In Vitro Interactions of Asian Freshwater Clam (*Corbicula fluminea*) Hemocytes and *Cryptosporidium parvum* Oocysts

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***Corbicula fluminea* hemocytes phagocytosed infectious oocysts of *Cryptosporidium parvum* in vitro. After 15, 30, 60, 90, and 120 min of incubation, averages of 35.8, 58.0, 69.7, 77.7, and 81.6% of the oocysts were phagocytosed by 24.3, 70.0, 78.5, 87.3, and 93.0% of the hemocytes, respectively. A single clam can retain by phagocytosis an average of 1.84×10^6 oocysts per ml of hemolymph. *C. fluminea* bivalves can serve as biological indicators of contamination of wastewaters and agricultural drainages with *Cryptosporidium*.**

Cryptosporidiosis is a zoonotic diarrhoeal disease caused by an intestinal protozoan, *Cryptosporidium parvum*. The infectious stage, the oocyst, is transmitted via water. The pathogen represents a global public health threat, having caused massive waterborne epidemics (10). As many as 5,500 oocysts per liter in agricultural drainage have been reported (14). The Asian freshwater clam (*Corbicula fluminea*) is well adapted to life in unstable and unpredictable habitats and is highly successful in waters receiving agricultural and industrial pollution and urban waste (13). For the past 15 years, this benthic species has served as a bioindicator of water pollution (9, 12, 15, 17). *C. fluminea* is a suspension feeder able to filter unicellular algae, bacteria, and detrital particles 1.5 to 10.0 μm in diameter. The clams inhabit agricultural drainage systems and wastewaters (13) known to carry high numbers of *Cryptosporidium* oocysts (14). The purpose of the present study was to describe in vitro interactions between hemocytes of *C. fluminea* and *C. parvum* oocysts.

The oocysts of *C. parvum* (strain AUCP-1) were obtained as described previously (2). Oocyst infectivity was determined in a mouse infectivity bioassay (1), and a phosphate-buffered saline (PBS) suspension of 1.13×10^7 oocysts/ml was prepared. Hemolymph (0.1 to 0.5 ml) was aspirated (16) from 14 wild-collected *C. fluminea* clams (0.8- to 2.0-cm shell length). The hemolymph was pooled, and the hemocyte concentration was determined (8). Fifty microliters of undiluted hemolymph was placed in each of 30 wells (12-mm diameter) on slides of the MERIFLUOR *Cryptosporidium*/*Giardia* test (Meridian Diagnostic, Inc., Cincinnati, Ohio). The hemocytes were allowed to adhere for 30 min in a humidified chamber at 21°C. Twenty hemocyte monolayers each received 10 μl of suspension with 1.13×10^5 oocysts, and 10 monolayers that received 10 μl of PBS served as unexposed controls. The incubation of six mono-

layers (four oocyst-exposed and two control monolayers) was stopped at 15, 30, 60, 90, and 120 min. Following incubation, the wells were washed three times with PBS and incubated for 10 min with 1.0% glutaraldehyde, air dried, and fixed with methanol. A minimum of 100 hemocytes were examined for evidence of phagocytosis with the aid of bright-field and phase-contrast microscopes with a 40 $\times$ objective. Three hemocyte monolayers from each time point (two oocyst-exposed and one control monolayer) were acid-fast stained (AFS), and the three monolayers were stained with the immunofluorescent antibody of the MERIFLUOR test. The immunofluorescence-stained slides were examined, and the fluorescence was scored (6, 7). The number of nonphagocytosed oocysts was determined for the entire well area using a 40 $\times$ objective, and the number of phagocytosed oocysts (per hemocyte) was determined at a magnification of $\times 100$ for at least 100 phagocytes.

The mean hemocyte concentration of the pooled *C. fluminea* hemolymph was 4.50×10^5 cells/ml; individual hemocyte monolayers contained an average of 2.25×10^4 cells. The proportion of hemocytes to oocysts in the assay was approximately 1/5. Phagocytosed oocysts were clearly visible by bright-field and phase-contrast microscopy at 15-, 30-, and 60-min time points and became less visible at 90- and 120-min time points. In AFS preparations, the phagocytosed oocysts displayed nonuniform, bright red coloration with characteristic black granules (Fig. 1). Viewed by fluorescence microscopy, nonphagocytosed oocysts and the hemocyte-internalized oocysts at the 15-min time point had the highest (5+) bright apple-green fluorescence, and it significantly decreased over time (Spearman rank correlations; $R = -0.97$, $P < 0.01$) (Table 1). The number of hemocytes showing phagocytosis increased significantly over time ($R = 0.93$, $P < 0.03$). The numbers of nonphagocytosed oocysts significantly decreased ($R = -0.88$, $P < 0.05$), whereas the mean number of phagocytosed oocysts per cell significantly increased ($R = 0.90$, $P < 0.04$) (Table 1). After 15, 30, 60, 90, and 120 min of incubation, averages of 35.8, 58.0, 69.7, 77.7, and 81.6% of the oocysts were phagocytosed by 24.3, 70.0, 78.5, 87.3, and 93.0% of the hemocytes, respectively. The kinetics of phagocytosis was given

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FIG. 1. Photomicrograph showing migrating hemocyte of the Asian freshwater clam (*C. fluminea*) with a phagocytosed *C. parvum* (strain AUCP-1) oocyst (arrow) and free, nonphagocytosed oocysts (arrowheads). Bar = 10 μ m.

by a binomial time-dependent curve, $y = 24.8 + 1.1x - 5.0^{-3x^2}$, where y is the percent phagocytosed oocysts and x is the interaction time (in minutes).

The present study is the first report of interaction between hemocytes of a freshwater benthic bivalve, *C. fluminea*, and *C. parvum* oocysts. *C. fluminea* hemocytes have been shown to be capable of erythrophagocytosis (16), and *Corbicula japonica* hemocytes have been chemotactically attracted to *Vibrio parahaemolyticus* and *Escherichia coli* (11). In vitro phagocytosis of *C. parvum* oocysts by Eastern oyster (*Crassostrea virginica*) hemocytes has been demonstrated in vitro and in vivo (3, 4, 8).

TABLE 1. Results of interactions between *C. fluminea* hemocytes and *C. parvum* AUCP-1 oocysts in an in vitro slide phagocytosis assay^a

Interaction time (min)	No. of nonphagocytosed oocysts ^b		No. of phagocytosed oocysts/cell (mean $\pm$ SD) ^c	Mean fluorescence score ^d
	Range	Mean $\pm$ SD		
15	69–111	87.0 $\pm$ 9.9	1.8 $\pm$ 0.3	5+
30	31–57	45.0 $\pm$ 6.1	3.1 $\pm$ 0.9	4+
60	27–41	32.5 $\pm$ 4.1	3.4 $\pm$ 1.1	3+
90	17–30	22.8 $\pm$ 3.5	3.9 $\pm$ 1.4	3+
120	13–22	15.4 $\pm$ 3.2	4.1 $\pm$ 1.7	1+

^a Data for each time point were derived from four replicates in which hemocyte monolayers of 2.25×10^4 cells each received 1.13×10^5 oocysts.

^b Oocysts adhering to the glass. Values were determined by a 10-min examination with a 40 $\times$ objective.

^c Determined with a 100 $\times$ objective for 100 cells.

^d Hemocyte monolayers were processed with the MERIFLUOR *Cryptosporidium*/*Giardia* test for direct immunofluorescence, which was scored on a scale from 1+ to 5+, where 5+ is the brightest.

We conclude that waterborne oocysts of *Cryptosporidium* are filtered from the water by *C. fluminea* and are then phagocytosed by hemocytes. As various environmental factors may affect the recovery and phagocytosis of particles by freshwater benthic bivalves (13), the kinetics of phagocytosis demonstrated in vitro may differ in vivo.

C. fluminea from agricultural drainage systems has been used as a bioindicator for agricultural pollutants, contaminants, and toxicants (9, 12, 15, 17) but not for *Cryptosporidium*. Ecological and physiological features make this benthic clam attractive for biomonitoring of water contamination with *Cryptosporidium*. This bivalve is abundant in wastewaters and has a wide geographical range (13), which would allow direct comparison of water contamination at different sites within North America. The species is resistant to environmental variations and habitat disturbances induced by adverse weather conditions which facilitate contamination with *Cryptosporidium* (14). The clam is collectible through the year and can be readily held in field enclosures (13). The morphology of the AFS phagocytosed oocysts did not change over 2 h. This indicates the usefulness of a low-cost and time-efficient AFS procedure which can be applied for screening of *C. fluminea* hemocytes.

C. fluminea has the highest filtration rate of all freshwater bivalves (up to 2.50 liters/hr) and is capable of developing dense populations (up to 3,750 clams/m²) (13). In the Potomac River, phytoplankton density declined up to 75% after water passed over the *C. fluminea* beds (13). In the Chowan River, the entire water column overlying *C. fluminea* beds has been filtered every 1.0 to 1.6 days (13). In the Trinity River, which averages 0.25 m in depth, the entire water volume overlying *C. fluminea* beds was filtered every 16 min (13). *C. fluminea* develops particularly high-density beds in agricultural drainages

which carry high loads of *Cryptosporidium* oocysts, in certain locations as high as 5,500 oocysts/liter (14). The clam, because of its important ecological functional role in reducing the amount of seston, has significant epidemiological and epizootiological importance in recovery of *C. parvum* and reduction of the oocyst load.

At a concentration of 4.50×10^5 /ml, the hemocytes were able to phagocytose on average 81.6% of the *C. parvum* oocysts. By extrapolation, each milliliter of *C. fluminea* hemolymph could internalize 1.84×10^6 oocysts. A single medium-size clam (13) might retain in its tissue, by hemocyte internalization alone, an average of 3.68×10^6 waterborne oocysts.

In the United States, *Corbicula* clams, which are not commercially offered for human consumption, are collected from the wild and consumed raw by minority ethnic groups (5). Thus, *C. fluminea* clams may play an epidemiological role in food-borne cryptosporidiosis.

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