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GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Occurrence of a *Cryptosporidium xiaoi*–like genotype in peafowl (*Pavo cristatus*) in China

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Abstract

The aim of this study was to survey the *Cryptosporidium* species in peafowls (*Pavo cristatus*) in Henan Province, China. A total of 143 fecal specimens collected from a breeding farm were tested for *Cryptosporidium* by nested PCR targeting the small subunit rRNA (SSU rRNA), 70-kDa heat shock protein (HSP70), and actin genes of *Cryptosporidium* followed by sequence analysis. Only one isolate from an asymptomatic host was obtained, and the isolate differed from a new *C. xiaoi*–like genotype by one nucleotide and from *C. xiaoi* or *C. bovis* at the SSU rRNA locus by six nucleotides. Likewise, the actin gene shared 99% identity with the *C. xiaoi*–like genotype, accompanied by four nucleotide mutations. A complete sequence of the HSP70 gene was obtained, and exhibited 96% similarity with that from *C. xiaoi* and differed by one nucleotide from that with the *C. xiaoi*–like genotype. Phylogenetic analysis of the current isolate revealed genetic relatedness to the *C. xiaoi*–like genotype and distinction from *C. xiaoi* and *C. bovis*. Therefore, our results provided the first documentation of avian infection with a *C. xiaoi*–like genotype in China and further insight into the diversity of *Cryptosporidium* spp. in avians.

Keywords Cryptosporidium · C. xiaoi · like genotype · Genetic diversity · Peafowl

Introduction

Cryptosporidium, an intracellular and parasitic protist, can infect a wide variety of vertebrates, including humans (Ryan and Hijjawi 2015). The various transmission modes for *Cryptosporidium* spp. between humans and other vertebrates represent an important public health threat (Xiao 2010). To date, of the near 40 valid *Cryptosporidium* species, at least nine have been documented in a wide range of birds worldwide, including *C. meleagridis*, *C. baileyi*, *C. galli*, *C. avium*, *C. proventriculi*, *C. hominis*, *C. parvum*, *C. andersoni*, and *C. muris* (Condlova et al. 2018; Cui et al. 2018; Holubova et al. 2019; Kvac et al. 2018; Ryan et al. 2016). Only the first five are bird-specific species causing natural infections. In addition, more than 14 *Cryptosporidium* genotypes have been recorded, comprising avian genotypes I–II, IV, and VI–IX; black duck genotype; goose genotypes I–V; and Eurasian woodcock genotype (Cui et al. 2018). Recently, a new *Cryptosporidium* genotype was described in chicken from Brazil, temporarily named as *C. xiaoi*–like genotype (Ewald et al. 2017; Santana et al. 2018). However, there are no data on infection by this genotype in other bird species.

Because research on avian *Cryptosporidium* spp. is scarce compared to that on mammals, our understanding of the genotypic diversity of the pathogens in birds, especially in wild species, is poor (Baroudi et al. 2013; Elkarim Laatamna et al. 2017). Previously, an asymptomatic white peafowl was diagnosed as *Cryptosporidium*-positive through immunofluorescence microscopy and PCR assays in China, but the genotype was unknown; afterwards, *Cryptosporidium* was reported in an Indian peafowl with no clinical symptoms and the parasite was avian genotype I (Karanis et al. 2007; Nakamura et al. 2009). Recently, Feng et al. (2019) identified *C. proventriculi*

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and goose genotype I in peafowl in Beijing and Jiangxi. However, to the best of our knowledge, limited data are available on the prevalence and genetic characteristics of *Cryptosporidium* infection in peafowl in Henan Province. Therefore, this study employed molecular tools to determine the occurrence and genetic identity of *Cryptosporidium* in peafowl in captivity in Henan Province, China.

Materials and methods

Fecal samples

This study was performed in compliance with the Chinese law on the Protection of Wildlife (modified by Henan Province, no. 005253090). The enclosure of each peafowl was cleaned before sample collection. From every peafowl, the feces that had not contacted the ground was collected using a medical cotton swab, placed in sampling bags, and stored at 4 °C for PCR detection. Synchronously, the clinical symptoms of each peafowl were recorded.

A total of 143 fecal samples were obtained from a breeding farm in Henan Province, China, and no clinical signs were noted for all peafowls at the time of sampling. Genomic DNA was extracted immediately in our laboratory using a Stool DNA Kit (Omega Bio-Tek Inc., D4015-2, USA) following the manufacturer's instructions and stored at – 20 °C for subsequent PCR assay.

Species determination and genotyping

Genomic DNA was used to detect *Cryptosporidium* by nested PCR amplification targeting the small subunit rRNA (SSU rRNA) gene followed by amplification of the 70-kDa heat shock protein (HSP70), oocyst wall protein (COWP), and actin genes to further determine species/genotype. Primers and amplification conditions were chosen according to previous studies (Sulaiman et al. 2002; Sulaiman et al. 2000; Xiao et al. 1999; Xiao et al. 2000). To neutralize PCR inhibitors, non-acetylated bovine serum albumin (400 ng/mL, TaKaRa, China) was used in both PCRs. The nucleotide sequences of secondary PCR products of the four loci were obtained directly in both directions using secondary PCR primers. To verify sequence accuracy, a new secondary PCR product was sequenced again.

DNA sequence analysis

Nucleotide sequences obtained from the nested PCR amplicons in this study were aligned with homologous sequences and reference sequences published in the NCBI GenBank database at each locus using ClustalX 1.83 software (ftp://ftpigbmc.u-strasbg.fr/pub/ClustalX/) and adjusted manually by BioEdit 7.04 software (www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic relationships were inferred to support the grouping of *Cryptosporidium* species/genotypes in MEGA 7.0 using neighbor-joining analysis (Kumar et al. 2016). Based on the estimated evolutionary distances, phylograms were constructed for each genotyped locus using the Kimura 2-parameter model and drawn by MEGA 7.0 software, with bootstrap values of greater than 50% reported using 1000 pseudoreplicates.

The unique partial nucleotide sequences of the SSU rRNA, HSP70, and actin genes generated in this study were deposited in the GenBank database under accession numbers MK095941–MK095943.

Results and discussion

In the current study, only one sample (isolate HN74) was found to be positive by PCR amplification of the SSU rRNA gene. Similarly, one case of an Indian peafowl was found to be infected with *Cryptosporidium*in Brazil, along with a white peafowl in Qinghai Province, China (Karanis et al. 2007; Nakamura et al. 2009). Recently, *Cryptosporidium* prevalences of 4.58% and 9.52% were reported in Beijing and Jiangxi Province, China (Feng et al. 2019).

Several molecular tools, especially the SSU rRNA, HSP70, COWP, and actin genes, are typically used to identify and differentiate the genetic characteristics of species/genotypes (Xiao 2010). In our study, no COWP fragments were detected, and the sequences of other loci were amplified successfully, indicating infection by only one *Cryptosporidium* sp./genotype in peafowl. DNA sequencing and a nucleotide BLAST search identified sequence heterogeneity with 6 sporadically distributed nucleotide substitutions and insertions into *C. xiaoi* and *C. bovis* (FJ896050 and JX51556), which shared 99% genetic similarity. However, compared to an emerging *C. xiaoi*–like genotype (MF627417 and MG243625 from a chicken isolate, EU825742 from a water isolate), there was only one mutation (T to C) at nucleotide 447 in the SSU rRNA gene, resulting in 99.9% homology through manual alignment. Likewise, for the actin gene, 4 nucleotide mutations were observed in the *C. xiaoi*–like genotype (MF627416 from the chicken isolate OPSJ1), and a maximum similarity of 99.6% was noted. Significantly, the complete HSP70 gene obtained from this study exhibited at least 78 nucleotide changes, with a maximum identity of 96% (*C. xiaoi* Tibetan sheep isolate, KF907826), followed by an identify of 90% with other reported HSP70 sequences based on BLAST results. In addition, the incomplete HSP70 fragment published for *C. xiaoi* (FJ896041) and the *C. xiaoi*–like genotype (MG243695) shared 5 nucleotide changes and no differences with the current HN74 isolate, respectively, based on manual

comparative analysis. Therefore, the peafowl isolate was likely the *C. xiaoi*–like genotype.

In the phylogenetic tree of the three genes, the similar topological structures determined the genetic relationship and revealed that the peafowl isolate was most closely related to the *C. xiaoi*–like genotype and formed a sperate branch independent of *C. xiaoi* and *C. bovis*, with strong bootstrap support (Fig. 1). Therefore, our isolate, HN74, was demonstrated to be the most recent *C. xiaoi*–like genotype. Prior to this identification, a nearly identical SSU rRNA gene from a water sample (W26 isolate) was genotyped as *C. bovis*–like (Yang et al. 2008). Nevertheless, in free-range chickens in Brazil, the same sequence was identified recently and considered to be the first case of the *C. xiaoi*–like genotype in chickens; the genotype was subsequently reported in domestic chickens (Ewald et al. 2017; Santana et al. 2018). Significantly, the occurrence of the *C. xiaoi*–like genotype in peafowl strongly suggests that the emerging *C. xiaoi*–like genotype infects other avians.

Based on the surveys of *Cryptosporidium* infection in China, five bird-adapted species, including *C. baileyi*, *C. galli*, *C. meleagridis*, *C. avium*, and *C. proventriculi*, were reported from 25 bird species. In addition, goose genotype I,

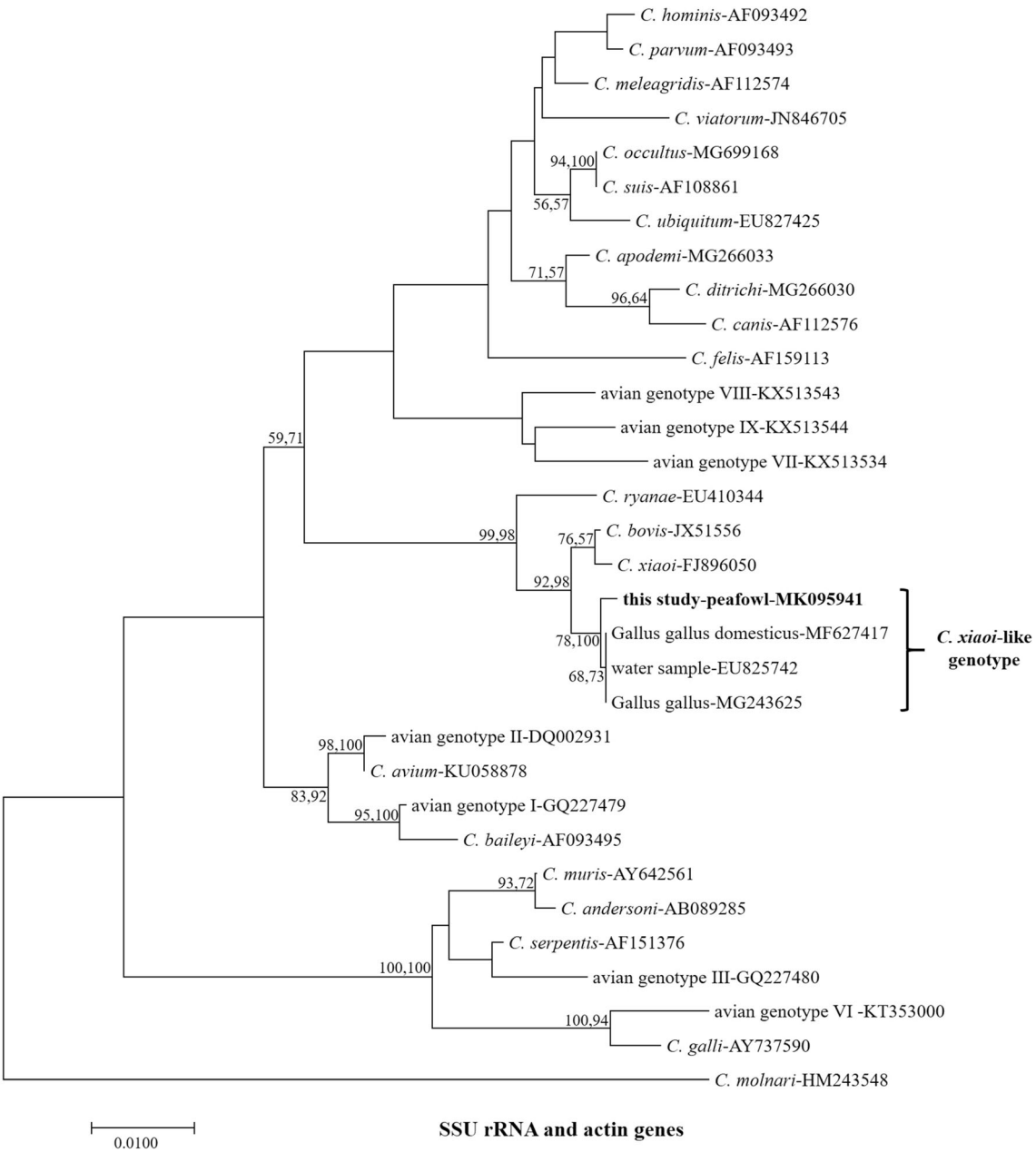

Fig. 1 Phylogenetic relationship of the SSU rRNA and actin genes of *Cryptosporidium* peafowl isolate in this study to other known *Cryptosporidium* species/genotype as inferred by a neighbor-joining analysis based on evolutionary distances calculated using the Kimura two-parameter model. Bootstrap values were obtained using 1000 pseudoreplicates. Bar = substitutions/site

ferret genotype, avian genotype I, and *C. ubiquitum* have been found occasionally in cockatiel, red-billed blue magpie, budgerigar, peafowl, and hill myna (Amer et al. 2010; Feng et al. 2019; Li et al. 2015; Li et al. 2016; Nakamura et al. 2009; Qi et al. 2011; Wang et al. 2011; Wang et al. 2012; Zhang et al. 2015). Combined with those findings, our results expand the *Cryptosporidium* range in birds.

In conclusion, infection by a *C. xiaoi*–like genotype was confirmed in peafowl for the first time in China, and the emerging genotype provided further insight into *Cryptosporidium* in avian species. However, the host specificity of the *C. xiaoi*–like genotype remains unclear and requires further research.

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Compliance with ethical standards

This study was performed in compliance with the Chinese law on the Protection of Wildlife (modified by Henan Province, no. 005253090).

Competing interests The authors declare that they have no competing interests.

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