Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

e-ISSN: 1806-9061 2024 / v.26 / n.3 / 001-008

http://dx.doi.org/10.1590/1806-9061-2023-1836

Original Article

Blastocystis sp. of Subtypes 2, 4 and 9 in Selected Avian Species, Brazil, 2009-2018

■Author(s)

Marin SYG[I] https://orcid.org/0000-0002-9374-5591
Silva CR[I] https://orcid.org/0009-0001-3753-1708
Andrade JP[I] https://orcid.org/0000-0003-2108-9748
Soares CF[I] https://orcid.org/0009-0002-2837-7603
Rodrigues PHC[I] https://orcid.org/0009-0009-2672-609X
Oliveira HCAM[I] https://orcid.org/0009-0009-9580-782X
Vilela DAR[II] https://orcid.org/0000-0001-5707-3233
Coelho HLG[I] https://orcid.org/0000-0003-1460-0202
Freitas Neto OC[I] https://orcid.org/0000-0002-1437-069X
Martins NRS[I] https://orcid.org/0000-0001-8925-2228

[I] Avian Diseases, Departamento de Medicina Veterinária Preventiva, UFMG, Brazil.
[II] Wild Animal Triage Center, CETAS-IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis), Belo Horizonte, Brazil.

■Mail Address

Corresponding author e-mail address
Nelson Rodrigo S. Martins
Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Campus Pampulha, Belo Horizonte, Minas Gerais, 31270-901
Phone: +55 31 3409-2075
Email: nelsonrodrigoaves@gmail.com

■Keywords

Stramenopiles; Gallus gallus domesticus; Guaruba guarouba; Saltator similis; Sicalis flaveola.

Section editor: Ramon Malheiros

Submitted: 15/August/2023
Approved: 30/May/2024

ABSTRACT

Avian species of the metropolitan region of Belo Horizonte, Brazil, submitted to laboratory routine due to a variety of morbid conditions, were diagnosed as carriers of *Blastocystis* sp. All samples were examined under light microscopy and those with structures suggestive of *Blastocystis* sp. were tested by PCR. The total DNA was purified from feces and/or intestinal mucosa and used as a template for PCR, targeting the small subunit (SSU) rRNA gene, and the products were sequenced. The sequences detected in two chickens, one passerine (*Saltator similis*, Thraupidae) and a golden parakeet (*Guaruba guarouba*, Psittacidae) were phylogenetically characterized and shown to cluster with sequences of subtypes 2, 4 or 9. The occurrence of *Blastocystis* sp. in domestic and local native avifauna is described and the perspective of a complex local interspecies epidemiology is considered of risk, including to humans.

INTRODUCTION

Blastocystis sp. is polymorphic and existing in different forms, including amoeboid, cyst, granular, vacuolar and others. It occurs in more than one billion humans, with certain subtypes (ST) considered pathogenic (Parija *et al.*, 2013). The microorganism is a member of *Stramenopiles*, a group containing 21 classes and more than 100.000 species of eukaryotic organisms, mainly represented by photosynthetic algae. However, five classes are devoid of chloroplasts, including the etiology of a potato disease (*Phytophthora infestans* HERB-1), described in the great famine in Ireland when potato cultures were destroyed between 1845-1852 (Yoon *et al.*, 2009).

A very wide range of species has been described as hosts for *Blastocystis* sp., in phyla Arthropoda, Chordata and Mollusca, including classes Amphibia, Aves, Insecta, Mammalia and Reptilia [Stenzel & Boreham, 1996; Tan, 2008]. *Blastocystis* sp. was initially considered commensal, and is known to be widespread in humans. It is especially prevalent in suboptimal public health conditions, while also acting opportunistically in cases of immunodeficiency and being associated to gastrointestinal diseases (Wawrzyniak *et al.*, 2013). However, *Blastocystis* may possibly have a balancing role towards a healthy microbiome (Kodio *et al.*, 2019). Regarding human intestinal microbiome health, clinically normal Malian (West Africa) children with intestinal colonization by *Blastocystis* had a higher diversity and evenness of bacterial species, with a higher count of beneficial bacteria (Kodio *et al.*, 2019; Aykur *et al.*, 2024). The celiac disease and colorectal cancer have been associated with low bacterial diversity and increased *Bacteroides* (De Palma *et al.*, 2010). Subtypes ST1, ST3, and ST4 may increase the diversity of the gut microbiome and promote an anti-inflammatory state in the intestinal mucosa (Aykur *et al.*, 2024).

At least 46 subtypes (ST) of *Blastocystis* have been described (Koehler at al., 2024), and the genetic diversity was initially proposed despite the identical phenotypes (Clark, 1997). The genetic diversity of *Blastocystis* ST in stool samples of human patients with chronic urticaria revealed ST3 (allele 34) as the most prevalent, with a significant total IgE associated with the presence of ST2 and ST3 (Aykur *et al.*, 2022). The ST3 infection in humans was described as restricted to one of four mitochondrion-like organelles (MLO) clades, ST4 almost exclusively to one of two SSU rRNA clades, and only five multilocus sequence types (MLST) were detected among 50 ST4 of clade 1 (Stensvold *et al.*, 2012). The occurrence of *Blastocystis*, mostly ST7 or ST9, was associated with irritable bowel syndrome and a history of contact with pigs and poultry (Stensvold *et al.*, 2009).

In Italy, isolates were subtyped from fallow deer (*Dama dama*), goats, and pigs (ST5), pheasants (ST6), chickens (ST7), sheep (ST10) and water buffaloes (ST14), suggesting animal-to-human spillover for ST6 and ST7 (Gabrielli *et al.*, 2021). The organism was detected in 1.3% of humans of Rio de Janeiro, along with cestodes, nematodes and protists, and was identified as ST1 (42%), ST2 (7%), ST3 (49%), ST4 (2%) and mixed infections (14%) (Valença Barbosa *et al.*, 2017). Also in Brazil, *Blastocystis* sp. was visualized in feces of poultry (chicken 22.9-42.9%, mallard 37.1-55.3% and quail 5,5-42,9%) in live markets (Bomfim *et al.*, 2013).

The marine fauna has been studied in the Northeastern Atlantic (France), detecting six different ST with three potentially new ones, with a prevalence of 3.5% in fish and 13.8% in mammals (Gantois *et al.*, 2020). Zoo animals were also investigated in France, and the organism was detected in 37.9% of species, suggesting avian and artiodactyl species as reservoirs to humans, but not mammalian carnivores, insects or reptiles (Cian *et al.*, 2017). Reptiles of 28 species were investigated in Singapore, and *Blastocystis* sp. was detected in three snake species, three tortoises, one lizard (iguana), and one crocodile species (Teow *et al.*, 1992).

We describe the investigation of *Blastocystis* in exotic and native avian species received for diagnosis of diverse and unrelated morbid conditions.

MATERIALS AND METHODS

Samples

Intestinal mucosal scrapings were obtained at necropsy of one *Pionopsitta pileata* (Psittaciformes), five *Nymphicus hollandicus* (Psittaciformes), one *Guaruba guarouba* (Psittaciformes), one *Saltator similis* and one *Sicalis flaveola* (Passeriformes), one *Aix sponsa* (Anseriformes), and chickens of the family poultry (n=10) and layer industry (n=10). Most samples were taken by scraping off the duodenum/jejunum mucosa, except for sampling of ceca of the industrial layer chickens (Table 1 and Figure 1).

After the demonstration of typical *Blastocystis* spp. structures at microscopy, the total DNA was extracted and used as template in a PCR targeting the SSU rRNA gene. The amplified products were sequenced and four obtained sequences were aligned with sequences published in the GenBank.

Microscopy

Samples were investigated fresh under optical microscopy for cyst structures typical of *Blastocystis* sp. at 100 and 400-time magnifications (Olympus CBB, São Paulo, Brazil), and positive specimens were submitted to further molecular studies.

DNA extraction and PCR

After the microscopic demonstration of typical cystic *Blastocystis* sp. cells, the positive samples were submitted to DNA extraction. The total DNA was extracted and employed as a template for the PCR. The total DNA was extracted using 6M sodium iodide (Vogelstein & Gillespie, 1979; Boom *et al.*, 1990) and adsorption to silicon dioxide (Boom *et al.*, 1990), and the concentration and purity were determined (NanoVue®, GE, Healthcare, UK).

The PCR reactions were performed in a thermocycler (Axygen-Maxygene, USA) for the detection of the small subunit (SSU) of the rRNA encoding gene of *Blastocystis* sp., using the oligonucleotides *Blastocystis* Ext rRNA Fwd 5'-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3' and *Blastocystis* Ext rRNA Reve 5'-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3' for the amplification of a 310 bp product. PCR conditions were as previously described (Stensvold *et al.*, 2006) (Table 2).

Table 2 – *Blastocystis* PCR cycles protocol*.

Cycle	Step	Temperature	Time
1	1	94.0	4:00
2	1	94.0	0:30
2	2	54.0	0:30
2	3	72.0	0:30
3	1	72.0	5:00
3	2	4.0	Hold

*Based on Stensvold *et al.* (2006)

Table 1 – Selected hosts and morbid cases with the detection of *Blastocystis* sp.

Protocol number	Host	Clinical-pathological state of host at examination	Status	Notes
117 (06/03/2009)	*Saltator similis* (Passeriformes). (n=1)	Death	Adult. Captive at triage center.	Fatal cases associated to *Isospora* spp. Accession No. MK880048.1
479 (28/02/2011)	*Gallus gallus domesticus* (n=5)	Recovery. Flaccid paralysis diagnosed as botulism. *Clostridium botulinum* type C serum treatment.	Adult. Captive family poultry.	Total recovery. Accession No. MK880049.1
543 19/06/2012	*Aix sponsa* (Anseriformes) (n=1)	Death.	Adult. Captive ornamental species.	Fatal traumatic illness.
804 (12/09/2014)	*Guaruba guarouba* (n=1)	Death	Adult; poor feathering;	Diagnosis of avian *Bornavirus* infection.
932 (08/05/2015)	*Pionopsitta pileata* (Psittaciformes) (n=1)	Death.	Captive at a commercial and conservation center.	Cachexia. Vascular congestion of ingluvius.
1564 (11/10/2017)	*Guaruba guarouba* (n=1)	Death	Captive at a conservation center.	Cachexia, feces adhered to the cloaca, dark feces; dark spot in the gizzard. Accession No. MK880050.1
1565 (16/10/2017)	*Gallus gallus domesticus* (n=5)	Curved toes associated with hypovitaminosis B; Advanced morbidity culminated in death, despite prior medication with vitamin B complex, ivermectin and metronidazole.	Captive Family poultry.	Intestinal *Ascaridia galli, Histomonas meleagridis.* Accession No. MK880051.1
Unregistered 25/04/2008	*Forpus xanthopterygius* (n=1)	Death. Nestling; airsacculitis; venous blood congestion in the cranial bones.	Captive at a commercial and conservation center.	Nestling hand fed.
Unregistered	*Nymphicus hollandicus* (Psittaciformes).(n=5)	Dysbiosis.	Pet	Microbiome balance.
Unregistered	*Sicalis flaveola* (Passeriformes)	Death.	Captive at a triage center.	Fatal cases associated with *Isospora* sp., with intestinal distension.
Unregistered	*Gallus gallus domesticus* (n=10)	Clinically normal white leghorn layer chickens.	Normal.	Routine monitoring.

The electrophoresis of products was performed in 1% agarose gel at 100V/40 min (Life Technologies, Gaithersburgh, MD, USA), revealed by GelRed® (Biothium, CA, USA) and visualized in a UV transilluminator (Hoefer, San Francisco, CA, USA). The molecular mass of products was estimated by comparing with a 100 bp ladder (Promega, Fitchburg, WI, USA).

Sequencing and phylogeny

The obtained PCR amplification products were submitted to sequencing (Sanger *et al.*, 1977), and sequences were phylogenetically analyzed. To obtain the products for sequencing, the previously described primers were employed in a reaction using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, Brazil; https://www.thermofisher.com/br/en/home.html), and sequences were determined bidirectionally. The forward and reverse consensus sequences were obtained using BioEdit v. 7.7.1.0 (Hall, 1999; https://bioedit.software.informer.com/7.7/). The alignment of sequences was determined by ClustalW, and the evolutionary history was inferred using the Minimum Evolution method (Rzhetsky & Nei, 1992), both in MEGA11 (Tamura *et al.*, 2021; https://www.megasoftware.net/).

RESULTS

Typical *Blastocystis* structures were detected in species of *Anseriformes*, *Galliformes*, *Passeriformes* and *Psittaciformes* (Table 2 and Figure 1). The *Aix sponsa* individual was gravely affected by a fatal traumatic disease. In the chicken, mostly in family poultry cases, other gastrointestinal parasites, such as cestodes (*Davainea* spp., *Hymenolepis* spp., *Raillietina* spp.), nematodes (*Ascaridia galli*, *Heterakis gallinarum*), and protists (*Eimeria* spp.) were eventually present; and in a few cases, such as case 479 (Table 2), were concomitant with avian botulism. The *S. similis* (microscopy not shown) and *S. flaveola* cases were associated with a fatal disease caused by *Isospora* sp. in a triage center. Among the psittacines of the commercial and conservation institutions, the *P. pileata* had cachexia and vascular congestion of the ingluvius and the *Guaruba guarouba* had cachexia, dark feces adhered to the cloaca, and a dark spot in the gizzard. The cockatiel evaluated was not fatally affected and had altered to pasty fecal consistency.

New hosts for *Blastocystis* sp. are described in this study, including the captive native passerine species *S. similis* and *S. flaveola* (*Passeriformes*) and the

captive native psittacine species *G. guarouba* and *Pionopsitta pileata* (*Psittaciformes*). The avian species were examined due to a diversity of morbid conditions, which may also favor an enhanced infection, replication and transmission of *Blastocystis* sp. In chickens, the intestinal presence of nematodes, cestodes and coccidia was common. In the native passerines, such as in *Saltator similis* and *Sicalis flaveola*, cases involved coccidiosis by *Isospora* sp.

The domestic avian species were chickens of the family or industrial poultry and pet cockatiels, and the native avifauna species were captive in commercial, conservation or triage facilities. Captivity, proximity and intensification are considered conditions of infection risk, which typically favor prevalence and transmission. However, none of the native species here described were previously investigated in a previous study in Brazil (Maloney *et al.*, 2020).

The microscopy of feces and/or the intestinal mucosa at the routine laboratory diagnosis in the investigated species enabled the demonstration of typical *Blastocystis* sp. cyst structures (Figure 1). The four obtained sequences of PCR products (small subunit rRNA) were aligned with sequences of the GenBank and the identities to subtypes ST2, ST4 and ST9 were demonstrated (Figure 2). In humans in Brazil, studies revealed ST3 to have the highest occurrence, followed by ST1 and ST2 (Melo *et al.*, 2021).

Sequences of the small subunit (SSU) rRNA of *Blastocystis* sp. were detected in two chickens, one passerine (*S. similis*) and one psittacine (*G. guarouba*) of the metropolitan region of Belo Horizonte, and analyzed phylogenetically. The amplified products were sequenced and four obtained sequences were aligned with sequences published in the GenBank. The sequences detected in one chicken (MK880049.1) and one *G. guarouba* (MK880050.1) were grouped with a subtype 2 (ST2) sequence of *Coturnix japonica* (AF408426.2) described in Japan. The sequence detected in *S. similis* (MK880048.1) was clustered with a ST4 strain (AY135411.1) detected in *Meleagris gallopavo* in France. Another sequence found in *Gallus gallus domesticus* (MK880051.1) was clustered with a ST9 sequence of *Homo sapiens sapiens* (AF408426.2) of Japan. All STs have been previously described in humans in Brazil (Malheiros *et al.*, 2011; Valença Barbosa *et al.*, 2017; Melo *et al.*, 2021). The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed

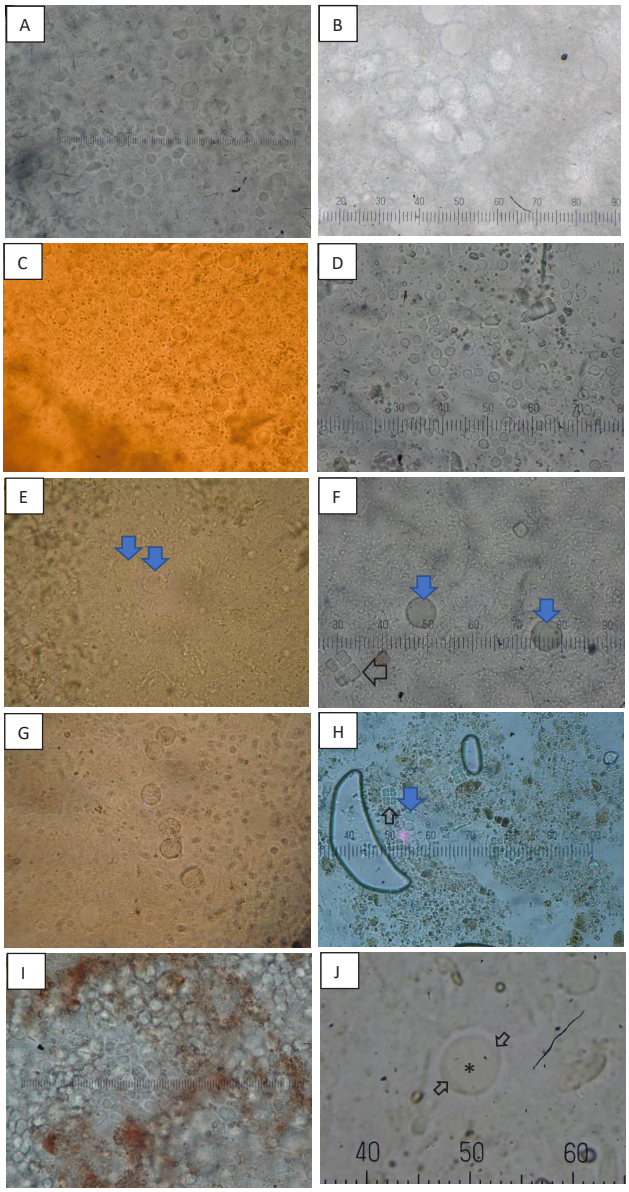

Figure 1

A. *Blastocystis* sp. in *Pionopsitta pileata* (*Psittaciformes*) case 932. Note the diversity in diameters. Each division = 10 µm. 40X.

B. *Blastocystis* sp. in the small intestine of free-range chickens. Each division = 10 µm. 100X.

C. *Blastocystis* sp. in free-range chicken, case 458. Note the apparent uniformity of diameters. 100X.

D. *Blastocystis* sp. in *Nymphicus hollandicus* (*Psittaciformes*). Note the apparent uniformity of diameters. Each division = 10 µm. 400X.

E. *Blastocystis* sp. in the cecum of an industrial layer chicken. Note the two large round central cells (arrows). 400X.

F. *Blastocystis* sp. in the cecum of an industrial layer chicken. Note the two large round cells (arrows). Groups of *Sarcina* sp. (Clostridiaceae) are at left (hollow arrow). 400X.

G. *Blastocystis* sp. in the small intestine of *Sicalis flaveola* (*Passeriformes*). 400X.

H. *Blastocystis* sp. in the cecum of *Aix sponsa* (*Anseriformes*) case 543. A. Note the two round cells (blue arrow). Groups of *Sarcina* sp. (*Clostridiaceae*) are above the ruler at 50 (hollow arrow). 100X.

I. *Blastocystis* sp. in the small intestine of *Guaruba guarouba* (*Psittaciformes*), case 804. 400X.

J. Typical *Blastocystis* sp. vacuolate form in the small intestine of chicken. Cell with large vacuole formation with cytoplasm (*) and nuclei (arrows) are indicated. 1000x.

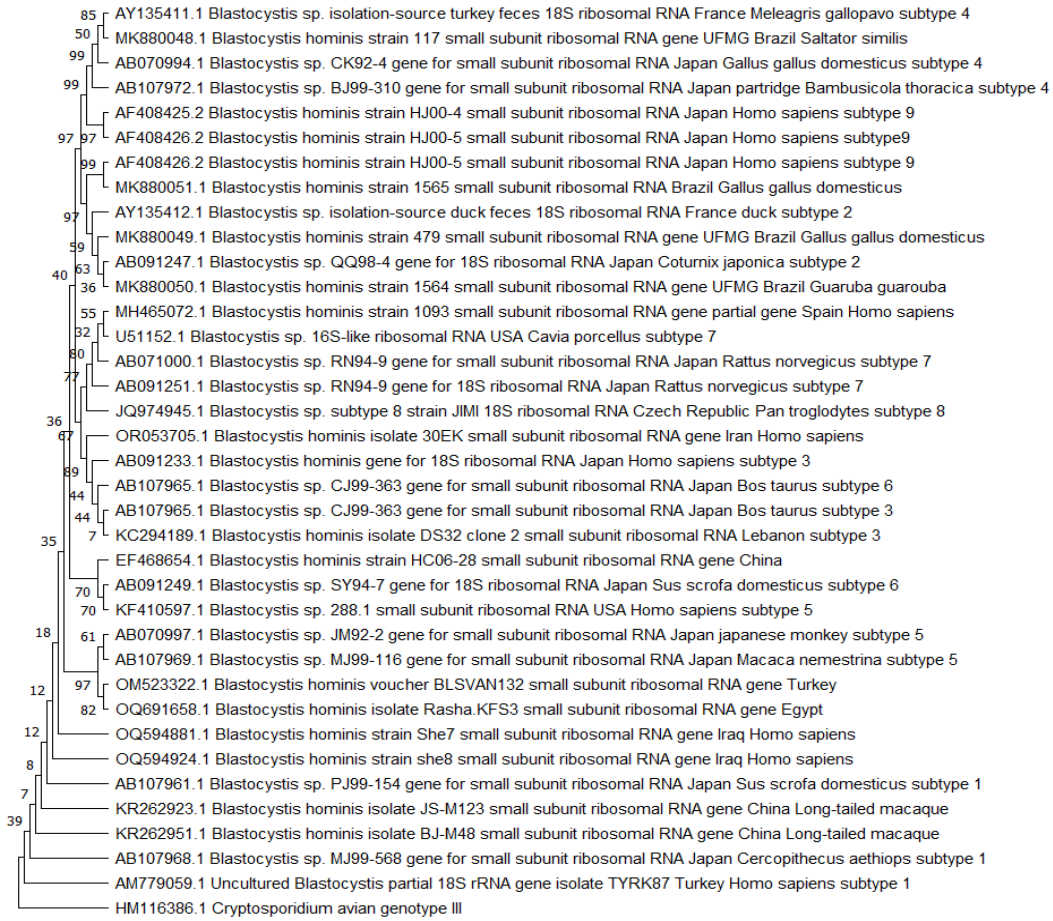

Figure 2 – Evolutionary relationships of avian *Blastocystis* sp.

using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm [Saitou & Nei, 1987] was used to generate the initial tree. The evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021)(Figure 2).

We describe the detection of ST2 and ST4, both previously detected in humans in Brazil, and additionally describe ST9 for the first time. The avian species with the detected STs may be participating in a complex and potentially significant epidemiology. The occurrence of ST1 to ST9 were previously described in humans elsewhere (Skotarczak, 2018).

The biological samples have chronological differences. ST2 was detected in chicken in 2011 (MK880049.1) strain 479, and *G. guarouba* in 2017 (MK880050.1) strain 1564. ST4 was detected in *S. similis* in 2009 (MK880048.1) strain 117, and ST9 was detected in free-range chicken in 2017 (MK880049.1) strain 1565.

ST2 was previously detected in humans (7%), in Brazil (Rio de Janeiro), as well as ST1 (42%), ST3 (49%), ST4 (2%) and mixed infections (14%) (Valença-Barbosa *et al.*, 2017), along with cestodes, nematodes and protists. In a previous study in humans, ST3 was detected with the highest positivity, followed by ST1 and ST2 (Melo *et al.*, 2021). ST4 was previously detected in humans in Rio de Janeiro (Valença Barbosa *et al.*, 2017), although it was not detected in an indigenous group of the Tapirapé ethny in the Amazon basin (Mato Grosso) (Malheiros *et al.*, 2011). A previous investigation in feces of poultry revealed the occurrence in chicken (22.9-42.9%), mallard (37.1-55.3%) and quail (5,5-42,9%) in live markets (Bomfim *et al.*, 2013). ST1 was described to occur in swine in Uberaba (Brazil) (Moura *et al.*, 2018). A previous local study through the microscopy of feces in cracids has revealed the occurrence of an untyped *Blastocystis* sp. in *Aburria jacutinga* (Marques *et al.*, 2013). Our unpublished routine diagnosis results suggest that *Blastocystis* sp. might be widespread in local domestic avian species, especially prevalent in free-range chicken, with some flocks acting as potential reservoirs.

Poultry has been suggested as reservoirs for *Blastocystis* sp. (Gabrielli *et al.*, 2021). A very wide range of host species has been described to harbor *Blastocystis* sp., including most classes of *Animalia* (Liu *et al.*, 2022; Stenzel & Boreham, 1996; Tan, 2008). In Italy, STs were identified in fallow deer, goat, and pig (ST5), pheasant (ST6), chicken (ST7), sheep (ST10) and water buffalo (ST14), and animal-to-human spillover was suggested for ST6 and ST7, with a high similarity (99–100%) when comparing human to other animal isolates (Gabrielli *et al.*, 2021). The natural occurrence was described in the marine fauna of the Northeastern Atlantic ocean (France), and six different subtypes (ST) were detected in fish and mammals (Gantois *et al.*, 2020). The occurrence was previously investigated in France, suggesting avian and artiodactyl species as reservoirs (Cian *et al.*, 2017); and reptilian host species were investigated in Singapore (Teow *et al.*, 1992). In humans, the occurrence of *Blastocystis*, mostly ST7 or ST9, has been associated with irritable bowel syndrome and the history of contact with pigs and poultry (Stensvold *et al.*, 2009). A very wide range of species has been described as hosts for *Blastocystis* sp. (Stenzel & Boreham, 1996; Tan, 2008), which are known to be widespread in humans and associated to gastrointestinal diseases, especially in immunocompromised patients (Wawrzyniak *et al.*, 2013). Such diversity of host taxa encourages broadening local investigation, as it is possible that additional subtypes are occurring in other avian hosts.

In this study, *Blastocystis* sp. was detected by microscopy and by PCR, along with additional untyped strains demonstrated only at microscopy. The detection of *Blastocystis* in local chicken hosts may have a higher epidemiological and public health significance due to its socioeconomic importance, large populations and for being a popular protein source. It may be cause for concern as potential risk to immunocompromised humans. The subtypes ST2, ST4 and ST9 may be also be locally relevant to different animal species, such as swine (Moura *et al.*, 2018), in addition to humans.

CONCLUSION

Our study has shown the occurrence of three subtypes (ST2, ST4 and ST9) of *Blastocystis* sp. in different domestic and wild avian species in Brazil (Belo Horizonte). The obtained subtypes have chronological (2009-2017), host, and, potentially, pathogenicity differences. *Blastocystis* subtypes may have been circulating among different avian host species in the local environment.

ACKNOWLEDGMENTS

Author contributions

Conceptualization, S.Y., N.R.S., O.C.; Methodology, S.Y., H.L.; Investigation: C.R., J.P., C.F., P.H., H.C.G., D.A.R.; Formal analysis: S.Y., N.R.S.; Resources: N.R.S., O.C., D.A.R.; Data curation: S.Y., N.R.S.; Writing: N.R.S., S.Y.; Project administration: N.R.S.; Funding acquisition: N.R.S., O.C.; Supervision: N.R.S.

Funding

The authors thank CAPES, CNPq and FAPEMIG for scholarships, and to FEPE (Fundação de Apoio ao Ensino Pesquisa e Extensão, Escola de Veterinária, UFMG) for administrative support.

Data availability statement

Data will be available upon request.

Conflicts of interest

There are no conflicts of interest in the design, collection, analysis, interpretation of data, or the writing of the manuscript.

REFERENCES

Aykur M, Camyar A, Türk BG, *et al.* Evaluation of association with subtypes and alleles of *Blastocystis* with chronic spontaneous urticaria. Acta Tropica 2004;231:106455. https://doi.org/10.1016/j.actatropica.2022.106455

Aykur M, Malatyalı E, Demirel F, Cömert-Koçak B, *et al. Blastocystis*: a mysterious member of the gut microbiome. Microorganisms 2024;12(3):461. https://doi.org/10.3390/microorganisms12030461

Barbosa CV, Barreto MM, Andrade RD, *et al.* Intestinal parasite infections in a rural community of Rio de Janeiro (Brazil): prevalence and genetic diversity of *Blastocystis* subtypes. Public Library of Science One 2018;13(3):e0193860. https://doi.org/10.1371/journal.pone.0193860

Cian A, El Safadi D, Osman M *et al.* Molecular epidemiology of *Blastocystis* sp. in various animal groups from two French zoos and evaluation of potential zoonotic risk. Public Library of Science One 2017;12(1):e0169659. https://doi.org/10.1371/journal.pone.0169659

De Palma G, Nadal I, Medina M, *et al.* Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiology 2010;10:1-7. https://doi.org/10.1186/1471-2180-10-63

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783-91.

Gabrielli S, Palomba M, Furzi F, *et al.* Molecular subtyping of *Blastocystis* sp. isolated from farmed animals in southern Italy. Microorganisms 2021;9(8):1656. https://doi.org/10.3390/microorganisms9081656

Gantois N, Lamot A, Seesao Y, *et al.* First report on the prevalence and subtype distribution of *Blastocystis* sp. in edible marine fish and marine mammals: a large scale-study conducted in Atlantic Northeast and on the coasts of Northern France. Microorganisms 2020;8(3):460. https://doi.org/10.3390/microorganisms8030460

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 1999;41:95-8.

Kodio A, Coulibaly D, Koné AK, *et al. Blastocystis* colonization is associated with increased diversity and altered gut bacterial communities in healthy Malian children. Microorganisms 2019;7(12). https://doi. org/10.3390/microorganisms7120649

Koehler AV, Herath HD, Hall RS, *et al.* Marked genetic diversity within *Blastocystis* in Australian wildlife revealed using a next generation sequencing–phylogenetic approach. International Journal for Parasitology: Parasites and Wildlife 202;23:100902. https://doi.org/10.1016/j.ijppaw.2023.100902

Liu L, Sun H, Wang Y, *et al. Blastocystis* is widely distributed in mammals and aves. Durham: Research Square; 2022. https://doi.org/10.21203/rs.3.rs-1467419/v1.

Malheiros AF, Stensvold CR, Clark CG, *et al.* Molecular characterization of *Blastocystis* obtained from members of the indigenous Tapirapé ethnic group from the Brazilian Amazon region, Brazil. The American Journal of Tropical Medicine and Hygiene 2011;85(6):1050. https://doi.org/10.4269/ajtmh.2011.11-0481

Maloney JG, Molokin A, Cunha MJ da, *et al. Blastocystis* subtype distribution in domestic and captive wild bird species from Brazil using next generation amplicon sequencing. Parasite Epidemiology and Control 2020;9:e00138. https://doi.org/10.1016/j.parepi.2020.e00138

Marques MV, Junior FC, Assis AD de, *et al.* Serologic, parasitic, and bacteriologic assessment of captive cracids (Aves: Galliformes: Cracidae) in Brazil. Journal of Zoo and Wildlife Medicine 2013;44(1):27-34. https://doi.org/10.1638/1042-7260-44.1.27

Melo GB, Bosqui LR, Costa IN, *et al.* Current status of research regarding *Blastocystis* sp., an enigmatic protist, in Brazil. Clinics 2021;76:e2489. https://doi.org/10.6061/clinics/2021/e2489

Moura RG, Oliveira-Silva MB, *et al.* Occurrence of *Blastocystis* spp. in domestic animals in Triângulo Mineiro area of Brazil. Revista da Sociedade Brasileira de Medicina Tropical 2018;51:240-3. https://doi.org/10.1590/0037-8682-0484-2016

Nei M, Kumar S. Molecular evolution and phylogenetics. Oxford: Oxford University Press; 2000.

Rzhetsky A, Nei M. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution 1992;9:945-67. https://doi.org/10.1093/oxfordjournals.molbev.a040771

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 1987;4:406-25. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences 1977;74(12):5463-7. https://doi.org/10.1073/pnas.74.12.5463

Skotarczak B. Genetic diversity and pathogenicity of *Blastocystis*. Annals of Agricultural and Environmental Medicine 2018;25(3):411-6. https://doi.org/10.26444/aaem/81315

Stensvold CR, Alfellani M, Clark CG. Levels of genetic diversity vary dramatically between *Blastocystis* subtypes. Infection, Genetics and Evolution 2012;12(2):263-73.

Stensvold CR, Lewis HC, Hammerum AM *et al. Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite. Epidemiology & Infection 2009;137(11):1655-63. https://doi.org/10.1017/S0950268809002672

Stensvold R, Brillowska-Dabrowska A, Nielsen HV, *et al.* Detection of *Blastocystis* hominis in unpreserved stool specimens by using polymerase chain reaction. Journal of Parasitology 2006;92(5):1081-7. https://doi.org/10.1645/GE-840R.1

Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 2004;101:11030-11035. https://doi.org/10.1073/pnas.0404206101

Tamura K, Stecher G, Kumar S. MEGA 11: molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution 2021; 38(7):3022-7. https://doi.org/10.1093/molbev/msab120

Tan KSW. *Blastocystis* in humans and animals: new insights using modern methodologies. Veterinary Parasitology 2004;126(1-2):121-44. https://doi.org/10.1016/j.vetpar.2004.09.017

Teow WL, Ng GC, Chan PP, *et al.* A survey of *Blastocystis* in reptiles. Parasitology Research 1992;78:453-5. https://doi.org/10.1007/BF00931705

Valença Barbosa C, Batista RJ, Igreja RP, *et al.* Distribution of *Blastocystis* subtypes isolated from humans from an urban community in Rio de Janeiro, Brazil. Parasites & Vectors 2017;10:1-9. https://doi.org/10.1186/s13071-017-2458-0

Wawrzyniak I, Poirier P, Viscogliosi E *et al.Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis. Therapeutic Advances in Infectious Disease 2013;1(5):167-78. https://doi.org/10.1177/2049936113504754

Yoon HS, Andersen RA, Boo SM, *et al.* Stramenopiles. In: Schaechter M, editor. Encyclopedia of microbiology. 3rd ed. London: Academic Press; 2009. p.721-31. https://doi.org/10.1016/B978-012373944-5.00253-4

Disclaimer/Publisher's Note