Contamination of River Water by Cryptosporidium parvum
Oocysts in Western Japan

KAZUO ONO,1* HIDETAKA TSUJI,1 SHIBA KUMAR RAI,2† AKIO YAMAMOTO,1
KUNIYOSHI MASUDA,1 TAKURO END0,3 HAK HOTT4 TAKASHI KAWAMURA,1
AND SHOJI UGA5

Division of Microbiology, Hyogo Prefectural Institute of Public Health,1 Department of Medical Zoology,2
Department of Microbiology,4 and Faculty of Health Science,5 Kobe University School of
Medicine, Kobe, and National Institute of Infectious Diseases, Tokyo,3 Japan

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In Japan, only a few rivers have been inspected for Cryptosporidium parvum contamination, and the methods
used had low sensitivity. In 1998 and 1999, we used a method with higher sensitivity to examine all large rivers
used as sources of water supply in one prefecture (which we divided into four areas) in western Japan for Cryptosporidium oocysts. One sample was collected at each of 156 sites along 18 rivers, and samples were tested
for Cryptosporidium oocysts by immunomagnetic separation. Samples were classified as being obtained on an
island with livestock and fishing industries, a densely populated urban area, a western region including
farming villages, or a still more rural northern area with agriculture and fishing. Restriction fragment length
polymorphism analysis was used for identification of the C. parvum found as the bovine or human type. C. parvum was detected in at least one sample from 13 of the 18 rivers and in 47% (74 of 156) of the samples.
One-third to all of the samples from each area contained C. parvum oocysts. The number of C. parvum oocysts per 20 liters of river water varied in the same pattern as the number of cattle kept in the four kinds of areas
(as determined by the Mantel extension test). Oocysts isolated were of the bovine type; the C. parvum detected
in rivers probably came from cattle kept in that valley. As we had expected, when tested with a more sensitive
method, river water in western Japan was found to be greatly contaminated with C. parvum oocysts, as reported
in other countries.

The protozoa Cryptosporidium parvum and Giardia intestinalis (lamblia) are enteric parasitic pathogens found in water
systems (17). Their oocysts and cysts, respectively, are environmentally robust, resisting disinfectants. C. parvum is small
enough to be difficult to remove by filtration. It can cause outbreaks more readily than G. intestinalis because it produces
many more oocysts in the host. The infective dose of C. parvum is low. Okhuysen et al. (19) found the 50% infective dose of the
most virulent of their three isolates to be nine oocysts for healthy adults. Calculation on the basis of an epidemiological
infection model showed that infection may occur with even one oocyst (9).

Since the first report of an outbreak of C. parvum infection caused by a contaminated water supply in 1985 (6), epidemi-
ological studies of such contamination have been done in many countries, including the United States (20), Scotland (27), Aus-
tralia (32), and South Africa (12). The methods used were diverse, so the results varied widely. Nevertheless, C. parvum
was detected in almost all of the environmental waters tested (see reference 28 for a review). Groundwater and lake water have
been examined, but river water used as the source of public water supplies has been surveyed more than other water
sources in such studies.

In a study of surface water in 17 states in the United States, Rose et al. (23) reported finding oocysts in 93 (51%) of the 181
samples tested, and the mean number of oocysts detected was 4.3 per 10 liters. LeChevallier et al. (13) did a similar study in
one province of Canada and 14 states in the United States and found oocysts in 74 (87%) of the 85 samples tested. In contrast,
Roach et al. (22), in a study in the Yukon, Canada, found Cryptosporidium oocysts in only 3 (5%) of the 63 samples tested.
These differences in the percentage and intensity of contamination of surface waters by Cryptosporidium oocysts
may be related to the season (23), rainfall (7), or the presence of untreated feces of humans (16) or domestic animals such as
cattle (26). The methods used to detect C. parvum are complicated, and results may be difficult to interpret because of low
recovery at dilute concentrations and other problems (5).

In Japan, only two studies of river water have appeared; none has been done in western Japan or by the immunomag-
netic separation assay based on method 1622 of the U.S. En-
nvironmental Protection Agency (EPA) (30). By immunomag-
netic separation, Cryptosporidium oocysts can be isolated from
water. Recovery is poor when the sample is contaminated with
organic particles or protozoa. The method has not been evalu-
ated thoroughly for detection of C. parvum oocysts in water in
field studies. We recently improved recovery in the field by
adding three steps to this procedure. Therefore, we used our
modified method to establish the present state of river con-
tamination by Cryptosporidium oocysts in an entire prefecture,
and we examined the relationship between the intensity of
contamination and the numbers and varieties of domestic an-

* Corresponding author. Mailing address: Division of Microbiology, Hyogo Prefectural Institute of Public Health, 2-1-29 Arata-cho, Hyogo-ku, Kobe 652-0032, Japan. Phone: 81 78 511 6784. Fax: 81 78
531 7080. E-mail: ono@iph.pref.hyogo.jp.
† Present address: Department of Microbiology, Nepal Medical Col-
lege, Kathmandu, Nepal.
of the 1 to 29 samples was found to contain an oocyst(s). Results were classified
parvum oocysts found. A river was considered to be contaminated when even one
be contaminated, a separate sample was taken for use in restriction fragment
sampling sites were 4 and 10 in the island area, 5 and 87 in the eastern area, 4
mainly of small communities involved in extensive farming. The northern area
Prefecture in the Kansai area of western Japan were sampled twice. The areas
studied were classified as island, eastern, western, and northern (Fig. 1). The
animals kept in the valleys of the rivers in question. We also examined whether
examined whether Escherichia coli could be used as an index of Cryptosporidium contamination.
MATERIALS AND METHODS
Water sampling. From July to November in 1998 or 1999, 18 rivers in Hyogo Prefecture in the Kansai area of western Japan were sampled twice. The areas studied were classified as island, eastern, western, and northern (Fig. 1). The island area, in the southernmost part of the prefecture, was an island in the Inland Sea, with flourishing livestock and fishing industries. The eastern area was an urban area with a high population density. The western area was a region mainly of small communities involved in extensive farming. The northern area was a still more rural region with much agricultural and fishing activity and with heavy snowfall in winter. The number of rivers sampled and the number of sampling sites were 4 and 10 in the island area, 5 and 87 in the eastern area, 4 and 40 in the western area, and 5 and 19 in the northern area, respectively, and the total number of samples taken was 156 (Fig. 1). One sample was taken from each sampling site.
In addition, in all areas but the northern one, from one large river judged likely to be contaminated, a separate sample was taken for use in restriction fragment length polymorphism (RFLP) analysis for identification of the genotype of any C. parvum oocysts found. A river was considered to be contaminated when even one of the 1 to 29 samples was found to contain an oocyst(s). Results were classified as to whether the sampling site was upstream (total number of sampling sites, 35), midstream (54 sites), or downstream (67 sites). For this classification, a straight line connecting the beginning and end of the river on a map was divided into three equal lengths, regardless of the winding of the river.
For water sampling, a bucket or a combination of a generator (Honda, Tokyo, Japan) and a water pump (Terada Pump, Tokyo, Japan) was used for collection of 20 liters of water from roughly 50 cm below the water’s surface; pumping took about 3 min. The sample was put into a polyethylene container. When the sampling site was far from a road, the same amount was concentrated while being collected with a capsule filter (Envirotech; Gelman Sciences, Ann Arbor, Mich.). This kind of filter was also used when an extra sampling of 100 liters was done for examination by RFLP analysis.
In 1998, at the same time as the sampling for C. parvum, 100-ml samples were collected in sterilized polyethylene test tubes for E. coli testing. All water samples were stored at 4°C in an insulated container with ice for immediate transportation to the laboratory and were processed within 24 h as described below.
Sample processing. We used a pressure device (model XXS82 001 15; Millipore Corp., Bedford, Mass.) to filter the water samples through a disk-filter holder fitted with a nitrocellulose membrane (CF; pore size, 1.2 μm; Millipore) and retrieved the material caught on the surface of the membrane by use of ultrasound (Heat Systems Ultrasorics, Farmingdale, N.Y.) in the first step that we added. In the second step that we added, the material retrieved was suspended in 0.15 M phosphate-buffered saline (pH 7.6), and the mixture was sonicated for 1 min. A kit for immunomagnetic separation with Dynabeads coated with anti-Cryptosporidium antibodies (G-C Combo; Dynal A.S., Oslo, Norway) was used to treat the suspension. Preliminary experiments had shown that some captured oocysts remained attached to the beads, so we repeated this step (our third modification). The material that had bound to the antibodies was separated from them by treatment with 50 μl of 0.1 N HCl; the beads were held aside with a magnet, and the remaining suspension was removed from the tube with a pipette, placed on a slide, dried, and labeled with anti-Cryptosporidium monoclonal antibod- ies (Cellabs Pty. Ltd., Brookvale, New South Wales, Australia) conjugated with fluorescein so that oocysts could be identified (the first method [see below]). Water samples concentrated before transportation were first eluted into a buffer as described elsewhere (18) and then processed by immunomagnetic separation as described above.
Identification of oocysts from water by microscopy. Three methods were used for identification of oocysts from water, with the exception of the 100-liter samples mentioned in the next section, and when results were positive by all three methods, the oocysts were identified as being of C. parvum. In the first method, oocysts stained with the labeled monoclonal antibodies mentioned above were checked for having a diameter of 4 to 6 μm at magnifications of ×400 to ×1,000 with a 400- to 440-nm (blue-violet) filter. With the second method, microscopy was used for examination of the same stained oocysts for sporozoites at a magnification of ×1,000 with a Nomarski interference contrast filter. With the third method, fluorescent staining with 6-diamino-2-phenylindole (Sigma, St. Louis, Mo.) was used to look for nuclei in sporozoites detected at magnifications of ×400 to ×1,000 with a 330- to 385-nm (UV) filter.
Identification of oocysts by PCR. We used oocysts collected in capsule filters from 100-liter samples for analysis of RFLP by the method of Carranay et al. (4). Using primers that flanked a 515-bp region of an open reading frame in the C. parvum polythene locus, we amplified oocyst DNA from about 10 oocysts isolated from river water. The amplification product was digested with Rsal, and the pattern obtained by electrophoresis of the digest was compared with patterns of three strains of C. parvum of known origin and with the pattern of Crypto- sporidium andersoni as well (15). For this comparison, we isolated one strain of C. parvum (diameter, 4.5 to 5.0 μm) from a calf and one strain of C. andersoni (5.5 to 7.5 μm) from a Guernsey cow. In addition, we used purified DNAs of C. parvum isolated from two patients with diarrhea. One strain had been identified as having the human profile by RFLP (only strains from humans have been found as having this genotype), and the other strain had been identified as having the bovine profile (isolates from human patients may have either the bovine or human profile). The National Institute of Infectious Diseases, Tokyo, Japan, provided both strains from patients.
Other. Testing for E. coli was done by the method used by public water departments in Japan (11). This qualitative method is a combination of the defined-substrate method and the standard method. In brief, a 30-ml water sample is examined for coliforms by a method that uses ortho-nitrophenyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-galactoside (Collert; Idex Lab Inc., Westbrook, Maine). Culture is done at 44.5°C for 24 h in two tubes con- taining a broth for E. coli (Nissui, Tokyo, Japan), and bacteria that produce gas are further cultured at 36°C for 20 h in a medium that uses 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranos-
the numbers of domestic animals being kept (10).

Statistical analysis of differences between proportions of samples with C. parvum and E. coli contamination was done with the $\chi^2$ test of proportions. A possible trend for areas with larger numbers of livestock to have greater intensities of C. parvum contamination, in terms of oocysts per 20 liters, was evaluated with the Mantel extension test. The Kruskal-Wallis test was used to examine the differences in the intensities of contamination in different sections of the rivers. Differences with $P$ values of $<0.05$ were defined as being statistically significant.

RESULTS

Contamination of rivers and areas. C. parvum contaminated 13 (72%) of the 18 rivers tested, and E. coli contaminated 9 (69%) of the 13 rivers tested (Table 1). C. parvum and E. coli were detected in all areas tested. The proportions of samples with C. parvum were 10 of 10 (100%) in the island area, intermediate values in the eastern and western areas, and 7 of 19 (37%) in the northern area (Table 2; $P < 0.005$). The proportions with E. coli changed in the same pattern ($P < 0.001$).

Relationship of contamination by oocysts to numbers of domestic animals in the area. Table 3 shows the mean number of C. parvum oocysts found per 20 liters of water, referred to as the intensity of contamination, and the numbers of cattle, pigs, and chickens in each area. The mean concentration of oocysts and the number of cattle, pigs, and chickens in each area. The mean concentration of oocysts was largest (2.4 per 20 liters) in the island area and smallest (1.4 per 20 liters) in the northern area. The intensity of contamination, and the numbers of livestock in four areas.

<table>
<thead>
<tr>
<th>Area</th>
<th>No. of livestock</th>
<th>Mean no. of oocysts per 20 liters (range)</th>
<th>No. of livestock (to nearest hundred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Island</td>
<td>10 (100)</td>
<td>2.4 (1–6)</td>
<td>Cattle: 43,100, Pigs: 23,600, Chickens: 26,900</td>
</tr>
<tr>
<td>Eastern</td>
<td>36 (41)</td>
<td>1.8 (1–8)</td>
<td></td>
</tr>
<tr>
<td>Western</td>
<td>21 (53)</td>
<td>1.6 (1–3)</td>
<td></td>
</tr>
<tr>
<td>Northern</td>
<td>7 (37)</td>
<td>1.4 (1–4)</td>
<td></td>
</tr>
</tbody>
</table>

* Mantel extension test: cattle, $\chi^2 = 9.56$, df = 1, and $P < 0.005$; pigs, $\chi^2 = 1.03$, df = 1, and $P = 0.31$; chickens, $\chi^2 = 0.80$, df = 1, and $P = 0.37$.

Relationship of location along the river and intensity of contamination. The percentages of samples with C. parvum and the number of cattle in the different areas varied in the same pattern ($P < 0.005$).

Relationship of location along the river and intensity of contamination. The percentages of samples with C. parvum and the number of cattle in the different areas varied in the same pattern ($P < 0.005$).

<table>
<thead>
<tr>
<th>Part of river</th>
<th>No. of samples tested</th>
<th>No. (%) of samples with oocysts</th>
<th>Mean no. of oocysts detected per 20 liters (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream</td>
<td>35</td>
<td>13 (37)</td>
<td>1.9 (1–6)</td>
</tr>
<tr>
<td>Midstream</td>
<td>54</td>
<td>25 (46)</td>
<td>1.5 (1–6)</td>
</tr>
<tr>
<td>Downstream</td>
<td>67</td>
<td>36 (54)</td>
<td>2.1 (1–8)</td>
</tr>
</tbody>
</table>

* The beginning and end of each river was marked on a map, a straight line was drawn between the two points, and the line was divided into three sections of equal length.

and the intensities of contamination in different sections of the rivers are shown in Table 4. Of the samples from the 156 sampling sites, 37% of the upstream samples, 46% of the midstream samples, and 54% of the downstream samples were contaminated. The differences were not significant.

Identification of oocysts by PCR. Results of electrophoresis of PCR products are shown in Fig. 2. With undigested DNAs from the three C. parvum strains of known origin and from the three large water samples, there was a single band at about 510 bp, but C. andersoni was not amplified (Fig. 2A). After digestion, DNAs from five of the six strains of C. parvum had three main bands, at 270, 130, and 50 bp. However, the C. parvum strain of the human type had two main bands, at about 400 and 50 bp. The oocysts that we detected in the water samples were from C. parvum of the bovine type.

**DISCUSSION**

The results of two epidemiological studies of C. parvum in Japan have been published. In a study done by Hashimoto and Hirata (8) in central Japan, from a total of 13 sites along the Sagami River and its tributaries, water was sampled one to four times, with a large sample size (100 liters), and contamination was found at 97% of the 12 sites examined in 1996 and at 46% of the 6 sites examined in 1997. The rate of contamination that we found was also high, in agreement with their results, although they used an earlier method still used by the Ministry of Health and Welfare of Japan and we used a method more popular outside of Japan (method 1622 of the U.S. EPA).

Groups in Japan generally use a provisional method published in Japan in 1996 and based on an earlier EPA procedure, the information collection rule, with filtration usually of only 10 liters of water through a nitrocellulose membrane and dissolution of material caught on the filter in acetone. With
this method, in a 1997 survey of sources of water supply that examined 277 sites in 94 rivers throughout Japan, the Ministry of Health and Welfare found Cryptosporidium oocysts in only 8 (2.9%) of the 277 sites tested (31). The different volumes of the samples (10 and 100 liters) used is the probable cause of the discrepant results in the two surveys mentioned above, rather than differences in the sites or seasons. The method that both surveys used, which is complicated, is termed provisional because there are doubts about recovery and sensitivity (21); reliability and validity also are questionable. We used immunomagnetic separation, an unrelated method, adding three modifications to improve recovery. Our results suggest again that the method would be useful in epidemiological studies as found earlier by Campbell and Smith (3) and Bukhari et al. (2).

Possible sources of contamination of rivers by Cryptosporidium are raw or treated sewage from humans and feces of domesticated and wild animals (15, 25). C. parvum has a wide range of hosts, including 79 species of mammals. Atwill et al. (1) found that 5.4% of 221 wild pigs tested in California were shedding C. parvum oocysts. Hashimoto and Hirata (8) suggested that uncomposted feces from a large-scale pig-breeding facility on the upper reaches of the river studied might be contaminating the river. In the areas we studied, however, there were no such large-scale facilities, and the incidence of parasitism was low (there were no positive results for any of the 567 pigs tested); pigs were not a likely source of contamination in our study. Uga et al. (data not shown) found that 8.9% of 418 chicken tested were shedding oocysts of Cryptosporidium baileyi in our survey areas. Wild animals such as deer and boar live in the western and northern areas we surveyed, but in numbers too small to be a problem. In rural parts of the prefecture, human waste is gathered in underground domestic tanks emptied regularly by collection trucks. In those areas, raw sewage that accidentally overflows may end up in a river, but sewage is almost always properly treated and there is strict water quality control for treated sewage in Japan, so contamination probably does not arise in this way.

In small farms in Japan, cattle feces are not composted, and Cryptosporidium oocysts shed by these cattle may contaminate rivers. The island area, the smallest of the four, had the most cattle and the highest density. That the percentage of samples contaminated was highest in the island area and lowest in the northern area suggested that the percentage of contaminated samples was related to the number of cattle in that area, or in other words, that oocysts shed by cattle are contaminating the environment. Sacki et al. (24) detected C. parvum in 1 (0.2%) of 582 adult cattle in the same prefecture, and we (29) reported that 24 (80%) of 30 calves tested shed C. parvum oocysts during the first month of life. Our RFLP results showed that the genotype of the C. parvum oocysts retrieved from river water was that found in cattle. Humans can be infected with C. parvum of the genotype originally isolated from cattle and still called the bovine type; nevertheless, it is likely that cattle had shed a large majority of the oocysts that we found in river water.

In our study, the percentage of contaminated samples was higher downstream than upstream, and the intensity of contamination also was greater downstream. We estimated that about half of the farms with cattle were upstream, making it likely that contamination started upstream and later affected downstream areas. Our findings suggest that C. parvum oocysts do not decrease in number by settling out while moving downstream but that the intensity of contamination instead increases downstream, because of the confluence of contaminated tributary streams.

Studies of C. parvum in river water involve complex procedures and various kinds of equipment. Evaluation of the data obtained also requires skill. Accordingly, attempts have been made to find an index of contamination other than C. parvum oocysts, one for which testing is easier. LeChevallier and Norton (14) found that turbidity (which increases after a heavy rainfall) may be one index of contamination by C. parvum oocysts. In addition, protozoa in the water come from animal feces, so E. coli and coliform bacilli in general have been suggested for use as an index of protozoan contamination. However, such bacilli die more quickly in water than protozoa, preventing their detection by culture, and a satisfactory method for their use as an index of contamination has not been established. Rose et al. (23) found no correlation between the concentration of Cryptosporidium (or Giardia) and coliform bacilli in their samples of surface water, but LeChevallier et al. (13) reported a significant correlation between the numbers of Cryptosporidium oocysts and the numbers of total coliform bacilli in samples of raw water. We surveyed contamination by
organisms other than *C. parvum* (*G. intestinalis* and *Clostridium perfringens* [data not shown], in addition to *E. coli*) and found that the percentage of samples contaminated by *E. coli* was closest to the percentage contaminated by *C. parvum*. Many samples were contaminated with both organisms, more so than for the two other combinations. In this survey, we used a qualitative method for detection of *E. coli*. In our previous quantitative study, we found no relation between the number of *E. coli* cells and the number of *Cryptosporidium* oocysts detected (data not shown). A similar observation was reported by Simmons et al. (25). If *E. coli* is an index for *Cryptosporidium* contamination, a simple qualitative test may be sufficient.

The results of our study showed that *Cryptosporidium* contamination is widespread in the rivers of Japan and that immunomagnetic separation is a useful method for detection of this protozoan. *E. coli* may be an index of *Cryptosporidium* contamination.

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**REFERENCES**


