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Real-time reverse transcription-polymerase chain reaction assays for identification of wild poliovirus 1 & 3

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***Background & objectives*: The poliovirus serotype identification and intratypic differentiation by real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay is suitable for serotype mixtures but not for intratypic mixtures of wild and vaccine poliovirus strains. This study was undertaken to develop wild poliovirus 1 and 3 (WPV1 and WPV3) specific rRT-PCR assays for use.**

***Methods*: Specific primers and probes for rRT-PCR were designed based on VP1 sequences of WPV1 and WPV3 isolated in India since 2000. The specificity of the rRT-PCR assays was evaluated using WPV1 and WPV3 of different genetic lineages, non-polio enteroviruses (NPEVs) and mixtures of wild/wild and wild/Sabin vaccine strains. The sensitivity of the assays was determined by testing serial 10-fold dilutions of wild poliovirus 1 and 3 stock suspensions of known titre.**

***Results*: No cross-reactivity with Sabin strains, intertypic wild poliovirus isolates or 27 types of NPEVs across all the four *Enterovirus* species was found for both the wild poliovirus 1 and 3 rRT-PCR assays. All WPV1 and WPV3 strains isolated since 2000 were successfully amplified. The rRT-PCR assays detected $10^{4.40}CCID_{50}$/ml of WPV1 and $10^{4.00}CCID_{50}$/ml of WPV3, respectively either as single isolate or mixture with Sabin vaccine strains or intertypic wild poliovirus.**

***Interpretation & conclusions*: rRT-PCR assays for WPV1 and WPV3 have been validated to detect all the genetic variations of the WPV1 and WPV3 isolated in India for the last decade. When used in combination with the current rRT-PCR assay testing was complete for confirmation of the presence of wild poliovirus in intratypic mixtures.**

Key words rRT-PCR - VP1 region - wild poliovirus 1 - wild poliovirus 3

Poliovirus belongs to the genus *Enterovirus*, family *Picornaviridae*[1]. There are three serologically distinct poliovirus types, all capable of causing paralytic poliomyelitis[1,2]. At the genomic level, the three serotypes show >30 per cent nucleotide divergence from each other[3]. Within each poliovirus serotype there exist several genotypes[4]. Global polio eradication initiative (GPEI) has made significant progress; four out of the six WHO Regions have been certified polio free[5]. The last case of wild poliovirus 2 (WPV2) was reported in 1999 and the last case of wild poliovirus 3 (WPV3) was reported in Nigeria in 2012[6,7]. In September 2015, Nigeria has been removed from the list of polio-endemic countries and presently Pakistan and Afghanistan are the only polio-endemic countries in the world[8].

Risk to global polio eradication continues to exist in the form of *(i)* undetected/putative circulation of indigenous wild poliovirus in recently polio free countries, *(ii)* importation of WPV from polio endemic countries, and *(iii)* release of WPV from laboratory stocks into the environment[9,10]. High quality sensitive surveillance is, therefore, necessary in every country to identify WPV circulation at the earliest. In many countries including India, acute flaccid paralysis (AFP) surveillance and/or *Enterovirus* surveillance is augmented by establishing environmental sample surveillance as supplementary surveillance for WPV[11-13]. In countries using oral polio vaccine (OPV), polioviruses isolated from environmental samples (mainly community sewage samples) are complex mixtures of vaccine viruses. Identification of WPV in the presence of vaccine viruses requires development of new molecular reagents. Presently used real-time reverse transcription polymerase chain reaction assays (rRT-PCR) for polioviruses are based on identification of Sabin OPV derived strains[14,15]. These assays are not suitable to investigate samples containing intratypic mixtures of wild and vaccine poliovirus strains. The objective of this study was to develop rRT-PCR assays for identification of WPV1 and WPV3 strains.

Material & Methods

This study was conducted in the Enterovirus Research Centre (ERC), Mumbai, India.

Polioviruses: Live attenuated oral poliovirus vaccine (Sabin OPV) strains were obtained from National Institute of Biological Science and Control (NIBSC), UK. Virus stocks were prepared in Hep-2 Cincinnati cell line (obtained from Centres for Disease Control and Prevention, Atlanta, USA). WPV1 and WPV3 strains isolated from AFP cases as well as environmental samples were obtained from collection of viruses at the Enterovirus Research Centre, Mumbai. Complete VP1 sequences of WPV1 and WPV3 isolated since 2000 were determined at the time of their isolation.

WPVs were inoculated in human Rhabdomyosarcoma (RD) cell line. The cells were observed for cytopathic effect (CPE). When all cells were infected, the culture was frozen, thawed three times and culture fluid was collected after centrifugation at 1500 x *g* for 10 min. One µl of the sample was used for testing in rRT-PCR assay.

Non-polio enteroviruses (NPEV): Enterovirus types belonging to all four *Enterovirus* species A to D were obtained from the Enterovirus Research Centre's collection. Virus types were confirmed by partial VP1 sequencing. Virus stocks were prepared in human RD cell line.

Wild poliovirus specific primers and probes: VP1 sequences of WPV1 and WPV3 were aligned using ClustalW (*http://www.ebi.ac.uk*). Primers and TaqMan MGB Probes were designed using Primer Express software version 3.0 (Applied Biosystems, Foster City, CA). Primers and probes sequences were subjected to BLAST search (*www.ncbi.nlm.nih.gov*). Nucleotide sequences of selected primers and probes were provided to the Applied Biosystems (Foster City, CA) for synthesis. Three sets of primers and probes of WPV1 and four sets of primers and probes of WPV3 were designed and evaluated.

Real-time reverse transcription-polymerase chain reaction assays: TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City) was used. Each 25 µl reaction mixture contained 12.5 µl of 2X Master mix without UNG, 0.625 µl of 40X MultiScribe and RNase inhibitor (Applied Biosystems, Foster City), 1.25 µl of primer-probe mix containing 22.5 pmol forward and reverse primers and 6.25 pmol TaqMan MGB Probe, 1µl of test sample (virus positive cell culture fluid) and 9.625 µl of nuclease free water.

rRT-PCR reactions were performed in ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) as follows: reverse transcription reaction at 48°C for 30 min, inactivation at 95°C for 10 min followed by 40 cycles of PCR at 95°C for 15 sec and 60°C for one min. Data were captured during amplification step and analyzed using the SDS Software v2.0.5 (Applied Biosystems, Foster city, USA) of the rRT-PCR assay. Amplification after 36 cycles was considered as non-specific and thus interpreted as a negative reaction.

Specificity of the rRT-PCR assays was determined by testing *(i)* WPV isolates belonging to various genetic lineages isolated in previous years, *(ii)* Sabin vaccine strains 1, 2 and 3, and *(iii)* different NPEV types. Stock preparations of WPV1 and WPV3 of known titre were diluted (10^{-1} to 10^{-8}) in Eagle's minimum essential medium (MEM, Sigma, USA) containing 2 per cent foetal bovine serum (FBS, Gibco BRL, Life Technologies, India). One µl of each dilution was tested in WPV1 and WPV3 detection assays, respectively. The highest dilution giving positive amplification was determined as sensitivity of the assay.

Interference was determined by diluting test virus in MEM containing approximately 10^8 $CCID_{50}$ (cell culture infectious dose 50%)/ml of other poliovirus types. WPV1 assay was tested against WPV3 and Sabin OPV strains. Similarly, WPV3 assay was tested using WPV1 and Sabin OPV strains. The highest dilution giving amplification of the test virus was determined and compared with the control.

Results

Complete VP1 sequences of WPV1 and WPV3 were aligned in ClustalW to identify the different genetic lineages and stretches of conserved sequence suitable for development of primers and probes for rRT-PCR assays. Three different regions of VP1 were identified for designing primers and probes for WPV1. A 71 bp segment from nucleotide 436 to 506 gave most promising results and was selected for further evaluation. For WPV3, of the four selected region of VP1, a 62 bp segment from nucleotide 709 to 770 showed promising results.

As shown in the Table, the WPV1 rRT-PCR assay consisted of forward primer 21 bp (genomic position 436 to 456), reverse primer 18 bp (genomic position 506 to 489) and 17 bp TaqMan MGB probe (genomic position 457 to 473) labelled at the 5' end with reporter dye FAM (6- carboxyfluorescein) and the non-florescent quencher (NFQ) at the 3' end.

WPV3 rRT-PCR assay consisted of forward primer 25 bp (genomic position 709 to 733), reverse primer 22 bp (genomic position 770 to 749) and 13 bp TaqMan MGB probe (genomic position 735 to 747) labelled at the 5' end with reporter dye FAM (6-carboxyfluorescein) and NFQ at the 3' end.

Melting temperature (Tm) values of the primers were similar to each other and Tm values of the probes were about 10°C higher making the assay highly suitable for cycling protocols for the One-Step RT-PCR Master Mix (Applied Biosystems, Foster city, USA).

Evaluation of real-time reverse transcription-polymerase chain reaction assays for wild poliovirus 1 and 3: WPV1 isolates representing genetically divergent groups of sequences were used to test the ability of the WPV1 rRT-PCR assay to detect all genetic lineages isolated since 2000. As shown in Fig. 1a, all the WPV1 isolates (n=19) tested were successfully amplified by the assay. Similarly, a large number of WPV3 belonging to different genetic lineages were used to evaluate the WPV3 assay. As shown in Fig. 1b, all the genetically divergent viruses (n=46) were positively amplified by the WPV3 rRT-PCR assay.

Specificity, sensitivity and interference: Poliovirus 1, 2 and 3 Sabin OPV strains (titers 10^7 to 10^8 $CCID_{50}$/ml) and 27 different NPEVs were tested in WPV1 and WPV3 rRT-PCR assays. As shown in Fig. 2a and 2b, only the positive control (PC) of WPV1 and WPV3 was amplified. The Sabin OPV strains and 27 different NPEVs did not show any amplification.

Fig. 3a shows amplification curves for 10-fold serially diluted WPV1 suspensions. A dilution effect can be inferred from the increasing cycle threshold (C_T) values. Amplification was obtained for 10^{-4} dilution representing $10^{4.40}$ $CCID_{50}$/ml, thus the assay detected approximately 25 infectious particles/reaction. Fig. 3b shows the amplification curves for 10-fold serially diluted WPV3 suspensions. Amplification was obtained for 10^{-4} dilution representing $10^{4.00}$ $CCID_{50}$/ml. Thus the assay detected approximately 10 infectious particles/reaction.

Table. Characteristics of primers and TaqMan MGB probes used for detection of WPV1 and WPV3 by rRT-PCR

Primer/probe name	Sequence 5'→3'	Direction	Genomic position	GC content (%)	Tm (°C)	Amplicon size (bp)
WPV1-F	AACAATGGGCATGCTTTGAAT	Sense	436	38	58.1	71
WPV1-R	TTTTCTGGCACTGGTGCG	Antisense	506	56	57.9	
WPV1-PR	CAGGTCTATCAAATCAT	Sense	457	35	68.0	
WPV3-F	GGTGTTCTTGCTGTAAGAGTTGTGA	Sense	709	44	58.5	62
WPV3-R	CGCACCTTGGATGTAACTTTTG	Antisense	770	45	58.3	
WPV3-PR	CGATCACAACCCC	Sense	735	62	68	

F, forward; R, reverse; PR, probe; Tm, melting temperature

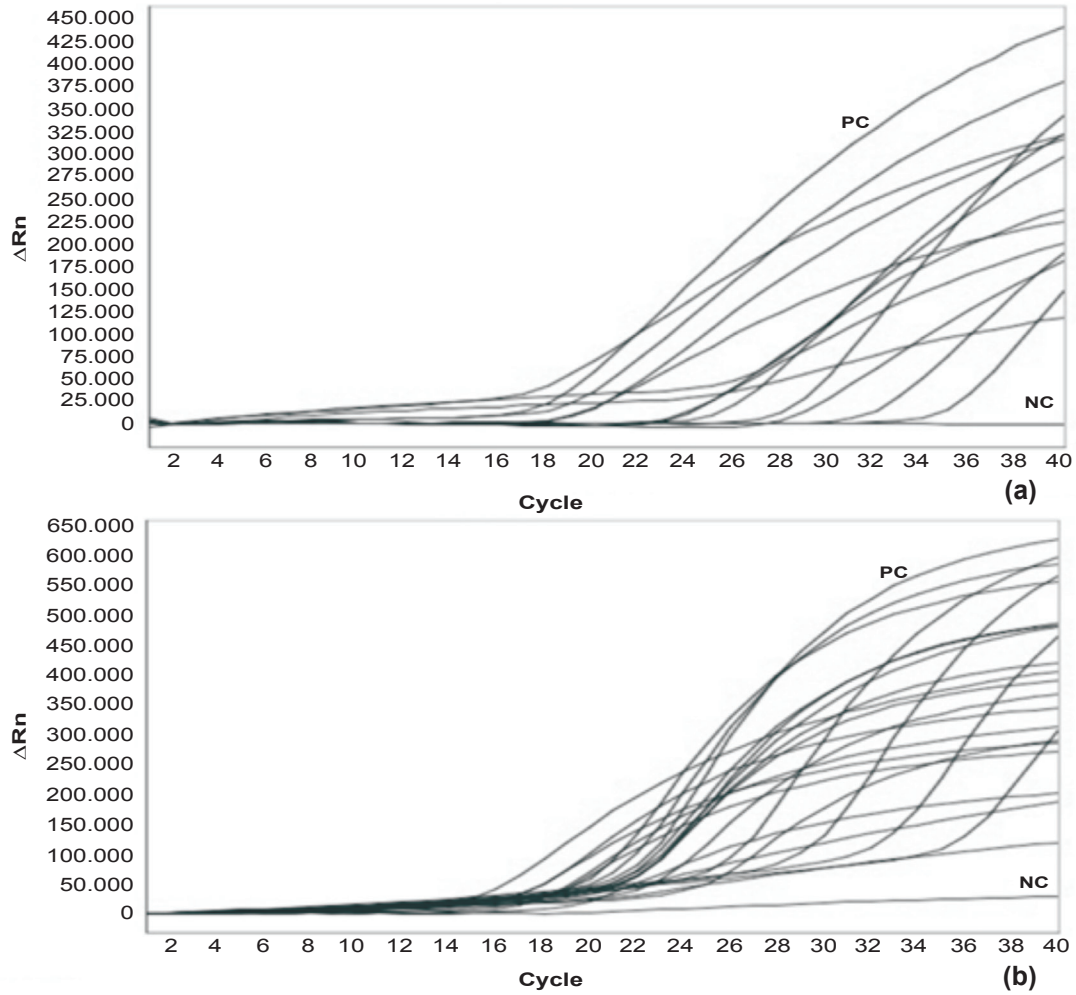

Fig. 1. Evaluation of WPV1 and WPV3 rRT-PCR assays for amplification of different genetic lineages. **(a)** WPV1 isolates (n=19) representing different genetic lineages isolated during 2000 to 2011 were tested. All isolates were positively amplified. **(b)** WPV3 isolates (n=46) representing different genetic lineages isolated during 2000 to 2011 were tested. All isolates were positively amplified. NC, negative control; PC, positive control.

Sensitivity of the PCR may be adversely affected if any one of the probes or primers reacts with heterologous RNA in the samples. Sensitivity of the WPV1 and WPV3 rRT-PCR assays was tested in the presence of large excess (10^7 to $10^{8.40}$ $CCID_{50}$/ml) of intertypic WPV as well as intratypic and intertypic Sabin OPV strains. The experiments were aimed at determining interference due to the presence of enteroviral RNA molecules. No reduction was observed in the sensitivity of both WPV1 and WPV3 rRT-PCR assays (data not shown). None of the heterologous RNAs tested interfered with the rRT-PCR reagents.

Discussion

Substantial progress has been made towards global polio eradication with only three countries (Pakistan, Afghanistan and Nigeria) remaining polio endemic. The South East Asia Region of the WHO was certified polio-free in March 2014[5]. However, the risk of WPV importation and resurgence of indigenous WPV from unknown sources continues.

Polioviruses isolated from any sources need to be differentiated as wild virus or Sabin-like. Various methods based on biological as well as physico-chemical properties of the virus have been

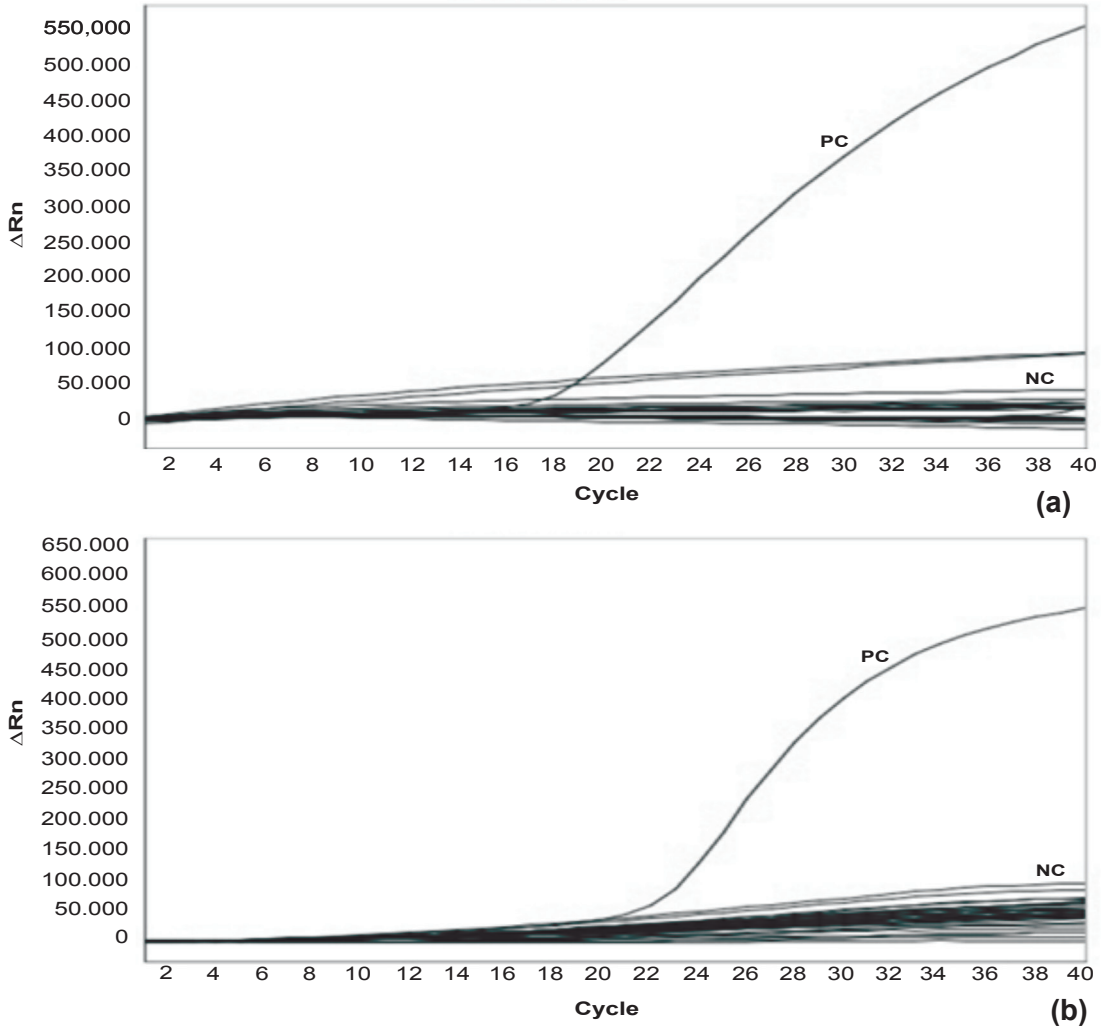

Fig. 2. Specificity of WPV1 and WPV3 rRT-PCR assays was tested using intertypic WPV1, WPV3, Sabin 1, 2, 3 and 27 different NPEVs. **(a)** Amplification plot for WPV1 assay **(b)** Amplification plot for WPV3 assay. NC, negative control; PC, positive control.

described since the introduction of OPV for polio immunization[16]. In recent years five different assays *viz.* the enzyme-linked immunosorbent assay (ELISA), using highly specific cross-absorbed antisera[17]; nucleic acid hybridization, using Sabin vaccine strain specific RNA probes[18,19]; RT-PCR, using vaccine strain-specific primers[20-22]; the neutralization test that uses monoclonal antibodies[23,24]; and RT-PCR followed by restriction fragment length polymorphism analysis[25] were used to differentiate wild and vaccine strains.

In the Global Polio Laboratory Network (GPLN) a combination of ELISA with cross-absorbed intratype-specific polyclonal rabbit antisera (antigenic) and strain specific RNA probe hybridization (genetic) was used for several years for intratypic differentiation of polioviruses[26].

The ELISA test indicated whether the virus reacted with Sabin strain specific antiserum or wild virus specific antiserum. In 2008, the probe hybridization assay was replaced by RT-PCR[27]. The complete RT-PCR assay contained primers for amplification of any enteroviruses (pan EV); any polioviruses whether Sabin-like or wild (panPV); serotype specific (PV1, PV2 and PV3 specific primers) and multiplexed reaction specific to amplify Sabin 1, Sabin 2 and Sabin 3. An isolate would be differentiated by amplification in RT-PCR reaction as an enterovirus (panEV positive), a poliovirus (panPV positive) and serotype identification

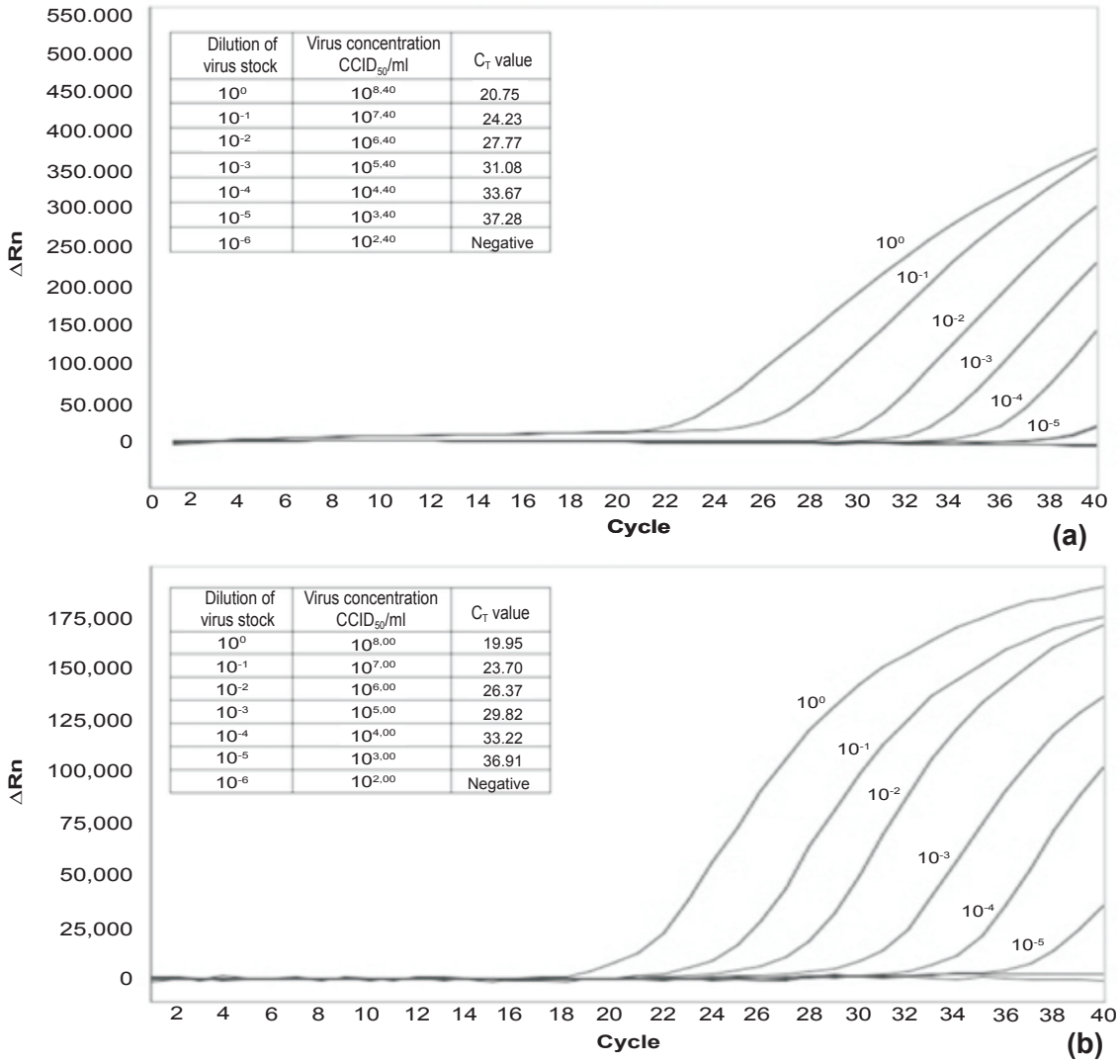

Fig. 3. Sensitivity of WPV1 and WPV3 rRT-PCR assays was determined by amplification of 10-fold dilution series of virus suspensions. WPV1 ($10^{8.40}$$CCID_{50}$/ml) and WPV3 ($10^{8.00}$ $CCID_{50}$/ml) stocks were diluted 10^{-1} through 10^{-8}. Sigmoidal curves obtained until 36 cycles were considered as positive amplification. Negative control was included in the experiment. **(a)** Amplification plot for WPV1 assay **(b)** Amplification plot for WPV3 assay.

according to the genotype primers (PV1/PV2/PV3)[21,22]. The poliovirus was identified as Sabin-like if any of the Sabin specific primers showed amplification. Presence of wild virus was deduced by amplification in serotype specific primer and absence of amplification by corresponding type specific Sabin primer. The presence of WPV was inferred by non-amplification of virus isolate by Sabin specific primers.

In 2009, real-time RT-PCR intratypic differentiation and real-time RT-PCR vaccine derived poliovirus assays were introduced[14,15]. The new methods completely replaced ELISA. The new intratypic differentiation methods were completely driven by Sabin specific primers and probes, and, therefore, it was impossible to comprehend the presence of wild virus in intratypic mixtures.

AFP surveillance is supplemented by initiating surveillance of environmental samples (community waste water/sewage)[11-13]. Most countries focus on maintaining high OPV coverage as supplementary immunization to maintain population immunity. Under such circumstances it is possible that wild virus

and Sabin OPV strains are present as mixtures. As discussed previously, it is very difficult to deduce the presence of wild virus in such samples. To overcome this problem we have developed real-time RT-PCR assay for detection of WPV1 and WPV3. Isolates can be screened directly and circulation of WPV can be monitored. The two assays were found to be sensitive, specific and able to detect WPVs of all genetic lineages isolated in India since 2000. No interference was observed when WPV was present as mixtures of different serotypes or homologous vaccine strains. No false positive reactions were observed when more than 300 Sabin 1 and 900 Sabin 3 poliovirus isolates were tested.

Our reagents used in combination with the current assay will allow "rule-in" identification of WPV1 and WPV 3. This is especially important when testing isolates from sewage samples which may contain intratypic mixtures of wild and vaccine strains.

Polioviruses evolve rapidly due to absence of proof reading mechanism during RNA replication[28]. Human-to-human transmission of WPV results in high sequence diversity making it difficult to design WPV specific primers/probes[19]. Our WPV assays have the following limitations. Though our WPV1 and WPV3 assays are highly sensitive and detect all genetic lineages found in India, their performance with genotypes circulating in other parts of the world needs to be evaluated. Also we have not tested reagents against WPV2 isolates as this serotype is considered eradicated globally.

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