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

Tahir Ahmad[1], Sadia Anjum[1], Muhammad Sohail Afzal[2], Hamid Raza[2], Najam-us-Sahar Sadaf Zaidi[1] and Najma Arshad[2]

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

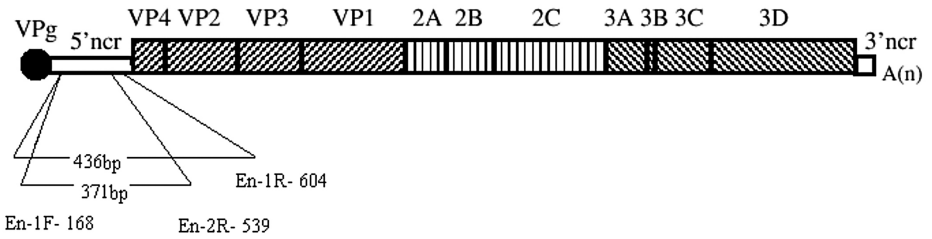


Fig 1–Schematic diagram of Enterovirus single strand RNA genome. Regions VP1-VP4 encode open reading frame 1 and code for capsid proteins VP1, VP2, VP3 and VP4, respectively. Regions 2A-3A encodes open reading frame 2 and regions 3A-3D open reading frame 3. 3'ncr and 5'ncr represents non-coding region at 3' and 5' end, respectively. A(n) is poly A sequence.

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; Suffredini *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 ($n = 2$l) were collected from the same localities. Aliquots of 50 ml of waste samples were centrifuged at 10,000$g$ 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

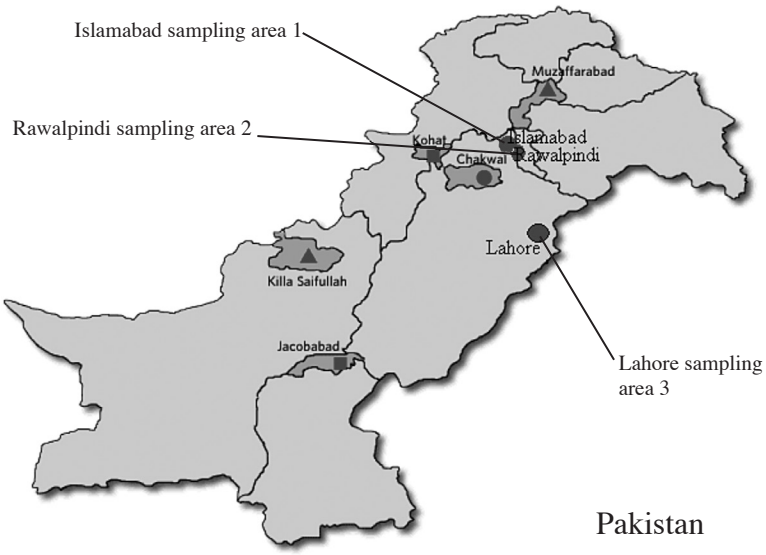


Fig 2–Location of water sampling sites in three metropolitan cities of Pakistan.

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 4ºC. The solutions were centrifuged at 10,000*g* for 90 minutes and pellets was re-suspended in 4 ml aliquots of 0.1 M phosphate buffer pH 8.0 and kept at -80ºC 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 $MgCl_2$ 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 $H_2SO_4$ (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 $H_2SO_4$ and 50 μl of 100X TE buffer (pH 8.0) were added. The samples then were centrifuged at 1,500*g* for 15 minutes at 4ºC in a vacuum concentrator (Eppendorf, Hamburg, Germany) to reduce the final volume to 50 μl.

**Virus propagation in cell culture**

HeLa cells ($10^5$ 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 37ºC under an atmosphere of 6% $CO_2$. Cells were sedimented at 4,000*g* for 1 minute at 4ºC, 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'ATTGTCACCATAAGCAGCCA3'; 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 42°C for 1 hour. The 25 µl first round PCR mixture contained 1X buffer (Invitrogen), 0.4 mM $MgCl_2$, 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: 95°C for 3 minutes; 35 cycles of 94°C for 40 seconds, 50°C for 45 seconds, and 72°C for 45 seconds; and a final step of 72°C 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 *Eco*R1 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 *Eco*R1 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 phylogenic tree was constructed using PhyML and

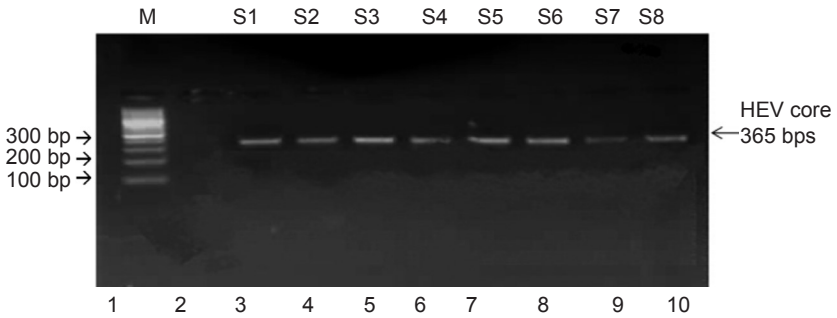


Fig 3–Agarose gel-electrophoresis of 365 bp amplicon from 5'UTR of Enterovirus. RNA extracted from isolated virus samples using either PEG/NaCl precipitation or negatively charged membrane filtration technique were amplified by nested RT-PCR and analyzed by 2% agarose gel-electrophoresis. Lane 1, M: 100 bp DNA size markers; lane 2, negative control; lanes 3 and 4, duplicate sample amplified after PEG/NaCl precipitation method; lanes 5 and 6, s duplicate sample amplified after negatively charged membrane filtration; lanes 7 and 8, duplicate sample amplified after cell culture propagation for 4 days; lanes 9 and 10, duplicate sample amplified after cell culture propagation for 2 days.

Maximum likelihood approach on ATGC bioinformatics platform. The phylogenic tree generated with 100 bootstraps was viewed by Archaeopteryx (Guindon and Gaseuel, 2003). HCV isolate AJ621219 5UTR 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 fecally 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.

| Sample number (drinking water) | Nested PCR results/drinking water (%) | Sample number (sewage water) | Nested PCR results/sewerage water (%) |
|---|---|---|---|
| Islamabad (*n*=7) | 2/7 (8) | Islamabad (*n*=15) | 4/15 (27) |
| Lahore (*n*=14) | 3/14 (21) | Lahore (*n*=27) | 17/27 (30) |
| Rawalpindi (*n*=21) | 4/21 (19) | Rawalpindi (*n*=36) | 7/36 (47) |

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.

| Sample number | CPE | Subsequent RT-PCR |
|---|---|---|
| Isl-1 (drinking) | - | - |
| Isl-3 (drinking) | - | - |
| Isl-4 (sewage) | + | + |
| Isl-5 (sewage) | - | + |
| Isl-7 (drinking) | - | - |
| Lah-4 (drinking) | - | + |
| Lah-5 (drinking) | - | - |
| Lah-6 (drinking) | + | + |
| Lah-9 (drinking) | - | + |
| Lah-22 (sewage) | + | + |
| Lah-23 (sewage) | + | + |
| Lah-27 (sewage) | - | - |
| Raw-11 (drinking) | + | + |
| Raw-12 (drinking) | + | + |
| Raw-17 (drinking) | + | + |
| Raw-18 (drinking) | + | + |
| Raw-27 (sewage) | - | - |
| Raw-33 (sewage) | + | + |
| Raw-37 (sewage) | - | - |

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.

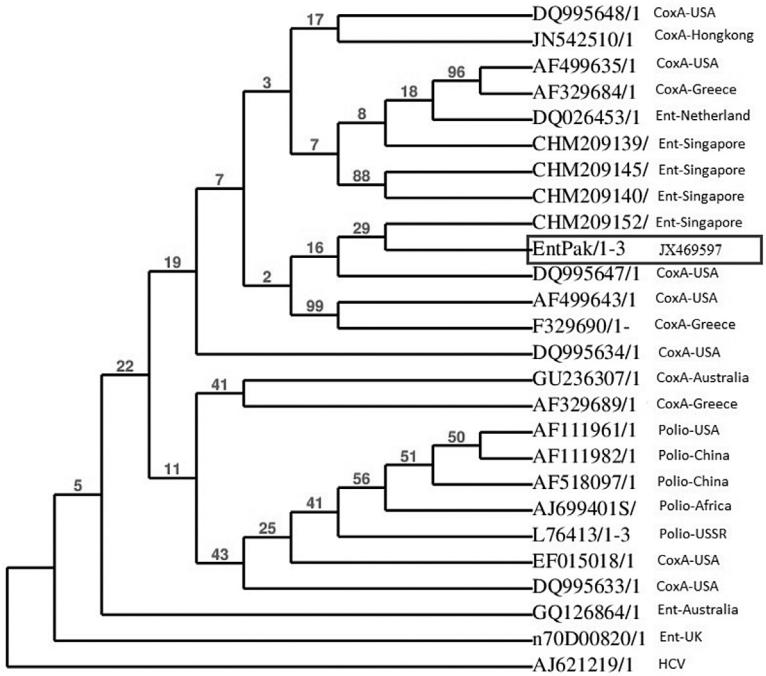


Fig 4–Phylogram of Enterovirus. The phylogenetic tree was constructed using PhyML and maximum likelihood approach using ATGC bioinformatics platform; tree generated with 100 bootstraps was viewed by Archaeopteryx. Numbers represent node support and horizontal lines evolutionary events in terms of base 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 mediator 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.

## REFERENCES

Anjum s, Ali S, Ahmad T, *et al*. Sequence and structural analysis of 3′ untranslated region of hepatitis C virus, genotype 3a, from Pakistani isolates. *Hepa Mon* 2013 May 9; 13 (5): e8390.

Azizullah A, Khattak MNK, Richter P, Häder D-P. Water pollution in Pakistan and its impact on public health–a review. *Environ Int* 2011; 37: 479-97.

Benjamin L, Kelly M, Cohen D, *et al*. Detection of herpes viruses in the cerebrospinal fluid of adults with suspected viral meningitis in Malawi. *Infection* 2013; 41: 27-31.

Blomqvist S, El Bassioni L, Nasr EM, *et al*. Detection of imported wild polioviruses and of vaccine-derived polioviruses by environmental surveillance in Egypt. *Appl Environ Microbiol* 2012; 78: 5406-9.

Chung JY, Han TH, Park SH, Kim SW, Hwang ES. Detection of GII-4/2006b variant and recombinant noroviruses in children with

acute gastroenteritis, South Korea. *J Med Virol* 2010; 82: 146-52.

Connell C, Tong H-I, Wang Z, Allmann E, Lu Y. New approaches for enhanced detection of enteroviruses from Hawaiian environmental waters. *PLoS ONE* 2012; 7: e32442.

Centers for Disease Control and Prevention (CDC). Enterovirus surveillance--United States, 2002-2004. *MMWR Morb Mortal Wkly Rep* 2006; 55: 153-6.

Duintjer Tebbens RJ, Pallansch MA, Kim JH, *et al*. Oral poliovirus vaccine evolution and insights relevant to modeling the risks of circulating vaccine-derived polioviruses (cVDPVs). *Risk Anal* 2013; 33: 680-702.

Froger A, Hall JE. Transformation of plasmid DNA into *E. coli* using the heat shock method. *J Vis Exp* 2007; (6): 253.

Guerrero-Latorre L, Carratala A, Rodriguez-Manzano J, Calgua B, Hundesa A, Girones R. Occurrence of water-borne enteric viruses in two settlements based in Eastern Chad: analysis of hepatitis E virus, hepatitis A virus and human adenovirus in water sources. *J Water Health* 2011; 9: 515-24.

Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 2003; 52: 696-704.

Gwimbi P. The microbial quality of drinking water in Manonyane community: Maseru District (Lesotho). *Afr Health Sci* 2011; 11: 474-80.

He Y-Q, Chen L, Xu W-B, *et al*. Emergence, circulation, and spatiotemporal phylogenetic analysis of Coxsackievirus A6-and Coxsackievirus A10-associated hand, foot, and mouth disease infections from 2008 to 2012 in Shenzhen. *Chin J Clin Microbiol* 2013; 51: 3560-6.

Hovi T, Blomqvist S, Nasr E, *et al*. Environmental surveillance of wild poliovirus circulation in Egypt–balancing between detection sensitivity and workload. *J Virol Methods* 2005; 126: 127-34.

Hunter P. Waterborne disease: epidemiology and ecology: Chichester: John Wiley & Sons, 1997.

Joffret M-L, Jégouic S, Bessaud M, *et al*. Common and diverse features of cocirculating type 2 and 3 recombinant vaccine-derived polioviruses isolated from patients with poliomyelitis and healthy children. *J Infect Dis* 2012; 205: 1363-73.

Khetsuriani N, LaMonte-Fowlkes A, Oberst S, Pallansch MA. Enterovirus surveillance–United States, 1970-2005. *MMWR Surveill Summ* 2006; 55: 1-20.

Liu J, Kibiki G, Maro V, *et al*. Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. *J Clin Virol* 2011; 50: 308-13.

Martinelli D, Prato R, Chironna M, *et al*. Large outbreak of viral gastroenteritis caused by contaminated drinking water in Apulia, Italy, May-October 2006. *Euro Surveill* 2007; 12: E070419.

Mellou K, Katsioulis A, Potamiti-Komi M, *et al*. A large waterborne gastroenteritis outbreak in central Greece, March 2012: challenges for the investigation and management. *Epidemiol Infect* 2014; 142: 40-50.

Pérez-Sautu U, Sano D, Guix S, Kasimir G, Pintó RM, Bosch A. Human norovirus occurrence and diversity in the Llobregat river catchment, Spain. *Environ Microbiol* 2012; 14: 494-502.

Phillips G, Lopman B, Rodrigues LC, Tam CC. Asymptomatic rotavirus infections in England: prevalence, characteristics, and risk factors. *Am J Epidemiol* 2010; 171: 1023-30.

Poddar SK. Influenza virus types and subtypes detection by single step single tuble multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. *J Virol Methods* 2002; 99: 63-70.

Sinclair R, Jones E, Gerba C. Viruses in recreational water-borne disease outbreaks: a review. *J Appl Microbiol* 2009; 107: 1769-80.

Suffredini E, Magnabosco C, Civettini M, Ros-

setti E, Arcangeli G, Croci L. Norovirus contamination in different shellfish species harvested in the same production areas. *J Appl Micorbiol* 2012; 113: 686-92.

Sun Q, Zhang Y, Zhu S, *et al*. Complete genome sequence of two coxsackievirus A1 strains that were cytotoxic to human rhabdomyosarcoma cells. *J Virol* 2012; 86: 10228-9.

Thao NTT, Ngoc NTK, Tú PV, *et al*. Development of a multiplex polymerase chain reaction assay for simultaneous identification of human enterovirus 71 and coxsackievirus A16. *J Virol Methos* 2010; 170: 134-9.

Tong H-I, Lu Y. Effective detection of human adenovirus in Hawaiian waters using enhanced PCR methods. *Virol J* 2011; 8: 57.

Wringe A, Fine PE, Sutter RW, Kew OM. Estimating the extent of vaccine-derived poliovirus infection. *PLoS ONE* 2008; 3: e3433.

Wu W, Guo X, Yang Y, *et al*. The prevalence of functional gastrointestinal disorders in the Chinese air force population. *Gastroenterol Res Pract* 2013 (2013). doi10.1155/2013/497585.

Zhang Y, Riley LK, Lin M, Purdy GA, Hu Z. Development of a virus concentration method using lanthanum-based chemical flocculation coupled with modified membrane filtration procedures. *J Virol Methods* 2013; 190: 41-8.