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The first study of molecular prevalence and species characterization of *Cryptosporidium* in free-range chicken (*Gallus gallus domesticus*) from Brazil

Primeiro estudo de prevalência molecular e caracterização de espécies de *Cryptosporidium* em galinhas (*Gallus gallus domesticus*) colonial/caipiras do Brasil

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Abstract

Rearing free-range chicken is based on grazing feeding patterns, and these animals could be potential environmental contaminants of *Cryptosporidium* oocysts for humans and other animals. Therefore, the present study aimed to evaluate the molecular prevalence of *Cryptosporidium* spp. in free-range chickens from Brazil. A total of 351 fecal samples from chickens were examined from 20 farms. For detection of *Cryptosporidium* spp., 18S rRNA gene fragments were amplified using a nested PCR reaction. Positive samples were sent for sequencing. The overall prevalence of *Cryptosporidium* was 25.6% (95% CI = 21.2% - 30.6%). Sequencing of the amplified fragments allowed for the identification of three species: *C. meleagridis* in 57 (62.6%), *C. baileyi* in 15 (16.4%), *C. parvum* in 3 (3.2%) samples, and a new *Cryptosporidium* genotype (*C.* genotype BrPR1) in 3 (3.2%) samples. *Cryptosporidium* genotype BrPR1 has not yet been classified as a species, and its host spectrum is not known. *Cryptosporidium*, including zoonotic species, exists at a high prevalence in free-range chickens within the region studied.

Keywords: Cryptosporidiosis, zoonosis, public health, epidemiology.

Resumo

A criação de galinhas no estilo colonial/caipira é baseada em padrões de alimentação de pastagem, o que as torna potenciais contaminantes ambientais de oocistos de *Cryptosporidium* para humanos e outros animais. Portanto, o presente estudo teve como objetivo avaliar a prevalência molecular de *Cryptosporidium* spp. em galinhas criadas em sistema colonial/caipira. Um total de 351 amostras de fezes de frangos foram examinadas em 20 fazendas. Para a detecção de *Cryptosporidium* spp., os fragmentos do gene rRNA 18S foram amplificados utilizando-se a reação de *nested*-PCR. A prevalência global de *Cryposporidium* foi de 25,6% (IC 95% = 21,2% - 30,6%). O sequenciamento dos fragmentos amplificados permitiu a identificação de três espécies que infectam aves: *C. meleagridis* em 57 (62,6%), *C. baileyi* em 15 (16,4%), *C. parvum* em 3 (3,2%) amostras, bem como, um novo genótipo de *Cryptosporidium* (*C.* genótipo BrPR1) foi identificado em 3 (3,2%) amostras. *Cryptosporidium* genotipo BrPR1 não foi ainda classificado como uma espécie, e seu espectro de hospedeiros é desconhecido. O presente trabalho permitiu concluir que *Cryptosporidium*, incluindo espécies zoonóticas, existe com alta prevalência em galinhas criadas em sistema colonial/caipira na região estudada.

Palavras-chave: Criptosporidose, zoonoses, saúde pública, epidemiologia.

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Introduction

Cryptosporidium can infect a wide variety of vertebrate animals, including mammals, birds, amphibians, reptiles, and fish (RYAN & HIJJAWI, 2015). Three species of *Cryptosporidium* have been identified in chickens: *C. meleagridis, C. baileyi,* and *C. galli* (CURRENT et al., 1986; PAVLÁSEK, 2001; SLAVIN, 1955). Of these three, *C. meleagridis* has zoonotic potential and causes watery diarrhea, abdominal pain, nausea, vomiting, and fever in humans (CHAPPELL et al., 2011; CHAPPELL et al., 2015). Additionally, in some studies of HIV-patients, the prevalence of *C. meleagridis* is similar to that of *C. parvum* (GATEI et al., 2003; CAMA et al., 2007).

Cryptosporidiosis can be acquired through several routes, including person-to-person contact, ingestion of contaminated food, drinking, and recreational water, as well as contact with companion and farm animals (GRIFFITHS, 1998; MAcKENZIE et al., 1994; SHIELDS et al., 2008; SMITH et al., 2007; TANGERMANN et al., 1991). Therefore, because the production of free-range chickens is based on grazing feeding patterns, these animals are potential biological vectors for the environmental contamination of oocysts (THAMSBORG et al., 1999).

In considering the demands of consumers in industrialized nations who desire meat raised without chemical agents in welfare-friendly and organic systems, there is an association with the popular concept that animals raised under free-range conditions are sanitary and safer than those reared indoors (WANG et al., 2009). However, considering host-pathogen interactions, outdoor animals may have inherently more risk to be infected with zoonotic parasites or act as infection sources for several animals and humans (THAMSBORG et al., 1999). Despite this perception, there is no study focusing on characterizing *Cryptosporidium* spp. from free-range chicken production systems.

Therefore, the present study is aimed at evaluating the molecular prevalence of *Cryptosporidium* spp. in chickens raised under free-range systems in southern Brazil.

Materials and Methods

Study population and sampling

Fecal samples from 351 free-range chickens, aged between 90 and 190 days, were collected between August 2011 to February 2014. Samples were acquired from 20 farms located in 10 different cities from the northern Parana state, southern Brazil. All animals were raised in free-range system and slaughtered at farms for human consumption. Conglomerate sampling was carried out proportional to the number of animals slaughtered in the period. The sample size was calculated assuming a prevalence of 35% (BAROUDI et al., 2013) with a confidence level of 95% and efficacy of 5% by Epi Info 3.5.4. A questionnaire including several factors, such as, presence of other animals, water source, presence of fence, and population density, was applied to all farmers to determine possible risk association.

Fecal samples were collected from the distal portion of the rectum and stored at -20 °C until DNA extraction. This study was approved by Ethics Committee of the Universidade Estadual de Londrina (Protocol n° 206/12).

DNA extraction and nested-PCR

DNA extraction was performed using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. For detection of *Cryptosporidium* spp., 18S rRNA gene fragments were amplified using a nested PCR (n-PCR) reaction using primers previously described by (XIAO et al., 1999). The first PCR reaction consisted of 9.25 µL of autoclaved ultrapure water, 2.5 µL of buffer 10X concentrate, 0.5 µL dNTP (10 mM), 1.25 µL $MgCl_2$ (50 mM), 0.5 µL of each primer 20 pmol/uL (forward and reverse), Platinum™ Taq DNA Polymerase (Invitrogen™, Carlsbad, CA, USA) 0.25 µL (5 U/µL), and 2.0 µL of genomic DNA, reaching a final volume of 25 µL. To reduce unused primers from the primary PCR, the first round of PCR products was diluted 1:5 with dH_2O. The conditions for each second-round reaction were identical to the first. Initial heating was carried out at 95 °C for 5 minutes followed by 35 cycles, each cycle consisting of 94 °C for 45 seconds (denaturation), 55 °C for 45 seconds (annealing) and 72 °C for 60 seconds (extension). One final extension step at 72 °C for 5 minutes was included. The PCR products were subjected to electrophoresis on 1.5% agarose gel (Ultrapure™ Agarose, Invitrogen™, Carlsbad, CA, USA) stained with SYBR® Safe (Invitrogen, Carlsbad, CA, USA), visualized by ultraviolet light and photodocumented using program LPix Image ST (Loccus Biotecnologia, Cotia, SP, Brazil). Negative and positive controls consisting of ultrapure water and *C. muris* DNA respectively, were included in each batch.

Sequence analysis of SSU rRNA gene

The positive PCR products were purified from agarose gel using a PureLink™ Quick Gel Extraction Kit (Invitrogen™, Carlsbad, CA, USA) and quantified using a Picodrop™ Spectrophotometer (Invitrogen™, Carlsbad, CA, USA). The sequencing was performed using the BigDye Terminator™ v.3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA) with second reaction forward and reverse primers using a 3500 Genetic Analyzer (Applied Biosystems™, Carlsbad, USA) according to the manufacturer's instructions. The obtained sequences were analyzed in Chromas Lite v.2.6.1, and the contigs were generated in BioEdit (Biological Sequence Alignment Editor) v.7.2.5 (HALL, 1999). dentity was verified with sequences deposited in the GenBank© using BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and by manual alignment using the program BioEdit (Biological Sequence Alignment Editor). Phylogenetics analysis were carried out using neighbor-joining method with bootstrap values derived from 1000 replicates in MEGA v7.0.14, evolutionary distances were calculated by Kimura 2-parameter method. When uncommon *Cryptosporidium* species were identified on 18S n-PCR, we performed n-PCR for *actin* and *hsp70* genes and posterior sequencing at the same conditions as described above for 18S rRNA n-PCR (SULAIMAN et al., 2000, 2002).

Statistical analysis

Association of the risks between variables and positivity to *Cryptosporidium* spp. was performed using the chi-square test or Fisher's exact test, and a 5% significance level was adopted. The measure of association Odds Ratio was calculated with a 95% confidence interval. The statistical package Epi 3.5.4. (DEAN et al., 2011) was used for analysis.

Results

18S rRNA n-PCR reactions were positive for *Cryptosporidium* spp. in 91 (25.9%) of 351 fecal samples (Table 1). The prevalence at the farms ranged from 0 to 80%, and 16 (80%) of them were positive for *Cryptosporidium* spp.. Those positive samples tested at 18S rRNA n-PCR were submitted for sequencing (Table 1), and 57 (62.6%), 15 (16.4%), 3(3.2%), and 3 (3.2%) were detected as *C. meleagridis, C. baileyi,* and *C. parvum,* and a new genotype named *C.* genotype BrPR1 (n=3, 3.2%), respectively. Thirteen samples did not have good amplification results due to low DNA quality. The identities on BLAST were confirmed as 99 to 100% for *C. meleagridis* (KT151551.1, EU814432.1) and *C. baileyi* (GQ426096.1, AF093495.1, KT151550.1, EU814432.1), 100% for *C. parvum* (KU892559.1, AF164102.1), and 99% for *C. bovis* (KT922231.1). Phylogenetically, three from the 18S rRNA n-PCR (Figure 1) showed three clusters that were very well defined. Three isolates CryBrFRChi98/104/106 clustered with *C. parvum*, CryBrFRChi 24/plus 54 isolates clustered

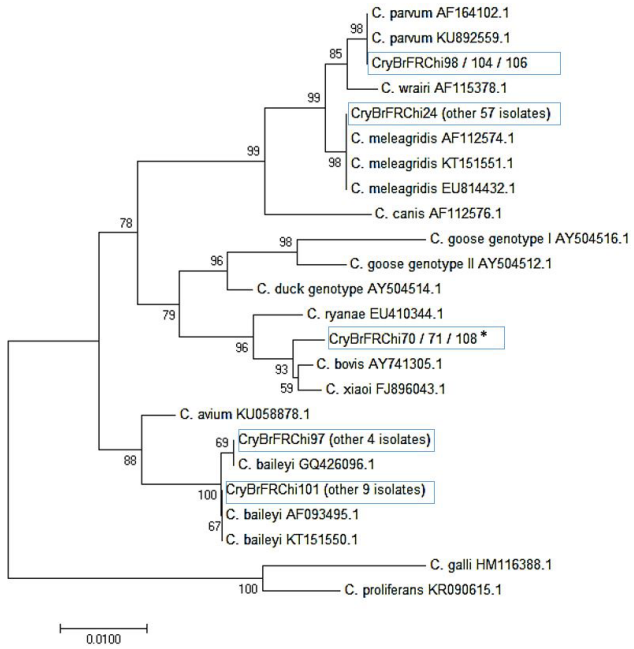

Figure 1. Phylogenetic relationships between *Cryptosporidium* spp. found in this study by 18S rRNA and selected *Cryptosporidium* spp. from GenBank inferred by Neighbor-joining (ML). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *Cryptosporidium galli* and *C. proliferans* were used as outgroup. Isolates obtained in the present study were marked as CryBrFRCh# (***Cry****ptosporidium* **Br**azil **F**ree **R**ange **Ch**ickens and the number of respective sample). *New genotype (*Cryptosporidium* genotype BrPR1).

Table 1. Results of positivity in 18S rRNA nested-PCR for *Cryptosporidium* from fecal samples in free-range chickens from the northern Paraná state, Brazil.

Farms	Cities	n	n-PCR	Sequencing result				
			+/%	*C. meleagridis*	*C. baileyi*	*C. parvum*	*C. bovis*	NAQ*
1	A	70	10/ 14.3	9				1
2		15	5/ 33.3	4				1
3	B	5	4/ 80		2			2
4		59	29/ 49.1	23	2	2		2
5	C	8	0/0					
6	D	10	2/20	1	1			
7	E	35	2/ 5.7	1	1			
8		5	1/20					1
9		6	0/0					
10		20	1/5	1				
11		18	2/11.1	2				
12		10	7/70		6			1
13	F	37	13/35.1	11				2
14	G	2	0/0					
15		3	2/66.7	1				1
16	H	12	2/16.7		2			
17		15	6/40	3	1			2
18		11	0/0					
19	I	5	3/60	1		1	1	
20	J	5	2/40				2	
Total		351	91/25.9	57(62.6%)	15(16.5%)	3(3.3%)	3(3.3%)	13(14.3%)

*NAQ: Not sequenced due to low quality of DNA.

with *C. meleagridis*. However, isolates characterized as *C. baileyi* showed two clusters one for CryBrFRChi 97/plus four isolates which clustered with GQ426096.1 and CryBrFRChi 101/plus nine isolates which clustered with AF093495.1 GenBank isolates. The isolates CryBrFRChi 70/71/108 showed higher evolutionary genetic distances (egd) = 0.006/ 100 base pairs (bp) with *C. bovis* (AY741305.1), egd = 0.007/100bp for *C. xiaoi* (FJ896043.1), and egd = 0.014/100bp for *C. ryanae.*

The isolates CryBrFRChi 70/71/108 were then n-PCR tested for *hsp70* and actin genes. One sample (CryBrFRChi 70) did not have clear amplification in the *actin* gene and therefore was not analyzed; the other isolates were sequenced, and demonstrated low identities on BLAST for *C. bovis* in HSP70 gene (98%, AY41306.1) and for actin gene (95%, AY41307.1). Two isolates came from farm 20 (CryBrFRCh70, and CryBrFRCh71) and one from farm 19 (CryBrFRCh108), which were located in different cities. A phylogenetic tree is shown in Figure 2. Evolutionary genetic distances (egd)/100bp for *C. bovis*, *C. ryanae,* and *C. xiaoi* using the *actin* gene was 0.049, 0.115, and 0.042 and 0.022, 0.131, 0.027 using *hsp70*, respectively. Differences among isolates in regions of 18S rRNA n-PCR, *hsp70* and *actin* genes are shown in Table 2. There were six, seven, and 43 differences in nucleotide sequences for 18S, *hsp70*, and *actin* genes, respectively.

The nucleotide sequences were submitted at GenBank with accession numbers; MG209076-MG209080 and MG243623- MG243695 for 18S rRNA gene, and MG209081-MG209082 for actin gene, and MG209083-MG209085 for HSP70 gene.

The association of risk between the positivity from the 18S rRNA n-PCR, and characteristics of the raising system, such as water source, population density, and presence of livestock, showed no significant differences in all variables analyzed (p>0.06).

Discussion

To the best of the author's knowledge, this study shows, for the first time, the molecular prevalence and characterization of *Cryptosporidium* spp. in fecal samples from free-range chickens. Here,

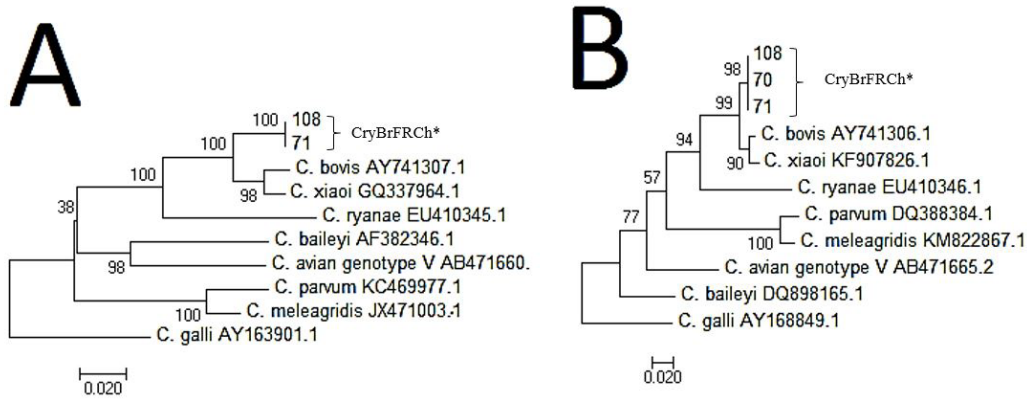

Figure 2. Phylogenetic relationships between *Cryptosporidium* spp. found in this study by partial DNA sequences of actin (A), and HSP70 (B) loci and selected *Cryptosporidium* spp. from GenBank inferred by model Kimura 2-parameter. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Cryptosporidium galli* was used as outgroup. Isolates obtained in the present study were marked as CryBrFRCh# (***Cry****ptosporidium* **Br**azil **F**ree **R**ange **Ch**ickens and the number of respective sample). *New genotype (*Cryptosporidium* genotype BrPR1).

Table 2. Nucleotide substitutions at 18S rRNA, HSP70, and actin genes from isolates of present study (CryBrFRChi 70/71/108).

18S rRNA	**14**	**395**	**396**	**426**	**442**	**570**																	
CryBrFRChi70 / 71 / 108	G	T	A	C	G	G																	
C. bovis AY741305.1	A	C	.	T	A	.																	
C. xiaoi FJ896043.1	A	.	T	T	A	A																	
HSP70	**3**	**15**	**33**	**63**	**114**	**135**	**213**																
CryBrFRChi70, 71 / 108	C	T	G	T	G	C	T																
C. bovis AY741306.1	T	.	.	C	A	T	C																
C. xiaoi FJ896041.1	T	C	A	C	.	T	C																
Actin	**36**	**117**	**129**	**147**	**150**	**153**	**198**	**216**	**219**	**249**	**279**	**303**	**321**	**366**	**369**	**372**	**387**	**393**	**414**	**438**	**447**	**453**	
CryBrFRChi71 / 108	A	A	T	A	A	T	G	C	C	A	G	T	T	A	G	C	T	T	G	G	C	G	
C. bovis AY741307.1	G	G	G	G	G	A	A	T	T	T	A	A	C	G	.	T	C	A	A	A	T	A	
C. xiaoi GQ337964.1	.	.	G	G	.	A	A	T	T	G	A	.	C	G	A	T	C	A	A	.	T	.	
	483	**501**	**507**	**540**	**555**	**558**	**576**	**585**	**618**	**624**	**633**	**639**	**666**	**687**	**714**	**723**	**732**	**735**	**744**	**759**	**786**		
CryBrFRChi71 / 108	T	C	G	G	C	G	G	A	A	A	A	C	C	A	T	T	A	A	A	T	C		
C. bovis AY741307.1	.	.	A	A	T	A	A	G	G	G	.	T	.	G	C	C	G	T	G	C	T		
C. xiaoi GQ337964.1	C	T	.	A	T	A	A	G	G	.	G	T	T	.	C	.	G	T	G	C	T		

the 18S rRNA n-PCR reactions were positive for *Cryptosporidium* spp. in 91 (25.9%) of 351 fecal samples

There are few reports of *Cryptosporidium* molecular characterization from chickens in Brazil (HUBER et al., 2007; NAKAMURA et al., 2009) and worldwide (HSU et al., 2008; NG et al., 2006; SILVERLÅS et al., 2012; WANG et al., 2014), however, none of the previously studies reported studies worked with free-range chickens. In Brazil, Huber et al. (2007) and Nakamura et al. (2009) worked with chickens from indoor public markets and chickens under captivity, respectively. The authors from other regions of world isolated *Cryptosporidium* spp. from commercial poultry farms, layer chickens, and pet shops.

In the present study, all positive samples tested at 18S rRNA n-PCR were submitted to sequencing, and 62.6%, 16.4%, 3.2%, and 3.2% were detected as *C. meleagridis, C. baileyi, C. parvum,* and a new genotype named *C.* genotype BrPR1, respectively. Similar results were observed by Baroudi et al. (2013), who studied 90 broiler chickens from 23 farms in Algeria; which found by 18S rRNA gene 28.9% of *C. meleagridis,* and 5.5% of *C. baileyi.* Conversely, *C. baileyi* had been previously reported as the most common *Cryptosporidium* species in chickens (FAYER, 2010). There are few reports in Brazil on the occurrence of *C. baileyi* infecting domestic chickens (CARDOZO et al., 2005; MEIRELES & FIGUEIREDO, 1992). Herein, we observed two clusters in 18S rRNA gene phylogenetic tree from *C. baileyi*, this can be explained by genetic diversity observed in sequences of GenBank.

In a previous study by Nakamura et al. (2009), *C. meleagridis* was found in one sample of a domestic chicken in Brazil. Previous studies performed outside Brazil affirm that infections by *C. baileyi, C. galli* and other parasite genotypes are present in several avian cases. While the number of reports of infection by *C. meleagridis* was low (SRÉTER & VARGA, 2000; WANG et al., 2014), Ng et al. (2006) did not find this species in 430 avian fecal samples, and described that *C. meleagridis* infection has low prevalence in avian hosts. This finding is the opposite what we observed in our results. *C. meleagridis* has a wide host range, and it is the third most prevalent species infecting humans (CAMA et al., 2003; ELWIN et al., 2012; GATEI et al., 2003; GUYOT et al., 2001; LEONI et al., 2006; PEDRAZA-DÍAZ et al., 2000; XIAO, 2010). This species is considered an important human pathogen, accounting for several reports of human infection (DARABUS, 1997; DARABUS & OLARIU, 2003; SRÉTER & VARGA, 2000; XIAO & FENG, 2008). A study in England found *C. meleagridis* in 0.9% of 2,414 human cases (LEONI et al., 2006). Sequence analysis of the 18S rRNA gene and *hsp70* loci has been used to provide evidence of zoonotic transmission of *C. meleagridis* from chickens to humans on a Swedish farm (SILVERLÅS et al., 2012).

C. parvum was detected in three animals from two farms; therefore, animals could be acting as an environment spread host, considering that viable oocysts of *C. parvum* can pass undamaged through the digestive system of several avian hosts (GRACZYK et al., 1996, 1997). Oocysts of *C. parvum* were detected in feces of wild Canada geese (*Branta canadensis*) (GRACZYK et al., 1998), and it was demonstrated experimentally to cause a weak tracheal infection in 1- and 7-day-old chickens (LINDSAY et al., 1987). However, molecular detection of *C. parvum* in feces of chickens is uncommon (BAROUDI et al., 2013; HUBER et al., 2007; NAKAMURA et al., 2009; NG et al., 2006)

In the present work, sequencing of 18S rRNA amplicons allowed the identification of a new *Cryptosporidium* genotype in three samples, and consequently we performed *actin* and *hsp70* n-PCR and sequencing of the identified isolates. The results showed identities of these genes with *C. bovis* between 95% to 98%. Thus, because of such genetic distance with *C. bovis, C. ryanae,* and *C. xiaoi* we named this new genotype as *Cryptosporidium* genotype PR1.

There were no significant differences in all variables studied here that could be associated to cryptosporidiosis. It is known that water can act as a transmission route of oocysts to birds and acts as a major source of oocysts and human outbreaks in developed countries, including North America, the United Kingdom, and Japan (FAYER, 2004). In the present work, although no significance was observed, the farms that were provided with deep water showed a reduction in positive animals (20%) when compared with surface water (30%).

Conclusion

Cryptosporidium, including zoonotic species, exists at a high prevalence in free-range chickens. A high prevalence of farms positive for *Cryptosporidium*, mainly *C. meleagridis*, shows us the importance of the chicken as an environmental indicator for this zoonotic protozoa. Other studies must be performed to better elucidate the role of chickens in the dissemination of *Cryptosporidium* species that infect cattle. We described a new genotype of C*ryptosporidum* which was named *C.* genotype BrPR1.

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