

Cryptosporidiosis in broiler chickens in Zhejiang Province, China: molecular characterization of oocysts detected in fecal samples

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Abstract – *Cryptosporidium* is one of the most important parasites in poultry, and this pathogen can infect more than 30 avian species. The present study investigated the infection rate of *Cryptosporidium* among broiler chicken flocks. A total of 385 fecal samples from broiler chickens in 7 regions of Zhejiang Province collected from November 2010 to January 2012 were examined by microscopy. Thirty-eight (10%) samples were positive for *Cryptosporidium* infection, and 3 genotypes (*Cryptosporidium baileyi*, *Cryptosporidium meleagridis*, and avian genotype II) were identified by PCR and sequencing. A phylogenetic tree of the isolates was analyzed. These results suggest that cryptosporidiosis is widespread in poultry in Zhejiang Province, and is a potential threat to public health as well as the economy. This is the first report about the infection rate and molecular characterization of *Cryptosporidium* in broiler chickens in Zhejiang.

Key words: *Cryptosporidium*, Infection rate, China, Chicken, Epidemiology.

Résumé – *Cryptosporidiose chez les poulets de chair dans la province du Zhejiang, Chine : caractérisation moléculaire des oocystes détectés dans les échantillons fécaux.* *Cryptosporidium* est l'un des parasites les plus importants de la volaille, et cet agent pathogène peut infecter plus de 30 espèces aviaires. La présente étude a examiné le taux d'infection de *Cryptosporidium* chez les poulets de chair. Un total de 385 échantillons fécaux de poulets de chair dans 7 régions de la province du Zhejiang, recueillis de novembre 2010 à janvier 2012, ont été examinés par microscopie. Trente-huit (10 %) étaient positifs pour l'infection à *Cryptosporidium*, et 3 génotypes (*Cryptosporidium baileyi*, *Cryptosporidium meleagridis*, génotype aviaire II) ont été identifiés par PCR et séquençage. Un arbre phylogénétique des isolats a été analysé. Ces résultats suggèrent que la cryptosporidiose est répandue chez les volailles dans la province du Zhejiang, et est une menace potentielle pour la santé publique ainsi que l'économie. Ceci est le premier rapport sur le taux d'infection et la caractérisation moléculaire de *Cryptosporidium* dans les poulets de chair dans le Zhejiang.

Introduction

Cryptosporidiosis is caused by *Cryptosporidium*, which has a broad range of hosts and worldwide distribution. The *Cryptosporidium* species can infect human beings as well as other animals; it is mainly located in the epithelial cells of the gastrointestinal tract, and is likely in the respiratory tract [7], particularly in the case of infection by *C. meleagridis*, a bird species.

The main clinical manifestation is persistent diarrhea, and respiratory disturbances can occasionally be observed [5, 12].

Cryptosporidium is one of the most common and important parasites in poultry, and this pathogen can infect more than 30 avian species. Traditionally, three different species of *Cryptosporidium* (*C. baileyi*, *C. meleagridis*, and *C. galli*) were considered to be the major species in birds [4, 6, 17, 20, 23, 30]. Recently, more than 11 novel genotypes have been described which are genetically different from the above three species by means of molecular analysis, including avian genotypes (I–V), goose genotypes (I–IV), the Eurasian woodcock

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genotype and the black duck genotype [1, 9, 14–17, 20, 31]. *C. meleagridis*, *C. parvum*, and *C. hominis* carried by poultry may infect human beings through the oocysts in contaminated water or vegetables eaten uncooked [1, 13, 20, 31, 32]. Considering that the possibility of exposure to *Cryptosporidium* of human beings still exists, the prevention of Cryptosporidiosis is important for public health [10].

Up to now, there is little information regarding the distribution of *Cryptosporidium* spp. in Zhejiang Province, and also limited data about its molecular characterization in poultry, particularly in chickens. The aim of the present study was to estimate the infection rate of *Cryptosporidium* in poultry in Zhejiang Province, and to determine the genotypes in the area.

Materials and methods

Sample collection

Three hundred and eighty-five fecal samples were randomly collected from broiler chickens that were around 90 days old in seven regions (Hangzhou, Huzhou, Jiaxing, Jinhua, Ningbo, Quzhou, and Shaoxing) in Zhejiang Province during the period from November 2010 to January 2012. Chickens were reared in steel cages; each contained 5 ~ 7 birds whose feces were collected on the tray under the cages gathered as a mixture. Therefore, the feces in one cage were considered as one sample, collected by the use of disposable plastic gloves, marked with the region name, serial number, and collection date. The sampling process was conducted to collect the fresh droppings to the best of our ability. The weight of each sample was approximately equal to about 50 g. Samples were kept in ice boxes until they were transported to the laboratory, then stored in the refrigerator at 4 °C and processed as soon as possible.

Microscopy detection

Samples were handled by Sheather's sugar flotation method as previously described by Huber et al. [8], and *Cryptosporidium* oocysts were examined by optical microscopy observation under 400× magnification based on the shape of oocysts and the shape index measured. Subsequently, the positive samples containing oocysts were stored in 2.5% potassium dichromate and kept at 4 °C until DNA extraction.

DNA extraction

Oocysts in positive samples were purified by discontinuous sucrose density gradient centrifugation [3]. For genomic DNA extraction, 100 µL of suspension liquid containing oocysts was frozen-thawed for 5 min in liquid nitrogen and then kept in a 65 °C water bath kettle for 5 min. The process was repeated three times, then the treated samples were centrifuged at 12 000 × g for 5 min. The genomic DNA was extracted using a Genomic DNA Extraction Kit (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China) in accordance with the manufacturer's instructions, and kept at -20 °C until detected by the PCR method.

Nested-PCR amplification and sequencing

A nested PCR was done in order to amplify a fragment of approximately 830 bp [30]. Then, the secondary purified PCR product was sequenced (BGI sequencing) to confirm the species/genotype identification.

Cryptosporidium genotyping and phylogenetic analysis

The acquired sequences were submitted to a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to initially define the species/genotypes and to confirm the high similarity and homology with other known sequences of *Cryptosporidium* spp. in GenBank. All sequences were multiple-aligned and analyzed by Bioedit and MEGA 4.0 software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html> and <http://www.megasoftware.net/>). A neighbor-joining cladogram was built using MEGA 4.0. To assess the reliability of this tree, bootstrap analysis was done with 1000 replicates using the Kimura 2-parameter logarithm.

The partial 18S rRNA nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers [JX548291-JX548300](#).

Statistical analysis

The infection rate of *Cryptosporidium* was analyzed using the chi-square test. Differences were considered significant when $P < 0.05$.

Results

Infection rate of *Cryptosporidium* in Zhejiang Province

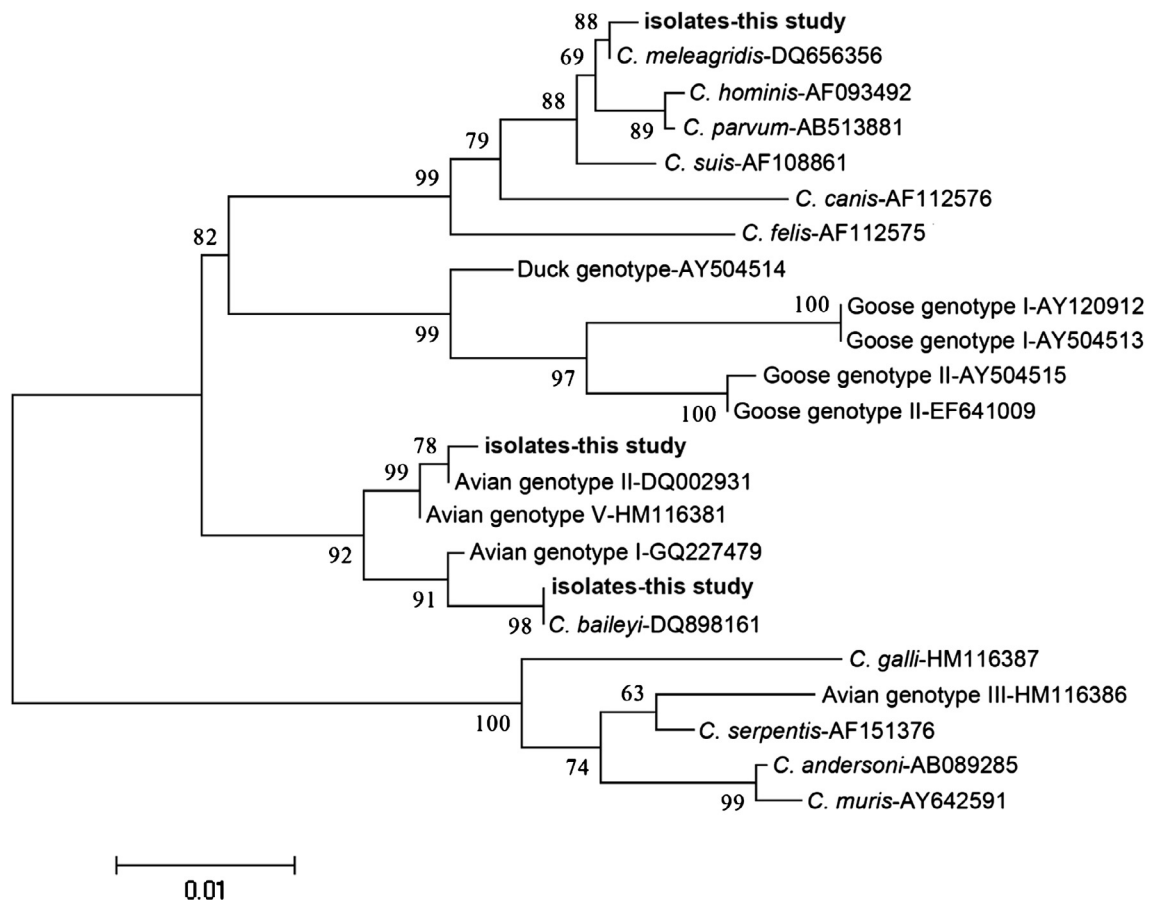
Of the 385 fecal samples from broiler chickens examined in this study, 38 samples were positive, and the overall infection rate of *Cryptosporidium* was 9.9%. The infection rate of *Cryptosporidium* varied among the seven regions. The highest infection rate was 16.9% in Huzhou, followed by 16.7% in Hangzhou, 12.1% in Ningbo, 7.7% in Shaoxing, 5.3% in Jiaxing, 4.0% in Quzhou, and 2.3% in Jinhua (Table 1). Significant statistical differences were not found in relation to the geographical provenance of chickens and infection rates in each region.

Distribution of *Cryptosporidium* species

Two species and one genotype, including *C. baileyi*, *C. meleagridis*, and the avian genotype II, were identified through morphological observation and sequencing. *C. baileyi* was widely distributed in the seven areas. 33 of 38 positive samples were for *C. baileyi* infection (86.8%), only two samples for *C. meleagridis* infection (5.3%), and three samples for avian genotype II infection (7.9%). Moreover, the average size of oocysts for *C. baileyi* was 5.96 µm × 4.73 µm

Table 1. Infection rate of *Cryptosporidium* in different source supplied for the native chicken.

Region	No. of cages examined	No. of cages <i>Cryptosporidium</i> positive	Infection rate (%)	<i>Cryptosporidium</i> species/genotypes (No. of isolates)
Hangzhou	66	11	16.7	<i>C. baileyi</i> (10), <i>C. meleagridis</i> (1)
Huzhou	59	10	16.9	<i>C. baileyi</i> (7), <i>C. meleagridis</i> (1), Avian genotype II (2)
Shaoxing	52	4	7.70	<i>C. baileyi</i> (4)
Ningbo	58	7	12.1	<i>C. baileyi</i> (6), Avian genotype II (1)
Jiaxing	57	3	5.26	<i>C. baileyi</i> (3)
Jinhua	43	1	2.33	<i>C. baileyi</i> (1)
Quzhou	50	2	4.00	<i>C. baileyi</i> (2)
Total	385	38	9.87	<i>C. baileyi</i> (33), <i>C. meleagridis</i> (2), Avian genotype II (3)

**Figure 1.** Phylogenetic tree of *Cryptosporidium* spp. built by the neighbor-joining method using Mega 4.0, based on 18S rRNA. To assess the reliability of this tree, bootstrap analysis was done with 1000 replicates.

by measuring 35 oocysts, for *C. meleagridis* it was $4.96 \mu\text{m} \times 4.15 \mu\text{m}$, and for avian genotype II it was $6.02 \mu\text{m} \times 5.47 \mu\text{m}$. The neighbor-joining among these species/genotypes and other previously described species and genotypes of *Cryptosporidium* is shown in Figure 1.

isolates showed 99.5% sequence similarity with a GenBank sequence from Iran (DQ656356). Three avian genotype II isolates showed 99.2% sequence similarity with a GenBank sequence (DQ002931).

Sequence and phylogenetic analysis

The 18S rRNA gene of *C. baileyi* isolates showed high sequence homology with each other by direct sequence alignment, and exhibited 99% sequence similarity with a GenBank sequence of quail origin (DQ898161). Two *C. meleagridis*

Discussion

Cryptosporidium is widespread around the world except Antarctica, and is most common in developing countries and less developed countries [22]. Thus far, there are few data available about the infection rate of *Cryptosporidium* in poultry,

especially in chickens in China. It was reported that the infection rate of *Cryptosporidium* was 3.4% in Henan and 23.8% in Shandong, China [26, 27]. Moreover, the rate was 3.75% in the Garmsar region, Iran, and 4.5% in Tunisia [21, 24]. It was clear that the overall rate in this study was much higher than in other countries. We speculate that this difference may be attributed to the different approaches in the sample collection. In our study, the fecal sample was collected from a pool of animals; thus, it is possible to find a higher rate of infection when compared with individual samples. Moreover, the various environmental factors and host species may also account for the difference [2]. The differences between different studies may be due to various factors such as seasonality, the age of chickens, the breeding environment, etc. Given the similar climate of the regions in Zhejiang Province, the interregional variation in infection rates observed could be due to the nutrient levels, breeding conditions and the density of animals [11–25].

In China, the infection rate of *Cryptosporidium* derived from poultry has been reported so far in almost 10 provinces. However, most researchers were focused on the prevalence in the region through traditional morphological observation, rather than identifying the species or genotypes of the *Cryptosporidium* by modern molecular biology approaches. One exception is Henan Province. In more recent studies, the prevalence of *Cryptosporidium* spp. has been reported in detail in quails, chickens, Pekin ducks, pet birds, and ostriches in Henan, and most species have been identified as *C. baileyi* and *C. meleagridis* [18, 27–29]. The epidemiological investigation of *Cryptosporidium* in poultry in Zhejiang Province was carried out in our research.

Among the three *Cryptosporidium* species/genotypes identified in this study, *C. baileyi* was the primary species of the broiler chickens, for the reason that it was not only widely distributed in the seven areas, but also had the highest percentage of positive samples (33/38). Thus, the phenomenon of *C. baileyi* infection in broiler chickens in this study confirmed the following views. *C. baileyi*, which is the most common species in poultry, has more than 20 kinds of hosts including chickens [19]. In addition, studies in China showed that most cases in chickens were diagnosed as *C. baileyi* infections, which can slow down the growth rate in broilers and decrease egg production of layer chickens. Because the poultry industry plays a critical role in the agricultural economy in China, it is essential to pay sufficient attention to this pathogen. Only two isolates were identified as *C. meleagridis* in our investigation. Meanwhile, *C. meleagridis* had extensive hosts too, and it could cause deadly diseases in immunocompromised persons [19]. People could be infected with *C. meleagridis* by potential zoonotic transmission when the chickens carry the pathogen. Therefore, it has a great influence on public health. Three isolates were identified as avian genotype II in the present study. Avian genotype II was first described in turkey and was reported in parrot in the same year [14, 16]. Afterward, avian genotype II was found in ostriches [17]. Recently, chickens have also appeared to be a new host for avian genotype II. Perhaps we could make a bold conjecture that avian genotype II also has a range of hosts like *C. baileyi* and *C. meleagridis*. In addition, according to the phylogenetic analyses of 18S rRNA, together with the analysis based on actin and HSP70 in a previous study by

Abe and Makino [1], it is further assumed that avian genotype II has a close relationship with avian genotype V at a genetic level. The cladogram in the present study proved the above points, which demonstrated the reliability of the tree.

In conclusion, this is the first report about the infection rate and molecular characterization of *Cryptosporidium* in broiler chickens in Zhejiang. The results suggest that *Cryptosporidium* is widespread in poultry in Zhejiang Province, and may pose a potential threat to public health as well as the economy.

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