Molecularand Phylogenetic Characterization Of Cryptosporidium Species In The Saffron Finch Sicalis Flaveola

Francisco Carlos Rodrigues de Oliveira
Universidade Estadual de Norte Fluminense Darcy Ribeiro
Samira Salim Mello Gallo (samiragallo@yahoo.com.br)
Universidade Estadual de Norte Fluminense Darcy Ribeiro
Taynara Kerolayne Santos Elizeu
Universidade Estadual de Norte Fluminense Darcy Ribeiro
Nicole Brand Ederli
Universidade Federal Fluminense, Santo Antônio de Pádua

Research Article

Keywords: Protozoan, Cryptosporidium galli, Cryptosporidium andersoni, molecular biology, Sicalis flaveola

Posted Date: March 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1463016/v1

Abstract

Cryptosporidium is the most common protozoan that can infect a wide variety of animals, including mammals and birds. Fecal samples of saffron finches, *Sicalis flaveola,* from a commercial establishment were screened for the presence of *Cryptosporidium* by the modified Ziehl–Neelsen technique and nested PCR of the 18S rRNA gene followed by sequencing of the amplified fragments. The species *Cryptosporidium galli* was identified in six saffron fiches, in addition to *Cryptosporidium andersoni* in one of the birds, indicating a mixed infection. Only two birds had feathers that were ruffled and dirty with feces. Concomitant infection with *Isospora* spp. was observed in all birds. In conclusion, this is the third report of parasitism by *C. galli* in *S. flaveola* and that this bird is a possible host of *C. andersoni*.

1. Introduction

Protozoa of the genus *Cryptosporidium* belong to the phylum Apicomplexa, class Coccidea, order Eucoccidiorida and family Cryptosporidiidae (Fayer 2007). Cryptosporidiosis has been described in several bird species on several continents (Ryan 2009), and to date, four species of *Cryptosporidium* have been described in birds: *Cryptosporidium meleagridis* (Slavin 1955), *Cryptosporidium baileyi* (Current et al. 1986), *Cryptosporidium galli* (Ryan et al. 2003a) and *Cryptosporidium avium* known as Avian genotype V (Holubová et al. 2016). In addition, 21 *Cryptosporidium* genotypes have been identified in over 30 bird species worldwide (Ryan 2010), and these appear to be bird specific. They include bird genotypes I-IV and VI-IX (Chelladurai et al. 2016; Helmy et al. 2017), goose genotypes I-IV (Zhou et al. 2004) and Id (Cano et al. 2016), duck genotypes I–II (Jellison et al. 2004; Morgan et al. 2001; Zhou et al. 2004) and b (Cano et al. 2016), a Eurasian woodcock genotype (Ryan et al. 2003b), finch genotypes I–III (Morgan et al. 2000) and the YS-2017 genotype of owls (Makino et al. 2018). Recently, *Cryptosporidium* genotype III was suggested as a new species named *Cryptosporidium proventriculi* (Holubová et al. 2019).

Cryptosporidium baileyi infects the epithelium of a wide variety of organs, such as the trachea and the bursa of Fabricius, while *C. meleagridis* infects the small intestine and cecum (Zha and Jiang 1994; Bermudez et al. 1987). *Cryptosporidium galli* causes changes in the proventriculus as the parasite develops in the epithelial cells of this organ and does not affect either the intestines or the respiratory tract (Pavlásek 1999).

The aim of the present study was to determine the prevalence, identify and molecularly characterize *Cryptosporidium* species in fecal samples of saffron finches, *Sicalis flaveola*, from a commercial farm in the city of Campos dos Goytacazes, state of Rio de Janeiro, Brazil.

2. Methods

2.1 Fecal samples and the Ziehl–Neelsen method

Fecal samples were collected from six adult saffron finches, *Sicalis flaveola*, from a commercial establishment in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil. The six birds were in separate cages, and all fecal content deposited at the bottom of the cage during a 24-hour period was collected and placed in a sterile collection tube. The tubes were identified and transported in isothermal boxes with ice to the Center for Advanced Research in Parasitology of the Universidade Estadual do Norte Fluminense Darcy Ribeiro. A part of the fecal content was examined for the presence of oocysts of *Cryptosporidium* spp. by microscopy of fecal smears stained by the modified Ziehl–Neelsen technique according to Angus (1987).

2.2 DNA extraction and nested PCR

From the other part of the fecal content, genomic DNA was extracted using a DNA and tissue kit (QIAGEN) with some modifications of the manufacturer's protocol (Santín et al., 2004). DNA samples were stored at − 20°C, and all samples were screened for *Cryptosporidium* using nested PCR (n-PCR) to amplify fragments of the 18S subunit of the rRNA gene (Xiao et al. 1999, 2000), with subsequent sequencing of amplified fragments. Primers P1: 5-TTTCTAGAGCTAATACATGCG-3, P2: 5-CCCATTTCCTTCGAAACAGGA-3 and P3: 5-GGAAGGGTTGTATTTATTAGATAAAG-3, P4: 5-AAGGAGTAAGGAACAACCTCCA-3 were used for the primary (~ 1325 bp) and secondary (~ 830 bp) reactions, respectively. In addition, *Cryptosporidium parvum* DNA was used as a positive control, and ultrapure water was used as a negative control.

2.3 Sequencing and phylogenetic analysis

The amplified fragment (~ 830 bp) resulting from the secondary reaction of n-PCR was purified using the GFX PCR DNA Band Purification® Kit (GE Health Sciences, Champaign, IL, USA) and sequenced with the aid of DYEnamic® ET Kit Cycle Sequencing® Terminator Dye (GE Health Sciences, Champaign, IL, USA) on a MegaBACE® sequencer (GE Health Sciences, Champaign, IL, USA). Sequencing reactions were performed at least three times in both directions with the n-PCR secondary reaction primers. The consensus sequence was analyzed using CodonCode Aligner v.2.0.4 software (CodonCode Corp., Dedham, MA) and aligned with *Cryptosporidium* reference sequences published in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using MEGA X software (Kumar et al. 2018) by the neighbor-joining method (Saitou and Nei, 1987) after estimating the distance using the Kimura 2-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (full delete option). In the construction of the phylogenetic tree, *Eimeria tenella* (KT184354) was used as an outgroup. Group confidence was assessed by bootstrap values using 1000 replicates.

The following sequences were used to construct the phylogenetic tree: MK311144 (*C. baileyi*) from *Erythrura gouldiae*, GQ227475 (*C. baileyi*) from *Sicalis flaveola*, MK311145 (*C. baileyi*) from *Carduelis psaltria*, MK311141 (Avian genotype I) from *Serinus canaria*, GQ227479 (Avian genotype I) of *Serinus canaria*, HM116381 (Avian genotype V) of *Nymphicus hollandicus*, DQ650341 (Avian genotype II) of *Eolophus roseicapilla*, DQ002931 (Avian genotype II) of *Struthio camelus*, HM116382 (*C. meleagridis*) of *Columba livia*, HM116383 (*C. meleagridis*) from *Bombycilla garrulus*, HM116384 (*C. meleagridis*) from

Streptopelia orientalis, DQ650344 (Avian genotype IV) from *Zosterops japonica*, MK311135 (*C. proventriculi*) from *Poicephalus gulielmi*, MK311136 (*C. proventriculi*) from *Agapornisico rosellis*, GU816048 (*C. galli*) from *Sicalis flaveola*, GU816049 (*C. galli*) from *Saltator similis*, GU816054 (*C. galli*) from *Sporophila angolensis*, KT175411 (*C. andersoni*) from slaughterhouse wastewater and MT648437 (*C. andersoni*) isolated from *Cygnus* sp.

2.4 Accession numbers of nucleotide sequences

The 18S rRNA gene nucleotide sequences were deposited in the GenBank database under accession numbers OM436006-OM436011 (*C. galli*) and OM491513 (*C. andersoni*).

3. Results

Cryptosporidium oocysts were detected in all fecal samples by microscopic analysis of smears stained using the Ziehl–Neelsen technique (Fig. 1).

Molecular analysis revealed amplification of *Cryptosporidium* DNA in all samples analyzed. By sequencing the fragment amplified by n-PCR, the species *C. galli* was identified in all samples, but in one of the birds, a mixed infection was detected since in one of the sequencing runs, the species *C. andersoni* was identified. The *C. galli* isolates from the present study shared 92.54–96.41% similarity with other *C. galli* isolates according to nBlast analysis, and the *C. andersoni* isolate from one of the birds shared 98.85% identity with *C. andersoni* from slaughterhouse wastewater. Molecular characterization of the seven *Cryptosporidium* samples was performed with phylogenetic reconstructions of the 18S rRNA gene using a total of 329 positions in the final dataset. The phylogenetic reconstruction of *Cryptosporidium* isolates from *S. flaveola* can be seen in Fig. 2.

The sporulated oocysts of *C. galli* (n = 117) were 5.81 ± 0.78 (3.97–8.09) by 4.86 ± 0.66 (3.3–7.23) µm on average, with a length/width ratio of 1.20 ± 0.12 (0.95–1.50). *C andersoni* oocysts were not measured. The microscopic analysis also detected oocysts of *Isospora* spp. Two birds had feathers ruffled and soiled with feces.

4. Discussion

Common techniques used to diagnose *Cryptosporidium* infection are microscopic analysis and n-PCR (Jex et al. 2008). Despite microscopy being an intensive procedure that demands time and experience, the extraction of DNA from fecal samples of *S. flaveola* was performed only by means of previous microscopy. As discussed by Nakamura et al. (2009), performing PCR on samples previously identified as positive by microscopy implies a lower cost, as the reagents are expensive. Microscopy is an affordable and quick technique; however, it does not identify *Cryptosporidium* species and is less sensitive and specific. Therefore, n-PCR was performed to allow the identification of the species after amplicon sequencing.

In the present study, we identified only 1 of the 4 species of *Cryptosporidium* commonly found in birds. The 100% positivity of saffron finches, family Emberizidae, was high, but the number of samples collected and analyzed was low, making it difficult to compare the prevalence with that reported in most studies of *Cryptosporidium* in captive and wild birds. One factor that may interfere with the rate of *Cryptosporidium* infection is a difference in the age of the animals (de Graaf et al., 1999; Nichols, 2008), although almost all reports of *Cryptosporidium* infections in captive or wild birds do not specify the age range of the animals examined.

Silva et al. (2010) carried out a study in which 480 samples of passerine feces were collected from Araçatuba, São Paulo. Of these samples, 105 were positive for *Cryptosporidium*, with n-PCR and sequencing revealing all infections to be of the species *C. galli*. Similarly, Antunes et al. (2008) detected the species *C. galli* in all samples studied through molecular analysis, with four canaries (*Serinus canaria*) and eight cockatiels (*Nymphicus hollandicus*) in captivity. These works corroborate the finding of the present study in which *C. galli* was detected in all PCR-positive samples.

The mean size of *C. galli* oocysts obtained in the present study was smaller than the mean size of *C. galli* reported by Ryan et al. (2003a) and by Qi et al. (2011). Since the sizes of the oocysts of different species of *Cryptosporidium* are very similar, oocyst morphometry alone is not sufficient to distinguish the species, making molecular studies necessary for accurate identification.

Among the species/genotypes of *Cryptosporidium* in birds, only two named species, *C. baileyi* and *C. galli*, were identified in the saffron finch, *S. flaveola,* in previous studies (Nakamura et al. 2009; Sevá et al. 2011; Nakamura et al. 2014). Nakamura et al. (2009) conducted a study of 966 stool samples from birds belonging to 18 families. These captive or wild birds came from three Brazilian states: Goiás, Paraná and São Paulo. In a specimen of *S. flaveola*, the species *C. baileyi* (GQ227475) was diagnosed through PCR and sequencing of the 18S rRNA gene. In 2012, Sevá and colleagues analyzed 242 fecal samples from wild birds seized by the environmental control agency of São Paulo State. Four *S. flaveola* were positive for *Cryptosporidium,* three birds harbored the species *C. galli* (GU816048, GU816069, HM126668), and one was positive for the species *C. baileyi* (GU816042). Nakamura et al. (2014) collected a total of 1027 fecal samples from birds of the orders Psittaciformes and Passeriformes. These birds were from captivity or the wild and came from Divisão Técnica de Medicina Veterinária e Manejo da Fauna Silvestre (DEPAVE-3) of São Paulo. Of the 108 positive samples, 40 were sequenced, one of which was from *S. flaveola* and was positive for *C. galli* according to n-PCR sequencing (accession number in GenBank not available). Even though our research represents the third diagnostic report of *C. galli* in *S. flaveola*, further studies are still needed on species or genotypes of *Cryptosporidium* that can infect this species of Passeriformes.

A coinfection of *C. galli* and *C. andersoni* occurred in one of the birds in the present study, although only monoinfections were previously found in *S. flaveola* (Nakamura et al. 2009; Sevá et al. 2011; Nakamura et al. 2014). According to Máca and Pavlásek (2016), the intensive rearing of birds in breeders can be problematic, as it is associated with a large number of birds in a relatively small area, increasing

the possibility of bacterial, viral and parasitic diseases and their rapid spread compared to those in wild birds.

The birds in the present study lived in separate cages but were kept in the same environment and close to each other. As reported by Nakamura et al. (2014), this can result in the spread of infection through direct contact with feces or human transport of oocysts during routine management related to cleaning. In addition, the saffron finch cages were close to the cages of other bird species, which may have contributed to the interspecific spread of *Cryptosporidium* infections.

Specifically, *C. galli* infections are associated with other pathogens (Lindsay et al. 1991), and these associations can lead to weight loss, lameness, pelvic limb joint swelling and high mortality in captive birds (Antunes et al. al. 2008). Although the birds in the present study were infected with *Isospora* oocysts, they did not show any of these clinical symptoms. Due to the association of infections by *C. galli* and *Isospora* in the birds of the present study, it was not possible to determine which agent was responsible for the feathers ruffled and soiled with feces observed on two of the birds, since both infections can cause the observed characteristics. According to Cox et al. (2001), in mixed infections, the burden of one or both infectious agents may be increased, that of one or both may be suppressed, or that of one may be increased and that of the other suppressed.

Passerines infected with *C. galli* can shed oocysts intermittently for 12-13 months (Antunes et al. 2008; Silva et al. 2010). The determination of intermittent and prolonged shedding of *C. galli* oocysts in fecal samples, in addition to demonstrating that this species causes chronic infection in birds, also maintains the species between generations of birds through contact between parents and progeny. In view of this, it is necessary to adopt strict sanitary management measures to prevent the occurrence of infections in breeders, commercial establishments and nongovernmental organizations that receive apprehended wild birds.

The *C. andersoni* isolate from *S. flaveola* (Isolate 4b) clustered with the other isolates of the same species from previous studies (MT648437 and KT175411) with high (80%) bootstrap support (Figure 2). The branch of the *C. andersoni* species clustered with the isolates of *C. galli,* which is also a gastric parasite, suggesting that these two *Cryptosporidium* species are close relatives. *Cryptosporidium andersoni* is a species found mainly in cattle and humans (Chalmers and Katzer 2013; Ryan et al. 2014) but was previously reported in the bird *Podargus strigoides* in an Australian study (Ng et al., 2006) and in an ostrich, *Struthio camelus*, from a zoo in southwestern France (Osman et al. 2017). Similar to Ng et al. (2006), we were unable to determine whether the presence of *C. andersoni* oocysts in the fecal samples of birds analyzed in the present study was due to a real infection or accidental contamination by mechanical transport, since the birds in the present study had close contact with humans. In addition, animals can also be infected indirectly after drinking water contaminated with *Cryptosporidium*. In view of the above, studies are needed to discover whether birds are natural hosts or only carriers of *C. andersoni*, since studies have already reported that a species of *Cryptosporidium* may have a wider host range than originally assumed (Widmer and Sullivan 2012).

5. Conclusion

In conclusion, the high *C. galli* parasite load in all birds in this research shows that the saffron finch, *S. flaveola*, is a host of this protozoan species, although this is the third report of parasitism in this bird species. In addition, *S. flaveola* may contribute to the maintenance of intraspecific and interspecific infections in environments with large numbers of birds. We can also conclude that *C. andersoni* parasitizes *S. flaveola*; however, the low prevalence in our studies and the few reports of coccidia from this species in birds prevent us from inferring that this species of passerine is a good host or if it is simply a carrier of the protozoan in environments with high fecal contamination.

Declarations

Funding Financial support was received from FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro) through Scientist of Our State scholarship (grant number 232568).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments The authors would like to thank CAPES (Coordination for the Improvement of Higher Education Personnel) for granting the scholarship to T.K.S., Elizeu during the development of this research.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SSMG, FCRO, TKSE, NBE. The first draft of the manuscript was written by SSMG and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Angus KW (1987) Cryptosporidiosis in domestic animals and humans. In Pract 9:47–49
2. Antunes RG, Simões DC, Nakamura AA, Meireles MV (2008) Natural infection with *Cryptosporidium galli* in canaries (*Serinus canaria*), in a cockatiel (*Nymphicus hollandicus*), and in lesser seed-finches (*Oryzoborus angolensis*) from Brazil. Avian Dis 52:702–705.
3. Bermudez AJ, Ley DH, Levy MG, Barnes HJ, Gerig TM (1987) Experimental cryptosporidiosis in turkey poults. In: Proceedings Meeting American Veterinary Medical Association, vol. 124, Chicago, Annals.
4. Cano L, de Lucio A, Bailo B, Cardona GA, Muadica ASO, Lobo L, Carmena D (2016) Identification and genotyping of *Giardia* spp. and *Cryptosporidium* spp. isolates in aquatic birds in the Salburua wetlands, Alava, Northern Spain. Vet Parasitol 221:144–148.

5. Chalmers RM, Katzer F (2013) Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. Trends Parasitol 29:237–251.
6. Chelladurai JJ, Clark ME, Kváč M, Holubová N, Khan E, Stenger BLS, Giddings CW, McEvoy J (2016) *Cryptosporidium galli* and novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds (*Agelaius phoeniceus*). Parasitol Res 115:1901–1906.
7. Cox FEG (2001) Concomitant infections, parasites and imune responses. Parasitol. 122:S23–S38.
8. Current WL, Upton SJ, Haynes TB (1986) The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. J Protozool 33:289–296.
9. de Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE (1999) A review of the importance of cryptosporidiosis in farm animals. Int J Parasitol 29:1269–1287.
10. Fayer R (2007) *Cryptosporidium* and cryptosporidiosis. RC, Boca Raton, pp 1–42.
11. Helmy YA, Krucken J, Abdelwhab EM, von Samson-Himmelstjerna G, Hafez HM (2017) Molecular diagnosis and characterization of *Cryptosporidium* spp. in turkeys and chickens in Germany reveals evidence for previously undetected parasite species. PLoS One 12:e0177150.
12. Holubová N, Sak B, Horˇciˇcková M, Hlásková L, Kvˇetoˇnová D, Menchaca S, McEvoy J, Kváˇc M (2016) *Cryptosporidium avium* n. sp. (Apicomplexa: Cryptosporidiidae) in birds. Parasitol Res 115:2243–2251.
13. Holubová N, Zikmundová V, Limpouchová Z, Sak B, Konečný R, Hlásková L, Rajský D, Kopacz Z, McEvoy J, Kváč M (2019) *Cryptosporidium proventriculi* sp. n. (Apicomplexa: Cryptosporidiidae) in Psittaciformes birds. Eur J Protistol 69:70–87.
14. Jellison KL, Distel DL, Hemond HF, Schauer DB (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.
15. Jex AR, Smith HV, Monis PT, Campbell BE, Gasser RB (2008) *Cryptosporidium* – biotechnological advances in the detection, diagnosis, and analysis of genetic variation. Biotechnol Adv 26:304–317.
16. Kimura MA (1980) Simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.
17. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35:1547–1549.
18. Lindsay DS, Blagburn BL, Hoerr FJ, Smith PC (1991) Cryptosporidiosis in zoo and pet birds. J Protozool 38:180S–181S.
19. Máca O, Pavlásek I (2016) *Cryptosporidium* infections of ring-necked pheasants (*Phasianus colchicus*) from an intensive artificial breeding programme in the Czech Republic. Parasitol Res 115:1915–1922.
20. Makino I, Inumaru M, Abe N, Sato Y (2018). A new avian *Cryptosporidium* genotype in a 1-month-old caged brown wood owl (*Strix leptogrammica*) with severe dehydration and diarrhea. Parasitol Res 117:3003–3008.

21. Morgan UM, Xiao L, Limor J, Gelis S, Raidal SR, Fayer R, Lal A, Elliot A, Thompson RC (2000) *Cryptosporidium meleagridis* in an Indian ring-necked parrot (*Psittacula krameri*). Aust Vet J 78:182–183.
22. Morgan UM, Monis PT, Xiao L, Limor J, Sulaiman I, Raidal S, O'Donoghue P, Gasser, R, Murray A, Fayer R, Blagburn BL, Lal AA, Thompson RC (2001) Molecular and phylogenetic characterization of *Cryptosporidium* from birds. Int J Parasitol 31:289–296.
23. Nakamura AA, Homem CG, da Silva AM, Meireles MV (2014) Diagnosis of gastric cryptosporidiosis in birds using a duplex real-time PCR assay. Vet Parasitol 205:7–13.
24. Nakamura AA, Simões DC, Antunes RG, da Silva DC, Meireles MV (2009) Molecular characterization of *Cryptosporidium* spp. from fecal samples of birds kept in captivity in Brazil. Vet Parasitol 166:47–51.
25. Ng J, Pavlasek I, Ryan U (2006) Identification of novel *Cryptosporidium* genotypes from avian hosts. Appl Environ Microbiol 72:7548–7553.
26. Nichols G (2008) Epidemiology. In: Fayer R, Xiao L (Eds.), *Cryptosporidium* and Cryptosporidiosis. CRC Press and IWA Publishing, Boca Raton, pp. 79–118.
27. Osman M, El Safadi D, Benamrouz-Vanneste S, Cian A, Moriniere R, Gantois N, Delgado-Viscogliosi P, Guyot K, Bosc S, Chabé M, Petit T, Viscogliosi E, Certad G (2017). Prevalence, transmission, and host specificity of *Cryptosporidium* spp. in various animal groups from two French zoos. Parasitol Res 116:3419–3422.
28. Pavlásek I (1999) Cryptosporidia: biology, diagnosis, host spectrum, specificity, and the environment. Klin Mikrobiol Infekcni Lek 3:290–301.
29. 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 128:336–40.
30. Ryan UM (2009) *Cryptosporidium* in birds, fish and amphibians. Exp Parasitol 124:113–120.
31. Ryan UM (2010) *Cryptosporidium* in birds, fishand amphibians. Exp Parasitol 124:113–120.
32. Ryan UM, Fayer R, Xiao L (2014) *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitol 141:1667–1685.
33. Ryan UM, Xiao L, Read C, Sulaiman IM, Monis P, Lal AA, Fayer R, Pavlásek I (2003a) A redescription of *Cryptosporidium galli* Pavlásek, 1999 (Apicomplexa:Cryptosporidiidae) from birds. J Parasitol 89:809–813.
34. Ryan U, Xiao L, Read C, Zhou L, Lal AA, Pavlásek I (2003b). Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl Environ Microbiol 69:4302–4307.
35. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
36. Santín M, Trout JM, Xiao L, Zhou L, Greiner E, Fayer R (2004) Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. Vet Parasitol 122:103–117.

37. Silva DC, Homem CG, Nakamura AA, Teixeira WF, Perri SH, Meireles MV (2010) Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. Parasitol Res 107:271–277.
38. Sevá AP, Funada MR, Richtzenhain L, Guimarães MB, Souza SO, Allegretti L, Sinhorini JA, Duarte VV, Soares RM (2011) Genotyping of *Cryptosporidium* spp. from free-living wild birds from Brazil. Vet Parasitol 175:27–32.
39. Slavin D (1955) *Cryptosporidium meleagridis* (sp. nov.). J Comp Pathol 65:262–266.
40. Zha HB, Jiang JS (1994) Life cycle of *Cryptosporidium meleagridis* in quails. Acta Vet Zootechn 25:273–278.
41. Zhou L, Kassa H, Tischler ML, Xiao L (2004) Host-adapted *Cryptosporidium* spp: in Canada geese (*Branta canadensis*). Appl Environ Microbiol 70:4211–4215.
42. Widmer G, Sullivan S (2012) Genomics and population biology of *Cryptosporidium* species. Parasite Immunol 34:61–71.
43. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA (1999) Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol 65:1578–1583.
44. Xiao L, Alderisio K, Limor J, Royer M, Lal AA (2000) Identification of species and sources of *Cryptosporidium* oocysts in storm waters using a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol 66:54926–55498.

Figures

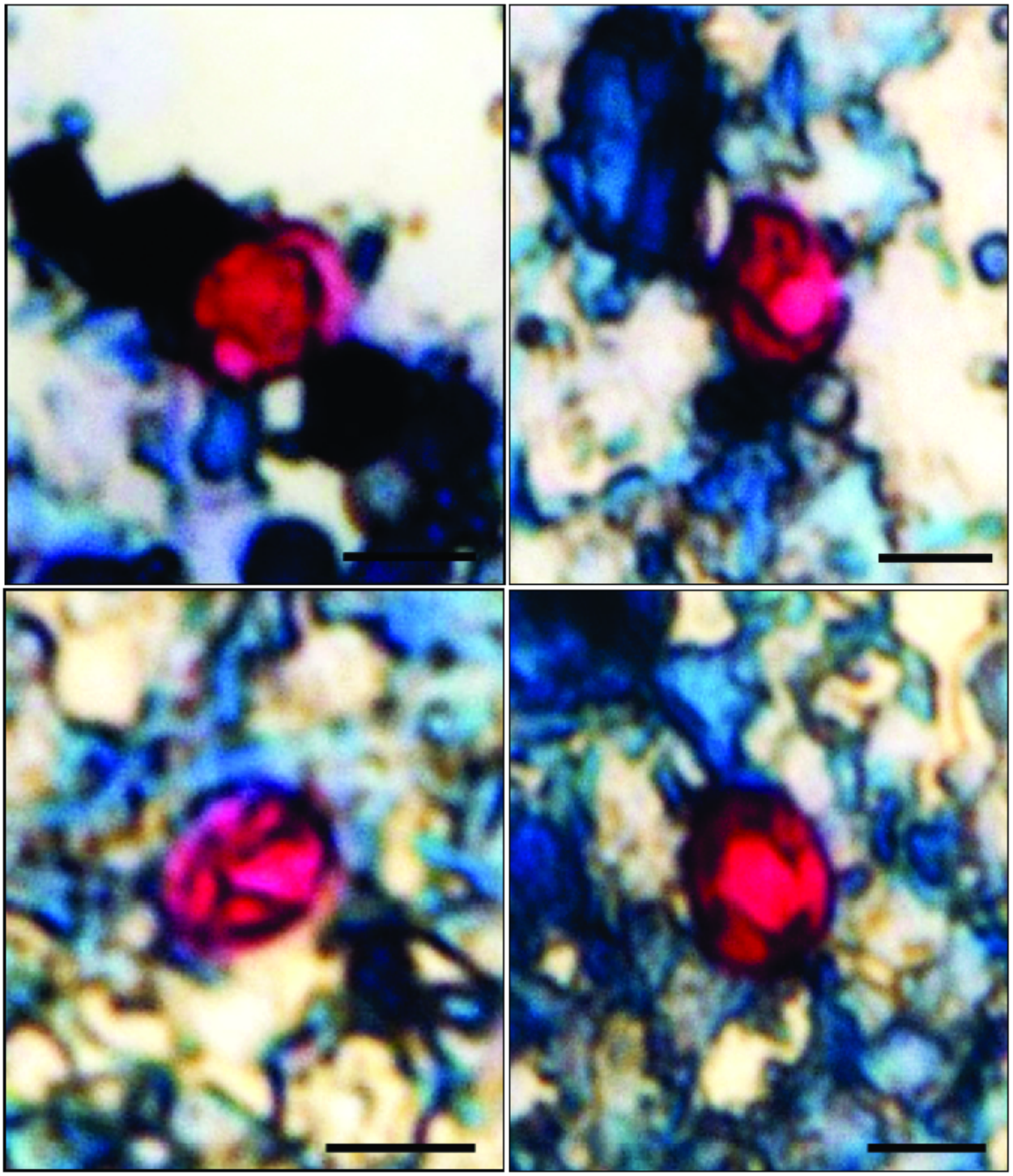

Figure 1

Sporulated oocysts of *Cryptosporidium galli* stained by the modified Ziehl–Neelsen technique. Bars: 5 µm.

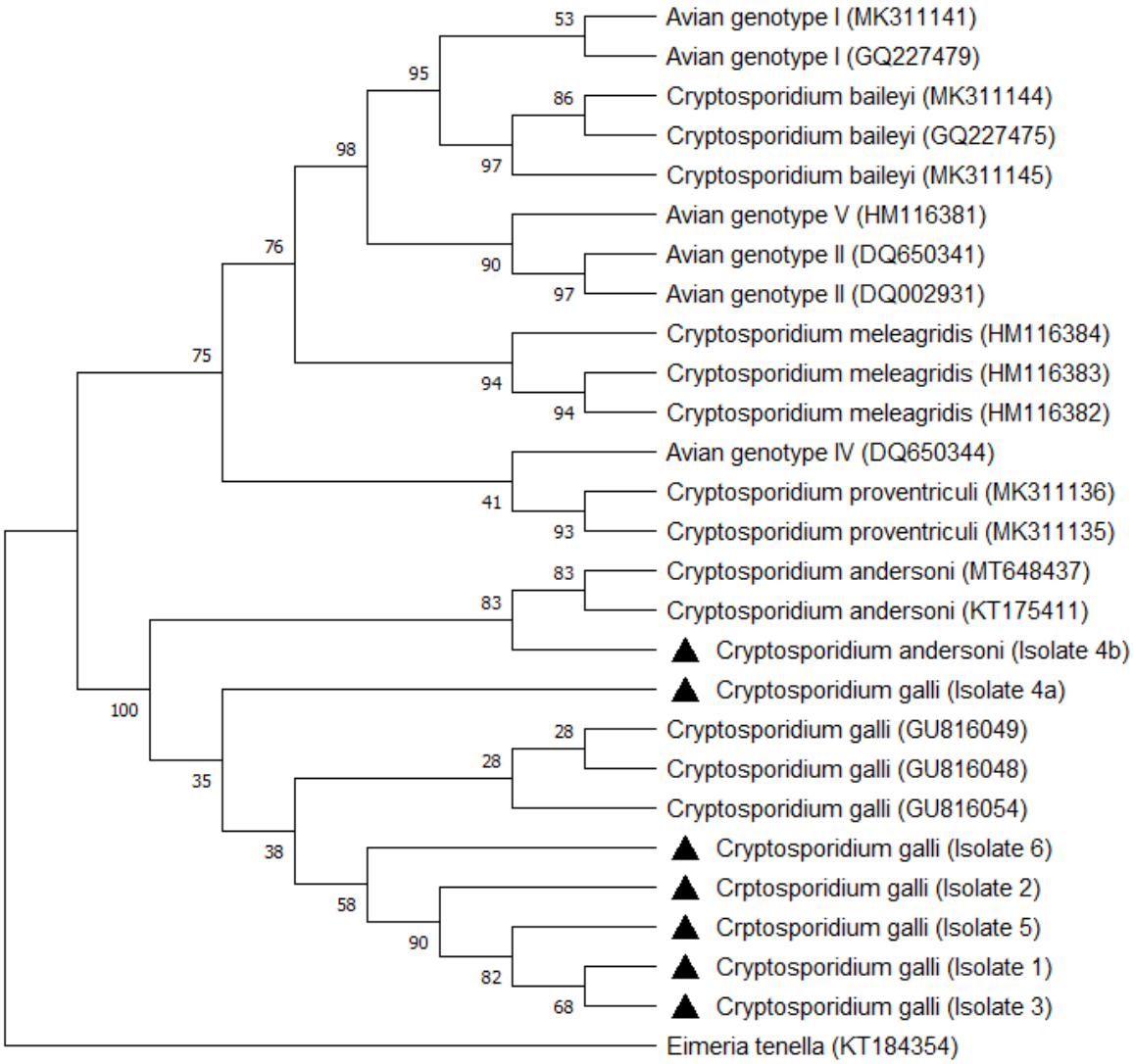

Figure 2

Phylogenetic analysis of *Cryptosporidium* spp. using the neighbor-Joining method and the Kimura 2-parameter model based on isolated sequences of the 18S rRNA gene of *Cryptosporidium* from this experiment and other *Cryptosporidium* species.