Molecular identification of *Cryptosporidium* isolates from pet birds in Japan

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ABSTRACT

*Cryptosporidium* spp. are important pathogens for humans and animals. Cases of infection by *C. parvum*, *C. hominis*, *C. meleagridis*, *C. andersoni*, and *C. muris* with zoonotic potential have also been reported in domestic and wild birds. Recent studies have revealed the presence of at least 13 host-adapted genotypes in birds. It is generally difficult to discern their oocysts accurately by morphology among *Cryptosporidium* species and genotypes. In Japan, 2 species (*C. baileyi* and *C. meleagridis*) and 2 genotypes (avian genotypes III and V) have been identified molecularly in pet (cockatiels and peach-faced lovebirds) and domestic (chickens) birds, but the presence of other species and/or genotypes in birds remains unclear. In this study, we attempted to identify 7 isolates from 3 cockatiels, 1 budgerigar, 1 masked lovebird, 1 Pacific parrotlet, and 1 Java sparrow, which were raised by individual owners, using sequence analysis of *Cryptosporidium* actin locus. Analysis identified avian genotype V in cockatiels and a budgerigar, avian genotype III in a masked lovebird, *C. galli* in a Pacific parrotlet, and *C. baileyi* in a Java sparrow. This report is the first of a study identifying the presence of avian genotype V and *C. baileyi* in budgerigar and Java sparrow in Japan, respectively. This study also demonstrated Pacific parrotlet (*Forpus coelestis*) as a new host record of *C. galli*.

Key words: *Cryptosporidium*, avian genotype V, avian genotype III, *C. baileyi*, *C. galli*.

Seven species of *Cryptosporidium*, an important pathogen in humans and animals, have been reported in domestic birds: *C. meleagridis*, *C. baileyi*, *C. galli*, *C. parvum*, *C. hominis*, *C. muris*, and *C. andersoni* [17]. The five species aside from *C. baileyi* and *C. meleagridis* have also been recognized as zoonotic *Cryptosporidium* [21]. In addition to these valid species, the following 13 genotypes are known: avian genotypes I–VI (identified in many avian species), goose genotypes (*Branta canadensis*) I–V, black duck (*Anas rubripes*) genotype, and Eurasian woodcock (*Scolopax rusticola*) genotype [6, 17]. Among these species and genotypes, *C. meleagridis*, *C. baileyi*, *C. galli*, avian genotype III, and possibly avian genotype V are recognized as important pathogens associated with mortality, weight loss, diarrhea, respiratory illness, chronic vomiting, and renal or cloacal illness [7, 13, 17]. It is generally difficult to discriminate accurately among *Cryptosporidium* species and genotypes using light microscopy because of the morphological similarity of their oocysts. The host specificity of *Cryptosporidium* genotypes found in birds remains unclear. Moreover, it is important to identify the avian isolates accurately, particularly pet birds that are in close contact with humans in everyday life and which could be a source of human infection. *Cryptosporidium* parasite was first isolated from chickens in domestic birds [10]. Later, this isolate was identified as *C. baileyi* using multilocus sequence analysis [11]. *Cryptosporidium* parasites were also found histopathologically in Japanese quail [14], but they have not been identified. Recently, several isolates from pet birds in Japan were identified molecularly.
Infection of *C. baileyi*, *C. meleagridis*, avian genotype III or V in cockatiels and peach-faced lovebirds has been reported [1-3, 13]. This study identified seven isolates from five species of pet birds using sequence analysis of *Cryptosporidium* actin locus.

Fecal samples from seven pet birds (three cockatiels, one budgerigar, one masked lovebird, one Pacific parrotlet, and one Java sparrow) were collected during 2012–2014 at Little Animal and Bird Clinic Little Bird and Fujisawa Avian Clinic (Table 1). They were found to be positive for *Cryptosporidium* infection by light microscopy using sucrose centrifugal flotation. All birds had been kept at separate households. The two cockatiels (original hosts of isolate codes 0306-01 and 120420-1) died during treatment. One cockatiel (0306-01) was provided for autopsy. Small pieces of each internal organ (crop, proventriculus, small intestine, cloaca, trachea, kidney, liver) collected using disposable tweezers were put into individual 1.5-ml tubes containing 70% ethanol. These samples were provided for identification of the location of *Cryptosporidium* found in a fecal sample of this cockatiel by PCR sequence analysis, as reported previously [3]. DNAs were extracted and purified from fecal and tissue samples using a QIAamp DNA Stool Mini Kit and DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), respectively, according to the manufacturer’s instructions. The *Cryptosporidium* actin gene fragment (approximately 1100 bp) was amplified following the nested PCR protocol [23]. PCR amplification was performed in a volume of 50 μl containing 1× PCR buffer, 2 mM MgCl₂, 250 μM of each dNTP, 0.5 μM of each primer, 1.25 units of TaKara Ex Taq Hot Start Version (Takara Shuzo Co. Ltd., Otsu, Shiga, Japan), and 5 μl of DNA sample. Reactions were performed using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, CA, U.S.A.). The PCR products were purified using the QIAquick Gel Extraction or QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Mettmann, Germany), and were sequenced in both directions on an automated sequencer (ABI 3130; Applied Biosystems, Foster City, Carlsbad, California, U.S.A.). Sequence chromatograms from each strand were inspected using the SEQUENCHER Version 4.1 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). Nucleotide similarity searching of the obtained partial gene sequences was performed using the FASTA program (EMBL; http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html).

Table 1. *Cryptosporidium* isolates from pet birds identified in the present study

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Host details</th>
<th>Identification at actin locus</th>
<th>Most highest nucleotide sequence identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0306-01</td>
<td><em>Nymphicus hollandericus</em>; cockatiel; 2 months; emaciation, diarrhea and death</td>
<td>avian genotype V</td>
<td>100% identity (978/978 bp) to those from avian genotype V (AB471660, AB471661, JQ320301)</td>
</tr>
<tr>
<td>0016-01</td>
<td><em>Nymphicus hollandericus</em>; cockatiel; 3 months; dispepsia</td>
<td>avian genotype V</td>
<td>100% identity (978/978 bp) to those from avian genotype V (AB471660, AB471661, JQ320301)</td>
</tr>
<tr>
<td>120420-1</td>
<td><em>Nymphicus hollandericus</em>; cockatiel; 2 months; emaciation, diarrhea and death</td>
<td>avian genotype V</td>
<td>100% identity (978/978 bp) to those from avian genotype V (AB471660, AB471661, JQ320301)</td>
</tr>
<tr>
<td>3503-58</td>
<td><em>Melopsittacus undulatus</em>; budgerigar; 2 months; diarrhea and vomiting</td>
<td>avian genotype V</td>
<td>100% identity (978/978 bp) to those from avian genotype V (AB471660, AB471661, JQ320301)</td>
</tr>
<tr>
<td>733-3</td>
<td><em>Agapornis personata</em>; masked lovebird; 6 years; no symptom</td>
<td>avian genotype III</td>
<td>100% identity (1000/1000 bp) to those from avian genotype III (AB471655-AB471659)</td>
</tr>
<tr>
<td>0018-03</td>
<td><em>Forpus coelestis</em>; Pacific parrotlet; &gt;2 years; diabetes and proventriculus distension</td>
<td><em>C. galli</em></td>
<td>100% (987/987 bp, 921/921 bp) or 99.9% (961-962 bp, 927/928 bp) identity to those from <em>C. galli</em> (AY163901, EU532655-EU543267, EU543265)</td>
</tr>
<tr>
<td>0224-01</td>
<td><em>Padda oryzivora</em>; Java sparrow; 2 months; diarrhea</td>
<td><em>C. baileyi</em></td>
<td>100% identity (990/990 bp) to those from <em>C. baileyi</em> (AF382346, EU741840-EU741852)</td>
</tr>
</tbody>
</table>

*Described in order of scientific name, common name, age of the bird, and clinical symptoms.*
Partial actin gene nucleotide sequences (978–1000 bp) were obtained from all isolates examined in this study. Sequences from four isolates from three cockatiels and one budgerigar were identical among those isolates and were also identical to those of Cryptosporidium avian genotype V (Table 1). The PCR was positive in six samples (crop, proventriculus, small intestine, cloaca, trachea, kidney) except for the liver. The sequences from those amplicons were mutually identical and also identical to that of avian genotype V (AB471660). The sequence of the isolate from a masked lovebird was identical to that of avian genotype III. The sequences of the two isolates from a Pacific parrotlet and a Java sparrow were also identical to those of C. galli and C. baileyi, respectively (Table 1). Results demonstrated that intragenotype or intraspecies variations are low or absent at the actin locus in Cryptosporidium [23]. Therefore, we identified the present isolates as Cryptosporidium avian genotypes III, V, C. galli, and C. baileyi.

Cryptosporidium avian genotype V, recently proposed as a new Cryptosporidium species (C. avium) [9], was first found in fecal samples from two cockatiels caged at the same pet shop in Japan [2]. Subsequently, it has been identified in cockatiels and a budgerigar in China [19, 24] and in a blue-fronted parrot in Brazil [16], in a pet Major Mitchell’s cockatoo in U.S.A. [7]. Recently, we also identified this genotype in a cockatoo [3]. In the present study, the infection with this genotype was confirmed in the three cockatiels. Although we were unable to perform a follow-up survey of the origin, avian genotype V might be widely distributed throughout the cockatiel population of Japan. The pathogenicity of this genotype in avian hosts has remained unclear because of a lack of clinical reports associated with this genotype infection [2, 9, 16, 19, 24]. All birds infected with this genotype examined in the present study showed mainly digestive symptoms (Table 1). A recent report has also described a fatal case with renal and cloacal cryptosporidiosis caused by this genotype in a Major Mitchell’s cockatoo (Cacatua leadbeateri) [7], suggesting possible pathogenicity of avian genotype V. However, it was not possible to ascertain whether the digestive symptoms found in the present study resulted from an infection of avian genotype V because we were unable to examine other pathogens such as Candida spp., Escherichia coli, Salmonella spp., and various viruses that are recognized as concurrent pathogens of intestinal cryptosporidiosis [5, 12]. The site of infection in avian hosts of this genotype has been found by scanning electron microscopical or histopathological examination to be the ileum, cecum, ureter, and cloaca [7, 9]. In the present study, Cryptosporidium DNA was detected in the trachea, crop, proventriculus, small intestine, cloaca, and kidney collected from a dead cockatoo (0306-01). Recently, we also identified the trachea and cloaca as the possible location of this genotype in avian hosts by PCR for actin and 18S ribosomal DNA loci using the tissue DNA samples from a severely emaciated dead cockatiel [3]. Considering both the present and the previous [3] PCR data, we strongly presume the possible location of this genotype as the trachea, which is true also for C. baileyi. Further histological examination of trachea in birds infected with avian genotype V must be conducted to examine this possibility.

In Japan, C. baileyi has been identified only in domestic chickens and pet cockatiels [1, 11]. In addition, the avian genotype V has been identified only in pet cockatiels [2, 3]. Therefore this report is the first of a study identifying the presence of C. baileyi and avian genotype V in Java sparrow and budgerigar, respectively in Japan. Reports of the literature show that C. galli has been identified in at least 32 avian species [4, 6, 8, 15, 16, 18-20, 22], but it has not been found in the Pacific parrotlet (Forpus coelestis). Therefore, the present report also identified a new host of C. galli.

About 5,000 psittaciform birds have been introduced annually into Japan during the most recent five years (2011–2015). These birds are mainly imported from Belgium, the United States of America, the Philippines, and Singapore (This information is available at the following URLs, but all descriptions at these sites are in Japanese: http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/0000069864.html). In addition, a few birds are bred in households or breeder farms in Japan. Because no survey of Cryptosporidium infection in birds has...
been performed in exporting countries and/or in Japan, the origins of Cryptosporidium- parasite-infected pet birds in Japan remain unclear. Global epizootiological studies of Cryptosporidium infection in pet birds must be undertaken to control Cryptosporidium infection among pet bird populations, and to clarify the infection sources and routes of transmission.

REFERENCES
遺伝子解析による小鳥由来 Cryptosporidium 株の同定

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要 約

クリプトスポリジウムはヒトと動物に寄生する原虫で、鳥類では人獣共通寄生性5種と少なくとも13の遺伝子型の報告がある。日本では、C. baileyi（オカメインコ、鶏）、C. meleagridis（オカメインコ）、avian genotype Ⅲ（コザクラインコ）、Ⅴ（オカメインコ）が確認されていたが他種寄生状況は不明であった。今回、本原虫のactin遺伝子領域における塩基配列の相同性検索により鳥類由来7株の同定を試みたところ、オカメインコ由来3株とセキセイインコ由来1株をavian genotype Ⅴ、キエリクロボタンインコ由来1株をavian genotype Ⅲ、マメルリハ由来1株をC. galli、文鳥由来1株をC. baileyi と同定した。国内のセキセイインコと文鳥にavian genotype ⅤとC. baileyiが寄生していることを初めて確認し、マメルリハをC. galliの新宿主として報告した。

Key words: Cryptosporidium、avian genotype Ⅴ、avian genotype Ⅲ、C. baileyi、C. galli