Bioconcentration of hepatitis A virus by the zebra mussel, *Dreissena polymorpha* under laboratory conditions

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Abstract

The ability of the zebra mussel, *Dreissena polymorpha*, to concentrate hepatitis A virus, a prevalent human pathogen with a history of bioconcentration by bivalves was investigated. Under laboratory conditions and using rt-PCR for detection, zebra mussels were shown to be able to quickly bioconcentrate hepatitis A from inoculated water, and to maintain levels of detectable virus for at least one week. However, no virus could be detected from 45 field samples taken from the south shore of Lake Erie between Presque Isle, PA and Monroe, MI. Despite the negative field data, our study shows that the testing of zebra mussels for such human pathogens as hepatitis A by means of rt-PCR could be used to monitor for the presence of these pathogens in aquatic environments.

Introduction

Since its introduction into Lake Erie, the filtering abilities of the zebra mussel *Dreissena polymorpha*, has had a profound impact on many aspects of the lake ecosystem. Although this filtering capacity has caused significant elevation in lake clarity (Leach, 1993), it also has significant detrimental effects, including the removal of the algael base of the lake foodchain, and the cycling of environmental contaminants. While the role of zebra mussels in the concentration of such contaminants as PCBs, PAHs, PCDDs, and heavy metals has been well established, little attention has been given to the role they may play in the concentration of biotic contaminants such as human pathogens which may pollute the waters they inhabit.

The accumulation of human pathogens by bivalves has been studies for several marine species. They have been shown to be efficient concentrators of both bacteria (Dore and Lees, 1995) and viruses (Enriquez et al., 1992), exhibiting an ability to concentrate such contaminants by up to 100-fold over the levels seen in their surroundings. Of great interest to human health is the ability of marine shellfish to concentrate hepatitis A virus, which can then be passed on to humans when shellfish are eaten raw. Several incidents of HAV outbreaks originated from sewage contaminated shellfish have been documented (Gard, 1957, Mason and Lean, 1962, Rudy et al., 1969, Hamilton, 1973).

Little attention has been given to the abilities of freshwater bivalves, including zebra mussels, to concentrate human pathogens. Recently, data has shown that giardia, cryptosporidium, and *e.coli* (Selegean et.al, 2001) can be found in zebra mussel samples, but the ability to concentrate other pathogens, including much smaller viral particles, has yet to be established. Towards this end, we have investigated the ability of zebra mussels to concentrate live hepatitis A virus in the laboratory, as well at their ability to clear the virus over time.

Additionally, we attempted to detect the virus in zebra mussels collected along the south shore of Lake Erie. While our field collected samples showed no signs of HAV contamination, our laboratory results show that zebra mussles rapidly concentrate virus from inoculated water, and that virus can remain in the zebra mussel tissues for an extended period of time.

Materials and Methods

Field Collection

Field collections were made along the south shore of Lake Erie between Presque Isle, PA and Monroe, MI. A total of 45 samples were taken between August and October of 1997. Field sites were selected by their proximity to sewage treatment stations, especially those with recent bypass events or those with a history of such events. From each field site, we collected 20-30 zebra mussels, a 2 liter sample of water, and a 100ml sample of sediment. Zebra mussels were placed in 1 qt ziplock bags, water samples were collected in alcohol sterilized polypropylene bottles, and sediment was collected in two 50ml polypropylene tubes. All samples were placed on ice for transfer to the laboratory, where they were frozen at -70^{o}C until processed.

Zebra Mussel Infection and Clearing

Standard hard reference water was inoculated with 1.0×10^{6} PFU/L of a laboratory strain of hepatitis A virus obtained from Dr. John Sheridan at the Ohio State University. One liter of the inoculated water was then aliquoted into each of a series of 30 1 liter beakers. One adult zebra mussel was added to each beaker and incubate without food to ensure maximum filtering rates. Each zebra mussel was observed hourly for filtering activity, with those not filtering being removed from the experiment. At 1, 2, 4, 8, 16, and 24 hours after the start of the experiment, 5

zebra mussels from inoculated water and 1 from uninoculated water was removed from the beakers, washed three times with dH_2) and then processed for RNA extraction.

To determine the amount of time required for clearing HAV from zebra mussel tissue, individuals were first incubate for 24 hrs in inoculated water before transfer to beakers containing uncontaminated standard hard reference water. Water was changed daily, and zebra mussels were harvested at 1, 3, 5, and 7 days post inoculation and processed for the detection of HAV.

Sample Processing

Prior to isolation of RNA, collected water samples were first filtered, with extractions made from the filters. Vacuum filtration was used to progressively pass the samples through Whatmann standard filter paper, and then through 0.45uM and 0.025uM filters. Sediment samples were concentrated to the smallest volume possible via centrifugal filtration using centriprep-100 molecular cut-off filters.

RNA isolation

Total RNA was isolated from field and lab samples by homogenization in TRIzol® reagent (Invitrogen Inc.) using standard protocol. For zebra mussel samples, individual mussels were opened, and placed into 1ml of TRIzol® and homogenized. For water samples, filters were immersed in TRIzol® reagent and allowed to incubate with periodic vortexing until residue could no longer be detected on the filter. For sediment samples, 1g of the concentrated sediment was placed in 1ml TRIzol®. Each sample then received 200ul of chloroform, followed by brief vortexing and centrifugation at 12,000g for 15 minutes. The upper aqueous phase was decanted

into a fresh tube, to which isopropanol was added to precipitate the RNA. This was centrifuged at 12,000g for 10 minutes to pellet the RNA, washed once with 70% EtOH to remove residual protein, and was finally resuspended in 50ul nuclease free water for use in rt-PCR.

RT-PCR

Due to the fact that HAV is an RNA virus, reverse transcription was required prior to amplification. This was accomplished by adding 5ug of sample RNA to a buffered solution containing reverse transcriptase, and incubating for 1hr at 37^{o}C, followed by a 10min incubation at 90^{o}C to melt the resulting RNA-DNA hybrid molecules as well as any secondary structures that might affect PCR reaction efficiency.

Polymerase chain reaction was conducted using the resultant DNA as a template, published primers specific to HAV (Jaykus et al, 1996), and optimized PCR buffer (Invitrogen, Inc.). The PCR reaction was carried out for 30 cycles, with 30 secs for disassociation at 94^{o}C, reannealing for 30 secs at 55^{o}C, and extension for 45 secs at 72^{o}C.

Electrophoresis

After PCR, samples were loaded onto a 1.5% agarose gel containing ethidium bromide for separation by electrophoresis. Gels were observed under ultraviolet light, and those PCR reactions which resulted in a PCR product of 500bp were considered to be positive. After electrophoresis, samples were transferred to nitrocellulose filters by downward capillary action for use in Southern blotting.

Probe development

In order to probe our Southern blots of the PCR products, we first developed a partial clone of the HAV genome. This was accomplished by rt-PCR of a sample of laboratory reared HAV, using the same primers as were used for the detection of HAV in experimental samples. The 500bp product of this reaction was inserted into a cloning vector which was incorporated into *e. coli* cells using a TA cloning kit (Invitrogen, Inc). Sequence analysis of this clone was conducted at the University of Georgia using an ABI373A DNA sequencer and dye terminator chemistry as according to standard protocol. Using the BLAST program for comparison against all Genbank sequences, the clone was confirmed to be identical to bases 2000-2500 for the HAV genome.

Southern blotting

To confirm the identity of the PCR products as well as to provide a method of detection from with relative assessment can be made, Southern blotting was performed on all samples/ The afore mentioned probe was labeled with ^{32}P-CTP using the RTS DNA labeling system (Gibco BRL). Prior to application of the probe, filters were first exposed to UV irradiation to cross-link the samples to the nitrocellulose. The southern blotting protocol consisted for prehybridization for 3hrs at 65^{o}C in standard hybridization buffer (0.5M NaCl, 0.1M $NaPO_4$, 6mM EDTA, 1%SDS). Hybridization was then performed overnight at 65^{o}C with the same buffer containing the radioactively labeled probe. The filters were washed one with ¼ strength hybridization buffer for 45 min at 65^{o}C and twice with ¼ strength hybridization lacking SDS for 30 min each, again at 65^{o}C. Afterwards, Fuji x-ray film (Fisher Scientific) was exposed to the filters at –70^{o}C overnight. In order to determine relative signal levels, the radiographs were

digitized by a flatbed scanner, and the average gray scale density value of each band was determined by using NIH Image version 1.61 software (Masters et al, 1992).

Results

Virus Uptake

When incubated under laboratory conditions with artificially inoculated water, zebra mussels exhibited the ability to bioconcentrate HAV virus (fig 1.). As little as one hour of exposure resulted in a small amount of virus being detected in mussel tissues. This level increased substantially at four hours of incubation, with the increase continuing through 18 hours of incubation. Southern blot hybridization confirmed the identity of these bands as HAV. Densitometry of the blots indicates a similar pattern as seen in the ethidium bromide staining, with a maximum concentration attained by sixteen hours of incubation, after with a plateau is established through 24hrs (fig 2.). Data shown is representative of three replicates of the procedure.

Virus Clearing

When returned to uninoculated water after a 24hr incubation in inoculated water, zebra mussels were shown to clear the virus quite slowly. After 7 days of incubation, mussel tissues still showed detectable, although decrease levels of HAV signal (Fig 3). Again, the data shown is representative of three replicates of the procedure.

Field Samples

All 45 zebra mussel, water, and sediment samples failed to show detectable PCR product by ethidium bromide staining or Southern blotting (data not shown). Hence, all of our samples were either free of HAV or the levels were below the limits of our assay.

Conclusions

Although no virus was found in the 45 samples collected in the field during August through October of 1997, our laboratory data demonstrates that zebra mussels have the ability to bioconcentrate HAV from contaminated water. The lack of HAV in field samples may be due to the timing of the collection process. Further field studies should be conducted during periods of documented HAV infections in areas of the Lake Erie basin. Retrospective data from the Centers of Disease Control indicate that Ohio had less than 5 reported cases of HAV infection per 100,000 people in 1997 (CDC, 1997). Additionally, a search of more prevalent viruses, such as rotaviruses, which affect 95% of the human population as opposed to HAV, which shows 33% seropositivity in adults (Fields et al, 1996) would more likely show positive results in the field.

Our findings in the laboratory demonstrate the ability of zebra mussels to bioconcentrate HAV from water. The dynamics of this bioconcentration closely mirror those of HAV in other bivalves. In the mussel *Mytilus chilensis,* maximum concentrations of HAV were realized between three and twenty-four hours of incubation in contaminated water (Enriquez, 1992). The same study showed a similar rate of clearing as well, with diminished but still detectable levels present seven days after removal from contaminated water.

One possible conclusion from our study is that zebra mussels could be used as a tool to monitor water quality with respect to human pathogens. This function of zebra mussels has been

suggested for the monitoring of other human pathogens including *e. coli* (Selegean, 2001) in water. Our study shows that this concept could be extended to viral contaminants as well, even though they have a much smaller particle size than do other biocontaminants previously shown to be concentrated by zebra mussels (27nM diameter for HAV particles as compared to 5um for cryptosporidia oocytes and 10um for giardia cysts). Additionally, we have demonstrated that this virus can be easily isolated from zebra mussel tissue, and by use of rt-PCR, samples can be assayed easily, quickly, and economically for the presence of HAV and perhaps other pathogens. The monitoring of either endemic populations or caged zebra mussels placed in locations of interest to human health could be used as a water quality monitoring tool.

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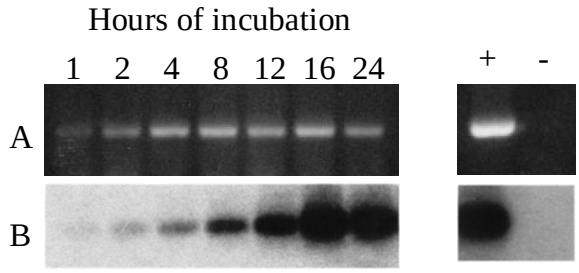

Figure 1: The uptake of hepatitis A virus by zebra mussels from inoculated water as detected by A.) ethidium bromide staining of PCR product and B.) Southern blotting of PCR reactions. Positive controls refer to zebra mussels tissue harvested and then seeded with HAV. Negative controls indicate samples from zebra mussels incubated in uninoculated water.

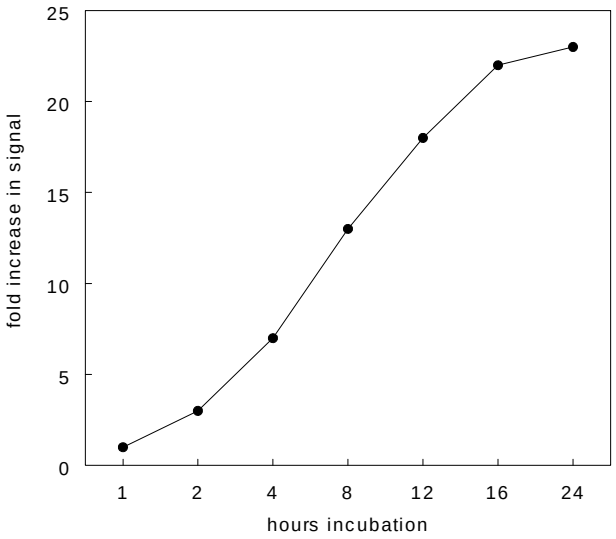

Figure 2: Graph of the densitometry analysis of Southern blot from figure 1, with y-axis as fold increase in signal over the value at 1 hour incubation.

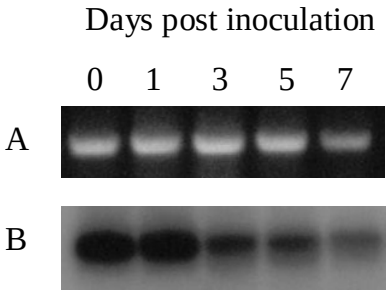

Figure 3: The persistence of hepatitis A virus in zebra mussels as detected by A.) ethidium bromide staining of PCR product and B.) Southern blotting of PCR reactions. Both methods show strong signals at 24 hrs on incubation, with noticeable reductions by 7 days post inoculation.