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ORIGINAL ARTICLE

Assessment of the risk for human health of Enterovirus and Hepatitis A virus in clinical and water sources from three metropolitan cities of Pakistan

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Abstract

Introduction. Molecular studies have confirmed the silent circulation of enterovirus (EntV) and hepatitis A virus in the environment, even in the absence of clinical manifestation. Viral pathogens are among the major causes of disease outbreaks, particularly in the bigger cities and both in the developed and underdeveloped nations.

Materials and method. Between June 2016 – June 2017, 97 samples of drinking water, river water polluted with sewage and blood were selected and obtained from high risk communities in Pakistan. Negatively charged membrane filters were used to concentrate the virus, followed by the use of specific PCR primers set for quick identification of the waterborne viruses.

Results. Enteroviruses were recovered from 40%, 28.57% and 33.33% of river water polluted with sewage samples in Lahore, Islamabad and Rawalpindi, respectively, while the presence of 13.13% and 11.76% of viral load was also confirmed in the drinking water of Lahore and Rawalpindi, respectively. A high prevalence of HAV (12.5% and 21.05%) was also verified in the clinical samples. Phylogenetic analysis indicated close resemblance of HAV isolates with the Indian strains. This study is the first ever comparative analysis of the EntV and HAV isolated from environmental samples and clinical specimen on a molecular level.

Conclusions. The parallel surveillance of EntV and HAV in the river water polluted with sewage, and clinical samples is quite helpful for controlling and reducing the disease burden of the waterborne illnesses.

Key words

epidemiology, Enterovirus (EntV), HAV, PCR, virology

INTRODUCTION

Viral pathogens are among the major causes of disease outbreaks, particularly in the bigger cities of both developed and under-developed nations. However, the burden of viral disease is more severe in the highly polluted environments [1]. The mixing of pollutants with the natural environment leads to the spread of viruses [2]. Direct or indirect exposure to both contaminated drinking and recreational waters, results in acute to mild viral gastroenteritis [2]. The virus directly sheds into the water bodies via the faecal/oral route and its presence is directly related to the efficiency of the sewage treatment process [3].

The problem of gastroenteritis is the most common among the Pakistani population. Almost all metropolitan cities, but especially Faisalabad, Peshawar, Rawalpindi, Lahore and Karachi, face a high prevalence and severity of waterborne gastroenteritis [4]. The enteroviral group is the one which is most frequently associated with gastroenteritis illness in children under the age of 5 years, irrespective of their socio-economic status [5]. It causes more than 300 deaths and 39,000 hospitalizations each year [6].

Most of the current studies have focused on the epidemiology and control of enteroviral infections, especially with reference to rotavirus and poliovirus [6]. Few studies exist for human adenovirus (HAds), human norovirus (HNoVs) and hepatitis A virus (HAV), but even less attention has been given to viral gastroenteritis in China [7]. Information regarding the incidence of viral gastroenteritis and its rate of prevalence has not yet been reported in the Pakistani population. Most of the data is available with respect to bacterial contamination through water related environmental routes. Pakistan is facing huge public health problems which are directly associated with the discharge of treated and untreated sewage water. Few treatment plants are working in Pakistan which has been considered as having a below average monitoring system with respect to viral surveillance [8]. Environmental pollution is worsening as more people are migrating towards cities and are settling in slums, especially in Karachi. This is leading to more

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mismanagement in waste disposal and it is being dumped into rivers and the coastal environment [9].

The molecular analysis and characterization of the viruses commonly found/circulating in the urban waters polluted with sewage have been widely studied across the globe, yet this area has not been explored in Pakistan [10]. This study targets human enterovirus (EntV) and the hepatitis A virus directly from blood and water sources which are associated with public health risks. River water polluted with sewage and blood samples were collected from the Rawalpindi, Islamabad and Lahore districts of Pakistan. Molecular characterization and identification of Enterovirus (EntV) and hepatitis A virus (HAV) was performed with the assistance of specific PCR nested primers and further confirmed by nucleotide sequence analysis. Phylogenetic analyses were also performed to confirm the ancestor of these viruses with the help of data available on the NCBI gene data bank.

MATERIALS AND METHOD

Drinking water, river water polluted with sewage and blood samples. Three cities were selected for the sample collection; Rawalpindi, Islamabad and Lahore. During the one year sampling period, different samples of river water polluted with sewage from Rawalpindi, Islamabad and the Canals of Lahore were collected in sterilized bottles 2–3 times per month, from June 2016 – June 2017. 44 drinking water (2L) and 26 river water samples polluted with sewage were collected from the urban water supplies. Lahore Canal runs from the middle of this metropolitan city, and the uncontrolled sewage discharged directly into the water causes high levels of contamination (10^5 faecal coliforms/100 ml). Approximately 2 liters of canal water was also collected randomly, especially from the close vicinity of the informal settlements' discharge into the canal water. Most of the samples were collected during the dry period, i.e. April – June. Blood samples (27) were taken in ethylenediaminetetraacetic acid (EDTA) tubes. The blood and stool samples were collected from the Pakistan Institute of Medical Sciences (PIMS) Hospital and Polyclinic in Islamabad, the General Hospital and Jinnah Hospital in Lahore and Holy Family Hospital in Rawalpindi. The patients were suffering from gastroenteritis and diarrhea.

Recovery of viral particles. All EntV detected in this study were isolated from different sources of water bodies. The samples were collected from different cities and then pretreated to remove unwanted solid wastes. This was performed through the process of centrifugation at 10,000 rpm for 10 minutes, followed by the pH maintenance to 7.2–7.3 by using 1M NaOH or, in some cases, 1M HCl. After the removal of solid wastes, the supernatant was again centrifuged at 10,000 rpm for 90 minutes and the lower 2/3 phase of the supernatant was collected for further processing.

Concentration of viruses using negatively charged membrane filters. Duplicate samples of each 100 ml were centrifuged at 3,000 rpm for 45 minutes to remove the solid particles from the raw water. HA (mixed cellulose esters) negatively charged filters (Millipore, USA) with a pore size of 0.45 µm and 0.22 µm and, 47 mm diameter, were placed in a vacuum pump. First, the samples were centrifuged at 4,000 × g in a refrigerated centrifuge for the removal of suspended solid and fine particles. Briefly, 25 mM $MgCl_2$ was added to the water samples and passed through a filtration assembly. Filtration time was regulated by the vacuum pump attached to the filtration assembly. To rinse out the cations, 100 ml of 0.5 mM H_2SO_4 was passed through the membrane filter, then 5 ml of 1 mM NaOH (pH 10.5–10.8) was passed through the membrane filters and the filtrate was recovered in a falcon tube. The filtrate was neutralized by adding 0.1 ml of 100 × TE buffer and 0.1 ml of 50 mM H_2SO_4. The eluted liquid was further concentrated to 2 ml by using DNA concentrator (Eppendorf, Germany). These samples were then stored at -20 °C till the further processing.

Nucleic acid extraction. The samples were concentrated by using negatively charged membrane filters and then subjected to the viral extraction procedures. They were mixed with 500 µl TRizol reagent (Life Technology, USA), incubated for 5 minutes, after which 100 µl chloroform was added and the samples further incubated for 5 minutes at room temperature. Samples were centrifuged at 1,200 × g at 4 °C for 20 minutes. 300 µl supernatant was transferred to 1.5 ml Eppendorf tubes, 3 M sodium acetate (pH 5.2) was added at 1/10 of the supernatant volume and 100% ethanol (chilled) added at twice the volume of supernatant. This mixture was then incubated overnight at -20 °C. The overnight incubated sample was centrifuged at 1,200 × g at 4°C for 10 minutes; the obtained supernatant was discarded and the pellet was washed with 250 µl of 70% ethanol and centrifuged again at 1,200 × g at 4 °C for 10 minutes to obtain a completely dry pellet. The dry pellet was resuspended in 40 µl nucleus free water. Quantity of RNA was tested by Biophotometer (Eppendorf, USA). Aliquots of samples were stored at -20 °C. Total RNA and viral RNA extraction kits (QIAmpUltrasens virus kit, Qiagen, USA; Nucleospin total RNA/DNA extraction kit, Invitrogen, UK; virus DNA/RNA extraction kit, Genead, UK) were used for direct extraction from the environmental water samples. The extracted nucleic acid was then measured by spectrophotometer.

Molecular detection and characterization of viruses. Before proceeding to PCR, the genomes of EntV and HAV were reverse transcribed to form the cDNA. A variety of parameters were used to optimize the synthesis of the EntV cDNA. Both gene specific primers (Tab. 1) were used to make the first strand for the cDNA synthesis. Specific primers were designed by using the available sequences of the selected enteric viruses from a conserved region of the virus. These primers were optimized to detect various viruses using PCR for the DNA viruses, and RT-PCR for the RNA viruses.

The recipe for the EntV cDNA amplification is as follows: 4 µl M.Mulv Buffer, 2 µl 10 mM dNTPs, 0.2 µl RNase

Table 1. Primer sets used for PCR amplification. Reverse primer indicated as "R" in the primers code was used for cDNA synthesis

Organisms	Primers	Sequence 5′ – 3′	Nucleotide Position	Product Size
Enterovirus	En-1F	CAAGCACTTCTGTTTCCCCGG	164–184	362bp
	En-1R	ATTGTCACCATAAGCAGCCA	599–580	
	En-2R*	CTTGCGCGTTACGAC	562–512	
Hepatitis A virus	HAV FP	GTTTTGCTCCTCTTTATCATGCTATG	2109–2135	246bp
	HAV RP	GGAAATGTCTCAGGTACTTTCTTTG	2330–2355	

Inhibitor, 1.5 µl Reverse Primer, 0.6 µl RT- enzyme, 10 µl RNA template and the thermal conditions were 42 °C for 1 hour. For PCR, the following recipe was used: 5–8 µl cDNA template, 2 µl 2 mM dNTPs, 2 µl 2.5 mM $MgCl_2$, 2 µl PCR buffer, 2 µl 2 pmole F- Primer, 2 µl 2 pmole R- Primer and 0.5 µl Taq polymerases were used. Nuclease free (N/F) water was used to maintain the final volume up to 20 µl. Esco thermal cycler (Esco, USA) was used to maintain the thermal cycling conditions during the PCR. A total of for 35 cycles were carried out at 95 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1 minute, followed by the final gap filling round which was carried out at 72°C for 10 minutes.

For the molecular amplification of the hepatitis A virus (HAV), the following conditions were used for obtaining the cDNA: 4 µl M.Mulv Buffer, 2 µl 10 mM dNTPs, 1.5 µl HAV R- Primer, 0.6 µl M.Mulv Enzyme and 5 µl RNA template. The thermal conditions were 42°C for 1 hour. For the PCR contents of HAV, the following recipe was used: 5 µl cDNA template, 2.5 µl Dream Taq Buffer (Fermentas, UK), 1.5 µl 2 mM dNTPs, 1.5 µl Forward Primer, 1.5 µl Reverse Primer, 0.5 µl Dream Taq Enzyme and 7.5 µl NF- Water.

For the first round of PCR Amplification of HAV, a total of 35 cycles were carried out at 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 45 seconds, followed by the final gap filling round which was carried out at 72 °C for 7 minutes. The same concentration of reagents was used for the nested PCR and the following conditions were used in this process: after the initial denaturation step, DNA was amplified for 35 cycles with a final extension period of 7 minutes at 72 °C. Each cycle consisted of 45 seconds of denaturation at 94 °C, 45 seconds of annealing at 52 °C, and 1 minute of extension at 72 °C.

The amplified product obtained was then visualized under an ultraviolet lamp with a gel documentation system (Whealtec Dolphin, Germany) using ethidium bromide staining, and the viruses were observed according to the expected size of the PCR product. In the case of nested PCR, especially for the RNA viruses, the PCR product obtained from one round was used as a template for the other round of amplification and was then visualized on the gel documentation system.

Sequencing and phylogenetic analysis. The amplified product obtained from the second round of PCR of both HAV and EntV were purified by using a PCR purification kit (Invitrogen, UK). Purified products were directly sequenced by using a Beckman coulter Genetic Analyzer SEQ 8000 (USA). The sequences were assembled using the software CLC sequence viewer (version 7.6) and MEGA6. The sequences were aligned and a phylogenetic tree was constructed using maximum likelihood method against the other strains data obtained from NCBI GenBank.

Statistical analysis. This was performed by using IBM SPSS software version 20.0. The Spearman test for correlation and chi-square test were applied to test the relationship between the values of viral prevalence in samples collected from the various environments (drinking water, sewage polluted water and human blood) in 3 cities of Pakistan. Mann Whitney analysis was also conducted to test the differences between the prevalence of both viruses in particular cities. The p-value smaller or equal to 0.05 was assumed as significant, while the p-value between 0.05–0.1 was assumed as weakly significant.

RESULTS

A total of 44 drinking water, 26 river polluted water with sewage and 27 blood samples were collected from 3 metropolitan cities of Pakistan to test the prevalence of the 2 most common viruses, i.e. EntV and HAV (Tab. 2). The epidemiological pattern of waterborne gastroenteritis and hepatitis A virus, commonly found in some of Pakistan's major urban areas, was investigated through the use of a combination of different molecular techniques, such as polymerase chain reaction and sequencing followed by phylogenetic analysis by using CLC software and MEGA6. Drinking water and the river water polluted with sewage were used to detect the molecular surveillance of the viruses. Blood samples were also collected from the same areas in order to determine the distribution pattern of these viruses in the general population. Table 2 summarizes the distribution of waterborne viruses in the surrounding communities affected directly or indirectly by river water polluted by sewage discharge. Results indicated that out of total 44 drinking water samples, as much as 13.13% (2/15) of the drinking water samples from Lahore and 11.76% (2/17) of the samples from Rawalpindi showed the presence of EntV. However, no samples were positive for HAV from Lahore and Islamabad, whereas only 5.8% (1/17) of HAV was recovered from Rawalpindi. Similarly, in river water polluted by sewage samples, EntV has been recovered in high amounts from Lahore, Islamabad and Rawalpindi with 40%, 28.57% and 33.33% respectively. As much as 10% and 11.11% HAV was found in the samples of river water polluted with sewage in Lahore and Rawalpindi, but no HAV was recovered from Islamabad. 27 blood samples were also collected from

Table 2. Prevalence of enterovirus and hepatits A virus in samples of drinking water, river water polluted with sewage and blood samples from patients with gastroenteritis and diarrhea, collected in three metropolitan cities of Pakistan. In each field of the Table the numbers of positive/examined samples and percentage (in parentheses) are given

	Drinking water			River water polluted with sewage			Blood		
Cities	No.	EntV	HAV	No.	EntV	HAV	No.	EntV	HAV
Lahore	15	2/15 (13.13%)	0/15 (0)	10	4/10 (40%)	1/10 (10%)	0	NT	NT
Islamabad	12	0/12 (0)	0/12 (0)	7	2/7 (28.57%)	0/7 (0)	8	0/8 (0)	1/8 (12.5%)
Rawalpindi	17	2/17 (11.76%)	1/17 (5.8%)	9	3/9 (33.33%)	1/9 (11.11%)	19	2/19 (10.52%)	4/19 (21.05%)
Total	44	4 (9.09%)	1 (2.27%)	26	9 (34.61%)	2 (7.69%)	27	2 (7.40%)	5 (18.51%)

*NT: not tested

different clinics and laboratories in Islamabad, Rawalpindi and Lahore. HAV was found positive with 12.5% (1/8) from Lahore and 21.05% (4/19) from Rawalpindi.

Figures 1 and 2 show the positive amplification of both EntV and HAV with 362 bp and 246 bp, respectively, for HAV by using 2% agarose gel electrophoresis and 1% TAE buffer. Two HAV samples were sequenced by using Beckman Coulter DNA Genetic Analyzer (USA), sequences were submitted in the Genbank with KX095244 and KX093245 accession numbers. A phylogenetic tree was constructed by using the CLC Bio sequence viewer and MEGA6 software.

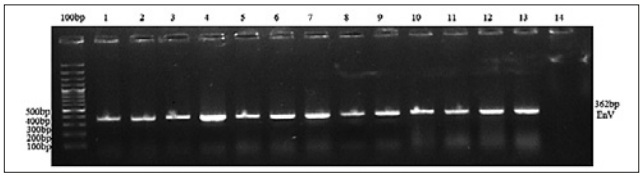

Figure 1. Representative results of 2% agarose gel electrophoresis showing amplified fragment of enterovirus. First row from left shows 100 base pair ladder, lanes 1–13 show enterovirus positive results, lane 14 is a negative control

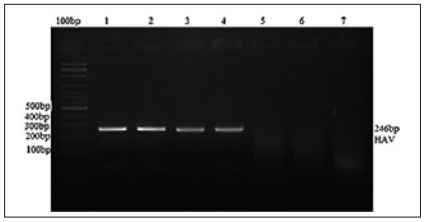

Figure 2. Representative result of 2% agarose gel electrophoresis showing PCR amplification of HAV. Extreme left lane represents 100 bp ladder, lanes 1–4 represent positive results with 246 bp PCR product, while lanes 5–7 represent negative results with no amplification

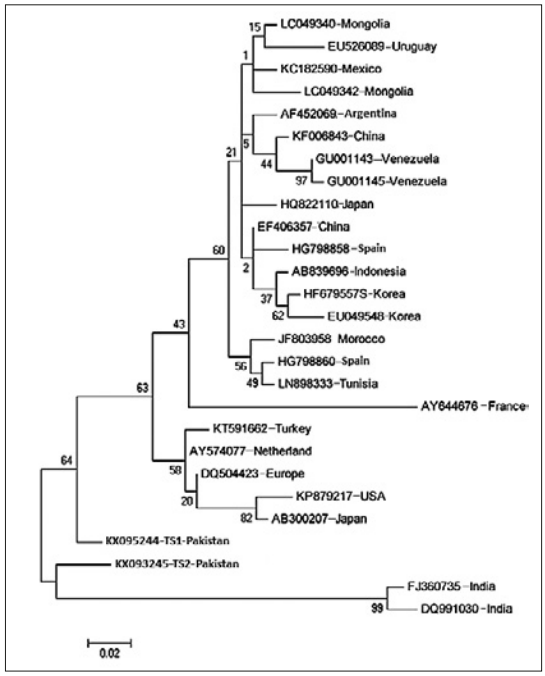

Figure 3. Phylogenetic tree based on the partial sequence of HAV strains from clinical samples. Both strains were indicated by TS1-Pak and TS2-Pak. The Indian sequences FJ36073 and DQ991030 were used as an out group. Phylogenetic analysis was based on a 186-nucleotide fragment of the open reading from of HAV by using MEGA6

The evolutionary history was inferred by using maximum likelihood method which is based on the Tamura-Nei model [11]. According to this model, both HAV strains from Pakistan are phylogenetically very close not only to the Indian strains but also to the strains found in Turkey and Japan (Fig. 3). The EntV serotype 71 and HAV serotype 1 (subgenotype IA) were found in the results of this study.

A Spearman correlation analysis with the use of Cohen's standard [12] and nonparametric chi-square test were applied to test the relationship between the prevalence of viruses in drinking water, sewage polluted water and human blood. A significantly moderate positive correlation was found between the prevalence of Hepatitis A Virus (P-HAV) in drinking water and in sewage polluted water within all the cities of Lahore, Islamabad and Rawalpindi ($r=0.33$; $p=0.023$). A stronger positive correlation was observed between prevalence of Enterovirus (P-EntV) in drinking water and in sewage polluted water ($r=0.44$; $p=0.002$). A weakly significant positive correlation was observed between P-EntV in drinking water and human blood ($r=0.281$; $p=0.059$). No significant correlation was found between P-HAV in drinking water and human blood ($r=-0.15$; $p=0.9$), nor between P-HAV in sewage polluted water and human blood ($r=-0.4$; $p=0.79$).

Another test, a nonparametric chi-square analysis, was applied to test the relationship between the prevalence of viruses in drinking water, sewage polluted water and human blood within the particular cities. A significant correlation between the prevalence of P-EntV in these environments was found for Islamabad and Rawalpindi ($X2=32.7$ and 64.3, respectively, $p=0.000$ for both cities), but not for Lahore ($X2=12.3$; $p=0.15$). In the case of P-HAV, this correlation was stronger and significant for all cities ($X2=64.1$, 69.3 and 47.7, respectively, $p=0.00$ for all cities).

Summarizing, the analysis showed that the values of virus prevalence in drinking water, sewage polluted water and human blood were dependent on each other [13].

Mann Whitney analyses were also conducted to test the differences between the prevalence of both viruses in particular cities. It was observed that the prevalence values of P-EntV and P-HAV were significantly, or weakly significantly higher in Lahore compared to Islamabad ($U=609.0$ and 635; $p=0.050$ and 0.055, respectively. The differences between the prevalence values of both viruses in Lahore versus Rawalpindi and in Rawalpindi versus Islamabad were not significant ($p>0.05$).

DISCUSSION

This study helped in gaining an overview about the epidemiology of the waterborne viral diseases spread by contaminated water in the 3 metropolitan cities of Pakistan. The presence of 2 viruses was investigated by molecular method followed by sequence analysis. This allowed a highly precise and sensitive identification of the human pathogenic viruses found naturally in the urban sewers. Specific samples were obtained from highly congested areas from the 3 selected cities. Previously, there was no data about the genetic diversity of the viruses from the geographical areas targeted in this study.

The prevalence of EntV and HAV enabled comparison of the data on prevalence of the enteric viruses in Pakistan with

that of the river water polluted by sewage and the polluted (or sewage mixed) aquatic environment worldwide. The results indicate that EntV is present in almost all samples throughout the year, which is in accordance with the results obtained for the other geographical regions reported previously [14]. Statistical analysis revealed that the most significant results were obtained for the presence of enterovirus among the polluted samples, with a correlation of 0.678. Overall, EntV were the most commonly detected viruses in the highly polluted sites of the 3 metropolitan cities of Pakistan. These areas received untreated household waste. The average population of Lahore, Islamabad and Rawalpindi is about 8.741, 1.386 and 2.506 million inhabitants, respectively, with the majority belonging to low income households [15]. The organic and viral pathogens received from these inhabitants were the EntV, therefore these viruses are very important from an epidemiological point of view.

The RT-PCR methods utilized in this study by using specific primers allowed the characterization of EntV and HAV, not only in the urban river water polluted with sewage, but also from those which were recovered from drinking water sources. These findings are in accordance with the results obtained from previous studies conducted elsewhere [16].

There are very few epidemiological reports presently available on EntV and HAV in Pakistan, therefore, the presented data represents the first documentation of EntV and HAV strain circulating in this geographical area. The HAV load was found to be highest in the blood samples obtained from Rawalpindi (21.05%), having a correlation of 0.664 at a p value less than 0.01.

The EntV reported worldwide have been associated with a significantly large number of gastroenteritis cases [17]. In this presented study, EntV is the predominant virus which is prevalent not only in river water polluted with sewage (40%) but also in the drinking water (13.13%). EntV are the small RNA viruses which have been divided into 5 groups and many serotypes (EV-68 – EV-71). Serological studies indicate that EntV could be divided into 71 serotypes on the basis of the antibody neutralization test [18]. PCR sequencing is a more reliable technique compared to the serotyping method because some EntV cannot be identified by serotyping as the specific antibodies are not present in the antisera panel [19]. Such a limitation can be overcome by molecular methods, e.g. RT-PCR. Therefore, the true importance of evolutionary mechanisms of EntV cannot be fully understood by the serotyping method.

HAV, like EntV, has been among the public health concerns in many countries. Brazil has been reported to be an area having a high prevalence of these viruses, especially in children up to 5 years of age [20]. However, EntV and HAV infections have dramatically declined due to improvement in the standards of hygiene and sanitation in recent years [21]. The new epidemiological pattern has increased the potential risk for large scale epidemics [22]. Phylogenetic analysis was performed on the basis of maximum likelihood method by using MEGA6 which indicate that Pakistani strains are very much close to the Indian strains (Fig. 3). A previous report for the similarity of the strains was reported with reference to Hepatitis E virus (which is also an enteric virus). A similar strain was prevalent throughout Asia, with highly similar strains being present in India, China and Pakistan [23]. According to the PCR results and phylogenetic Analysis, the EntV serotype 71 [24] and HAV serotype 1 (subgenotype IA) [25] are the most prevalent serotypes in Asia and match the results presented in the current study.

Different methods of viral RNA extraction were used to evaluate the diagnosis of EntV and HAV by PCR. The virus shed to the environment is diluted many times and there are many PCR inhibitory substances. The adsorption elution method by using negatively charged membrane filters is the best choice for the concentration of viruses from water sources [26]. For the easy recovery and removal of inhibitory substances, the proteinase K method was used to eliminate the inhibitory factors [27]. Compared to blood, other environmental samples contained diverse flora which have different dietary components in them that make the RNA extraction difficult. The purity of RNA extraction from heterogeneous material is critical for the downstream process. RNA extraction with phenolchloroform is the most labour intensive method which requires safety measures for the proper handling and disposal of phenol. In the presented study, the TRizol method was used for the easy recovery of viral RNA from serum samples, as previously demonstrated by other researchers [28].

Pakistan does not have a proper programme for the disease surveillance. The molecular detection and characterization by using the PCR method not only provides relevant location and specific epidemiological data, but also gives an alternative strategy to establish the health-based targets. These findings are in accordance with the water quality limits for pathogen presence, as recommended by the World Health Organisation (WHO) [29].

There is no proper sewage disposal system in the studied cities, and sewage is discharged directly into the open rivers and canal with subsequent adverse effects on human and ecological life [30]. Therefore, the health risks associated with the transmission of viruses through water bodies or the sewage discharges needs to be addressed by proper monitoring programmes. There is no legal system for the regulation of viruses in Pakistan such as exists among the developed countries of the world [31]. EntV have been suggested as the viral makers that are used to identify and monitor the faecal contamination in water [32]. The anthropogenic impact on the aquatic environment may play an important role in the epidemiology of waterborne diseases in the 3 studied cities of Pakistan. The detection and identification of these viral problems associated with urban sewage discharge in Pakistan could be addressed through national and international scientific cooperation, thus providing assistance in reducing the global burden of waterborne diseases and environmental degradation.

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Competing interest

The author declares that they have no competing interest and all authors contributed equally.

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