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Design and Development of a Simple Method for the Detection and Quantification of Residual Host Cell DNA in Recombinant Rotavirus Vaccine

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Abstract

Rotavirus recombinant vaccine is usually produced in Vero cells. Residual host DNA may reside in the final product and is considered a source of contamination. WHO protocols indicate that biological products should be free of any type of impurity such as nucleic acids, endotoxins, and host cell intermediate materials. Therefore, all recombinant biological therapeutics should be assessed for residual host DNA. In the present study, a sensitive and specific real-time PCR method was developed to detect residual host cell DNA in the final product. The Beta-actin gene of Vero cells was selected to detect residual host cell DNA. One set of primers and a TaqMan probe were designed for the gene using AlleleID 6 software. Real-time PCR reactions were set up, and efficiency of 84% was obtained. The sensitivity and limit of detection of the assay were determined to be 0.176 Fg/ μ l and 0.044 Fg/ μ l, respectively. The intra-assay and inter-assay variations were 4.4% and 1.04%, respectively. Furthermore, the specificity and sensitivity of the assay were high enough, and the detection limit was lower than that of the FDA and WHO standards. This indicates that our assay is highly specific and sensitive to detect residual host DNA of Vero cells in the recombinant rotavirus vaccine.

Keywords: Rotavirus, Recombinant vaccine, Residual DNA, RT-qPCR

1. Introduction

The application of recombinant therapeutics such as vaccines has recently grown for the treatment of various diseases [1]. Rotavirus A (RVA) is the main cause of diarrhea in infants and young children accounting for a substantial number of child deaths in developing countries [2]. However, this disease is not confined to developing countries. In fact, 50% of hospitalized children and 50% of patients admitted in emergency rooms in the USA have been infected with rotavirus before vaccination [3]. The most effective way of controlling the disease is by complying with hygiene standards and vaccination [4].

Vero cells are usually used as the host to develop vaccines. Therefore, the final product may be contaminated with impurities such as nucleic acids, endotoxins, and host cell proteins. Residual host cell DNA (HCD) is the most important impurity in recombinant products such as vaccines and should be assessed during quality control processes [5]. The most important problematic outcomes of HCD in viral vaccines are infections and carcinogenesis. Thus, recombinant products should be assessed for HCD to ensure the final product is free of impurities [6-8]. WHO and FDA have determined the acceptable level of HCD in recombinant products. In 1987, the acceptable HCD cut-off was less than 100 pg/dose to reduce the risk of oncogenicity of continuous cell lines HCD. Then, the WHO increased the acceptable level of HCD to less than 10 ng/dose [7]. Most pharmacologists confirm these cut-offs. In 2010, the FDA suggested that residual DNA fragments should be smaller than nearly 200 nucleotides, which is less than the length of a functional gene. Regarding live attenuated vaccines such as the rotavirus vaccine, the HCD should be less than 100 μ g/dose [9].

Nowadays, FDA, WHO, and European Pharmacopoeia suggest that risk assessment should be performed for each step of the production process. Impacts of several items on the amount and biological activity of HCD should be considered. These items are (i) cellular substrate, (ii) the application of the product, and most importantly, (iii) production steps such as inactivation. If it is impossible to reduce HCD levels, alkylating agents such as β -Propiolactone can be used to reduce the HCD level and its risks [9].

There are several methods to quantitatively detect HCD in recombinant products. However, few of them are routinely used in biotechnological industries. Some of these methods are Threshold assay, Hybridization assay, qPCR, and PicoGreen analysis. Hybridization assay is a very

sensitive and specific, yet an expensive assay. It is also a complicated method and difficult to interpret [10, 11]. Although the threshold assay is cost-effective, it is not sensitive and specific enough. It cannot be easily used for fragments of less than 800 nucleotides long. Finally, it cannot be used when DNA concentration is more than 1 ng/ml [12, 13]. PicoGreen is a simple and inexpensive method, yet lacking specificity[9]. It detects all dsDNA and cannot detect low levels of HCD [14]. In recent years, Nucleic Acid Amplification tests (NAT) have been widely used for the detection of HCD in recombinant products since they are specific, sensitive, and rapid. Also, the required equipment and reagents are provided by numerous companies. If the target sequence has several copies in the host genome, the HCD quantification will be more accurate and precise. However, contamination is always a potential problem. For example, if mammalian HCD is quantified, and the target sequence is a highly conserved mammalian sequence such as 18s rDNA or Alu repeats, contamination with human DNA may occur.

PCR is the most sensitive and specific molecular assay and is used as a high-throughput method. Furthermore, its results are highly accurate and can be interpreted rapidly [15]. Analytical laboratories of Sanofi Pasteur used hybridization and Threshold Assays in 1980 and 1990, respectively. Then, in 2006, Quantitative Real-time PCR (qPCR) was used to quantify the HCD of Vero cells in biological products [9].

In the present study, Vero cells' HCD in rotavirus recombinant vaccine was detected using qPCR, which is a rapid, sensitive, specific, and cost-effective method.

2. Materials and methods

2.1. Samples

Samples were taken during the production process before and after purification steps. Samples were also taken after the final formulation.

2.2. Primer and probe design

The Beta-actin gene of Vero cells was selected based on the analysis of the NCBI database. Primers and a TaqMan probe were designed using AlleleID 6 software (Premier Biosoft) and synthesized by Metabion (Germany). FAM and BHQ1 were used to label the probe at 5' and 3' end, respectively (Table 1).

2.3. Extraction of Vero cell DNA

Standard DNA from Vero cells that were used for vaccine production was extracted after cell lysis and RNase treatment using the High Pure PCR Template Preparation Kit (Roche). The quantity of the extracted DNA was determined using spectrophotometry at 260 nm. The purity of the extracted DNA was determined using $OD_{260/280}$. The DNA was stored at -70 °C until use.

2.4. Cloning and standard preparation

After amplification of the extracted DNA, the PCR product was cloned into PTG19-T vector using T/A vector cloning kit (Banqiuo, Taiwan). The recombinant vector was transformed into Top10 competent *E. coli*. White colonies were selected and subcultured. The recombinant plasmids were extracted using alkaline lysis. The concentration of extracted plasmids was determined using NanoDrop (Thermo scientific, Waltham, MA, USA). Ten-fold serial dilutions were prepared from the stock plasmid solution.

2.5. Vero ABCT qPCR

qPCR was performed on the extracted DNA samples of Vero cells. qPCR reactions contained 5 µl of extracted DNA or the standard plasmids (serial dilutions from 0.716 Fg/µl to 1760 Fg/µl), 10 µl of master mix (RealQ plus 2x master mix probe High Rox, Ampliqon), 0.4 µl of each primer (20 µM), and 0.2 µl of the probe (20 µM) in a final volume of 20 µl. Nuclease-free water was used as a negative control. StepOne Real-time PCR instrument (Applied Biosystems, USA) was used with the following program: 95°C for 15 min, 40 cycles of 95°C for 10 sec, and 60°C

for 40sec. Absolute quantification module of StepOne Real-time PCR software was used to quantify Vero cells HCD. A standard curve was developed by plotting the logarithm of standard plasmid concentration versus Cq values.

2.6.qPCR validation

Validation of accuracy, precision, reproducibility, specificity, dynamic range, and linearity was performed based on the guidelines of the Q2 (R1) International Conference on Harmonization (ICH).

2.7.Specificity

Specificity assessment was performed in two steps. First, the specificity of the primers and the probe was evaluated using NCBI nBlast. Second, qPCR was performed on the genome samples of HBV, HPV, HCV, Toxoplasma, TB, HSV, EBV, and Adenovirus. Vero cells were used as the positive control. No template control (NTC) was also included in the experiment.

2.8.Intra- assay and inter-assay variations

Serial dilutions of the standard plasmids from 0.176 to 1760 Fg/ μ l were used to determine the intra-assay and inter-assay variations based on the protocol mentioned in the Vero ABCT qPCR section. Each standard was tested in triplicate to assess the intra-assay variations. Each standard was tested in triplicates in three consecutive days to determine the inter-assay variations.

2.9.LOD and sensitivity

Serial dilutions from 0.022 to 0176 Fg/ μ l were tested using the developed assay to determine the limit of detection (LOD) and sensitivity of the assay. The test was performed in triplicates on three different days.

3. Results

3.1.qPCR validation

The results indicated that the assay was specific. The assay was also linear in the concentration range of 0.176 to 1760 Fg/ μ l.

3.2.Efficiency and standard curve

Table 1 shows the standard curve of the assay. The R² value was determined to be 0.991, and the slope of the curve was -3.783. The efficiency of the reaction was 83.684. (Figure 1)

The intra-assay and inter-assay variations were 4.4% and 1.04%, respectively and show the assay is reproducible. The Standard deviations (SD) and coefficient of variations (CV) are shown in tables 2 and 3.

3.3.LOD and sensitivity

The sensitivity and LOD of the assay were 0.176 and 0.044 Fg/ μ l, respectively, and were less than FDA and WHO standards. This indicates the high sensitivity of the assay.

3.4.Specificity

To ensure the specificity of the assay, genome samples of *Hepatitis B virus*, *Human Papilloma virus*, *Hepatitis C virus*, *Toxoplasma*, *Mycobacterium tuberculosis*, *Herpes simplex virus*, *Epstein-Bar virus*, and were used. The assay only detected Vero cells' DNA. This indicated a 100% specificity of the assay.

4. Discussion

In the present study, we developed a qPCR assay to detect Vero cells' HCD in recombinant rotavirus vaccines. The assay was linear in a wide range, *i.e.* from 0.176 to 1760 Fg/ μ l. The detection limit was also 0.176 Fg/ μ l. The intra-assay and inter-assay variations were acceptable based on the standards of the College of American Pathologists (CAP). The assay was also shown to be highly specific and sensitive. Thus, it can be used for the HCD analysis of a variety of live and inactivated vaccines produced in Vero cells. This qPCR method can be used to detect small amounts of DNA in highly pure vaccines and should be used instead of other methods such as Threshold DNA and hybridization assay to detect HCD of vaccines produced in Vero cells. Thus, we suggest that specific qPCR assays should be developed for the detection of HCD in products developed in mammalian systems. The standard DNA that is used for standard curve generation should be similar to the HCD DNA. If the number of the DNA fragments in standard samples is not similar to the HCD of the product, quantification would be biased. Furthermore, to increase the sensitivity of the assay, HCD should be assessed before final formulation when the concentration of the HCD is high.

In molecular diagnostic laboratories, sensitivity, specificity, and rapidity are the major factors. In addition, the cost and advantages/disadvantages of the methods are important [16-19]. Thus, using a specific and sensitive method is essential.

There are several methods to detect HCD in recombinant products. Hybridization dot-blot assay is sensitive and specific, yet time-consuming [20]. Probe hybridization is also a sensitive method, in which probes are labeled with radioactive compounds. It can detect fewer HCD levels than the Hybridization dot-blot assay [11]. PicoGreen method can detect dsDNA and is simple and cost-effective. However, it cannot detect HCD specifically [21].

Molecular methods such as qPCR and Threshold analysis are other methods of detecting HCD. qPCR is a rapid, sensitive, and specific method [22]. Although threshold DNA assay is a simple method, it is not sensitive enough [23, 24] and requires expensive equipment (9). The high dynamic range of qPCR makes it a suitable method for DNA detection. In addition, it needs less volume of samples than the threshold DNA assay (500 μ l) [25]. Our method was developed as a sensitive and specific method to detect HCD in the recombinant rotavirus vaccine.

To obtain precise results and better interpretation as well as validation of HCD detection methods, the CAP has proposed guidelines. According to CAP guidelines, acceptable CV for intra-assay and inter-assay should be less than 5% and 10%, respectively [6]. In our developed method, the CV of intra-assay and inter-assay was 4.4% and 1.04%, respectively. In addition, the LOD of the assay was 0.176 Fg/ μ l, which was less than the standards of the FDA and WHO. The efficiency and specificity of the assay were 84% and 100%, respectively.

Authorship contribution statement

Farzaneh Amourizi Varnamkhasti: Writing - original draft. Vahid Kia: Data curation. Rahman Shokri: Supervision. Mohammad Javad Mehdipour Moghaddam: Methodology. Mahdi Paryan: Validation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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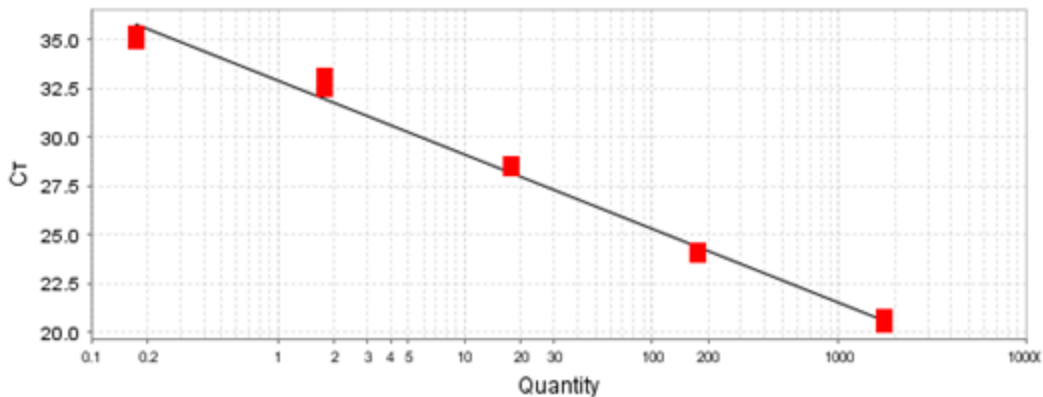
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Figure Legend:

Figure 1: The standard curve obtained from standard plasmids analysis. R², Slope and efficiency of the assay are within the acceptable range.

Standard Curve



Target: Verocell Slope: -3.783 Y-Inter: 32.915 R²: 0.991 Eff%: 83.798

Table 1. The primers and the probe sequences used in the study.

Primer	Original Name Sequence 5'-3'
ABCT F Cholorocebus	5'-TGA CAG GAT GCA GAA GGA GAT TAC-3'
ABCT R Cholorocebus	5'-CAG TGA GAA CCC CGG ATG TG-3'
Probe	Oligo Name Sequence 5'-3'
ABCT P Cholorocebus	5'-FAM-CAC ACA CCA CAG GGC ACC ACG-BHQ1-3'

Table 2. The table shows the values of inter-assay of the developed method. The mean C_q values of each run, SD, and CV are shown in the table. The highest CV value was 1.04%, which was less the cut-off suggested by CAP.

Mean of Each Run	SD of Each Run	CV of Each Run
20.56	0.19	0.92
24.08	0.09	0.39
28.50	0.13	0.46
32.74	0.34	1.04
35.15	0.20	0.57

Table 3. The table shows the values of intra-assay of the developed method. The mean C_q of the three runs for each standard, SD, and CV are shown in the table. The highest CV value was 4.4%, which was less the cut-off suggested by CAP.

Mean of Three Runs for Each Concentration	SD of Three Runs for Each Concentration	CV of Three Runs for Each Concentration
21.07	0.51	2.43

Mean of Three Runs for Each Concentration	SD of Three Runs for Each Concentration	CV of Three Runs for Each Concentration
25.08	0.96	3.83
29.86	1.31	4.40
33.75	0.88	2.61
36.04	0.78	2.16