensure that none of the duplex real-time PCR positive reactions detected this genotype since it presents the same annealing regions on the 18S rRNA gene for primers and probes used for duplex real-time PCR, except one and two substitutions in the *C. galli* and avian genotype III forward primers, respectively, and one substitution in the avian genotype III probe.

There was no significant difference between nested PCR and microscopic analysis results ($p = .1237$) by the McNemar test, and the Kappa correlation coefficient showed a fair agreement (Kappa = 0.242) (Landis and Koch, 1977) between them. The rates of positive results obtained by duplex real-time PCR and nested PCR/sequencing for the diagnosis of gastric cryptosporidiosis were significantly different ($p < .0001$). The concordance between duplex real-time PCR and nested PCR/sequencing was also considered fair (Kappa = 0.317).

Parrots seem to be infected mainly by host-adapted *Cryptosporidium* species/genotypes (Xiao et al., 2002). Avian genotype III is common in psittacines (Makino et al., 2010; Nakamura et al., 2014; Ng et al., 2006; Ravich et al., 2014), as demonstrated in this study in both molecular tests. The clinical significance of gastric cryptosporidiosis in parrots is still poorly understood and has been associated with clinical and subclinical infections (Makino et al., 2010; Ravich et al., 2014). More sensitive and specific molecular techniques have become essential for the specific diagnosis of gastric cryptosporidiosis in birds.

In several countries, *C. parvum* is the main causative agent of human cryptosporidiosis (Xiao and Feng, 2008). There is only one report of *C. parvum* causing clinical disease in birds (Zylan et al., 2008). *C. parvum* is not considered to be a major cause of disease in birds, although there are many reports of detection of *C. parvum* DNA in fecal samples of asymptomatic birds from several orders (Nakamura et al., 2009; Reboredo-Fernández et al., 2015; Oliveira et al., 2017). In parrots, *C. parvum* has been found in *Nymphicus hollandicus* (Nakamura et al., 2009) and now, we describe it in three *Forpus* sp. and in one *Psittacula krameri*.

The possible transmission of *C. canis* among children and a dog has been suggested (Xiao et al., 2007), and its occurrence ranges from 0.04% (1/2414) in individuals with diarrhea (Leoni et al., 2006) to 4.0% (12/302) in immunosuppressed individuals (Cama et al., 2003). *C. canis* DNA was found in a fecal sample from *P. krameri*. *C. canis* is commonly associated with infection in dogs (Fayer et al., 2001) and has never been found in birds.

The coexistence of birds and other domestic animals in the same environment is common and facilitates the transmission of *Cryptosporidium* spp. oocysts among different host species. All the samples positive for *C. canis* and *C. parvum* DNA tested negative by microscopy. The presence of DNA from *Cryptosporidium* species adapted to mammals in feces from parrots may indicate an absence of proper management practices in the aviary, which may have allowed the psittacines to ingest oocysts from contaminated food or water and shed oocysts mechanically.

4. Conclusion

Zoonotic *Cryptosporidium* species, such as *C. parvum* and *C. canis*, can be observed in fecal samples from psittacines. Nested PCR and duplex real-time PCR were the best options for the detection of *Cryptosporidium* spp. and gastric *Cryptosporidium*, respectively, in psittacines. Although several *Cryptosporidium* species/genotypes have been found in this study, avian genotype III was the most commonly detected in the feces of captive exotic psittacines from the Southern and Southeastern regions of Brazil.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2017.12.004.

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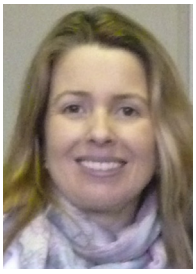

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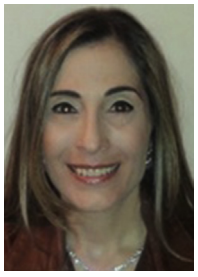

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