RECOVERY OF WATERBORNE OOCYSTS OF CYCLOSPORA CAYETANENSIS BY ASIAN FRESHWATER CLAMS (CORBICULA FLUMINEA)

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Abstract. Asian freshwater clams (Corbicula fluminea) were exposed for 24 hr in 38 liters of water contaminated with 1.0 × 10^3 Cyclospora cayetanensis oocysts (2.6 × 10^2 oocysts/L). The hemolymph and gill smears of 30 clams were examined by acid-fast stain on days 1, 3, 5, 7, 10, 13, and 18 postexposure (PE). Since no oocysts were detected in the water 24 hr after contamination by the membrane filter-dissolution method, the oocyst retention rate was 4.6 × 10^2 oocysts/clam. The prevalence of oocyst-positive clams significantly decreased (P < 0.01) from 93% to 47% during 13 days PE. None of the clams contained oocysts on day 18 PE; no oocysts were detected in the clam feces. The numbers of oocysts recovered from six clam size classes varied and significantly decreased with smaller clam size (P < 0.01). The lowest prevalence values of oocyst-positive clams, 45% and 34%, were observed in the two lowest size classes: 12.1–14.0 mm and 14.1–16.0 mm, respectively. The prevalence values in the remaining four classes ranged from 84% to 100%. The sampling program demonstrated that the population of 180 clams examined during the study up to 13 day PE could be assessed for C. cayetanensis positivity by random testing of a minimum of 75 clams (42%). When the two lowest clam size classes are eliminated, the population of 114 clams could be assessed by sampling a minimum of 32 clams (28%). The results demonstrate that Corbicula fluminea can recover waterborne oocysts of C. cayetanensis, and could be used as biological indicators of contamination of water with C. cayetanensis oocysts.

The Asian freshwater clam Corbicula fluminea, which was introduced to North America in the early 1900s, is well adapted to life in unstable and unpredictable habitats. The species is highly successful in agricultural drainage systems and can survive in waters receiving agricultural and industrial pollution, and urban waste. Corbicula fluminea serves as a biological indicator of water pollution with organochlorine insecticides, metals, and waterborne mutagens. It is an efficient suspension feeder able to filter detrital particles of 10-μm-diameter at a rate up to 2.50 L/hr.

Cyclospora cayetanensis is an intestinal coccidian protozoan that causes prolonged diarrheal illness in adults and children worldwide. Waterborne transmission of C. cayetanensis is believed to be the main mode of transmission; however, with the exception of one confirmed drinking water outbreak, it remains epidemiologically unproven. The outbreaks of cyclosporiasis epidemiologically linked to contamination of berries or vegetables have been consequently classified as foodborne, although contamination of field watering systems with the oocysts was possible. Epidemiologic circumstances presented in the cases, outbreaks, or non-outbreak settings of C. cayetanensis infections, together with the prolonged pathogen sporulation period, strongly indicate waterborne transmission of the oocysts.

It has been demonstrated that Corbicula fluminea clams are capable of recovery and sedimentation of infectious waterborne oocysts of Cryptosporidium parvum, which is a pathogen with an oocyst diameter of 3.5–6.0-μm. In addition, these benthic clams can recover infectious waterborne cysts of Giardia duodenalis (length range = 8.0–12.0 μm). These clams play important epidemiologic/epizootiologic functions by reducing waterborne cyst/oocyst load, and can also serve as biological indicators of contamination of freshwater reservoirs with Cryptosporidium and Giardia.

Waterborne Cryptosporidium parvum oocysts filtered by marine shellfish, e.g., oysters, retained their infectivity in the oyster tissues. Shellfish harvested from contaminated waters were incriminated in numerous massive foodborne epidemics.

The only exogenous stage of C. cayetanensis, the oocyst, is spherical in shape, and ranges in diameter from 8.0 to 10.0 μm. The oocysts become sporulated (infectious) within 7–13 days after being released into the environment with the feces. Although foodborne cyclosporiasis outbreaks have not been linked to the consumption of any of the seafood items, most of the reports originate from coastal regions near both freshwater and marine waters. The size and shape of C. cayetanensis oocysts are very similar to the unicellular Chlorella algae on which Corbicula fluminea filter-feed; however, there are no data on interaction(s) between the infectious stages of the pathogen and any of the bivalve mollusks.

The purpose of the present study was to determine if Corbicula fluminea clams can recover and sediment waterborne oocysts of C. cayetanensis, and if the water-recovered oocysts can be subsequently detected in the clam tissue.

MATERIALS AND METHODS

Cryptospora cayetanensis oocysts were collected from the feces of patients with cyclosporiasis. Feces were sieved and stored in 2.5% potassium dichromate. An initial concentration was performed by using a modified ethyl acetate method. Pellets were diluted in distilled water and layered over a primary discontinuous sucrose gradient (densities = 2.064 and 1.103 g/L) and centrifuged (1,500 × g for 25 min). Oocysts were stored in 2.5% potassium dichromate until used.

A 38-liter (approximately 10-gallon) aquarium was filled with dechlorinated drinking water filtered with the Filterite 10-μm-pore yarn-wound cartridge (Mentec America Corp., Baltimore, MD). The aquarium was equipped with two air-
stones working continuously for one week prior to clam introduction. Two hundred-fifty Corbicula fluminea clams, 1.4–2.4 cm shell length, collected from Lake Cheston (Franklin County, TN) were placed in the aquarium. The clams were fed every day with a 400-ml suspension of algae (Chlorella pyrenoidosa; Carolina Biological Supply Company, Burlington, NC), cultured in a 3.8-liter all-glass aquarium according to the manufacturer’s instructions. The aquarium was attached to a Magnum 220 filter (Aquaria, Inc., Moonpark, CA) equipped with activated carbon and ammonia-removing resin cartridge (Aquarium Pharmaceuticals, Inc., Chalfont, PA). The Magnum filter was working daily for 1 hr before addition of the algae.

Thirty randomly selected control clams were removed from the aquarium and the water was seeded with $1 \times 10^6$ C. cayetanensis oocysts (2.6 $\times$ 10$^3$ oocysts/L). After 24 hr (one day postexposure [PE]), five water samples of 3.8 liters (approximately one gallon) each were collected from the aquarium and the remaining water was removed. The aquarium was filled with 38 liters of dechlorinated drinking water processed as described previously, and the clams were maintained as prior to the exposure to C. cayetanensis oocysts.

The water samples were individually processed by the cellulose acetate membrane (CAM)-filter dissolution method for detection of waterborne Cryptosporidium parvum oocysts. Recovery efficiency of C. cayetanensis oocysts was determined by spiking individually of 10 3.8-liter water samples (each sample contained 0.2 liters of the eluting fluid) with 1.0 $\times$ 10$^3$ C. cayetanensis oocysts. The retention rate of the oocysts by clams was determined based on comparison of the number of oocysts added to the water and the number recovered from the water.

Thirty randomly selected clams were examined on days 1, 3, 5, 7, 10, 13, and 18 PE. While kept on ice, the anterior and posterior adductor muscles were cut by a scalpel inserted gently below the anterior and posterior lateral teeth. The shell was opened, and the hemolymph (approximately 0.2 ml) was aspirated from blood sinuses with a 1.0-ml pipette. The hemocyte monolayer was prepared by spreading evenly on a glass slide. Excised gills were placed on the hemocyte monolayer, disrupted with two forceps while submerged in the hemolymph, and the remaining gill tissue fragments were removed from the slide. Individual slides were acid-fast stained following air-drying and fixation with methanol. A hemacytometer was used to determine the concentration of hemocytes for the first 10 control clams and the first 10 oocyst-exposed clams at each time point.

Clam feces were aspirated daily in the morning from the bottom of the aquarium and efforts were made to collect all feces. Water (approximately 0.5 liters) with the feces was left overnight at 4°C in a Kimax conical graduate (VWR Scientific, Piscataway, NJ). Each daily feces sediment collection was used for preparation of up to 10 acid-fast stained slides.

The number of C. cayetanensis oocysts was counted during a 10-min slide examination with a 40× objective. To ensure that Chlorella pyrenoidosa algae were not mistakenly identified as C. cayetanensis oocysts, direct wet smears of Chlorella pyrenoidosa were acid-fast stained and examined microscopically. Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, MN). The variables, i.e., numbers of hemocytes and oocysts, were examined by the Runs test to determine if their distribution conformed to a normal distribution and, if not, nonparametric tests were used to assess the significance of the differences among variables. Nonparametric test included the Kruskal-Wallis analysis of variance (ANOVA). The degree of linear association between variables was assessed using the regression test. The minimal number of clams to be sampled to accurately determine their positivity for C. cayetanensis oocysts after the exposure to oocyst-contaminated water was computed according to the sampling program equation $N = F/p 	imes q/D^2$ applied previously to human cryptosporidiosis where $t$ is Student’s $t$, $p$ is a fraction of oocyst-positive clams, $q = 1 - p$, and $D$ is the predetermined half-width of the confidence limits, $D = 0.1$ (Table 1).

The equation was applied to the results obtained for 180 clams that showed oocyst-positivity up to 13 days PE, and to the 114 clams that remained after extraction of the two clams that showed oocyst-positivity up to 13 days PE, and to the 114 clams that remained after extraction of the two lowest clam-size classes (12.1–14.0 and 14.1–16.0 mm) with the lowest oocyst-positivity (Figure 1). The mean values were associated with the standard deviation (SD) and the coefficient of variation (CV). Statistical significance was considered to be a $P$ value $\leq 0.05$.

**RESULTS**

The mean hemocyte concentrations varied from $3.7 \times 10^4$ to $7.0 \times 10^5$ cells/ml, with an overall mean of $5.1 \times 10^4$ cells/ml ($\pm SD = 8.7 \times 10^4$ cells/ml). Fluctuations in the mean hemocyte concentration were not significant (Kruskal-Wallis ANOVA $F = 0.79$, $P > 0.05$). No clam mortality was observed during the experiment.

Recovery efficiency of C. cayetanensis oocysts by the CAM-filter dissolution method ranged from 66.0% to 82%, mean $\pm SD = 74.7 \pm 5.5\%$, $CV = 7.0\%$. No C. cayetanensis oocysts were detected in the aquarium water samples. The filtration rates were considerably accelerated, compared with the drinking water filtration rates, indicating that the filtered water was substantially depleted of any particulate matter. Based on the negative readings of the CAM-filter dissolution method, the retention rate of C. cayetanensis oocysts by Corbicula fluminea clams was approximately $4.6 \times 10^2$ oocysts/clam. Chlorella pyrenoidosa algae did stain with the acid-fast stain, and the control clams were negative for C. cayetanensis oocysts.

**Table 1**

<table>
<thead>
<tr>
<th>Size of clams (mm)</th>
<th>Sampled clams</th>
<th>Student’s $t$-test value*</th>
<th>Model-derived minimal number of clams to be sampled*</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.1–24.0</td>
<td>180</td>
<td>0.72</td>
<td>1.94</td>
</tr>
<tr>
<td>16.1–24.0</td>
<td>114</td>
<td>0.91</td>
<td>1.98</td>
</tr>
</tbody>
</table>

*According to the equation $N = F/p \times q/D^2$ where $t$ is Student’s $t$, $p$ is a fraction of oocyst-positive clams, $q = 1 - p$, and $D$ is the predetermined half-width of the confidence limits, $D = 0.1$. **WATERBORNE C. CAYETANESIS OOCYSTS AND FRESHWATER CLAMS**


Clam feces collected at all timepoints were negative for C. cayetanensis oocysts. The oocysts of C. cayetanensis were detected in the hemolymph and gill smears up to 13 days PE (Figure 2). The number of C. cayetanensis oocysts detected in the clam tissue did not differ significantly up to first 10 days PE (Kruskal-Wallis ANOVA; $F = 1.12$, $P > 0.05$), and then significantly decreased on day 13 PE (Kruskal-Wallis ANOVA; $F = 3.22$, $P > 0.05$). None of the clams was oocyst-positive on day 18 PE (Figure 2). The prevalence of oocyst-positive clams was within the limits of 47–93% and significantly decreased over time (regression test; $R = 0.88$, $P < 0.01$) (Figure 2).

The size of 30 clams sampled on days 1, 3, 5, 7, 10, 13, and 18 PE did not differ significantly (Kruskal-Wallis ANOVA; $F = 2.92$, $P > 0.05$). However, when clams were categorized by their size into six classes (Figure 1), the numbers of C. cayetanensis oocysts recovered from various class-size clams differed significantly (Kruskal-Wallis ANOVA; $F = 45.53$, $P < 0.01$). The mean number of detected oocysts significantly decreased when the size of the clams decreased whether all sampled clams were analyzed or only the oocyst-positive clams (regression test; $R = 0.61$, $P < 0.01$). The lowest numbers of oocyst-positive clams, 14 (of 31; 45%) and 12 (of 35; 34%), were observed in the two lowest size classes: 12.1–14.0 mm and 14.1–16.0 mm, respectively (Figure 1). In contrast, the prevalence of oocyst-positive clams in the remaining four size classes ranged from 84% to 100% (Figure 1).

As demonstrated by the sampling program equation, the population of 180 clams examined during this study could be accurately assessed for oocyst-positivity by random sampling of only 75 clams (42% of the total sampled) (Table 1). Moreover, if the two lowest clam size classes, which displayed the lowest oocyst-positivity fractions, are eliminated,
the population of 114 clams could be accurately assessed by sampling of 32 clams (28% of the total sampled) (Table 1).

**DISCUSSION**

As demonstrated in the present study, the Asian freshwater clam *Corbicula fluminea* can recover waterborne oocysts of *C. cayetanensis*. Based on negative findings from the CAM-filter dissolution method, 220 clams removed $1.0 \times 10^3$ oocysts from 38 liters of water within 24 hr. To achieve such removal, the oocyst retention rate had to be approximately $4.6 \times 10^2$ oocysts per clam. Since *Corbicula fluminea* can filter as much as 2.5 L/hr, under the conditions given in the experiment (approximately 0.17 L/clam), the aquarium water was filtered approximately 14 times. A similar phenomenon was observed in the clam’s natural habitat the Trinity River. In this river, with an average depth of 0.25 m, the water volume overlaying *Corbicula fluminea* beds was filtered every 16 min.

*Corbicula fluminea* clams initially exposed to waterborne oocysts of *C. cayetanensis* retained the pathogen in its tissue for at least 13 days (since the experimental design excluded re-exposure to the oocysts). In *Corbicula fluminea*, the water-filtered particles pass several times through the clam broncho-intestinal system before they are extracellularly digested, phagocytosed, or rejected as feces. This particle-circulation mechanism, which extends the presence of the water-recovered matter in clam tissue, actually facilitates detection of the particles of interest, e.g., *Cryptosporidium parvum* or *C. cayetanensis* (present study) oocysts, or *Giardia duodenalis* cysts.

*Corbicula fluminea* clams have been used for years as a bio-indicator of water pollutants, contaminants, and toxicants of mainly agricultural origin. The present study demonstrated that in addition to in vivo recovery of infectious freshwater *C. parvum* oocysts, these clams can also recover waterborne oocysts of *C. cayetanensis*. However, since *Corbicula fluminea* are preferential filter feeders (but not detritus feeders), the presence of oocysts in the clam tissue is indicative of water contamination rather than contamination of sediments. An exception would be in situations (e.g., variable-flow rate environments) in which episodic sediment disturbance might resuspend oocysts at a frequency greater than once every 14 days. However, because the study presents results of experiments carried out in a very controlled environment, extrapolation to natural circumstances have to be made cautiously.

The ecology and biology of *Corbicula fluminea* make this benthic clam attractive for biomonitoring of waterborne pathogens such as *Cryptosporidium, Giardia*, or *Cyclospora*, of which infectious stages are within the size range of unicellular algae on which the clams feed. These bivalves are abundantly prevalent in a wide geographic range, facilitating comparison of water contamination. They can survive in agricultural drainage and waste waters, and they are collectible throughout the year and can be held in field enclosures. Also, small clam size and consequently, the small amount of tissue to be tested, facilitates more accurate detection of the waterborne pathogens.

The present study offers some practical indications that can considerably reduce the excessive amount of time, material, and labor, and consequently decrease expenses so important in long-term field projects. The processing of a single sample would require 30 min, 10 min for microscopic slide examination and 20 min for processing and staining of clam tissue. Costs of the acid-fast stain are low, and the processing can be considerably shortened by bathing the samples. Also, the slides can be prepared and fixed with methanol at the site of clam collection; transport of wild-collected clams to the laboratory would require moist coolers, in which clams can survive up to two days. It is not necessary to collect all clam sizes, or as many clams as possible. Clam collection should target only the biggest bivalves in the bed because such clams yield a average of six times more *C. cayetanensis* oocysts in their tissue. The minimal number of clams to be collected can be determined based on the fraction of oocyst-positive clams from the pilot study, e.g., by sampling and testing the 50 biggest clams. As demonstrated, the population of 180 clams in this study could have been assessed by testing a minimum of 75 clams. When the smallest clams are eliminated, the population of 114 clams could be assessed by testing a minimum of 32 clams. Some natural populations of *Corbicula fluminea* include individuals even larger than those studied here; presumably, these would carry an even higher load of oocysts.

The lack of *C. cayetanensis* oocysts in *Corbicula fluminea* feces may indicate that the clams did not ingest and sediment waterborne oocysts. The question of whether *C. cayetanensis* oocysts resist extracellular or intracellular digestion by clams remains valid. Based on the oocysts found in the hemolymph and on gills 13 days PE to the oocyst-contaminated water, we conclude that some of the oocysts were not digested by the clams. Although the overall fraction of the oocysts recovered from clam tissue was low, it may represent low sensitivity of the current detection method since the pathogen stains variably with acid-fast stain. The sentivity of detection of *Cryptosporidium* oocysts or *Giardia* cysts is close to 100% due to the use of fluorescein-labeled monoclonal antibodies, which are, unfortunately, not yet available for *Cyclospora* oocysts. Further studies are necessary to assess the threshold of oocyst detection in the clam tissue when water is contaminated with a low number of the oocysts.

*Corbicula fluminea* clams can substantially reduce (up to 75%) the amount of particulate matter in the water. The results of our previous study on recovery of waterborne *C. parvum* oocysts by these bivalves, together with the results of the present study, indicate that *Corbicula fluminea* may have significant epidemiologic and epizootiologic importance in the recovery of waterborne oocysts.

In some parts of the world, *Corbicula* bivalves are consumed raw and serve as reservoirs of intestinal helminths. In the United States, *Corbicula fluminea* clams are not commercially offered for human consumption; however, they are collected by minor ethnic groups and consumed raw. We demonstrated previously that *Cryptosporidium parvum* oocysts recovered from water by *Corbicula fluminea* retain their infectivity in clam tissue. Based on the results obtained for *C. cayetanensis*, we suspect that other species of bivalve mollusks, which are consumed raw, can also recover this waterborne pathogen, and thus, may play a role in foodborne cyclosporiasis.
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