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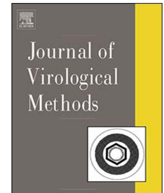

Quantifying low-frequency revertants in oral poliovirus vaccine using next generation sequencing

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A B S T R A C T

Spontaneous reversion to neurovirulence of live attenuated oral poliovirus vaccine (OPV) serotype 3 (chiefly involving the n.472U > C mutation), must be monitored during production to ensure vaccine safety and consistency. Mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC) has long been endorsed by the World Health Organization as the preferred *in vitro* test for this purpose; however, it requires radiolabeling, which is no longer supported by many laboratories. We evaluated the performance and suitability of next generation sequencing (NGS) as an alternative to MAPREC.

The linearity of NGS was demonstrated at revertant concentrations equivalent to the study range of 0.25%–1.5%. NGS repeatability and intermediate precision were comparable across all tested samples, and NGS was highly reproducible, irrespective of sequencing platform or analysis software used. NGS was performed on OPV serotype 3 working seed lots and monovalent bulks ($n = 21$) that were previously tested using MAPREC, and which covered the representative range of vaccine production. Percentages of 472-C revertants identified by NGS and MAPREC were comparable and highly correlated ($r \geq 0.80$), with a Pearson correlation coefficient of 0.95585 ($p < 0.0001$). NGS demonstrated statistically equivalent performance to that of MAPREC for quantifying low-frequency OPV serotype 3 revertants, and offers a valid alternative to MAPREC.

1. Introduction

The live attenuated oral poliovirus vaccine (OPV), developed by Albert Sabin in the 1950s and commercially available since 1961 (Sabin, 1985), has played a pivotal role in the World Health Organization (WHO)'s Global Program of Polio Eradication, achieving a > 99% decrease in polio cases since 1988. The last acute flaccid paralysis (AFP) case due to wild type 2 poliovirus was reported in 1999 (Centers for Disease Control and Prevention (CDC), 2001). AFP cases due to wild type 3 and type 1 poliovirus are still reported in Afghanistan, Pakistan, and Nigeria, but the numbers for 2016 are the lowest yet recorded cumulatively for one year (Global Polio Eradication Initiative, 2016). Vaccination against serotype 2 has now been discontinued in OPV-using countries; in parallel, the inactivated polio vaccine (IPV) is being introduced in these countries (Miller and John, 2016). The continued use of serotypes 1 and 3 in OPV also remains important in the face of the ongoing risks of poliovirus transmission and importation from areas where poliovirus circulation has not yet been eradicated (World Health Organization, 2016).

During the OPV manufacturing process, the Sabin polivirus strains may spontaneously mutate, driven by the inherent genetic instability of RNA viruses. Potential point mutations in the 5′ non-coding regions of the viral RNA may result in reversion to full or partial virulence, constituting a safety risk (Neverov and Chumakov, 2010). In particular, the uracil to cytosine reversion at position 472 (n.472U > C) in the 5′-untranslated region (UTR) of the attenuated Sabin OPV serotype 3 strain (Laassri et al., 2006) has been associated with increased neurovirulence (Chumakov et al., 1991; Evans et al., 1985; Miller and John, 2016). The potential accumulation of these revertants during the manufacturing replication runs must be closely monitored to ensure not only the consistency of the product but also the safety of the vaccine.

As a potential substitute for time-consuming and costly animal-based neurovirulence testing, a quantitative molecular procedure called mutant analysis by polymerase chain reaction (PCR) and restriction

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enzyme cleavage (MAPREC) has been used for many years to monitor the presence of neurovirulent revertants during OPV production (mainly for quantification of n.472U > C revertants in OPV serotype 3) (Chumakov, 2016; Global Polio Eradication Initiative, 2016; Neverov and Chumakov, 2010). MAPREC shows high sensitivity and accuracy, but requires radioisotope labeling which may limit its use in some laboratories (Bidzhieva et al., 2011), and the imaging platform required to visualize radiolabeled DNA is no longer supported. The use of chemiluminescent instead of radioactive labeling in MAPREC has also been explored to address these issues (Bidzhieva et al., 2011).

Next generation sequencing (NGS) has recently been proposed as a replacement to MAPREC for monitoring of 472-C revertants following the rapid evolution in genomic sequencing platform technology and availability of validated protocols. NGS does not require radioisotope use, can be used to identify very small amounts of mutant viruses in live viral vaccine preparations, and enables accurate quantification. It also allows for the rapid generation of very large amounts of sequence data (Neverov and Chumakov, 2010; Schirmer et al., 2015).

Here, we describe the NGS methodology, present results of correlation studies and performance testing between NGS and MAPREC, and evaluate the suitability of NGS as a replacement for MAPREC for the quantitation of 472-C revertants during the manufacturing of OPV serotype 3.

2. Materials and methods

2.1. Samples and test references

The following samples of the Sabin type 3 OPV virus (Sabin Original Leon 12a1b/KP3) (Order *Picornavirales*, Family *Picornaviridae*, Genus *Enterovirus*, Species *Human enterovirus C*, Subtype Poliovirus) were included in this study: two monovalent OPV serotype 3 bulks representing lots with 'routine' levels of 472-C revertants as measured by MAPREC (Lot A; mean MAPREC result: 0.5%), and those with the highest historically measured levels of 472-C revertants among OPV lots at Sanofi Pasteur (Lot B; mean MAPREC result: 0.9%); and an OPV serotype 3 panel comprising of working seed lots (n = 2) and monovalent bulks (n = 19), representative of the historical range of production at Sanofi Pasteur (previously tested using MAPREC). We also used two commercial international references developed by the National Institute for Biological Standards and Control (NIBSC) throughout the study: the WHO 1st International Low Virus Reference Reagent for MAPREC analysis of poliovirus type 3 NIBSC code 96/572 (MAPREC reference titer: 0.7% 472-C nucleotide content) and the WHO 1st International High Virus Reference Reagent for MAPREC analysis of poliovirus type 3 NIBSC code 96/578 (MAPREC reference titer: 1.1% 472-C nucleotide content). OPV lots and WHO references were stored at or below −70 °C.

cDNA plasmids encoding the vaccine 472-T or the revertant 472-C variant of Sabin serotype 3 poliovirus, were constructed using standard molecular biological techniques. In brief, full-genome poliovirus cDNA was purified and cloned into linearized pCR®-XL-TOPO® plasmid vector (TOPO® XL PCR Cloning kit, ThermoFisher Scientific). Competent *Escherichia coli* One Shot® TOP10 cells (ThermoFisher Scientific) were transformed with 2 μL of ligation product using the heat shock method, whereby the transformation mixture was incubated on ice, submitted to heat shock at 42 °C, followed by a cold shock. Luria-Bertani (LB) broth culture was then added and cells incubated for 1 h at 37 °C. 100 μL of bacterial suspension was plated on an LB-agar plate containing appropriate antibiotic, and incubated overnight at 37 °C. Bacterial colonies were directly screened using PCR, and where positive, the entire plasmid was sequenced to verify that sequences were correct. Mixtures of these two plasmid constructs were used to provide theoretical mutant concentrations ranging from 0% (472-T plasmid only) to 1.5% 472-C.

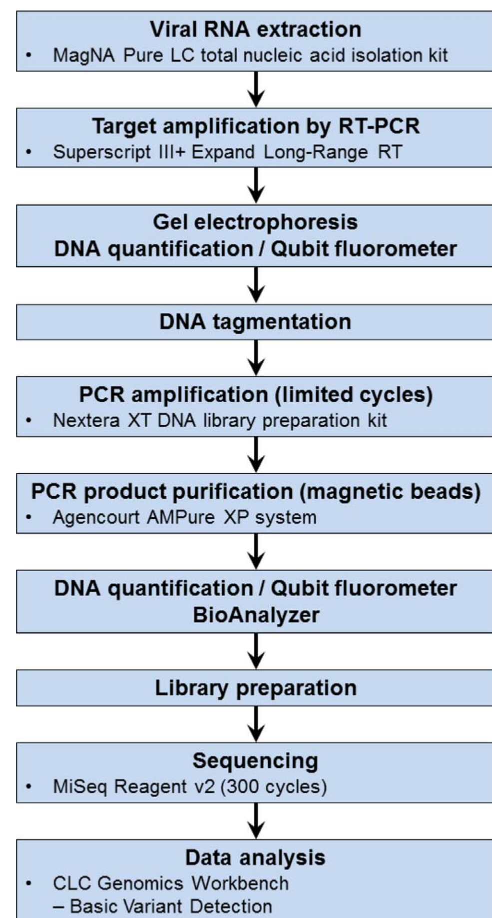

Fig. 1. Flow diagram of the stages during NGS using the MiSeq System.NGS, next generation sequencing; PCR, polymerase chain reaction; RT, reverse transcriptase.

2.2. Next generation sequencing (NGS)

The NGS methodology used was adapted from that of Chumakov and colleagues (Neverov and Chumakov, 2010) (Fig. 1).

2.2.1. Preparation of DNA for analysis

RNA was extracted from cell culture supernatants or monovalent vaccine lots using the MagNA Pure LC total nucleic acid isolation kit (Roche) and KingFisher system (ThermoFisher Scientific). Isolated RNA was reverse transcribed with Superscript III + Expand Long-Range reverse transcriptase (ThermoFisher Scientific) according to manufacturer's instructions, using a modified A3-As primer lacking the poly-T tail (CCTCCGAATTAAAGAAAAATTTACCCCTAC).

Amplicons corresponding to the first ~3.8 kb of the poliovirus genome containing the mutation site were PCR amplified from 5 μL full-length cDNA using 0.8 μM of primer pairs S1-T7 (5′-GCGGCCGCTAATACGACTCACTATAGGTTAAAACAGCTCTGGGGTTG-3′) and 3797R (5′-CCCTGCTCCATGGCCTCTTCCTCGTAAGC-3′) in 1X Expand Long Range Buffer, 0.5 mM deoxynucleotide mix, 6% (v/v) dimethyl sulfoxide and 0.07 U/μL Expand Long-Range enzyme mix (ThermoFisher Scientific) according to the following protocol: 2 min denaturation at 92 °C, then 10 cycles of [10 s at 92 °C, 15 s at 55 °C, 12 min at 68 °C], 10 cycles of [10 s at 92 °C, 15 s at 55 °C, 15.5 min at 68 °C], 10 cycles of [10 s at 92 °C, 15 s at 55 °C, 19 min at 68 °C], 10 cycles of [10 s at 92 °C, 15 s at 55 °C, 22.5 min at 68 °C], and a final extension step of 7 min at 68 °C.

PCR products were purified and their quality assessed by agarose gel electrophoresis and spectrophotometry, quantified with a Qubit fluorometer (ThermoFisher Scientific) and prepared to a final concentration of 0.1 ng/μL. DNA tagmentation using the Nextera XT DNA library

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