Detection of bacteria and enteric viruses from river and estuarine sediment

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ABSTRACT
River and estuarine sediment is suggested to play an important role in transmission of microbes in the water environment. However, although effective methods to recover bacteria from sediment are available, preparation methods for viruses, especially using molecular detection methods, are still under development. In this study, preparation methods for viruses in sediment were evaluated by qPCR methods. Thirty-six sediment samples were collected from the Takagi River and the Matsushima Bay receiving the Takagi River from December 2007 to May 2008 and tested for fecal coliforms, *Bacteroides* spp., human adenoviruses and *Cryptosporidium* spp. As the results, recovery rate of a preparation method for RNA viruses was low (Geometric mean: 3.3%, n=11), while that for DNA viruses was relatively high and stable (Geometric mean: 37%, n=6). The detection rate was the highest for fecal coliforms (92%, 33/36), followed by *Bacteroides* spp. (61%, 22/36). Human adenoviruses and *Cryptosporidium* spp. were not detected partly due to the limited sediment volume (0.5 g) applicable to the DNA extraction kit. Although the high positive rates of fecal coliforms and *Bacteroides* showed that the preparation methods for fecal indicator bacteria were applicable for environmental application, it was recommended that more effective methods for enteric viruses and protozoa be developed for direct monitoring of pathogens in sediment.
Keywords: *Bacteroides*, enteric viruses, fecal indicator, sediment.

INTRODUCTION

Sediment has been demonstrated a significant relationship with fecal indicator bacteria which indicate the presence of pathogens. The concentrations of fecal indicator bacteria were shown to be much higher in different kinds of sediments than those in the adjacent water (Craig *et al*., 2002; Alm *et al*., 2003; Mimura *et al*., 2005; Bissett *et al*., 2006). It is also reported that fecal coliforms, *Escherichia coli* and enterococci in the estuarine sediment were found to survive at least 7 days longer than those in a water column (Jeng *et al*., 2005), suggesting that sediment might provide a more stable indicator of long-term fecal contamination (Craig *et al*., 2002). When storms, tides, or strong winds cause sediment resuspension, fecal bacteria survived in sediment would also be resuspended, resulting high fecal bacteria levels in the water column (Jeng *et al*., 2005; Dorner *et al*., 2006).

Furthermore, recent studies have suggested important roles of sediment in pathogen contamination. Bacterial, protozoan and viral pathogens have been detected from sediment: *Mycobacterium avium* (Whittington *et al*., 2005), *Clostridium botulinum* type E (Perea-Fuentetaja *et al*., 2006), *Cryptosporidium* (Searcy *et al*., 2006), enteroviruses

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(Gerba *et al*., 1977), *Hepatitis A Virus* (Le Guyader *et al*., 1994) and *Rotavirus* (Green and Lewis, 1999). Survival of *Mycobacterium avium* in sediment was 12 to 26 weeks longer than that in the water column (Whittington *et al*., 2005). These data suggest that sediment may act as a reservoir of pathogens (Alm *et al*., 2003; Salvo and Fabiano, 2007). It is suggested that pathogen-sediment interactions be taken into consideration when predicting the fate of pathogens in the environment (Searcy *et al*., 2006).

The sample preparation methods which have been used for viruses in sediment consist of dispersing sediment particles in various buffer solutions, centrifugation to remove the sediment and purification of the supernatant (Gerba *et al*., 1977; Bitton *et al*., 1982; Wait and Sobsey, 1983; Lewis *et al*., 1985; Green and Lewis, 1999). Recovery rates of the preparation methods have been evaluated only using cell-culture based plaque assay (Gerba *et al*., 1977; Bitton *et al*., 1982; Wait and Sobsey, 1983; Lewis *et al*., 1985), which ranged from 8 to 50 % for estuarine sediment. Johnson *et al*. (1984) showed a negative correlation between virus recovery and ratio of clay in sediment, which suggested that preparation methods should be evaluated using the sediment collected from the target area. Although molecular detection methods such as PCR have been developed and widely used especially for viruses without cell lines (e.g. *Norovirus*), sample preparation methods have not been evaluated using molecular methods partly because some of the preparation methods use beef extract which is known to inhibit PCR (Wait and Sobsey, 1983; Lewis *et al*., 1985). Moreover, it is reported that humic substances which are extracted from soil and sediment inhibit nucleic acids extraction (Zhou *et al*., 1996) and Taq DNA polymerase in PCR (Tsai and Olson, 1992; Watson and Blackwell, 2000). Due to the knowledge mentioned above, it is necessary to evaluate recovery rates of preparation methods using sediment collected in the target area by molecular detection methods.

In this study, a field survey was conducted at the Takagi River and the Matsushima Bay receiving the Takagi River from December 2007 to May 2008. Sediment samples were collected and tested for fecal coliforms, *Bacteroides* spp. which is used for microbial source tracking, human adenoviruses and *Cryptosporidium* spp. There have been no reports which explored the three types of microbes, bacteria, virus and protozoa, in the same field, and there have been no study detecting fecal indicators and pathogens in sediment in Japan. Recovery rates of preparation methods for both DNA and RNA viruses in sediment were evaluated by qPCR methods. For DNA virus preparation, a commercial kit for direct DNA extraction from soil was used. For RNA virus, the sample preparation method developed by Gerba *et al*. (1977), which can process large amount of sediment and which does not use beef extract for elution, was used with some modification.

MATERIALS AND METHODS

Sample collection

Sediment samples were collected in the Takagi River estuary during the ebb tide monthly from November 2007 to May 2008. Locations of sample sites are shown in Figure 1: St.A, St.B and St.C were located in the bay where oyster beds are placed (Figure 1 & 2); St. D was located at the river mouth; and St.E and St.F were located in the river downstream. There is a small dam to control the river flow at St.F (Figure 3).

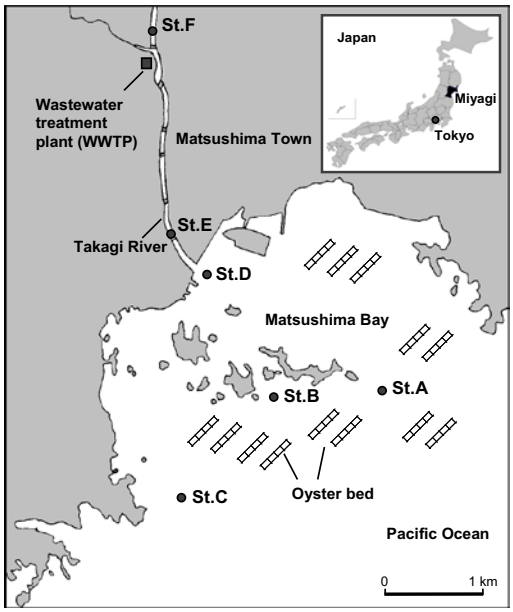

Figure 1. Locations of sample sites

Figure 2. Oyster beds at the bay

Figure 3. Dam at the Takagi River (St.F)

Table 1. Primer and probe sequences for detection of enteroviruses, human adenoviruses, *Bacteroides* spp. and *Cryptosporidium* spp.

Microbes	Primer & probe	Sequence (5' - 3')	Annealing temp.	References
Enteroviruses (*Poliovirus*)	Ev1 (Forward) Ev2 (Reverse) Ev-probe (TaqMan probe)	GATTGTCACCATAAGCAGC CCCCTGAATGCGGCTAATC (FAM)GGAACCGACTACTTTGGGTG TCCGT(TAMRA)	60 °C	Monpoeho *et al*., 2003
Human adenoviruses	AQ1 (Forward) AQ2 (Reverse) AP (TaqMan probe)	GCCACGGTGGGGTTTCTAAACTT GCCCCAGTGGTCTTACATGCACATC (FAM)TGCACCAGACCCGGGCTCA GGTACTCCGA(TAMRA)	55 °C	Heim *et al*., 2003
Bacteroides	BacUni_520F BacUni_690R1 BacUni_690R2	CGTTATCCGGATTTATTGGGTTTA CAATCGGAGTTCTTCGTGATATCTA AATCGGAGTTCCTCGTGATATCTA	63 °C	Kildare *et al*., 2007
Crypto-sporidium	Forward Reverse	CGCTTCTCTAGCCTTTCATGA CTTCACGTGTGTTTGCCAAT	60 °C	Fontaine *et al*., 2002

The sample obtained by an Ekman-Birge type bottom sampler covered a square area of 15 by 15 cm, and the top layer of 1 cm was collected. The samples were transported to the laboratory on ice in sterile containers and processed within a few hours of collection. Thirty-six samples were collected in total from 6 sample sites for 6 months.

Sample preparation for RNA viruses

Sediment samples were processed following the method by Gerba *et al*. (1977) with one modification: a vortex mixer was used for 15 sec instead of a shake table for 10 min to prevent conformational change in capsid protein caused by high pH of elution buffer (pH 11.5). The modified procedure is as follows. Five grams of wet sediment were placed in 50 mL centrifuge tube with 15 mL of 0.25 M glycine-NaOH buffer (pH 11.5) containing 0.05 M EDTA. The tube was vortexed for 15 sec and centrifuged for 4 min at

2,500 x *g* to remove the sediment. The supernatant was collected and pH was adjusted to 3.5 by addition of 1 M glycine-HCl buffer (pH 2.0). Aluminum chloride (1 M) was then added to yield a 0.06 M final concentration, and the solution was passed through a HA membrane filter (0.45 μm pore size and 90 mm diameter; Millipore, Tokyo). Virus was eluted from the filter by passage of 10 mL volumes of 0.25 M glycine-NaOH buffer (pH 11.5) and the eluate was immediately neutralized by addition of 1 M glycine-HCl buffer (pH 2.0). Viral RNA was extracted using QIAamp RNA mini kit (QIAGEN, Tokyo), and cDNA was obtained from 5 μL out of 60 μL of the extracted RNA with reverse transcription reaction using First Strand cDNA Synthesis Kit for RT-PCR (Roche, Tokyo).

Sample preparation for *Bacteroides* spp., *Cryptosporidium* spp. and *Adenovirus*

DNA of *Bacteroides* spp., *Cryptosporidium* spp. and *Adenovirus* were extracted directly from 0.5 g wet sediment samples using ISOIL for Beads Beating (Nippon Gene, Tokyo).

Detection of pathogens and fecal indicators by PCR and qPCR methods

The concentration of (c)DNA of *Poliovirus* and *Adenovirus* was determined using real-time qPCR methods with LightCycler ST300 (Roche, Tokyo). Each 20 μL PCR mixture contained 5 μL of cDNA or DNA, 4 μL of LightCycler TaqMan Master (Roche, Tokyo), 1.5 μL of 10 pM primers and 0.4 μL of 10 pM TaqMan probe listed in Table 1. The PCR condition including a denaturing step at 95 ^{o}C for 10 min, followed by 50 cycles of 95 ^{o}C for 3 sec, annealing temperature specified in Table 1 for 10 sec, and 72 ^{o}C for 30 sec.

For detection of *Bacteroides* spp. and *Cryptosporidium* spp., PCR was carried out with Veriti 96-Well Thermal Cycler (Roche, Tokyo). Each 20 μL PCR mixture contained 5 μL of the extracted DNA, 10 μL of master mix (Roche, Tokyo), 0.5 μL of 10 pM primers specified in Table 1. The PCR conditions included a denaturing step at 95 ^{o}C for 5 min, followed by 40 cycles of 95 ^{o}C for 1 min, annealing temperature (Table 1) for 1 min, and 72 ^{o}C for 20 sec, followed by a final extension step of 72 ^{o}C for 30 sec. The PCR products were electrophoresed in 1.5% (w/v) agarose gel stained with ethidium bromide, and visualized by UV illumination.

Evaluation of recovery rate of preparation method for viruses in sediment

As a surrogate of RNA viruses, Sabin strain of *Poliovirus* type 1 was spiked into the sediment taken at St.F (n=11) and recovered following the procedure by Gerba *et al*. (1977). *Poliovirus* belongs to genus *Enterovirus* and has positive-sense single-stranded RNA genome surrounded by a non-enveloped, icosahedral capsid of approximately 25 nm diameter. This virus was also used by Gerba *et al*. (1977) for evaluation of the method. Recovery rate was evaluated by dividing amount of spiked *Poliovirus* by amount of detected *Poliovirus* from spiked sediment samples.

For DNA viruses, 0.5 g of the sediment samples (n=6) collected at St. F was spiked with *Adenovirus* type 41 and was applied to ISOIL for Beads Beating.

Quantitative detection of fecal coliforms from sediment

Fecal coliforms in the sediment samples were recovered following the procedure of Craig *et al*. (2002) with some modifications: 5 mL double-distilled water was used instead of 9 mL 0.1% peptone water; a vortex mixer was used for 15 sec instead of hand

shaking for 1 min. The modified procedure for fecal coliforms isolation is as follows. One gram of well-mixed wet sediment sample was placed in a 15 mL centifuge tube with 5 mL double-distilled water. The tube was vortexed for 15 sec and left to settle for 10 min prior to aspirating the supernatant. Five milliliters of the supernatant were collected, diluted if necessary, and passed through a HA membrane filter (0.45 μm pore size and 47 mm diameter; Millipore, Tokyo) The membrane filter was placed on m-FC agar (Merck, Tokyo) and incubated at 44.5 ^{o}C for 24 hr. Results were recorded as CFU/100 g (dry weight) of sediment following previous studies (Craig *et al*., 2002; Alm *et al*., 2003).

Particle size distribution of sediment samples

Particle size distribution of sediment from each sampling point was measured by Microtrac (9320HRA (X-100); Nikkiso, Tokyo) and composition of sediment was classified based on the standard of The Japanese Geotechnical Society (JGS 0051-2000) as follows; clay, < 0.005 mm; silt, 0.005 – 0.075 mm; fine sand, 0.075 – 0.25 mm; medium sand, 0.25 – 0.85 mm. The samples were collected on May 13th, 2008.

RESULTS AND DISCUSSION

Evaluation of recovery rate of preparation method for viruses in sediment

Figure 4 shows recovery rates of RNA and DNA viruses. Recovery rate (geometric mean) of *Poliovirus* (RNA virus) was 3.3 % (geometric standard deviation (GSD) = 0.6, n = 11) and it was much lower than the recovery rate by Gerba *et al*. (1977) (50 %). Figure 5 shows composition of particle size of sediment from each sampling point. Gerba *et al*. (1977) mentioned that their sediment was largely composed of organic mud and sand, while the sediment sample taken at St.F was mainly consisted of silt (67 %) and clay (13 %). As mentioned by Johnson *et al*. (1984), the difference in the recovery rates may be because of the difference of particle size distribution of the sediment, especially composition of silt and clay. The low recovery rate was also because of the inhibition of nucleic acids extraction and PCR by humic substances extracted from sediment by high pH as Tsai and Olson (1992), Zhou *et al*. (1996) and Watson and Blackwell (2000) reported. The color of supernatant after vortex mixing and centrifugation was brown and that of the concentrate after membrane filtration was yellow, both of which indicates the presence of humic substances. Therefore, the method by Gerba *et al*. (1977) was not sensitive enough for the sediment in the Takagi River and it is suggested that more reliable and sensitive preparation method for RNA viruses in sediment with high proportion of silt and clay and with presence of humic substances should be developed for environmental monitoring. Based on this result, naturally occurring RNA viruses were excluded from the field monitoring.

Recovery rate (geometric mean) of *Adenovirus* type 41 (DNA virus) using ISOIL for Beads Beating was 37 % (GSD = 0.03, n = 6) and it was comparable with reported virus recoveries, such as 50 % reported by Gerba *et al*. (1977), 8 % to 22 % by Bitton *et al*. (1982), 31 % by Wait and Sobsey (1983), and 18 ± 20 (% ± SD) by Lewis *et al*. (1985), although there are differences in viruses, sediment, extraction and detection methods. The relatively high and stable recovery may be because of efficient extraction of viral DNA with removing humic substances.

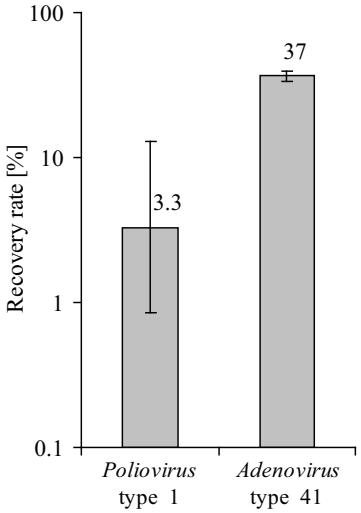

Figure 4. Recovery rates (geometric mean) of *Poliovirus* type 1 (RNA viruses) and *Adenovirus* type 41 (DNA viruses) in sediment. Error bars show geometric standard deviation of recovery rates.

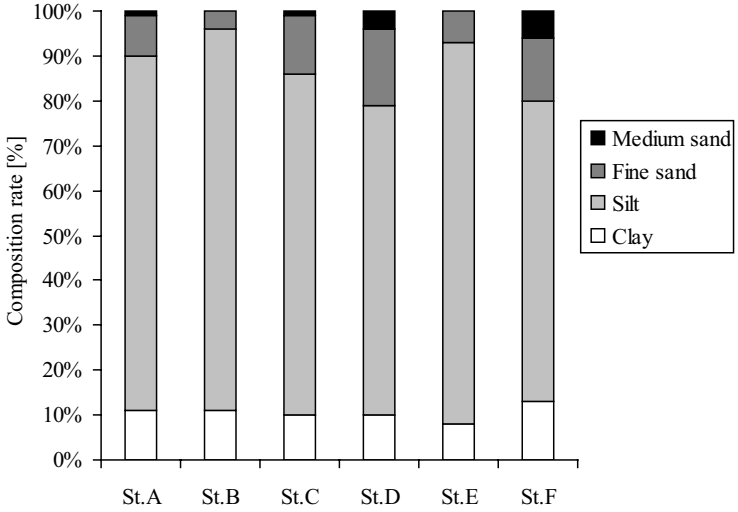

Figure 5. Composition of particle size of sediment from each sampling point. The samples were collected on May 13th, 2008.

Fecal coliforms, *Bacteroides*, human adenoviruses, *Cryptosporidium* in sediment

Figure 6 shows detection rates of fecal coliforms, *Bacteroides* spp., human adenoviruses, *Cryptosporidium* spp. in sediment samples. The detection rate was the highest for fecal coliforms (92%, 33/36), followed by *Bacteroides* (61%, 22/36). The high prevalence of these fecal indicators suggested that part of the fecal indicator bacteria that flew into the river from various contamination sources settle on river and estuarine sediment, and that the sediments may protect the bacteria from rapid decay as suggested by Gerba and McLeod (1976) and Anderson *et al*. (2005). On the contrary, human adenoviruses and *Cryptosporidium* were not detected from any samples. It is unlikely that protozoa (*Cryptosporidium*) which are larger than bacteria and have been found in water column (data not shown) is completely absent in sediment where high prevalence of bacteria was observed. Thus the negative results may be because of low detection efficiency

caused by the very small portion of sediment samples (0.5 g wet weight) applicable for the DNA extraction kit.

Figure 7 shows concentration of fecal coliforms in sediment samples from each sampling point. The range of the concentration was comparable with previous study (Craig *et al*., 2002). The concentration in river sediments (St. F; GM = 7.5 x 10^4 CFU/100 g dry weight) was the highest, followed by estuarine sediments (St. D and E; GM = 4.7 x 10^3 and 4.9 x 10^3 CFU/100 g dry weight, respectively) and then marine sediments (St. A, B and C; GM = 7.3 x 10^2, 4.6 x 10^2 and 4.5 x 10^2 CFU/100 g dry weight, respectively), while the sediment particle size composition (Figure 5) was similar and mainly consisted of silt. This may be because fecal coliforms cannot survive for a long period of time in seawater (Anderson *et al*., 2005).

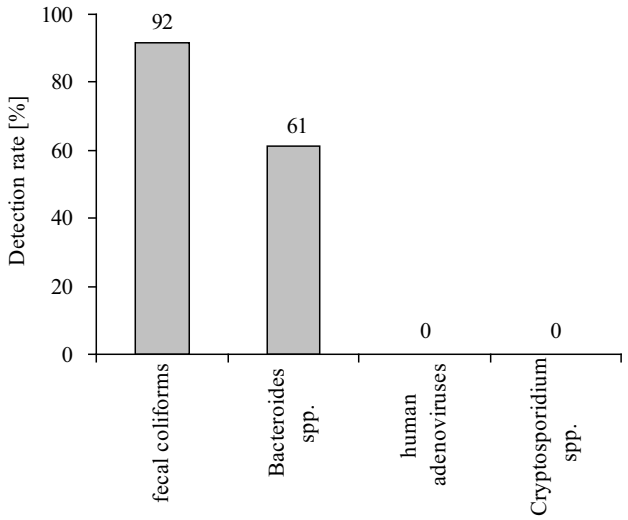

Figure 6. Detection rates of fecal coliforms, *Bacteroides* spp., human adenoviruses and *Cryptosporidium* spp. in sediment samples.

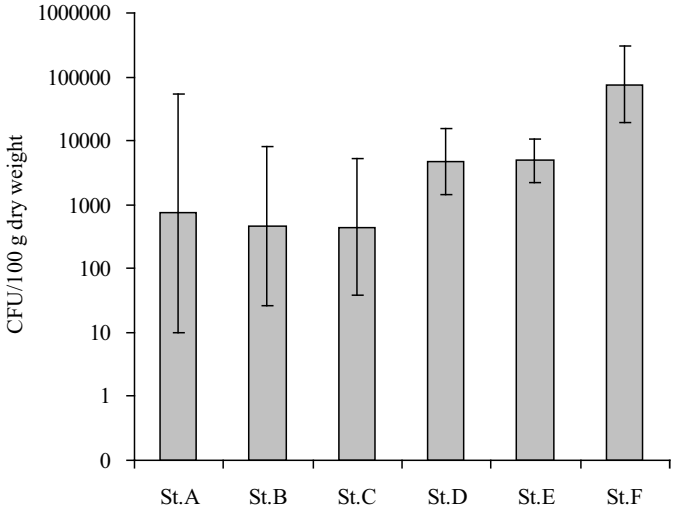

Figure 7. Concentrations of fecal coliforms in sediment samples. Values are geometric mean and error bars show geometric standard deviation.

CONCLUSIONS

In this study, sample preparation methods for viruses in sediment which have not been well investigated were evaluated using molecular methods. Recovery rate of *Poliovirus* (RNA viruses) using the preparation method developed by Gerba *et al*. (1977) was low for sediment collected in the Takagi River partly because of high proportion of fine particles such as silt and clay and presence of humic substances. Direct DNA extraction method using DNA extraction kit for soil (ISOIL for Beads Beating) showed relatively stable recovery rate for *Adenovirus* type 41 (DNA viruses). The sediment samples collected in the Takagi River watershed and the Matsushima Bay contained fecal indicator bacteria (fecal coliforms and *Bacteroides* spp.), thus the methods used in this study would be applicable for monitoring these bacteria in the sediment. However, no pathogens (human adenoviruses and *Cryptosporidium* spp.) were found in any sediment samples using ISOIL, suggesting that more effective methods for enteric viruses and protozoa be necessary for direct monitoring of these pathogens in the sediment.

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