FULL PAPER
Parasitology

Molecular characterization of *Cryptosporidium* spp. from migratory ducks around Tokachi subprefecture, Hokkaido, Japan

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ABSTRACT. *Cryptosporidium,* a waterborne protozoan parasite, has a substantial veterinary and medical impact worldwide. This parasite is more often recognized during waterborne outbreaks because of its resistance to chlorine disinfection, small size making it difficult to inactivate/ eliminate through filtration, and presence in many animal species including humans. Migratory waterfowl, in addition to acting as mechanical carriers of *Cryptosporidium* oocysts, can also serve as natural reservoirs of infection by host-specific *Cryptosporidium* species. For better understanding of the extent of genetic diversity and inter-relationships among avian isolates of *Cryptosporidium,* 200 fecal samples of migratory ducks from the Tokachi subprefecture, Hokkaido, Japan were collected and analyzed by nested PCR (N-PCR) at the 18S rRNA gene. N-PCR revealed that 11.5% (23/200) were positive for *Cryptosporidium* spp. Among all samples, sequence analysis identified that 10% (20/200) were 98–100% identical to *Cryptosporidium* avian genotype III. On the other hand, 1.5% (3/200) were 99–100% identical to *C. baileyi*. This is the first molecular study reporting the prevalence of *Cryptosporidium* in migratory ducks in Japan. Genetic diversity among *Cryptosporidium* isolates from humans and birds has been reported worldwide. Nevertheless, further studies are important to assess genetic variety and to elucidate the transmission dynamics of *Cryptosporidium* parasites.

KEY WORDS: *Cryptosporidium*, genotype, migratory duck

Cryptosporidium is a cosmopolite waterborne enteric protozoan that infects a wide range of animals including humans, causing diarrheal illness in the host [8]. Its infection takes a significant toll on public health, and *Cryptosporidium* is a common leading cause of childhood morbidity and mortality in developing countries [11]. Additionally, cryptosporidiosis is one of the most common protozoal infections in wild and domesticated birds [26]. Avian cryptosporidiosis was first recorded in 1929 [28]. To date, the parasite has been reported in over 30 avian species worldwide [22]. However, studies about the genetic diversity of *Cryptosporidium* spp. among avian hosts are limited. Migratory waterfowl act as mechanical carriers of *Cryptosporidium* oocysts, as they can pick up the infection from their habitat, carry and spread them in the environment, including drinking water sources and also domestic animals. They can also act as natural reservoirs of the infection by host-specific *Cryptosporidium* species [10]. A few reports have investigated the prevalence of cryptosporidiosis in ducks [2, 7, 12, 13, 17, 21, 29]. Nevertheless, to the best of our knowledge, no previous studies explored cryptosporidiosis infection rates in migratory ducks in Japan. In the current study, fecal samples obtained from 200 migratory ducks around the Tokachi subprefecture, Hokkaido, Japan were analyzed for *Cryptosporidium* using nested PCR (N-PCR). Furthermore, *Cryptosporidium* isolates demonstrated in this study were characterized genetically to expand our knowledge on their distribution in migratory ducks and their genetic relatedness to other *Cryptosporidium* spp. in humans and animals.

MATERIALS AND METHODS

Materials

Sampling area and sampling methods: Two hundred fecal samples were collected from migratory ducks around the Tokachi subprefecture, Hokkaido, Japan in 2018 and stored at −80°C until use. The Tokachi area has water bodies such as rivers and ponds but not wetlands. The area also has many forests, fields, and pastures. The fecal samples used in this study were collected at a river bank 5 m north of the Obihiro River, 400 m north of the Satsunai River, and 350 m south of the Tokachi River. All three rivers join about 800 m further downstream. The site is located near a residential area and is adjacent to a park and soccer field. The water in the river is not intended for human consumption. Latitude and longitude coordinates for the sampling spot are 42.932096°N and 143.234799°E. Many waterfowl can be seen in and around the river only during the migratory season (autumn to winter). Those birds are mainly ducks, specifically Order *Anseriformes*, Family *Anatidae*, Genus *Anus*. Other birds such as Whooper swans *Cygnus cygnus*, Grey heron *Ardea cinerea* are sometimes found there, but these were not present during the sampling for this study. We visited the site often to check the number of birds and found that those birds are very likely to be migratory birds not the resident birds. Therefore, we concluded that the fecal samples originated from migratory ducks which stopped there.

The sampling was conducted in October 2018. The samples were collected at the concrete bank along the river where birds rest. Before the sampling started, we kept a distance from the birds and watched them to evaluate the approximate number of the birds. Once we got closer to the birds, they flew away, and then we collected only the fecal samples which looked fresh. The number of samples collected was kept proportionally below the number of birds found on the day of collection. We cannot exclude the possibility that any given duck was sampled twice, but we believe that a prevalence could be estimated by using the samples used in this study. We have been conducting such surveillance studies for wild waterfowl to conduct research on viruses carried by them, and the findings have been reported previously [1]. Fecal samples were placed in plastic bags using plastic spoons and kept cold during the return to the laboratory. The samples were given ID numbers and stored at −80°C until use. The storage period was from a week to one month before processing.

The fecal samples were suspended in a medium containing antibiotics to prepare 10–20% homogenates and kept at room temperature for 2 hr as previously described [1]. In brief, the fecal samples were dissolved in a lysis solution and then subjected to three cycles of freezing-thawing. Freezing was done in liquid nitrogen, and thawing was performed at 90°C in a Dry Thermo unit (DTU-1B, Taitec, Koshigaya, Japan) to rupture the *Cryptosporidium* oocysts. From the homogenate supernatants, the extraction of DNA was performed using a QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's guidelines. The extracted DNA samples were stored at −20°C prior to analysis. Extracted DNA concentration was measured by a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

N-PCR

The N-PCR was carried out by employing primers directed to the 18S rDNA of *Cryptosporidium* spp. The primers used for the first amplification were SHP3 and SHP2 [25]. CPB-DIAGF and SSU-R3 [30] were used for the second amplification (Table 1). For the primary reaction, the PCR mixture was comprised of 2.5 μl of ready master mix (KAPA 2G Robust Hot Start Ready Mix PCR Kit, KAPA Biosystems), 2.0 μl of water, 0.1 μl of 50 mM each primer (forward, reverse) and finally 0.5 μl of DNA template to form a 5.0 μl reaction mixture for PCR. For the secondary reaction, the PCR mixture was the same as that for the primary one, except the amplicons from the primary PCR reaction were used as the template. The following cycling parameters were used for the primary reaction: after an initial denaturation for 3 min at 95°C, 40 cycles were performed, each consisting of 15 sec at 95°C for denaturation, 15 sec at 50°C for annealing, and 45 sec at 72°C for extension, followed by a final extension step of 1 min at 72°C. The cycling parameters for the secondary reaction were the same. The N-PCR products were detected on an ethidium bromide-stained 1.8% agarose gel by visualization with UV light [4, 24].

Sequencing and phylogenetic analysis

The N-PCR products were purified by ExoSAP-IT PCR Product Cleanup kit (Thermo Fisher Scientific, Inc., Tokyo, Japan), and direct sequencing of both forward and reverse directions was done by the dideoxy chain-termination method using the original primers (Table 1). Gene sequencing was performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). The results of sequencing were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/) in order to identify their similarities with sequences of the GenBank. Sequence alignment was carried out using CLUSTAL W [27] with manual adjustments. For phylogenetic analyses, the MEGA X software program was used. Phylogenetic relatedness of *Cryptosporidium* spp. detected in this study to other *Cryptosporidium* from animals and humans was analyzed by retrieving relevant gene sequences

Table 1. Primers used in this study

Name	Description	Primers Sequences
SHP3	Outer-forward	5′-acagggaggtagtgacaagaaataaca-3′
SHP2	Outer-reverse	5′-ttctcataaggtgctgaaggagtaagg-3′
CPB-DIAGF	Inner-forward	5′-aagctcgtagttggatttctg-3′
SSU-R3	Inner-reverse	5′-aaggagtaaggaacaacctcca-3′

from the GenBank database.

The phylogenetic analyses were performed by the Maximum Likelihood Estimation (MLE) method with Kimura 2-parameter. The consensus tree was obtained after bootstrap analysis, with 1,000 replications. For the construction of the phylogenetic tree, *Cryptosporidium molnari* (HM243548.1) was used as the outgroup. For comparative phylogenetic analysis, the following sequences were retrieved from the GenBank: *C. andersoni* (MK982464.1), *Cryptosporidium* avian genotype II (JX548292.1), *Cryptosporidium* avian genotype III (KX668210.1)., *Cryptosporidium* avian genotype V (HM116381.1), *Cryptosporidium* avian genotype VIII (KX513543.1), *C. baileyi* (MF276919.1), *C. bovis* (KJ020904.1), *C. canis* (AB210854.1), *C. hominis* (DQ286403),, *C. muris* (AF093496.1), *C. parvum* (AY204234.1), and *C. suis* (JQ936502.1).

Representative nucleotide sequences obtained in this investigation were deposited in the GenBank database under accession numbers MN988644 to MN988666.

RESULTS

In the current study, the overall prevalence of *Cryptosporidium* spp. was 11.5% (23/200) as detected by N-PCR targeted to the gene encoding the small ribosomal subunit 18S rDNA, using specific primers (Table 1). The N-PCR demonstrated an expected band on agarose gel with amplicon size about 410 bp (data not shown).

Sequencing of the variable region of the 18S rDNA [6, 20] may be a reliable typing (species identification) technique. As shown in this study, direct sequencing of N-PCR products revealed that 10% (20/200) was 98–100% identical to *Cryptosporidium* avian genotype III isolate N422, which was isolated from India with accession No. KX668210 (Supplementary Fig. 1). On the other hand, 1.5% (3/200) was 99–100% identical to *C. baileyi* isolates of Brazilian origin with accession No. MF276919.1 (Supplementary Fig. 2). To establish the relationship between current isolates and other *Cryptosporidium* spp., the 18S rDNA gene sequences of *Cryptosporidium* spp. were aligned with the previously published sequences of *Cryptosporidium* spp. (Fig. 1).

Topology of the phylogenetic tree was obtained by the MLE method, showing the taxonomic position of the isolates obtained in the current study in relation to the sequences deposited in the GenBank. We could observe that 20 isolates were clustered together in the same clade with the *Cryptosporidium* avian genotype III, while the other 3 isolates were clustered together in the group belonging to *C. baileyi*.

DISCUSSION

Limited epidemiological data concerning *Cryptosporidium* in wild birds in Japan warranted the need for more relevant research. In the present study, the mobility of migratory birds was an important factor related to the potential spread of pathogens near easy access to drinking water supplies that flow through populated city regions. This danger to humans motivated us to conduct a preliminary investigation of cryptosporidiosis in migratory ducks around Tokachi subprefecture, Hokkaido, Japan as one waterborne infection that might affect human health. This research also afforded the opportunity to genetically characterize *Cryptosporidium* isolates. Our investigation demonstrated a *Cryptosporidium* prevalence of 11.5% (23/200) in migratory wild ducks in Tokachi subprefecture, Hokkaido, Japan. These results were higher than those reported in the previous studies in which the prevalence rate was 1% (1/97) in Australia [13], 3.4% (5/148) in the Qinghai province, China [2], 2.3% (6/265) in northern Spain [7], and 9.1% (1/11) in Nigeria [17]. On the other hand, prevalence of *Cryptosporidium* was lower than those of other previous investigations, in which the infection rate was 57% (73/128) in Germany [21], 49% in the USA [12], and 16.3% (92/564) in the Henan province, China [29]. The differences in prevalence rates obtained among different countries may be attributed to the use of different methods of detection (such as serology, fecal examination, histology, and other methods) or differences in animal and hygiene management practices [5, 9, 29].

Little is known regarding *Cryptosporidium* species and subtypes in wildlife populations [3, 14, 23, 31]. Therefore, we further performed sequence analysis and identified two *Cryptosporidium* spp.: *Cryptosporidium* avian genotype III and *C. baileyi*. *Cryptosporidium* avian genotype III was previously reported among several species of Passeriformes and Psittaciformes (reviewed in Nakamura and Meireles [18]). In this study, *Cryptosporidium* avian genotype III was the predominant species in which 20 samples were identical to *Cryptosporidium* avian genotype III (20 out of 200). These rates were higher than those of previous investigators [7], where only six samples out of 265 were identical to *Cryptosporidium* avian genotype III. Our finding extends the distribution of this genotype in avian species that have been characterized as *Cryptosporidium* avian genotype III.

C. baileyi is generally reported to be the most prevalent species in domestic poultry resulting in considerable morbidity and mortality worldwide mostly due to respiratory disorders [15, 16, 19]. Our findings were in the same line with those reported in Ruddy Shelduck in the Qinghai province, China [2] where five samples (5 out of 148) were identical to *C. baileyi*. In contrast, our results were lower than those shown in Pekin ducks in China (92 out of 564) [29].

In conclusion, the current investigation is the first report in migratory ducks in Japan showing the presence of *Cryptosporidium* avian genotype III and *C. baileyi*. Also, it seems that ducks are not relevant as a natural source of human cryptosporidiosis. Further epidemiological studies should be undertaken to uncover the distribution of *Cryptosporidium* genotypes in wild ducks in Japan, which could contribute to assessing the zoonotic potential of *Cryptosporidium* from migratory ducks and their possible role in dissemination of *Cryptosporidium* species.
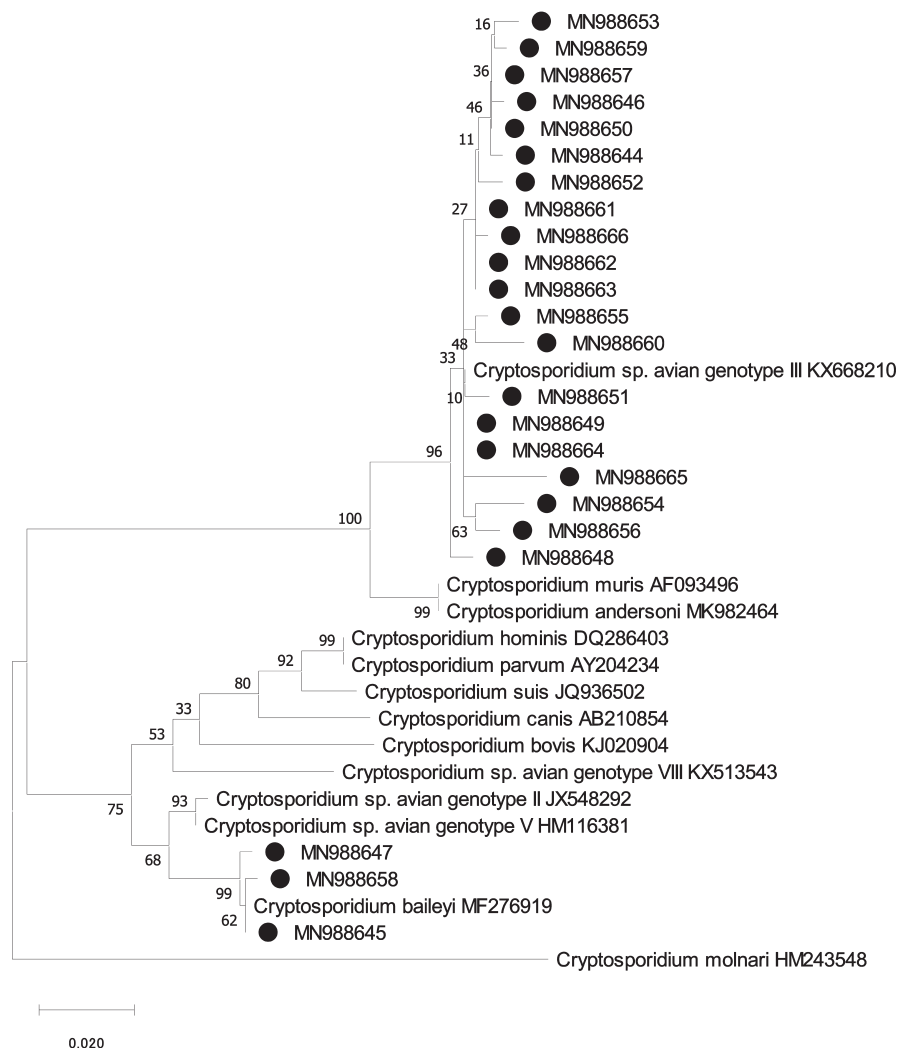
Fig. 1. The evolutionary relationship among *Cryptosporidium* sequences retrieved from the GenBank database and sequences data in this study (accession numbers MN988644 to MN988666, marked by bullets) was inferred by using the Maximum Likelihood Estimation method based on the Kimura 2-parameter model derived from partial 18S rDNA sequence data. Values on branches are the percent of bootstrapping using 1,000 replicates. All positions containing gaps and missing data were trimmed. Evolutionary analyses were conducted in MEGA X.

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