MOLECULAR CONFIRMATION OF ENTEROVIRUS FROM SEWAGE AND DRINKING WATER SAMPLES FROM THREE CITIES, PAKISTAN: A POTENTIAL RISK FACTOR FOR PUBLIC HEALTH

Tahir Ahmad1, Sadia Anjum1, Muhammad Sohail Afzal2, Hamid Raza2, Najam-us-Sahar Sadaf Zaidi1 and Najma Arshad2

1Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad; 2Department of Chemistry, School of Sciences, University of Management and Technology (UMT), Lahore; 3Department of Zoology, University of the Punjab, Lahore, Pakistan

Abstract. Gastroenteritis causes from 4 to 10 million children deaths every year worldwide, mainly from infection with water-borne Enteroviruses, which consist of 67 diverse serogroups. Forty-two sewage and drinking water samples from three metropolitan cities of Pakistan were analyzed for the occurrence of Enterovirus by nested RT-PCR amplification. Molecular detection was based on amplification of a part of 5’UTR region of the viruses. Our results revealed an alarming situation in densely populated areas of the three main cities of Pakistan: 28%, 19% and 21% of drinking water samples were positive for enteroviruses in Islamabad, Rawalpindi and Lahore, respectively. Sequence analysis and phylogenetic study of the amplified region of the virus revealed its close relationship with Coxsackie A strains reported from Greece, Singapore and USA.

Keywords: enterovirus, epidemiology, molecular phylogeny, nested RT-PCR, water, Pakistan

INTRODUCTION

Contamination of surface water with enteric viruses through disposal of human waste water is of grave concern for civic health, especially if these water resources are utilized for the production of drinking water. Water-borne outbreaks of enteric viruses have been reported frequently with symptoms ranging from mild febrile illness, common cold, to severe illnesses such as hand, foot and mouth disease, acute flaccid paralysis, myocarditis, aseptic meningitis, neonatal sepsis-like disease, and acute hemorrhagic conjunctivitis (Martinelli et al, 2007; Chung et al, 2010; Pérez-Sautu et al, 2012). In USA alone, these viruses (Picornaviridae) are responsible for 30,000 to 50,000 patients hospitalized per year for meningitis (Khetsuriani et al, 2006). The risk of infection is directly proportional to the poor hygienic and sanitary conditions in addition to overcrowding, with outbreaks mostly occurring in rainy seasons (CDC, 2006). Viral infection is acquired mostly through fecal-oral or respiratory route. Occurrence of gastroenteritis outbreaks}

Correspondence: Tahir Ahmad, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan.
Tel: +92 51 9085 6144
E-mail: baig42@gmail.com
within communities can be anticipated by careful monitoring of the local sewage treatment system for the presence of pathogenic viruses and hence appropriate preventive strategies can be adopted to protect inhabitants (He et al., 2013).

Enteroviruses have been classified classically into four groups, namely, echovirus, Coxsackie A and B viruses, and poliovirus, but due to closely overlapping biological properties recently discovered Enteroviruses are named with a system of consecutive numbers EV68, EV69, EV 70 and EV 71 (Blomqvist et al., 2012). Enteroviruses comprise more than 70 distinct serotypes, but vaccines are not available for many serotypes except poliovirus (Benjamin et al., 2013), and thus avoidance of the pathogens is very essential for community health.

Enterovirus genome is a single-stranded positive sense RNA, 7.5 kb in length, with 5’ UTR consisting of ~750 nucleotides (nts); followed by a long open reading frame (ORF) coding a protein of 2,100 amino acids, a short 3’ UTR and a polyA tail (Fig 1) (Sun et al., 2012). Variations in Enterovirus 5’UTR sequences have provided a means for molecular typing of Enteroviruses (Joffret et al., 2012).

In water samples, viruses are usually present in very low numbers. Thus large volumes of water samples are needed to be analyzed. A variety of concentration techniques are available, including direct concentration using ultracentrifuge, two phase separation using Dextran T40 and polyethylene glycol (PEG) 6000 and adsorption/elution glass powder method (Hovi et al., 2005; Wringe et al., 2008; Sufredini et al., 2012; Duintjer Tebbens et al., 2013). In this study Enteroviruses in drinking water and sewerage water samples from urban areas of Pakistan were isolated by physical techniques and amplified by culturing in a human cell line, followed by identification employing nested RT-PCR and DNA sequencing.

MATERIALS AND METHODS

Specimen collection

Sewerage water samples (1 liter) from 47 different water disposal localities of Islamabad, Rawalpindi, and Lahore, Pakistan (Fig 2) were collected from April 2013 to August 2013. The major portion of these samples contained household and industrial waste water. In addition, 25 drinking water samples (n = 2l) were collected from the same localities. Aliquots of 50 ml of waste samples were centrifuged at 10,000g for 10 minutes to sediment solid material, and the pH of supernatants were adjusted to 7.2-7.3 using either 1 M NaOH or 1 M
HCl. Then the samples were centrifuged at 10,000 g for 90 minutes and upper third of the supernatants was discarded.

**Virus isolation**

Supernatants were adjusted to contain 10% PEG 6000 (Sigma-Aldrich, St Louis, MO) and 0.4 M NaCl and then incubated overnight at 4°C. The solutions were centrifuged at 10,000 g for 90 minutes and pellets were re-suspended in 4 ml aliquots of 0.1 M phosphate buffer pH 8.0 and kept at -80°C for further use. The samples were restored to room temperature and centrifuged at 80,000 g for 90 minutes and the pellets re-suspended in 1 ml aliquots of nuclease free water.

Viruses were also isolated from PEG/NaCl-treated samples using negatively charged membrane filters (Millipore, Billerica, MA) as previously described (Tong and Lu, 2011; Zhang et al, 2013). In brief, 1.2 g of MgCl₂ were added to 1 liter samples, pH was adjusted to 5 and then the solutions were filtered through Whatman grade 2 qualitative filter paper (ProSource, Ottawa, Canada). Samples then were passed through negatively charged membrane (Millipore, Billerica, MA) of 0.45 μm pore size using a vacuum pump. Membranes were rinsed with 350 ml of 0.5 mM H₂SO₄ (pH 3.0), after which viruses were eluted using 10 ml of 1 mM NaOH (pH 10.8). In order to neutralize the solution 50 μl of 50 mM H₂SO₄ and 50 μl of 100X TE buffer (pH 8.0) were added. The samples then were centrifuged at 1,500g for 15 minutes at 4°C in a vacuum concentrator (Eppendorf, Hamburg, Germany) to reduce the final volume to 50 μl.

**Virus propagation in cell culture**

HeLa cells (10⁵ cells) were seeded in 12-well plates (Invitrogen, Paisley, UK) containing 1.5 ml Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St Louis, MO) containing 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich), and 1% penicillin (Sigma-Aldrich) and cultured for 24 hours at 37°C under an atmosphere of 6% CO₂. Cells were sedimented at 4,000g for 1 minute at 4°C, washed with phosphate-buffered saline solution (PBS), transferred to fresh medium and allowed to grow to 75% confluency. Then cells were inoculated with 500 μl aliquot of isolated virus sample (concentrated) and grown for 2 days as described above. Cytopathic effect (CPE) of the virus was recorded under microscopic examination. Cells were sedimented as described above and the medium containing released viruses was
used to isolate viral RNA.

**Nested RT-PCR**

Enteroviral RNA was extracted using an RNA extraction kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and used as template for nested RT-PCR using forward primer Ent1F (5’CAAGCACTTCTGTTTCCCCGG3’; starting from nt position 168) and reverse primer Ent1R (5’ATTGTCACCATAGGCAGCCA3’; starting from nt position 605) generating an amplicon of 436 base pairs (bp). The nested primer Ent2R (5’CTTGCGCGTTACGAC3’; starting from nt position 533) produced an amplicon of 365bp. The 20-µl reaction mixture for cDNA synthesis, containing 1X MMLV-RT buffer (Invitrogen), 2 mM dNTPs, 1.5 µM Ent1R, 40 U reverse transcriptase (Invitrogen), 20 U RNase inhibitor (Invitrogen), and 13 µl of RNA template, was incubated at 42ºC for 1 hour. The 25 µl first round PCR mixture contained 1X buffer (Invitrogen), 0.4 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM Ent1F and Ent1R primers, 100 ng of cDNA template, 5 µl Taq DNA polymerase (Farmentas, Hanover, MD). Thermocycling (carried out in Esco thermocycler) conditions were as follows: 95ºC for 3 minutes; 35 cycles of 94ºC for 40 seconds, 50ºC for 45 seconds, and 72ºC for 45 seconds; and a final step of 72ºC incubation for 10 minutes. PCR amplicon was separated by 2% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light. The second round 20 µl PCR mixture contained 2 µl of first round PCR product and 0.5 µM Ent1F and Ent2R primers. The other components of the PCR mixture and thermocycling conditions were the same as described above. The nested PCR amplicon was visualized as described previously (Poddar, 2002).

**DNA sequencing**

Amplicons were purified from gels using a commercial kit (QIAGEN, Hilden, Germany) according to manufacturer’s protocol and was inserted into pTA plus vector (TA Cloning Kit, Invitrogen, Carlsbad, CA). Recombinant plasmids were used to transfect Escherichia coli Top10 competent cells (LifeTechnology, Delhi, India) by a heat-shock method (Froger and Hall, 2007). Cells were streaked on solid agar (Invitrogen) and selection of transformants was made initially by selecting white colonies (blue and white selection). Final confirmation was made by EcoR1 restriction digestion of the plasmids isolated from these colonies. For this purpose plasmids were isolated using a plasmid isolation kit (Mini Prep, QIAGEN, Helden, Germany) according to manufacturer’s protocol. Approximately 200 ng of plasmid were digested with EcoR1 and the digestion products were analyzed by agarose gel-electrophoresis. Purified recombinant plasmids containing Enterovirus amplicon inserts were sequenced (DNA Genetic analyzer CEQ8000; Beckman Coulter, Fullerton, CA) using universal M13 sequencing primer according to the protocol described previously (Anjum et al, 2013). The sequences were aligned and compared using CLC sequence viewer software (CLCbio; Qiagen, Valencia, CA) with those available in NCBI GenBank.

**Phylogenetic tree construction**

Phylogenetic tree was conducted using partial sequence of 5’UTR obtained and submitted to NCBI GenBank (Enteropak accession no. JX469597). NCBI Blast results were used to obtain related sequences from NCBI data base reported from various countries. The sequences were aligned using T-coffee software, and phylogenetic tree was constructed using PhyML and
Maximum likelihood approach on ATGC bioinformatics platform. The phylogenetic tree generated with 100 bootstraps was viewed by Archaeopteryx (Guindon and Gaseuel, 2003). HCV isolate AJ621219 5'UTR sequence was used as out group.

RESULTS

In this study urban waste and drinking water samples were used as source of Enteroviruses for subsequent identification by nested RT-PCR. A 371 bp fragment located in the 5'UTR of the virus genome (Fig 1) was amplified and nucleotide sequencing of the cloned amplicons was analyzed. Representative results of RT-PCR detection of Enteroviruses are shown in Fig 3. Percentage of EnV-positive drinking waters was much lower as expected when compared to sewage. However 2/7 (28%), 4/21 (19%) and 3/14 (21%) drinking water samples were positive for EnV in Islamabad, Rawalpindi and Lahore, respectively (Table 1).

Our results showed a slightly enhanced Enterovirus detection following cell culture propagation of virus isolated from environmental samples (Fig 3). It was clear from the CPE data that 3-4 days of incubation in culture was required for raising virus concentration (Fig 3). Virus from sampling sites 11, 12, 17 and 18 (drinking water) of Rawalpindi District showed a considerable level CPE, which was confirmed by RT-PCR analysis. Some of the cell cultures with no CPE also showed RT-PCR-positive results (Table 2) indicating low virus levels released into the culture medium or the presence of non-viable viruses.

One of the objectives of present study was to identify Enterovirus type on the basis of nucleotide sequence of the virus 5'UTR region. 5'UTR fragment amplified from a virus sample obtained from Rawalpindi (Raw 17/27) was cloned and sequenced. The sequenced fragment from the study, named “EnteroPak” (NCBI GenBank accession no. JX469597) was used for the construction of a phylogenetic tree. The results revealed that the isolated virus was closely related to Coxsackievirus A isolates of Greece, USA and Singapore (Fig 4).

DISCUSSION

Multiple enterovirus strains are feecally shed in high loads from infected...
Table 1
Analysis for Enterovirus by negatively charged membrane filtration followed by nested RT-PCR in water samples from Pakistan.

<table>
<thead>
<tr>
<th>Sample number (drinking water)</th>
<th>Nested PCR results/drinking water (%)</th>
<th>Sample number (sewage water)</th>
<th>Nested PCR results/sewerage water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islamabad (n=7)</td>
<td>2/7 (8)</td>
<td>Islamabad (n=15)</td>
<td>4/15 (27)</td>
</tr>
<tr>
<td>Lahore (n=14)</td>
<td>3/14 (21)</td>
<td>Lahore (n=27)</td>
<td>17/27 (30)</td>
</tr>
<tr>
<td>Rawalpindi (n=21)</td>
<td>4/21 (19)</td>
<td>Rawalpindi (n=36)</td>
<td>7/36 (47)</td>
</tr>
</tbody>
</table>

Table 2
Cytopathic effect (CPE) of virus inoculation in HeLa cells and subsequent RT-PCR results of randomly selected drinking and sewage water samples from Islamabad, Rawalpindi and Lahore, Pakistan.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>CPE</th>
<th>Subsequent RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl-1 (drinking)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isl-3 (drinking)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isl-4 (sewage)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isl-5 (sewage)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Isl-7 (drinking)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lah-4 (drinking)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lah-5 (drinking)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lah-6 (drinking)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lah-9 (drinking)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lah-22 (sewage)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lah-23 (sewage)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lah-27 (sewage)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raw-11 (drinking)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raw-12 (drinking)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raw-17 (drinking)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raw-18 (drinking)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raw-27 (sewage)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raw-33 (sewage)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raw-37 (sewage)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Isl, Islamabad; Lah, Lahore; Raw, Rawalpindi.

individuals (Zhang et al., 2013). The enteric viruses survive in the environment and retain the capability to cause infection, which depends on such factors as type of water and season (Hunter, 1997).

Human enteric viruses are anticipated as alternative indicators of sewage contamination; however, their detection is difficult owing to their low viral concentrations in the environment. Previously described methods for the recovery of Enteroviruses in environmental samples are often incompatible with the subsequent PCR-based detection of viruses.
Fig 4–Phylogram of Enterovirus. The phylogenetic tree was constructed using PhyML and maximum likelihood approach using ATGC bioinformatics platform; tree generated with 100 bootstraps was viewed by Archaeopteryx. Numbers represent node support and horizontal lines evolutionary events in terms of base substitutions. Box is the sequence from current study.

Suspended solids could also reduce virus recovery during PEG precipitation. New approaches are required for concentrating virus and removal of inhibitory compounds, especially in the case of detection of low levels of viruses (Connell et al., 2012). In the present study integration of the initial steps of sewage solids separation and virus removal from separated solids resulted in an improved recovery of the viruses. Results of this study are in line with previous reports documenting a much high titer in cell culture experiments for enterovirus (Liu et al., 2011).

The high percentage of EnV-positive drinking water is an alarming situation and is indicative of a high risk of infection in these metropolitan areas. In all these three cities sewage treatment plants are either nonexistent or considered to be insufficient for the removal and inactivation of enteric viruses. Nevertheless, tap water is anticipated to be exposed to contamination originating from seepage of the sewerage distribution system, leading to gastroenteritis (Mellou et al., 2014). This study highlights the high frequency of EnV in the area. EnV were detected almost every month in sewage waste water, indicating a constant potential threat to the inhabitants of the sampling site. A risk assessment study of rotavirus and adenovirus has shown that one infection unit of rotavirus could cause infection, whereas for adenovirus an annual risk of infection in drinking water is 1 pfu/1,000 liters (Sinclair et al., 2009; Phillips et al., 2010). According to US EPA recommendation, viral load in tap water should not present a risk of infection > 1:10,000 per year (Gwimbi, 2011). Our results indicate significant risk to human health. Though this study comprises of samples from a limited geographical area and the numbers of tested samples were not very extensive but the percentage of viral occurrence especially of enteric viruses in drinking water was very high and explains the high frequency of viral gastroenteritis outbreaks among these communities.

On average Pakistan Institute of Medical Sciences (PIMS) receives 70 to
90 adult patients with gastroenteritis in its emergency department, and 25 to 30 patients in the children emergency every 24 hours, with over 10,000 gastroenteritis patients in six months (Wu et al, 2013). An estimated 90 to 100 patients suffering from gastroenteritis visit the Emergency Department of PIMS and the Federal Government Services Hospital (FGSH) (Azizullah et al, 2011). An estimated 6,000 gastroenteritis patients report daily to public sector hospitals of Punjab, with 3,000 patients reported in the provincial capital Lahore alone (Azizullah et al, 2011). The observed contamination of drinking water can be anticipated as one of the major possible mediators of these incidents in addition to several undocumented reports of viral gastroenteritis in these vicinities (Azizullah et al, 2011).

5'UTR is a highly conserved region of the viral genome and it plays a vital role in the regulation of transcription and translation of viral genome and hence this region is used as the target for comparative sequence analysis, allowing rapid PCR-based detection of the majority of human Enteroviruses (Thao et al, 2010; Guerrero-Latorre et al, 2011). Our sequence results indicate that the local isolate enteroPK was related to an Enterovirus isolate from Singapore and Coxsackievirus A2 isolate from Greece, Singapore and USA. As the node support value of EnteroPak is not very strong, this indicates that despite the close clustering the local isolate might have evolved further. More extensive studies comparing several sequences from local virus isolates are recommended.

In conclusion, the observed high rate of contamination by Enteroviruses of untreated sewage water and unlined dumping channels poses a potential threat to close lying drinking water distribution systems especially in highly populated areas of Pakistan. Enterovirus contamination of drinking water is one of the major causes associated with frequent gastroenteritis in these areas. We suggest that cell culture or negatively charged membrane filter method can be confidently used for viral detection. However, the membrane filter method is much more cost effective and time saving and we recommend this protocol for regular monitoring of viruses in environmental, sewage and drinking water samples. We further recommend continuous monitoring of a wide range of target viruses especially in drinking water. We also propose installation of more sewage treatment units and proper filtration systems for drinking water resources in the studied areas. A more detailed extensive study is required to establish the true spectrum of human enteric viruses that constitute a potential threat to human life.

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