FULL PAPER *Parasitology*

Viability and Infectivity of *Cryptosporidium parvum* Oocysts Detected in River Water in Hokkaido, Japan

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ABSTRACT. The viability and infectivity of *Cryptosporidium parvum* (*C. parvum*) oocysts, detected in water samples collected from river water in Hokkaido, were investigated using Severe Combined Immunodeficient (SCID) mice. The water samples collected from September 27 through October 10, 2001 by filtration using Cuno cartridge filters were purified and concentrated by the discontinuous centrifugal flotation method. From 1.2×10^5 liters of the raw river water, approximately 2×10^4 oocysts were obtained and designated as Hokkaido river water 1 isolate (HRW-1). Oocyst identification was carried out using microscopic and immunological methods. Six 8-week-old female SCID mice were each inoculated orally with 1×10^3 oocysts. Infection was successfully induced, resulting in fecal oocyst shedding. Oocysts were then maintained by sub-inoculation into SCID mice every 3 months. Infectivity was evaluated by m aking comparisons with two known *C. parvum* stocks, HNJ-1 and TK-1, which were bovine genotypes detected in fecal samples from a cryptosporidiosis patient and young cattle raised in Tokachi, Hokkaido respectively. The oocyst genotypes were determined from a small subunit ribosomal RNA (SSU-rRNA) gene by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis. No significant differences were observed in the average number of oocysts per gram of feces (OPG) in any of the isolates. Our data indicates that the *C. parvum* oocysts detected in the sampled river water were of *C. parvum* genotype 2. Moreover, our data on the continued isolation, detection and identification of the *C. parvum* isolates is consistent with the available epidemiological data for the Tokachi area.

KEY WORDS: *Cryptosporidium parvum* oocyst, infectivity, SCID mouse, viability, water.

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Cryptosporidiosis is recognized as one of the main causes of diarrhea in immunologically healthy and immunocompromised humans and animals of veterinary importance throughout the world. However, it was only after the 1983 outbreak in Texas [3] that cryptosporidiosis became known as a common disease that infects humans drinking contaminated tap water. The symptoms of *Cryptosporidium* infection are diarrhea, stomachache, nausea, fever and fatigue. It has been reported that the average pre-patent period ranges from 1 to 12 days in humans [4]. Because of the variable pre-patent period, identification of the cause of outbreaks is usually difficult.

Since the 1980s, outbreaks have been reported in several countries and cryptosporidiosis has now been identified as one of the emerging diseases in the last two decades. During the Milwaukee cryptosporidiosis outbreak, 400,000 cases were reported [9]. Two outbreaks of waterborne cryptosporidiosis have occurred in Japan. The first was reported at Hiratsuka in Kanagawa prefecture in 1994 with about 500 cases and the second at Ogose in Saitama prefecture in 1996 with 8,000 cases. Previously, environmental waters in Japan were considered cleaner and safer than in other countries. However, the waterworks system in Japan appears to have entered a critical phase. Recently, in 2002, two outbreaks occurred in Hokkaido. The need to promptly carry out measures to deal with the impending problem of cryptosporidiosis in Hokkaido cannot be over emphasized.

Monitoring of *Cryptosporidium* oocysts is one of the most important steps in dealing with cryptosporidiosis. In monitoring *Cryptosporidium* oocysts, it is important that species are determined. In view of the potential harm to humans, it is especially important to investigate the genotype, viability and infectivity of *Cryptosporidium* species in environmental waters.

Studies have involved various polymorphic gene loci of the two major genotypes of *C. parvum* in humans [1, 2, 11, 14, 16, 23]. Genotype 1, or the human genotype of *C. parvum*, has been isolated almost exclusively from humans and is associated mainly with anthroponotic (human to human) transmission cycles [15]. Experiments using animals such as cattle and mice infected with the human genotype have been unsuccessful, and the only *in vivo* model that exists for this genotype is a gnotobiotic piglet model [22]. So far, the only reported animals infected with *C. parvum* genotype 1 are monkeys in the United States [19] and dugongs (*Dugong dugon*) in Australia [12]. In contrast, genotype 2 or the bovine genotype of *C. parvum* infects a wide range of mammals, including humans [14, 16]. Genotype 2 isolates have been associated with the zoonotic (animal to human) trans-

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mission cycle. Furthermore, the genotype 2 isolates were infective to Severe Combined Immunodeficient (SCID) mice or calves. However, genotype 1 isolates were not [15].

Recently, various species of *Cryptosporidium* and genotypes of *C. parvum* were determined using large-scale genomic sequencing. Taking into account the pathogenicity and infectivity to humans, this study aimed at examining whether the oocysts detected in river water were *C. parvum* and whether the detected *C. parvum* oocysts were genotype 1 or 2, using microscopic and DNA based techniques [24].

MATERIALS AND METHODS

Oocysts: Three stocks of *C. parvum* oocysts were used in this study. The first stock, designated Hokkaido River Water - 1 (HRW-1), was detected and isolated in the Obihiro River in Hokkaido using microscopic and immunological techniques. From September 27 through October 10, 2001, approximately 1.2×10^5 liters of river water was filtered and 2×10^4 oocysts were detected by indirect an immuno-fluorescence antibody test (IFAT) using the IFAT kit (Cellabs DIF, Cellabs Pty. Ltd., Sydney/Australia) and 4', 6-diamidino-2-phenylindole (DAPI).

The second stock, TK-1, was obtained from fecal samples of cattle raised in the Tokachi area [18]. The third stock, HNJ-1, was *C. parvum* genotype 2 detected in fecal samples from a patient with cryptosporidiosis [10]. All stocks and strains were maintained by sub-inoculation into SCID mice every three months.

Collection, purification and concentration of oocysts from river water: Collection, purification and concentration of oocysts from river water were carried out using previously described methods [20]. Briefly, filtration using a Cuno cartridge filter, the discontinuous sucrose gradient method, and IFAT and DAPI staining were carried out. After staining, samples were analyzed using a UV-light microscope. The UV excitation bands ranged from 450 to 490 nm for IFAT staining and from 310 to 395 nm for DAPI staining. The number of oocysts was counted and then the oocysts were stored in 2.5% (w/v) solution of $K_2Cr_2O_7$ until further use.

Mouse infectivity: Six 8-week-old female SCID mice (the CP group) were each orally inoculated with $\times 10^3$ *C. parvum* oocysts from the river water. The mice were each raised in their own wire netted cage. Feces were collected every two days starting on Day 2 post infection. The oocysts were purified using the sucrose floatation method as previously reported [8] and identified using IFAT and DAPI staining. Oocysts per gram of feces (OPG) were recorded for 30 days. On Day 30 post infection, the mice were sacrificed, and their intestines removed and the contents examined by IFAT staining.

Three parallel experiments were conducted involving three groups of 6 8-week-old female SCID mice, the HRW-1, HNJ-1 and TK-1 groups. Depending on the group, each mouse was orally inoculated with 1×10^4 oocysts from either the HRW-1, HNJ-1 or TK-1 stock. The mice were treated and the oocysts investigated as in the above experiment.

PCR-RFLP analysis: Oocyst genotype was determined using the PCR-RFLP analysis for a small subunit ribosomal RNA (SSU-rRNA) gene as previously reported [33]. Oocyst species determination was carried out by digesting the secondary PCR product with the Ssp I. *C. parvum* generated three visible bands at 448, 247 and 106 bp. To differentiate human and bovine genotypes of *C. parvum*, the secondary PCR product was digested with *Ase* I (*Vsp* I). *C. parvum* bovine genotype exhibited two visible bands at 628 and 104 bp.

RESULTS

UV light microscopy: *C. parvum* oocysts detected in the river water and in feces from cattle were stained by IFAT and DAPI staining and observed under a UV light microscope. IFAT and DAPI staining of the oocysts are summarized in Fig. 1. The oocysts were spherical and 3 – 5 μm in diameter. Moreover, they had a suture line with apple-green fluorescence when stained with IFAT as shown in Fig. 1A, and they had four sporozoite nuclei with pale fluorescence when stained with DAPI as shown in Fig. 1B. Taken together, these characteristics indicate that the isolated

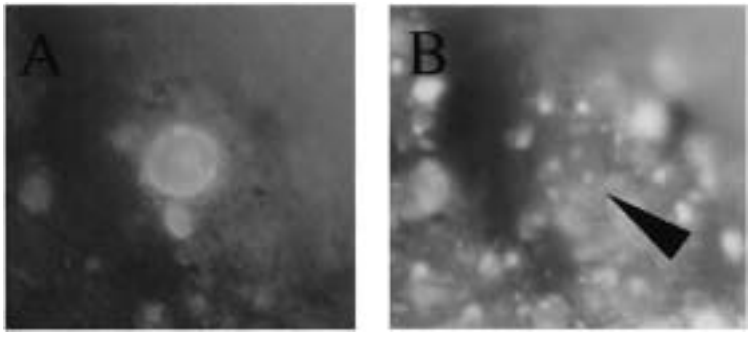

Fig. 1. *C. parvum* oocysts isolated from river water samples as shown by: (A) IFAT and (B) DAPI staining. Note - The apple-green color staining of the *C. parvum* oocysts in (A) and the 4 pale colored sporozoite nuclei (B).

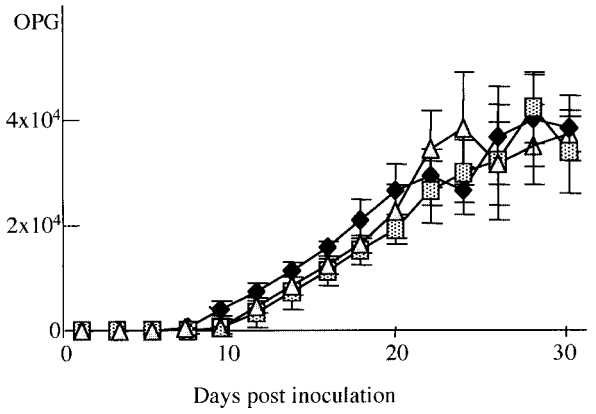

Fig. 2. OPG values from 3 groups of oocyst stocks (HNJ-1, TK-1 and HRW-1) at 2 days intervals in SCID mice orally inoculated with 1×10^4 *C. parvum* oocysts. ◆: HNJ-1, △: HRW-1, ▦: TK-1

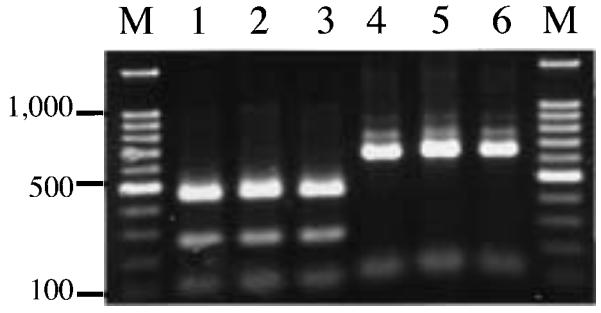

Fig. 3. Molecular diagnosis of *C. parvum* oocysts by PCR-RLFP procedure based on the SSU rRNA gene sequence. Species diagnoses are shown in Lane 1 to 3. *C. parvum* genotype differentiation is shown in Lanes 4 to 6. *C. parvum* bovine genotype HNJ-1 strain is shown in Lanes 1 and 4. *C. parvum* TK-1 stock is shown in Lanes 2 and 5. *C. parvum* HRW-1 stock is shown in Lanes 3 and 6.

oocysts in the river water were *C. parvum*.

Mouse infectivity: In the CP group, mouse infectivity was successful, with oocyst appearing in the feces in 2 SCID mice. The pre-patent periods were 10 and 12 days post infection. Throughout the 30 days in which the OPG was observed, the SCID mice continued to shed oocyst in their feces.

The average OPG of the feces of the HRW-1, HNJ-1 and TK-1 groups of mice is summarized in Fig. 2. No significant differences were observed in the average OPG of any of the isolates. Upon inoculation, infection was successfully induced, indicating that the isolated oocysts were alive and infective.

PCR-RFLP analysis: Results of the PCR-RFLP analysis are shown in Fig. 3. The three samples tested, HNJ-1, TK-1 and HRW-1, generated three visible bands of 448, 247 and 106 bp, respectively, following digestion of the secondary PCR product with *Ssp* I (Lane 1 to 3). When the secondary PCR product was digested with *Ase* I, two visible bands at 628 and 104 bp were generated in Lanes 4 to 6, suggesting that oocysts detected in the water sample were of *C. parvum* genotype 2.

DISCUSSION

Our study has shown that the *C. parvum* oocysts detected in the sampled river water in Hokkaido are viable and infective to susceptible hosts. Moreover, by using a DNA based technique, we were also able to demonstrate that the isolated oocysts belong to the bovine genotype. Our data on the continued isolation, detection and identification of the *C. parvum* isolates is consistent with available epidemiological data and has shown that the animal-inoculation assay used in this study is an effective tool for monitoring *Cryptosporidium* in environmental water.

Monitoring *C. parvum* oocysts in environmental water involves determining the viability, infectivity and genotype of oocyst species, in connection with their reported pathogenicity to humans.

When detected oocysts are viable, oocyst infectivity to hosts becomes an important concern. Recently, DAPI/PI staining has been used in viability assays. However, there are problems associated with DAPI/PI staining. For instance, the vast amount of debris in the water samples makes it difficult to discriminate specific fluorescence from non-specific fluorescence from the four nuclei under the UV light microscope. Identification of the 4 sporozoites in the stained nuclei was difficult, as shown in Fig. 1B.

To date, several different assays to investigate oocyst viability and infectivity have been reported in previous related studies [5]. In this study, the mouse-inoculation assay was used for two reasons. First, because it was easy to determine viability and easy to demonstrate infectivity following oocyst shedding and, second, because it increased the number of oocysts.

Infection of the isolated oocysts in SCID mice was successfully demonstrated by oocyst shedding in feces of inoculated SCID mice. The oocysts were shown to be viable and infective to the SCID mice. To compare the patterns of oocyst shedding, *C. parvum* HNJ-1, TK-1 and HRW-1 oocyst stocks were used. Patterns of oocyst shedding showed a similar trend in all three oocyst stocks.

The pattern of increase in oocyst numbers was similar in all three oocyst stocks. In this study, we obtained a yield of approximately 2×10^4 oocysts, indicating that only a small number of oocysts can be isolated from environmental waters. The small number of oocysts may make it difficult to conduct DNA and RNA based techniques that require large numbers of oocysts.

The results of PCR-RFLP analysis indicated that oocysts in river water belong to the *C. parvum* genotype 2. This finding is consistent with the existing and previously reported epidemiological data for the Tokachi area in Hokkaido [18]. From our results, cattle may be the source of the *C. parvum* polluting river water. Many types of *Cryptospo-*

ridium species have been detected in feces of humans and animals [3]. More studies regarding the epidemiology of *Cryptosporidium* species need to be carried out in the Tokachi area of Hokkaido and in Japan as a whole.

In conclusion, the results of this study have shown that *C. parvum* oocysts detected in sampled river water in the Tokachi area of Hokkaido are viable and, therefore, may be infective to susceptible hosts, as demonstrated by sub-inoculation into SCID mice. Moreover, by using a DNA based technique, we have shown that the isolated oocysts belonged to *C. parvum* genotype 2. Our results appear to be consistent with the current prevalence of bovine cryptosporidiosis. The success in the use of SCID mice to demonstrate *C. parvum* genotype 2 infection indicates that laboratory animals are helpful in the analysis of viability and infectivity. Recently, it has been reported that *C. parvum* genotype 1 does not infect SCID mice [16] and that *C. parvum* genotype 1 can infect not only humans but also pigs [15]. In the laboratory, gnotobiotic pigs were used for maintaining *C. parvum* genotype 1 oocyst [1]. Studies have shown that *C. parvum* genotype 1 infects not only humans but also pigs, primates, and other animals, making the identification of the cause of *C. parvum* genotype 1 outbreaks difficult [16]. Further studies need to be carried out to investigate the application of other laboratory animals, for example, pigs, in order to develop monitoring systems for *C. parvum* genotype 1.

Our results also send a warning that the Tokachi area of Hokkaido, Japan may harbor a significant presence of *C. parvum* oocysts capable of transmitting infection to human hosts. In view of the two recent outbreaks reported in Hokkaido in 2002, it is imperative that prompt measures be taken to deal with the impending threat of human cryptosporidiosis.

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