Current Research in Parasitology & Vector-Borne Diseases 1 (2021) 100025

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.editorialmanager.com/crpvbd/default.aspx

Cryptosporidium of birds in pet markets in Wuhan city, Hubei, China

Cong Liao [a], Tao Wang [b,*], Anson V. Koehler [b], Min Hu [a], Robin B. Gasser [b,**]

[a] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, 430070, China
[b] Department of Veterinary Biosciences, Melbourne Veterinary School, The University of Melbourne, Parkville, Victoria, Australia

ARTICLE INFO

Keywords:
Pet bird
Cryptosporidium
Zoonotic potential
PCR-based sequencing

ABSTRACT

Cryptosporidium is a group of protistan parasites of a range of vertebrates including mammals and birds. Stimulated by previous work that revealed "zoonotic" *Cryptosporidium meleagridis* subtypes (i.e. IIIbA26G1R1b and IIIbA22G1R1c) in diarrhoeic children and domestic chickens in Wuhan city and environs in Hubei Province, China, here we explored whether zoonotic *C. meleagridis* subtypes might also occur in pet birds in Wuhan city. From 11 bird markets in this city, we collected 322 faecal samples from 48 species of birds (representing six taxonomic orders), isolated genomic DNA and then conducted PCR-based sequencing of genetic markers in the small subunit (*SSU*) of the nuclear ribosomal RNA and the 60 kDa glycoprotein (*gp60*) genes of *Cryptosporidium*. Using *SSU*, *Cryptosporidium* was detected in 55 (17%) of the 322 samples. *Cryptosporidium avium*, *C. baileyi*, *C. meleagridis*, *C. muris* and *C. proventriculi* were characterised in 18%, 47%, 11%, 2% and 20% of the 55 samples, respectively, and a novel *Cryptosporidium galli*-like taxon in one sample. Using *gp60*, only one subtype (IIIeA17G2R1) of *C. meleagridis* was identified, which had not been detected in a previous study of diarrhoeic children in Wuhan. However, IIIe subtypes have been found in both humans and birds around the world. The relatively high prevalence and genetic diversity of *Cryptosporidium* recorded here in pet birds raise awareness about possible reservoirs of zoonotic variants of *Cryptosporidium* in birds in Wuhan, and potentially, other provinces in China.

1. Introduction

Species of *Cryptosporidium* (Phylum Apicomplexa) infect vertebrates, including amphibians, fish, reptiles, birds and mammals (Santín, 2013). Currently, approximately 40 species and more than 70 genotypes are recognised (Zahedi et al., 2016; Holubová et al., 2019). *Cryptosporidium* species can cause intestinal or respiratory disease, called cryprosporidiosis (Bouzid et al., 2013). Cryptosporidiosis is a leading cause of diarrhoea and death in children (Striepen, 2013). Disease can be self-limiting in healthy hosts, but is life-threatening, particularly in young, old, or immuno-compromised individuals, such as those affected by HIV/AIDS (Bouzid et al., 2013).

The application of molecular epidemiological tools for the genetic identification and characterisation of *Cryptosporidium* (to the species, genotype and/or subtype levels) has improved our understanding of the distribution and transmission of cryptosporidiosis. *Cryptosporidium* species and genotypes vary in their host ranges, and some are recognised as zoonotic (Xiao & Feng, 2008; Feng et al., 2018; Khan et al., 2018), for example, with transmission occurring between mammalian species (sheep, cattle, dog or cat; Alves et al., 2003; Chalmers et al., 2005; Lucio-Forster et al., 2010) or bird species (Nakamura et al., 2009; da Silva et al., 2010; Qi et al., 2011; Li et al., 2016; da Cunha, 2018).

In a previous epidemiological survey of diarrhoeic children in Wuhan, we characterized molecular subtypes of *C. meleagridis* (IIIbA22G1R1c and IIIbA26G1R1b) by PCR-based sequencing of part of the *gp60* gene (Wang et al., 2017) which matched those recorded in chickens in Hubei Province (Liao et al., 2018). The findings indicated that, in Wuhan and environs, chickens may contribute to the transmission of *C. meleagridis* to humans. It was also suggested that wild or other domestic birds (such as pets) might be involved in such transmission, warranting further investigation. In this study, we explore the occurrence of *Cryptosporidium* of pet birds sold at animal markets in Wuhan city in Hubei Province, China.

2. Materials and methods

Between August and December 2018, a total of 322 fresh faecal samples were obtained from pet birds of different breeds from 11 pet bird markets in Wuhan city, Hubei Province, China. Pet shop managers

* Corresponding author.
** Corresponding author.
E-mail addresses: tao.wang1@unimelb.edu.au (T. Wang), robinbg@unimelb.edu.au (R.B. Gasser).

https://doi.org/10.1016/j.crpvbd.2021.100025
Received 23 March 2021; Received in revised form 13 April 2021; Accepted 14 April 2021

donated the samples for testing. The identification of bird species was performed using field guides to Australian and Chinese birds (Qian, 1995; MacKinnon, 2000; Slater et al., 2009). Single samples were collected from individual cages (containing 1–20 birds of a similar age). In total, 48 species, representing six orders of birds, were studied (Supplementary Table S1).

Genomic DNA was isolated from individual faecal samples using the PowerSoil DNA isolation kit (MoBio, Carlsbad, USA), according to the manufacturer's protocol, and frozen at −20°C. Then, aliquots (2 μl) of individual DNA samples were subjected to nested PCR-based amplification and sequencing, targeting a ~830 bp fragment of the small subunit (*SSU*) rRNA gene (Xiao et al., 2001). For classification of *C. meleagridis* to the subtype level in samples test-positive for *SSU*, a ~900–1100 bp fragment of the 60 kDa glycoprotein (*gp60*) gene was amplified by nested PCR (cf. Stensvold et al., 2014). Each PCR run included known positive, negative, and no-template controls. Individual PCR products were examined *via* 1.5% agarose gel electrophoresis (Liao et al., 2018). Following treatment with the enzyme *Exo*I plus FastAP thermosensitive alkaline phosphatase (ThermoFisher Scientific, USA), amplicons were subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) using the same internal primers (individually) as employed in nested PCR.

SSU and *gp60* sequences obtained were aligned using the program MAFFT (Katoh et al., 2002), and alignments manually adjusted employing the program Mesquite v.3.61 (Maddison & Maddison, 2018). Sequences were compared with reference sequences available from GenBank (NCBI) using BLASTn. Separate phylogenetic analyses of the *SSU* (840 bp) and *gp60* (869 bp) sequence alignments were conducted using the neighbour-joining (NJ) distance method (Saitou & Nei, 1987) in the program MEGA X v.10.1.8 (Stecher et al., 2020). Evolutionary distances were computed using the 'number of differences' method (Nei & Kumar, 2000), including 'transitions and transversions' for the nucleotide data. Rates of evolution among sites were considered uniform, and gaps were treated using pairwise deletion. Bootstrap replicates ($n = 10{,}000$) were performed, and bootstrap support (%) recorded. The outgroups used in the phylogenetic analyses of *SSU* and *gp60* sequence data sets were *Cryptosporidium molnari* (GenBank: HM243547) and *C. meleagridis* subtype IIId (GenBank: DQ067570), respectively.

3. Results and discussion

From the 322 faecal DNA samples tested, p*SSU* was amplified from 55 (17%) of them. The 55 p*SSU* amplicons represented 14 bird species (i.e. crested myna, Indian myna, golden-crested myna, Java sparrow, spotted munia, Gouldian finch, zebra finch, Japanese white-eye, budgerigar, cockatiel, Fischer's lovebird, rosy-faced lovebird, chicken and pigeon) of four orders (Table 1). The overall prevalence of 17% is comparable or higher to findings for previous studies of wild and zoo birds (Ng et al., 2006; Nakamura et al., 2009; Qi et al., 2011; Nakamura & Meireles, 2015; Máca & Pavlásek, 2015; Reboredo-fernández et al., 2015; Helmy et al., 2017; Iijima et al., 2018).

The p*SSU* sequences of the 55 amplicons were compared with reference sequences from GenBank (see Fig. 1). This comparison allowed us to identify seven distinct p*SSU* sequences representing six taxa (i.e. *C. avium*, *C. baileyi*, *C. galli*-like, *C. meleagridis*, *C. muris* and *C. proventriculi*; GenBank accession numbers MW783459-MW783465). The prevalences of these respective species were 3% ($n = 10$), 8% ($n = 26$), 0.3% ($n = 1$), 2% ($n = 6$), 0.3% ($n = 1$) and 3% ($n = 11$), with *C. baileyi*, *C. proventriculi* and *C. avium* being the predominant species (Table 1). The record of *C. muris* in pigeons is new, but may relate to pseudoparasitism (Xiao et al., 2004). Passeriforms were infected mostly with *C. baileyi*, whereas *C. avium*, *C. meleagridis* and *C. proventriculi* were detected mostly in psittaciforms (Table 1); similar findings have been reported previously (Ng et al., 2006; Nakamura et al., 2009; Sevá et al., 2011; Iijima et al., 2018).

Table 1
Cryptosporidium taxa molecularly identified in 55 of 322 faecal DNA samples from individual pet birds from markets in Wuhan city, China

Bird species	*Cryptosporidium* species/taxon (number of samples)
Passeriformes	
Crested myna (*Acridotheres cristatellus*)	*C. baileyi* (2)
Indian myna (*Acridotheres tristis*)	*C. baileyi* (2)
Golden-crested myna (*Ampeliceps coronatus*)	*C. baileyi* (1)
Java sparrow (*Lonchura oryzivora*)	*C. baileyi* (1)
Spotted munia (*Lonchura punctulata*)	*C. baileyi* (1)
Gouldian finch (*Erythrura gouldiae*)	*C. baileyi* (2)
Zebra finch (*Taeniopygia guttata*)	*C. baileyi* (10)
Japanese white-eye (*Zosterops japonicus*)	*C. galli*-like (1)
Psittaciformes	
Budgerigar (*Melopsittacus undulatus*)	*C. avium* (8); *C. baileyi* (4); *C. meleagridis* (1)
Cockatiel (*Nymphicus hollandicus*)	*C. avium* (2); *C. baileyi* (1); *C. meleagridis* (2); *C. proventriculi* (7)
Fischer's lovebird (*Agapornis fischeri*)	*C. baileyi* (1); *C. meleagridis* (2); *C. proventriculi* (1)
Rosy-faced lovebird (*Agapornis roseicollis*)	*C. proventriculi* (3)
Galliformesx	
Chicken (*Gallus gallus*)	*C. baileyi* (1); *C. meleagridis* (1)
Columbiformes	
Pigeon (*Columba livia*)	*C. muris* (1)

Phylogenetic analysis (Fig. 1) determined that all but one of the distinct p*SSU* sequences matched, with 100% identity, known sequences in GenBank for *C. avium* (HM116381), *C. baileyi* (KY352489 and JX548296), *C. meleagridis* (KY352486), *C. muris* (GQ121018) and *C. proventriculi* (HM116385). Additionally, the sequence from a Japanese white-eye was 99% identical (748 of 755 bp) to *C. galli* from an ibis in Australia (GenBank: MG516766). Here, we refer to it as *C. galli*-like, but the extent of sequence variation (7 bp) suggests that it could be a novel species; this proposal warrants further investigation using multiple genetic markers.

The five p*SSU* amplicons that were classified as *C. meleagridis* were further subtyped using *gp60* primers (Stensvold et al., 2014). Sequence alignment and phylogenetic analysis of these five p*gp60* sequences and reference sequences revealed a novel variant of the *C. meleagridis* IIIe subtype family (IIIeA17G2R1; GenBank accession no. MW810675) in Fischer's lovebirds ($n = 2$) and cockatiels ($n = 3$). This subtype (IIIeA17G2R1) has been detected previously in a farmed chicken in Hubei, China (GenBank: MG969388) and from humans in China (GenBank: KU852726) and India (or Nepal) (GenBank: KJ210608). Despite having a slightly different subtype, the present sequence (accession no. MW810675) is more closely related to a human sample from Australia, IIIeA18G2R1 (GenBank: MK165992) than to the other IIIeA17G2R1 subtypes (Fig. 2). Nevertheless, subtypes from group IIIe have been detected in both birds and humans (e.g. Stensvold et al., 2014; Máca & Pavlásek, 2015; Liao et al., 2018; Braima et al., 2019), including possible cases of transmission from poultry to immunosuppressed people (Wang et al., 2013). Although this subtype (IIIeA17G2R1) of *C. meleagridis* has not been detected previously in humans in Wuhan, other subtypes (IIIbA21G1R1, IIIbA22G1R1, IIIbA26G1R1) have been recorded previously in diarrhoeic children in this city (Wang et al., 2017).

In conclusion, we investigated here the presence and genetic identity of *Cryptosporidium* in birds (48 species of 6 orders) in 11 pet markets in Wuhan, Hubei Province. The prevalence (17%) and genetic diversity in species established here and the detection of some taxa, such as *C. meleagridis* subtype IIIe, that might be zoonotic emphasize the need to

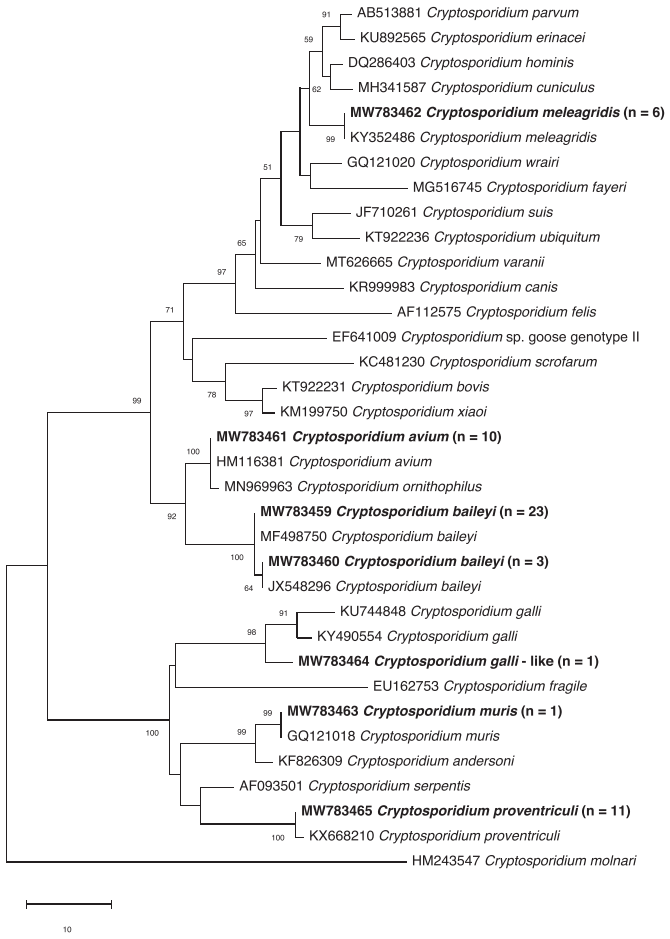

Fig. 1 Phylogenetic relationships of *Cryptosporidium* taxa constructed using the neighbour-joining distance method, employing nucleotide sequence data from a portion of the small subunit of the nuclear ribosomal RNA gene (*SSU*). *Cryptosporidium* species or genotypes characterised in the present study are in bold-type. The GenBank accession number precedes the species designation; the number of samples of a particular species/genotype is indicated in parentheses. The scale-bar represents the number of substitutions per site. *Cryptosporidium molnari* (GenBank: HM243547) was used as an outgroup. Bootstrap support is indicated at the nodes

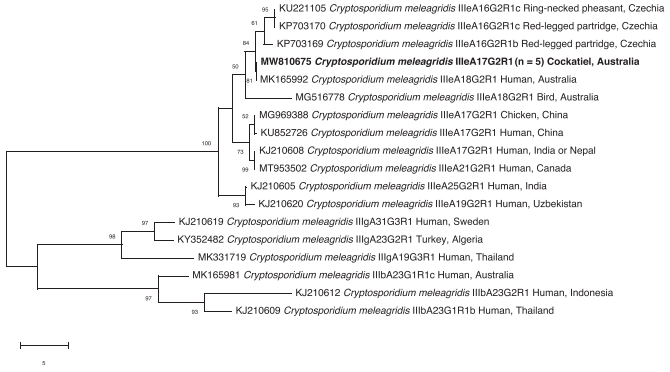

Fig. 2 Phylogenetic relationships of *Cryptosporidium meleagridis* constructed using the neighbour-joining distance method, employing nucleotide sequence data from fragment of the 60 kDa glycoprotein (*gp60*) gene. *Cryptosporidium meleagridis* sequence generated in the present study is in bold-type. The GenBank accession number precedes the species designation; the number of samples of a particular species/genotype is indicated in parentheses. The scale-bar represents the number of substitutions per site. *Cryptosporidium meleagridis* subtype IIIb (GenBank: KJ210609) was used as the outgroup. Bootstrap support is indicated at the nodes

undertake more detailed investigations in humans and animals in Wuhan and other provinces in China, in order to infer zoonotic transmission patterns of cryptosporidiosis.

Funding

This project was supported by Huazhong Agricultural University Scientific & Technological Self Innovation Foundation (program no. 2015RC005).

Ethical approval

The study was approved (permit no. HZAUBI-2018-001) by the Animal Management and Ethics Committee of the Huazhong Agricultural University, China.

CRediT author statement

Cong Liao: Investigation, Original draft preparation. Tao Wang: Conceptualisation, Validation, Writing - Reviewing and Editing. Min Hu: Investigation, Writing - Reviewing and Editing. Anson V. Koehler: Visualisation, Software, Validation, Writing-Reviewing and Editing. Robin B. Gasser: Conceptualisation, Supervision, Writing - Reviewing and Editing.

Data availability

The newly generated sequences were deposited in the GenBank database under the accession numbers MW783459-MW783465 (p*SSU*) and MW810675 (p*gp60*).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This project was supported by Huazhong Agricultural University Scientific & Technological Self Innovation Foundation (Program no. 2015RC005). We thank the pet market managers for donating faecal samples from birds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2021.100025.

References

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J. Clin. Microbiol. 41, 2744–2747.

Bouzid, M., Hunter, P.R., Chalmers, R.M., Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26, 115–134.

Braima, K., Zahedi, A., Oskam, C., Reid, S., Pingault, N., Xiao, L., Ryan, U., 2019. Retrospective analysis of *Cryptosporidium* species in Western Australian human populations (2015–2018), and emergence of the *C. hominis* IfA12G1R5 subtype. Infect. Genet. Evol. 73, 306–313.

Chalmers, R.M., Ferguson, C., Caccio, S., Gasser, R.B., Abs, E.L.O.Y.G., Heijnen, L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M., Stevens, M., 2005. Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. Int. J. Parasitol. 35, 397–410.

da Cunha, M.J.R., Cury, M.C., Santín, M., 2018. Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil. Res. Vet. Sci. 118, 331–335.

da Silva, D.C., Homem, C.G., Nakamura, A.A., Teixeira, W.F., Perri, S.H., Meireles, M.V., 2010. Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. Parasitol. Res. 107, 271–277.

Feng, Y., Ryan, U.M., Xiao, L., 2018. Genetic diversity and population structure of *Cryptosporidium*. Trends Parasitol. 34, 997–1011.

Helmy, Y.A., Krücken, J., Abdelwhab, E.M., von Samson-Himmelstjerna, G., 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, e0177150.

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 psittaciforme birds. Eur. J. Protistol. 69, 70–87.

Iijima, Y., Itoh, N., Phrompraphai, T., Ito, Y., Kimura, Y., Kameshima, S., 2018. Molecular prevalence of *Cryptosporidium* spp. among companion birds kept in pet shops in Japan. Korean J. Parasitol. 56, 281–285.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30, 3059–3066.

Khan, A., Shaik, J.S., Grigg, M.E., 2018. Genomics and molecular epidemiology of *Cryptosporidium* species. Acta Tropica 184, 1–14.

Liao, C., Wang, T., Koehler, A.V., Fan, Y., Hu, M., Gasser, R.B., 2018. Molecular investigation of *Cryptosporidium* in farmed chickens in Hubei Province, China, identifies 'zoonotic' subtypes of *C. meleagridis*. Parasit. Vectors 11, 484.

Li, Q., Li, L., Tao, W., Jiang, Y., Wan, Q., Lin, Y., Li, W., 2016. Molecular investigation of *Cryptosporidium* in small caged pets in northeast China: Host specificity and zoonotic implications. Parasitol. Res. 115, 2905–2911.

Lucio-Forster, A., Griffiths, J.K., Cama, V.A., Xiao, L., Bowman, D.D., 2010. Minimal zoonotic risk of cryptosporidiosis from pet dogs and cats. Trends Parasitol. 26, 174–179.

Máca, O., Pavlásek, I., 2015. First finding of spontaneous infections with *Cryptosporidium baileyi* and *C. meleagridis* in the red-legged partridge *Alectoris rufa* from an aviary in the Czech Republic. Vet. Parasitol. 209, 164–168.

MacKinnon, J., 2000. *A Field Guide to the Birds of China*. Oxford University Press, Oxford, UK.

Maddison, W., Maddison, D.R., 2018. Mesquite: A modular system for evolutionary analysis. Version 3.40. https://mesquiteproject.org/.

Nakamura, A.A., Meireles, M.V., 2015. *Cryptosporidium* infections in birds - a review. Rev. Bras. Parasitol. Vet. 24, 253–267.

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.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, Oxford, UK.

Ng, J., Pavlasek, I., Ryan, U., 2006. Identification of novel genotypes from avian hosts. Appl. Environ. Microbiol 72, 7548–7553.

Qian, Y., 1995. Atlas of Birds of China. Henan Science and Technology Press, Henan, China.

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 142, 917–925.

Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Santín, M., 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J. 61, 1–10.

Sevá, A.P., Funada, M.R., Richtzenhain, L., Guimarães, M.B., Souza Sde, O., Allegretti, L., Sinhorini, J.A., Duarte, V.V., Soares, R.M., 2011. Genotyping of *Cryptosporidium* spp. from free-living wild birds from Brazil. Vet. Parasitol. 175, 27–32.

Slater, P., Slater, P., Slater, R., 2009. The Slater Field Guide to Australian Birds, 2nd ed. New Holland Publishers, Sydney, Australia.

Stecher, G., Tamura, K., Kumar, S., 2020. Molecular evolutionary genetics analysis (MEGA) for macOS. Mol. Biol. Evol. 37, 1237–1239.

Stensvold, C.R., Beser, J., Axén, C., Lebbad, M., 2014. High applicability of a novel method for *gp60*-based subtyping of *Cryptosporidium meleagridis*. J. Clin. Microbiol. 52, 2311–2319.

Striepen, B., 2013. Parasitic infections: Time to tackle cryptosporidiosis. Nature 503, 189–191.

Wang, T., Fan, Y., Koehler, A.V., Ma, G., Li, T., Hu, M., Gasser, R.B., 2017. First survey of *Cryptosporidium, Giardia* and *Enterocytozoon* in diarrhoeic children from Wuhan, China. Infect. Genet. Evol. 51, 127–131.

Wang, L., Zhang, H., Zhao, X., Zhang, L., Zhang, G., Guo, M., Liu, L., Feng, Y., Xiao, L., 2013. Zoonotic *Cryptosporidium* species and *Enterocytozoon bieneusi* genotypes in HIV-positive patients on antiretroviral therapy. J. Clin. Microbiol. 51, 557–563.

Xiao, L., Fayer, R., Ryan, U., Upton, S.J., 2004. *Cryptosporidium* taxonomy: Recent advances and implications for public health. Clin. Microbiol. Rev. 17, 72–97.

Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol. 52, 309–323.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., Lal, A., 2001. Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Appl. Environ. Microbiol. 67, 1097–1101.

Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016. Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management. Int. J. Parasitol. Parasites Wildl. 5, 88–109.