Preventive Veterinary Medicine 151 (2018) 73–78

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Detection and characterization of *Cryptosporidium* species and genotypes in three chicken production systems in Brazil using different molecular diagnosis protocols

Bruna Nicoleti Santana[a], Brayan Kurahara[a], Alex Akira Nakamura[b], Vinícius da Silva Camargo[a], Elis Domingos Ferrari[a], Giane Serafim da Silva[c], Walter Bertequini Nagata[a], Marcelo Vasconcelos Meireles[a,*]

[a] *Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba, Rua Clóvis Pestana, 793, CEP 16050-680, Bairro Dona Amélia, Araçatuba, SP, Brazil*

[b] *Faculdades Adamantinenses Integradas (FAI), Adamantina, Brazil*

[c] *Instituto Biológico, Agência Paulista de Tecnologia Agropecuária, Votuporanga, Brazil*

ARTICLE INFO

Keywords:
Cryptosporidiosis
Diagnosis
Molecular characterization
Domestic chicken
Brazil

ABSTRACT

The objective of this study was to determine the occurrence of *Cryptosporidium* spp. in domestic chickens raised in different chicken production systems in Brazil using three nested PCR protocols. The purification and concentration of oocysts present in 190 fecal samples from chickens raised in extensive, semi-intensive and intensive production systems were accomplished by centrifugal flotation in Sheather's solution and were followed by the extraction of genomic DNA. The detection and molecular characterization of *Cryptosporidium* species and genotypes were performed using three nested polymerase chain reaction (nested PCR) protocols targeting the 18S rRNA gene followed by sequencing of the amplified fragments. Subgenotyping of *C. meleagridis* was performed using a nested PCR reaction targeting the gp60 gene. Sample identified as *Cryptosporidium* sp. genetically similar to *Cryptosporidium xiaoi* and *Cryptosporidium bovis* by 18S rRNA gene sequencing were further analyzed by nested PCR targeting the actin gene and subsequent sequencing of the amplified fragment. Positive amplification for *Cryptosporidium* spp. was observed in 12.6% (24/190) of the samples, including *C. baileyi* (9.8%; 18/190), *C. meleagridis* (0.5%, 1/190), *C. parvum* (2.1%; 4/190) and *Cryptosporidium* sp. (0.5%; 1/190). Subgenotyping of *C. meleagridis* revealed the presence of the zoonotic subtype IIIgA23G3R1. Sequencing of the 18S rRNA gene and the actin gene fragments revealed a *Cryptosporidium* genotype in an extensive poultry system genetically related to *C. xiaoi* and *C. bovis*. There was no significant difference in the frequency of positive results obtained by the three nested PCR protocols ($p > 0.05$); additionally, the agreement obtained by Kappa index ranged from substantial (0.70) to almost perfect (0.9).

1. Introduction

Cryptosporidiosis is one of the most prevalent protozoan infections in birds, and it manifests as a respiratory or digestive disease (Ryan, 2010; Nakamura and Meireles, 2015). Among the 31 species of *Cryptosporidium* that infect several vertebrates, only four infect birds: *C. meleagridis*, *C. baileyi*, *C. galli* and *C. avium* (previously known as avian genotype V) (Holubová et al., 2016; Ryan 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 (Ng et al., 2006), IV (Ng et al., 2006), VI (Chelladurai et al., 2016), VII, VIII and IX (Helmy et al., 2017), the goose genotypes I–IV (Jellison et al., 2004; Zhou et al., 2004), the black duck genotype (Morgan et al., 2001) and the Eurasian woodcock genotype (Ryan et al., 2003) infecting birds.

The literature related to the occurrence of *Cryptosporidium* infection in avian species is scarce, most likely because the techniques used to diagnose cryptosporidiosis are not routinely adopted in avian pathology laboratories. Infections by *C. baileyi*, *C. galli,* and other avian *Cryptosporidium* species/genotypes are present in a wide variety of bird species; in contrast, *C. meleagridis* has a more restricted number of avian hosts (Sréter and Varga, 2000; Ng et al., 2006; Qi et al., 2011; Nakamura and Meireles, 2015). There is only one report of *C. parvum* infection associated with clinical signs in birds (Zylan et al., 2008).

* Corresponding author.
E-mail address: marcelo@fmva.unesp.br (M.V. Meireles).

https://doi.org/10.1016/j.prevetmed.2018.01.007
Received 12 September 2017; Received in revised form 23 January 2018; Accepted 26 January 2018

Table 1
Nested PCR protocols used for the detection of *Cryptosporidium* spp. in domestic chicken fecal samples.

Protocols		Primers 5′-3′ sequences	Amplified product (bp)	References
Protocol 1	PCR	GGAAGGGTTGTATTTATTAGATAAAG CTCATAAGGTGCTGAAGGAGTA	~ 848	Xiao et al. (1999) Jiang et al. (2005)
	Nested PCR	AAGCTCGTAGTTGGATTTCTG TAAGGTGCTGAAGGAGTAAGG	~ 425	Johnson et al. (1995)
Protocol 2	PCR	GACATATCATTCAAGTTTCTGACC CTGAAGGAGTAAGGAACAACC	~ 761	Ryan et al. (2003)
	Nested PCR	CCTATCAGCTTTAGACGGTAGG TCTAAGAATTTCACCTCTGACTG	~ 585	
Protocol 3	PCR	TTCTAGAGCTAATACATGCG CCCATTTCCTTCGAAACAGGA	~ 1318	Xiao et al. (1999, 2000)
	Nested PCR	GGAAGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACCTCCA	~ 835	

Most reports of cryptosporidiosis in domestic chickens are related to *C. baileyi*, which infects the respiratory tract, bursa of Fabricius and cloaca (Current et al., 1986), and less frequently to *C. meleagridis*, which is restricted to the small intestine (Sréter and Varga, 2000). *C. galli* and *C. avium*, as well as the avian genotypes of *Cryptosporidium*, are rarely described in domestic chickens (Nakamura and Meireles, 2015).

The importance of cryptosporidiosis in domestic chickens is unclear. Snyder et al. (1988) reported that the prevalence of antibodies to *Cryptosporidium* sp. in broiler chickens was 24% per flock in 18 flocks. Infection with *C. baileyi* in broiler chickens has been related to decreased weight gain, higher incidence of airsacculitis, increased mortality, and higher carcass condemnation rates in slaughterhouses (Gorham et al., 1987; Goodwin et al., 1996). *C. meleagridis* infection in domestic chickens is asymptomatic (Nakamura and Meireles, 2015).

In Brazil, there have been several reports of natural infection by *C. baileyi* in chickens (Meireles and Figueiredo, 1992; Huber et al., 2007). Using experimental infection with a Brazilian isolate of *C. baileyi*, Meireles et al. (1998a, b, 1999) found that domestic chickens developed clinical signs and macroscopic lesions related to the respiratory tract, as well as macroscopic lesions in the bursa of Fabricius.

C. meleagridis is a parasite of the intestinal epithelial cells in several species of birds, particularly turkeys (Slavin, 1955; Sréter and Varga, 2000), and it is the only avian species of *Cryptosporidium* with zoonotic potential (Chalmers and Giles, 2010). In some countries, the occurrence of *C. meleagridis* infection in humans is similar or superior to that of *C. parvum* infections (Xiao et al., 2001; Cama et al., 2003). In Brazil, *C. meleagridis* has been found in fecal samples from domestic chickens (Huber et al., 2007; Nakamura et al., 2009) and humans (Araújo et al., 2008).

Cryptosporidium species can be identified using molecular methods, such as polymerase chain reaction (PCR), followed by sequencing of the amplified fragments. The most commonly used protocol is nested PCR targeting the 18S rRNA gene, developed by Xiao et al. (1999, 2000). However, Mirhashemi et al. (2015) compared three nested PCR protocols using equine, bovine and ovine samples and found that the protocol developed by Ryan et al. (2003) was the most sensitive. There are no studies on the effectiveness of different molecular diagnostic techniques for the detection of *Cryptosporidium* spp. in avian fecal samples.

Also, there are few studies related to the prevalence of *Cryptosporidium* spp. in chickens from extensive, semi-intensive and intensive production systems (Nakamura and Meireles, 2015). In addition to presenting clinical disease when infected with *Cryptosporidium*, domestic chickens may be a source of infection of zoonotic species of this protozoan, such as *C. meleagridis* and *Cryptosporidium* species and genotypes adapted to mammals. The present work aimed to determine the occurrence of *Cryptosporidium* spp. in domestic chickens raised in different production systems and to perform molecular characterization of *Cryptosporidium* spp. using three nested PCR protocols and sequencing of the amplified fragments.

2. Material and methods

2.1. Fecal samples

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 domestic chickens (*Gallus gallus domesticus*) in 20 municipalities of the State of São Paulo, Brazil. According to availability, the samples were collected in 61 chicken farms with extensive (24), semi-intensive (8), intensive-layers (25), and intensive-broilers (4) production systems. Mixed types of chickens (broilers and layers) were raised in farms with extensive and semi-intensive production systems. Samples consisted of 190 fecal pools (each containing 10–20 g of feces) formed from the collection of up to 5–10 single fecal droppings per farm with extensive and semi-intensive production systems. In intensive production systems, one fecal pool was collected from up to four flocks.

In the extensive poultry production systems, it was not possible to identify the age of the birds at the time of collection. In the semi-intensive and intensive-layers production systems, the birds were all adults or were 8–16 weeks old, respectively. Broiler chickens from intensive production systems were 35–46 days old.

The samples consisted of freshly discarded feces collected using a disposable wooden spatula and were preserved in 2.5% potassium dichromate at 4 °C. The concentration and purification of oocysts were performed by centrifugal-flotation using Sheather's sugar flotation solution.

2.2. Molecular characterization

Genomic DNA from oocysts was extracted from purified fecal sediment (Boom et al., 1990; McLauchlin et al., 2000). Three nested PCR protocols targeting the 18S rRNA gene were used for the detection of *Cryptosporidium* spp. (Table 1). Samples that were identified as *C. meleagridis* and *Cryptosporidium* sp. by sequencing of the 18S rRNA amplicons were subjected to nested PCR targeting the gp60 gene (Stensvold et al., 2014) or the actin gene (Sulaiman et al., 2002), respectively. Genomic DNA from *C. parvum* and ultrapure water were used as positive and negative controls, respectively.

2.3. DNA sequence analysis

The nested PCR amplicons were purified using the Illustra ExoProStar 1-Step® (GE Healthcare Life Sciences) or the QIAquick®Gel Extraction Kit (Qiagen), following the manufacturer's guidelines. Sequencing was accomplished using the ABI Prism® Dye Terminator Cycling Sequence kit (Applied Biosystems) in an Automatic sequencer ABI 3730XL (Applied Biosystems).

DNA sequences were assembled with Codoncode Aligner version

7.1.1 software (CodonCode Corporation). The consensus sequences were aligned with homologous sequences published in GenBank using Clustal W (Thompson et al., 1997) and BioEdit Sequence Alignment Editor (Hall, 1999). Phylogenetic trees were generated using Maximum Likelihood analysis based on the General Time Reversible model (Nei and Kumar, 2000) in MEGA7 (Kumar et al., 2016) using *Cryptosporidium molnari* as an outgroup.

2.4. Nucleotide sequence accession number

Nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MF627416 through MF627421.

2.5. Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2010, and results were considered significant when $p < 0.05$. Frequency analysis (McNemar test) and the Kappa correlation coefficient (Landis and Koch, 1977) were calculated to verify the difference in positive rates between each paired nested PCR protocols and the agreement between the diagnostic methods, respectively. Prevalence rates with 95% confidence intervals were calculated using Wilson (score) intervals (Sergeant, 2017).

3. Results

The frequency of positive *Cryptosporidium* spp. detection by the three nested PCR protocols was 24/190 (12.6%; 95% confidence interval: 8.6–18.1%). Sequencing of the 18S rRNA amplicons identified *C. baileyi* (9.5%; 18/190), *C. meleagridis* (0.5%; 1/190), *C. parvum* (2.1%; 4/190) and *Cryptosporidium* sp. (1/190). The sequences from *C. baileyi*, *C. meleagridis* and *C. parvum* had 100% genetic similarity with sequences previously published in GenBank (AJ276096, AF112574, AF093490). The sequence from *Cryptosporidium* sp. had 100% genetic similarity with a sequence from environmental *Cryptosporidium* sp. (EU825742) (Yang et al., 2008).

Two out of four valid *Cryptosporidium* species from birds, in addition to *C. parvum* and *Cryptosporidium* sp., were identified in farms with different production systems (Table 2). In farms with extensive systems, *C. parvum* (16.7%; 4/24), *C. baileyi* (4.2%; 1/24), and *Cryptosporidium* sp. (4.2%; 1/24) were identified. In the semi-intensive systems, 25% of farms (2/8) were positive for *C. baileyi*. In the intensive systems (layers and broilers), 51.7% of farms (15/29) were positive for *C. baileyi*, and 3.4% of farms (1/29) were positive for *C. meleagridis*.

Statistical analyses related to variables type of chickens, production systems or *Cryptosporidium* species were not performed, since the convenience sampling adopted in our study resulted in small and very different sample sizes, among production systems and chicken types, to provide the statistical power required to detect a meaningful difference between study variables. Moreover, any statistically significant difference would be of very little use owing to the wide confidence intervals (Table 2).

GP60 subgenotyping of *C. meleagridis* revealed the zoonotic subtype IIIgA23G3R1. Ribosomal RNA gene sequence from *Cryptosporidium* sp. found in an extensive production system has already been described in an environmental sample (Yang et al., 2008). Here, we designated this genotype as *Cryptosporidium xiaoi*-like, owing to its genetic similarity at the actin gene with *C. xiaoi* (GQ337964) and *C. bovis* (AY741307) of 96% and 95%, respectively. At the 18S rRNA gene, *C. xiaoi*-like presented genetic similarity with *C. xiaoi* (FJ896043) and *Cryptosporidium bovis* (DQ871346) of 99% and 99%, respectively, and it clustered in a separate clade from those two species in the phylogenetic tree (Fig. 1).

The results from the McNemar test showed no significant differences in the number of samples diagnosed as positive for *Cryptosporidium* spp. among the three nested PCR protocols (Table 3): protocols 1 and 2 ($p = 0.3438$), 1 and 3 ($p = 0.1797$) and 2 and 3 ($p = 1$). The agreement obtained by Kappa correlation coefficient ranged from substantial to almost perfect: protocols 1 and 2 (0.70), 1 and 3 (0.74) and 2 and 3 (0.90).

4. Discussion

In the present study, the two most frequently reported species in Galliformes, *C. baileyi* and *C. meleagridis,* were identified in domestic chickens. Although there have been reports in Brazil concerning the occurrence of *C. baileyi* in domestic chickens (Meireles and Figueiredo, 1992; Cardozo et al., 2005; Nakamura et al., 2009), this is the first report of molecular identification in chickens reared under extensive, semi-intensive and intensive production systems in Brazil.

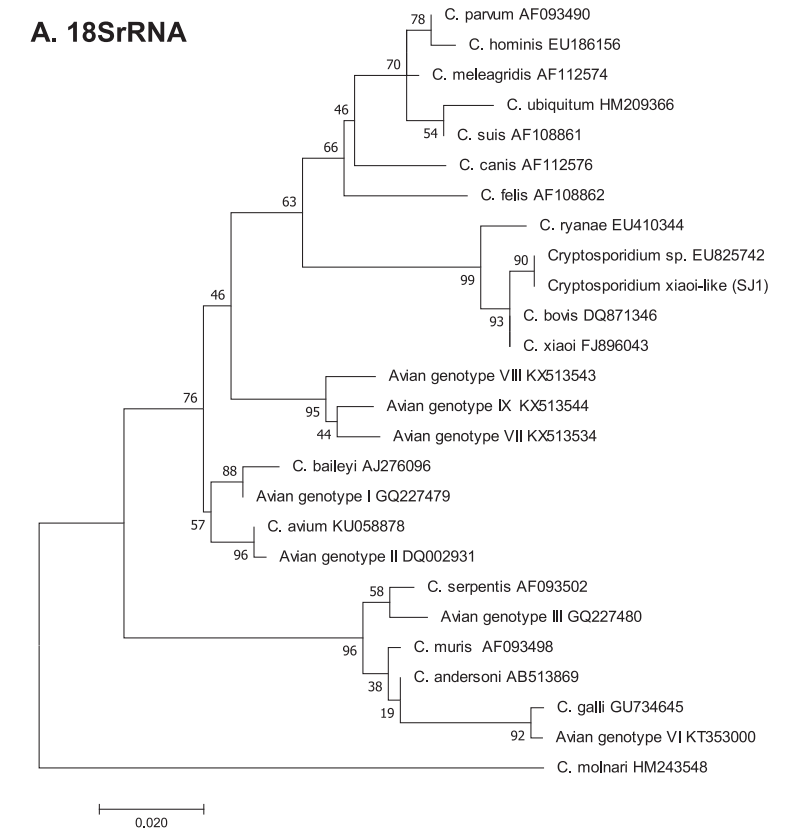
In Brazil, *C. parvum* has been found in fecal samples from *Nymphicus hollandicus* (Nakamura et al., 2009), *G. g. domesticus, Coturnix coturnix japonica* (Bomfim et al., 2013), *Lonchura striata domestica* (Gomes et al., 2012) and *Columba livia* (Oliveira et al., 2017). *C. parvum* is not a major cause of disease in birds, although there are many reports of detection of *C. parvum* DNA in fecal samples of asymptomatic birds from several avian species (Nakamura et al., 2009; Reboredo-Fernández et al., 2015; Helmy et al., 2017; Oliveira et al., 2017). The detection of *C. parvum*, the most frequent species found in ruminants, and *C. xiaoi*-like suggests that chickens, in both cases, were probably mechanical carriers of oocysts (Majewska et al., 2009), since they were raised in extensive production systems and cohabited with cattle. However, *C. parvum* has also been detected in fecal samples from chickens and turkeys raised under intensive production systems with no previous contact with fecal samples from mammals (Helmy et al., 2017), which raises concern that poultry could act as a source of infection of other zoonotic *Cryptosporidium* species, in addition to *C. meleagridis*.

The low rate of detection of *C. meleagridis* corroborated the results of Wang et al. (2014) who reported a prevalence of 5.3% (2/38) for *C. meleagridis* infection in domestic chickens in China. However, Laatamna et al. (2017) found a prevalence of 35.5% (11/31) for *C. meleagridis* in domestic chickens in Algeria. In Brazil, *C. meleagridis* has been

Table 2
Detection of *Cryptosporidium* spp. in fecal samples from domestic chickens raised in different production systems in the State of São Paulo, Brazil using nested PCR targeting the 18S rRNA gene and the subsequent sequencing of amplified fragments.

Production system	N° positive farms/n° sampled (% positive; 95% CI*)	Species identification – n° positive farms/n° sampled (% positive; 95% CI)
Extensive	6/24 (25; 12–44.9%)	*C. parvum* – 4/24 (16.7; 6.9–35.8%) *C. baileyi – 1/24 (4.2; 0.7–20.2%)* *C. xiaoi-like – 1/24 (4.2; 0.7–20.2%)*
Semi-intensive	2/8 (25; 7.2–59.1%)	*C. baileyi* – 2/8 (25; 7.15–59.1%)
Intensive-layers	15/25 (60; 40.7–76.6%)	*C. baileyi* – 14/25 (51.7; 37.1–73.3) *C. meleagridis – 1/25 (3.4; 0.7–19.5)*
Intensive-broilers	1/4 (25; 4.6–69.9%)	*C. baileyi* 1/4 – (25; 4.6–69.9)
Total	24/61 (39.3; 28.0–51.8%)	

* 95% Confidence Interval.



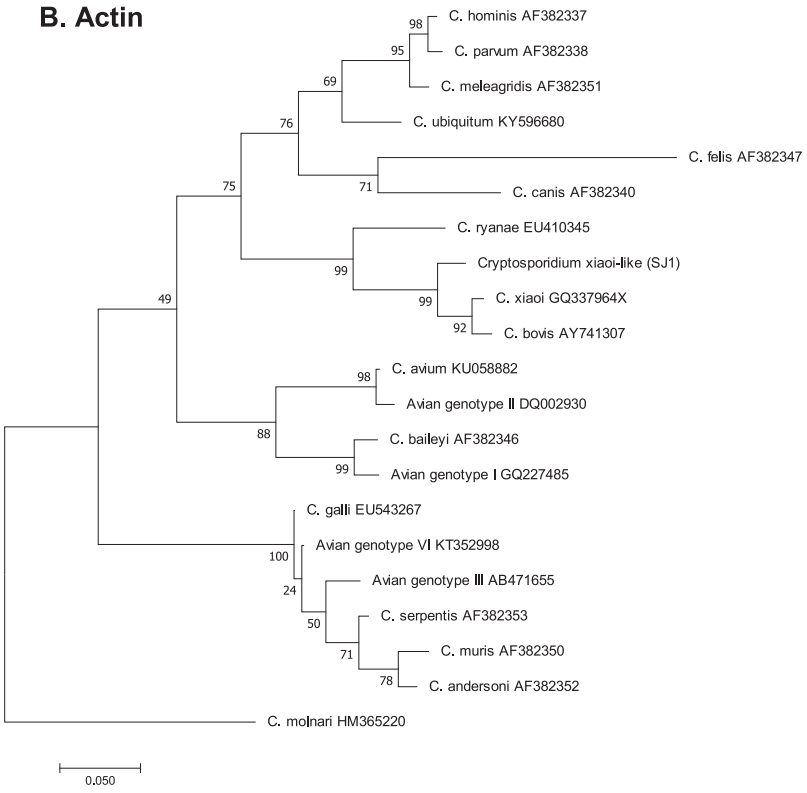


Fig. 1. Phylogenetic trees of the 18SrRNA (A) and actin (B) genes sequences (477 and 721 base positions in the final 18S rRNA and actin datasets, respectively) from *Cryptosporidium xiaoi*-like (SJ1) and selected *Cryptosporidium* species. The evolutionary history was inferred by a Maximum Likelihood analysis based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log-likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The trees were rooted with sequences from *Cryptosporidium molnari*. Numbers on the left of the supported nodes indicate the bootstrap values (1000 replicates). Scale-bar represents the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

described in domestic chickens (Huber et al., 2007; Nakamura et al., 2009) and in humans (Araújo et al., 2008).

C. meleagridis is an important zoonotic species that infects the intestinal epithelial cells in both birds and mammals (Chalmers and Gilles, 2010). GP60 subgenotyping demonstrated that the *C. meleagridis* subgenotype described in this report, which was previously detected

Table 3
Identification of *Cryptosporidium* spp. in fecal samples from domestic chickens using three nested PCR protocols targeting the 18S rRNA gene followed by the sequencing of the amplified fragments.

Sample	Species identification		
	Protocol 1	Protocol 2	Protocol 3
SJ1	–	*Cryptosporidium* sp.	*Cryptosporidium* sp.
P3 26	*C. baileyi*	–	–
A02	*C. meleagridis*	–	–
NS7	*C. baileyi*	–	–
SL1	*C. parvum*	–	*C. parvum*
NS6	*C. parvum*	–	–
NS4	*C. parvum*	–	–
P3 02	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 01	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 03	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 09	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 10	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 11	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 08	*C. baileyi*	*C. baileyi*	*C. baileyi*
P320	*C. baileyi*	–	–
P3 07	*C. baileyi*	*C. baileyi*	*C. baileyi*
P62	*C. baileyi*	*C. baileyi*	*C. baileyi*
P62FR	*C. baileyi*	*C. baileyi*	*C. baileyi*
P68 Mal	*C. baileyi*	*C. baileyi*	*C. baileyi*
SM9	*C. parvum*	*C. parvum*	*C. parvum*
P21	*C. baileyi*	*C. baileyi*	–
P30	*C. baileyi*	*C. baileyi*	*C. baileyi*
P3 04	–	*C. baileyi*	*C. baileyi*
P3 05	–	*C. baileyi*	*C. baileyi*

only in humans (Abal-Fabeiro et al., 2013; Stensvold et al., 2014), also infects Brazilian chickens.

In previous works developed in our laboratory, we found nonspecific amplification of *Eimeria* and *Isospora* DNA using the nested PCR protocol described by Ryan et al. (2003), in DNA extracted from fecal samples from rabbits and canaries, respectively. Those findings motivated our comparison of Ryań protocol with other nested PCR protocols for the detection of *Cryptosporidium* spp., but in the present study, no nonspecific amplification was observed in any of the protocols tested.

The forward primer used in the nested PCR of protocol 1 (Johnson et al., 1995) contains four substitutions in the annealing region with *C. bovis*, *C. xiaoi,* and *Cryptosporidium ryanae* sequences, which may explain the negative amplification obtained with the use of DNA from *C. xiaoi*-like genotype.

Despite the absence of statistical significance, protocol 1 (Johnson et al., 1995; Xiao et al., 1999, 2000; Jiang et al., 2005) detected slightly more positive samples than the other two protocols (Table 3). In contrast to the report of Mirhashemi et al. (2015), we found no difference in the positivity rates between protocols 2 (Ryan et al., 2003) and 3 (Xiao et al., 1999, 2000). Protocol 3 is the most widely used for the detection of *Cryptosporidium*. However, since the amplified fragments in the primary and secondary reactions are long, ~1318 bp and ~835 bp, respectively, the efficiency of this method is supposedly inferior to those that amplify smaller fragments (Piyamongkol et al., 2003; Chakravorty et al., 2006). The difference in the size of amplicons justifies the results obtained by Mirhashemi et al. (2015) and the results observed in this experiment with the use of protocol 1, which amplifies fragments of ~848 and ~425 bp in the primary and secondary reactions, respectively.

5. Conclusion

C. meleagridis was genotyped for the first time in Brazil, revealing the zoonotic subtype IIIgA23G3R1 in an intensive poultry production system. *C. baileyi* was the most frequently detected species in the feces of domestic chickens. Domestic chickens from extensive production systems may carry the zoonotic species *C. parvum* in feces. A genotype of *Cryptosporidium* genetically similar to two *Cryptosporidium* species of ruminants, *C. xiaoi* and C. *bovis*, was found in chickens from an extensive production system. No significant differences were observed among the three nested PCR protocols for the detection of *Cryptosporidium* spp.

Acknowledgements

We would like to thank the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP) for the financial support (2015/26334-8), the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES) for a master's degree scholarship awarded to B.N. Santana and Daiane Mompean Romera for the assistance during the collection of fecal samples.

References

Abal-Fabeiro, J.L., Maside, X., Bello, X., Llovo, J., Bartolomé, C., 2013. Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the gp60 locus. Mol. Ecol. 22, 4723–4732. http://dx.doi.org/10.1111/mec.12425.

Araújo, A.J., Kanamura, H.Y., Almeida, M.E., Gomes, A.H., Pinto, T.H., Da Silva, A., 2008. Genotypic identification of *Cryptosporidium* spp: isolated from HIV-infected patients and immunocompetent children of Súo Paulo, Brazil. Rev. Inst. Med. Trop. 50, 139–143.

Bomfim, T.C.B., Gomes, R.S., Huber, F., Couto, M.C.M., 2013. The importance of poultry in environmental dissemination of *Cryptosporidium* spp. Open Vet. Sci. J. 7, 12–17.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wetheim-van Dillen, P.M.E., Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. Clin. Microbiol. 28, 495–503.

Cama, V.A., Bern, C., Sulaiman, I.M., Gilman, R.H., Ticona, E., Vivar, A., Kawai, V., Vargas, D., Zhou, L., Xiao, L., 2003. Cryptosporidium species and genotypes in HIV-positive patients in Lima, Peru. J. Eukaryot. Microbiol. 50, 531–533. http://dx.doi.org/10.1111/j.1550-7408.2003.tb00620.x.

Cardozo, S.V., Teixeira Filho, W.L., Lopes, C.W.G., 2005. Transmissão experimental de *Cryptosporidium baileyi* (Apicomplexa: Cryptosporidiidae) isolado de frango de corteà codorna Japonesa (*Coturnix japonica*). Rev. Bras. Parasitol. Vet. 14, 119–124.

Chakravorty, S., Pathak, D., Dudeja, M., Haldar, S., Hanif, M., Tyagi, J.S., 2006. PCR amplification of shorter fragments from the devR (Rv3133c) gene significantly increases the sensitivity of tuberculosis diagnosis. FEMS Microbiol. Lett. 257, 306–311. http://dx.doi.org/10.1111/j.1574-6968.2006.00187.x.

Chalmers, R.M., Giles, M., 2010. Zoonotic cryptosporidiosis in the UK – challenges for control. J. Appl. Microbiol. 109, 1487–1497. http://dx.doi.org/10.1111/j.1365-2672.2010.04764.x.

Chelladurai, J.J., Clark, M.E., Kváč, M., Holubová, N., Khan, E., Stenger, B.L., Giddings, C.W., McEvoy, J., 2016. *Cryptosporidium galli* and novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds (*Agelaius phoeniceus*). Parasitol. Res. 115, 1901–1906. http://dx.doi.org/10.1007/s00436-016-4930-8.

Current, W.L., Upton, S.J., Haynes, T.B., 1986. The life cycle of *Cryptosporidium baileyi* n.s. (Apicomplexa: Cryptosporidiidae) infecting chickens. J. Protozool. 33, 289–296.

Gomes, R.S., Huber, F., Silva, S., Bomfim, C.B., 2012. *Cryptosporidium* spp. parasitize exotic birds that are commercialized in markets, commercial aviaries, and pet shops. Parasitol. Res. 110, 1363–1370. http://dx.doi.org/10.1007/s00436-011-2636-5.

Goodwin, M.A., Brown, J., Resurrecion, R.S., Smith, A.J., 1996. Respiratory coccidiosis (*Cryptosporidium baileyi*) among Northern Georgia broilers in one company. Avian Dis. 40, 572–575. http://dx.doi.org/10.2307/1592266.

Gorham, S.L., Mallinson, E.T., Snyder, D.B., Odor, E.M., 1987. Cryptosporidia in the bursa of Fabricius – a correlation with mortality rates in broiler chickens. Avian Pathol. 16, 205–211. http://dx.doi.org/10.1080/03079458708436369.

Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Helmy, Y.A., Krücken, J., Abdelwhab, E.M., von Samson-Himmelstjerna, V., Hafez, H.M., 2017. Molecular diagnosis and characterization of *Cryptosporidium* spp. in turkeys and chickens in Germany reveals evidence for previously undetected parasite species. PLoS One 12 (6), e0177150.

Holubová, N., Sak, B., Horčičková, M., Hlásková, L., Květoňová, D., Menchaca, S., McEvoy, J., Kváč, M., 2016. *Cryptosporidium avium* n. sp. (Apicomplexa: cryptosporidiidae) in birds. Parasitol. Res. 115, 2243–2251. http://dx.doi.org/10.1007/s00436-016-4967-8.

Huber, F., da Silva, S., Bomfim, T.C.B., Teixeira, K.R.S., Bello, A.R., 2007. Genotypic characterization and phylogenetic analysis of *Cryptosporidium* sp. from domestic animals in Brazil. Vet. Parasitol. 150, 65–74.

Jellison, K.L., Distel, D.L., Hemond, H.F., Schauer, D.B., 2004. Phylogenetic analysis of the hypervariable region of the 18S rRNA gene of *Cryptosporidium* oocysts in feces of Canada geese (*Branta canadensis*): evidence for five novel genotypes. Appl. Environ. Microbiol. 70, 452–458. http://dx.doi.org/10.1128/AEM.70.1.452-458.2004.

Jiang, J., Alderisio, K.A., Xiao, L., 2005. Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl. Environ. Microbiol. 71, 4446–4454.

Johnson, D.W., Pieniazek, N.J., Griffin, D.W., Misener, L., Rose, J.B., 1995. Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl. Environ. Microbiol. 61, 3849–3855.
Kumar, S., Stecher, G., Tamura, K., 2016. Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Laatamna, A.E., Holubová, N., Sak, B., Kvác, M., 2017. *Cryptosporidium meleagridis* and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria. Folia Parasitol. 64, 018.
Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.
Majewska, A.C., Graczyk, T.K., Słodkowicz-Kowalska, A., Tamang, L., Jędrzejewski, S., Zduniak, P., Solarczyk, P., Nowosad, A., Nowosad, P., 2009. The role of free-ranging, captive, and domestic birds of Western Poland in environmental contamination with *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts. Parasitol. Res. 104, 1093–1099.
McLauchlin, J., Amar, C., Pedraza-Díaz, S., Nichols, G.L., 2000. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. J. Clin. Microbiol. 38, 3984–3990.
Meireles, M.V., Figueiredo, P.C., 1992. Isolamento e identificação de *Cryptosporidium baileyi*, current et alii, 1986 (Apicomplexa: Cryptosporidiidae) em frangos de corte. Rev. Bras. Parasitol. Vet. 1 (2), 125–130.
Meireles, M.V., Paulillo, A.C., Silva, G.S., Costa, A.J., Doretto Júnior, L., Machado, R.Z., 1998a. Infecção experimental por *Cryptosporidium baileyi* em aves de corte – aspectos clínicos, parasitológicos e zootécnicos. Rev. Bras. Parasitol. Vet. 7, 11–14.
Meireles, M.V., Paulillo, A.C., Silva, G.S., Costa, A.J., Luvizotto, M.C.R., Júnior, Doretto, Ávila, F.A., 1998b. Influência da infecção experimental com *Cryptosporidium baileyi* na resposta imune de frangos de corte vacinados contra a doença de Newcastle. Rev. Bras. Cienc. Vet. 5, 15–19.
Meireles, M.V., Paulillo, A.C., Silva, G.S., Luvizotto, M.C.R., Costa, A.J., Andreatti Filho, R.L., 1999. Experimental infection with *Cryptosporidium baileyi* in floor-pen raised broilers. Rev. Bras. Cienc. Avic. 1, 37–42.
Meireles, M.V., Soares, R.M., dos Santos, M.M.A.B., Gennari, S.M., 2006. Biological studies and molecular characterization of a *Cryptosporidium* isolate from ostriches (*Struthio Camelus*). J. Parasitol. 92, 623–626.
Mirhashemi, M.E., Zintl, A., Grant, T., Lucy, F.E., Mulcahy, G., De Waal, T., 2015. Comparison of diagnostic techniques for the detection of *Cryptosporidium* oocysts in animal samples. Exp. Parasitol. 151–152, 14–20. http://dx.doi.org/10.1016/j.exppara.2015.01.018.
Morgan, U.M., Monis, P.T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B.L., Lal, A.A., Thompson, R.C.A., 2001. Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. Int. J. Parasitol. 31, 289–296. http://dx.doi.org/10.1016/S0020-7519(00)00164-8.
Nakamura, A.A., Meireles, M.V., 2015. Cryptosporidium infections in birds – a review. Rev. Bras. Parasitol. Vet. 24, 253–267. http://dx.doi.org/10.1590/S1984-29612015063.
Nakamura, A.A., Simões, D.C., Antunes, R.G., da Silva, D.C., Meireles, M.V., 2009. Molecular characterization of *Cryptosporidium* spp. from fecal samples of birds kept in captivity in Brazil. Vet. Parasitol. 166, 47–51. http://dx.doi.org/10.1016/j.vetpar.2009.07.033.
Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.
Ng, J., Pavlasek, I., Ryan, U., 2006. Identification of novel *Cryptosporidium* genotypes from avian hosts. Appl. Environ. Microbiol. 72, 7548–7553. http://dx.doi.org/10.1128/AEM. 01352-06.
Oliveira, B.C.M., Ferrari, E.D., Panegossi, M.F.C., Nakamura, A.A., Corbucci, F.S., Nagata, W.B., Santos, B.M., Gomes, J.F., Meireles, M.V., Widmer, G., Bresciani, K.D.S., 2017. First description of *Cryptosporidium parvum* in carrier pigeons (*Columba livia*). Vet. Parasitol. 243, 148–150.
Piyamongkol, W., Bermudez, M.G., Harper, J.C., Wells, D., 2003. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. Mol. Hum. Reprod. 9, 411–420.
Qi, M., Wang, R., Ning, C., Li, X., Zhang, L., Jian, F., Sun, Y., Xiao, L., 2011. *Cryptosporidium* spp. in pet birds: genetic diversity and potential public health significance. Exp. Parasitol. 128, 336–340.
Reboredo-Fernández, A., Ares-Mazás, E., Cacciò, S.M., Gómez-Couso, H., 2015. Occurrence of *Giardia* and *Cryptosporidium* in wild birds in galicia (Northwest Spain). Parasitology 146, 917–925.
Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307. http://dx.doi.org/10.1645/GE-74RI.
Ryan, U., Zahedi, A., Paparini, A., 2016. *Cryptosporidium* in humans and animals – a one health approach to prophylaxis. Parasite Immunol. 38, 535–547. http://dx.doi.org/10.1111/pim.12350.
Ryan, U., 2010. *Cryptosporidium* in birds, fish and amphibians. Exp. Parasitol. 124, 113–120.
Santos, M.M.A.B., Peiró, J.R., Meireles, M.V., 2005. *Cryptosporidium* infection in ostriches (*Struthio camelus*) in Brazil: clinical, morphological and molecular studies. Rev. Bras. Cienc. Avic. 7, 113–117.
Sergeant, E.S.G., 2017. Epitools Epidemiological Calculators. Ausvet Pty Ltd.. Available at: http://epitools.ausvet.com.au.
Slavin, D., 1955. *Cryptosporidium meleagridis* (sp.nov.). J. Comp. Pathol. 65, 262–266. http://dx.doi.org/10.1016/S0368-1742(55)80025-2.
Snyder, D.B., Current, W.L., Russek-Cohen, E., Gorham, S.L., Mallinson, E.T., Marquardt, W.W., Savage, P.K., 1988. Serologic incidence of *Cryptosporidium* in Delmarva broiler flocks. Poult. Sci. 67, 730–735. http://dx.doi.org/10.3382/ps.0670730.
Sréter, T., Varga, I., 2000. Cryptosporidiosis in birds – a review. Vet. Parasitol. 87, 261–279. http://dx.doi.org/10.1016/S0304-4017(99)00178-8.
Stensvold, C.R., Beser, J., Axen, C., Lebbad, M., 2014. High applicability of a novel method for gp60-based subtyping of *Cryptosporidium meleagridis*. J. Clin. Microbiol. 52, 2311–2319. http://dx.doi.org/10.1128/JCM.00598-14.
Sulaiman, I.M., Lal, A.A., Xiao, L., 2002. Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. J. Parasitol. 88, 388–394. http://dx.doi.org/10.1645/0022-3395(2002)088[0388:MPAERO]2.0. CO;2.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Wang, L., Xue, X., Li, J., Zhou, Q., Yu, Y., Du, A., 2014. Cryptosporidiosis in broiler chickens in Zhejiang Province, China: molecular characterization of oocysts detected in fecal samples. Parasite 21, 36. http://dx.doi.org/10.1051/parasite/2014035.
Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C.A., Fayer, R., Lal, A.A., 1999. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. Appl. Environ. Microbiol. 65, 3386–3391.
Xiao, L., Alderisio, K., Limor, J., Royer, M., Lal, A.A., 2000. Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl. Environ. Microbiol. 66, 5492–5498.
Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H., Lal, A.A., 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. 183, 492–497. http://dx.doi.org/10.1086/318090.
Yang, W., Chen, P., Villegas, E.N., Landy, R.B., Kanetsky, C., Cama, V., Dearen, T., Schultz, C.L., Orndorff, K.G., Prelewicz, G.J., Brown, M.H., Young, K.R., Xiao, L., 2008. *Cryptosporidium* source tracking in the Potomac river watershed. Appl. Environ. Microbiol. 74, 6495–6504. http://dx.doi.org/10.1128/AEM. 01345-08.
Zhou, L., Kassa, H., Tischler, M.L., Xiao, L., 2004. Host-adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). Appl. Environ. Microbiol. 70, 4211–4215. http://dx.doi.org/10.1128/AEM.70.7.4211-4215.2004.
Zylan, K., Bailey, T., Smith, H.V., Silvanose, C., Kinne, J., Schuster, R.K., Hyland, K., 2008. An outbreak of cryptosporidiosis in a collection of Stone curlews (*Burhinus oedicnemus*) in Dubai. Avian Pathol. 37, 521–526. http://dx.doi.org/10.1080/03079450802357019.