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# Assay for identification of heterozygous single-nucleotide polymorphism (Ala67Thr) in human poliovirus receptor gene

Shyam Sundar Nandi, Deepa Kailash Sharma & Jagadish M. Deshpande

*Enterovirus Research Centre (ICMR), Mumbai, India*

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***Background & objectives*: It is important to understand the role of cell surface receptors in susceptibility to infectious diseases. CD155 a member of the immunoglobulin super family, serves as the poliovirus receptor (PVR). Heterozygous (Ala67Thr) polymorphism in *CD155* has been suggested as a risk factor for paralytic outcome of poliovirus infection. The present study pertains to the development of a screening test to detect the single nucleotide (SNP) polymorphism in the *CD155* gene.**

***Methods*: New primers were designed for PCR, sequencing and SNP analysis of Exon2 of *CD155* gene. DNAs extracted from either whole blood (n=75) or cells from oral cavity (n=75) were used for standardization and validation of the SNP assay. DNA sequencing was used as the gold standard method.**

***Results*: A new SNP assay for detection of heterozygous Ala67Thr genotype was developed and validated by testing 150 DNA samples. Heterozygous *CD155* was detected in 27.33 per cent (41/150) of DNA samples tested by both SNP detection assay and sequencing.**

***Interpretation & conclusions*: The SNP detection assay was successfully developed for identification of Ala67Thr polymorphism in human *PVR*/*CD155* gene. The SNP assay will be useful for large scale screening of DNA samples.**

**Key words** Genetic polymorphism - poliomyelitis - poliovirus receptor - *PVR* gene - SNP

Global Polio Eradication Initiative (GPEI) was launched by the World Health Assembly at its 41st meeting in Geneva in 1988[1]. Although the goal is not completely achieved as yet, significant progress has been made. Wild poliovirus transmission has been eliminated in four out of the six WHO Regions. As of 2014, Pakistan, Afghanistan and Nigeria remain the only three polio-endemic countries in the world[2]. Notably, last case of polio of wild poliovirus 2 was detected in November 1999 and of wild poliovirus 3 in 2012[3, 4]. Thus global eradication of two of the three poliovirus serotypes has been achieved[4]. Though wild poliovirus is on the verge of eradication, poliovirus pathogenesis and susceptibility to the disease has not been understood completely[5, 6].

Poliovirus belongs to the genus *Enterovirus* in family *Picornaviridae*[7]. There are three distinct poliovirus serotypes, each capable of causing paralytic poliomyelitis in susceptible individuals[7]. Humans are the only natural host for poliovirus infection and

non-human primates can be infected experimentally[8]. Poliovirus receptor (PVR/CD155), a type I transmembrane glycoprotein in the immunoglobulin super family is the poliovirus cell surface receptor[9]. *CD155/PVR* gene is specific to the primate lineage[10-12]. It is located on the chromosome 19q13.2 (*http://www.ncbi.nlm.nih.gov/gene/5817*). PVR has three extracellular immunoglobulin-like domains (D1-D3), a transmembrane region and a cytoplasmic tail[13]. Poliovirus interaction with D1 domain is the first step in the virus replication[14,15]. No other *Enterovirus* uses *CD155/PVR* as its cellular receptor[16]. Four transcript variants encoding different isoforms have been found for the CD155 gene[17]. Normal cellular function of CD155 is in the establishment of intercellular adherens junctions between epithelial cells. It may be involved in intestinal humoral immune responses[18].

In recent years investigators have tried to explore interest is growing to investigate genetic basis of susceptibility to infectious diseases[19-21]. Saunderson *et al*[22] found significantly higher frequency of heterozygous Ala67Thr change in *PVR* in patients with progressive muscular atrophy than in controls. Kindberg *et al*[23] reported an increased risk of developing paralytic poliomyelitis in heterozygous individuals carrying the Ala67Thr mutation in *PVR*. Genetic predisposition to poliomyelitis due to heterozygous (Ala67Thr) has neither been supported nor refuted by case-control studies in other countries[22-24].

Polio eradication efforts in India faced several challenges including poor personal hygiene and environmental sanitation and low oral polio vaccine (OPV) immunization coverage in stopping wild poliovirus transmission in Uttar Pradesh and Bihar[25,26]. Low per dose efficacy of OPV has been found especially in Uttar Pradesh[25]. Increasing the number of supplementary immunization campaigns and introduction of monovalent/bivalent OPV were some of the important innovations introduced to improve population immunity and break all chains of transmission of wild poliovirus[25,27]. It would be interesting to investigate whether genetic predisposition to paralytic outcome of poliovirus infections due to Ala67Thr heterozygous polymorphism played any role in delaying polio eradication in the Indian population.

DNA sequencing and PCR followed by restriction fragment length polymorphism (RFLP) have been used, so far, to detect the single nucleotide polymorphism (SNP, GCG→ACG) in *CD155/PVR* gene[23]. Both these methods are slow and require processing to obtain final results. The objective of the present study was to develop an assay suitable for large scale screening of samples for identification of heterozygous SNP in the *CD155/PVR* gene.

## Material & Methods

This study was conducted in the Enterovirus Research Centre, Mumbai, India, after prior approval from the institutional ethics committee. The assay involved *(i)* isolation of DNA from blood leukocytes and/or oral cavity cells, *(ii)* PCR amplification of a segment of exon2 of *CD155* gene containing the nucleotide of interest, *(iii)* designing primers for the new SNP detection assay, and *(iv)* identification of the single nucleotide of interest by using the SNapShot method. DNA sequencing of the PCR amplicon was used as the gold standard method for confirmation of the nucleotide of interest.

Samples were collected from three different sources.

(1) Institutional staff members were informed about the development of the new assay and the need for DNA samples to test and validate the assay. Blood samples were collected from consenting members (n=46). Blood sample (100µl) was obtained by pricking the middle finger-tip with a sterile lancet. Blood drops were collected using a sterile micro-tip and pipette in a vial containing 200µg EDTA as anticoagulant; 2.5µl of the blood sample was used for PCR reaction.

(2) Blood samples (n=29) were drawn from a collection of samples for polio antibody serologic survey done in Bihar in 2010.

(3) Oracol sponges were used for collection of crevicular fluid for testing mucosal antibody response to poliovirus vaccines during a polio vaccine campaign conducted in Moradabad, UP, in 2011. Seventy five Oracol sponges were used for obtaining cellular DNA for this study. Briefly, for obtaining crevicular fluid, the Oracol sponge was rubbed on the molar teeth area for one min. The sponge was placed in a tube containing one ml PBS with antibiotics and 0.2 per cent Tween 20. In the laboratory, crevicular fluid was extruded from the sponge by squeezing it on the inner wall of the tube and centrifugation. Crevicular fluid (supernatant) was collected aseptically for polio antibody testing and the cell debris was used for DNA extraction.

*DNA extraction*: Phusion blood direct PCR kit (FINZYMES, Thermo Scientific, USA) does not require DNA extraction step. DNA was extracted from

cells of oral cavity using reagents from the Fast Tissue to PCR kit (Fermentas, Thermo Scientific, USA). Briefly, 100 µl of tissue lysis buffer and 5 µl Proetinase K were added to 50 µl of cell pellet. The mix was heated to 95°C for 3 min followed by addition of 100 µl of neutralization solution. All required reagents are supplied with the kit.

*Primers*: CD155 GenBank sequence NM001135770 and OligoPerfect Designer Software (*http://tools.lifetechnologies.com*) were used for identifying primer regions. Primers were designed for *(i)* PCR amplification of a 234 bp segment of Exon2 of *CD155* gene, *(ii)* sequencing of the 234 bp amplification product, and *(iii)* identification of the nucleotide at the first base of the codon (GCG/ACG) for the 67$^{th}$ amino acid (Ala/Thr). Details of the primers designed for this study are given in Table I. Primers were self designed and got synthesized from SIGMA Chemical (USA).

*PCR*: Phusion Blood Direct PCR Kit was used as per the manufacturer's instructions. Briefly, each 50 µl reaction contained 2.5 µl whole blood sample in EDTA, 25 µl of 2× Phusion Blood PCR Buffer, 1 µl each forward and reverse primers (1 µM/µl), 1 µl Phusion Blood DNA Polymerase, and 19.5 µl nuclease free water. Amplification was carried out in a 9700 PCR Cycler (Applied Biosystems, Foster City, CA) at 98°C for 5 min (initial denaturation), followed by 40 cycles of 98°C 30 for 30 sec (denaturation), 67°C for 30 sec (annealing), and 72°C for 30 sec (extension). The last cycle of extension was at 72°C for 10 min.

Fast Tissue to PCR kit (Fermentas, Thermo Scientific, USA) was used for PCR of DNA extracted from the oral cavity cells. Briefly, each 50 µl reaction contained 5 µl neutralized tissue extract (DNA sample), 25 µl Tissue Green PCR 2× Master Mix, 1 µl each of forward and reverse primers (10 pmole/µl) and 18 µl nuclease free water. Amplification was carried out in 9700 PCR Cycler at 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec. The last cycle of extension was at 72°C for 10 min.

PCR amplification was detected by electrophoresis in 2 per cent Agarose (Roche, Germany) gel stained with ethidium bromide. Amplification product (234 bp DNA) was purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) as per the manufacturer's instructions.

*SNP assay*: SNapShot Multiplex reagent (Applied Biosystems, Foster City, CA) was used for single nucleotide polymorphism analysis of the 234 bp DNA amplified product. Briefly, 1 µl of the 234 bp amplification product, 1.5 µl (2.5 uM/µl) of either forward or reverse SNP primers and 2.5 µl SNaPshot Multiplex Reagent were reacted in a 200 µl PCR tube and 25 cycles of denaturation, annealing and extension were carried out at 96°C for 10 sec, 50°C at 5 sec, and 60°C for 30 sec in a 9700 PCR Cycler. At the end of the 25 cycles, 0.5 µl Shrimp alkaline phosphatase and 0.5 µl buffer (Fermentas, Thermo Scientific, USA) were added and the reaction was incubated at 37°C for 15 min. The reaction mix was heated to 75°C for 10 min to inactivate the enzyme to stop the reaction; 0.5 µl of the reaction product was mixed with 9 µl HiDi-Formamide and 0.5 µl GeneScan 120 Liz (Applied Biosystems, Foster City, CA) for electrophoresis at

**Table I.** Details of primers used for PCR, single nucleotide polymorphism assay and sequencing

| Primer | Sequence (5'-3') | Position (nt) | Length (nt) | Tm° C |
|---|---|---|---|---|
| Primers for PCR | | | | |
| CD155Exon2F | GCTACCTACAGGTGCCCAAC | 445-464 | 20 | 63.9 |
| CD155Exon2R* | TGACGAACAGGCAGGTGTAG | 678-659 | 20 | 63.9 |
| Primers for SNP | | | | |
| CD155SNP_F | gactgactCACAGCTGACTTGG | 484-497 | 22 | 59 |
| CD155SNP_R | gactCTGCCAGATTCACCATGCCGCG | 520-499 | 26 | 79.8 |
| Primers for sequencing | | | | |
| CD155 Exon2 F20 | gactgactgactgactgactGCTACCTACAGGTGCCCAAC | 445-464 | 40 | 64.8 |

Position of the primers is relative to CD155, transcript variant 4 mRNA sequence (Gene Bank Accession Number NM_001135770)
*PCR primer CD155Exon2R was used also for sequencing of the PCR product in reverse direction
Tm, melting temperature

60°C at 15KV and 5 µAmp current for 45 min in ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). Results were analyzed using GeneMapper Software v4.0 (Applied Biosystems, Foster City, CA).

*DNA sequencing*: The 234 bp PCR amplification product was sequenced in both the directions. The 8 µl sequencing reaction contained 2 µl of 234 bp PCR amplification product, 1 µl BigDye Ready Reaction v.3.1 mix (Applied Biosystems, Foster City, CA), 1 µl primer (3.2pmole), 1.5 µl 5× sequencing buffer and 2.5 µl nuclease free water. After initial denaturation at 95°C for 3 min, 25 cycles of sequencing were performed at 95°C for 20 sec, 57°C for 20 sec, and 62°C for 1 min. The last cycle of extension was at 62°C for 7 min. Cycle sequencing product was purified using BigDye X terminator Purification Kit (Applied Biosystems, Foster City, CA). Sequences were resolved on ABI 3130*xl* Genetic Analyzer. Sequences were analyzed by Sequencher v.4.10.1 (Gene Codes, USA).

## Results

*CD155* gene is situated on the chromosome 19q13.2 and 29332 bp (Accession No. NG_008781). Four transcript variants (mRNAs) have been identified. The first four exons are common to all the four variants. Exon1 is 1$^{st}$nt to 378nt, exon 2 is 379 to 726nt (shown in blue colour in Fig. 1), exon3 is 727 to 1023nt and exon4 is 1024 to 1141nt. Nucleotides 1 to 299 constitute the 5' non-translated region. Translation of the mRNA was initiated from the AUG codon at nucleotide 300. A nucleotide change (G→A) at position 499 led to an amino acid substitution Ala67Thr in the D1 domain of *CD155/ PVR*. The SNP assay was designed to target the first nucleotide coding for the 67$^{th}$ amino acid (GCG/ ACG) of CD155/ PVR protein. Transcript variant 4 mRNA (1643nt long; Accession no. NM_001135770) sequence was used to design the primers in this study. At the gene level this nucleotide is situated in the exon2.

Positions and sequences of the primers designed for PCR, sequencing and the SNP assay with respect to the mRNA transcript4 are presented in Fig. 1. In this study the assays targeted the gene (DNA) and not the mRNA.

The PCR primers (CD155 exon2F and CD155 exon2R) amplified a 234 bp segment from exon2 (66-


**Fig. 1.** Systematic representation of genomic positions of primers for PCR, sequencing and SNP assay on poliovirus receptor (*PVR/CD155*) transcript variant 4, mRNA (Gene Bank accession no. NM_001135770). Exon 2 sequence (379 to 726 nt) is shown in blue colour. The initiation codon at 300 nt is shown in bold, underlined and in yellow colour. The SNP target nucleotide G is shown in bold, underlined and in yellow colour.

300nt) in *CD155/PVR* gene. PCR primers were used for sequencing with some modifications. We added 20 non-complementary bases at the 5' end of the forward PCR primer (CD155 exon2 F20) so that the nucleotide of interest was not very close to the starting point of the sequencing reaction, thus improving the quality of the sequences. PCR reverse primer was used for sequencing without any modification.

The 22nt long forward SNP primer was designed with eight non-complementary bases at the 5' end and 14nt complementary to the target sequence (484 to 497nt). The reverse SNP primer was 26nt long consisting of four non-complementary bases at 5'end and 22nt complementary to the gene sequence (499 to 520nt).

*SNP detection by SNapShot*: SNapShot reaction adds a single base to the primer. The single base is detected by capillary electrophoresis of the reaction. Each base is colour coded (blue:G; black:C; green:A and red:T).

SNapShot results of a DNA sample with the forward SNP primer is shown in Fig. 2a. The blue peak indicated "nucleotide G" at the targeted position. The same DNA sample tested with the reverse SNP primer showed "nucleotide C" in the complementary strand (Fig. 2b). SNP test results of DNA sample of another person showed two peaks blue and green (G and A) at the targeted nucleotide, revealing the heterozygous nature of the sample. Reaction with the reverse SNP primer also showed two peaks, black (C) and red (T) which confirmed the heterozygous nature of the DNA sample (Fig. 2c and 2d). Results of the SNapShot reactions were unambiguous and easy to interpret.

*SNP detection by sequencing*: DNA sequence of the 234bp PCR amplification product of the DNA sample used for the SNP assay as shown in Fig. 2a and 2b is given in Fig. 3A (forward direction) and 3B (reverse direction), respectively. The codon GCG for the target (Ala67) is highlighted. Sequence of the other DNA

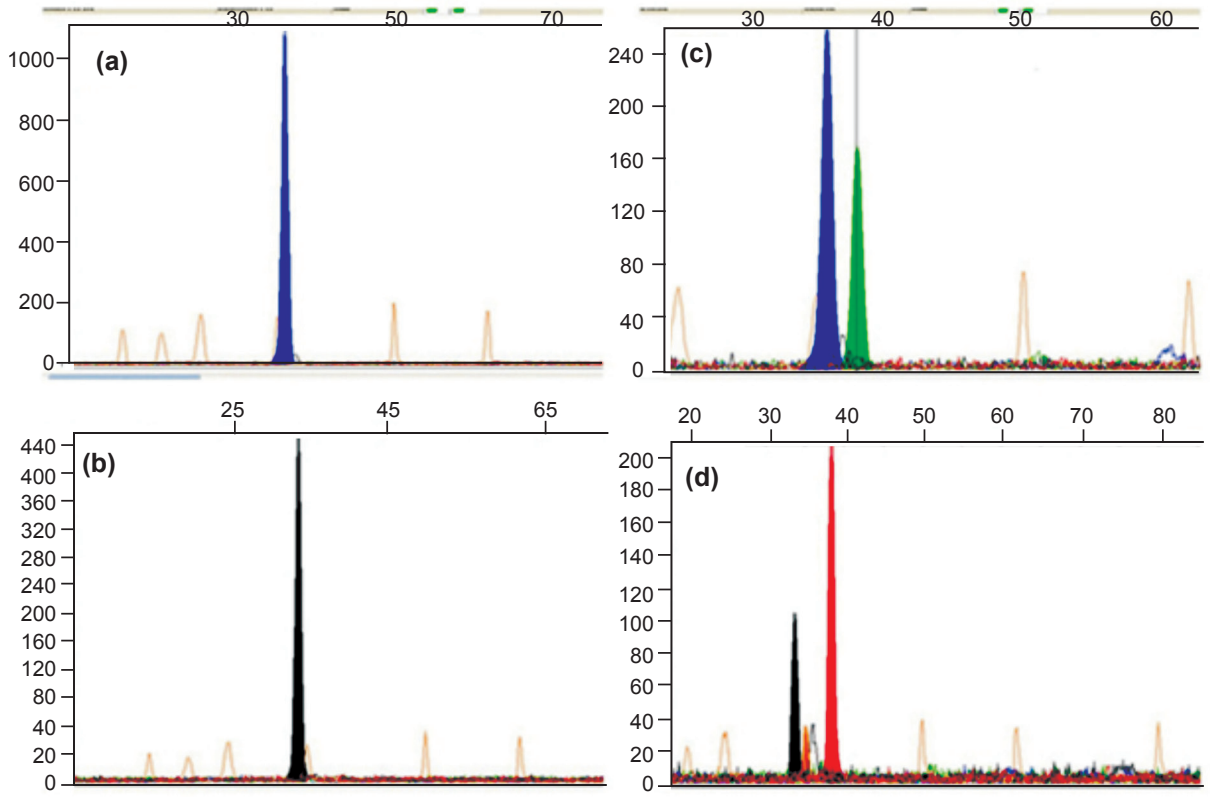


**Fig. 2.** Results of SNP assay of DNA samples containing homozygous and heterozygous genotype (Ala67Thr) at nucleotide position 499 in poliovirus receptor gene: **(a)** homozygous sample with forward primer showing a single peak of G (Blue), **(b)** with reverse primer showing a single peak of C (Black), **(c)** heterozygous sample with forward primer showing two peaks G/A (Blue/Green), and **(d)** with reverse primer showing two peaks C/T (Black/Red).

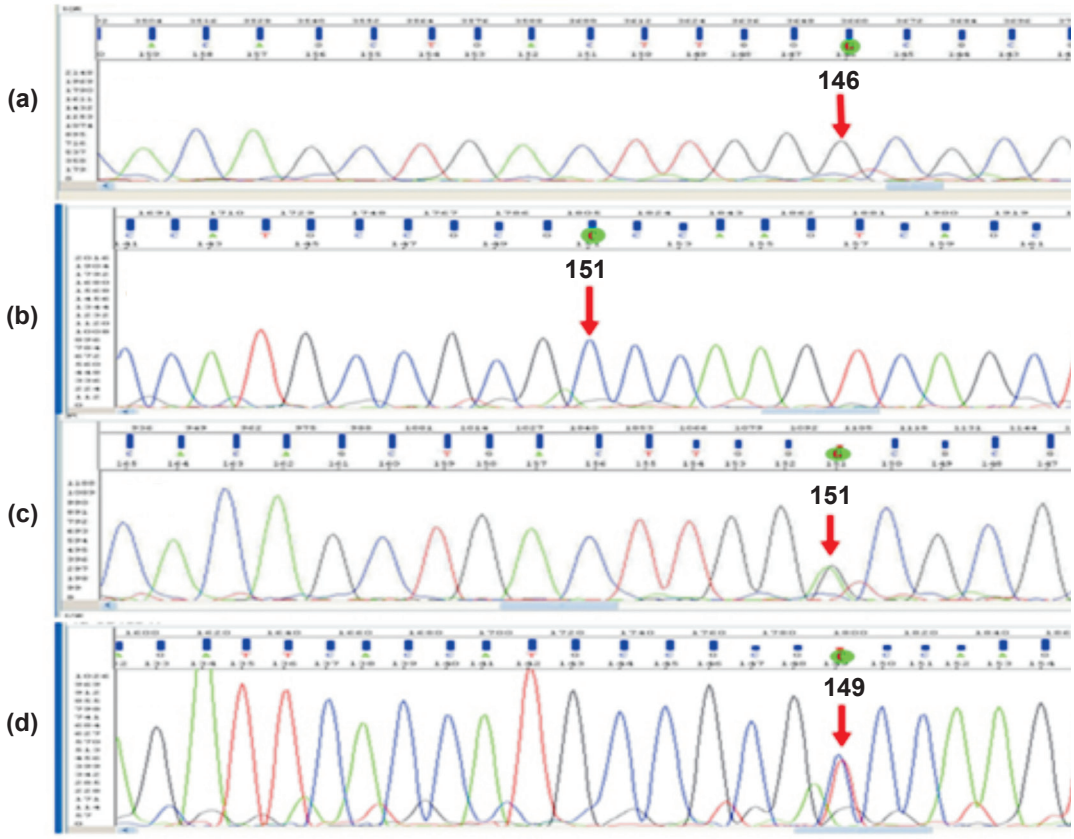


**Fig. 3.** Sequencing of DNA containing homozygous and heterozygous (Ala67Thr) genotypes in poliovirus receptor. Sequences of fragment flanking nucleotide 499 of a homozygous sample with forward and reverse primers are presented in 3a, 3b and a heterozygous sample in 3c, 3d.

sample showing two peaks (as shown in Fig. 2c and 2d) is presented in Figure 3c and 3d. Both the forward and reverse sequences confirmed the heterozygous nature of the sample.

We tested DNA samples of 150 different individuals consisting of 75 DNA from blood samples and 75 DNA samples from cells of oral cavity by the SNapShot method. The 150 samples were then sequenced in both forward and reverse directions to confirm and validate the results of the newly developed SNP assay. Results obtained by both the methods were 100 per cent concordant for all 150 samples. Heterozygous single nucleotide polymorphism (Ala67Thr) was detected in 41 of 150 (27.33%) samples (Table II).

## Discussion

Cell surface molecule CD155 serves as the receptor for the three polioviruses. Poliovirus initiates infection when it recognizes and binds to CD155/PVR, the only poliovirus receptor known to date[16]. Humans are the

**Table II.** Detection of single nucleotide polymorphism (Ala67Thr) in DNA samples using the new SNP assay

| Sample type | No. tested | No. homozygous (Ala67Ala) | No. heterozygous (Ala67Thr) | Per cent heterozygous |
|---|---|---|---|---|
| Whole blood | 75 | 56 | 19 | 25.33 |
| Oral cavity cells | 75 | 53 | 22 | 29.33 |
| Total | 150 | 109 | 41 | 27.33 |

only natural host of poliovirus, although chimpanzees and Old World monkeys, who express receptors closely related to human CD155, can be experimentally infected[28]. When poliovirus recognizes its receptor, the virus changes from a 160S to a 135S particle before releasing its genome into the cytoplasm[7]. The other cellular functions of CD155 are not fully understood[9,16].

Heterozygous SNPs (Ala67Thr) in *CD155* gene was found to be between 6.8 to 8.5 per cent in normal healthy population. It was significantly higher in patients with polio paresis (13.3%) and progressive muscular atrophy (20%). Ala67Thr polymorphism was proposed as a genetic risk factor for susceptibility to poliomyelitis[22-24]. However, the hypothesis has not been tested in other studies involving large numbers of polio patients.

Presently, DNA sequencing and PCR followed by RFLP have been used to detect the SNPs (GCGàACG) in *CD155/PVR* gene in the human sample[23]. Both these methods are slow and require processing to obtain final results. We developed and validated an SNP assay to detect the single nucleotide substitution (GCG→ACG) in *CD155/PVR* gene. The assay may be suitable for the large scale screening of samples for identification of heterozygous SNP in the *CD155/PVR* gene in human DNA samples. The SNP assay results were confirmed by DNA sequencing of all the samples.

Our aim was to develop a simple assay for the large scale screening of the SNP. It was found that the carrier rate of heterozygous (Ala67Thr) among the 150 anonymous DNA samples was about 27 per cent which was higher than that reported for normal healthy persons. Bhattacharya *et al*[29] reported prevalence of heterozygous Ala67Thr genotype in normal population to be 5-7.5 per cent and 45 per cent in patients with post-polio syndrome in India. These findings also indicate that large scale screening for the heterozygous *CD155/PVR* may be needed amongst the Indian population. Our SNP assay would be useful for such screening. It would be interesting to investigate the prevalence of heterozygous carrier rate in India and whether it had any bearing on the success of polio eradication programme in certain populations.

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***Conflicts of Interest*:** None.

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*Reprint requests*: Dr Jagadish Deshpande, Enterovirus Research Centre (ICMR), Haffkine Institute Compound, A.D. Marg, Parel, Mumbai 400 012, Maharashtra, India
e-mail: deshpandejm@gmail.com