NOTE Parasitology

Identification of Genotypes of Cryptosporidium parvum Isolates from a Patient and a Dog in Japan

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ABSTRACT. Cryptosporidium parvum (C. parvum) is recognized as a significant pathogen in humans and animals, primarily as a cause of diarrheal illness. Recent genetic and biological studies indicate that C. parvum is not a single species but composed of genetically distinct multiple genotypes. Thus, it is valuable to distinguish between genotypes in the epidemiology of Cryptosporidium infection in humans and animals. Although C. parvum has been detected in humans and animals in Japan, the genotype of isolates remains unclear because identification has been performed only by conventional microscopy. We report herein the genotypes of C. parvum isolates distinguished by the polymerase chain reaction (PCR)-based diagnostic method. C. parvum isolates, originally obtained from a patient and a pet dog, were found to have cattle and dog genotypes, respectively.

KEY WORDS: canine, Cryptosporidium parvum, genotype.

Cryptosporidium is a protozoan parasite that is ubiquitous in its geographic distribution and is wide in the range of vertebrate hosts. Transmission of this parasite is direct, by either the fecal-oral route or the contamination of water supplies with the infective oocyst. In humans and many other mammals, Cryptosporidium parvum is recognized as a significant pathogen, primarily as a causative agent of acute, severe diarrheal illness.

Recent molecular studies indicate that C. parvum is not a single species, but composed of at least 8 genetically distinct but morphologically identical genotypes (human, monkey, cattle, mouse, pig, marsupial, dog, and ferret genotypes) which may each constitute a separate species of Cryptosporidium [11, 13, 24]. Molecular epidemiological studies show that the human genotype has so far been found only in humans, while the cattle genotype has been found in humans, as well as in domestic livestock such as cattle, sheep, and goats [11, 13]. The other six genotypes, monkey mouse, pig, marsupial, dog, and ferret genotypes, have been found only in the respective hosts but not so far in humans [11, 13]. However, recently the dog genotype was found in the feces of HIV-positive and immunocompetent individuals [16, 17, 25].

Although C. parvum and C. parvum-like organism have been detected in humans and animals in Japan, the genotypes and species of isolates remain unclear because the identification of Cryptosporidium has been performed only by conventional microscopy [1, 7–9, 20, 21]. Recently, polymerase chain reaction (PCR) primers that can be used to distinguish between the genotypes and/or species of Cryptosporidium on the basis of sequencing of the PCR product have been developed [10]. In this paper, we reveal the genotypes of C. parvum isolates originating from a patient and a dog in Japan using a PCR-based diagnostic method to distinguish between genotypes and/or species of Cryptosporidium.

In this study, we used the fecal samples obtained from severe combined immunodeficient (SCID) mice infected with C. parvum strain HNJ-1, and from a dog infected with a Cryptosporidium-like organism. C. parvum strain HNJ-1 was originally isolated from a Japanese patient in 1989 and has been maintained by subinoculation into SCID mice [9]. Although this strain has been used for the genetic study of Cryptosporidium, its genotype was unclear [22, 23]. The dog was a 9-week-old female Maltese which was purchased from a pet shop in Niigata city in June 2000. Fecal samples were collected from the dog by the owner for 2 days after purchase and were sent to our laboratory for examination. The dog showed no clinical symptoms such as diarrhea when the fecal samples were collected.

The samples were stored in plastic bottles containing 2.5% potassium dichromate solution at 4°C for several weeks until used in the other examination. Each sample was washed three times with phosphate buffered saline (PBS) by centrifugation to remove preservatives and filtered through gauze. The filtrate was centrifuged at 750 × g (2,000 rpm) for 5 min and the supernatant was discarded. A sucrose solution with a specific gravity of 1.2 was added to the sediment, mixed thoroughly, and centrifuged at 750 × g (2,000 rpm) for 5 min. Oocysts floating on the surface were recovered using a bacteriological loop and observed under a light microscope at 400 × magnification.

To the other examination was applied the indirect fluorescence antibody test (IFA). The floating material obtained by the sucrose centrifugal-flotation method was transferred to a 2 ml tube containing PBS using a bacteriological loop and washed with PBS by centrifugation. After centrifugation, the supernatant was discarded and the sediment was suspended in 50 μl of PBS. The concentrated sample was stained for IFA with a commercially available kit (Hydrof-
This kit is used for the detection of *Cryptosporidium* spp. oocysts in the field with a monoclonal anti-cryptosporidial antibody that attaches to the wall of oocysts. According to the instruction of manufacturer, the monoclonal antibody to *Cryptosporidium* is directed against *C. parvum*, but will also cross-react to *C. meleagris* and *C. baileyi*. The reactivity to the other *Cryptosporidium* species is unclear.

For the extraction of DNA from fcal sample, the concentrated sample was obtained by the method mentioned above and was washed with PBS by centrifugation. After centrifugation, the supernatant was discarded and the sediment was suspended in 200 µl of PBS and subjected to freezing and thawing five times. Subsequently, the suspension was boiled for 5 min and centrifuged. The supernatant was purified using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

A region of the *Cryptosporidium* 18S ribosomal RNA (18S rRNA) gene was amplified using the primers 18S1F (forward primer; 5’-AGTGACAGAAATAACAAATACGGA 3’) and 18S1R (reverse primer; 5’-CCTGCTTTAAAGCAGCTATTTTTCC 3’) as previously described [10]. This area includes a variable region which can be used to distinguish among *Cryptosporidium* species, as well as the genotypes within *C. parvum*. PCR amplification was performed in a volume of 50 µl containing 1X PCR buffer, 2 mM MgCl₂, 250 µM each of dNTP, 0.5 µM of each primer, 1.25 units of Ex Taq DNA polymerase (TAKARA Shuzo Co., Ltd., Otsu, Japan), and 5 µl of the DNA sample. We used the PCR buffer and dNTP mixture appended to Ex Taq DNA polymerase. Reactions were performed on a TAKARA PCR Thermal Cycler MP (TAKARA Shuzo Co., Ltd., Otsu, Japan). Samples were denatured at 96°C for 2 min, and then subjected to 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 7 min. Amplification products were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

The PCR products were gel purified using a QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Corp., U.S.A.) on an automated sequencer (ABI PRISM 310 model; Perkin-Elmer Corp., U.S.A.). PCR products were sequenced in both directions using either 18S1F or 18S1R. The nucleotide sequences of 2 samples were aligned by the Clustal X [18].

A *Cryptosporidium*-like organism was found in the fcal sample of a dog by the sucrose centrifugal-flotation method but other intestinal parasites were not. This organism bright pink in color and nearly ovoid, and measured from 4 to 5 µm in diameter (Fig. 1A). It was stained with monoclonal antibody specific to *Cryptosporidium* oocysts (Fig. 1B). Based on the results of light microscopy and IFA, we identified the organism as *Cryptosporidium*. Agarose gel visualization of PCR products revealed the same sized diagnostic fragments (approximately 300-bp) among the samples examined (Fig. 2). Sequence analysis revealed two types of sequences (Fig. 3). The DNA sequence of the diagnostic fragment of *C. parvum* strain HNJ-1, originally isolated from the patient, was 295 bases long and was identical to that of the cattle genotype (GenBank accession no. AF108864). However, the sequence of the 18S ribosomal RNA gene isolated from the dog was 290 bases long and was found identical to that of the dog genotype (GenBank accession no. AF112576).

In this study, it was found possible to distinguish between genotypes of *C. parvum* by a PCR-based diagnostic method. *C. parvum* strain HNJ-1 and *Cryptosporidium* sp. originally isolated from a patient and a dog were found to have the cattle and dog genotypes, respectively. Cross-transmission

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**Fig. 1.** *Cryptosporidium*-like organism (arrowhead) found in the fecal sample of a pet dog. Light micrograph of the organism obtained by the sucrose centrifugal-flotation method (A). Apple green fluorescence was found by IFA at the periphery of the organism (B). Scale bar: 10 µm.
studies of genotyped isolates indicated that the cattle genotype readily infected other animals such as laboratory rodents, while the human genotype did not [11, 15]. Although C. parvum strain HNJ-1 was found to have the cattle genotype, this result is supported by the biological characteristic of the strain. Namely, this strain has been maintained through mice for many years. Although C. parvum was detected in humans and animals such as cattle, dogs, pigs and rodents in Japan, the genotypes of isolates were unknown [1, 7–9, 20, 21]. Thus, this is the first report of the genotype of C. parvum isolates in Japan.

At present, the genotype of dog-derived C. parvum isolates was analyzed for about 9 isolates only from Australia and the United States and all the isolates examined were found to have the dog genotype [2, 14]. Therefore, this study indicates that dogs harbor the dog genotype which is sustained in different geographical areas. We collected fecal samples from the same dog again in August 2000 for two weeks and tried to purify oocysts to infect laboratory animals experimentally because the host range of the dog genotype was not clarified. However, we could not find oocysts in the samples and reveal the host range of this genotype. The patent period of C. parvum was determined experimentally in humans and animals and ranged from 3 to 33 days in dogs [4]. Thus, C. parvum may disappear from the dog during the period from June to August.

The dog genotype of C. parvum is a causative agent of diarrhea in human HIV-infection. However, the pathogenicity in immunocompetent patients is unclear because those infected with the dog genotype are asymptomatic [16, 25]. The clinical significance of cryptosporidial infection is unclear in dogs but the concurrent infections with distemper virus can lead to clinical illness [5, 19]. The dog examined in this study showed no clinical symptoms after purchased from a pet shop in June 2000, although it may be symptom-

Cattle genotype

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Fig. 3. Alignment of the Cryptosporidium 18SrRNA gene diagnostic fragments obtained with primers 18SiF and 18SiR for C. parvum strain HNJ-1 (HNJ-1) and Cryptosporidium sp. from the dog (D). Dashes indicate gaps, and dots indicate bases that are identical to the cattle genotype bases. The GenBank accession number for the cattle and dog genotypes of C. parvum is AF108864 and AF112576, respectively.
atic while reared in the pet shop. Epidemiological surveys of zoonotic pathogens in animals reared in pet shops or by breeders have been overlooked, and thus, periodical examinations of pets are needed to prevent infections with zoonotic pathogens.

Molecular epidemiological studies revealed that different genotypes or species of Cryptosporidium occurred in different host groups [3, 6, 12, 16, 17, 25]. Thus, the PCR-based diagnostic method that can distinguish between genotypes and/or species of Cryptosporidium used in this study may be useful for the epidemiological survey of cryptosporidial infections in humans and animals.

REFERENCES