

Detection of *Cryptosporidium parvum* Oocysts in Environmental Water in Hokkaido, Japan

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ABSTRACT. Control of cryptosporidiosis is important in public health. Rivers that are polluted with *Cryptosporidium* and drinking water that is treated for drinking water production from polluted rivers could result in the waterborne disease of cryptosporidiosis. We carried out an epidemiological study of natural water supplies in Hokkaido, one of the largest dairy prefectures in Japan. To detect *Cryptosporidium* oocysts in environmental water, the filtration method was used for 28 samples, which were collected from 10 rivers. A method adapted from the United States Environmental Protection Agency (U.S. EPA) filtration method using a cartridge filter has been used for the collection of samples. Oocysts were separated from a pellet by discontinuous sucrose gradient method. Twelve samples were collected from 10 rivers and parasites were purified by iron (III) flocculation method. *Cryptosporidium parvum* oocysts were identified with the immunofluorescence antibody technique using DIF kit (Cellabs Pty. Ltd., Sydney/Australia). We detected *Cryptosporidium* oocysts in 6 out of 10 rivers sampled. Fifty percentage (14/28) of the samples were *Cryptosporidium*-positive. The average number of *Cryptosporidium* oocysts was 16.73/100 L (max. 80 /100 L).

KEY WORDS: cryptosporidiosis, *Cryptosporidium*, detection, Hokkaido, waterborne disease.

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The protozoan parasite *Cryptosporidium parvum* has been recognized as common pathogenic protozoa of the gastrointestinal tract of humans and animals [4]. Several outbreaks of cryptosporidiosis have been reported in the last few decades [7, 22]. Human infection with the pathogen is usually directly fecal-oral and by ingestion of contaminated food or water [5]. Water is perhaps the major route for massive outbreaks of the infection, as a result of contamination of either raw or treated water [6]. Despite the difficulties in analytical procedures, the number of *Cryptosporidium* oocysts has been detected in different countries of the world varied from 0.005 to 252.7 oocysts/L for surface waters [21, 24]. Information pertaining to *Cryptosporidium* in the drinking water supply systems in Japan is very limited in comparison with other countries. Moreover, recently, the cryptosporidiosis of cattle has occurred sporadically [12]. Two outbreaks of waterborne cryptosporidiosis have been described in Japan, the first one in Kanagawa in the year 1994 with 461 infections and the second one in Saitama in the year 1996 with 8,000 infections.

In Hokkaido, the animal husbandry was a basic industry and developed in using natural resources and enlarging the scale. According to the report from Livestock Industry's Environmental Improvement Organization [10], the number of raised cattle was 1,297,300 in 1998. The quantity of feces from cattle was 15,850,000 t. Almost of 90% feces from cattle returned to soil. In 1997, the result of the research of the farm near the river was that feces might flow into the river at about 2,700 farms in Japan. In the investigation of the actual conditions of fecal treatment in 1998, 46% of all farms give a bad influence to the environment or are possi-

ble to give. In 50% of the 46% farms, feces directly may flow into the rivers. Agricultural activities are very common in Hokkaido and infected livestock result to the pollution of rivers with *Cryptosporidium* oocysts. In this study, we conducted an epidemiological study of the environmental waters to detect *Cryptosporidium* oocysts from rivers in Hokkaido.

MATERIALS AND METHODS

Geography: In this study, *Cryptosporidium parvum* oocysts were collected at the area described by diagonal lines in the Fig. 1.

Water samples: The procedure for water sample collection, particle separation, and detection of parasites is the United States Environmental Protection Agency (EPA)

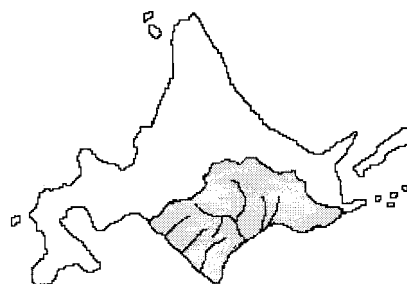


Fig. 1. Sampling area in Hokkaido, Japan. Samples were collected from rivers running at the closed area.

method modified by Karanis *et al.* [13–15]. The EPA method has been used in several studies in the U.S.A. and England [3, 17, 19] and Karanis *et al.* have adapted this method for analysis of samples in Germany [13, 15] and Greece [16]. The filtration method involved filtering large quantities of environmental water using Micro Wynd Cuno yarn wound cartridge filters. Typically 300 L of water was filtered with average flow rates between 2 and 5 liters/min. Filter fibers were entirely hand washed in 3 L of 0.1% Tween 80 solution to retrieve *Cryptosporidium*. Wash-water was concentrated by centrifugation (10 min, 1,040 g) of the Tween 80 solution. The volumes of 50 ml were transferred in 50 ml capacity conical tubes, vortexed, and centrifuged (10 min, 1,040 g). After centrifugation, the supernatant was discarded, pellets were vortexed, sonicated, resuspended in distilled water (DW) and pooled in one tube. The final volume of sample was 5 ml, which was served to the discontinuous sucrose gradient method for concentration and purification of oocysts. A sucrose gradient was prepared from Sheather's solution (500 g sucrose, 6.5 g phenol, 320 ml DW) and 0.025 M phosphate buffered saline with 1.0% Tween 80. Two solutions with specific gravities of 1.103 and 1.064 were prepared and used to build up a discontinuous gradient [2]. The volume of resulting pellet for sample was 100 μ l. The volume of 100 μ l was split as follows: routinely the 30 μ l was examined under microscope with Kinyoun staining and immunofluorescence assay (IFA), and the 70 μ l was stored in 2.5% (w/v) solution of $K_2Cr_2O_7$.

The flocculation method was adapted from the protocol using in the Institute for Medical Parasitology of University of Bonn. It has been shown that the rate of detection of *Cryptosporidium* oocysts depends on the method to draw the environmental water sample. Oocysts were not detected in the shallow waters of the river and were detected in the deep waters. We used Fe (III) sulfate-solution and adjusted pH 6 in order to examine 3 L of environmental water. The 750 μ l Fe (III) sulfate solution was put into 3 L water sample. After 24 hr, particles in the water were sedimented. Supernatant was discarded using a special pump and the remaining volumes of 200 ml were transferred in 50-ml tubes (4x). The water was concentrated by centrifugation (10 min, 2,100 g). Supernatant from each tube was discarded by a special pump, and remaining volume of 5 ml were transferred in a 50 ml tube after vortexing. After centrifugation (10 min, 2,100 g), supernatant was discarded completely and 6 ml lysis buffer (8.4 g citric acid monohydrate, 17.64 g trisodium citrate di-hydrate, 100 ml DW, adjusted pH 4.7) was added to the sample. After one hour, 44 ml distilled water was added to the sample and the sample was centrifuged (10 min, 2,100 g). After washing at three times with 50 ml DW, supernatant was discarded. The pellet was resuspended in 5 ml DW. The final volume of 5 ml was purified by the discontinuous sucrose gradient method. Finally, *Cryptosporidium parvum* oocysts were

detected by Kinyoun staining and the kit of IFA (Cellabs DIF, Cellabs Pty. Ltd., Sydney/Australia), which were ranged from 4 to 6 μ m in diameter and approximately spherical.

RESULTS

We examined 28 samples by the filtration method and 12 samples by flocculation method from 10 rivers in Hokkaido at August, September, and November in 1999. The frequency of detection of oocysts in raw water samples (from No. 4 to No. 31) by filtration and flocculation was 50% (14/28) and 67% (8/12), respectively (Table 1). The rivers from B to G running in the main dairy areas tested were positive for *Cryptosporidium* oocysts, whilst the rivers from H to K running in the horse rising areas tested were negative. In addition we investigated 3 samples of tap water in Obihiro City. All of the tap water samples of the Obihiro by three-time filtration and flocculation (Table 1) were negative. The number of oocysts detected by the flocculation method was (range 1.7 to 330 times) higher than the numbers of oocysts detected by the filtration method.

Table 1. Detection of *Cryptosporidium* oocysts in environmental water of Hokkaido

No.	Sample name	Filtration oocysts/L	Flocculation oocysts/L	Date
1	Tap water A	0.00	0.00	11/08/99
2	Tap water A	0.00	0.00	12/08/99
3	Tap water A	0.00	0.00	13/08/99
4	River B	0.74	2.80	23/08/99
5	River B	0.80	3.33	25/08/99
6	River B	0.07	2.70	25/08/99
7	River C	0.04	5.00	07/09/99
8	River D	0.01	3.33	07/09/99
9	River D	0.00	ND ^{a)}	07/09/99
10	River D	0.03	ND	07/09/99
11	River E	0.06	0.60	08/09/99
12	River E	0.03	ND	08/09/99
13	River E	0.03	ND	08/09/99
14	River F	0.07	0.60	08/09/99
15	River F	0.07	ND	08/09/99
16	River F	0.07	ND	08/09/99
17	River G	0.02	0.60	08/09/99
18	River G	0.00	ND	08/09/99
19	River G	0.03	ND	08/09/99
20	River H	0.00	0.00	06/11/99
21	River H	0.00	ND	06/11/99
22	River H	0.00	ND	06/11/99
23	River I	0.00	0.00	06/11/99
24	River I	0.00	ND	06/11/99
25	River I	0.00	ND	06/11/99
26	River J	0.00	0.00	07/11/99
27	River J	0.00	ND	07/11/99
28	River J	0.00	ND	07/11/99
29	River K	0.00	0.00	07/11/99
30	River K	0.00	ND	07/11/99
31	River K	0.00	ND	07/11/99

a) ND; No Data (Not examined).

DISCUSSION

In this study, we found that *Cryptosporidium parvum* oocysts were prevalent in surface waters especially in dairy area of Hokkaido, but were not detected in the areas that mainly raised horses. We presumed feces of infected cattle and wild animals as sources of *Cryptosporidium* oocysts. Because the assay for pathogen detection does not indicate parasite viability, it is hard to determine if the water sources serve of risk from waterborne pathogens. A comparison of the two collection methods showed that the number of oocysts detected by flocculation method was 1.7 to 330 times higher than the numbers detected by the filtration method. Moreover there was a correlation between *Cryptosporidium* positive rivers detected by filtration method and by flocculation method. It can be deduced from the efficiency of detection and the correlation of two methods that *Cryptosporidium* monitoring as best carried out by the flocculation method. Water from deep sections of the river was best collected using a pump. The disadvantage of using fewer quantities of water sample (3L) is that *Cryptosporidium* oocysts may be undetected. Then we can conclude that a combination of both the filtration and flocculation methods be used in monitoring *Cryptosporidium* oocysts in environmental water samples.

Table 2 lists the level of *Cryptosporidium* oocysts in Middle Eastern and Asian surface waters recently reported [1, 9, 11, 20, 23, 25], respectively. Our data are also included in this table for comparison. Although data in Table 2 were not adjusted for recovery efficiency, mean levels of *Cryptosporidium* oocysts from American, European, Middle Eastern and other Asian areas were more than those found in Hokkaido, Japan. No oocysts were found in the water samples from Malaysia [1]. The U.S. EPA Federal Register (U.S. EPA, 1989) shows that one case of microbiologically caused illness per year per 10,000 individuals is acceptable as a drinking water standard. To achieve this goal, Haas and Rose stated that an outbreak would probably occur if tap water contained more than 10 to 30 *Cryptosporidium* oocysts/100L [8]. Because the information about dose-

response relationship for this parasite is insufficient in Hokkaido, results predicted from this model are variable and uncertain. In consideration of the possibility of an outbreak of cryptosporidiosis, infectivity, pathogenicity, and viability of oocysts need to be examined, and studied great detail. On the other hand, because of several factors involved, it is difficult to prove the relationship between the oocysts in environmental water and in feces of cattle. We recommend that appropriate point-of-use equipment should be used to ensure safety and quality of drinking water.

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REFERENCES

1. Ahmad, R. A., Lee, E., Tan, I. T. L. and Mohamad-Kamel, A. G. 1997. Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in raw and treated water treatment plants in Selangor, Malaysia. *Water Res.* 31: 3132–3136.
2. Arrowood, M. J. and Sterling, C. R. 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic percoll gradients. *J. Parasitol.* 73: 314–319.
3. Badenoch, J. 1990. *Cryptosporidium* in water supplies. pp. 1–230. In: Report of the Group of the Experts (Badenoch, J. ed.), London, HMSO.
4. Cook, G. C. 1995. *Entamoeba histolytica* and *Giardia lamblia* infection: Current diagnostic strategies. *Parasite* 2: 107–112.
5. Donnelly, J. K. and Stentiford, E. I. 1997. The *Cryptosporidium* problem in water and food supplies. *Food Sci. Technol.* 30: 111–120.
6. Eisenberg, J. N. S., Seto, E. Y. W., Colfold, J. M., Olivieri, A. and Spear, R. C. 1998. An analysis of the Milwaukee cryptosporidiosis outbreak based on a dynamic model of the infection process. *Epidemiology* 9: 255–263.
7. Frost, F. J., Craun, G. F. and Calderon, R. L. 1996. Waterborne disease surveillance. *J. Am. Water Works Assoc.* 88: 66–75.
8. Haas, C. N. and Rose, J. B. 1995. Developing an action level for *Cryptosporidium*. *J. Am. Water Works Assoc.* 87: 81–84.
9. Ho, B. S. W. and Tam, T.-Y. 1998. *Giardia* and *Cryptosporidium* in sewage contaminated river waters. *Water Res.* 32:

Table 2. The Range and mean level of *Cryptosporidium* in different Middle Eastern, Asian, European and American areas

Areas	Sample type	Range of <i>Cryptosporidium</i> oocysts/ L	Reference
USA Washington	Rivers	2.0–112.0	Ongerth <i>et al.</i> (1987)
California			
Arizona	Surface water	0.005–252.7	Sterling <i>et al.</i> (1987)
Germany	Raw water	0.0–13.1	Karanis <i>et al.</i> (1998)
Hong Kong	Rivers	0.3–3.0	Ho and Tam (1998)
Israel	Surface water	0.0–1.9	Zuckerman <i>et al.</i> (1997)
	in northern part		
Taiwan	Raw water of	<0.009–8.0	Hsu <i>et al.</i> (1999)
	water treatment		
	plant		
Japan	Surface water	0.0–0.8	present work

- 2860–2864.
10. Chikusan Kankyo Joho. Vol. 3. pp. 23–25. 1998. Livestock Industry's Environmental Improvement Organization (in Japanese).
11. Hsu, B. -M., Huang, C., Jiang G. -Y. and Hsu. C. L. 1999. The prevalence of *Giardia* and *Cryptosporidium* in Taiwan water supplies. *J. Toxicol. Environ. Health* 56: 149–160.
12. Kaneta, Y. and Nakai, Y. 1998. Survey of *Cryptosporidium* oocysts from adult cattle in a slaughter house. *J. Vet. Med. Sci.* 60: 585–588.
13. Karanis, P., Schoenen, D. and Seitz, H. M. 1996. *Giardia* and *Cryptosporidium* in backwash water from rapid sand filters used for drinking water production. *Zentralbl. Bakteriol.* 284: 107–114.
14. Karanis, P., Opiela, K., Renoth, S. and Seitz, H. M. 1996. Possible contamination of surface waters with *Giardia* spp. through muskrats. *Zentralbl. Bakteriol.* 284: 302–306.
15. Karanis, P., Shoenen, D. and Seiz, H. M. 1998. Distribution and removal of *Giardia* and *Cryptosporidium* in water supplies in Germany. *Water Sci. Technol.* 37: 9–18.
16. Karanis, P., Papadopoulou, C., Kimura, A., Economou, E. and Sakkas, H. 2000. Distribution of *Cryptosporidium* and *Giardia* in natural and drinking water supplies in Northwestern Greece (in press).
17. Madore, M. J., Rose, J., Gerba, C. P., Arrowood, M. J. and Sterling, C. R. 1987. Occurrence of *Cryptosporidium* oocysts in swage effluents and selected surface waters. *J. Parasitol.* 73: 702–705.
18. Morris, R. D., Naumova, E. N. and Griffiths, J. K. 1998. Did Milwaukee experience waterborne cryptosporidiosis before the large documented outbreak in 1993. *Epidemiology* 9: 264–270.
19. Musial, C. E., Arrowood, M. J., Sterling, C. R. and Gerba, C. P. 1987. Detection of *Cryptosporidium* in water by using polypropylene cartridge filters. *Appl. Environ. Microbiol.* 53: 687–692.
20. Ongerth, J. E. and Stibbs, H. H. 1987. Identification of *Cryptosporidium* oocysts in river water. *Appl. Environ. Microbiol.* 53: 672–676.
21. Rose, J. B. 1988. Occurrence and significance of *Cryptosporidium* in water. *J. Am. Water Works Assoc.* 80: 53–58.
22. SoloGabriele, H. and Neumeister, S. 1996. US outbreaks of cryptosporidiosis. *J. Am. Water Works Assoc.* 88: 76–86.
23. Sterling, C. R., Arrowood, M. J., Marshall, M. M. and Stetzenbach, L. D. 1987. The detection of *Giardia* and *Cryptosporidium* from water sources using monoclonal antibodies. *Proc. Am. Water Works Assoc. Water Quality Technical Conf.* 15: 271.
24. Stezenbach, L. D., Arrowood, M. J., Mershell, M. M. and Sterling, C. R. 1988. Monoclonal antibody based immunofluorescent assay for *Giardia* and *Cryptosporidium* detection in water samples. *Water Sci. Technol.* 20: 193–198.
25. Zuckerman, U., Gold, D., Shelef, G. and Armon, R. 1997. The presence of *Giardia* and *Cryptosporidium* in surface waters and effluents in Israel. *Water Sci. Technol.* 35: 381–384.