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

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A B S T R A C T

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 (IleA17G2R1) of C. meleagridis was identified, which had not been detected in a previous study of diarrhoeic children in Wuhan. However, Ille 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 cryptosporidiosis (Bouzid et al., 2013). Cryptosporidiosis is a leading cause of diarrhoea and death in children (Santín, 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., 2012; Qi et al., 2013; Li et al., 2016; da Cunha, 2018).

In a previous epidemiological survey of diarrhoeic children in Wuhan, we characterized molecular subtypes of C. meleagridis (IllbA22G1R1c and IllbA26G1R1b) 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
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 glycophorin (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 reversions’ 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 C. meleagridis (GenBank: DQ067570), respectively.

| Bird species | Cryptosporidium species/taxon (number of samples) |
|--------------|--------------------------------------------------|
| Passeriformes |                                                  |
| Crested myna (Acrocephalus cristatus)             | C. bailey (2)                                   |
| Indian myna (Acrocephalus tristis)               | C. bailey (2)                                   |
| Golden-crested myna (Amphipithecus coronatus)    | C. bailey (1)                                   |
| Java sparrow (Lonchura oryzivora)               | C. bailey (1)                                   |
| Spotted munia (Lonchura punctulata)              | C. bailey (2)                                   |
| Gouldian finch (Erythrura gouldiae)              | C. bailey (1)                                   |
| Zebra finch (Taeniopygia guttata)                | C. bailey (2)                                   |
| Japanese white-eye (Zosterops japonicus)         | C. galli-like (1)                               |
| Psittaciformes                                   |                                                  |
| Budgerigar (Melopsittacus undulatus)              | C. avium (8); C. bailey (4); C. meleagridis (1) |
| Cockatiel (Nymphicus hollandicus)                 | C. avium (2); C. bailey (1); C. meleagridis (2); C. proventriculi (7) |
| Fischer’s lovebird (Agapornis fischeri)         | C. bailey (1); C. meleagridis (2); C. proventriculi (1) |
| Golden-crested lovebird (Agapornis roossouwii)   | C. proventriculi (3)                            |
| Galliformes                                      |                                                  |
| Chicken (Gallus gallus)                         | C. bailey (1); C. meleagridis (1)               |
| Columbiformes                                    |                                                  |
| Pigeon (Columba livia)                           | C. murs (1)                                     |

3. Results and discussion

From the 322 faecal DNA samples tested, pSSU was amplified from 55 (17%) of them. The 55 pSSU 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; Mäca & Pavlasek, 2015; Re suggested in 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; Mäca & Pavlasek, 2015; Re备oro-férdinández et al., 2015; Helmy et al., 2017; Iijima et al., 2018).

The pSSU sequences of the 55 amplicons were compared with reference sequences from GenBank (see Fig. 1). This comparison allowed us to identify seven distinct pSSU sequences representing six taxa (i.e. C. avium, C. baileyi, C. galli-like, C. meleagridis, C. murs and C. proventriculi; GenBank accession numbers MW783459-MW783465). The prevalence of these respective species was 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. murs in pigeons is new, but may relate to pseudoparasitism (Xiao et al., 2004). Patteriformes were infected mostly with C. baileyi, whereas C. avium, C. meleagridis and C. proventriculi were detected mostly in psittaciformes (Table 1); similar findings have been reported previously (Ng et al., 2006; Nakamura et al., 2009; Sevá et al., 2011; Iijima et al., 2018).

Phylogenetic analysis (Fig. 1) determined that all but one of the distinct pSSU sequences matched, with 100% identity, known sequences in GenBank for C. avium (HM116381), C. baileyi (KJ352489) and C. meleagridis (KY352486), C. murs (QG121018) 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 pSSU 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 pp60 sequences and reference sequences revealed a novel variant of the C. meleagridis Ile subtype family (IleA17G2R1; GenBank accession no. MW810675) in Fischer’s lovebirds (n = 2) and cockatiels (n = 3). This subtype (IleA17G2R1) 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, IleA18G2R1 (GenBank: MKI65992) than to the other IleA17G2R1 subtypes (Fig. 2). Nevertheless, subtypes from group Ile have been detected in both birds and humans (e.g. Stensvold et al., 2014; Mäca & Pavlasek, 2015; Liao et al., 2018; Bramia et al., 2019), including possible cases of transmission from poultry to immunosuppressed people (Wang et al., 2013). Although this subtype (IleA17G2R1) of C. meleagridis has not been detected previously in humans in Wuhan, other subtypes (IleA21G1R1, IleA22G1R1, IleA26G1R1) 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 Ile, that might be zoonotic emphasize the need to
undertake more detailed investigations in humans and animals in Wuhan and other provinces in China, in order to infer zoonotic transmission patterns of cryptosporidiosis.

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Ethical approval
The study was approved (permit no. HZAUUB-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 (pSSU) and MW810675 (pgp60).

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.

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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 (pgp60) 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
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