Journal of Virological Methods 201 (2014) 86–92

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Allele-specific PCR for quantitative analysis of mutants in live viral vaccines

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Article history:
Received 14 December 2013
Received in revised form 7 February 2014
Accepted 11 February 2014
Available online 7 March 2014

Keywords:
Oral Polio Vaccine
Virulent revertants
Consistency monitoring
MAPREC
Alternative methods

A B S T R A C T

Monitoring consistency of genetic composition of oral polio vaccine (OPV) is a part of its quality control. It is performed by mutant analysis by PCR and restriction enzyme cleavage (MAPREC) used to quantify neurovirulent revertants in the viral genome. Here an alternative method based on quantitative PCR is proposed. Allele-specific quantitative polymerase chain reaction (asqPCR) uses a "tethered" oligonucleotide primer consisting of two specific parts connected by a polyinosine stretch. Homogeneous DNA from plasmids containing wild Leon/37 and attenuated Sabin 3 sequences with 100% 472_C and 100% 472_T could only be amplified using homologous primers. Real-time implementation of the allele-specific PCR resulted in sensitive detection of 472_C revertants with the limit of quantitation of less than 0.05%. Monovalent vaccine batches and international viral references for MAPREC test were used to validate the method. asqPCR performed with the WHO references and monovalent batches of vaccine showed that the new method could measure accurately and reproducibly the content of revertants producing values comparable to MAPREC results. This suggests that asqPCR could be used as an alternative to MAPREC for lot release of OPV. The method could also be used for the quantitation of other mutants in populations of microorganisms.

1. Introduction

Viral replication is inherently error-prone and leads to emergence of mutants. Their accumulation during manufacture of live viral vaccines can lead to reversion of the attenuated phenotype and also to reduced immunogenicity by altering protective epitopes. Therefore changes in genetic composition of vaccine preparations could affect their safety and efficacy. Even if present at a very low level, mutants can change significantly the biological properties of viruses. By using sensitive mutant analysis by PCR and restriction enzyme cleavage (MAPREC) it was shown that all batches of oral poliovirus vaccine (OPV) contain a small fraction of revertant viral particles with increased neurovirulence (Chumakov et al., 1991). In some batches the content of these revertants was increased, resulting in failure of the monkey neurovirulence test used for vaccine lot release. Therefore MAPREC was recommended as a quality control procedure to identify vaccine batches with unacceptably high content of neurovirulent mutations.

Despite its high sensitivity, MAPREC has a number of shortcomings: it is laborious and technically challenging, and requires the use of radioactive isotopes. Therefore finding alternatives remains a desirable objective. The main challenge is the difficulty of detection and determining quantity of a very small fraction of mutants in heterogeneous viral populations. A number of techniques were used for this purpose, including matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Amexis et al., 2001), hybridization with microarrays of short oligonucleotides (Laassri et al., 2011), and massively parallel or deep sequencing (Neverov and Chumakov, 2010). These methods were used successfully for detecting minor quantities of revertants in the entire viral genomes. However they require sophisticated and expensive equipment and the assays take a significant time to complete.

Quantitative PCR (qPCR) with Taqman probes has also been used for studies of vaccine virus revertants in stool of vaccine recipients and sewage samples (Gnanashanmugam et al., 2007; Troy et al., 2011). However, the sensitivity of qPCR as measured by the minimum detectable percentage of mutants is relatively low. While being acceptable for analysis of field virus isolates that contain a significant proportion of mutants, it is not sufficient for discriminating acceptable batches of vaccine that contain less than 1% of mutants

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http://dx.doi.org/10.1016/j.jviromet.2014.02.012

from unacceptable that contain slightly more than 1%. PCR procedures for discrimination between mutants are based on primers the 3′-end of which matches the nucleotide of interest. Terminal mismatches reduce the ability of DNA-polymerase to initiate synthesis, and therefore reduce efficiency of PCR. However, this method is not absolutely allele-specific and even two terminal mismatches do not guarantee full selectivity of the primers. Improvement of binding specificity of PCR primers is an important goal for many applications requiring discrimination between closely related sequences. Various primer modifications that decrease their melting temperatures (Tm) and thus improve selectivity were tested (Christopherson et al., 1997; Day et al., 1999; Kwok et al., 1990; Newton et al., 1989). However, while low-Tm primers may discriminate better between point mutants, their specificity is lower and they tend produce additional artifactual DNA products.

To minimize the non-specific amplification, a dual priming oligonucleotide (DPO) system was proposed (Chun et al., 2007). In the DPO system each primer consists of two segments, one being longer than another, joined by a poly (I) linker. The shorter 3′-terminal segment serves as a sensor of mutations similar to low-Tm primers, while the longer 5′-terminal segment increases the overall stability of primer-template complexes and serves as an "anchor" to prevent the allele-specific 3′-end from binding unspecifically and priming polymerase reaction at other loci. Polyinosine forms weak complexes with DNA, which are not sequence-specific. Therefore the poly (I) linker serves as a flexible connector between discriminating and stabilizing parts of the primer. Such "tethered" primers have a higher selectivity than primers without poly-I stretch. This system was successfully used for detection of single-nucleotide polymorphisms in cytochrome genes, but its results were qualitative.

Here we propose an adaptation of this principle to create an allele-specific quantitative real-time method that we call asqPCR for accurate measurement of very small quantities of mutants in batches of OPV. We found that tethered primers have about 8-fold higher selectivity and lead to a similar increase in the sensitivity of mutant detection than regular primers. The content of virulent reversion $U_{472} \rightarrow C$ in Sabin 3 poliovirus determined by asqPCR matched closely the values determined by the industry-standard MAPREC method. The new protocol is faster and simpler than MAPREC assay and does not require the use of radioactive isotopes. It could be used as an alternative for routine lot release of OPV, as well as for measuring quantities of mutants in other applications.

2. Materials and methods

2.1. Viruses and plasmids

To develop and assess the sensitivity of the asqPCR method, plasmids containing the complete genome of Sabin 3 strain and 5′ genome region (1–1567 nucleotide) of Leon/37 virus (wild type 3 poliovirus) were used. Several viral preparations were used for validation of the asqPCR method. They included US National neurovirulence reference NC2 containing about 0.9% of $U_{472} \rightarrow C$ mutants, revertant Sabin 3 strain DM Pg35aP-396 isolated from a vaccine recipient containing 100% of $U_{472} \rightarrow C$ (kindly provided by Dr. Philip Minor, NIBSC, UK), two WHO references (96/572 and 96/578) representing OPV lots that passed and failed MAPREC, and one batch of OPV3 that failed the monkey neurovirulence test.

2.2. Construction of plasmid that contains $U_{472} \rightarrow C$ mutation

To construct a plasmid that contains $U_{472} \rightarrow C$ mutation, a 1567 bp DNA fragment was amplified from 5 prime of Leon/37 virus genome with US-SP6 (Forward) and 1567Sab3RT7 (Reverse) primers (Table 1). The PCR amplicons were purified through 1.5% agarose gel, extracted with QIAquick Gel Extraction kit (Qiagen, Valencia, CA), and cloned into plasmid vector pCR4-TOPO by using TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA). Approximately 100 ng of target amplicon was used in cloning procedure. Transformed TOP10 *Eschrichia coli* cells were grown overnight at 37 °C using an agar plates containing ampicillin, IPTG (isopropyl-b-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-b-dgalactopyranoside) (Fermentas, Hanover, MD). White colonies were selected for the following analysis. A QIAprep Spin MiniPrep kit (Qiagen, Valencia, CA) was used for isolation of plasmid DNAs in accordance with the manufacturer's protocol. Isolated plasmid DNA samples were analyzed by Sanger method to confirm their sequences.

2.3. Viral RNA extraction and cDNA preparation

Viral RNA was isolated from 140 μl of virus containing medium using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA was eluted to a final volume of 60 μl in sterile, RNase-free water. The cDNA were prepared with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using the manufacturer's protocol with specific reverse primer 1567Sab3RT7 (Table 1).

2.4. Design of allele specific primers

The tethered allele primers specific for 472_C and 472_T were designed to contain 3 parts: a longer 5′-segment targeting the primer to a specific region on the template, polyinosine linker between the specific segment and discriminating 3′-segment designed for identification of mutants by virtue of the 3′-terminal nucleotide being complementary to the nucleotide of interest (Fig. 1A, Table 1). Since the 3′ segment is responsible for primer specificity, different sizes of this part were tested and the length of 5–6 nucleotides was found to produce the best results. In addition, a standard allele-specific primer without polyinosine liner was made to evaluate the impact of primer modifications on the discrimination between alleles (Table 1). Another forward primer ("allele-nonspecific forward primer") located immediately downstream from the allele specific primer was designed to be used for measuring DNA quantity for data normalization. The common for both strains reverse primer ("unmodified reverse primer") was used to produce 72 bp amplicon with allele specific primers and 45 bp amplicon with allele-nonspecific forward primer. Both forward and reverse allele-nonspecific primers had complete complementarity to both mutant and wild type virus. Sequences of all primers and their location on Sabin 3 genome are presented in Table 1, and in the layout of primers in Fig. 1B.

2.5. Evaluation of discriminating ability of the allele-specific PCR method

The developed allele-specific PCR was designed to be very selective because of high specificity of primers used as well as the optimized PCR conditions. Briefly, Sabin 3 and Leon/37 plasmid DNAs were used as a template in a total reaction volume of 10 μl containing 5 μM of each of the above primers (Table 1), 50 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.25 U HotStarTaq DNA Polymerase (Qiagen, Valencia, CA). PCR was performed using a 9700 GeneAmp® PCR System (Applied Biosystems, Foster City, CA) under the following dual stage conditions: 95 °C for 15 min, followed by 10 cycles of 95 °C for 15 s, 68 °C for 50 s, 72 °C for 40 s, then 20 cycles of 95 °C for 20 s, 62 °C for 50 s, 72 °C for 50 s. 5 μl of PCR product was separated by electrophoresis in 2% agarose gels with ethidium bromide (Lonza, Rockland, ME). DNA bands in stained gels were assessed using Kodak Gel Logic 200 Imaging

Table 1
Primers used for identification of 472U → C mutants Sabin 3 virus.

Name	Nucleotide (nt) position	Polarity	Sequence (5′ → 3′)	Length, nt	Tm (°C)
Regular allele specific C primer	450–472	Forward	CCGGCCCCTGAATGCGGCTAATC	23	65
Tethered allele specific C primer	450–472	Forward	CCGGCCCCTGAAIIIIICTAATC	23	65
Tethered allele specific T primer	450–472	Forward	CCGGCCCCTGAAIIIIICTAATT	23	65
Allele-nonspecific primer (F)	477–496	Forward	CCATGGAGCAGGCAGCTGCA	20	63
Unmodified primer (R)	501–521	Reverse	CGTTACGACAGGCTGGCTGCT	21	62
US-SP6	1–20	Forward	ACCGGACGATTTAGGTGACACTATAGTTAAAACAGCTCTGGGGTTG	46	69
1567Sab3RT7	1567–1590	Reverse	ACGCGTTAATACGACTCACTATAGGCATTCACA TATGGTAGGACAATA	48	67

Note: The mismatch nucleotide is shown in bold; underlined nucleotides correspond to the SP6 and T7 promoter sequences.

System and Kodak Molecular Imaging Software (Carestream Health, Inc., Rochester, NY). The Sabin 3 plasmid was used as a negative control for PCR with tethered 472_C-specific primer and Leon/37 plasmid as negative control for PCR with tethered 472_T-specific primer.

2.6. Sensitivity of the allele-specific quantitative PCR to evaluate the revertant contents

To perform a quantitative analysis of revertants in addition to discriminating primers we have used an allele-nonspecific forward primer (Table 1). PCR products made from Leon/37 and from Sabin 3 plasmids that contain 100% and 0% of $U_{472} \rightarrow C$ revertants respectively were spiked in range 0.05–2% of Leon/37 plasmid and were run in triplicate in 5 separate experiments. The spiked samples were amplified by both pair of primers: tethered 472_C-specific forward/non-modified reverse primers and allele-non-specific forward/reverse primers, the amplification with the later pair of primers served for data normalization. The QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) was used for real-time PCR amplification. The asqPCR procedure was performed using Fast Real-Time PCR System 7900HT (Applied Biosystems, Foster City, CA) under the following dual stage PCR conditions: 95 °C for 5 min, followed by 10 cycles of 95 °C for 15 s, 68 °C for 50 s, 72 °C for 40 s, then 20 cycles of 95 °C for 20 s, 62 °C for 50 s, 72 °C for 50 s. 1 mM EDTA was added in the reaction mix to increase the specificity of amplification. The obtained Ct values were used to quantify the mutant's content (in percent) using the following formula: revertant contents = [(quantity of revertant contents in the sample)/(quantity of revertant contents in 100% revertant control)] × 100. Since the efficiency of PCR amplification using 472_C-specific and 472_T-specific primers was slightly different, the results had to be normalized by using data obtained from amplification reactions with allele-insensitive forward and reverse primers.

2.7. Validation of asqPCR method by the analysis of OPV references

Two WHO viral references (96/572 and 96/578) representing oral polio vaccine lots that passed and failed the MAPREC test, and containing about 0.7% and 1.1% of revertants respectively were used for the asqPCR. To construct standard curves we used revertant Sabin 3 strain DM Pg35aP-396 containing 100% 472_C. Samples tested include the US National neurovirulence reference NC2 with about 0.9% of revertants and a failed vaccine batch (Lot F) contained about 2.5% of 472_C revertants. cDNA were synthesized from all viral samples using the above protocol and amplified in the asqPCR assay.

3. Results

3.1. Specificity of the allele specific primers

The general design of tethered allele-specific forward primers is illustrated in Fig. 1A. The primers consist of three parts: the 3′-end "sensor" segment is made of 6 nucleotides complementary to the template. The 3′-terminal nucleotide of the segment matches the mutation of interest, and therefore its ability to prime polymerase reaction is affected significantly by the mutation. The second part is the flexible five-nucleotide poly (I) linker connecting to the third "anchor" segment that is complementary to the template and serves to increase the stability of primer-template complexes. This forward allele-specific primer is used together with a reverse primer that does not have any modifications. Together they produce a 72 bp DNA amplicon (Fig. 1B).

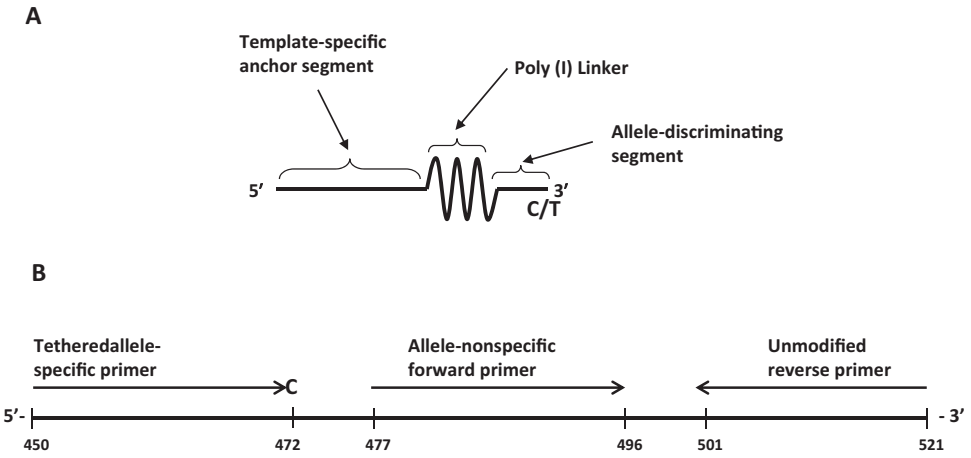

Fig. 1. (A) Design of tethered allele-specific primers consisting of 3′-terminal sensor segment discriminating between alleles connected through poly-I linker to the anchor segment increasing stability of the primer-template complex. (B) Layout of tethered and allele-nonspecific primers used in asqPCR assay.

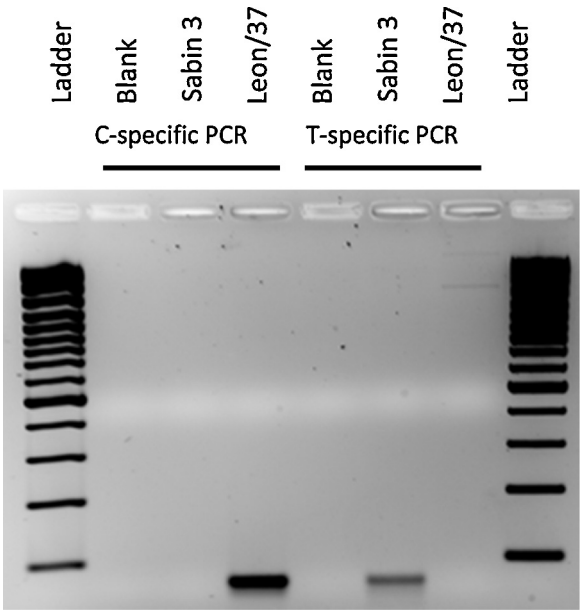

Fig. 2. Specificity of the tethered allele specific primers. PCR DNA amplicons were analyzed by electrophoresis in 2% agarose gel. Lanes 2–4: specific amplification of revertant type 3 poliovirus using tethered allele-specific forward primer for 472_C with Universal reverse primer: Lane 2, water (negative control); Lane 3, Sabin 3 strain; Lane 4, Leon/37 strain. Lanes 5–7: specific amplification of type 3 vaccine poliovirus using tethered allele-specific forward primer for 472_T with unmodified reverse primer: Lane 5, water (negative control); Lane 6, Sabin 3 strain; Lane 7, Leon/37 strain. Lanes 1 and 8 contained 100 bp DNA Ladder.

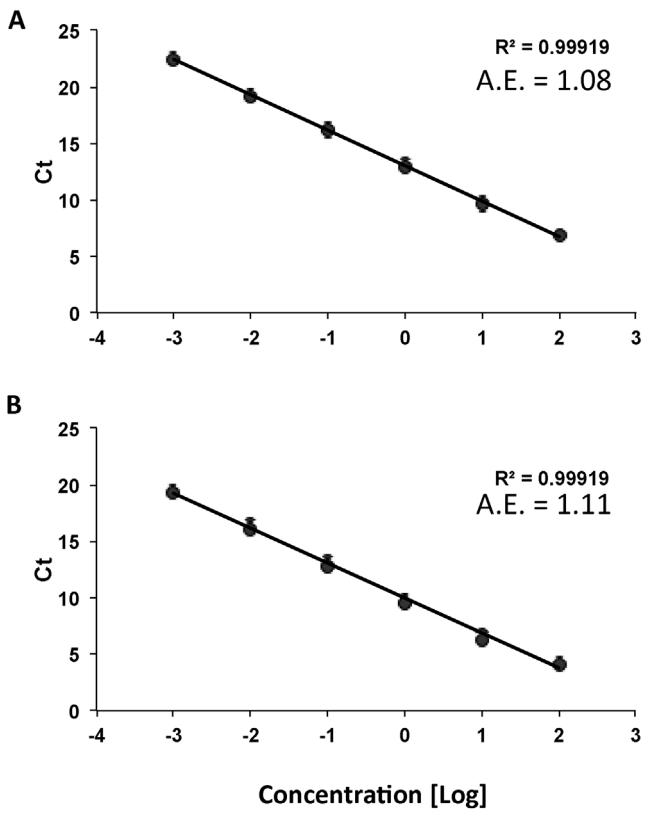

Fig. 3. Dose–response curves of asqPCR assay: the Leon/37 plasmid DNA at concentrations ranging from 100 ng to 1 pg was quantified by asqPCR using tethered allele-specific 472_C forward/unmodified reverse primers (panel A) and allele-insensitive forward/unmodified reverse primers (panel B).

To assess the discrimination of C revertant from non-revertant T at position 472 plasmids with cDNA of Leon/37 (wild poliovirus) containing 100% 472_C and Sabin 3 strain containing 100% 472_T were used. PCR was conducted using the dual temperature cycle as described in Materials and Methods and primers specific for 472_C and 472_T. The results in Fig. 2 show that the 472_C-specific primer generated one DNA band of proper size with the Leon/37 plasmid while the 472_T-specific primer produced one specific DNA band with Sabin 3 plasmid. No cross amplification of mismatched templates was observed. The Sabin 3 DNA band had a lower intensity probably because of a lower melting temperature of the T-specific primer compared to the C-specific primer. These results demonstrated that the tethered allele-specific primers used in the optimized dual temperature cycle produced a good specificity for 472_T and 472_C.

3.2. Use of allele-specific primers for quantitative PCR (asqPCR)

The above results are in good agreement with the previously published use of dual-priming oligonucleotides (Chun et al., 2007). Since the limit of detection is determined by cross-amplification because of the insufficient primer specificity, better discrimination between the two alleles could be used to lower the limit of detection and accurate quantitation of mutants. Therefore allele-specific quantitative PCR (asqPCR) was developed in which the above tethered 472_C and 472_T-specific forward primers were used in pair with the unmodified reverse primer (Table 1). PCR with another forward primer ("non-discriminating forward primer") located inside the amplicon was also performed to determine the quantity of the template that is used as a reference to normalize the results and make them comparable.

The asqPCR was performed in a 96-well plate that was divided in two equal parts: the first included samples amplified by tethered allele-specific primer, while the second part amplified with the unmodified primers. The samples included a positive control with 100% 472_C (wild-type or revertant), a negative control with 100% 472_T (vaccine strain), a reference with known content of mutants, and up to sixteen test samples. Each sample was tested in duplicate. The asqPCR was performed in real-time format using the ABI 7900HT thermocycler, and DNA amplification monitored by SYBR Green dye fluorescence. The copy numbers were calculated individually for each sample using parallel line analysis relative to the reference standard, and normalized by dividing by copy numbers obtained using non-discriminating primers.

The efficiency of PCR using C-allele-specific primer was higher than with T-allele-specific primer (Fig. 2), making it difficult to compare their results quantitatively. Therefore, the content of revertants was determined using only the primer specific to 472_C by calculating the ratio between the copy number in the sample with unknown percent of revertants to the copy numbers of 100% revertants control, rather than the ratio between copy numbers of revertant and non-revertant measured in experiments with two different allele specific primers.

Dose–response curves were generated to determine the efficiency and the limits of quantitative detection for both 472_C-specific and allele-non-specific primers (Fig. 3A and B). For both primer sets Ct values increased linearly with template DNA in the entire range tested between 1 pg and 100 ng. The amplification efficiency (A.E.) was calculated as: A.E. = $10^{(-1/b)} - 1$ (where b is the slope of the linear regression). The A.E. was 1.08 and 1.11 for 472_C-specific and allele-nonspecific primer sets, respectively. Determination coefficients (R^2) were higher than 0.99 for both primer sets. Since the linearity range was at least 5 orders of magnitude for both primer sets, the limit of the quantitative detection could potentially be as low as 0.001% of revertants.

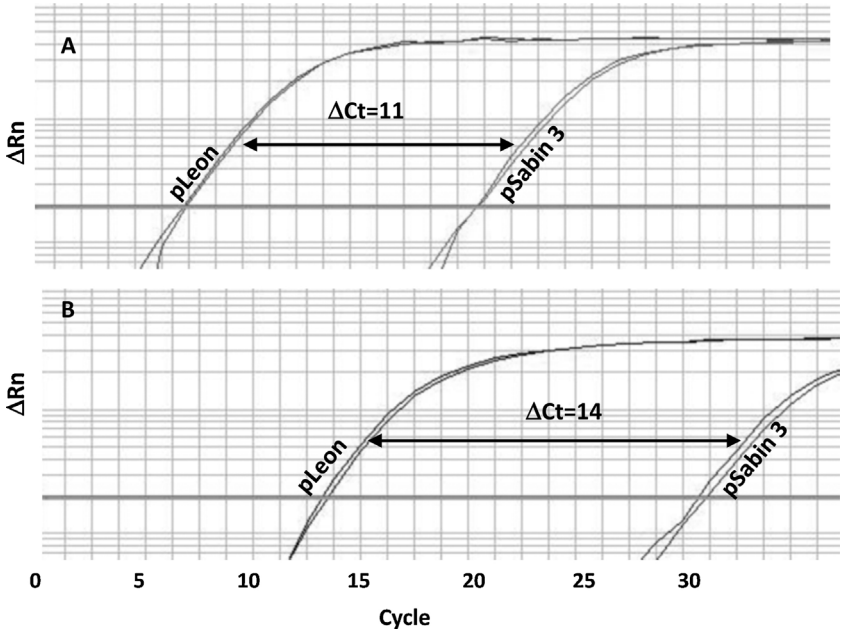

Fig. 4. Allele discrimination between revertant (472_C) and vaccine (472_T) viruses by asqPCR method. Equal copy numbers of Leon/37 and Sabin 3 plasmids were amplified with a regular allele-specific 472_C forward/unmodified reverse primers (panel A) and tethered allele-specific 472_C forward/unmodified reverse primers (panel B).

To evaluate the ability of the tethered 472_C allele-specific primer and the regular 472_C specific primer (without the polyinosine segment) (Table 1) to discriminate between C and T at nucleotide 472, both primers were used in separate reactions to amplify plasmid DNAs with 100% 472_C and 100% 472_T. The results presented in Fig. 4A and B demonstrated that the difference between Ct values (ΔCt) in amplification plots of revertant DNAs and non-revertant DNAs was about 11 for the regular allele-specific primer (Fig. 4A) and about 14 for the tethered allele-specific primer (Fig. 4B). Thus cross-amplification of non-mutant DNA occurred at the frequency of 2^{-11} for the regular allele-specific primer and 2^{-14} for tethered allele-specific primer. Therefore the cross-amplification due to mis-priming results in the lower limit of detection of $2^{-11} = 0.05\%$ for regular, and $2^{-14} = 0.006\%$ for tethered allel-specific primers, respectively, increasing the sensitivity of detection by about 8 fold.

3.3. Sensitivity of mutants quantitation by asqPCR assay

To evaluate the sensitivity of the asqPCR assay to quantify mutants, samples with different content of revertants were prepared by spiking samples of Sabin 3 plasmid containing no 472_C with varying quantities of Leon/37 plasmid containing 100% of 472_C. The contents of mutants ranged between 0.05% and 2% were prepared and analyzed by asqPCR as described above. The results of five independent experiments presented in Fig. 5 showed a good correlation between the expected mutant contents and the values obtained experimentally by asqPCR assay. We found that the assay was linear with $R^2 = 0.99$ in the entire tested range, suggesting that the range in which asqPCR may be used for quantitative analysis is at least 0.05%.

3.4. asqPCR assay for the analysis of revertants in batches of OPV3

To validate the asqPCR assay and to determine whether its results are consistent with the MAPREC assay results, well-characterized viral and DNA reference samples were analyzed. They included the US neurovirulence reference NC2 and two WHO references for MAPREC assay of type 3 OPV. Each sample was tested in triplicate in each experiment, and a total five independent experiments were performed. The results presented in Table 2 show the averages and the 95% confidence intervals of the results. There was a very good agreement between MAPREC and asqPCR results with the correlation coefficient of 0.999 (Fig. 6A). WHO viral references 96/572 and 96/578 were selected based on their 472_C content so that they pass and fail, respectively, when tested in MAPREC assay against the reference with the mutant content equal to the content of 472_C in the US neurovirulence reference NC2. Indeed, when we have analyzed asqPCR results for these samples (Table 2 and Fig. 6B) and used mutant content in NC2 as the reference (0.58 ± 0.03, C.I. 0.55–0.61), 96/572 passed ($p = 0.033$) and 96/578 failed ($p = 0.007$). These results confirm that asqPCR assay can be used as a simple and rapid alternative to evaluate the quantity of the virulent mutation (472_C) in Sabin 3 of the oral polio vaccine.

4. Discussion

Virus strains used in the manufacture of viral vaccines undergo mutational changes, which may cause reversion of attenuated

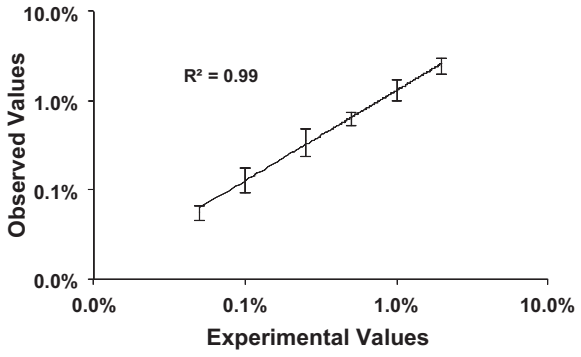

Fig. 5. Reproducibility and sensitivity of asqPCR for poliovirus type 3 revertants ($T_{472} \rightarrow C$) quantitation: the Leon/37 plasmid spiked into Sabin 3 plasmid samples (in the 0.05–2% range) were subjected to asqPCR analysis. Each sample was analyzed in triplicates in five independent experiments.

Table 2
Contents of 472-C in OPV 3 reference vaccine lots.

Sample ID	Description	Nominal 472-C content, %[a]	Values observed in independent experiments, %					Mean	95% CI
			exp 1	exp 2	exp 3	exp 4	exp 5		
WHO 96/572	WHO reference passed vaccine	0.70	0.42	0.45	0.44	0.47	0.49	0.45 ± 0.03	0.42–0.49
NC-2	US neurovirulence reference	0.90	0.61	0.58	0.61	0.55	0.56	0.58 ± 0.03	0.55–0.61
WHO 96/578	WHO reference failed vaccine	1.10	0.85	0.72	0.74	0.96	0.85	0.83 ± 0.10	0.70–0.95
Lot F	Failed vaccine lot	2.30	2.09	1.91	2.06	2.02	2.05	2.03 ± 0.07	1.94–2.11

exp: Experiment.
[a] Nominal mutant content was determined by MAPREC test.

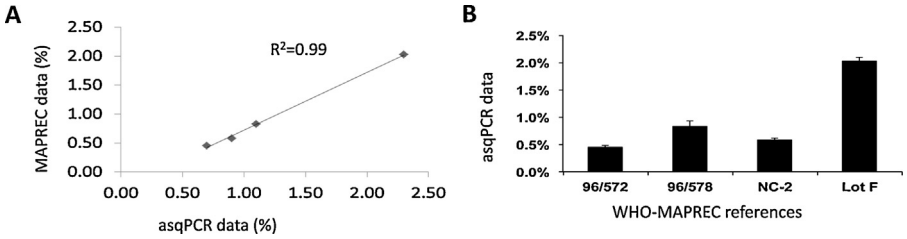

Fig. 6. asqPCR assay for quantitation of 472_C-revertants in type 3 OPV references. Each reference sample was tested by asqPCR in triplicates in five independent experiments. (A) correlation between the revertant contents in the WHO references determined by MAPREC and asqPCR. (B) Contents of 472_C-revertants in reference samples.

strains to virulence as well as to change the antigenic properties that affects the immunogenicity of inactivated vaccines (Gambaryan et al., 1998; Hughes et al., 2001; Schild et al., 1983). Therefore monitoring of the genetic consistency of vaccines is an important task to ensure their quality, safety, and potency.

Sabin strains used in the manufacture of OPV represent a case study of reversion of attenuated vaccine strains to virulence. Molecular studies performed in the 1980s showed that $U_{472} \rightarrow C$ mutation in Sabin type 3 virus (Cann et al., 1984), $A_{481} \rightarrow G$ mutation in type 2 virus (Macadam et al., 1993), and $G_{480} \rightarrow A$ and $U_{525} \rightarrow C$ mutations in type 1 virus (Christodoulou et al., 1990) accumulate during virus growth *in vitro* and *in vivo* and are responsible for reversion to virulence. Similar mutations are found in vaccine-derived strains isolated from cases of vaccine-associated paralytic polio, and therefore the presence of these revertants in batches of polio vaccine is considered a safety risk. A method based on PCR and restriction enzyme cleavage (MAPREC) was developed to identify batches of Oral Polio Vaccine (OPV) with unacceptable levels of neurovirulent revertants (Chumakov, 1999; Chumakov et al., 1991), and was recommended by WHO as an *in vitro* method of choice for lot release of OPV.

As mentioned in the introduction, MAPREC suffers from some shortcomings including the need for radioactive isotopes and various technical challenges at multiple steps of its complex protocol. Quantitative PCR is an obvious alternative, but most quantitative PCR-based genotyping protocols either require expensive TaqMan probes (Delobel et al., 2011; Germer et al., 2000; Kianianmomeni et al., 2007; Laassri et al., 2005; Punia et al., 2004; Schwarz et al., 2004; Taira et al., 2011) or are not sensitive enough to quantity low levels of mutants (less than 2%). This level of sensitivity is insufficient for analysis of viral vaccines with mutant contents of less than 1%.

The method proposed in this communication is based on allele-specific primers the specificity of which was enhanced by their special design. The primers consist of three segments: the mutant-discriminating 3′-end linked by a flexible poly-I stretch to a specific segment that serves as an anchor to increase stability of binding and prevent non-specific priming. Such tethered allele-specific primers were developed previously for identification of SNP (Chun et al., 2007) but were not used for quantitative analysis. Here we demonstrate that their specificity is about 8-fold higher than of regular allele-specific primers, and consequently the lower limit of reliable detection and quantitation of mutants is also about 8-fold lower. This improvement is significant because the concentration of mutants in OPV batches can be in the range where regular primers demonstrate cross-amplification. The proposed method is simple, sensitive, and could be used to test 16 samples simultaneously along with appropriate controls and reference samples.

Here we describe the use of asqPCR for determining small quantities of virulent mutants $U_{472} \rightarrow C$ in batches of type 3 OPV. Close agreement between the results of asqPCR and MAPREC suggests that asqPCR assay can be used as an alternative of MAPREC assay for lot release of OPV. Importantly, the existing validated WHO references for MAPREC assay could be used for asqPCR test also. In addition, the use of SYBR Green dye instead of expensive fluorescent probes makes this method much easier to perform. The asqPCR method is reproducible and is able to quantify with high accuracy revertants present at a level as low as 0.05% and possibly beyond. Similar primers were also developed for quantitation of mutants in two other Sabin strains and are undergoing evaluation.

In conclusion, asqPCR could be considered as an alternative to MAPREC for lot release of OPV. It also could be implemented for monitoring consistency of other live viral vaccines as well as for other applications requiring measuring low quantities of mutants. For example, it could be used for discovery and quantitation of mutants determining resistance of microorganisms to drugs and antibodies. Sensitive quantitative analysis of mutants could be also used in population and metagenomic studies.

Acknowledgements

We thank Mr. Antony DiPiazza and Mrs. Tatiana Zagorodnyaya for their help in quantitative PCR experiments. B.B. and M.L. contributed equally to this work.

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