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

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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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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