Application of Real-Time PCR method for evaluation of measles vaccine heat stability

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ABSTRACT

The Plaque Forming Unit(PFU) and Tissue Culture Infectious Dose50($TCID_{50}$) methods are used for evaluation of vaccine heat stability and effect of various stabilizers on thermal stability of vaccines. The aim of present study is using Real-Time PCRtechniquefor estimation of vaccine degradation rate and thermal stability of measles vaccines. Lyophilized measles vaccines containing three various stabilizers were reconstituted with distilled water. Three vial of each vaccine incubated at25˚C for 0, 4 and 8 hours. Titer of virus in vaccines calculated by $TCID_{50}$ method. Also after RNA Extraction and cDNA synthesis, the RNA copy numbers of viruses in vaccines were estimated by absolute quantitative Real-Time PCRtesting. The data were analyzedby SPSS 19 and Sigma Plot 11 software.The result of this study showed there is a significant relationshipbetween vaccine degradation rate calculated with $TCID_{50}$ and Real-Time PCR method ($p<0.05$). ThereforeReal-Time PCR is a good complement or appropriate replacement to traditional methods.Titration methods based on cell culture are gold tests for titration of viral vaccines and estimation of heat stability but Real-Time PCR technique can also be used for this goals. This method is faster, cheaper and easier than $TCID_{50}$.

Keywords: Real-Time PCR; Measles vaccine; Stability

INTRODUCTION

Measles is one of the most contagious viral diseases[1]. Vaccination against measles is the best method for disease eradication[2]. Today many live attenuated measles vaccinesare using for this goal[3]. These vaccines are producing with various formulations containing stabilizers and measles virus strains[4].

Previous research showed that heat stability of live vaccines dependent to vaccine components[5, 6]. The producing more stable live vaccines are important for manufactures. For reaching to thisgoal, researchers try to increase the thermal stability of vaccines by changing the vaccine formulation [3, 7, 8].

Up to present, the determination of vaccine potency by tissue culture infectious dose ($TCID_{50}$) is used for evaluation of measles vaccine heat stability[9]. In this method, lyophilized or reconstituted live vaccines were kept at various temperatures for many hours and vaccine titration was carried out by microtitration assay [5, 10]. In $TCID_{50}$ assay the vaccine content add to Vero cells monolayersin microplate wells.After 10 days, cytopathic effects (CPE) are visible that show virus replication[6, 11].It is true that $TCID_{50}$ assay is a suitable method for evaluation of vaccines thermal stability but this procedure is time-consuming because dependent on the CPEobservation[12].

At the present, Real-Time PCR method is using for virus diagnosis and estimation of viralRNA copy numbers[13]. This test is performable at short time. The aim of thisstudy is usingReal-Time PCR as a fast methodfor evaluation of measles vaccine thermal stability.

MATERIALS AND METHODS

Measles Vaccines and cell culture

Measles vaccines with various formulations containing AIK-c strain and 3 different stabilizers (Razi, SSG and TD) were obtained from Razi serum and research institute of Iran. Monolayers of African green monkey kidney cells (Vero) were grown in Dulbecco's Modified Eagle Medium(DMEM)(Sigma-Aldrich)supplemented with5% bovine calf serum and antibiotics (neomycin/kanamycin). Vero cells were used for microtitration assay.

Thermal stability testing for reconstituted vaccines

Three frizz-dried vaccinevials from each formulation were reconstituted with 1 ml sterile distilled water. Diluted vaccines divided in to three aliquots and exposed to 25ºC. Titration assay carryout at 0, 4 and 8hours after reconstitution by $TCID_{50}$ method.Also Real-Time PCR performed for determination of RNA copy numbers in vaccine vials.

Titration of vaccines

Suspension of Vero cells was prepared with concentration of 2×10^5 cell per milliliter. Also tenfold serial dilutions of reconstituted vaccines were prepared in Dulbecco's Modified Eagle Medium (DMEM) containing 2% bovine calf serum. 50µl virus dilutions and 100µl of Vero cell suspension added to wells of cell culture microplate. For each microplate a strilled cover were used to prevent microbial contamination and to maintain pH of medium constantly. The plates were incubated at 37ºC in an atmosphere of 5% CO_2 in incubator. A standard vaccine with determined titer was used for control of the Vero cell cultures and microtitration assays at any working days.Cytopathic effects of virus (CPE) were checked using invert microscope after the sixth day of titration to the tenth day. Estimation of the 50% tissue culture infectivity dose ($TCID_{50}$) was calculated using karber formula.

RNA extraction and cDNA synthesis

Firstly, thereconstituted vaccines, afterpreserving in 25ºC for 0, 4 and 8hours, collected.

Then,viral RNA extracted from reconstituted vaccines by Amplisens DNA/RNA extraction kit (Russia). cDNA synthesis from viral RNA carried out by using Fermentase kit. The reaction for synthesis of cDNA included 5 µ RNA, 1 µ random hexamer, 6.5 µ nuclease free water, 4 µ buffer 5x, 2µ dNTP, 0.5 µ RiboLockRNase Inhibitor and 1 µ RevertAid RT.Then transported at 65°C for 5 minute, 25°C for 10 minute, 42°C for 60 minute and 70°C for 10 minute.

Real-Time PCR test

The Real-Time PCR test performed using hydrolysis probe method.In this way, used from primers (forward primer:5´ TGG CAT CTG AAC TCG GTA TCA C3 and reverse primer:5´TGT CCT CAG TAG TAT GCA TTG CAA3´) and probe (5´ CCG AGG ATG CAA GGC TTG TTT CAG A3´) that were designed for N gene of measles virus (synthesized by Metabion: Germany).For any reaction with final volume of 50 µl, used25 µlTaqman universal PCR master mix(2x),5 µl of forward and reverse primers, 5 µlProbe(10 pm), 5 µl water and 5 µltemplate. Reactions carried out using thermal cycle including 10 minute holding time in 95 °C followed by 40 cycle including 15s in 95 °C and 1 minute in 60 °C.For achieve to standard curve, serial dilutions (tenfold) of counted plasmid containing measles N genewasprepared.

Data analyses

Sigma Plot 11 andSPSS 19 softwares were used for data analysis and statistical calculations.

RESULTS

Titration of reconstituted vaccines by $TCID_{50}$ method

Three measles vaccine, containing different stabilizers, reconstituted and preserving in 25 °C for 0, 4 and 8 hours. Then microtitration assay carried out and titer of vaccine calculated by $TCID_{50}$ method. Table 1 shows result of titrations.

Table1. Titer of 3 reconstituted measles vaccine after preserving at 25 °C for 0, 4 and 8 hours

$\log_{10}TCID_{50}$/ml			Hours after reconstitution (25 °C)
R Vaccine	SSG Vaccine	TD Vaccine	
5.38±0.38	5.13±0.38	4.8±0.25	0
4.63±0.38	4.8	4.07±0.04	4
4.24±0.29	4.13±0.38	3.80±0.25	8

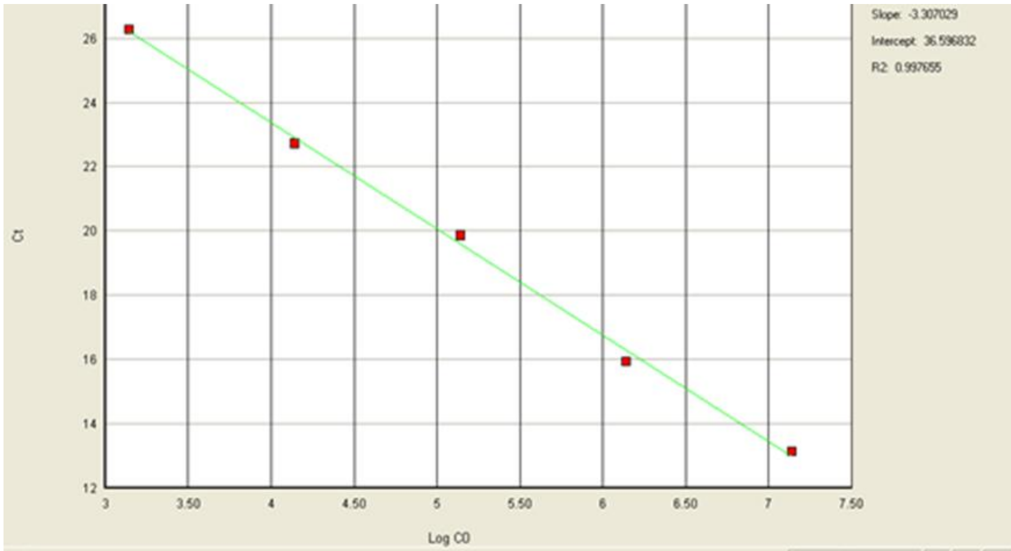

Figure1. Real-Time PCR standard curve for the estimation of measles virusN gene

Real-Time PCR standard curve

For the estimation of measles RNA copies, standard curve depicted using 5 dilution of plasmidscontaining N gene (1388×10^4 to1388×10^{-1}). Standard curve parameters that show in figure1 are in acceptable range.

Quantitative evaluation of measles virus in reconstituted vaccines

The number of measles virusgenomic RNA copies in 3 vaccine containing different stabilizers was estimated by Real-Time PCR method after vaccine reconstituting and preserving in 25 °C for 0, 4 and 8 hours and data summarized in table 2.

Table 2. measles virus genomic RNA copy numbers in 3 reconstituted vaccinesafter storing at 25 °C for 0, 4 and 8 hours

RNA copy numbers/ml			Hours after reconstitution (25 °C)
R Vaccine	**SSG Vaccine**	**TD Vaccine**	
462417108	332079228	234045960	0
11689476	133806828	141922812	4
1338984	12003240	7609992	8

Table 3. Regression calculations and estimation of degradation rate for 3 reconstituted measles vaccineswith two assay methods (Real-Time PCR and$TCID_{50}$)

Vaccine	Regression Equation	Correlation Coefficient	Assay method	Degradation Rate(log/hours)
R	y = 4220870 – 56128408.5 x y = 5.252 - 0.14 x	0.955 0.962	Real-Time PCR $TCID_{50}$	0.2 0.14
SSG	y = 319334426– 40009498.5 x y = 5.164 - 0.125 x	0.943 0.995	Real-Time PCR $TCID_{50}$	0.18 0.125
TD	y = 241077572 -28304496 x y = 4.592 - 0.12x	0.918 0.907	Real-Time PCR $TCID_{50}$	0.17 0.12

Calculation of degradation rate for reconstituted vaccines

Data analysis done by Sigma plot and SPSS software. Using result of titration assay ($TCID_{50}$) and copy number assay (Real-Time PCR) carried out Regression calculation for estimation of reconstituted measles vaccine degradation rate. This data summarized in table 3.

DISCUSSION

There are several methods for virus quantitation assay[1].$TCID_{50}$ and PFU (plaque forming unit) have been two assay method for titration and determination oflive vaccines heat stability [5, 14, 15].The plaque assay is a terrific method for determining virus titers, but it doesn't work for all viruses[16]. This two method are very useful but are classic and require to cell culture. Therefore, the procedures involved are often tedious and time-consuming and thus expensive in terms of laboratory time[17].

In recent years, researchers try to replace a modern method for viralquantitation assay.Flow cytometry method is used forrapid titration of measles,vesicular stomatitis virus and human immunodeficiency virus type 1 in 24-52 hours[18]. In 2012, quantitative PCR developed as an alternative $TCID_{50}$ read-out approach for assessing human herpesvirus 6 [19]. This method was valid and rapid.

Pourianfar and et al used a colorimetric-based method for the determination of enterovirus 71 titer [20]. At 2009, Jonsson did introduceReal-Time PCR as anacceptable alternative to estimation of picornavirus titers by $TCID_{50}$ and PFU [21].Researchers were used reverse transcriptase Real Time PCR method for quantitative evaluation of dengue virus. There are reported a significant correlation between result of virus titration by RT-PCR method and PFU assay [22]. Schalk for first time were estimated the number of infectious measles viruses in live virus vaccines using quantitative Real-Time PCR**.** The resultof this researcher showed in comparison to the plaque assay, the quantitative PCR infectivity assay was faster, while accuracy and intermediate precision were similar[23].Up to present, PFU and $TCID_{50}$ apply for estimation the degradation rate of vaccines and molecular technique not used for this aim [4, 7]. Today, Real-Time PCRis a rapid and cheap technique for detection and titration of many viruses. In this study, we compare $TCID_{50}$ to Real-Time PCR method for estimation of measles vaccine thermal stability. The result of evaluation of vaccine heat stability by Real Time PCR technique showed that measles vaccine containing TD stabilizer has less degradation rate than R and SSG vaccines and this vaccine is more stable.This data verified by $TCID_{50}$. Calculated degradation rate for 3 measles vaccine containing different stabilizers in Real-Time method was higher than $TCID_{50}$ method. The results of the present research showed that somewhat RT-PCR is reliable and practical for determination of live vaccine heat stability in comparison with $TCID_{50}$ method.

Titration methods based on cell culture are gold test for virus detection and vaccine titration. When stability of live vaccineis increased by stabilizers, therefore loss of virus titer is low per hour. In this case, degradation of virus is lessand release of RNA from virus particle occurs later.Genomic RNA degradation decrease Consistent with virus stability.

This study showed that Real-Time PCR method can use for estimation heat stability of vaccines and evaluation of effect various stabilizers on stability of vaccines. There is a significant relationship between vaccine degradation rate calculated with $TCID_{50}$ and Real-Time PCR method ($p<0.05$). Titration methods based on cell culture are gold tests for titration of viral vaccines and estimation of heat stability but Real-Time PCR technique can also be used for this goals. This method is faster, cheaper and easier than $TCID_{50}$.

CONCLUSION

Real Time PCR methodcan be used for evaluation of live vaccine thermal stability. This technique is cheaper and faster than titration methods. Although$TCID_{50}$ and PFU are gold tests for estimation of vaccine heat stability, Real Time PCR technique is applicable for this goal.

ACKNOWLEDGEMENT

The authors would like to thank Dr Abdolhamid Shoushtari for material support.

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