

## Environmental Surveillance of Polio and Non-Polio Enteroviruses in Sistan and Balouchestan Province

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### Abstract

**Background:** Enteroviruses can easily circulate in the population through sewage and they are suitable indicators for environmental surveillance. On the other hand, in some countries there are evidences of silent circulation of viruses in sewage specimens despite no virus isolation from clinical specimens. Therefore, WHO has suggested environmental surveillance using surface water and sewage specimens for final confirmation of Poliovirus eradication. In this research, according to wild Poliovirus circulation in Afghanistan and Pakistan and probability of virus entrance to Iran, and also to assure wild Poliovirus eradication, the environmental surveillance was performed in Sistan and Balouchestan Province of Iran.

**Methods:** From March 2004 to February 2005, 86 specimens from 2 sewage disposal systems, 5 hospitals and surface water from several villages were collected by Grab Sample method and tested for Enteroviruses directly and using 2 concentration methods: Pellet and Two-phase. Then Poliovirus and Non-Polio Enteroviruses (NPEV) were serotyped by microneutralization method and Polioviruses were intratypically differentiated using ELISA and Probe Hybridization techniques.

**Results:** From a total of 86 specimens, Enteroviruses and Non-Polio Enteroviruses were isolated from 49(56.98%) and 46(53.49%) of specimens respectively. Polioviruses were isolated from 18(20.93%) specimens and none of them was wild Poliovirus fortunately. 13(17.81%), 39(53.42%) and 57(78.08%) of enteroviruses were isolated using Direct, Pellet and Two-phase methods, respectively.

**Conclusions:** The results of this research confirm the validity of environmental surveillance and Polio eradication in Sistan and Balouchestan Province.

**Keywords:** Environmental surveillance, Sewage, Poliovirus, NPEV, Iran

### Introduction

Enteroviruses are a subgroup of the picornaviridae family. They are classified into polioviruses (3 serotypes), coxsackieviruses group A (23 serotype), coxsackieviruses group B (6 serotype), echoviruses (31 serotype) and new enteroviruses (1, 2). Transmissions of these viruses are usually by the fecal-oral or by the respiratory route (3). Enteroviruses infection typically occurs in outbreaks during the tropical rainy season, or the temperate summer and autumn, mainly affecting young children. The risk of infection is directly correlated with poor hygiene and poor sanitation and overcrowding, typically among inadequately vaccinated populations (4). Many enteroviruses are associated with specific syndromes:

for example, the viruses within the Human Enterovirus B species commonly cause meningitis, myopericarditis, spastic paralysis or meningoencephalitis and those within the Human enterovirus A species commonly cause hand-foot-mouth disease, hemorrhagic conjunctivitis, herpangina, acute flaccid paralysis (3, 5) while aseptic meningitis and diabetes are associated with both group A and group B. Echovirus infections range from the common cold and fever to aseptic meningitis and acute hemorrhagic conjunctivitis (5).

Polioviruses are important human pathogens causing the acute paralytic disease poliomyelitis (6). Poliovirus infection is clinically inapparent in 90-95% of cases. Some 4-8% of infections present as abortive illness characterised by upper res-

piratory infection, gastroenteritis or influenza like illness, and a further 1-2% present as aseptic meningitis (4).

In 1988, the World Health Assembly committed the World Health Organization to the global eradication of poliomyelitis by the year 2000 (7). Virtually all countries adopted the four principal strategies for eradication, namely high routine immunization coverage, national immunization days (NIDs), a surveillance system for acute flaccid paralysis (AFP) with laboratory investigation, and mopping-up immunization activities (8). Implementation of WHO-recommended strategies for poliomyelitis eradication resulted in a decrease in the number of globally reported poliomyelitis cases (7) and the number of countries in which polio is endemic declined from 125 to 6 (Afghanistan, Pakistan, Nigeria, Egypt, Niger, India) by 2003 (9, 10). The American, Western Pacific, and European regions of the WHO have been certified free from wild poliovirus (7).

Since 1992, Iran has consistently reported high routine vaccination coverage of infants (greater than or equal to 94%) with three doses of oral poliovirus vaccine (OPV3). Annual National Immunization Days since 1994 achieved high coverage (greater than 98%) among children aged less than 5 yr (11). The number of reported virologically confirmed cases of wild poliovirus was 13 in 1997 and 2 as of June 1998. During 1997-June 1998, a total of 13 of 15 wild-virus associated cases were reported from southeastern Iranian provinces and were frequently linked epidemiologically to Afghanistan and Pakistan (11). On the other hand, in several countries such as Israel and Egypt, wild polioviruses have been detected in the environment in the absence of reported AFP cases (12). Therefore, WHO has suggested environmental surveillance using surface water and sewage specimens in high risk regions (10).

Fortunately, since 2000, none of the wild polioviruses serotypes have been detected in Iran. Due to the neighborhood of Iran with Afghanistan and Pakistan in the eastern boarder

(which they have the circulating wild polioviruses), Sistan and Balouchestan province as for the wild poliovirus entrance are considered a part of the high risk areas.

The aim of this study was environmental surveillance of Sistan and Balouchestan Province using sewage and surface water to examine the existence of wild poliovirus and vaccine derived polioviruses (VDPV), and confirming the polio eradication. Also study of non-polio enteroviruses for evaluation of environmental circulation, and confirming of the concentration methods was performed.

## **Materials and Methods**

### ***Sampling***

In this study, from March 2004 to February 2005, 86 samples were collected from 2 sewage disposal systems, 5 hospitals and surface water from several villages in Sistan-Balouchestan Province using Grab Sampling procedure. All the samples were collected from the influent of raw sewage. Samples were collected in 1000 ml sterile bacteriological sampling bottles and were carried to National Polio Laboratory in Tehran University of Medical Science Research Institute. In all cases, the characteristics of sewage samples (place, date, pH, and temperature) were documented. The samples during transferring and before inoculation to cell culture, kept at 4° C (cold chain).

### ***Concentration***

The sewage samples were examined directly and also two concentration methods: Pellet and Two-phase. It is worthy to say that, the Pellet method, for the first time, is suggested by us.

To concentrate by this method the supernatant was transferred to a sterile flask. Then from the remainder of sewage, 75 ml was transferred to 5 sterile centrifuge tubes and it was centrifuged for 10 min with 5000 RPM at 5° C and the tubes were kept at 4° C. The Two-phase method was accomplished by using the suggested method of Hovi in 2001 (13).

For destroying the bacteria and fungus 1 ml of chloroform were added to 4 ml of the Direct, Pellet and Two-phase samples and were shake

for 20 min whit 200 RPM. The containers of the tubes were centrifuged in 2000 RPM at 5° C and supernatant was collected in 1.8 ml sterile cryotube.

#### **Cell culture method**

For isolation of polio and non-polio enteroviruses (NPEVs) the L20B, RD and Hep-2 cell lines are used.

The sewage inoculation rate to each tube of cell culture was 200 µl. After inoculation they were kept in 36° C for 7 d. To observe the CPE, the tubes were examined by inverted microscope every day and the positive samples were kept at -20° C. Also after 7 d, the negative tubes were Freezed & Thawed and repasaged. Any culture positive in RD cells but negative in L20B cells was re-passaged in L20B cells and examined for 7 d to exclude the possibility that they are polioviruses (14).

#### **Neutralization test**

For the identification of poliovirus isolates, samples of diluted isolate were mixed with equal volumes of a selected set of polyclonal antisera made in animals against poliovirus types 1, 2 and 3. Using the microneutralization technique, the antisera-virus mixtures were incubated for 1 h at 36° C to allow the antibodies to bind to the virus. Subsequently, suspensions of cells were added to the microplates which were examined daily for the presence of CPE. The antiserum that prevented the development of CPE indicated the identity of virus.

Identification of non-polio enterovirus isolates were similar to poliovirus, but the viruses were tested in duplicate against a trivalent pooled polio antiserum (PP), a coxsackievirus B1-B6 pool (CP), and seven pools against coxsackievirus A9 and 20 echoviruses (A-G) (14).

#### **ELISA method**

Wells of microtitre plates or strips coated with bovine IgG antibodies to poliovirus type 1, 2 and 3 were incubated with the identified and typed poliovirus strain to be tested. Incubation was then carried out with the type-specific, cross-adsorbed, rabbit antisera. After washing off any unbound rabbit sera peroxidase-labelled

anti-rabbit IgG antibody was added to detect bound rabbit sera. RIVM supplies a kit containing essential reagents to carry out the ELISA method which is obtainable through WHO (14).

#### **Probe hybridization method**

RNA probe hybridization was done on identified typed polioviruses of high titer. The viral RNA was extracted and immobilized on to filters. Digoxigenin-labelled enterovirus group; sabin type-specific; and wild virus genotype-specific probes were added and allowed to hybridize to the immobilized RNA. Unbound probe was removed by washing and bound probe was detected using a colorimetric reagent (14).

#### **Statistical analysis**

The statistical analysis of the results obtained was performed by SPSS13 software.

## **Results**

Eighty six samples were collected from two sewage disposal systems, 5 hospitals and number of villages in Zabol, Zahedan and Chabahar cities. The most population were related to Zabol and Zahedan Jame-Jam disposal systems, with 120000 (54.97%) and 50000 (22.90%) people, respectively. Distribution frequencies of sampling from researching place were approximately equal, and averages of 21 samples were collected in every season.

From the 86 collected samples, enteroviruses and NPEVs were isolated from 49(56.98%) and 46(53.49%) samples, respectively and a total of 18 polioviruses were isolated: 9 viruses in Zahedan, 5 in Zabol and 4 in Chabahar. Type analysis identified 13 polio II and 5 polio III. All polioviruses were characterized as Sabin-like. Most enteroviruses (28.77%) were isolated from Zahedan Jame-Jam disposal system (Fig.1) and Echo4 (15.07%) and Cox-B (12.33%) were identified most frequently NPEV serotypes during this research (Table 1).

Statistical analysis with SPSS13 software were showed significant different (in 0.05 level) between Direct and Two-phase concentration methods, in detection of poliovirus. This difference, also, were showed between direct and two con-

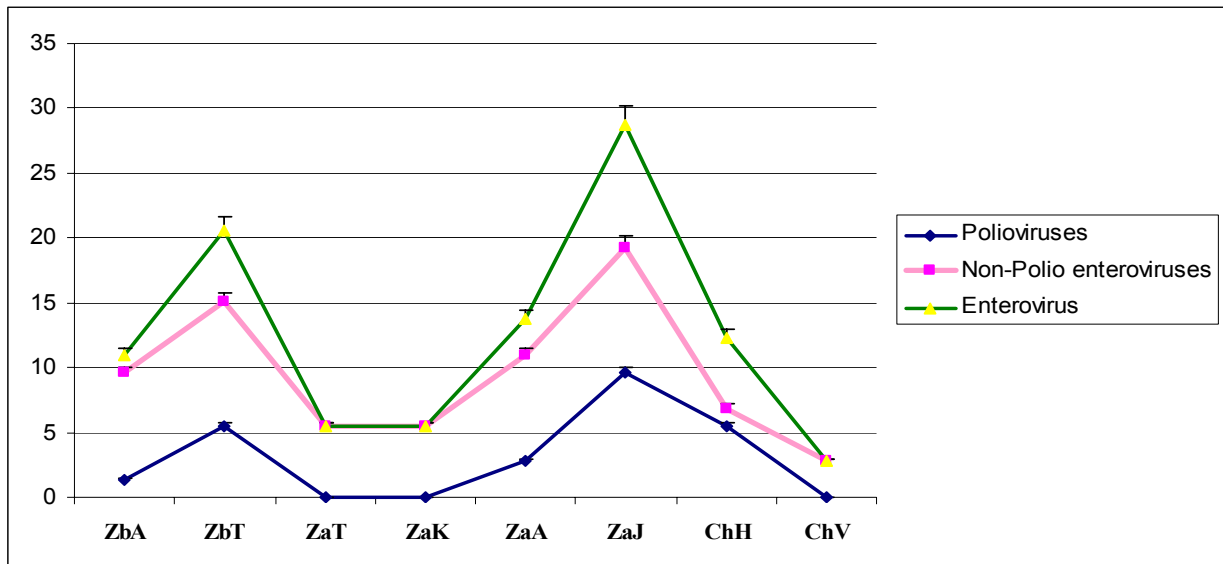
centration methods, in detection of non-polio enterovirus, and between L20B and Hep-2 cells, in poliovirus isolation, and RD and Hep-2, in non-polio enterovirus isolation. Peak detection of polioviruses using Pellet (5.48%) and Two-phase (6.85%) concentration methods

were occurred during autumn and summer, and the most isolation of non-polio enteroviruses using Pellet (17.81%) and Two-phase (21.92%) concentration methods were documented in winter and autumn, respectively.

**Table 1:** Number of Polio and NTEVs that isolated in this study, Iran, Sistan and Balouchestan

Serotype	Polioviruses			Non – Polio enteroviruses										Total n (%)
	P1	P2	P3	E1	E3	E4	E6	E7	E11	E12	E33	Cox-B	NTEV	
Place	n (%)			n (%)										(%)
ZbA <sup>#</sup>	0(0)	1(1.37)	0(0)	0(0)	2(2.74)	2(2.74)	2(2.74)	0(0)	0(0)	0(0)	0(0)	1(1.37)	0(0)	8(10.96)
ZbD <sup>\$</sup>	0(0)	3(4.11)	1(1.37)	2(2.74)	1(1.37)	0(0)	0(0)	0(0)	2(2.74)	0(0)	0(0)	2(2.74)	4(5.48)	15(20.55)
ZaT <sup>†</sup>	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(2.74)	0(0)	0(0)	1(1.37)	1(1.37)	0(0)	0(0)	4(5.48)
ZaK <sup>*</sup>	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	3(4.11)	0(0)	0(0)	1(1.37)	0(0)	4(5.48)
ZaA <sup>‡</sup>	0(0)	2(2.74)	0(0)	0(0)	0(0)	2(2.74)	0(0)	3(4.11)	0(0)	1(1.37)	0(0)	1(1.37)	1(1.37)	10(13.70)
ZaJ <sup>§</sup>	0(0)	3(4.11)	4(5.48)	0(0)	0(0)	3(4.11)	0(0)	2(2.74)	3(4.11)	1(1.37)	0(0)	4(5.48)	1(1.37)	21(28.77)
ChH <sup>€</sup>	0(0)	4(5.48)	0(0)	0(0)	0(0)	3(4.11)	0(0)	0(0)	0(0)	1(1.37)	0(0)	0(0)	1(1.37)	9(12.33)
ChV <sup>±</sup>	0(0)	0(0)	0(0)	0(0)	0(0)	1(1.37)	0(0)	1(1.37)	0(0)	0(0)	0(0)	0(0)	0(0)	2(2.74)
Total	0(0)	13(17.81)	5(6.85)	2(2.74)	3(4.11)	11(15.07)	4(5.48)	6(8.22)	8(10.96)	4(5.48)	1(1.37)	9(12.33)	7(9.59)	73(100)

#: Zabol Amirolmomenin Hospital, \$: Zabol Disposal sewage system, †: Zahedan Tamine Ejtemaei Hospital, \*: Zahedan Khatamolambia Hospital, ‡: Zahedan Aliebneabitaleb Hospital, §: Zahedan Jame-Jam sewage disposal system, €: Chabahar Hospital, ±: Chabahar Villages



ZbA: Zabol Amirolmomenin Hospital, ZbD: Zabol Disposal sewage system, ZaT: Zahedan Tamine Ejtemaei Hospital, ZaK: Zahedan Khatamolambia Hospital, ZaA: Zahedan Aliebneabitaleb Hospital, ZaJ: Zahedan Jame-Jam sewage disposal system, ChH: Chabahar Hospital, ChV: Chabahar Villages

**Fig. 1:** Number of Enteroviruses isolated base sample place, Iran, Sistan and Balouchestan

## **Discussion**

In recent years substantial progress toward polio eradication has been made in the Eastern Mediterranean Region, where 18 of the 22 countries are polio-free and polio remains endemic in only three countries (Afghanistan, Egypt, and Pakistan) of this region (15).

One of the greatest risks to achieving the global polio eradication initiative is ongoing wild poliovirus transmission in any of the 6 remaining endemic countries (Afghanistan, Pakistan, Egypt, Nigeria, India and Niger) which it can be dangerous for the neighboring areas of those (10). For example, in 2004, polio cases caused by wild poliovirus originating from northern Nigeria were reported in 11 countries- Benin, Botswana, Cameroon, Guinea, Mali, Saudi Arabia, Burkina Faso, Central African Republic, Chad, Cote d'Ivoire, and Sudan- reestablishing transmission in the latter five countries (9).

In the first half of 2005, Pakistan had 6 active polio transmission zones that the most of those locates near the borders of Iran with Afghanistan and Pakistan, unfortunately (16). It is considerable, the last isolated wild polioviruses in Chabahar, the city in the Sistan and Balouchestan province of Iran, were linked epidemiologically to Pakistan.

On the other hand, in several countries wild polioviruses have been detected in the environment in the absence of reported AFP cases. Thus, after eradication of wild polioviruses from AFP cases in high risk areas, WHO has recommended the complementary surveillance by using sewage sample and stools of healthy children (12). Therefore, Sistan and Balouchestan province was selected for this research.

Based on the recommendation of WHO, a useful criterion of satisfactory overall performance of the surveillance is detection of non-polio enteroviruses in the samples. At least 30% of concentrated sewage from grab samples should reveal NPEV (12).

In this study, for the first time, we suggested the Pellet concentration method, and used the two-

phase concentration method, simultaneously. From the total samples, non-polio enteroviruses were isolated from 11(12.79%), 31(36.05%) and 44 (51.16%) samples by direct, pellet and two-phase methods, respectively. These results confirm the efficiency of concentration methods, in enterovirus surveillance.

Another purpose of this study was evaluation of distribution and analysis of environmental circulation of NPEVs. Japanese study on enteroviruses shows that E6, E17, Cox-B5 in 1999, E9, E71, E25, E11 in 2000 and E11 and Cox-B5 in 2001 have played the main role in aseptic meningitis outbreak. In 2002, also E11 and E13 were the most frequently isolated enteroviruses from aseptic meningitis patients (17).

During the seasons under study, E4 (15.07%), Cox-B (12.33%) and E11 (10.96%) were the predominant serotypes. Other non-polio enterovirus serotypes were isolated include Non-Typeable Enteroviruses (NTEVs) (9.59%), E7 (8.22%), E12 and E6 (5.48%), E3 (4.11%), E1 (2.74%) and E33 (1.37%).

The epidemiological pattern of enterovirus infections varies by geographical region, climate, age and season. Therefore, it is necessary to evaluate relationship between non-polio enterovirus disease and environmental circulation of these viruses in different part of Iran. Such studies can be performed to provide a suitable vaccine to prevent of enterovirus infections in high risk area.

Until now, the cell line that capable to isolation of all enteroviruses was not identified. Since 1998, L20B cells replace Hep-2 to reduce the workload of running three cell lines at once. The combination of L20B and RD cells should considerably shorten the time required for poliovirus isolation and identification. However, the use of L20B and RD cells without Hep-2, may have an impact on the non-poliovirus enterovirus isolation rate, especially during periods of Coxsackie B circulation in the community (14, 18, 19). Therefore, in this study, L20B, RD and Hep-2 cells were used for identification of more extended spectrum of enteroviruses. Overall, 18, 14 and

6 vaccine-like polioviruses were detected in L20B, RD and Hep-2 cells, and 46 and 9 NPEVs were detected in RD and Hep-2 cells, respectively.

Another risk to achieving the global polio eradication initiative is increase of polio outbreak due to vaccine derived polioviruses (10).

Recently, cVDPVs have been associated with four outbreaks of poliomyelitis. Type 2 VDPV circulated in Egypt for over 10 yr (1983 to 1993) (20, 21). An outbreak of poliomyelitis on the island of Hispaniola was associated with type 1 cVDPV (22). In the Philippines, type 1 cVDPV was involved in three poliomyelitis cases in 2001 (23), and in Madagascar in 2002, type 2 cVDPV was the causative agent of four paralytic cases (24).

Environmental surveillance is also a potential tool for monitoring circulating vaccine-derived poliovirus (12). For example, A type 3 VDPV was isolated from a single sewage sample collected in Estonia in 2003 (6). Type 2 VDPVs were isolated intermittently from sewage in Slovakia during Oct 2003-June 2004 and from a single sewage sample collected in Israel in Apr 2004 (25).

In this study, the isolated polioviruses were differentiated intratypically by ELISA and Probe Hybridization techniques. Fortunately, all 18 isolated polioviruses were Sabin-like. Not isolating wild and vaccine derived polioviruses, in this study, shows the proper AFP surveillance and vaccination coverage in our country, especially in high risk area.

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The authors declare that they have no Conflict of Interests.

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