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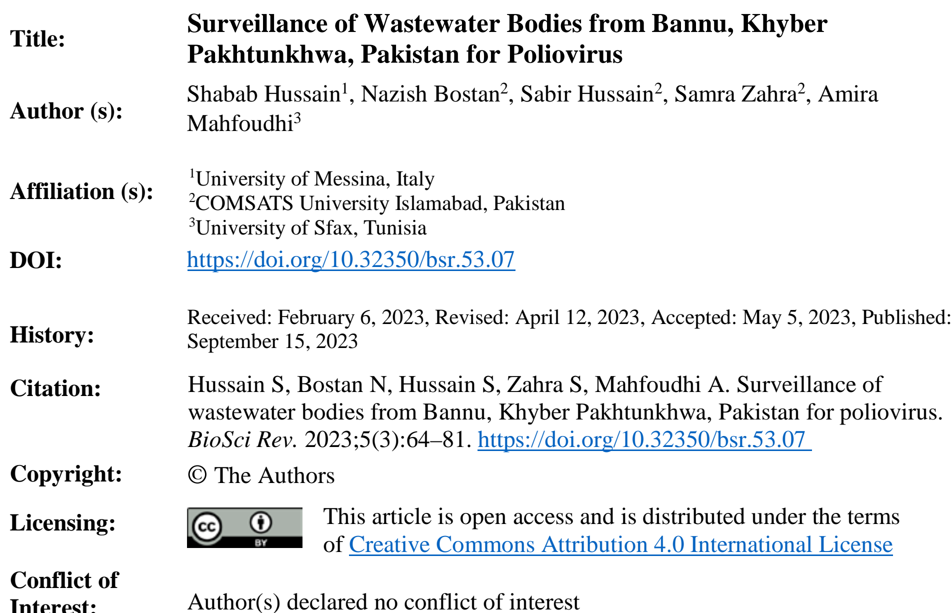

Title:	**Surveillance of Wastewater Bodies from Bannu, Khyber Pakhtunkhwa, Pakistan for Poliovirus**
Author (s):	Shabab Hussain[1], Nazish Bostan[2], Sabir Hussain[2], Samra Zahra[2], Amira Mahfoudhi[3]
Affiliation (s):	[1]University of Messina, Italy [2]COMSATS University Islamabad, Pakistan [3]University of Sfax, Tunisia
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Surveillance of Wastewater Bodies from Bannu, Khyber Pakhtunkhwa, Pakistan for Poliovirus

Shabab Hussain[1*], Nazish Bostan[2], Sabir Hussain[2], Samra Zahra[2], Amira Mahfoudhi[3]

[1]Department of Applied Biology and Experimental Medicine, University of Messina, Italy
[2] Department of Biosciences, COMSATS University Islamabad, Pakistan
[3]Labortory of Biochemistry, University of Sfax, Tunisia

ABSTRACT

Poliomyelitis is a viral disease caused by the poliovirus (PV), globally. This virus is responsible for Acute Flaccid Paralysis (AFP) in children in Pakistan. Due to this virus, thousands of children are paralyzed. This virus is transmitted through wastewater in a congested population. The target group of polioviruses comprises the children with no vaccination history. Different factors are involved which create hurdles in the process of vaccination. Some of them are false beliefs, as well as the lack of security for the vaccination team in different areas of Pakistan, especially in the Khyber Pakhtunkhwa (KPK) region. KPK is one of the few remaining regions in the world with a high rate of PV outbreaks. In KPK, District Bannu is among the districts with the highest number of cases of PV.

Keywords: Acute Flaccid Paralysis (AFP), poliomyelitis, poliovirus (PV), wastewater

1. INTRODUCTION

Poliovirus (PV) is the prototypic pathogenic agent responsible for poliomyelitis in children. Poliomyelitis is a life threating and disability causing disease. Polio virus damages the neurons and dysfunctional neurons cause paralysis, meningitis, and in some cases, paresthesia. Paralytic poliomyelitis is chronic and causes severe symptoms. More than 15 out of every 100 patients of poliomyelitis die due to breathing problems. In most early recovery cases, individuals of age group 20-40 years feel weakness, muscle pain, and paralysis, known as post paralytic syndrome. Asymptomatic cases of PV are about 26%, while others show mild symptoms. Globally, PV was controlled by vaccination initiated in the 1950s. PV has been almost completely eradicated form the world except for some countries. To eradicate PV from the globe, Global Polio Eradication Initiative (GPEI) has started routine immunization, worldwide. Environmental surveillance is used as a tool to find the eradication ration in high alert areas of polio. For environmental surveillance, sewage water is studied form the polio reservoirs and checked for the presence and absence of virus. Furthermore, differentiating serotypes are a part of the surveillance program [1].

1.1 History

In 1789, a British Physician observed children with devastating paralysis in the lower part of the body. In the 19th century, the first outbreak of PV was reported in

[*] Corresponding Author: shabab.jaffri@gmail.com

Europe and in 1843, the first case of PV was reported in United States. In 1952, the number of cases reported were more than twenty-one thousand in the U.S. [2].

Globally, the parameters to eradicate PV were set in 1988. The World Health Organization (WHO) claimed that this disease would be completely eradicated from the planet by the start of the 20th century. Still, PV continues to circulate in Pakistan, Afghanistan, and Nigeria. Environmental factors, poor hygiene, and socioeconomic values show the regional variations. Unvaccinated birth is also one of the factors which correlates with polio incidence among these three countries. To make the planet polio free, Global Polio Eradication Initiate (GPEI) was introduced in 1988, worldwide. The WHO, the Center of Disease Control and Prevention (CDC), Rotary International, and UNICEF work together to eradicate polio by supporting GPEI. Public-private organizations, NGOs, donors, and private foundations support GPEI in planning and financing. The main aim of GPEI is to eradicate all types of polioviruses, including the wild type, Sabin PV, and Vaccine Derived Poliovirus (VDPV), worldwide [3]. The eradication of PV from the affected countries is among the top priorities of GPEI. Environmental surveillance is the method used for the detection of wild poliovirus transmission. Environmental surveillance is important in the polio eradication free program to find the polio sensitive areas [4].

1.2. Poliovirus (PV)

Poliovirus (PV) is an enterovirus that belongs to the family Picornaviridea. It is a single stranded RNA virus with positive polarity. PV possesses a non-enveloped capsid. The genome contains the RNA of the complex secondary structure and protein capsid which play an important role in the replication and translation process. PV is the causative agent of poliomyelitis in children. Among animal viruses, PV is one of the simplest ones [5].

1.3. Structure

PV contains a small icosahedral (30 nm in diameter) structure having four capsid proteins. PV genome is composed of 7500 nucleotides, poly-adenylated at the 3' terminal and on the other side, covertly attached to VPg linked protein at 5' terminus. The capsid of PV functions in the viral assembly and provides suitable stability to the virus against hash environmental stress [6]. PV is a picornaviridea family member and it has three different serotypes, namely PV1, PV2, and PV3. These serotypes were observed through X-ray crystallography. There are also present some non-structural protein parts (2A, 2B, 2C, 3A, 3B, 3C, and 3D) at UTR and in poly A tail region. PV contains deep surface depression canyons on the surface of the cell [7].

PV has three wild types, namely WPV type 1, WPV type 2, and WPV type 3. Wild PV is responsible for Acute Flaccid Paralysis (AFP) which is mostly detected in environmental samples. Bulbar polio which affects the respiratory tract, breathing, and speaking causes the death of children [8]. PV is among the first viruses that was propagated in cell culture and its plaques were purified successfully. The virion of PV is composed of single stranded messenger sense RNA covered in a capsid made up of 60 different copies of structural proteins. The diameter of the mature virion is about 30 nm [9].

1.4. Transmission

Poliomyelitis is a communicable disease caused by PV in children. The mode of transmission is oral-fecal and the

respiratory tract route. The droplets of PV also become a source of transmission in the early stages of poliomyelitis. PV in infected individuals is shed in the feces through which it transmits to other individuals. The transmission of PV occurs through oral contact, through the saliva of infected persons, and via the exposure to the fecal material of infected individuals. It mainly enters through the mouth and spreads in the whole body [10].

Poliomyelitis is also known as the gray inflammation of the spinal cord and brain. In children, infantile paralysis is also common due to poliomyelitis [11]. In some cases, PV causes muscle weakness, abdominal paralysis, and even death. Before the polio attack, its symptoms appear including headache, fever, fatigue, pain in arm and legs, neck pain, and nausea [12]. Poor hygiene is the most common reason of PV transmission in underdeveloped countries. PV is found in drinking water, waste water, and soil. To overcome the issue of transmission, it is now mandatory to take its vaccine before travelling to a polio free country [13].

1.5. Clinical Feature

The incubation period of PV is 7 to 10 days with the development of some clinical symptoms, such as headache, fever, and occasionally, sore throat [14]. During the poliomyelitis state, PV replicates itself in the central nervous system, especially in motor neurons. During its replicating time, it harms the nerve cells in the spinal cord. As a result, the damaged muscle cells do not perform their functions, which develops a condition known as Acute Flaccid Paralysis (AFP). PV can tolerate acidic pH and mainly inhabits the gastrointestinal tract [15].

1.6. Vaccination

Thousands of children fell victim to poliomyelitis and died each year before the initiation of Global Polio Eradication Initiative (GPEI) program. Polio has been eradicated throughout the world except Pakistan and Afghanistan [16]. Vaccines used for the eradication of polio virus are of two types, namely Inactivated Polio Vaccine (IPV) and Oral Poliovirus Vaccine (OPV). To overcome this deadly disease in children, vaccines were successfully introduced globally in 1950 and 1960 against this virus [17]. Both types of vaccines namely IPV and OPV enhance the production of antibodies (anti PV) in the serum which helps to reduce the chances of poliomyelitis. Oral polio type 2 vaccines were drawn out of the global immunization program in 2016 and replaced by trivalent oral polio vaccine [18]. Unvaccinated children during the polio eradication program became the major reason behind the circulation of the wild type PV in Pakistan and Afghanistan. A large number of children in Pakistan during the rounds of the immunization program missed vaccination. The main reason behind this immunization gap is either that the children go out of town or to school. As a solution, polio teams visit each and every school during polio free week and remain present at each district station. They vaccinate children and mark their nails with specific ink. Genetic variation is also responsible for the emergence of new cases of polio caused by Vaccine Derived Poliovirus (VDPV) circulating strains [19]. PV has the potential of high mutation. A rapid mutation rate is also a major challenge to eradicate all types of PVs**.** For complete eradication of PV, VDPV and WPV should be eliminated from the globe. IPV has the ability to limit the transmission of these viruses. OPV was initiated in 2011, based

totally on new developments in OPV strains that caused new PV cases. IgG_3 antibodies are produced in response to PV infection in human beings. For the eradication of PV, Pakistan Polio Eradication Program conducted two sessions of vaccination. The first was known as National Immunization Days (NIDs) conducted with the help of polio workers. The second was sub national immunization day (, where a specific area was targeted based on cases or environmentally positive samples (Pakistan Polio Eradication Program).

1.7. Inactivated Polio Vaccine (IPV)

Jonas Salk developed for the first time an inactivated vaccine for polio virus in 1952. Salk vaccine is an Inactivated Polio Vaccine (IPV) which successfully prevented from three types of strains. IPV is injected in an intradermal and intramuscular fashion by trained health workers. Antibodies are produced after the injection of IPV and these antibodies stop the transmission of PV to the central nervous system. PV still has the ability to spread through the oral-fecal route after the injection of IPVs because of low immunity development in the intestine.

When GPEI was initiated in 1988, the number of cases decreased up to 80% in the population. This decrease in cases marked the fruitful use of live, attenuated polio vaccines which became a part of the global immunization program, worldwide. In this eradication program, mainly the serotypes WPV1 and WPV2 were targeted [3]. IP vaccine has been used in the U.S. since 2000. IPV has made a significant contribution in polio eradication but remains more expensive than OPV. Low-income countries use OPV in their routine immunization program. IPV is injectable alone and can also be administered along with other vaccines, such as influenza, measles, and hepatitis B vaccines [20].

1.8. Oral Polio Vaccine (OPV)

Albertin Sabin introduced attenuated Oral Polio Vaccine (OPV) in 1961. Live OPV are produced from the Sabin strain of poliovirus. OPV contain attenuated PV which can replicate in intestine but cannot transmit to CNS due to less power than the wild type poliovirus. OPV keep immune system strong against all serotypes for a long time. Mucosal immunity is activated by OPV and administered orally. Two and half billon children have been vaccinated since 1988, worldwide [21].

1.9. Replication

Poliovirus binds to immune-globin, such as the host cell receptor CD155, for replication. CD155 belongs to immune-globin super family. Viral particle changes its configuration upon binding. VP1 moves N-terminus to the exterior side of the capsid and sets free VP4 from the virion. The loss of VP4 is caused by the attachment of free CD155. Via receptor mediated endocytosis mechanism, PV enters the host cell which releases viral RNA into the cellular cytoplasm. Proteins such as 3AB, 3A, 3B, 2BC, 2B, and 2C are utilized in viral replication. PV genome functions as an mRNA. Capped mRNA differs from RNA which is not capped in the stages of translation. After reaching cytoplasm, the host cell machinery translates RNA into a single polyprotein. Encoded single polyprotein generates cleavages of structural and nonstructural proteins namely VP1, VP2, VP3, and VP4. Internal ribosomal entry site translates viral RNA directly [22].

RNA synthesized in replication complexes contains cell protein and viral protein. The RNA of eukaryotes replicates

in similar associate membrane complexes [23]. Multiple copies of (+) RNA replicates and (-) RNA are transcribed for infecting a cell. These (-) RNA are used as template for the (+) RNA synthesis. CIS acting replication element, where transcription takes place, occurs in a stem loop in a genome. (-) RNA uses UUVPg as a primer, while (+) RNA uses VPg or uridylated as a primer. Replicative complexes containing RNA are used as Replicative Intermediate (RI) that contains RNAs varying in length and also possesses template RNA. Infected PV is synthesized at 2500 RNA per minute. RNA act as a templet for (-) RNA and (+) RNA. The last step of PV replication is assembly and exit in which VP0, VP1, and VP3 assemble into 5 pentamers. For pro-capsids, 12 pentamers are assembled. PV infected cells produce 105 virion, while virion progeny leave the cell when lyses occur. PV replication lysis mainly occurs due to formaldehyde, ultraviolet rays, and chlorine [24].

1.10. Epidemiology

During 1950, PV vaccines were used in many countries to control poliomyelitis. More than sixty thousand children were infected with this deadly disease. In the same year, about one thousand children became paralyzed and up to 3000 died in the United States. Special iron lungs were kept in hospital wards to keep the victims alive and overcome the death rate in the U.S. In 1955, U.S. started vaccinating children the age of 1 to 5 years. Due to a successful vaccination campaign, PV was completely eradicated from the U.S. in 1979 [25].

'Eliminate polio' is an initiative taken by the WHO in 1988 to eradicate PV. Three million children were paralyzed per year by PV in 125 countries at that time. Based on the routine vaccination program, international commission declared the eradication of the wild poliovirus from the western hemisphere in 1994 [26]. Type 2 and type 3 PVs were eradicated from the globe in 2015 and 2019, respectively. About 99% eradication was achieved with the support of GPEI. The reemergence of VDPV type 2 is due to the poor implementation of the vaccination program and the decrease in routine immunization in different regions [27].

1.11. Disease Burden in Pakistan

In 2014, the number of infected individuals reported was 306, which decreased to 54 in 2015, 20 in 2016, 89 in 2017, and eventually, 12 cases were reported in Pakistan in 2018. The number of polio cases registered in 2017 were 89. The most affected area was the Khyber Pakhtunkhwa province with 65 cases, Sindh with 12 cases, Baluchistan with 7 cases, and Punjab with 5 cases. Health authorities are struggling to eradicate polio from Pakistan in order to make it a polio free country. In 2019, 144 active cases were reported in different provinces. Similarly, more than 80 cases were reported in 2020, with 22 cases in Khyber Pakhtunkhwa, 23 cases in Baluchistan, 13 cases in Punjab, and 22 cases reported in the Sindh province.

Polio is still endemic in Pakistan. There are still many barriers which create hurdles in the process of vaccination. Hurdles include health system, political commitment, some dogmatic beliefs about polio vaccines, and illiteracy and insecurity in border areas [28]. More than 25% of the population is affected with malnutrition. About 37.5 million people in Pakistan lack sufficient food for their survival. Terrorism also becomes a hurdle in the elimination of polio. Terrorist groups killed polio workers since they perceived them as government spies. In 2012, 74 polio workers were killed

by these terrorist militants. Some other factors include the fatwas declaring vaccination illegal based on the premise that vaccine drops are composed of pig blood [29].

WPV Polio Cases 2023 Across Districts in Pakistan

Province	District Name	Total Cases	District Total
KHYBER PAKHTUNKHWA	Bannu	1	1
2023 Total Cases		1	1

WPV Polio Cases 2022 Across Districts in Pakistan

Province	District Name	Total Cases	District Total
KHYBER PAKHTUNKHWA	North Waziristan	17	1
	Lakki Marwat	2	1
	South Waziristan	1	1
2022 Total Cases		20	3

Figure 1. Report of 2022, 2023 Issued by Pakistan Polio Eradication Program

1.12. Pathogenesis

PV infection starts from the oral cavity in human beings. After intake, the virus doubles its number in alimentary mucosa and tonsils. Through Peyer's patches, the virus moves to loci for further multiplication. A high amount of PV passes through cervical and lymph nodes and enters into the blood vessels. PV neutralizes antibodies in the blood which causes viremia in blood vessels. It circulates in the blood and ultimately reaches CNS. In CNS, the virus initiates replication in motor neurons. PV reaches CNS via two pathways. It either crosses the blood brain barrier to enter into CNS or uses peripheral nerves for transmission. Neural destruction in CNS allows the paralytic poliomyelitis disease to develop in children, damaging cells in CNS and generating apoptosis in mice in *in vitro* model experiment. As a result of this experiment, it was determined that increased viral load can lead to more apoptosis and cause paralysis [30]. There are three types of paralytic polio, namely spinal polio, bulbar polio, and bulbospinal polio. The incubation period of non-paralytic PV is 4-6 days, while it is 1-2 weeks for paralytic PV. There are chances of complete recovery of an individual affected with paralytic poliomyelitis. The death to case ratio of paralytic polio is approximately 3-6% in children and 16-31% in adults [31]. Morphological and metabolic variations occur in the host cell due to the PV infection known as the cytopathic effect. Host cell transcription and translation is cut off by the PV protease. Also, the inhibition of nuclear cytoplasmic trafficking induced by PV infection causes nuclear protein accumulations in cytoplasm [32].

1.13. Environmental Surveillance

For the detection of PV affected area, WHO has introduced the Environmental Surveillance (ES) program, globally. For the eradication of PV, AFP surveillance is the gold standard, with the aim to achieve the goal of a polio free world. ES helps to

identify high alert areas based on treating sewage water for the detection of PV. ES area selection depends on the cases ration reported population wise and the decrease in the AFP surveillance rate. In most cases, there are present asymptomatic individuals that become carriers of the virus and transmit it to healthy people. According to a WHO report, 1 out of 200 people show symptoms, while others remain asymptomatic. For the confirmation of the complete elimination of PV, ES is conducted three years after the last case reported. It has yielded fruitful results in 5 WHO regions out of 6, namely the European Region (EUR), South East Asian Region (SEAR), Eastern Mediterranean Region (EMR), American Region (AMR), and the African Region (AFR).

The eradication of PV is quite challenging. Teachers, health workers, traditional medical staff, family doctors, and polio surveillance teams need to work together to find the affected children and report them to the ES head office. To conduct ES in a specific area, ES team takes the recommendation from the WHO regional office. They issue proper guidelines according to the area and population. Before starting ES, a proper lab setup, trained team, and proper resources are arranged by the WHO regional team. ES successfully detects the wild type, VDPV, and IPV circulating in the sewage system. PV have been detected during ES even where AFP cases have not been reported [33].

PV strains commonly circulate in waste water. Waste water surveillance has a vital importance in GPEI. Also, routine immunization acts as a crucial support in PV eradication program [34]. This surveillance is the only means to identify the inaudible strains of PV circulating in the population. Through these approaches, it becomes easy to achieve the successful eradication of PV [35].

1.14. Temporal Pattern

Changes in atmospheric situation leads to seasonal variations which directly affect the virulence to host by PV. In the case of PV, pathogenic cycle and humidity, as well as temperature, wind, and rain show vital statistical correlation. Studying the seasonal variation of PV for the eradication of poliomyelitis is very important. Vaccines show an increased rate of prevention in winters as compared to summers. Due to seasonal variations, the rate of transmission also increases from person to person. PV was highly seasonal in the U.S. during the 20th century [36]. In high temperatures, PV appears as a mutation in the VP1 capsid coding protein sequence. These mutations protect virion assembly in wild type PV [24], which presumably spreads through the oral-fecal route and shows a high rate of cases in late summer and autumn. Poliomyelitis cases increase in the months from August to October and then their number gradually decreases. There is no specific seasonal variation pattern in tropical climates [4].

1.15. Diagnosis

For the detection of PV in paralytic poliomyelitis patients, WHO recommends virological testing and clinical course for the identification of symptoms. Polymerase Chain Reaction (PCR) is a suitable method to detect PV in throat swabs, stool, and the cerebrospinal fluid. Stool sample is the primary means for detection. For the measurement of polio antibodies, electromyography, nerve conduction test, and agnatic resource imaging are used. Real Time Polymerase Chain Reaction (RT-PCR) also differentiates between the wild type and VDPV. For intra-typic differentiation, RT-PCR is used in the

Global Polio Laboratory Network. Genome sequencing of cell line culture and serology tests are conducted by CDC for PV detection. Isolating virus from the cell line culture is a sensitive and expensive method. For serology testing, two specimens are needed, one form early illness and the second is collected after the third week of illness [37].

Figure 2. Flow Chart of Polio Virus Surveillance Study

2. MATERIALS AND METHODS

2.1. Sampling Sites

A total of 10 sewage water sites (Table 1) were chosen throughout Bannu, Khyber Pakhtunkhwa. These sites were previously reported for PV cases in 2019. Densely populated areas and hospitals were covered in this study. Sites were picked on the basis of high alert areas for PV in Bannu, Khyber Pakhtunkhwa, as reported by GPEI Pakistan. Densely populated areas and hospital sewage lanes were marked for sewage water sampling. Villages reported for polio cases in 2019 were also targeted for sampling.

2.2. List of Sites for Water Collection and Time

Sampling Site Sokary

Sampling Site DHQ Bannu Sewage

Table 1. Names of Sampling Sites and the Timing of Sampling

S. No	Site Name	Time
Site 1	Gari Mamash Khel Miran Shah Road	09:05 a.m
Site 2	Shaheen Adda	09:30 a.m
Site 3	Sokary	09:40 a.m
Site 4	Nurar Miryan Road	10:35 a.m
Site 5	Tanger	11:00 a.m
Site 6	Kauser Fateh Khel	11:20 a.m
Site 7	DHQ Bannu	01:05 p.m
Site 8	Mera Khel D.I khan Road	01:40 p.m
Site 9	Ismail Khani	02:00 p.m
Site 10	Salih khan kali Mandan Road	02:50 p.m

2.3. Sampling Methods

Sewage water samples were collected in 500 ml plastic bottles. Sterilized bottles were used for sample collection. Samples were collected from mid-stream using a bucket. After collecting the samples, the

bottles were closed tightly and cleaned with spirit. Each sample was labelled with proper details and stored in a cold container.

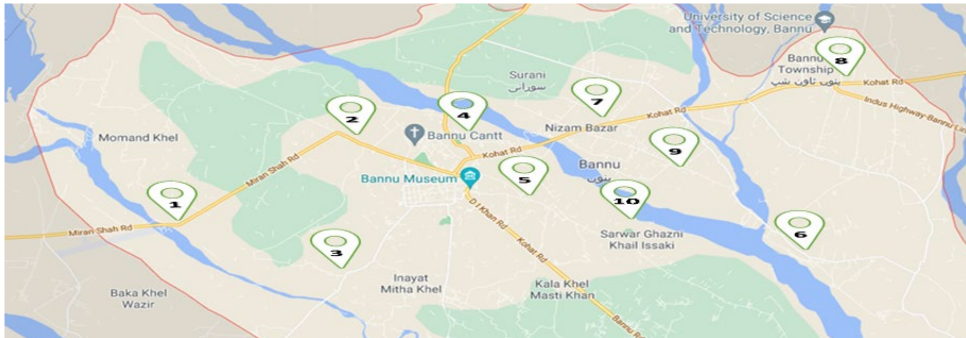

Figure 3. Map of Sampling Sites

2.4. Transport of Sample

Samples were transported to the laboratory in a cold container under 4°C temperature within 12 hours after their collection.

2.5. Sample Storage

Samples were stored at 4°C in a refrigerator at the Molecular Virology Laboratory, Biosciences Department, COMSATS University, Islamabad and processed within the next 24 hours.

2.6. Virus Concentration in Sewage Samples (PEG Precipitation)

A 200 ml aliquot of sewage water sample was centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was pooled in flask. Chloroform (1 ml) was added to the centrifuge tube and centrifuged for 10 minutes at 2000 rpm. The supernatant was discarded and the precipitate (pellet) was transferred to a separate conical flask. Afterwards, NaCl 0.5M (5.864 grams) and 10% PEG were added to the flask. It was magnetically stirred for 30 minutes. The sample was kept overnight at 4°C. The next day, the sample was transferred to a new 50 ml falcon tube. The overnight sample was centrifuged for 30 minutes at 4°C on 7000 rpm. The supernatant was removed from the direction opposite to the centrifugal force. Again, it was centrifuged at 7000 for 10 minutes. Then, the obtained supernatant was discarded and the pellet was dissolved in 4 ml of phosphate-buffered saline (PBS) and stored at -20°C [38].

2.7. RNA Extraction by TRIzol

A 250 µl aliquot of concentrated sample was transferred into the micro-centrifuge tube. Afterwards, 250 µl of TRIzol reagent was added and vortexed for 30 seconds. Then, 200 µl chloroform was added in the same tube and chilled for 30 seconds on vortex. Micro-centrifuge was centrifuged at 12000 rpm at 4°C for 5 minutes. The clear and transparent upper phase was gently transferred into the new sterilized Eppendorf. Isopropyl alcohol of 500-700 ml volume was added and mixed by turning the tube 5-7 times. Finally, the sample was centrifuged at 12000 rpm at 4°C for 15 minutes. The supernatant was discarded immediately and the pellet was dried in the air. The pellet was then re-suspended in 20 µl of DEPC water. The extracted RNA was stored at -20°C until used for PCR [39].

2.8. RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Wiz-script cDNA synthesis kit was used for the synthesis of cDNA following the manufacturer protocols. About 1 ul of purified RNA was reverse transcribed by following the recipe mixture, 10 µl reaction mixture by Oligo dT primer (1 µl), RT (10X) reaction buffer (2 µl), DNTP Mix (0.5 µl), RT Enzyme (1 µl), (0.5 µl) RNA Inhibitor, and RNase DNase free water (4 µl). The mixture obtained in the first step was heated at 25°C for 5 minutes, then for 60 minutes at 50°C, and finally at 85°C for 5 minutes.

2.9. cDNA Confirmation

cDNA was confirmed with the already confirmed housekeeping gene primer. The reaction mixture of 10 µl for PCR included Master Mix 2X, PCR water, Forward Primer 1 µl (AARAARTTYAAYGAYATGGC), Reverse Primer 1 µl (TTTGCTATTCTGGTTATAAC) and cDNA 1 µl. PCR was heated for 10 minutes at 25°C, then at 60°C for 30 minutes, and finally, at 85°C for 5 minutes. cDNA was checked using gel electrophoresis and the resulting pictures of bands are attached with the results (Table 2).

2.10. Gradient PCR

For the optimization of the reported primers (Table 2), gradient PCR was performed. For this purpose, 10 µl of total reaction was conducted which contained Master Mix 5X, forward and reverse primers, as well as PCR water and confirmed cDNA. Optimizing temperatures were kept between 52°C to 62°C. Gel was run for the visualization of bands attached with the results.

2.11. Gene Amplification of PV

PCR was performed for the amplification of the PV gene. For this purpose, 1 µl cDNA along with 5X master mix, 1 µl of each reverse and forward primer (Table 2.1), and PCR water with 10 µl of total volume was amplified by PCR. Primers EVB-4110-f and EVB-7400-r were used.

Table 2. List of Primers [40]

Primer Name	5'–3' Sequence	Genome Position
EVA-4110-F	AARAARTTYAAYGA YATGGC	4110–4130
EVA-7410-R	TTTGCTATTCTGGTTA TAAC	7410–7390

2.12. Electrophoresis

A total of 1% agarose gel was run for 10 µl PCR products. To estimate the PCR product size, 2 µl of 100 base pair (bp) DNA leader was kept in the extreme left side of the gel with 10X TAE loading buffer. Ethidium bromide was used for staining and the gel was run for 40 minutes on 80V and 150 watts. (Biorad) Gel documentation system was used to observe the results.

3. RESULTS

3.1. pH of Water Sample

According to the results, PV becomes inactive and degraded upon pH below 3 and above 8. At pH 10, PV shows a high rate of degradation of the genetic material. However, pH 7 is more favorable for the circulation of PV (Figure 4). This proves that high pH is directly proportional to PV inactivation rate.

Table 3. pH of Water Sample

Order	Site Name	pH of water Sample
1	Gari Mamash Khel Miran shah Road	7.06
2	Shaheen Ada	7.92
3	Sokary	7.00
4	Nurar Miryan Road	7.41
5	Tanger	6.34
6	Kauser Fateh khel	8.12
7	Ismail Khani	6.64
8	DHQ Bannu	7.89
9	MeraKhel D.I khan Road	7.02
10	Salih khan kali Mandan Road	7.72

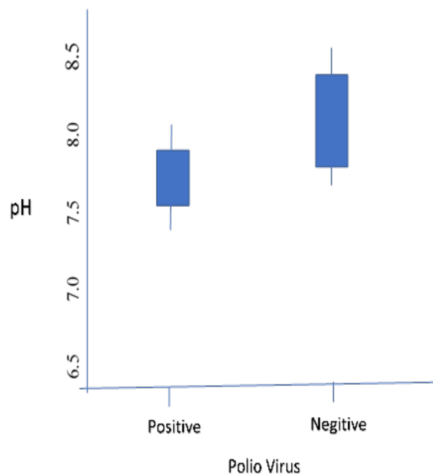

Figure 4. pH Value of the Obtained Waste Water

The sample, found positive for PV, had a range of pH between 7.06 and 7.89.

3.2. RT-PCR

RT-PCR product was confirmed (Figure 5) for the synthesis of cDNA by GAPDH primers. This step was performed only for the verification of steps used for the amplification of DNA. High yield results were obtained.

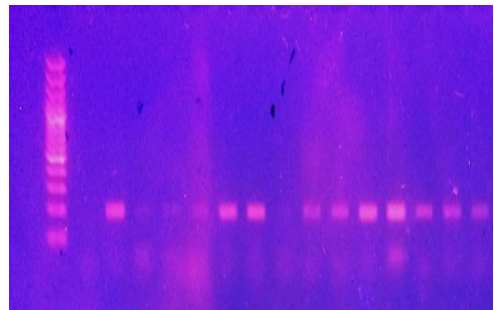

Figure 5. Gel Electrophoresis Results for cDNA Confirmation

3.3. Optimization of Primers

Primers (EVB-F and EVC-R) were optimized by using gradient PCR (Figure 6) from 52°C to 62°C. The resulting primers were optimized at 56°C.

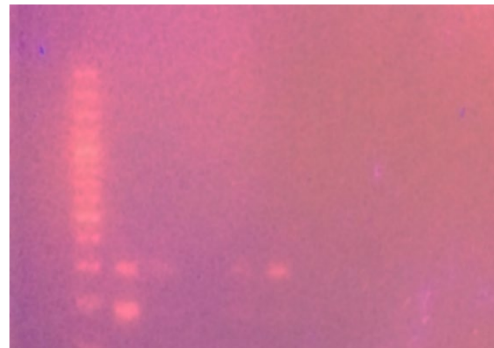

Figure 6. Optomized Primer Results

Primers were optimized in the Virology Laboratory, COMSATS University, Islamabad, Pakistan.

3.4. Gene Amplification

DNA was amplified at 56°C. Two samples were found positive out of 10 samples.

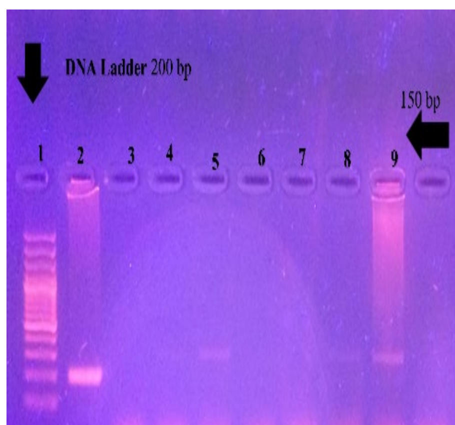

Figure 7. Amplified Gene by 200 bp Ladder

Table 4. Positive and Negative Sample Results

Sr. No.	Sample Site	Positive	Negative
1	Gari Mamash Khel Miran shah Road	+ve	
2	Shaheen Ada		-ve
3	Sokary		-ve
4	Nurar Miryan Road		-ve
5	Tanger		-ve
6	Kauser Fateh khel		-ve
7	Ismail Khani		
8	DHQ Bannu	+ve	
9	MeraKhel D.I khan Road		-ve
10	Salih khan kali Mandan Road		-ve

Out of the ten samples, two yielded positive results. These two areas are frequented by migrants from North and South Waziristan. DHQ is the only hospital in Bannu which facilitates the whole district as well as the people from Waziristan.

4. DISCUSSION

Poliovirus (PV) is a member of Picornaviridea. It is the main pathogen responsible for paralytic poliomyelitis in children. Motor neurons of the Central Nervous System (CNS) become nonfunctional due to PV. The mode of PV transmission is mouth to intestine, where it starts to replicate itself. In most cases, PV causes paralysis and casualties. On the other hand, about 25% of people affected by PV remain asymptomatic and act as carriers to healthy individuals. In the early PV infection, people feel weakness in muscles that slowly spreads to the whole body, completely affecting the spinal cord and brain. Headache, fatigue, and nausea are the side effects of PV infection [41]. In developing countries, the way of transmission is drinking sewage water. In 1999, the last case of WPV2 was recorded. Since then, the serotypes of WPV2 have been eradicated completely from the world. Globally, international health authorities struggle to eradicate polio from all countries. The strategies used by them are helpful in all other countries except Pakistan, Afghanistan, and Nigeria. There are several hurdles due to which PV still circulates in these countries, such as insecurity to polio eradication team, lack of education, false beliefs, and lack of a proper setup for vaccine transportation. Vaccine development is the only hope to eradicate PV from the globe. In the years 1950-60, scientists initiated two types of vaccines, namely oral poliovirus vaccines (OPV) and inactivated polio vaccines (IPV). These two are now part of the global immunization program. They support the antibodies production in the body against PV. Still, new strains are arising in different regions of the world due to vaccine derived poliovirus (VDPV) or Sabin VDPV. For the production of PV vaccines, WHO and

Biosafety Authority recommend a polio free environment. In the 20th century, the development of polio vaccines successfully prevented Acute Flaccid Paralysis (AFP) in children, globally [42].

Single stranded RNA genome of PV was cleaved into different structural and nonstructural proteins. Structural protein (P1) and non-structural proteins (P2 and P3) were generated by protease 3CD enzyme. P1 was further cleaved into VP0, VP1, and VP3, while VP0 was divided into VP4 and VP2. Before their exit from the host cell, structural proteins have the potential to self-assemble. Mutation in the PV genome mostly occurs in VP1, VP0, and VP3 regions [43].

For PV replication, specific receptors need to be present on host cells for attachment. In paralytic poliomyelitis, PV shows strong bonding with host cell receptors present on the cell surface, which act as entry sites for the viral genetic material. A genome of the virus activates the mRNA of the host cells by hijacking their replication machinery. Viral structural and nonstructural proteins are synthesized by the initiation of PV genomic RNA required for the replication and translation process in PV. In positive standard RNA virus, the protein of the host cell also aids in viral replication [44].

Replication mechanism and the factors needed for these mechanisms still remain unclear. PV has the ability to replicate thousands of copies within one hour. In viral replication, speed and replication efficacy play an important role. All three types of PV use CD155 cellular receptors for entry into the host cell. PV starts un-coating and enters into the viral cell after attaching with CD155 receptors. The virus contains canyon regions which are found also in HRV and used as attachment sites for CD155 receptors [45].

Wastewater surveillance has a vital importance in the Global Polio Eradication Initiative (GPEI). This surveillance is the only means to identify the inaudible strains of PV circulating in the population. Through these approaches, it becomes easy to achieve the successful eradication of PV [46]. Genetic sequencing and developing phylogenetic trees are important steps in the ES findings. Endemic of WPV1 in Pakistan has linkages to WPV1-SOAS cluster. The same linkages were also isolated from sewage water in Egypt in September 2012 [47].

Low-income countries face challenges in running the Environmental Surveillance (ES) program. In this program, waste and sewage water are used as sources of PV circulating in the environment. GPEI approved laboratory observed the sample for virus detection and concentration. For the surveillance-based study on wastewater, standard techniques were used in the laboratory. Standard protocol adopted by WHO and GPEI are recommended due to the low viral load in sewage. PV can easily be adsorbed in very small particles of feces [35]. GPEI is optimized to eradicate PV from underdeveloped countries. In this regard, Nigeria has been re-declared endemic for polio after one year of staying polio free. Pakistan and Afghanistan have a shared border, therefore, the same strains of WPV circulate in both countries. Most of the Afghan refugees have moved back to their country but still have relatives in Pakistan. They visit Pakistan without undergoing any specific vaccination process. This facilitates PV circulation between Pakistan and Afghanistan [48]. For total PV eradication, a high level of biosecurity has considerable importance in surveillance

studies. The WHO initiated Global Action Plan (Phase 3) along with BSL 3 for the eradication of PV globally in 2015 [49].

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