

Detection and molecular characterization of *Cryptosporidium* spp. in captive canaries (*Serinus canaria*) using different diagnostic methods

Detecção e caracterização molecular de *Cryptosporidium* spp. em canários mantidos em cativeiro (*Serinus canaria*) por diferentes métodos de diagnóstico

Vinícius da Silva Camargo¹; Bruna Nicoleti Santana¹; Elis Domingos Ferrari¹; Alex Akira Nakamura²; Walter Berthequini Nagata¹; Ana Rita Moraes Nardi³; Marcelo Vasconcelos Meireles^{1*}

¹ Faculdade de Medicina Veterinária, Universidade Estadual Paulista – UNESP, Araçatuba, SP, Brasil

² Curso de Medicina Veterinária, Faculdades Adamantinenses Integradas, Adamantina, SP, Brasil

³ Fundação Municipal de Ensino Superior, Bragança Paulista, SP, Brasil

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Abstract

This study used several diagnostic methods to examine the occurrence of and molecularly characterize *Cryptosporidium* spp. in captive canaries (*Serinus canaria*) in southern and southeastern Brazil. A total of 498 fecal samples were purified by centrifugal-flotation using Sheather's solution. *Cryptosporidium* spp. diagnosis was performed using three diagnostic methods: malachite green negative staining, nested PCR targeting the 18S rRNA gene, followed by sequencing the amplified fragments, and duplex real-time PCR targeting the 18S rRNA specific to detect *Cryptosporidium galli* and *Cryptosporidium* avian genotype III. The overall positivity for *Cryptosporidium* spp. (total samples positive in at least one protocol) from the microscopic analysis, nested PCR and duplex real-time PCR protocol results was 13.3% (66/498). The positivity rates were 2.0% (10/498) and 4.6% (23/498) for *Cryptosporidium* spp. by microscopy and nested PCR, respectively. Sequencing of 20 samples amplified by nested PCR identified *C. galli* (3.0%; 15/498), *Cryptosporidium* avian genotype I (0.8%; 4/498) and *Cryptosporidium avium* (0.2%; 1/498). Duplex real-time PCR revealed a positivity of 7.8% (39/498) for *C. galli* and 2.4% (12/498) for avian genotype III. Malachite green negative staining differed significantly from nested PCR in detecting *Cryptosporidium* spp. Duplex real-time PCR was more sensitive than nested PCR/sequencing for detecting gastric *Cryptosporidium* in canaries.

Keywords: Cryptosporidiosis, birds, diagnosis, epidemiology.

Resumo

Este trabalho teve como objetivos determinar a ocorrência e realizar a caracterização molecular de *Cryptosporidium* spp. em 498 amostras fecais de canários (*Serinus canaria*) criados em cativeiro, utilizando três métodos de diagnóstico: análise microscópica pela coloração negativa com verde malaquita, *nested* PCR seguida de sequenciamento dos fragmentos amplificados e PCR duplex em tempo real específica para detecção de *Cryptosporidium galli* e *Cryptosporidium* genótipo III de aves. A positividade total para *Cryptosporidium* spp. (total de amostras positivas em pelo menos um método de diagnóstico) obtida pela análise microscópica, *nested* PCR e PCR duplex em tempo real foi de 13,3% (66/498). As taxas de positividade para *Cryptosporidium* spp. foram 2,0% (10/498) e 4,6% (23/498) por microscopia e *nested* PCR, respectivamente. O sequenciamento de 20 amostras amplificadas pela *nested* PCR identificou *C. galli* (3,0%; 15/498), *Cryptosporidium* genótipo I de aves (0,8%; 4/498) e *Cryptosporidium avium* (0,2%; 1/498). A PCR duplex em tempo real revelou positividade de 7,8% (39/498) para *C. galli* e 2,4% (12/498) para *Cryptosporidium* genótipo III de aves. A análise microscópica diferiu significativamente da *nested* PCR para detecção de *Cryptosporidium* spp. A PCR duplex em tempo real apresentou maior sensibilidade que a *nested* PCR/sequenciamento para detectar as espécies/genótipos gástricos de *Cryptosporidium*.

Palavras-chave: Criptosporidiose, aves, diagnóstico, epidemiologia.

*Corresponding author: Marcelo Vasconcelos Meireles. Faculdade de Medicina Veterinária, Universidade Estadual Paulista – UNESP, Rua Clóvis Pestana, 793, Bairro Dona Amélia, CEP 16050-680, Araçatuba, SP, Brasil.
e-mail: marcelo@fmva.unesp.br



Introduction

Cryptosporidiosis is a major protozoan infection in birds, causing respiratory and gastrointestinal diseases in domestic and wild species (NAKAMURA & MEIRELES, 2015). Four *Cryptosporidium* species infect birds: *C. meleagridis* (SLAVIN, 1955), *C. baileyi* (CURRENT et al., 1986), *C. galli* (RYAN et al., 2003a) and *C. avium* (formerly avian genotype V) (HOLUBOVÁ et al., 2016). In addition to the avian *Cryptosporidium* species, many genotypes infect birds, mainly the avian genotypes I (NG et al., 2006), II (SANTOS et al., 2005; MEIRELES et al., 2006; NG et al., 2006), and III (NG et al., 2006).

Studies conducted in several countries, with several species of domestic and wild birds, reported that *Cryptosporidium* spp. prevalence ranged from 0.8% to 44.4%, and *C. andersoni*, *C. avium*, *C. baileyi*, *C. galli*, *C. muris*, *C. meleagridis*, and *C. parvum* and several avian genotypes, including the avian genotypes I, II, III and VI were identified (NAKAMURA & MEIRELES, 2015, CHELLADURAI et al., 2016).

In Brazil, *C. avium*, *C. baileyi*, *C. meleagridis*, *C. parvum*, the avian genotypes I, II and III and the duck genotype were reported to occur in fecal samples of domestic and wild birds (MEIRELES & FIGUEIREDO, 1992; SANTOS et al., 2005; MEIRELES et al., 2006; HUBER et al., 2007; NAKAMURA et al., 2009, 2014; SEVÁ et al., 2011; NARDI, 2015; CUNHA et al., 2017).

C. galli is the most frequent species in Passeriformes; however, there is controversy regarding its pathogenicity in birds (NAKAMURA & MEIRELES, 2015). *C. galli* or *Cryptosporidium* avian genotype III infections can result in diarrhea, anorexia, weight loss, and chronic vomiting (ANTUNES et al., 2008; MAKINO et al., 2010, SILVA et al., 2010; RAVICH et al., 2014).

In canaries (*Serinus canaria*), *C. galli*, *C. avium* and *C.* avian genotypes I and III infections have been described (NG et al., 2006; ANTUNES et al., 2008; NAKAMURA et al., 2009, 2014; NARDI, 2015). Most epidemiological studies on avian cryptosporidiosis were performed using convenience sampling with fecal samples from several bird species (NG et al., 2006; NAKAMURA et al., 2009, SEVÁ et al., 2011; BAMAIYI et al., 2013; REBOREDO-FERNÁNDEZ et al., 2015).

Parasite-host adaptation and co-evolution among *Cryptosporidium* spp. and their avian hosts are thought to occur (XIAO et al., 2002) since some *Cryptosporidium* species/genotypes are found almost exclusively in certain avian orders (NAKAMURA & MEIRELES, 2015). Therefore, epidemiological studies using samples representing avian orders or species would aid in investigating *Cryptosporidium* species evolution.

The common techniques used to diagnose *Cryptosporidium* infection are microscopic analysis and nested PCR. Microscopy is an inexpensive and fast technique; however, it does not identify the *Cryptosporidium* species and is less sensitive and specific. Nested PCR is more expensive than microscopy, despite being highly sensitive and specific and identifying the species after amplicon sequencing (JEX et al., 2008). Another option for specific species/genotype diagnosis of gastric cryptosporidiosis in birds is through duplex real-time PCR (NAKAMURA et al., 2014).

The aim of the present study was to determine the prevalence of *Cryptosporidium* spp. in fecal samples from captive canaries from southern and southeastern Brazil. Additionally, molecular characterization of positive samples was performed, and three techniques for detecting *Cryptosporidium* spp. were compared.

Materials and Methods

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 01022-2015.

Fecal samples

Fecal samples were obtained from asymptomatic captive canaries housed in 102 aviaries from six southern and southeastern Brazilian states, exhibited at the 64th Ornithological Championship 2015 of the Ornithological Federation of Brazil (FOB), from 09/07/2015 to 19/07/2015, in the city of Itatiba, state of São Paulo, Brazil.

The total population of canaries exhibited in the championship was approximately 40,000 birds. To determine the prevalence of *Cryptosporidium* spp. in these canaries, the sample number of 385 was calculated using Win Episcopo software (THRUSFIELD et al., 2001) considering a diagnostic test with 100% sensitivity and 100% specificity. As lower sensitivity and specificity rates are common when using the diagnostic protocols employed in this study (JEX et al., 2008), and because losses occur in sample storage and processing, 498 samples were collected.

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, transferred to a 2-ml microtube containing 0.9% sodium chloride solution in enough quantity to prevent dehydration, and stored at 4°C.

Purification of oocysts

Samples were fragmented and homogenized using a disposable wooden spatula in a 2 mL microtube containing Sheather's solution (g = 2.05) prepared with phosphate buffered saline and 0.1% Tween 20 (PBS-T). The contents of each microtube were homogenized, and half of the contents were transferred to another microtube so that each sample was purified in two tubes simultaneously. Each microtube was filled with 1.9 mL of Sheather's solution and vortexed and centrifuged at 800 g for 5 minutes. Four hundred µL of supernatant was transferred to a microtube containing 1,500 µL of PBS-T, homogenized by inversion and centrifuged at 10,000 g for 3 minutes. After discarding the supernatant, the microtubes were filled with 1.9 mL of PBS with 0.01% Tween 20 and centrifuged at 10,000 g for 3 minutes. The supernatant was discarded, preserving approximately 100 µL of solution and sediment. One hundred µL of 10% buffered formalin was added to one microtube for *Cryptosporidium* oocyst microscopy screening using malachite green negative staining (ELLIOT et al., 1999),

while the other microtube was frozen at -20°C for DNA extraction and amplification by nested PCR and duplex real-time PCR.

Genomic DNA extraction

Genomic DNA of *Cryptosporidium* spp. was extracted per the protocol adapted from McLaughlin et al. (2000) and Wang et al. (2011) using Zymo-Spin[®] IIIC silica columns (Zymo Research, Irvine, USA) to replace activated silica. The DNA was eluted in 100 μL of elution buffer (10 mM Tris, 0.5 mM EDTA, pH 9) and stored at -20°C in two 50 μL aliquots.

Nested PCR and sequencing

Nested PCR targeting the 18S rRNA gene was performed using the PCR primers, 5'-GACATATCATTCAAGTTTCTGACC-3' and 5'-CTGAAGGAGTAAGGAACAACC-3' (-761 bp), and the nested PCR primers, 5'-CCTATCAGCTTTAGACGGTAGG-3' and 5'-TCTAAGAATTTACCTCTGACTG-3' (-585 bp) (RYAN et al., 2003b). Genomic DNA from *C. parvum* and ultrapure water were used as positive and negative controls, respectively.

The reactions contained a volume of 25 μL , with 2.5 μL of 10x PCR buffer, 2.0 mM MgCl_2 , 0.5 U of JumpStart[®] Taq DNA Polymerase (Sigma-Aldrich, St. Louis, USA), 200 μM of each deoxyribonucleotide, 200 nM of each primer, 5 μL of target DNA in the PCR and 2.5 μL of DNA in the nested PCR. Samples were subjected to initial DNA denaturation at 94°C for 2 minutes, followed by 40 cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 58°C and extension at 72°C for 60 seconds (PCR) or 45 seconds (nested PCR), with a final extension at 72°C for 7 minutes. Amplified fragments were analyzed by GelRed[®] (Biotium, Fremont, USA) stained gel electrophoresis.

Duplex real-time PCR

Duplex real-time PCR was performed to simultaneously detect *C. galli* and *Cryptosporidium* avian genotype III (NAKAMURA et al., 2014), amplifying 134-bp and 138-bp amplicons, respectively (Table 1), under the following reaction conditions: 25 μL of solution containing 12.5 μL of JumpStart[®] TaqReady Mix (Sigma-Aldrich, St. Louis, USA), 4.5 mM MgCl_2 , 250 nM of each probe, 600 nM of each primer, 0.6 $\mu\text{g}/\mu\text{L}$ of non-acetylated bovine serum albumin (Sigma-Aldrich, St. Louis, USA), and 5 μL of target DNA. PCR cycles consisted of 2 minutes of denaturation at 94°C followed by 50 cycles of 30 seconds at 94°C and 1 minute

at 61°C in the CFX96[®] Real-Time PCR Detection System (Bio-Rad, Hercules, USA).

DNA sequencing

Identifying the *Cryptosporidium* species was performed by sequencing the nested PCR amplicons, after purification using the Illustra ExoProStar[®] 1-Step (GE Healthcare Life Sciences, Champaign, USA) or the QIAquick[®] Gel Extraction kit (Qiagen, Hilden, Germany), using ABI Prism[®] Dye Terminator 3.1, in an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, USA), at the Center for Sequencing and Functional Genomics of UNESP, Jaboticabal Campus, Brazil. Sequencing reactions were performed in both directions using nested PCR primers.

Consensus sequences were determined using the CodonCode Aligner v. 7.1.2 (CodonCode Corporation, Dedham, USA) and aligned with homologous sequences published in GenBank using Clustal X software (THOMPSON et al., 1997) and Bioedit Sequence Alignment Editor (HALL, 1999).

Nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MG832881-MG832883.

Statistical analysis

The McNemar test was used to compare diagnostic techniques, and the Kappa correlation coefficient test was used to evaluate the agreement between them. Statistical analyses were performed using the BioEstat 5.0 software (Analyst Soft Inc., Walnut, USA) and the results were considered significant when $p < 0.05$.

Results and Discussion

The overall positivity rates for *Cryptosporidium* spp. (total samples positive in at least one protocol) were 13.3% (66/498) and 40.2% (41/102) for fecal samples and aviaries, respectively (Table 2). By microscopy and nested PCR, the positivity rates for *Cryptosporidium* spp. were 2.0% (10/498) and 4.6% (23/498), respectively. There was a significant difference ($p=0.01$) and a fair agreement (Kappa=0.28) between nested PCR and microscopic analysis for detecting *Cryptosporidium* spp.

Only one study has reported specifically on *Cryptosporidium* infection in canaries (NARDI, 2015), with the reported positivity for *Cryptosporidium* spp. in asymptomatic canaries at 2% (8/394) in Ziehl-Neelsen stained fecal samples. This is consistent with

Table 1. Primers and probes used to detect *C. galli* and *Cryptosporidium* avian genotype III by duplex real-time PCR.

Species/Genotype	Primers/ Probes	Sequence 5'-3'	Amplicon (bp)
<i>C. galli</i>	Forward primer	CGTAGTTGGATTCTGTGTCATCA	134
	Probe	FAM AATATAATATCAACATCCTCCC MGB	
	Rewind primer	GCCAGTTGCCTGCTTTAAGC	
<i>Cryptosporidium</i> avian genotype III	Forward primer	GCTCGTAGTTGGATTCTGTGTTGATT	138
	Probe	VIC CATTATAATAACAACATCCTTCC MGB	
	Rewind primer	GCCAGTTGCCTGCTTTAAGC	

the results of the present study using malachite green negative staining (2%; 10/498).

Nested PCR/sequencing revealed the presence of *C. galli* (3%; 15/498), *Cryptosporidium* avian genotype I (0.8%; 4/498), and *C. avium* (0.2; 1/498) (Table 3). Sequences from *C. galli*, *Cryptosporidium* avian genotype I and *C. avium* were 100% similar to GenBank homologous sequences EU543269 (*S. canaria*), GQ227479 (*S. canaria*) and KJ487974 (*Amazona aestiva*), respectively. Unexpected nonspecific nested PCR amplification of *Isoospora* spp. amplicons of the predicted sizes occurred in six samples. Because nested PCR primers target conserved regions of a pan-eukaryotic gene (18S rRNA), diagnosis of *Cryptosporidium* spp. based only on 18S rRNA amplicon sizes should be confirmed with caution in Passeriformes, since *Isoospora* spp. is common in fecal samples from birds of this order (BERTO et al., 2011).

Duplex real-time PCR was positive for gastric *Cryptosporidium* in 10.2% (51/498) of the samples, 7.8% (39/498) for *C. galli* and 2.4% (12/498) for *Cryptosporidium* avian genotype III. For detecting the gastric species of *Cryptosporidium*, nested PCR/sequencing and duplex real-time PCR were significantly different ($p < 0.0001$), and the agreement between the two methods was fair (Kappa = 0.40).

C. galli is associated with chronic infection in the passerine proventriculus and is likely responsible for chronic gastric disease and predisposition to concomitant infections (ANTUNES et al., 2008; NAKAMURA & MEIRELES, 2015). The species most frequently detected by nested PCR/sequencing was *C. galli*, corresponding to 75% (15/20) of the sequenced samples. In addition, *Cryptosporidium* species identification by nested PCR/sequencing and duplex real-time PCR revealed results similar to those of other authors (NAKAMURA et al., 2014; NARDI, 2015), in which

Table 2. Results of positivity for *Cryptosporidium* spp., in at least one protocol, from canary fecal samples from southern and southeastern Brazil.

States of Brazil	Fecal samples		Aviaries	
	% positive (n° positive/n° sampled)	Species identification % positive (n° positive/n° sampled)	% positive (n° positive/n° sampled)	Species identification % positive (n° positive/n° sampled)
São Paulo	10.9 (29/267)	<i>C. galli</i> 48.2 (14/29) <i>C. avium</i> 3.4 (1/29) Avian genotype III 17.2 (5/29) Avian genotype I 3.4 (1/29) <i>Cryptosporidium</i> sp.* 27.5 (8/29)	32.8 (22/67)	<i>C. galli</i> 17.9 (12/67) <i>C. avium</i> 1.4 (1/67) Avian genotype III 6 (4/67) Avian genotype I 3 (2/67) <i>Cryptosporidium</i> sp. 7.4 (5/67)
Paraná	14 (18/128)	<i>C. galli</i> 50 (9/18) <i>Cryptosporidium</i> sp. 22.2 (4/18) Avian genotype III 16.6 (3/18) Avian genotype I 5.5 (1/18) <i>C. galli</i> and avian genotype III 5.5 (1/18)	58.8 (10/17)	<i>C. galli</i> 47 (8/17) <i>Cryptosporidium</i> sp. 17.6 (3/17) Avian genotype III 17.6 (3/17) Avian genotype I 5.8 (1/17) <i>C. galli</i> and avian genotype III 5.8 (1/17)
Rio Grande do Sul	9.3 (4/43)	<i>C. galli</i> 75 (3/4) Avian genotype I 25 (1/4)	33.3 (2/6)	<i>C. galli</i> 33.3 (2/6) Avian genotype III 16.6 (1/6)
Minas Gerais	17.1 (6/35)	<i>C. galli</i> 83.3(5/6) Avian genotype III 16.7(1/6)	100 (2/2)	<i>C. galli</i> 100 (2/2) Avian genotype III 50 (1/2)
Santa Catarina	53.8 (7/13)	<i>C. galli</i> 71.4 (5/7) Avian genotype III 14.3(1/7) <i>C. galli</i> and avian genotype III 14.3 (1/7)	75 (3/4)	<i>C. galli</i> 75 (3/4) Avian genotype III 25 (1/4) <i>C. galli</i> and avian genotype III 25 (1/4)
Rio de Janeiro	16.7 (2/12)	<i>C. galli</i> 100 (2/2)	33.3 (2/6)	<i>C. galli</i> 33.3 (2/6)
Total	13.3 (66/498)		40.2 (41/102)	

*Samples not sequenced.

Table 3. Detection and identification of *Cryptosporidium* species and genotypes in canary fecal samples using microscopy and molecular methods targeting the 18S rRNA gene.

Diagnostic methods	<i>Cryptosporidium</i> detection/identification (n° positive/n° sampled)				
	<i>Cryptosporidium</i> spp.*	Gastric <i>Cryptosporidium</i> species**		<i>Cryptosporidium</i> avian genotype I	<i>C. avium</i>
		<i>C. galli</i>	<i>Cryptosporidium</i> avian genotype III		
Malachite green negative staining	2.0 (10/498)	-	-	-	-
Nested PCR	4.6 (23/498)	-	-	-	-
Nested PCR/ sequencing	-	3.0 (15/498)	0.0 (0/498)	0.8 (4/498)	0.2 (1/498)
Duplex real-time PCR	-	7.8 (39/498)	2.4 (12/498)	-	-

*McNemar test ($p=0.01$); Kappa=0.28; **McNemar test ($p < 0.0001$); Kappa=0.4.

C. galli showed higher positivity than *Cryptosporidium* avian genotype III in Passeriformes, including canaries.

Cryptosporidium avian genotype VI, described by Chelladurai et al. (2016), is closely related to *C. galli* and *Cryptosporidium* avian genotype III and likely infects the gastric epithelia. Although we did not detect avian genotype VI in canary samples by nested PCR/sequencing, we cannot assure that duplex real-time PCR does not detect this genotype due to the high similarities in its primer and probe annealing regions.

Tissue tropism and the clinical importance of *Cryptosporidium* avian genotype I are undetermined (NAKAMURA & MEIRELES, 2015). Herein, *C. galli* was detected more frequently in canaries than *Cryptosporidium* avian genotype I. In contrast, Nardi (2015) found a 14.2% (29/204) positivity for *Cryptosporidium* avian genotype I in fecal samples and cloacal swabs from canaries. This higher positivity rate may have resulted from cloacal swab sampling and by including samples from symptomatic and dead birds. Although the tissue tropism of avian genotype I is undetermined, the close genetic similarity among this genotype, *C. avium*, *C. baileyi* and avian genotype II is evidence that avian genotype I exhibits tropism for the cloacal epithelia, the bursa of Fabricius or the respiratory tract (NAKAMURA & MEIRELES, 2015).

C. avium was identified in one sample and has been described in the trachea/lung and cloaca of a cockatiel (ABE et al., 2015), the kidney and cloaca (CURTISS et al., 2015) and in fecal samples of budgerigars, Amazon parrots, cockatiels and Major Mitchell's cockatoos (ABE & MAKINO; 2010; QI et al., 2011; NAKAMURA et al., 2014; ZHANG et al., 2015). Nardi (2015) also found low positivity (1.0%; 2/204) for *C. avium* in canary fecal samples.

In conclusion, nested PCR was more sensitive than microscopic analysis using malachite green negative staining to detect *Cryptosporidium* spp. in fecal samples from canaries. Duplex real-time PCR was more sensitive than nested PCR/sequencing for diagnosing gastric cryptosporidiosis in canaries. There was a higher prevalence of *C. galli*, and *Cryptosporidium* avian genotype III, *Cryptosporidium* avian genotype I and *C. avium* were detected at lower positivity rates.

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