Comparison of ELISA and dual stage real time RT-PCR to differentiate Sabin like and non-Sabin like poliovirus isolates

Nirmal Kaundal¹ · Purva Sarkate² · Charu Prakash² · Narayan Rishi³

Received: 8 December 2016 / Accepted: 20 April 2017 / Published online: 25 May 2017
© Indian Virological Society 2017

Abstract Environmental surveillance of polioviruses has been used as an important tool in monitoring circulation of wild polioviruses and/or Vaccine derived polioviruses in sewage samples. It is important to distinguish Sabin like isolates from non-Sabin like; ELISA & dual stage real time RT-PCR have been used for the same. Current study was carried out on sewage isolates to compare ELISA & RT-PCR with sequencing to distinguish Sabin like from non-Sabin like. Out of 468 sewage specimens, 91 (19.44%) were non-polio enteroviruses positive and 377 (80.56%) were polio positive by virus isolation method. A total of 488 polio virus isolates were detected by L20B and RD route which were further subjected to ELISA and RT-PCR. The results were compared with sequencing. On comparison, the specificity of ELISA was only 66.67% in spite of very low sensitivity (3.43%). The sensitivity of RT-PCR was 97.71% which makes it a good primary screening test for detection of non-Sabin like viruses. However, the specificity was only 33.33%. RT-PCR appears to be a sensitive tool for detecting non-Sabin like viruses however; the isolates which are non-Sabin like by RT-PCR may not necessarily be mutated viruses. ELISA cannot be used for

differentiation of Sabin likes from non-Sabin likes due to low sensitivity.

Keywords Poliovirus · Environmental surveillance · Non-Sabin like · Sabin like · ELISA · Real-time RT-PCR

Introduction

Eradication of polio is a public health program of unprecedented magnitude, complexity and cost. Tremendous progress has been made in the global fight against poliovirus. Cases of type 1 wild poliovirus are still being detected in 2016 in Afghanistan, Pakistan and Nigeria (<http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx>). Last wild poliovirus type 2 was found in the year 1999 in India and last wild poliovirus type 3 was found in the year 2012 in Nigeria (<http://www.searo.who.int/entity/immunization/topics/polio/eradication/sea-polio-type-2-free/en/>).

Environmental surveillance of polioviruses has been used as a supplementary tool in monitoring the circulation of wild PVs (Polioviruses) and/or VDPV (vaccine derived poliovirus) in sewage samples (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf) [5]. In India, environmental surveillance was initiated in 2001 in Mumbai. This was later extended to Delhi in 2010 [5]. This was further strengthened with the inclusion of Punjab and Gujarat in 2013 (<http://www.searo.who.int/india/topics/poliomyelitis/surveillance/en/>).

OPV (oral polio vaccine) has played an important role in the elimination of wild poliovirus disease. At the same time, there is increasing risk of Vaccine Associated Poliomyelitis resulting from vaccine-derived poliovirus. The attenuated live Sabin virus in OPV type is genetically unstable [1].

✉ Charu Prakash
drcharuprakash@gmail.com

¹ Virology-1 Laboratory, Microbiology Division, National Centre for Disease Control, 22-Shamnath Marg, Delhi 110054, India

² Microbiology Division, National Centre for Disease Control, 22-Shamnath Marg, Delhi 110054, India

³ Amity Institute of Virology and Immunology, Amity University Uttar Pradesh, Sector 125, Distt. Gautam BudhaNagar, Noida, Uttar Pradesh 201313, India

After cycles of reproduction in vaccinees and their contacts, it tends to lose its attenuating mutations evolving into Vaccine-Derived Polio Viruses (VDPV) which have virtually no phenotypic distinctions from wild polioviruses and have the ability to circulate, especially in under-immunized communities, causing sporadic cases of the disease and outbreaks [2]. Such VDPV are a serious threat to the global polio eradication initiative. Poliovirus genome generally evolves at a rate of 1% per year. Hence the World Health Organization classifies VDPV as those Sabin vaccine derivatives in which the genome segment encoding the capsid protein VP1 deviates from the parental vaccine strain by at least 1% of nucleotides [8]. The frequency of occurrence of circulating vaccine derived poliovirus is low but significant. A total of 23 cVDPV outbreaks have occurred between 2000–2013 resulting in 717 cases of VDPV [10]. The number of VDPV's reported is 32 in the year 2015 globally (<http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx>). It is important to distinguish Sabin like strains from non-Sabin like strains which can potentially give rise to Vaccine Associated Poliomyelitis. Several methods have been used to distinguish Sabin like viruses from non-Sabin like viruses such as ELISA, dual stage real-time RT-PCR and sequencing. Although sequencing is the gold standard to identify non-Sabin like strains, it is not possible to do sequencing for all isolates due to the large number of isolates obtained from sewage sample processing. Hence, efforts have been made to find out a screening test to distinguish non-Sabin from Sabin like. The current study was carried out on isolates obtained from environmental sewage specimens with the aim to compare ELISA & RT-PCR with sequencing to distinguish SL (Sabin like) from NSL (non-Sabin like).

Materials and methods

Sewage sample collection, sample processing and virus isolation was done as per the Environmental Poliovirus Surveillance (EPS) guidelines provided by World Health Organization (WHO) (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf). A generalized scheme of virus identification is given in Fig. 1.

Sewage sample collection

Sewage samples from 7 sites of Delhi (on a weekly basis) and 4 sites of Punjab (on a fortnightly basis) were collected by Grab method mentioned in WHO guidelines of Environmental surveillance 2015. Briefly, sewage sample was collected by lowering a clean bucket and the collected sample was filtered through muslin cloth into a pre-labeled sterile 1-liter bottle. The collected sewage sample was

transported to laboratory in cold chain as per WHO guidelines 2003 (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf).

Sample processing

To start with pH of the sample was adjusted to 7.2. One litre of sample was then centrifuged at 5000 rpm for 30 min. The supernatant (supernatant 1) is transferred to another container and sediment containing bottles were rinsed with phosphate buffer saline and the sediment was collected in 50 ml conical centrifuge tube. The resultant solution was diluted with 3% beef extract (five times of its volume) and chloroform containing 0.0001% of Dithizone (half of its volume). This was again centrifuged at 5000 rpm for 30 min and the supernatant was added to supernatant 1. To supernatant 1, PEG 8000 (80 g/l) and NaCl (17.5 g/l) was added. The mixture was mixed on a magnetic stirrer for 30 min and allowed to stand at 4 °C for 16 h. Next day, the mixture was centrifuged at 5000 rpm for 30 min. The supernatant (supernatant 2) obtained was discarded. The sediment containing bottles were rinsed with PBS. Final sediment was collected in 50 ml conical centrifuge tube and was re-suspended in 3% beef extract (five times of its volume) and chloroform (half of its volume). The sample was then centrifuged at 5000 rpm for 30 min. The upper aqueous phase was collected in 15 ml centrifuge tube and was labeled as supernatant 3. The sediment was re-suspended in 3% beef extract (half of its volume and centrifuged at 5000 rpm for 30 min. This supernatant was added to the supernatant 3 and sediment was discarded. 2% of fetal bovine serum and 1% of antibiotics was added to the final volume of the supernatant 3. Final supernatant 3 is called the sewage extract and was used for virus isolation.

Virus isolation

For virus isolation, two cell lines RD (Rhabdomyosarcoma) and L20B (mouse cell line expressing the gene for the human cellular receptor for poliovirus) were used. Adsorption method was used for each sewage extract, 0.4 ml of sewage extract was added to RD flask/L20B flasks. It was incubated 37 °C for 30 min. After this 4.6 ml of maintenance medium was added to the flasks.

Polio neutralization test

Polio neutralization test was performed as per the guidelines described in the WHO manual (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf). After confirmation of the poliovirus type, virus was further characterized by ELISA and RT-PCR to

Fig. 1 Scheme followed during the isolation and identification of poliovirus from sewage samples

classify virus as Sabin-like (SL), non-Sabin-like (NSL) or double-reactive (DR). Double-reactive is called when the isolate is showing positivity both as SL and NSL.

ELISA (Enzyme Linked Immunosorbant Assay) for intratypic differentiation

ELISA was used to determine if a particular type of poliovirus is sabin-like or non sabin-like. The ELISA for intratypic differentiation into SL, NSL and DR was performed on the single serotype isolates which were isolated from polio neutralization tests. Three different ELISAs were used for P1, P2 and P3 types respectively. The wells were coated with 100 µl of anti P1, anti P2 and anti P3 antibody respectively for intra-typic differentiation. In anti P1 plate, four wells were used per sample i.e. 1st as blank, 2nd as Anti-Total (Anti group specific enterovirus antibody), 3rd as Anti-SL and 4th as Anti-NSL. 100 µl of sample had to be dispensed in three wells except blank well. In blank well, wash buffer was added at each step. One SL and NSL control was used in each plate and incubated at 37 °C for 2 h. After washing, three different antibodies were added to three different wells for one sample (to the 2nd well 100 µl of Anti-Total antibodies, 3rd well Anti-SL antibodies and 4th well Anti-NSL antibodies) and incubated at 37 °C for 1 h. After washing, 100 µl of conjugate and substrate was added followed by incubation for 1 h. After adding stop solution reading was taken with the help of ELISA reader using filter of 450 nm. If the sample contained SL type virus, it will react with the anti-SL antibody of its type and will give a positive reaction. If the sample contains NSL virus, it will react with the

anti-NSL antibody of its type and will give a positive reaction. If the sample is both SL and NSL positive, it was considered as double reactive and referred for sequencing. The NSL and DR positive isolates were sent to Referral lab for sequencing of complete VP1 region of the viral genome.

Dual stage RT-PCR for intratypic differentiation

Dual stage RT-PCR was performed on isolates which were isolated from polio neutralization tests. The real-time PCR kit used for differentiating Sabin like from non-Sabin like viruses was developed by CDC and was performed as per the kit literature. Isolates were subjected to VDPV (vaccine derived poliovirus) screening to determine whether the type-specific isolate was Sabin like or Non-Sabin like. The NSL isolates were sent to referral lab for sequencing.

Results and discussion

Sample processing and virus isolation

The examination of composite human fecal samples through environmental surveillance links poliovirus isolates from unknown individuals to populations served by the wastewater system. Environmental surveillance can provide valuable supplementary information in addition to AFP surveillance. Environmental surveillance has crucial importance in the final stages of the WHO global polio eradication initiative [7].

The study was conducted on environmental sewage samples collected from Delhi and Punjab in the period March 2013 to March 2014. A total of 489 sewage samples were collected in this period. Out of 489 sewage samples, 21 (4.29%) samples were found to be toxic. Sewage is normally composed of many types of pollutants. Raw sewage may contain high levels of pollutants like nutrients, inorganic chemicals, organic micropollutants, microorganisms and microbial products like endotoxins [4, 6, 12]. In the present study 21 (4.29%) of the 489 specimens showed cell toxicity and could not be processed further. However, the problem was not restricted to one particular site but was seen in all sewage samples. On root cause analysis, the chloroform lot was found to be defective, as cell toxicity resolved after changing the lot number of chloroform. So, a total of 468 sewage samples were tested for poliovirus isolation and molecular characterization by ELISA and dual stage RT-PCR. Except 21 toxic samples, all samples had shown CPE in either of the cell lines used. CPE was seen in all the sewage samples (except the toxic ones) which indicate that the current sewage sample processing and isolation procedure is sensitive enough to detect the enteric viruses.

According to the WHO, the high rate of NPEV in the environment can be considered a proof that the environmental samples were processed and analyzed appropriately and that the cold chain used during specimen transportation was effective in preserving virus infectivity [11], (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf; World Health Organization, Polio Laboratory Manual, 4th edn. Department of Immunization, Vaccines and Biologicals 2004. WHO/IVB/04.10).

Out of the 468 sewage samples, 91 (19.44%) samples were positive for NPEV and 377 (80.56%) sewage samples were positive for polioviruses. There were 126 (26.92%) sewage samples in which poliovirus was obtained from L20B flask and NPEV was obtained from RD cell line flask. The positivity for NPEV was 19.44% (91/468) which is lower as compared to other studies on non-polio enterovirus isolation from sewage samples [13]. As per the algorithm in the present study which was intended to detect polioviruses, the sewage sample which had only non-polio enterovirus and no poliovirus gave result as NPEV positive however a sewage sample which contained a mixture of polio and non-polio enteroviruses gave results of poliovirus only. The other studies had provisions to detect NPEV isolates even in case of mixture. This must have resulted in difference in variation in the results (http://polioeradication.org/wp-content/uploads/2016/07/WHO_V-B_03.03_eng.pdf). A total of 660 poliovirus isolates were obtained from 377 positive sewage samples and subjected for neutralization assay for the detection of poliovirus type.

Polio neutralization test

Neutralization assay detected poliovirus types from L20B positive isolates. A total of 660 isolates were obtained from 468 sewage samples. On neutralization assay test, 172 (26.06%) isolates were non-polio enteroviruses and rest 488 isolates were found poliovirus strains. Out of 488 isolates, 45 (9.22%) were P1 type, 344 (70.49%) were P2 type and 228 were (59.01%) P3 type polioviruses which were subjected to ELISA as well as RT-PCR.

The percentage of isolation for polio type 2 virus (35.76%) was highest followed by polio type 3 (24.15%) and then by polio type 1 (3.85%). Joseanound Simone de Oliveira Pereira¹ in their study from Italy have also found isolation rates for P2 higher than those for P1 and P3 [7].

ELISA

All polio viruses were subjected to ELISA to determine if the isolate was Sabin-like, non-Sabin like or double reactive. ELISA was done on a total of 488 isolates (Table 1). Out of these, 481 (98.57%) were SL, 1 (0.20%) was NSL and 6 (1.23%) were DR. The NSL and DR isolates were referred for sequencing. On comparison, the sensitivity of ELISA was found to be very low. High number of false negatives makes ELISA inappropriate for primary screening of poliovirus isolates. Out of the seven samples which were non-Sabin like or double-reactive by ELISA, six samples (85.71%) showed nucleotide changes on sequencing however one sample (14.29%) did not show any nucleotide change. The specificity of ELISA was also not good (66.67%) in spite of very low sensitivity (3.43%). VDPV isolates can retain their “vaccine-like” antigenic properties in the ELISA, such that the routine screening algorithm does not identify the differences (<http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx>) [3]. This might have resulted in the low sensitivity of the assay.

Table 1 Comparison of ELISA and RT-PCR with sequencing results for the detection of NSL poliovirus

	Sequencing		
	Positive	Negative	Total
ELISA			
Positive	6 (True Positives)	1 (False Positives)	7
Negative	169 (False Negatives)	2 (True Negatives)	171
Total	175	3	178
RT-PCR			
Positive	171 (True Positives)	2 (False Positives)	173
Negative	4 (False Negatives)	1 (True Negatives)	5
Total	175	3	178

Dual stage RT-PCR

RT-PCR was also performed on these 488 isolates to determine whether the isolate was SL or NSL (Table 1). Out of these, 315 (64.55%) were SL and 173 (35.45%) were NSL. The NSL isolates were referred for sequencing. The sensitivity of dual stage RT-PCR (97.71%) is high, this makes it a good primary screening test for detection of non-Sabin like viruses. However, the specificity was only 33.33%. Other studies in literature also have similar findings [9]. Out of the 5 specimens which were negative (Sabin like) by RT-PCR, only one sample showed no nucleotide changes by sequencing however the remaining four had nucleotide changes. Thus, the negative predictive value was only 20% for RT-PCR. This indicates that a specimen which is positive (NSL) by RT-PCR is likely to be a strain with nucleotide changes however an isolate which is Sabin like by RT-PCR may also have mutations.

To conclude, the current method of environmental sewage sample processing appears to be satisfactory for isolation of polioviruses. Sabin type 2 was more commonly isolated as compared to Sabin 1 and Sabin 3. ELISA cannot be used for differentiation of SLs from NSLs due to low sensitivity. RT-PCR appears to be a sensitive tool for detecting non-Sabin like viruses however the isolates which are Sabin like by RT-PCR may not necessarily be unmutated viruses. Further studies may be required to confirm Sabin like isolates by sequencing as some isolates may be missed due to low specificity. Changes may be required in RT-PCR to increase the specificity.

Acknowledgements Guidance and support of HOD Microbiology Division, NCDC and Director, NCDC is gratefully acknowledged.

References

1. Agol VI. Molecular mechanisms of poliovirus variation and evolution. In: Domingo E (ed) Quasispecies: concept and implications for virology. Berlin Heidelberg: Springer; 2006. pp. 211–59.
2. Agol VI. Vaccine-derived polioviruses. *Biologicals*. 2006;34(2):103–8.
3. Burns CC, Shaw J, Jorba J, Bukbuk D, Adu F, Gumedé N, Pate MA, Abanida EA, Gasasira A, Iber J, Chen Q. Multiple independent emergences of type 2 vaccine-derived polioviruses during a large outbreak in northern Nigeria. *J Virol*. 2013;87(9):4907–22.
4. Carlson JC, Anderson JC, Low JE, Cardinal P, MacKenzie SD, Beattie SA, Challis JK, Bennett RJ, Meronek SS, Wilks RP, Buhay WM. Presence and hazards of nutrients and emerging organic micropollutants from sewage lagoon discharges into Dead Horse Creek, Manitoba, Canada. *Sci Total Environ*. 2013;445:64–78.
5. Chatterjee A, Vidyant S, Dhole TN. Polio eradication in India: progress, but environmental surveillance and vigilance still needed. *Vaccine*. 2013;31(9):1268–75.
6. da Silva AK, Amador J, Cherchi C, Miller SM, Morse AN, Pellegrin ML, Wells MJ. Emerging pollutants—part I: occurrence, fate and transport. *Water Environ Res*. 2013;85(10):1978–2021.
7. de Oliveira Pereira JS, da Silva LR, de MeirelesNunes A, de Souza Oliveira S, da Costa EV, da Silva EE. Environmental surveillance of polioviruses in Rio de Janeiro, Brazil, in support to the activities of global polio eradication initiative. *Food Environ Virol*. 2016;8(1):27–33.
8. Kew OM, Sutter RW, de Gourville EM, Dowdle WR, Pallansch MA. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. *Annu Rev Microbiol*. 2005;59:587–635.
9. Kilpatrick DR, Ching K, Iber J, Chen Q, Yang SJ, De L, Williams AJ, Mandelbaum M, Sun H, Oberste MS, Kew OM. Identification of vaccine-derived polioviruses using dual-stage real-time RT-PCR. *J Virol Methods*. 2014;197:25–8.
10. Kumar D, Kapoor N, Raghav PR, Gehlot MS. VDPV and polio end game strategy: the final frontier. *Int J Contemp Pediatr*. 2017;1(2):128–30.
11. Lewis GD, Metcalf TG. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. *Appl Environ Microbiol*. 1988;54(8):1983–8.
12. Makene VW, Pool EJ. The assessment of inflammatory activity and toxicity of treated sewage using RAW264. 7 cells. *Water Environ J*. 2015;29(3):353–9.
13. Ndiaye AK, Diop PAM, Diop OM. Environmental surveillance of poliovirus and non-polio enterovirus in urban sewage in Dakar, Senegal (2007–2013). *Pan Afr Med J*. 2014; 19.