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SHORT COMMUNICATION

Detection of Polioviruses in Sewage Using Cell Culture and Molecular Methods

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Abstract

The work presented here demonstrates the utility of a two-step algorithm for environmental poliovirus surveillance based on: preselection of sewage samples tested for the presence of enteroviral genetic material-RT-PCR assay and detection of infectious viruses by cell culture technique (L20B for polioviruses and RD for polio and other non-polio enteroviruses). RD and L20B cell lines were tested to determine their sensitivity for isolation of viruses from environmental samples (sewage). Finally, we wanted to determine if sewage concentration affects the results obtained for RT-PCR and cell cultures.

Key words: cell lines L20B and RD, environmental surveillance, poliovirus, sewage

Polioviruses (PVs) are small (30 nm in diameter), non-enveloped, icosahedral-shaped capsid viruses belonging to the *Picornaviridae* family. PVs possess an approximately 7.5-kilobase (kb) positive-sense single-stranded RNA genome. Poliovirus is a causative agent of *poliomyelitis*, commonly known as polio (Landsteiner and Popper, 1909). Polioviruses are transmitted by the fecal-oral route, they multiply in the gastrointestinal tract and are excreted in large numbers in the feces of infected persons, whether or not they are symptomatic. Virus infects sensitive cells of lymphoid tissue in the mouth, nose and throat. The incubation period lasts from 2 to 35 days. It leads to a transient viremia and the virus spreads to the reticuloendothelial system without causing clinical symptoms (Sabin, 1956; Bodian and Horstmann, 1965; Melnick, 1996). Most natural infections of humans end at this stage with a minor disease comprising nonspecific symptoms such as sore throat and fever. In very rare cases, 1–2% of infected individuals, the virus enters the central nervous system (CNS) and replicates in motor neurons within the spinal cord, brain stem, or motor cortex. All individuals infected with wild poliovirus or vaccinated with live, attenuated oral polio vaccine (OPV) excrete large number of virus particles in faeces for periods of up to several weeks. The rationale for environmental surveillance is based on the characteristic poliovirus excretion pattern. Viruses cannot replicate outside the host cell and therefore cannot multiply in the environment, however, they can survive and remain infectious in the environment for varying lengths of time, depending on the immediate conditions. For this reason environmental poliovirus surveillance (ENV) is recommended by the WHO (WHO, 2003).

ENV is based on detection of the presence of the virus in sewage samples by a variety of laboratory methods for concentration, separation and identification. Environmental surveillance has been used successfully in monitoring enteric viruses circulation and in assessing the extent or duration of epidemic poliovirus circulation in a specific population (AW and Gin, 2010; Kern *et al*., 2013; Parasidis *et al*., 2013). ENV currently plays an important role in the monitoring of circulating vaccine-derived polioviruses (VDPVs). In recent years, VDPV strains were isolated from sewage in Israel, Finland, Egypt, Greece, Switzerland and Slovakia in the years 1984–2010 (Vinje *et al*., 2004; Kew *et al*., 2004; 2005; Pavlov *et al*., 2005; Dedepsidis *et al*., 2007). VDPV strains are derived from attenuated vaccine-virus contained in oral polio vaccine (OPV). OPV strains can mutate in VP1 region during their replication in the human intestine, and some mutations may result in recovery of the capacity for higher neurovirulence. These neurovirulent revertants may cause paralysis in

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humans and develop sustained circulation (cVDPV). On rare occasions, if a population is seriously under-immunized, VDPVs can continue to circulate for an extended period of time.

In 2007 in Switzerland (certified by WHO as polio-free), testing of sewage samples revealed the presence of wild poliovirus (WHO, 2007). Wild polioviruses were also isolated from sewage samples in Egypt (2012) and Israel (2013) in the absence of reported AFP cases. The list of countries routinely employing ENV includes the Czech Republic, Egypt, Estonia, Finland, India, Japan, Latvia, the Netherlands, New Zealand, Pakistan, Russia, Slovakia and Switzerland. Our previous study confirmed that sewage is a rich source of enteroviruses circulating in the community (Wieczorek *et al.*, 2015). In Poland, systematic environmental poliovirus surveillance is planned. Accordingly, the aim of this study was to develop and optimize a method for the preparation of wastewater samples collected in Poland. The present study was also conducted to develop a two-step algorithm for ENV based on: 1) preselection of sewage samples tested for the presence of enteroviral genetic material-RT-PCR assay, 2) detection of infectious viruses by cell culture technique (L20B for polioviruses and RD for polio and other non-polio enteroviruses). Thus, we would obtain information about the sensitivity of RT-PCR assay for the detection of enteroviruses in sewage. RD and L20B cell lines were tested to determine their sensitivity for isolation of viruses from environmental samples (sewage). Finally, we wanted to determine if sewage concentration affects the results obtained for RT-PCR and cell cultures.

Sewage samples (pooled, homogeneous, tested negative for enteroviruses): raw (100%), diluted in PBS (80%, 50%, 20%) and PBS (0%) were contaminated with reference laboratory poliovirus strain – PV Sabin type 1 ($TCID_{50} = 10^{-8.2}$/1 ml calculated by Spaerman-Kärber formula). Virus decimal dilutions from 10^{-1} to 10^{-7} were used to contaminate sewage samples. Raw sewage was used as negative control. All samples were processed according to the protocol described earlier (Zubriggen *et al.*, 2008). The RT-PCR assays were performed using Pan-enterovirus primers for enterovirus detection based on the WHO manual (WHO, 2004). Viral RNA was extracted from 140 µl of sewage concentrate using Qiagen Viral RNA Kit following to the manufacturer's instructions. A volume of 200 µl of sewage concentrate was inoculated into tubes with L20B cells and RD cells. Each specimen underwent three passages according to WHO procedures (WHO, 2004).

In order to determine its sensitivity, RT-PCR was used for the detection of enteroviral RNA in sewage samples: raw (100%), diluted in PBS (80%, 50%, 20%) and PBS only (0%). Previously, samples were contaminated with PV Sabin type 1. The results of the RT-PCR assay are shown in Table I. The highest dilution of PV suspension, showing a visible band in RT-PCR in all three replicates of a sample, was taken as the detection limit. The amplicon was visible up to 10^{-5} dilution for each concentration of sewage. Viral genetic material was detected in concentration of ~0.5 $TCID_{50}$. The number of positive results and probabilities range from 9 to 10 and 0.60 to 0.67 respectively for each concentration of sewage. The concentration does not significantly affect the amplification results.

The cell lines: L20B for polioviruses and RD for polio and other non-polio enteroviruses were tested to determine their sensitivity for isolation of infectious viral particles from the environmental samples. The results of poliovirus isolation in L-20B and RD cells are

Table I
Detection of poliovirus genetic material in sewage samples by RT-PCR.

RT-PCR						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (50 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (5 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-5} (0,5 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-6} (0,05 $TCID_{50}$)	0 0 0	0 0 +	0 0 0	0 0 +	0 0	2 P(A) = 0.14
10^{-7} (0,005 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A) and probability P(A)	9 P(A) = 0.60	10 P(A) = 0.67	9 P(A) = 0.60	10 P(A) = 0.67	6 P(A) = 0.60	

"0" – negative RT-PCR result, "+" – positive RT-PCR result

Table IIA
Poliovirus isolation from sewage samples in L20B cells.

L20B						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (2000 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (200 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-5} (20 $TCID_{50}$)	0 + 0	+ + 0	+ + +	+ + +	+ 0	10 P(A) = 0.70
10^{-6} (2 $TCID_{50}$)	0 0 +	0 + +	+ + 0	0 + +	0 0	7 P(A) = 0.5
10^{-7} (0,2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A)	8 P(A) = 0.53	10 P(A) = 0.67	11 P(A) = 0.73	11 P(A) = 0.73	5 P(A) = 0.50	
Probability	P(A) = 0.63					

"0" – negative result, "+" – positive result

Table IIB
Poliovirus isolation from sewage samples in RD cells.

RD						
Poliovirus suspension	Sewage concentration					No of positive results (A)
	100%	80%	50%	20%	0%	
10^{-3} (2000 $TCID_{50}$)	+ + +	+ + +	+ + +	+ + +	+ +	14 P(A) = 1
10^{-4} (200 $TCID_{50}$)	+ + +	+ 0 +	+ + +	+ + +	0 0	14 P(A) = 0.79
10^{-5} (20 $TCID_{50}$)	0 0 +	0 0 0	0 0 +	0 0 +	0 0	3 P(A) = 0.20
10^{-6} (2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
10^{-7} (0,2 $TCID_{50}$)	0 0 0	0 0 0	0 0 0	0 0 0	0 0	0 P(A) = 0
No of positive results (A)	7 P(A) = 0.47	5 P(A) = 0.33	7 P(A) = 0.47	7 P(A) = 0.47	2 P(A) = 0.20	
Probability	P(A) = 0.39					

"0" – negative result, "+" – positive result

shown in Tables IIA and IIB, respectively. The highest dilution of virus suspension showing cytopathic effect in all three replicates of a sample (raw sewage, 100%) was 10^{-4} (200 $TCID_{50}$). The probability of poliovirus isolation in cell lines was 0.63 (L20B) and 0.39 (RD).

The highest sensitivity of poliovirus isolation in L20B cells was observed in 80%, 50% and 20% sewage at 10^{-6} dilution of the virus suspension (~2 $TCID_{50}$). For 80%, 50% and 20% sewage samples the number of positive results and probabilities range from 10 to 11 and P(A) = 0.67 to P(A) = 0.73, respectively.

The highest sensitivity of poliovirus isolation in RD cells was observed in 100%, 50% and 20% sewage at 10^{-5} dilution of the virus (20 $TCID_{50}$) and was ten times lower than the values obtained for L20B cell line. For 100%, 50% and 20% sewage samples the number of positive results and probability was A = 7 and P(A) = 0.47 respectively.

The efficiency of poliovirus isolation from control samples, containing only PBS, was significantly lower than the other samples and the probability of isolation in L20B and RD cells was P(A) = 0.5 and P(A) = 0.2 respectively.

Environmental surveillance is an effective approach in investigating the circulation of polioviruses and other human enteroviruses (HEV) in the population.

Both Finland and Israel have used ENV as the main approach to PV surveillance for decades (Hovi *et al*., 2010). The World Health Organization recommends regular testing environment for the presence of poliovirus as an important complement to the new strategy for Global Polio Eradicaction Initiative (GPEI). Criteria for considering environmental surveillance as a supplementary approach in GPEI were published in WHO Guidelines, which contain principles for selecting sampling sites, propose methods for sample processing and suggest possible program responses to PV detection in sewage (WHO, 2003).

In this paper we used the protocol based on concentration of sewage samples by precipitation. A rapid, efficient and inexpensive method was developed to concentrate viruses from sewage. The method consists of adsorbing the viruses to silicon dioxide (SiO_2) in the presence of 0.5 mM $AlCl_3$ and adjustment of the pH to 3.5. Because of its simplicity, the protocols based on silicon dioxide, becomes more often used in environmental virology, especially for large volumes of analyzed samples (Katayama *et al*., 2002; Kocwa-Haluch and Zalewska, 2002; Li *et al*., 2010). Furthermore Zubriggen *et al*. (2008) showed a higher efficiency of virus recovery using a precipitation technique (60%) than the two phase separation method (30%) recommended by the WHO (2003).

In this study we showed the high sensitivity of RT-PCR assay for detection of enteroviruses in sewage. The number of detected virus particles at 10^{-5} dilution was ~0.5 $TCID_{50}$. The RT-PCR assay gave identical results to those obtained in our previous study (Witek and Wieczorek, 2009). Viral RNA was extracted directly from the poliovirus suspension and identical sensitivity of RT-PCR assay was observed. The data suggest high efficiency of virus recovery from sewage samples. Our results also indicate that all steps of analytical process (*e.g.* preparation of sewage sample, amplification procedure) were properly selected.

Cell culture technique, however, in spite of some undeniable problems, is still the gold-standard method for virological surveillance of polioviovirus. According to WHO protocol two poliovirus-sensitive cell lines (RD, L20B) are recommended for virus detection from clinical and environmental samples (WHO, 2004). The results of the study show that L20B cells (~2 $TCID_{50}$ for 100% sewage) can provide greater sensitivity for detection of polioviruses than RD cells (~20 $TCID_{50}$ for 100% sewage). Similar results have been reported by other authors (Wood and Hull, 1999). Likewise, compounds of sewage that are toxic for cells can affect the probability of isolation of poliovirus in different cell lines. Certain substances have a stronger cytotoxic effect on RD cells than L20B cells (Al-Khayat and Ah mad, 2012).

The sewage concentration affected the results obtained for PV isolation in cell lines. The efficiency of virus isolation was lower in control samples containing only PBS. There is evidence that virus survival is enhanced in polluted waters, presumably as a result of some protective effect that the viruses may receive when they are adsorbed onto suspended solid particles in sewage (Feachem *et al*., 1983). The lack of these particles could reduce virus ability to survive outside the host organism.

Preparation of sewage samples according to the protocol based on silicon dioxide and sodium chloride seems to be very promising. Therefore, it is important to consider the limitations of this method such as concentration of sewage and its toxicity. Environmental toxicity and inhibitory factors often interfere with both molecular and cell culture assays. Molecular assays such as direct RT-PCR are sensitive and allow lower quantities of the original sample to be analysed. On the other hand, RT-PCR may provide inaccurate estimates of infectious virus contamination in the environment because they detect both inactivated viruses as well as infectious viruses. Cell culture assay is the only reliable method that will allow the detection of infectious virus in that case. In conclusion, our preliminary data suggest applying two-step algorithm for environmental monitoring: RT-PCR-based detection step and isolation of infectious viruses in cell cultures.

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