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Viral Etiology and Incidence of Acute Gastroenteritis in Water Bodies of Peshawar, Khyber Pakhtunkhwa (KPK), Pakistan

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ABSTRACT
Water contaminated with microbe's causes about 6,000 deaths of adults and children every day. According to the World Health Organization, diarrhea is responsible for the deaths of 1.8 million people every year around the world. In Pakistan around 30% to 40% of all reported diseases and deaths are due to poor water quality. Khyber Pakhtunkhwa (KP) is rather worst due to being amongst the backward and poverty stricken areas. In 1988, the World Health Assembly adopted the goal of global eradication of poliovirus by the year 2000. Polio remains endemic only in three countries i.e. Afghanistan, Pakistan and Nigeria. In the past, Pakistan has fought polio strongly but progress is now disappointing. Its progress lags far behind every other country in the world. It was the only endemic country to show an increase in cases in 2014. The greatest challenge lies in the Federally Administered Tribal Areas (FATA) and Peshawar. Population has substantially increased in Peshawar due to which it is unable to carry all the sewage water from the inner part of the city. Thirty drinking and sewage water samples with volume of 1liter were collected from different areas of Peshawar. The samples were analyzed through PCR technique for the detection of enteric viruses especially polio virus, rotavirus, adenovirus, hepatitis A virus and enterovirus. Although no sample of drinking or sewage water is found to be contaminated by polio virus but results show the presence of other enteric viruses i.e. rotavirus (6.66%), adenovirus (13.33%), HAV (10%) and enterovirus (20%) in the samples. The social impacts were also studied and the results revealed top challenges faced by polio workers i.e. security threats, inaccessible areas and interruption by the community during vaccination campaigns.

Key words: Water, Enteric viruses, molecular epidemiology, diagnostic, public health.

1. INTRODUCTION
Pathogenic microorganisms greatly affected the quality of both surface and subsurface water. Microorganisms are mostly derived from fecal contamination. Presently, the water quality is monitored by only the detection of present and absence of coliforms[1]. There are different human viruses which replicate in to the digestive tract also shed into the surrounding water by fecal oral route. These agents are collectively name as enteric viruses. Most of these viruses are highly resistant with non-enveloped structures which can live and survive many months in norovirus, Hepatitis A virus etc. diverse kind of environment[2]. The most common type of enterovirus is rotavirus, adenovirus[3]. Non-enveloped viruses are more resistant to decontamination process which can be used for the potential source tracking candidate better than coliforms, in drinking and waste water treatment process[4]. Enterovirus are belonging to genus picornaviridae family, most of these viruses remain asymptomatic infections in humans but some may be responsible with meningitis, acute flaccid paralysis, respiratory illness, myocarditis and others clinical outcomes[5]. Adenovirus is double standard DNA virus belongs to member of adenoviridae family, are most stable virus in environment. Adenovirus shows higher degree of resistant to UV light when compared to others enterovirus. It causes not only gastroenteritis but also involves conjunctivitis, cystitis and respiratory infections in humans[6]. Rotavirus is a double stranded RNA virus belongs to the family of Reoviridae. It causes server diarrhea among infants and young children. Nearly all children once at the age of five have been infected with rotaviral infection. In addition to its impact on human health, rotavirus can also infect livestock. There are 5 species (A-E) among which 90% infection is causes due to Rotavirus A species[7]. Like other enterovirus, Hepatitis A virus (HAV) is primarily transmitted through fecal oral route, diseases have been reported and associated with contaminated water supply in both urban and rural areas of Pakistan[8]. Peshawar is the largest and oldest city of Khyber Pakhtunkhwa (KPK) province with seven tribal agencies located around the city. The total population is around about 3575000 individuals. The old drainage system name Shahi Khata is still intact in a debilitated state. Population has substantially increased due to which it is unable to carry all the sewage water from the inner part of the city. These drainage systems are filled with dirt in the pipelines with little or no maintenance, which is unable to accommodate all the water, especially during rain. In this study, water samples were collected from different region of Peshawar, Pakistan. These samples were tested by Polymerase chain reaction (PCR) technique for the qualitative analysis of enterovirus, rotavirus, Hepatitis A virus and Adenovirus. Consensus primers were design for the efficient detection of these viruses from the environment. This is the first study on the contamination of water by enteric virus at Peshawar, Pakistan. Though predominantly Pashtun has evolved into one of Pakistan's most ethnically and linguistically diverse cities. In the last three decades, there has been a significant increase in urban population, in part due to internal migration

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of people in search of better employment opportunities, education, and services and in part because of the influx of Afghan refugees and other people internally displaced due to military operations and civil unrest in neighboring regions. Peshawar is the major educational, political, and business center of Khyber Pakhtunkhwa. Peshawar's recorded history goes back as far as at least 539 BC, making it one of the oldest living cities in South Asia[9].

2. MATERIALS AND METHODS

2.1 Study Area

Peshawar is the capital of Khyber Pakhtunkhwa (formerly called the North-West Frontier Province) and the administrative center and economic hub for the federally administered tribal areas (FATA) of Pakistan. Peshawar is situated near the eastern end of the Khyber Pass, close to Afghanistan border. This land is irrigated by various canals of the Pakhto and Bara rivers.

2.2 Water Sample Collection

The total of thirty drinking and sewage water samples were collected directly from surface and subsurface water in sterilized plastic bottles of capacity 1L. Physical parameter like pH, temperature, BOD and COD were measured directly from the source by multi pH meter (Thermo Scientific, USA). Samples were transported immediately and store in the laboratory at 4C until sample concentration. The samples were collected during May 2015 to March 2016 from district Peshawar.

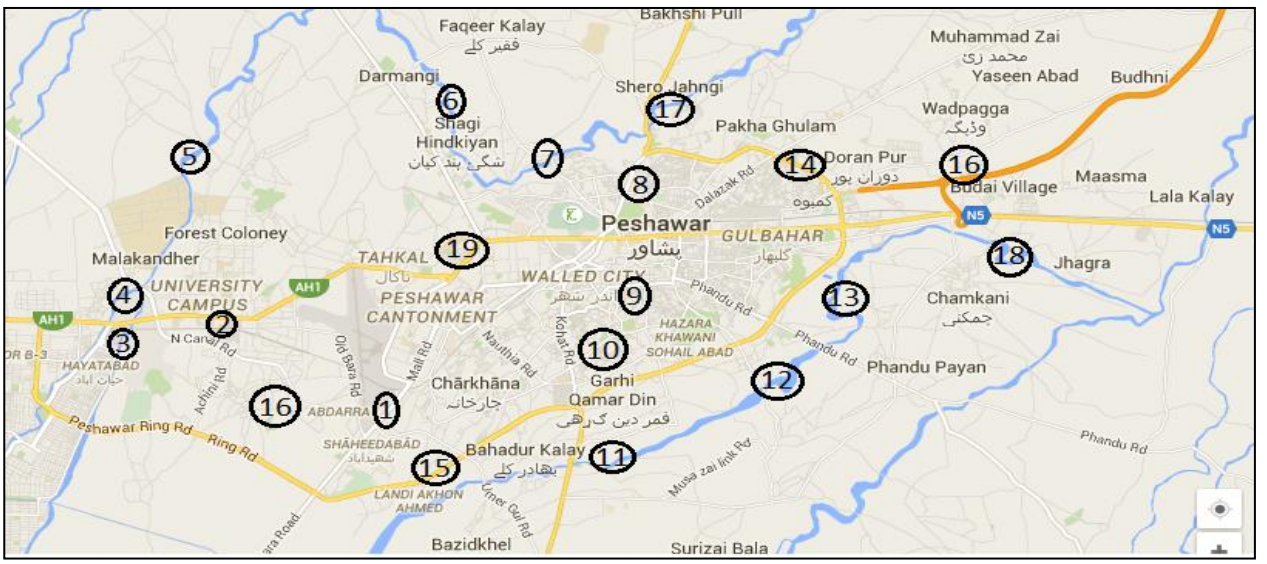

Fig.1: Map showing the sampling sites of Peshawar City of Pakistan.

2.3 Sample Concentration

Samples were concentrated by using adsorption elution method as previously described by Katayama et. al., with 0.45µm negatively charged membrane filters (HA, Millipore, USA)[10]. For sewage sample prior to filtration, samples were centrifuge at 4000 rpm for 5 minutes and then filter through whatmann filter paper no 10. 1L of water sample were mixed with 0.6g MgCl2, pH was adjusted to 5 with 10% HCl. Samples were pass through Vacuum filtration assembly by using 0.45µm negatively charged membrane filters. Discard the filtrate, rinse the membrane with 100ml of 0.5mm H2SO4 (pH: 3.0). 2.5ml of 1mmNaOH (pH: 10.5) was used for the elution of viral particle absorbed by the membrane. Filtrate was then mixed with 12.5 µl of 50mm H2SO4 and 12.5 µl of 100x Tris-EDTA (TA) Buffer. Mixture was stored at -80ºC until processing.

2.4 Viral Nucleic Acid Extraction

Viral RNA and DNA were extracted by using RNA/DNA extraction kit (Invitrogen, USA) according to given protocol. Viral RNA/DNA was kept at -80ºC until used.

2.5 PCR

The detection of Rotavirus, adenovirus, Hepatitis A virus and enterovirus from the water samples were amplified by using specific primers. Sequence of the primers, location and amplification band are described in Table 1. PCR Thermal conditions were optimized and reaction were standardized as follows: (a) RV: cDNA: M.Mulv Buffer: 4µl, 10mM dNTPs: 2µl, Rotavirus R- Primer: 1.5µl, M.Mulv Enzyme: 0.6µl, RNA Template: 8µl, cDNA thermal conditions: 42ºC

for 1hour, PCR recipe for Rotavirus: cDNA template: 6µl, Dream Taq Buffer: 2.5µl, 2m dNTPs: 2.5µl, Forward Primer: 1.5µl, Reverse Primer: 1.5µl, Dream Taq Enzyme: 0.5µl, NF- Water: 5.5µl, PCR were carried by ABI thermal cycler (ABI, UK) the conditions were as follows: 94ºC for 4min, 35cyles of 94ºC for 1min, 50ºC for 1.5min, 72ºC for 1min and extension at 72ºC for 7min. (b) HAV: cDNA: M.Mulv Buffer: 4µl, 10mM dNTPs: 2µl, HAV R- Primer: 1.5µl, M.Mulv Enzyme: 0.6µl, RNA Template: 5µl, cDNA thermal conditions: 42ºC for 1hour. PCR were carried by Esco thermocycler (ESCO, USA), PCR recipe was as follows: cDNA template: 5µl, Dream Taq Buffer: 2.5µl, 2mM dNTPs: 1.5µl, Forward Primer: 1.5µl, Reverse Primer: 1.5µl, Dream Taq Enzyme: 0.5µl, NF- Water: 7.5µl, Total volume: 20µl. PCR conditions were as follows: First Round of amplification: 94ºC for 3min followed by 35cyle at 94ºC for 1min, 55ºC for 1min, 72ºC for 45sec and extension 72ºC for 7min, then Second round of Amplification: 94ºC for 3min, 35 cycles at 94ºC for 45sec, 52ºC for 45sec, 72ºC for 1min and extension for 72ºC for 7min. (c) HAdV: PCR mixture of adenovirus consist of the following recipe: MgCl2:2.5µl, Taq Buffer: 2.5µl, 2mM dNTPs: 2µl, Primer Forward: 1µl, Primer Reverse: 1µl, NF-Water: 10.5µl, Tap Polymerase: 0.5µl, Total volume: 20µl. PCR were carried by ABI thermal cycler (ABI, UK) the conditions were as follows: 95ºC for 3min, 35cycles at 94ºC for 30sec, 54ºC for 30sec, 35 cycle 72ºC for 1min, and then extension at 72ºC for 7min. (d) EnV: The recipe for Enteroviurs cDNA was as follows: M.Mulv Buffer: 4 µl, 10mM dNTPs: 2µl, RNase Inhibitor: 0.2µl, Reverse Primer: 1.5µl, RT- Enzyme: 0.6µl, RNA Template: 10µl, Thermal condition: 42ºC for 1hour. PCR were carried out in ESCO Thermoscycler (Esco, USA), PCR recipe is as follows: cDNA Template: 5–8µl, 0.2mM dNTPs: 2µl, 2.5mM MgCl2: 2µl, PCR buffer: 2µl, 2pmole F-Primer: 2µl, 2 pmole R–Primer: 2µl, DNA Taq polymerases: 0.5µl, N/F water: volume up to 20 µl. PCR thermal cycling conditions was performed by using Esco thermal cycler (Esco, USA), conditions were as follows: 95ºC for 3min, 35cycles at 95ºC for 30sec, 58ºC for 30sec, 35 cycle 72ºC for 1min, and then extension at 72ºC for 7min.

Table1. Primer and conditions used for amplification of Rotavirus (RV), Human adenovirus (HAdV), Enterovirus (EV) and Hepatits A virus (HAV) in PCR.

Viruses	Primer Name	Sequence	Nucleotide Position	Product size
RV	RT-F	GTTGTTGTCATGCTGCCAT	169-187	322bp
	RT-R	AGTACAGTACCAAATTTCAT	471-490	
HAdV	Ad-1F	CATTGCCCAGGAATAAAGAA	32763-32782	400bp
	Ad-2F*	TCATATCATGGGTAACAGACAT	33000-33021	202bp
	Ad-2R*	CCCATGTAGGCGTGGACTTC	33182-33201	
EV	En-1F	CAAGCACTTCTGTTTCCCCGG	164-184	362bp
	En-1R	ATTGTCACCATAAGCAGCCA	599-580	
	En-2R*	CTTGCGCGTTACGAC	562-512	
HAV	HAV FP	GTTTTGCTCCTCTTTATCATGCTATG	2109-2135	246bp
	HAV RP	GGAAATGTCTCAGGTACTTTCTTTG	2330-2355	

2.6 Gel Electrophoresis

PCR results can be analyzed in 2% 1X TAE agarose gel containing 2ul of 10mg/ml ethidium bromide. 5- 8µl sample were mix with 5ul loading dye (fermentas) and load in the agarose gel along with reference ladder (fermentas) which is submerged in 1X TAE buffer pH7.0. Gels were electrophoresed for 25 to 35 minutes at 90Volts. The bands were visualized by gel documentation system (Dolphin Whealtec, USA).

3. RESULTS AND DISCUSSION

Viruses are common place in natural waters contaminated with human feces. Waterborne viruses cause severe infections such as myocarditis, hepatitis, diabetes, and paralysis and also mild conditions such as self-limiting gastroenteritis. It is not easy to identify the etiologic agent or agents responsible for community illness because the seclusion and identification of the causative agent is futile[26]. Thirty Water samples were taken from different sites of Peshawar, KPK Pakistan from May 2015 to March 2016. All samples were analyzed by conventional PCR for the prevalence of Rota virus, Human Adenovirus, Hepatitis A virus and Enterovirus. Out of these samples, the detection rate for Rotavirus was found only in 2 samples with ratio of 6.66%, while 4 samples show HAdV genome (13.33%), whereas only 3 samples show HAV positive results (10%). Among all the viruses present in water, enteroviruses were the highly prevalent genome with 6 samples showing their presence in water i.e. 20%.

3.1 Primers

Specific primers were designed which amplify the target sequence of the given viruses as shown in the Figure 2. Gel electrophoresis clearly shows the result of Rotavirus, Adenovirus and Hepatitis A virus with 322bp, 202bp, 246bp amplification respectively. Enterovirus with 362bp amplification was found from both in drinking and sewage water samples (Figure 3).

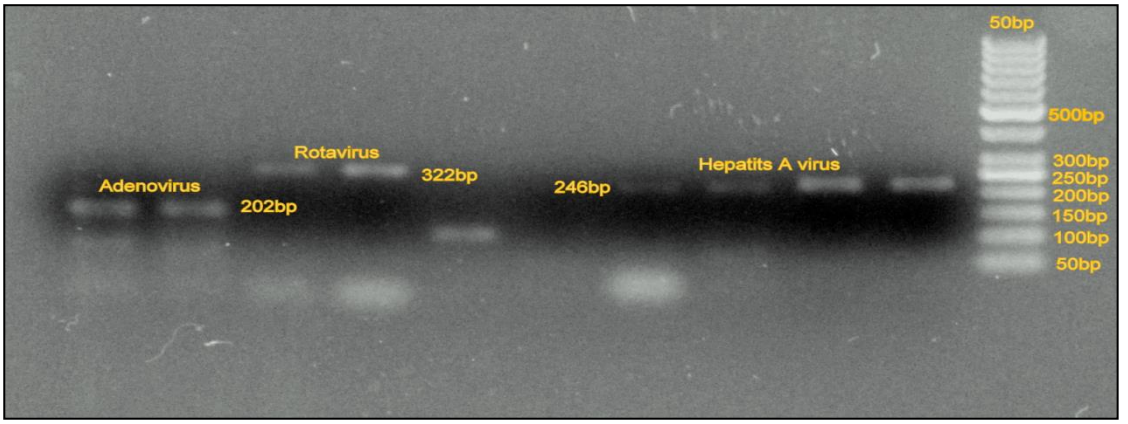

Fig.2: 1.5% Agarose Gel electrophoresis showing 50bp molecular marker with amplified product of Adenovirus, Rotavirus and Hepatitis A virus with 202bp, 322bp and 246bp respectively.

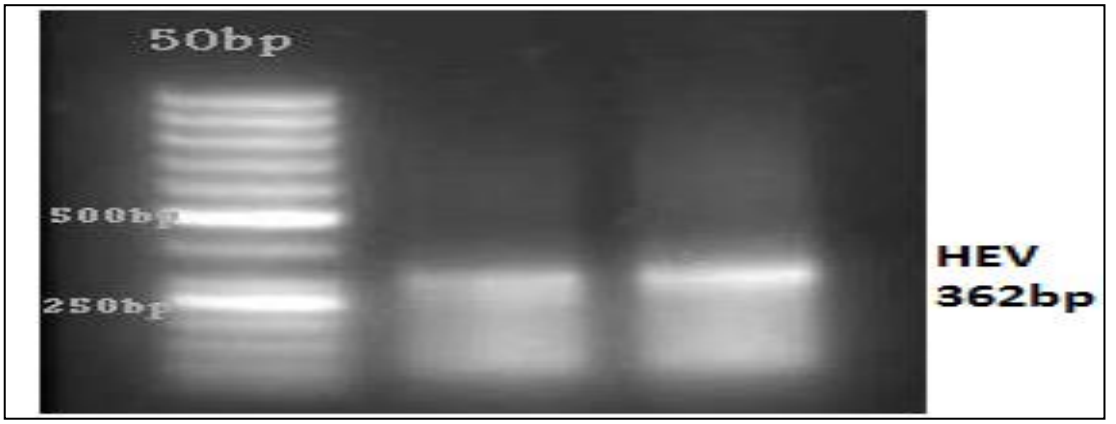

Fig.3: Enterovirus with 362bp amplification were found from both in drinking and sewage water samples

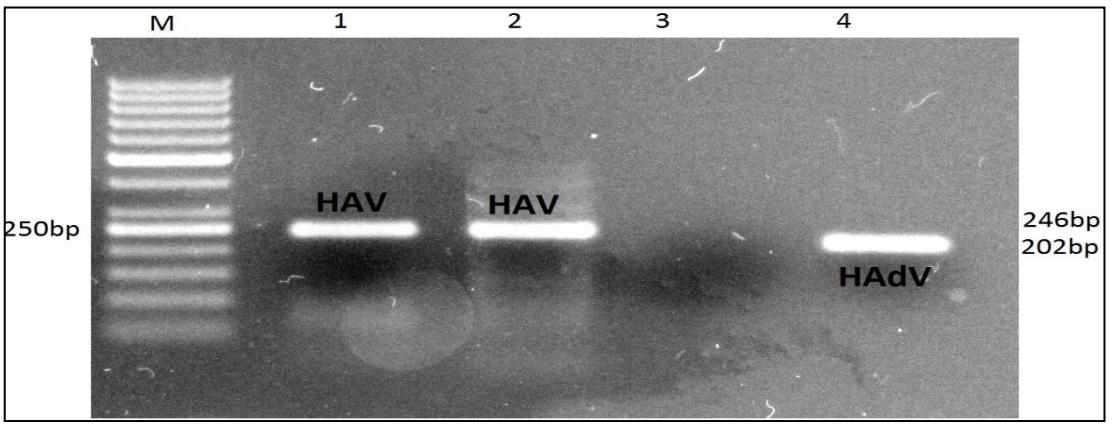

Fig.4: Ethidium bromide stained 1.5% agarose gel electrophoresis showing the amplified product of HAV and HAdV. Lane M showing 50bp molecular marker, Lane1 and 2 showing 246bp amplified product of HAV and lane 4 showing the 202bp amplified product of HAdV.

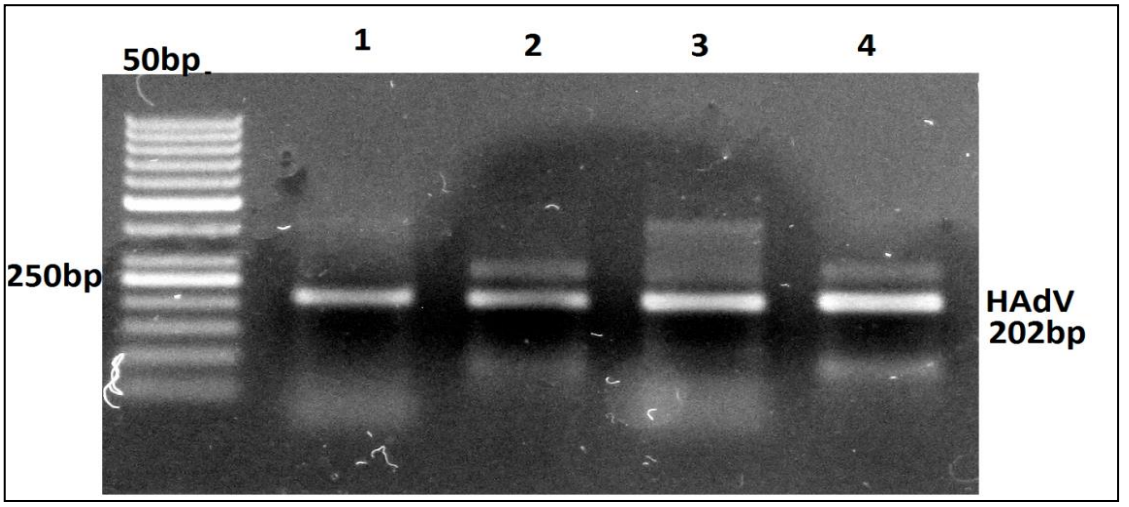

Figure5. Ethidium bromide stained 1.5% agarose gel electrophoresis showing the amplified product of HAdV. Lane M showing 50bp molecular marker, Lane1 to 4 showing nested PCR result for HAdV with 202bp amplified product.

3.2 Prevalence of Viruses in Rainy Season

Most of the samples positive were obtained during the months of June to September. June was the month with highest viral detection rate, while lowest viral detection rate was recognized in December to March (winter season). Almost all sampling points showed the presence of at least one group of viruses, but Adenovirus and enterovirus was showing with highest viral detection rate. Figure 1 indicates the sampling site; samples were obtained randomly from different locations of Peshawar. Sample no 1 and 3 were obtained from sewage water which receives waste water from all the major channels of the city. It shows the highest viral detection of all the said viruses. Most stable viruses which can sustain in diverse kind of environment are Adenoviruses and Enteroviruses. Adenovirus genome is composed of double stranded DNA so it is more resistant and stable to adverse conditions (UV irradiation) which are confirmed by its presence in water (13.33%). Fong et al., (2005) have evaluated the presence of enterovirus, adenoviruses, and bovine enterovirus (BEV) in water samples obtained from Georgia[12]. The authors detected Enterovirus in 20% of water samples, while only 6.66% of samples were positive for Rotavirus. Almost Similar results were also obtained from Seoul, Korea where 33.3% enterovirus samples were positive from Han River, Seoul, Korea[13-14]. Budhni stream usually overflow in rainy season resulting in filthy water flowing onto the streets outside their homes, whole area turns into a huge puddle, which can be the potential cause of viral gastroenteritis. The waste water receives untreated sewage of at least one third of the population of the city. In the current study the fecal contamination in surface and sub-surface water was evaluated by detection of rotavirus, human adenoviruses, hepatitis A viruses and Enteroviruses.

3.3 Most Prevalent Virus Detection

Enteroviruses and Human Adenoviruses were detected in June to September (Table 2). Apparently, there could be a relationship with the prevalence of different viruses with respect to meteorological data such as minimum and maximum temperature, monthly precipitation and annual rain fall pattern as shown in Figure 6. Other parameters like, air humidity, solar insulation, wind speed, wind direction, water temperature and turbidity may be associated with the viral detection pattern in the given areas[11].

Table2. Detection of rotavirus (RV), Human Adenovirus (HAdV), Hepatitis A virus (HAV) and Enterovirus (EV) genome in water samples collected from Peshawar Kahyber Pakhtunkhwa (KPK), Pakistan. (+ detected, - not detected, N/T-not tested)

Collection Date May 2015 to March 2016				
Sample ID	**Rotavirus (RV)**	**Human Adenovirus (HAdV)**	**Enterovirus (EV)**	**Hepatitis A virus (HAV)**
1 (Waste water)	+	+	+	-
3 (Waste water)	-	+	+	+
8 (Drinking Water)	-	+	+	-
11 (Drinking Water)	-	-	+	-
13	+	-	+	-

(Waste Water)				
23 (Drinking Water)	-	+	+	-
25 (Drinking Water)	-	-	-	+
28 (Drinking Water)	-	-	-	+

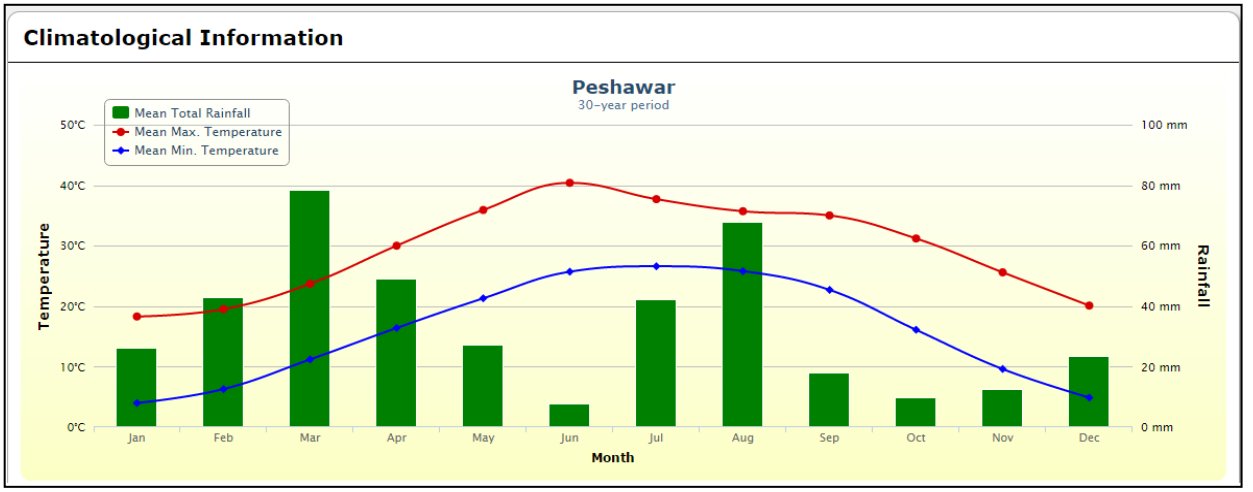

Fig. 6: Climatologically information of Peshawar KPK, Pakistan showing average temperature (°C) with rain fall pattern (mm), adapted from Pakistan meteorological department.[20]

3.4 Challenges in Polio Campaigns

Pakistan and Afghanistan form a common epidemiological block[21,23]. There had been 47 cases of polio reported in the first 10 months or so in 2012 in Pakistan. These countries had 42% of the 175 cases worldwide during this period.[22] Confined religious opinions of both the oral polio vaccine and immunization are closely interrelated to OPV refusal, which has been found in Pakistan, Afghanistan, Nigeria and India, particularly in polio-endemic areas[24-25]. In Pakistan, a study of parents revealed the data that 33% of the refusals are attributed to religious misconceptions[25].

4. CONCLUSION AND RECOMMENDATIONS

Pakistan is a developing country with high population density, experience myriad human health effects because of climate change. Most of the diseases are directly or indirectly concerned with the water especially in urban areas of KPK, Pakistan[15].

4.1 Scientific aspects of research

This article describes a pilot study to investigate the presence of different enteric viruses in stream water and sewage environment of the urban areas of Peshawar. Molecular based assay such as RT-PCR were applied for the detection of enteric viruses from water. There are different methods are available for the concentration of viruses from water, most of them are not cost effective. We use negatively charged membrane filters for the concentration of low level of viruses in water[10]. Despite the high prevalence of EV (20%) and HAdV (13.33%), HAV also present 10% which is higher than rotavirus (6.66%). Detection of these viruses even in low quantity pose a serious threat to general population especially children under the age of ten[16-17]. Poor distribution of water and open drainage system in Peshawar, especially during raining season, overflow drainage water usually contaminate not only drinking water pipe line but also viruses accumulate on the ground water resources[18]. The rates of detection of HAdV and EV on the present work are higher than those found on urban areas of central Punjab of Pakistan. Indeed, HAdV and other enteroviruses are often found as highly prevalent on environmental water but it should be less prevalent in low population density areas. Prevalence of these viruses is directly correlated with the meteorological data shown in Figure 6, which shows that maximum temperature and rain fall appears in March, July and August. Rain fall patterns reduce the fresh water supply as most of the surface water get contaminated with sewage water which definitely increase the risk factor for the general population[19]. High prevalence of EV and HAdV strains in surface water samples might be the potential cause of spreading of gastroenteritis in the urban population. Analysis of questionnaires indicated that most of the parents do not have enough information regarding polio and polio workers have security issues while giving vaccination to the children in remote areas of Peshawar. Enteric viruses represent a significant risk to public health. Although RT-PCR method does not directly used for the viral infectivity but our results shows that the water obtained from the urban city of KPK, Pakistan, were heavily contaminated with HAdV, EV but lesser extent with RV and HAV. Theprevalence of these viruses in water is therefore a

potential health risk for the rural communities that directly or indirectly exposed to these water sources. By improving the quality of drinking water and adequate sanitation will help to reduce the burden of diseases.

4.2 Social aspects of research

Polio eradication efforts face several serious challenges that hamper the drive to eliminate the virus from the Pakistan. A lack of oversight and accountability at all levels of government affects programming. Form the following research it has been revealed that the main reasons for not vaccinating children are:

- Vaccinator absent/not visiting home/vaccine not available
- Lack of awareness
- Child ill and other family problem
- Wrong ideas about vaccine such as it causes sterility in children

Many health personnel considered lack of awareness among people and low accessibility to vaccine as the main hurdles in immunization, besides the poor salaries and incentives. The security environment puts polio workers in danger and makes some areas inaccessible. They are frequently threatened by non-state armed groups and facing difficulty gaining entry to UN ―No Go‖ zones. Program staffers are under threats of kidnappings, beatings, harassment and even assassinations in conflict zones. A precarious security environment increases the number of missed children during vaccination rounds. This study helps to demonstrate the advantage of environmental surveillance as an additional tool for the prevalence of enteric viruses in water bodies of Pakistan.

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Conflict of interest

The author declares that they have no competing interest.

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