Detection of human adenovirus, rotavirus, and enterovirus in tap water and their association with the overall quality of water

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Running title: Detection of pathogenic viruses in drinking water

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Abstract

Drinking water supplies in the developing world often serve as a biosphere for various organisms. Viral gastroenteritis is a neglected area of research in Pakistan, there is no data for the prevalence of enteric viruses in drinking water of the largest city of Karachi. The present study aimed to provide a survey of the existence of enteric viruses: human adenovirus (HAdV), human enteroviruses (hEV), and genotype A rotavirus (GARV) in tap water. Using a simple PCR approach, we detected 20%, 43%, and 23% of HAdV, hEV, and GARV in tap water respectively. We have also shown an overall quality of tap water at the pumping station and consumer tap. Moreover, we assessed the efficiency of small-scale water treatment methods for the removal of viruses.

Keywords: Tap water; human adenovirus; enteroviruses; rotavirus; viral gastroenteritis

Introduction

Water a fundamental need for life has a significant contribution to human health. Access to clean and safe drinking water is considered a basic human right globally, however, more than 2 billion people around the world have no luxury of drinking safe water [1]. Approximately 3.4 million people lost their lives every year fighting water-related diseases, making it the leading cause of death around the world. Ingestion of filthy water kills 4,000 children each day and most of them are from continent Africa and Asia [2]. Waterborne viral diseases like Gastroenteritis has a significantly large contribution to water-related diseases and deaths. Contamination of water with enteric viruses generally transmitted by the fecal-oral route are common causes of diarrhea in infants and children [3]. Human adenovirus (HAdV), human enteroviruses (hEV), and genotype A rotaviruses (GARV) are commonly reported in drinking water and mostly associated with waterborne gastroenteritis [4]–[6].

HAdV is a tough non-enveloped, double-stranded DNA virus, having more than 57 serotypes out of which serotype 40/41 are the major causative agents of gastroenteritis. Since the survival characteristic of HAdV is not fully understood, the United States Environmental Protection Agency (USEPA) listed adenovirus as one of the nine microorganisms on the contamination candidate list for drinking water [7]. hEVs are positive-sense RNA viruses, replicate in the respiratory tract and the gut, and mostly transmitted through the fecal-oral route [8]. hEV Infections are often mild however they may cause problems in infants [9]. Rotavirus is a double-stranded, segmented RNA virus, classified in seven groups: A-G. Group A, B and, C are known to infect human, out of which group A is the most prominent cause of severe gastroenteritis [8], [10].GARV infections are the major cause of dehydrating gastroenteritis in neonates and the largest contributor to children's death in the developing world [11].

Karachi is the largest and the most populated city of Pakistan with more than 16 million inhabitants, in the total area of 3,780 km². Karachi is a highly urbanized and the most industrialized city, also known as the hub for the country's economy which attracts people from all over the country for earning their lives. The city has become a meshwork of concrete with diminishing green belts which is associated with several environmental and health hazards. Access to safe-water has emerged as the most important problem in recent years. Poor infrastructure for water supply, collapsed system for sanitation, and negligence in pre-water treatments are leading causes for several water-borne diseases in the community [12]. Unfortunately, there is no data available for the prevalence of potentially pathogenic viruses in water, people drinking at different parts of the city. We have performed a comprehensive study to evaluate the quality of water and detection of HAdV, hEV, and GARD

in water supplied in different parts of the city. We are providing experimental evidence that unsound and inadequate water supply system has serious health implications. We for the first time not only presenting prevalence data for pathogenic viral contaminants in tap water but also the efficiency of small-scale treatment procedures for the removal of viruses.

Materials and methods

Collection of water samples

From Pumping Stations: Water from three main pumping stations of Karachi: COD filter plant, NEK new filter plant, and PIPRI filter plant were collected before and after water treatment.

From General circulation: Only fresh tap water which was supplied from main lines operated and maintained by municipal authorities were collected during sampling. Water samples were collected mainly from food selling shops like restaurants, tea shops, juice shops, and dairy shops. A total of 30 samples were collected: 20 from food selling outlets, 5 from local medical care clinics, and 5 from residential houses. Each sample was collected in two separate bottles of 1 liter. Water samples were kept at 4°C until processed for water concentration.

Physical, chemical and bacteriological assessment of water

A pH meter, conductivity meter, and a turbidity meter were used for the determination of pH, total dissolved solids (TDS), Electrical Conductivity (EC) and turbidity in water samples. The amount of chlorine in the water was estimated by standard colorimetric method using N, N-dialkyl-1,4-phenylenediamine (Sigma-Aldrich, Germany) commonly known as the DPD method [13]. The total number of live and culturable microorganisms was measured by the heterotrophic plate count (HPC) method [14]. Briefly, ten-fold serial dilutions of water samples were prepared in sterile PBS. One ml of each dilution was mixed with liquefied plate count agar and poured onto Petri dishes. Petri dishes were incubated at 35 °C for 48 ±2 h. The total number of coliforms was determined by multiple-tube fermentation techniques [15]. Briefly, for the presumptive test, ten-fold serial dilutions of samples were prepared in peptone water (Sigma-Aldrich, Germany) and incubated for 24-48 hours at 35 °C. Subsequently, tubes showing lactose fermentation with gas production were considered positive for coliforms. For confirmation, loopful from each positive sample tube was inoculated into Brilliant Green Lactose Bile (BGLB) broth tubes, (Merck, Germany) and incubated at 35°C for 24 h. Bacterial growth and gas production in BGLB tubes confirmed the presence of coliforms.

Assessment of water treatment processes on viral detection

To understand how commonly used pre-water treatment processes affect the detection of HAdV, hEV, and GARV, we have performed the detection of viruses before and after the treatment. Five samples all collected from residential houses in different locations were analyzed for the effect of commercially available 3-stage water purification system which contains polypropylene yarn for first stage filtration of suspended particles by sedimentation, second stage granular activated carbon for the removal of taste and odor of most organic chemicals, chlorine, insecticides, pesticides, herbicides, followed by a third stage UV post-treatment to give pure, pleasant-tasting drinking water. Moreover, the effect of heat treatment at 100 °C for 1 min was also evaluated. Water samples were analyzed before and after each treatment process for the comparison.

Water concentration

Water samples were processed for the concentration of viral contents within 24 hours of sample collection by using a negatively charged membrane as previously described by [16], with few modifications suggested by Vecchia [17]. Briefly, Water samples were added with 10 ml of 2.5M $MgCl_2$ solution to obtain a final concentration of 25 mM, and the pH adjusted to 5 by adding 10% HCl. Subsequently, the resulting mixture was passed through a HA type negatively charged sterile membrane filter (45 mm diameter, 0.45 µm pore size; Merck Millipore), using a vacuum pump. The HA membrane filter was then rinsed with 100 mL of 0.5 mM H_2SO_4 (pH 3.0) to dissolve and remove the bound magnesium from the filter. Finally, the adsorbed virus particles were eluted with 2.5 mL of 1mM NaOH (pH 10.5). The elute was collected in a tube containing: 12.5 µL of 100 × Tris-EDTA buffer and 12.5 µL of 50 mM H_2SO_4 for neutralization. The elutes were stored at -80 °C until further process.

Extraction of viral nucleic acid

Viral nucleic acids: DNA and RNA were separately extracted using QIAamp® DNA mini kit and QIAamp® viral RNA mini kit respectively as per manufacturer's protocol. 280 µL of concentrated water samples were used as initial material for nucleic acid extraction and eluted nucleic acid samples were stored at -80 °C until further process.

Complementary DNA (cDNA) synthesis

Nucleic acid extracted from water samples were processed for cDNA synthesis for the detection of GARV and hEV before PCR amplification of target DNA. First-strand cDNA was synthesized using a commercially available kit (Revert-Aid, Thermo Scientific™ USA) as per manufacturer's protocol using random hexamer primers. DNA from each virus-type was included as a positive control. DNA from Human mastadenovirus 5, hEV, and GARV were generously provided by Dr. Fernando Rosado Spilki, Feevale University, Brazil.

PCR amplification

Targetted regions of viral nucleic acid were amplified using a commercially available PCR master mix (Thermo Scientific™ USA) according to the manufacturer's instructions. Reaction conditions were separately optimized for each primer set. The sequences of primers, their product size, and reaction conditions are mentioned in table 1. All the reactions were performed in triplicates.

Table 1. Specific primers, their target site, oligonucleotide sequences, their product size, and reaction conditions for amplification of each primer set is given in the table.

Human Adenovirus type 40/41	
Target gene	Hexon protein, GenBank accession number: X51782.1 and X51783.1
Forward Primer	VTB1-HAdVFf: 5'- GCCTGGGGAACAAGTTCAGA-3'
Reverse Primer	VTB2-HAdVCr: 5'-GATGAACCGCAGCGTCAA-3'
Product size	137 base pairs
Reference for primers	(Wolf *et al.* 2010)
Reaction conditions	98 °C (7 min)- 40x[94 °C (1 min)-55 °C (1 min)- 72 °C (1 min)] 72 °C (7 min)
Human Enteroviruses	
Target gene	5-UTR region, GenBank accession number: JX469597.1 and KF385944.1
Forward Primer	ENT-F1: 5'-CCTCCGGCCCCTGAATG-3'
Reverse Primer	ENT-R2: 5'-ACACGGACACCCAAAGTAG-3'
Product size	116 base pairs
Reference for primers	(Tsai *et al.* 1993; Vecchia *et al.* 2012)
Reaction conditions	98 °C (5 min)- 40x[94 °C (1 min)-56 °C (1 min)- 72 °C (1 min)]- 72 °C (7 min)
Genotype A Human Rotaviruses	
Target gene	VP6 protein, GenBank accession number HM348746.1
Forward Primer	ROTAFEEVALE-FW: 5'-GATGTCCTGTACTCCTTGT-3'
Reverse Primer	ROTAFEEVALE-REV: 5'-GGTAGATTACCAATTCCTCC-3'
Product size	160 base pairs
Reference for primers	(Vecchia *et al.* 2012)
Reaction conditions	94 °C (5 min)- 40x[94 °C (1 min)-50 °C (1 min)- 72 °C (1 min)])]- 72 °C (7 min)

Results

The quality of water was examined by different physical, chemical, and bacteriological tests. Physical parameters including color, turbidity, TDS, odor, and taste were recorded. Chemical tests including pH, Chlorine, Chloride, and, Electrical Conductivity (EC) were estimated. Bacteriological tests including Heterotrophic Plate Count (HPC) and Most Probable Number (MPN) tests were performed. The results are summarized in Table 2-4.

For the initial quality of water and efficiency of water treatment procedures at the pumping stations of water supply in the city of Karachi, three water samples were collected before and after the treatment procedure. Although the performance of treatment plants was not found perfect but they are working good and the effect of each treatment step including coagulation, sedimentation, filtration, and disinfection is visible. There are 66 and 96 percent reductions in turbidity and TDS of water respectively. Similarly, chloride and EC were also found to decline 50 and 70 percent respectively. Most importantly, the disinfection procedure eliminated 99.9 % HPCs and 96.6 % MPNs from water (Table.2A) and most of the viral contaminants (Table.2B). A similar water assessment was performed at the consumer level (Table 3). The parameters of drinking water quality at the consumer level were compared with permissible limits set by WHO [18], and National Standards for Drinking Water Quality (NSDWQ), Pakistan [19]. We found alarmingly high levels of all physical, chemical, and bacteriological parameters, far above the permissible limits.

A total of 30 tap water samples were analyzed, out of which hEV were present in most of the samples, achieving the highest detection rate of 43.33%, followed by GARV 23.33% and HAdV 20%. The results are summarized in Table 4. Seven samples (23.33%) were found positive for more than one type of the tested viruses, four samples (13.33%) were found co-positive for all the tested viruses (hEV, HAdV, and GARV), six samples (20%) were found co-positive for hEV and HAdV and five samples (16.66%) were found positive for hEV and GARV. Two samples were found positive for GARV alone and none was found positive for HAdV alone. Moreover, five samples were included for the assessment of water purification and/or boiling pre-treatments for their effectiveness to remove selected viral contaminants. Both treatments: 3-stage purification system and boiling were found effective to remove nucleic acid contaminants of hEV and GARV but not HAdV (Table 4).

Discussion

In the present study, we have presented a comprehensive picture of the quality of water which is supplied in the city of Karachi for more than 200 million inhabitants. Our study for the first time investigated the presence of pathogenic viruses in tap water, which is the most common source of drinking water in the city. Physical and chemical assessment of water has shown large variation in different parameters, within 30 samples of water collected from different parts of the city which indicate either poor standards of water treatment at pumping stations or poor physical containment of water between pumping stations to the consumer. TDS of 40% water samples were found higher than 600 ppm out of which 2 samples collected from different medical centers were found 1000 and 5000 ppm. As stated in the WHO report, the TDS value of 1000 ppm indicates poor quality of water, and above 1200 ppm is specified as unacceptable for drinking water [20]. Total chloride in tap water was found less than 300 mg/L except for one sample (the same with TDS 5000 ppm), where chloride was found very high up to 2848 mg/L. Deliberate or undelibrate infusion of boring water may be a possible reason for such a high level of TDS and chlorides in tap water. Similarly, there is a large diversity in EC of samples, 53% of water samples falling above 800 µS/cm while two samples were reported abnormally high EC of 1852 and 2000 µS/cm. EC of domestic tap water typically ranges between 500-800, these higher levels of EC imply the higher concentration of dissolved salts and has similarity to groundwater [21].

Bacteriological investigation of tap water samples revealed more inquisitive findings. Out of 30 samples, no sample was found negative for HPC and also for coliform. The ranges for a total number of HPC and coliform were found 8.7×10^{2}-4.5×10^{6} CFU/mL and 210 to uncountable coliforms/100mL respectively. These higher numbers of bacteria in tap water pose a serious health concern for the general population. Both national [19] and international [18], [22] guidelines are set on zero detectable organisms in treated water. These findings indicate serious negligence in the enforcement of SOPs for water treatment and processing, poor maintenance of water pipelines, and most importantly inappropriate planning of Water supply and Sewage disposal systems [23].

In this study, our focus was the detection of pathogenic viruses in tap water and their association with the quality of water. We were able to detect all targeted viruses (hEV, HAdV, and GARV). Out of 20 sampling food outlets, 60% were found with viral contaminants in their tap water. Overall, 30 samples were collected and the most common viral contaminant was found to be hEV (43%) followed by GARV (23%) and

HAdV (20%) in tap water samples from the city of Karachi. 13% of water samples were found contaminated with all three (hEV, HAdV, and GARV) viral genomes (Table 4). Most of the samples found contaminated with viruses were also recorded higher in HPCs and MNPs. 69% of the viral contaminated samples were found with 1100 coliforms/100mL, which is the highest possible count for MNPs. This situation of the city of Karachi is far worse than other metropolitan cities of Pakistan: Islamabad, Rawalpindi, Lahore [24], and Peshawar [25]. The detection of viral genome in water samples does not directly correspond to the quality of water treatment at remote water pumping station [26]–[28] but largely depends on the supply system. Leaky pipes, faulty water reservoirs, and illegal tapping of pipelines are very common in the network of Karachi water supply and are the main reasons for poor water quality and heavy viral contamination [29].

We have also examined consumer-based common water treatment procedures for their effectivity for the removal of viral contaminants. 3-stage water purification system and boiling water are the most common water treatment methods, we investigated both methods individually and in combination. We noticed no difference in the detection of any viral genomes after boiling, whereas, 3-stage water purification system can remove the viral genomes of hEV and GARV but not HAdV. Treatment in combination has no additional effect on the detection of the viral genome. These findings show that the boiling of water does not affect PCR based detection of viruses, however, the viability and infectivity of viruses have not been analyzed.

Our study has presented an overall picture of the quality of water supplied in Karachi. For the first time, we have experimentally shown that poor design and construction of the water supply system allow different pathogens to concentrate in water. A large number of detectable bacteria and pathogenic viruses in tap water is alarming and indicate serious environmental contamination of water. This data provide evidence that tap water in Karachi is not safe for drinking without treatment. The 3-stage water purification system is a good means for small scale water purification. The presence of hEV and GARV and HAdV genome in drinking water poses a serious health risk and provides a possible reason for the very high incidence rate of viral diarrhea in Karachi.

Conflicts of interests

The authors declared no potential conflict of interest concerning the research, authorship, and/or publication of this article.

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Table 2A. Assessment of different physical, chemical, and bacteriological parameters in water supplied to the city of Karachi, before and after the treatment process at main water pumping stations.

Sample type	Sample#	Physical Parameters	Chemical Parameters	Bacteriological Parameters

		Turbidity (NTU)	TDS (ppm)	pH	Chloride (mg/L)	EC (µS/cm)	HPC (CFU/mL)	MPN (Coliforms/ 100mL)
Before Water treatment	01	0.3	2580	7.7	119	557	8.52E+05	498
	02	0.3	3400	7.4	171	835	7.74E+05	514
	03	0.3	5000	7.5	163	785	8.54E+05	668
After Water treatment	01	0.1	112	7.2	73	201	2.80E+02	12
	02	0.1	165	7.0	56	198	1.40E+02	24
	03	0.1	175	7.1	89	245	2.03E+02	19
Gross effect (average % of difference)		**66% reduction**	**96% reduction**	**6% reduction**	**50% reduction**	**70% reduction**	**99.9% reduction**	**96.6% reduction**

Table 2B. Detection of selected viral contaminants in water supplied to the city of Karachi, before and after the treatment process at main water pumping stations.

Detection of viral contaminants			
Sample	**Before Water treatment**	**After Water treatment**	**Gross effect (average % of difference)**
01	hEV		**100% reduction**
02	hEV/HAdV/GARV	HAdV	**66% reduction**
03	hEV/GARV		**100% reduction**

Table 3. Assessment of different physical, chemical and bacteriological parameters in tap water samples, in comparison to maximum permissible limits set by WHO [18], [22] and NSDWQ, Pakistan [19]

Sample type	Sample#	Physical Parameters		Chemical Parameters			Bacteriological Parameters	
		Turbidity (NTU)	TDS (ppm)	pH	Chloride (mg/L)	EC (µS/cm)	HPC (CFU/mL)	MPN (Coliforms/ 100mL)
Food outlets	1	0.1	248	7.4	88	497	**6.70E+04**	**460**
	2	0.2	246	7.9	84	494	**8.94E+04**	**1100**
	3	0.1	247	7.8	88	495	**9.87E+05**	**210**
	4	0.5	**927**	7.9	237	**1852**	**8.92E+04**	**240**
	5	0.2	**628**	8	123	560	**9.86E+04**	**1100**
	6	0.2	345	7	82	456	**9.80E+02**	**>1100**
	7	0.5	415	8	89	634	**9.84E+03**	**>1100**
	8	0.5	449	8	83	791	**5.67E+03**	**460**
	9	0.1	**890**	8	78	902	**9.81E+02**	**1100**
	10	0.2	**913**	7	121	892	**9.83E+04**	**1100**
	11	0.1	**887**	8	191	805	**9.87E+05**	**>1100**
	12	0.1	**678**	8	132	876	**9.84E+05**	**460**
	13	0.2	**987**	8	213	982	**8.75E+05**	**460**
	14	0.1	465	8	121	982	**8.72E+05**	**>1100**
	15	0.5	**782**	8	100	983	**8.74E+05**	**>1100**
	16	0.2	**813**	8	129	567	**7.85E+05**	**460**
	17	0.3	298	7	178	821	**7.62E+05**	**460**
	18	0.2	363	7.2	183	727	**7.84E+05**	**>1100**
	19	0.1	358	7.7	146	714	**8.73E+05**	**1100**
	20	0.1	354	7.9	95	712	**8.74E+02**	**1100**
Medical Centers	21	0.1	358	7.7	95	721	**8.92E+04**	**460**
	22	0.1	**1000**	8.1	241	**2000**	**4.57E+04**	**240**
	23	0.2	**5000**	7.9	**2848**	1020	**2.30E+04**	**210**
	24	0.1	403	7.8	168	801	**6.74E+04**	**240**
	25	0.1	**654**	7.7	274	1307	**9.81E+05**	**1100**
Residential Houses	26	0.1	356	7.8	95	713	**8.73E+05**	**>1100**
	27	0.2	358	7.7	102	714	**9.83E+05**	**1100**
	28	0.1	471	8	153	943	**4.56E+06**	**1100**
	29	0.1	412	7.8	139	825	**5.62E+05**	**1100**
	30	0.1	444	8	124	926	**2.38E+05**	**>1100**
Permissible limits 1-WHO, 2-NSDWQ		**<1.5[1] <5[2]**	**<600[1] <1000[2]**	**6.5-8.5[1,2]**	**<600[1] <250[2]**	**<1400[1]** ----	**0[1,2]**	**0[1,2]**

Note: values above permissible limits are mentioned bold.
Samples found contaminated with viral genome are shown in gray color.

Table 4. Detectable prevalence of selected viral contaminants and the effect of common pre-treatment household methods on the detection of viral contaminants in tap water are shown.

Sample type	Sample#	Detection of viral contaminants						
		hEV	HAdV	GARV	co-positives	Water treatments		
Food outlets	1					After the 3-stage purification system	After boiling (100 °C for 1 min)	After the 3-stage purification system + boiling (100 °C for 1 min)
	2	Positive	Positive	Positive	hEV/HAdV/GARV			
	3							
	4	Positive						
	5	Positive	Positive	Positive	hEV/HAdV/GARV			
	6							
	7	Positive						
	8							
	9							
	10	Positive	Positive		hEV/HAdV			
	11			Positive				
	12							
	13	Positive		Positive	hEV/GARV			
	14							
	15	Positive						
	16							
	17	Positive	Positive	Positive	hEV/HAdV/GARV			
	18							
	19	Positive						
	20			Positive				
Medical Centers	21							
	22	Positive	Positive		hEV/HAdV			
	23							
	24							
	25	Positive						
Residential Houses	26							
	27							
	28	Positive	Positive	Positive	hEV/HAdV/GARV	HAdV	hEV HAdV GARV	HAdV
	29							
	30	Positive				None	hEV	None
Total		**13**	**6**	**7**	**7**			