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Brackish Water Benthic Shellfish (*Corbicula japonica*) as a Biological Indicator for *Cryptosporidium parvum* Oocysts in River Water

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The coccidian protozoan genus *Cryptosporidium* belonging to *Apicomplexa* is capable of infecting the gastrointestinal or respiratory tracts of a wide range of vertebrates (Spano *et al.* 1998). Six *Cryptosporidium* species are so far distinguished on the basis of differences in oocyst morphology, site of infection and host specificity (Morgan *et al.* 1995). *Cryptosporidium parvum* (*C. parvum*) is particularly considered a significant human and livestock pathogen causing cryptosporidiosis, severe diarrhea, and wasting which is not currently treatable with antimicrobial drugs, as well as no effective vaccine is available (Shianna *et al.* 1998).

Contamination of drinking water with *C. parvum* oocysts from human and animal feces occasionally causes massive outbreaks of cryptosporidiosis (Johnson *et al.* 1995). In present water purification systems, the removal of fine particles such as *C. parvum* oocysts is mainly based on the rapid filtration process utilized widely in Japan. However, the removal of oocysts by filtration is not necessarily complete, and *C. parvum* oocysts are resistant to chlorination (Carraway *et al.* 1996). If the amounts of oocysts in raw water are high and they cross through the filtration barriers, oocysts could become present in the finished water. Reported evidence supporting water-borne transmission of the parasite as an important mode of spreading is present for some documented epidemics (Awad-el-Kariem *et al.* 1994). There are several different methods available for detecting *C. parvum* oocysts in environmental water samples. Most commonly, direct and indirect microscopic visualization of oocysts is carried out using fluorescent dyes or fluorescein-conjugated antibodies (Johnson *et al.* 1995). The immunofluorescence assay (IFA) is a widely used method to detect oocysts in water, however the efficiency of the detection depends on the amount of suspended substances in the water, and this varies greatly according to the natural environmental conditions.

Benthic shellfishes, *Corbicula japonica* (*C. japonica*) live in restricted downstream brackish water areas where the terminal points of the rivers, and feed on suspended plankton by filtration with gills. Hence, *C. japonica* may be a possible biological indicator for net estimates of contamination levels in river water by *C. parvum* oocysts.
This study investigated and evaluated the role and the usefulness of *C. japonica* as a biological indicator or collection system for *C. parvum* oocysts, useful to explore effective, and stable oocysts gathering methods regardless of the surrounding natural conditions.

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MATERIALS AND METHODS

C. japonica with body size 33.3–43.1 × 29.6–37.1 × 18.0–24.1 mm and body weight 15.5–16.5 g were collected from the Ishikari River, Hokkaido, Japan. One hundred and fifty individual *C. japonica* clams were placed in a stainless steel cage in a 12 L aquarium which was filled with dechlorinated 10% artificial sea water (Tetra Marinsalt, Tetrawerke, Germany) of pH 7.4 and d = 1.002. The aquarium was kept at 15°C with a cooler (RZ-90, REI-SEA, Japan) and an air pump (Inno · β6000, Nisso, Japan) with two stick type air stones set under the stainless steel cage for aeration and circulation of the water. To remove ammonia and nitrites, an adequate amount of oxidizing bacteria (Nisso, Japan) was added to the water. The clams were acclimated for 6 weeks in the aquarium prior to the oocyst introduction, and they were fed daily with liquid-type chaw for invertebrates (Tetrawerke) and a proper amount of chlorella (*Chlorella pyrenoidosa*, Sun Chlorella, Japan) which had a negative IFA reaction against *C. parvum* oocysts. The water of the aquarium was changed daily.

Oocysts of *C. parvum* (bovine type, Lot No.01-6) suspended in storage solution (PBS containing penicillin, streptomycin and gentamicin) from Waterborne Inc. (USA) were stored at 4°C. After 6 weeks acclimation, twenty randomly selected control clams were removed from the aquarium, and the water had 8.75×10^6 oocysts of *C. parvum* (7.29×10^5 oocysts/L, 6.73×10^4 oocysts/clam) added. Ten randomly selected clams of the 130 remaining were examined at 2, 4, 6, 8, and 16 hrs after start of the exposure, and also 1, 2, 3, 4, 7, 10, and 14 days after the *C. parvum* addition. Clam feces at the bottom of the aquarium and 12 L water samples were collected at the time points detailed above.

The shells of the ten removed clams were opened by cutting the anterior and posterior adductor muscles with a scalpel, and the gills, gastrointestinal tract (GI tract) and mantle were excised in centrifuge tubes with PBS (GIBCO BRL, USA), followed by grinding with a mixer (IKA Labor Technik, Switzerland). According to the amount of contaminants, the ground fluids were diluted 100 to 1,000 times with a dilution buffer (B100-20, Waterborne, Inc.). For the DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma, USA) assay, samples were boiled for 5 min (Inomata *et al.* 1999), followed by staining with a direct immunofluorescent assay kit (Aqua-Glo G/C Direct, Waterborne, Inc.) and DAPI treatment in a shaking water bath incubator at 37°C. The treated samples were applied on four well microscope slides (C. A. Hendley Ltd, U.K.), dried in an incubator at 37°C, and DABCO (1,4-diazabicyclo-2,2,2-octane, Sigma)-glycerine was added dropwise. The identification and determination of *C. parvum* oocysts were carried out under a fluorescence microscope (ECLIPSE E800, Nikon, Japan) with 200 times magnification. The wavelength for detection of *C. parvum* oocysts were 450–490 nm (B excitation) for FITC (fluorescein isothiocyanate), 365 nm for DAPI, and 510–560 nm (G excitation) for chlorophyll in algaes.

The staining treatment of *C. parvum* oocysts in the collected clam feces sediment was conducted in the same manner as described above, while the water sample was processed by a mixed cellulose ester membrane filter (A100A090C, ADVANTEC, Japan) dissolution method, followed by acetone dissolution, the removal of contaminants by the Percoll (Pharmacia Biotech, USA)-sucrose density gradient method and the staining with

fluorescence dyes in a manner similar to that above. Recovery efficiency of the *C. parvum* oocysts in water samples was determined by adding 1.0×10^3 *C. parvum* oocysts to 6 L of water.

The *in vitro* qualitative detection of infectious *C. parvum* oocysts with cultured cells (HCT-8) was carried out according to the procedure reported previously (Hirata *et al.* 2001; Slifko *et al.* 1997; Upton *et al.* 1995). Thus, feces on day 3 pooled in PBS was prepared for purification of *C. parvum* oocysts by the density gradient method, thereafter the purified oocysts were processed with acid and a succeeding 30 min trypsin (DIFCO, USA) treatment, before subjection to the *in vitro* cultured cell method for the detection of infectious *C. parvum* oocysts. The sporozoits in HCT-8 cells were stained with immonofluorescent agents (Sporo-Glo, Waterborne, Inc., USA) and observed under the fluorescence microscope with 200 times magnification.

An aliquot of the fecal sample at 14 days was analyzed for *Cryptosporidium* DNA by PCR, using TRAP-C2-F (CAT ATT CCC TGT CCC TTG AG) and TRAP-C2-R (TGG ACA ACC CAA ATG CAG AC) primer pair (Science Tanaka, Japan) corresponding to positions 848-867 on the coding strand and 1180-1199 on the negative strand of GenBank sequence X77586, respectively. Purified oocysts were rinsed twice by repeated suspension in 100 μL sterile distilled water, and the aliquot (10 μL) was subsequently subjected to DNA extraction. The oocysts were ruptured by 1.0% SDS (GIBCO BRL) in TE buffer (Nippon Gene, Japan) at 100°C for 30 min and the supernatent was subjected to phenol /chloroform /isoamyl alcohol (24/24/1, GIBCO BRL) extraction, precipitated with absolute ethanol (Wako Pure Chemical Industries, Japan), washed with 70% ethanol, and resuspended in TE buffer. The PCR reaction in a total volume of 30 μL consisted of 1×PCR buffer (Clontech, USA), 0.2 mM each dNTP (Clontech), 0.2 μM of each specific primer, 2.5 U of Taq polymerase (Promega, USA) with antiTaq (Clontech), and 50 copies of internal control. After an initial hot start with both 5 min at 80°C and 5 min at 94°C, DNA amplification was carried out for 30 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extention at 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, USA). An additional cycle of 7 min at 72°C was added for strand completion. The PCR products (369 bp) were analyzed by 3.0% agarose gel electrophoresis and ethidium bromide staining. For the internal control (IC:542 bp), it was prepared from the PCR product (369 bp) and the fragment (173 bp) of the *Bss*HII digested and Klenow blunted multiple cloning site from pBluescript KS^- by the rapid DNA Ligation Kit (Roche, Switzerland).

The measurement of metals (Al, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Pb, and Zn) in the water sample was performed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, OPTIMA 3300DV, Perkin-Elmer) (Greenberk *et al.* 1992). The sample water was pretreated with 1% HNO_3 and 0.1 mg/L yttrium as the internal standard, then subjected to ICP-AES under recommended conditions. The determination of nitrogen derived from ammonia and nitrites in the aquarium was carried out according to the indophenol and colorimetric methods (Greenberk *et al.* 1992), respectively.

RESULTS AND DISCUSSION

It is not simple to keep benthic freshwater or brackish water shellfishes for appropriately long periods in an artificial environment. Therefore, several daily basic check points for shellfish keeping were observed, removal of ammonia and nitrites, the temperature and pH of the aquarium water, aeration and stirring of the water, the choice of clam chow, and the amount of daily chow consumption by clam *etc*. The clam mortality was below 2% for more than 4 months in this laboratory aquarium. In this study, clam mortality was zero during both the acclimation and experimental periods.

The metal concentrations, which might influence on the water organisms, in the aquarium water were maintained during the experimental period and were as follows: Al, 0.034 ± 0.005; Ba, 0.009 ± 0.001; Ca, 80.5 ± 0.550; Cd, <0.001; Cr, <0.001; Cu, 0.015 ± 0.003; Fe, 0.021 ± 0.001; Mg, 144 ± 5.00; Mn, <0.001; Pb, <0.001; and Zn, 0.010 ± 0.002 mg/L. The nitrogen values of ammonia and nitrites, which were both harmful to clam, were below the detection limit (0.05 and 0.005 mg/L, respectively) during the experiments. The filtration method could not be adopted for trapping of ammonia and nitrites to avoid the absorption of oocysts by filter fiber, while application of oxidizing bacteria might be considered to be adequate judging from the almost perfect removal of ammonia and nitrites in the aquarium during the experimental period.

Feeding to clam was conducted daily 30 min after water exchange. The chow was freshly prepared everyday by suspending powdered chlorella and liquid-type chow in water, while the appropriate daily amount of chow was presumed to be 1.5–2.0 mg/clam for powdered chlorella, and 1.0–1.5 μL/clam for liquid-type chaw, respectively, estimated by the chow consumption and residue in the aquarium.

Recovery efficiency of *C. parvum* oocysts in the water samples by the mixed cellulose ester membrane filter dissolution method was ranged from 71 to 78% (mean ± SD = 74.1 ± 5.8%, CV = 8.0%).

None of the control clams contained *C. parvum* oocysts, confirmed by both microscopic and PCR methods. The aquarium water was oocyst-negative before the start of the experiment.

The study showed that a single exposure of *C. parvum* oocysts (6.67×10^4 oocysts/clam) to *C. japonica* resulted in a relatively rapid intake and excretion of oocysts. Fig. 1 shows the results of the balance study on the percentage of *C. parvum* oocysts detected in clam bodies and feces. The oocyst intake to the clam body was rapid, and practically no oocysts were detected in the water 2 hrs after dose. The oocysts in the clams were almost all in the GI tract, and here reached the maximum at 2 hrs after dose, thereafter they gradually reduced (Fig. 1-A, and -B). The oocyst distribution in the shellfishs 2 hrs after exposure showed that about 90% of the dose was present in the GI tract, 5.0% in the mantle (Fig. 1-B, and -D) and 0.1% in the gills (data not shown). The excretion of oocysts into feces was the predominant route and about 85% (83.4 and 1.2% in the feces and water, respectively) of the oocysts were recovered 4 days after exposure (Fig. 1-E). The total recovery of oocysts was 90% 14 days after exposure. As shown in Fig. 1-F, the oocyst excretion in feces can be presumed to start more than 4 hrs after exposure, and reaches the maximum excretion level at 8 hrs after exposure. The biological half-life ($T_{1/2}$) for the *C. parvum* oocysts in the clams was estimated to be about 24 hrs. The oocysts were excreted into feces in several phases, more rapid in the first phase, and 6 days after exposure (end of the last phase), little of the oocysts

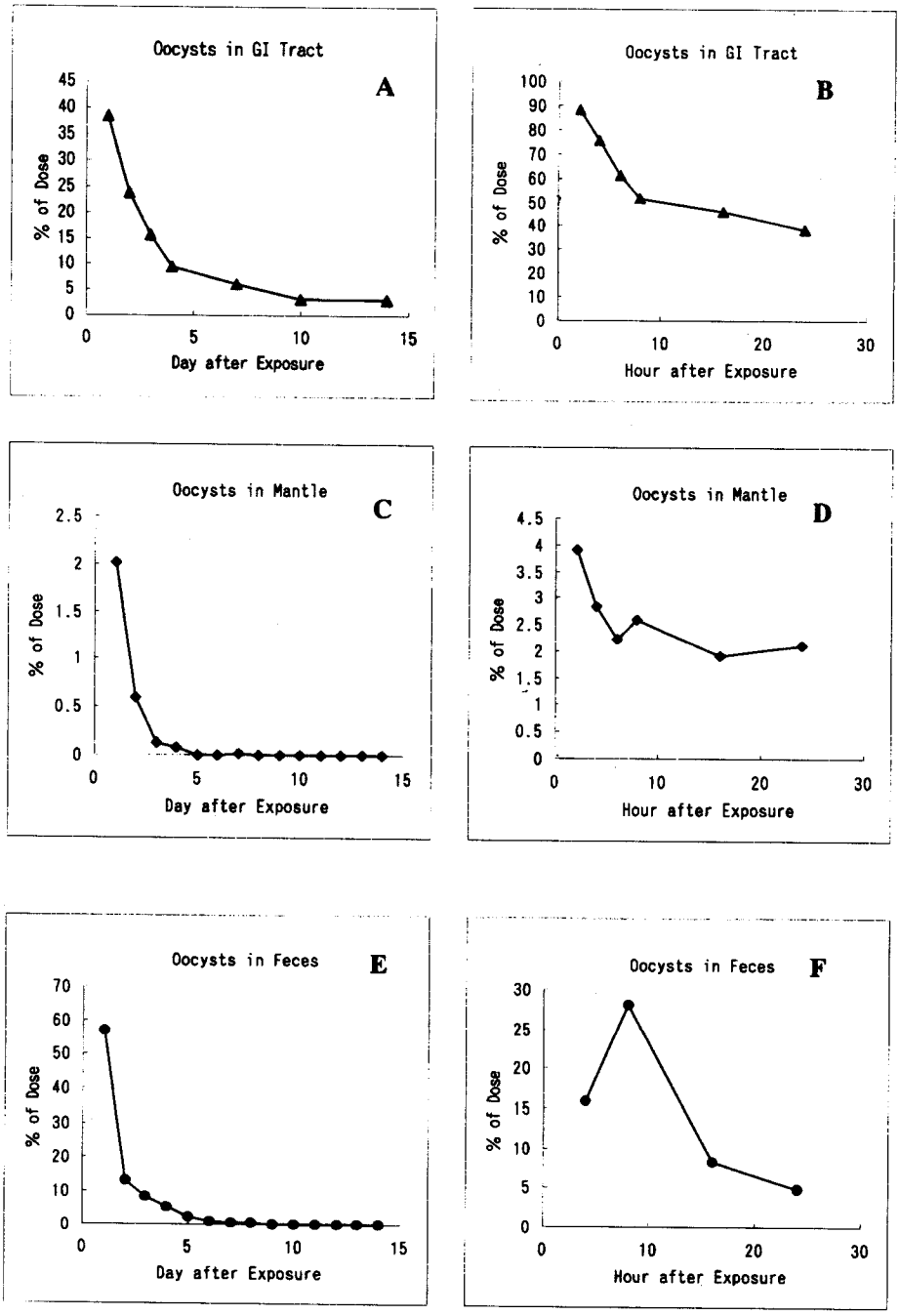

Figure 1. The relative amounts of oocysts detected in the clams and feces after a single exposure of *Cryptosporidium parvum* oocysts to *Corbicula japonica* at 6.67×10^4 /clam.

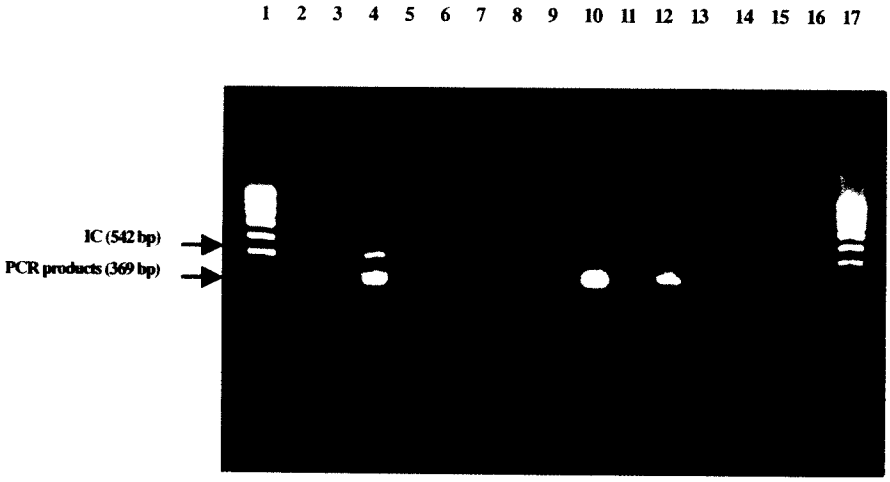

Figure 2. Ethidium bromide-stained 3.0% gel showing the amplification products using trap-C2 primers from clam fecal and GI tract samples.
Lane 1; molecular marker, **lane 2**; negative control, **lane 4**; fecal sample on 1 day after dose, **lane 6**; fecal sample on 6 days after dose, **lane 8**; fecal sample on 14 days after dose, **lane 10**; GI tract sample on 1 day after dose, **lane 12**; GI tract sample on 7 days after dose, **lane 14**; GI tract sample 14 days after dose, **lane 16**; positive control, **lane 17**; molecular marker

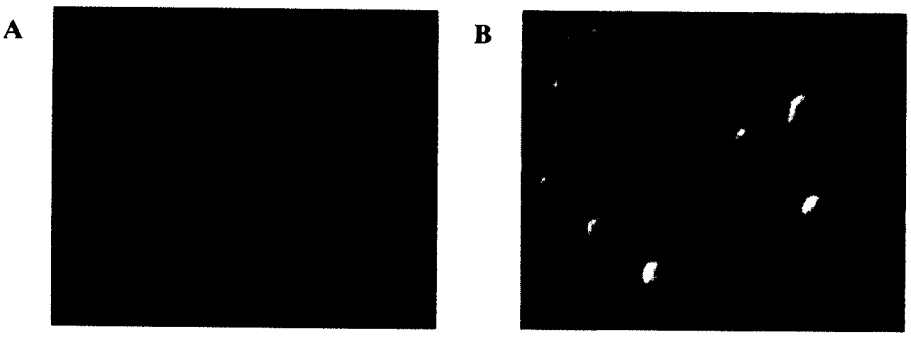

Figure 3. Photographs of developmental stage of *Cryptosporidium parvum* in HCT-8 cells 2 days after inoculation.
A: Fluorescence photograph of a field of foci after 2 days of inoculation.
B: Normarski interference-contrast photomicrograph of the field in panel **A**.

were detected, while, the DNA of *C. parvum* was detected by PCR (Fig. 2). The density of oocysts determined in the clam GI tract and mantle samples was highest 2 hrs after exposure (Fig. 1-B, and -D), almost no oocysts were detected in the gills (<0.01% of dose) during the whole of the experimental period. The oocyst decrease in the GI tract during the first 24 hrs showed two phases, with the decrease in the first 6 hrs steeper than later. One day (24 hrs) after the dose, the level of oocysts in the GI tract decreased further in two

phases, and the decrease in the first 3 day phase was more rapid than later. The slope of the curve in the second later phase was quite flat and appeared to reach a plateau level 10 days after exposure. Overall, the decrease in oocysts in the GI tract appeared to correspond to the fecal excretion in both magnitude and pattern of reduction. For the GI tract, the oocyst residue levels 7 and 14 days after exposure were 6.1 and 3.1% of the dose, and the DNA of *C. parvum* was identified by PCR (Fig. 2). The mantle showed very low oocysts residue levels, and the microscopic examination of the samples 4 days after exposure found no oocysts.

With respect to the infectious activity of *C. parvum* oocysts in the fecal samples, it was shown that the rate of excystation under the experimental condition here was about 70%, and that sporozoits were detected in HCT-8 cells 2 days after inoculation, proving that there are infectious oocysts in the clam feces (Fig. 3).

According to these results, it was presumed that a part of oocysts (about 3%) might nonspecifically adsorb to the GI tract. The present study also showed that the total amount of excreted and clam-retained oocysts was totally about 92%, which was about 8% less than the initial introduced oocyst dose. This may partly be accounted for by the absorption of oocysts by aquarium equipment. Thus far it was reported that *C. parvum* oocysts maintained infectivity for up to 1 year in seawater at 6–8°C, and oocysts captured by mussels, seawater shellfish, retained the infectivity for about 14 days in the clam body (Tamburrini and Pozio 1999), however, the infectivity of oocysts in the feces of shellfish was not yet clarified. As judged by a balance study and infection test to the cultured cells (HCT-8), most of the *C. parvum* oocysts appeared not to be readily digested by clams, and seemed to be excreted in feces with almost intact infectivity. These results suggest that estuarine shellfish may be useful to recover *C. parvum* oocysts from water samples by fecal sedimentation.
The present study indicated that *C. japonica* may serve as an effective scavenger of *C. parvum* oocysts, and that this brackish water clam could be employed as a biological indicator of contamination in river water by *C. parvum* oocysts. The optimum qualitative detection of *C. parvum* oocysts in *C. japonica* was demonstrated in the present study by screening feces and/or GI tract samples of clams with fluorescence-dye treatment. This could lead to improvements in the accuracy of detection of *C. parvum* oocysts especially in turbid water samples, where it is difficult to establish the presence of oocysts by routine filtration methods, because of the various other suspended substances.

A study on the recovery of *C. parvum* oocysts in Asian freshwater shellfish, *Corbicula fluminea* (*C. fluminea*) has been reported, however, no precise balance study of oocysts was carried out (Graczyk *et al.* 1998). The present study may be the first balance study report of *C. parvum* oocysts in bivalves. With respect to clam tissue residue of *C. parvum* oocysts, the present study showed much lower residual magnitudes in the GI tract and gills through the experimental period than in the previous reports on *C. fluminea* (Graczyk *et al.* 1998). Further, from the balance study, the *C. parvum* oocysts seemed to be harder to digest in *C. japonica* than in *C. fluminea* (Graczyk *et al.* 1998). A mortality of 10% in the above-mentioned study with *C. fluminea* seems too high. Under inadequate circumstances, shellfishes are liable to assume a fasting state and mortality would increase significantly, hence the high clam mortality (about 10%) may be presumed to be caused by inappropriate

conditions, and the differences in the retention and digestion of *C. parvum* oocysts in *C. japonica* and *C. fluminea* may be due to both biological metabolic species differences in metabolism and also in the aquarium conditions, involving the water-filtration system for removal of waste, which would affect the recovery of oocysts by its adsorptive function on glass fibers (Kawasaki 1998).

Several reports on the harboring of *C. parvum* oocysts in seawater clams, such as oysters, mussels, and cockles, were recently presented (Fayer *et al.* 1998; Gomez-Bautista *et al.* 2000), and it was shown that some tissue would be of retaining some oocysts. However, no quantitative time-course balance study has been carried out so far. Successive administration of *C. parvum* oocysts to shellfish would be required to clarify and assess water contamination by *C. parvum* oocysts as a bioindicator, as water pollution with *C. parvum* oocysts is not usually a short term phenomenon, but tends to last more than a few days. In addition, other experimental conditions, such as temperature, salt concentration of the water, and size of clams *etc*, that may have an effect on clam metabolism, should be taken into account. Further, other benthic organisms, e.g. *Corbiculidae* and *Unionidae* clams *etc* should be investigated as bioindicators. At present, we are investigating these subjects with *C. japonica* and other benthic freshwater organisms.
Corbicula clams are consumed after heat treatment, at least in Japan, so it may be assumed that there is practically no epidemiological danger of these shellfish to act as a reservoir of *C. parvum* and some helminths. However, some waterfowl, e.g. bay ducks and sea ducks *etc*, have been reported to feed on these shellfishes (Nakamura and Nakamura 1995; Robbins *et al.* 1983), and these wild animals may act as carriers of *C. parvum*. In the same manner, *C. japonica* may play an epidemiologically important role as a reservoir of *C. parvum* in the natural food chain in some cases.

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