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Detection and quantification of enteroviruses in coastal seawaters from Bohai Bay, Tianjin, China

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Abstract

An 8-month survey was conducted to detect and quantify enteroviruses in Tianjin coastal seawaters of Bohai Bay to assess coastal water quality. Ten water samples were collected from Bohai Bay for the detection and quantification of enteroviruses by conventional reverse transcription polymerase chain reaction (RT-PCR) and SYBR Green real-time quantitative RT-PCR (qRT-PCR). Total viral nucleic acid was extracted from 500 mL of seawater samples concentrated by Centricon plus-70 centrifugal filter devices. The viral recovery rate was 29.1% based on viral seeding study. The centrifugal ultrafiltration method applied is effective for viral recovery from small volume of polluted water, which may have broader applications to monitoring human virus in aquatic environment. Our results indicated that there was a severe viral contamination in seawater of Bohai Bay. Enteroviruses were detected at concentrations ranging from 1.7×10^6 to 6.3×10^7 copies/L by qRT-PCR. Sequencing analyses identified that all of the twenty clones as poliovirus type 2. This is the first quantitative report of human viruses in coastal waters of a metropolitan city in China. This study emphasized the importance for the local and central governments to monitor and assess the water quality.

Key words: seawater; enterovirus; SYBR Green; real-time quantitative RT-PCR

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Introduction

Enteroviruses have been found in rivers, coastal oceans, freshwater reservoir and even in treated drinking water (Lipp et al., 2002; Borchardt et al., 2003; Jiang and Chu, 2004; Fong et al., 2005; Lambertini et al., 2008). They are recognized as major public health hazards. Enterovirus infection can cause meningitis, encephalitis, hepatitis, gastroenteritis and/or other illnesses (Griffin et al., 2003). However, few studies reporting enterovirus in aquatic environment in China have been published so far, indicate a lack of microbiological and epidemiological investigations that can provide an assessment of human health risk from exposure to recreational waters in China.

Cell culture was traditionally used for detecting viruses in the environment. However, compared to PCR (polymerase chain reaction), cell culture techniques are more costly, time consuming and less sensitive. It requires large sample volumes and several days for results. In the past two decades, RT (reverse transcription)-PCR assays have been developed for the detection of norovirus, hepatitis A virus and enterovirus in sewage sludge, as well as in river and seawater, shellfish and other food products (Tsai et al., 1993; Katayama et al., 2002; Jothikumar et al., 2005; Ko et al., 2005; De Paula et al., 2007; Hwang et al., 2007). Also quantitative RT-PCR method was developed and applied to quantify viral concentration in environmental samples (Brooks et al., 2005; Bessaud et al., 2008).

The occurrence and concentration of enteroviruses in coastal seawaters of China have not been investigated. The present study reports the detection and quantification of enteroviruses in coastal seawater from Bohai Bay, Tianjin, China by RT-PCR and real-time quantitative RT-PCR.

1 Materials and methods

1.1 Sampling sites

Between December 2007 and July 2008, ten water samples were collected from seven different sampling sites along Tianjin coast of Bohai Bay (Fig. 1, BT1–BT7). Bohai is an important shipping harbor in northern China. Sampling sites are outside the shipping harbor, located on accessible beaches along the major highway. In addition, site BT3 is located at the mouth of Haihe River, a major

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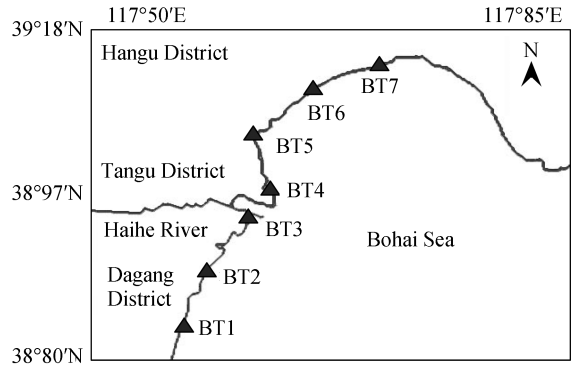

Fig. 1 Sampling locations in Bohai Bay, Tianjin, China. Among sampling locations, BT3 and BT4 are located near the mouth of Haihe River.

river run through the city of Tianjin. Water sample (500 mL) was taken from the surface of the water column from the beach. BT1 to BT3 were sampled once in winter 2007 and once in spring 2008. Only single samples were taken from the rest of the study sites. All sites were sampled in sterile bottles and collected samples were transported back to the laboratory within 1–2 hours for immediate processing.

1.2 Viral concentration and nucleic acid extraction

Five hundred milliliters of seawater samples were concentrated down to about 200 μL using a Centricon plus-70 centrifugal filter device with a 100-kDa molecular weight cutoff membrane (Millipore, USA). Approximately 70 mL of water was filled into the Centricon each time, the filtrate was discarded after each spin and filtration cup is refilled until 500 mL of water sample has passed through. Approximately 200 μL of concentrated samples were collected from the retention cup and frozen at –70°C until used for PCR analysis. Viral nucleic acid from concentrated seawater samples was purified using the method originally developed by Boom et al. (1990).

1.3 Detection of enteroviruses by RT-PCR

Primers for detection of enteroviruses are based on the conserved sequences at 5' untranscribed region of enteroviral genome (5'UTR). They are Env-1: 5'-CCCTGAATGCGGCTAAT-3'; Env-2: 5'-TGTCACCATAAGCAGCCA-3' as previously described by Gregory et al. (2006). The reaction mixture for cDNA synthesis contained 3.5 μg of viral RNA and 0.5 μg Random hexamers. This mixture was heated to 70°C for 5 min, immediately set on ice for 30 sec. Then, 10 μL of 5 × MMLV buffer, 10 μL of dNTPs (each 2.5 mmol/L), 25 U of RNase inhibitor (Promega, USA), and 200 U of reverse transcriptase (Promega, USA) were added to a total volume of 50 μL. The cDNA synthesis was carried out at 37°C for 1 hr. The reaction mixture for PCR contained 1 μL of cDNA, 2.5 μL of 10× Ex Taq buffer, 2.5 μL of dNTPs (each 2.5 mmol/L), 0.2 μmol/L of each of forward and reverse primer, and 0.625 U of Ex Taq HS (Takara Bio Inc., Japan) in a reaction volume of 25 μL. The thermal profile for RT-PCR was 95°C for 30 sec, followed by 45 cycles of 95°C for 30 sec, 53°C for 30 sec, and 72°C for 15 sec. Amplified cDNAs were separated in a 2% agarose gel.

1.4 DNA cloning and sequencing

The PCR amplified cDNA was cloned into a pMD 19-T cloning vector (Takara Bio Inc., Japan) following the manufacturer's protocol. Two to six white colonies from each sample were picked and cultured overnight in LB broth containing 50 mg/mL ampicillin. Plasmids were isolated using the plasmid purification kit (Takara Bio Inc., Japan). Two positive clones from each sample were randomly selected and submitted for sequence analysis. The sequencing was performed by Sangon Co. (China). Nucleotide sequences were submitted to BLAST search engine at NCBI GenBank and identified based on similarities.

1.5 Real-time quantitative RT-PCR analysis

cDNA was synthesized using random hexamers as described above for RT-PCR. SYBR Green real-time quantitative RT-PCR (qRT-PCR) was carried out in a 25-μL reaction volume containing 12.5 μL of 2× SYBR Green mix, 0.2 μmol/L each of the forward and reverse primers (same as in RT-PCR), and 1 μL of cDNA. The qRT-PCR analyses of all samples were performed in triplicates. The thermal profile for qRT-PCR was 95°C for 10 sec, followed by 45 cycles of 95°C for 5 sec, 53°C for 30 sec, and 72°C for 15 sec.

1.6 Standard curve for qRT-PCR

A standard curve was generated using a plasmid with a single copy of enteroviral 5' UTR insert. Five dilutions ranging from 0.001 to 100 pg/μL were used to create a standard curve. The DNA concentration was converted to genome copy and viral particle number based on the plasmid molecular mass. qRT-PCR was performed in triplicates for each dilution. The standard curve was created by plotting the log number of enteroviral particles versus their corresponding cycle threshold (C_T) value to create a best-fit line through these points. C_T value is defined as the PCR cycle at which an increase in the fluorescence above the baseline signal is first detected. The C_T value is inversely related to the viral particles. Enterovirus concentrations in the seawater samples were calculated by using the standard curve.

1.7 Viral recovery experiments

To determine the efficiency of filtration concentration procedure and viral RNA extraction, 500 mL seawater samples were seeded with a known amount of stock enterovirus prior to filtration. The same amount of virus was also spiked directly into a concentrated seawater sample after filtration but before RNA extraction. qRT-PCR was performed on both samples, and viral particle numbers were determined by using the standard curve. The recovery assay was performed in triplicates for each sample. The recovery from filtration was calculated using the ratio of the number of viral particles in the sample processed by filtration and the number of viral particles in the unfiltered

sample.

2 Results

2.1 Detection and identification of enterovirus

A 144-bp fragment was amplified from all collected samples. Figure 2 shows the expected enterovirus fragments by conventional RT-PCR. The DNA sequences retrieved from all samples aligned well with the 144-bp nucleotide sequences at the 5'UTR of enteroviral genome in the GenBank. The similarity values ranged from 99% to 100%. All of the twenty sequences (two from each sample) were identified as poliovirus type 2. Five of the sequences have a single nucleotide substitution (GenBank accession numbers: FJ492823, FJ492824, FJ492825, FJ492826, and FJ492827).

2.2 Standard curve of enterovirus

The enterovirus standard curve was obtained by using the plasmid with enterovirus 5'UTR insert derived from a serial dilution. A linear relationship was observed between the cDNA concentration and the C_T values with correlation coefficients (r^2) greater than 0.99 (Fig. 3). The melting temperature was 86.6°C. The melting curve indicated the excellent specificity of qRT-PCR.

2.3 Efficiency of enterovirus recovery

To determine the efficiency of our viral concentration protocol, the seawater samples from Bohai Bay were seeded with 1.1×10^8 particles of enterovirus in 500 mL seawater, and 3.3×10^7 particles of enterovirus were recovered after concentration. Concentrated and un concentrated samples were quantified using the qRT-PCR. The average percent of viral recovery is 29.1% from three trials.

2.4 Quantification of enterovirus in seawater samples

The qRT-PCR assay was performed to quantify enterovirus in the seawater samples. Enterovirus was detected in all of the ten seawater samples in concentration (corrected for recovery efficiency) range from 1.7×10^6 to 6.3×10^7 copies/L (Table 1).

3 Discussion

The results showed that Bohai Bay coastal water near the city of Tianjin was heavily polluted. The concentration

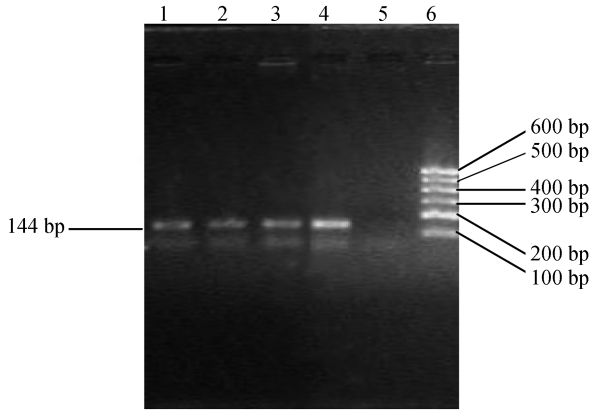

Fig. 2 Detection of enterovirus in seawater samples collected on Dec. 2007 by RT-PCR. Lane 1: BT1; lane 2: BT2; lane 3: BT3; lane 4: positive control; lane 5: negative control; lane 6: DNA markers.

Table 1 Levels of enterovirus in seawater determined by qRT-PCR

Sample ID	Sampling date (year-month)	Enterovirus detection by RT-PCR	Enterovirus concentration (copies/L)
BT1	2007-12	+	1.7×10^6
BT2	2007-12	+	5.2×10^7
BT3	2007-12	+	2.1×10^7
BT1	2008-04	+	1.1×10^7
BT2	2008-04	+	6.3×10^7
BT3	2008-04	+	4.4×10^7
BT4	2008-07	+	1.1×10^7
BT5	2008-07	+	2.3×10^7
BT6	2008-07	+	5.0×10^6
BT7	2008-07	+	1.9×10^7

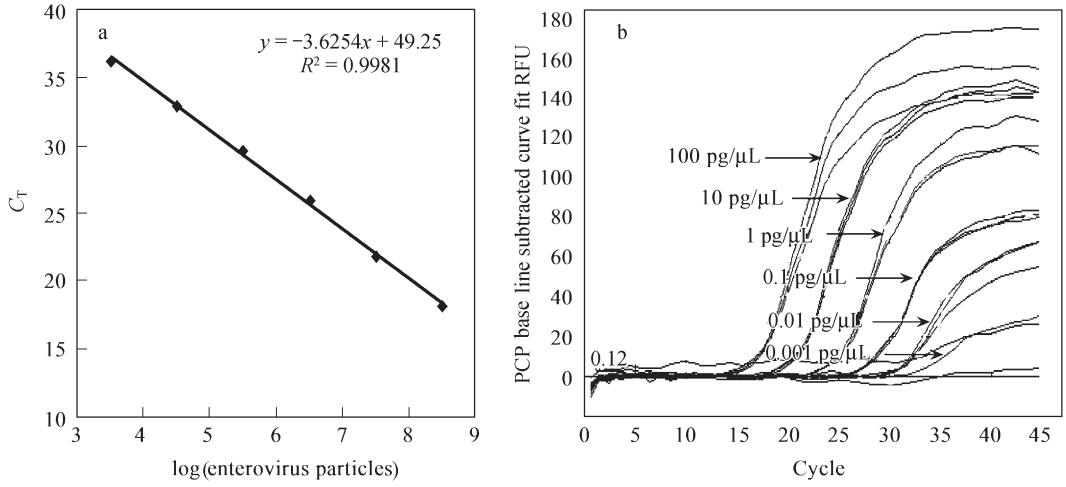

Fig. 3 qRT-PCR standard curve for enterovirus assay, showing efficiency and reproducibility. (a) standard curves were generated by plotting the C_T value versus the log of the number of viral genome copies; (b) detection of serial dilutions of plasmid with a single copy insert of enteroviral 5'UTR at 0.001–100 pg/μL.

of enterovirus ranges from 1.7×10^6 to 6.3×10^7 viral copies/L, which is much higher than any other studies previously reported using qRT-PCR (2.0×10^4 to 7.0×10^4 copies/L) (Donaldson et al., 2002; Rose et al., 2006). All of the twenty 5'UTR sequences indicated that the viruses detected in this study belong to poliovirus type 2. The high concentration is likely due to the discharge of industrial and domestic wastewater from the city and ships in the Tianjin Harbor. Tianjin is one of the most densely populated cities in China. The domestic sewage treatment has not reached 100% in the area. Haihe River, the major river empties into Bohai Bay, collects non-point source runoff as it cuts through the center of the city. Bohai Bay is also surrounded by heavy shipping industry. Our sampling sites are near a busy shipping harbor where domestic and international cargos were transported. Ship waste holding tanks can be another source of human viral contamination. To improve water quality along the Bohai coast, better wastewater treatment and management would be crucial. This study adds to the two previous studies (Fan et al., 2007; Zhang et al., 2008) reporting viral contamination in coastal waters and lakes of China, suggesting that there is a serious water quality problem in heavily populated regions of China.

The current study demonstrates that centrifugal ultrafiltration is an effective concentration method for viral recovery from 500 mL coastal water. Compared with the traditional sample volume that used 20–100 L water samples, ultrafiltration concentration requires minimal manipulation of the water sample, avoiding the complicated procedures that cause viral inactivation or PCR inhibition. In our preliminary study, we have used 20 L water and multiple steps for size fractionation, concentration and then purification. The final viral recovery was quite poor because of the loss during each additional step (data not shown). Our viral recovery rate using the single step viral concentration is comparable to that reported by Fuhrman et al. (2005) and Gersberg et al. (2006). Our centrifugal concentration method allows concentrating six water samples simultaneously. The total processing time is less than 2 hours. Reduction of sample holding time is critically important for detecting live viruses in environmental samples.

RT-PCR offers sensitivity and specificity over traditional viral culture, however, an inherent limitation of RT-PCR is its inability to discriminate between infectious and noninfectious viral particles. Gantzer et al. (1998) reported the ratio of viral particles determined by RT-PCR and plaque forming units (PFU) by viral culture is 66 to 1 PFU, and Shieh et al. (2008) found 91 viral particles to 1 PFU. Based on these reports, it may be reasonable to assume that the concentration of infectious enteroviruses in Bohai Bay in the range of 10^5 copies/L water. For some viruses that are difficult and time-consuming to culture, RT-PCR may be an invaluable tool for rapid environmental monitoring.

4 Conclusions

The present study described the detection and quantification of enteroviruses in Tianjin coastal seawaters from Bohai Bay by conventional RT-PCR and SYBR Green real-time quantitative RT-PCR. Enteroviruses were detected at levels ranging from 1.7×10^6 to 6.3×10^7 copies/L. To our knowledge, this is the first report to quantify enteroviruses in seawater samples in China. Our results indicated that there is a serious viral contamination in seawater of Bohai Bay.

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References

Bessaud M, Autret A, Jegouic S, Balanant J, Joffret M L, Delpeyroux F, 2008. Development of a Taqman RT-PCR assay for the detection and quantification of negatively stranded RNA of human enteroviruses: Evidence for false-priming and improvement by tagged RT-PCR. *Journal of Virological Methods*, 153(2): 182–189.

Boom R, Sol C J A, Salimans M M M, Jansen C L, Wertheim-vandillen P M E, Vandernoordaa J, 1990. Rapid and simple method for purification of nucleic-acids. *Journal of Clinical Microbiology*, 28(3): 495–503.

Borchardt M A, Bertz P D, Spencer S K, Battigelli D A, 2003. Incidence of enteric viruses in groundwater from household wells in Wisconsin. *Applied and Environmental Microbiology*, 69(2): 1172–1180.

Brooks H A, Gersberg R M, Dhar A K, 2005. Detection and quantification of hepatitis A virus in seawater via real-time RT-PCR. *Journal of Virological Methods*, 127(2): 109–118.

De Paula V S, Diniz-Mendes L, Villar L M, Luz S L B, Silva L A, Jesus M S et al., 2007. Hepatitis A virus in environmental water samples from the Amazon Basin. *Water Research*, 41(6): 1169–1176.

Donaldson K A, Griffin D W, Paul J H, 2002. Detection, quantitation and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using real-time RT-PCR. *Water Research*, 36(10): 2505–2514.

Fan J F, Song L C, Zhang X C, Liang Y B, Guan D M, 2007. Study on the distribution of hepatitis A virus in seawater and shellfish in the coastal area of Liaodong Bay. *Marine Sciences*, 31(2): 51–54.

Fong T T, Griffin D W, Lipp E K, 2005. Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. *Applied and Environmental Microbiology*, 71(4): 2070–2078.

Fuhrman J A, Liang X L, Noble R T, 2005. Rapid detection of enteroviruses in small volumes of natural waters by real-time quantitative reverse transcriptase PCR. *Applied and Environmental Microbiology*, 71(8): 4523–4530.

Gantzer C, Maul A, Audic J M, Schwartzbrod L, 1998. Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and bacteroides fragilis phages in treated wastewater. *Applied and Environmental Microbiology*, 64(11): 4307–4312.

Gersberg R M, Rose M A, Robles-Sikisaka R, Dhar A K, 2006. Quantitative detection of hepatitis a virus and enteroviruses near the United States-Mexico border and correlation with

levels of fecal indicator bacteria. *Applied and Environmental Microbiology*, 72(12): 7438–7444.

Gregory J B, Litaker R W, Noble R T, 2006. Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. *Applied and Environmental Microbiology*, 72(6): 3969–3967.

Griffin D W, Donaldson K A, Paul J H, Rose J B, 2003. Pathogenic human viruses in coastal waters. *Clinical Microbiology Reviews*, 16(1): 129–143.

Hwang Y C, Leong O M, Chen W, Yates M V, 2007. Comparison of a reporter assay and immunomagnetic separation real-time reverse transcription-PCR for the detection of enteroviruses in seeded environmental water samples. *Applied and Environmental Microbiology*, 73(7): 2338–2340.

Jiang S C, Chu W, 2004. PCR detection of pathogenic viruses in southern California urban rivers. *Journal of Applied Microbiology*, 97(1): 17–28.

Jothikumar N, Lowther J A, Henshilwood K, Lees D N, Hill V R, Vinje J, 2005. Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Applied and Environmental Microbiology*, 71(4): 1870–1875.

Katayama H, Shimasaki A, Ohgaki S, 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. *Applied and Environmental Microbiology*, 68(3): 1033–1039.

Ko G, Jothikumar N, Hill V R, Sobsey M D, 2005. Rapid detection of infectious adenoviruses by mRNA real-time RT-PCR. *Journal of Virological Methods*, 127(2): 148–153.

Lambertini E, Spencer S K, Bertz P D, Loge F J, Kieke B A, Borchardt M A, 2008. Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. *Applied and Environmental Microbiology*, 74(10): 2990–2996.

Lipp E K, Jarrell J L, Griffin D W, Lukasik J, Jacukiewicz J, Rose J B, 2002. Preliminary evidence for human fecal contamination in corals of the Florida Keys, USA. *Marine Pollution Bulletin*, 44(7): 666–670.

Rose M A, Dhar A K, Brooks H A, Zecchini F, Gersberg R M, 2006. Quantitation of hepatitis A virus and enterovirus levels in the lagoon canals and Lido beach of Venice, Italy, using real-time RT-PCR. *Water Research*, 40: 2387–2396.

Shieh Y C, Wong C I, Krantz J A, Hsu F C, 2008. Detection of naturally occurring enteroviruses in waters using direct RT-PCR and integrated cell culture-RT-PCR. *Journal of Virological Methods*, 149(1): 184–189.

Tsai Y L, Sobsey M D, Sangermano L R, Palmer C J, 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. *Applied and Environmental Microbiology*, 59(10): 3488–3491.

Zhang C M, Wang X C, Liu Y J, Xue X P, 2008. Characteristics of bacterial and viral contamination of urban waters: a case study in Xi'an, China. *Water Science and Technology*, 58(3): 653–660.