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## Original Article

# Development of qRT-PCR Test for Quantification of Rubella Virus in Commercially Available Vaccines

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## Abstract

**Background and Aims:** Determination of virions in live vaccines, especially multivalent vaccines is considered as a critical point and in-process of quality control (IPQC) test of vaccine production. Rapid tests, like real time PCR, are more appropriate when the production occurs at industrial scale because of the amounts of starting materials and the excess of consumed time required. In the current study, a real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was developed for Rubella virus (RV) and tested on a commercially available MMR vaccine.

**Materials and Methods:** The primers and Taq Man probe were designed by Gene runner version 5.0.63 software. Concentrations, as well as reaction temperatures were optimized to establish an efficient qRT-PCR assay for RV RNA. A RV-specific PCR amplicon (109 bp), conserved in all species, was made as an external standard to evaluate the linearity, amplification efficiency, analytical sensitivity and reproducibility of the qRT-PCR.

**Results:** The real time quantitative assay was shown to have good linearity ($R^2$=0.998), high amplification efficiency (E=96.18%), and high sensitivity ($7 \times 10^2$ copies/7 µl) for tested vaccine.

**Conclusion:** The established qRT-PCR method is a simple, rapid, quantitative, highly specific and sensitive assay for quantification of RV RNA copy numbers in IPQC tests at industrial scale.

**Keywords:** Rubella Virus, Mumps Measles Rubella (MMR) Vaccine, Quantitative Real-Time reverse transcription-polymerase chain reaction

## Introduction

Rubella virus (RV) as a member of Rubivirus genus of Togaviridea family contains a positive RNA genome of 10-12 Kb. RV encodes five proteins, including three structural proteins, E1, E2, and the capsid (C) protein. E1 is a structural glycoprotein with neutralizing and hemagglutinating epitopes (1-2). Rubella is a childhood endemic disease with slight fever, lymphadenopathy and short-live rash symptoms. The most serious complication of postnatal rubella is encephalomyelitis (3). Most adult patients with mild rubella symptoms recover without any health problems.

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RV has destructive and teratogenic effects on developing human fetus when acquired by the non-immune mothers in early pregnancy (4-5). It may cause congenital rubella syndrome (CRS) which is a severe illness and is characterized by deafness, cataract, glaucoma, retinopathy, neonatal thrombocytopenia, purpura, hepatosplenomegaly and intrauterine growth retardation, cardiac complications and neurological abnormalities (6- 9 ). After RV isolation, it was cultured in cell lines in 1962 for the first time (10). In 1965 rubella vaccine was presented 5 years after the last major epidemic of rubella disease in USA. Attenuated vaccines produced by serial passages in a variety of cell lines giving rise to several vaccine strains like HPV77/DK, TO336 and so on. Wistar R27/3 strain was derived from a rubella infected fetus kidney and was attenuated by passaging four times in human embryonic kidney (HEK) cells followed by 17-25 passages in WI-38 fibroblast cells (11). Rubella attenuated vaccine was US-FDA approved in 1979. CRS was eliminated in most developed countries because of precise vaccination schedules (6). Nowadays, wide spread vaccination programs have reduced the incidence of the disease to the low levels in developing countries. It is necessary to evaluate the precise titer of virus at a short period of time with a high efficacy in each phase of attenuated vaccine production. Moreover, virus isolation is commonly performed for the laboratory confirmation of RV infection, but the sensitivity is low because of poor conditions of samples transportation and time consuming process of samples collection. It takes 3 to 4 weeks to obtain the results of virus isolation after three blind passages in RK13 or Vero cells. Serological tests like the hemagglutination inhibition method are performed basically with paired sera from only the acute and convalescent phases (12). Although, IgM-based ELISA is employed for diagnosis of rubella using a single serum sample, but false-positive results are frequently occurres similar to other virological tests(13-15). As well, plaque assay was given a precise estimation of lived RV. In recent years, some novel tests have been developed for detection of RV in biological fluids by some research laboratories (16-20). In this work we established a quantitative Real-Time reverse transcription-polymerase chain reaction (qRT-PCR) method for fast and précise estimation of the copy numbers of RV in attenuated MMR vaccine.

## Methods

### Preparation of Rubella Amplicon

**RNA extraction and cDNA synthesis.** One container of commercial trivalent vaccine was dissolved in 400 µL of distilled water and 200 µL was used for RNA extraction using high pure viral nucleic acid extraction kit (Roche Molecular Biochemicals, Mannheim, Germany). Fifty µL of extracted total RNA was applied for cDNA synthesis using the first strand cDNA synthesis kit for-PCR (Roche Molecular Biochemicals, Mannheim, Germany) and random hexamer primers. The PCR reaction master mix was prepared in 25 µl final reaction volume for each sample as follows: 5 µl cDNA, 2.5 µl buffer 10X, 0.5 µl $MgCl_2$ 100 mM, 0.5 µl, dNTP 100 mM, , 0.4 µl of Taq DNA polymerase and 0.5 µl of each primer (100 mM concentration). The cDNA was purified by Roche high pure PCR clean up micro kit. One set primer was designed to amplify a conserved region in all species of rubella virus as follow: reverse primer: 5-AATTGCGTGGAGTGCTGG-3; and forward primer: 5-AGTGCGCGATGTTGTTTCC-3) (Copenhagen A/S Symbion, Denmark). The PCR program was as: step 1: 1 cycle, 94°C, 5 minutes; step 2: 30 cycles, denaturation at 94°C for 30 second, annealing at 56 °C for 35 second, and extension at 72°C for 60 sec/1000 bp; step 3: final extension,1 cycle at 72°C for 10 minutes. The PCR product was visualized and analyzed on 1% (w/v) agarose (Fermentas, Lithuania) gel by electrophoresis (Bio-Rad-USA).

**Cloning of Rubella Amplicon into *pTZ57R/T* cloning vector.** The PCR product was purified by high pure PCR clean up micro kit (Roche Molecular Biochemicals, Mannheim, Germany) and then cloned into a *pTZ57R/T* cloning vector (Fermentas, Lithuania). The

ligation reaction was prepared as follow; 3 µl 10X buffer, 9 µl PCR product, 3 µl *pTZ57R/T* cloning vector, 1.5µl T4 DNA ligase, 3µl Polyethylen glycol and 10.5µl distilled water. Simultaneously, competent cells were prepared by culturing a colony of *E.coli* Top10 in 5 ml of LB broth (Merck, Germany) containing tetracycline (50 µg/ml) at 37°C with shaking at 200 rpm, until OD600 was reached to 0.5. The culture was then centrifuged at 3500 rpm for 5 minutes at 4°C and the pellet was immediately dissolved in 1ml ice-cold CaCl2 (0.1M) and incubated on ice for 60 minutes. The tube contents were then centrifuged and the bacterial pellet was dissolved in 700µl ice-cold $CaCl_2$ for 30 minutes followed by centrifugation. Finally, the pellet was dissolved in 300 µl ice-cold $CaCl_2$ and incubated on ice for 15 minutes. The competent cells produced freshly were then divided into three tubes each of 100µl. One tube of competent cells was set as a negative control with no DNA. The second tube was used as the positive control with circular plasmid without an insert and to the third tube, 10 µl of the ligation reaction was added. All tubes were incubated on ice for 30 minutes followed by keeping at 37°C for 5 minutes and finally transferred on ice. After performing the heat shock, 1ml of Luria Broth medium without antibiotic was added to each tube and incubated at 37°C for 60 minutes. All tubes were centrifuged at 3500 rpm for 5 minutes and after removing 600µl of supernatant, the pellet was dissolved in remaining medium and then spread out on Luria Broth-agar plates containing antibiotic (Ampicillin, 50µg/ml) and incubated at 37°C overnight. Obtained colonies were picked up and used as a template in the colony PCR. First, 8 colonies were separately heated at 94°C for 5 minutes to destroy the bacterial cell walls resulted in release of plasmids which can be used as the template in the final PCR reaction. Positive colonies were subjected to plasmid extraction using Gene Jet TM Plasmid Miniprep Kit (Fermentas, Lithuania). The extracted plasmids were visualized on 1% agarose gel by electrophoresis (Bio-Rad, USA). Finally, 160 ng/µl of extracted plasmid was sent to Copenhagen A/S Symbion, Denmark for DNA sequencing. The plasmid DNA containing Rubella Amplicon (pRA) was used as standard for qRT-PCR.

**Preparation of test samples**

RNA was extracted from one vial of Rubella vaccine ($10^4$ copy numbers). The content of the vial was divided into two 200 µl parts and individually RNA-extracted using a high pure viral nucleic acid extraction kit (Roche Molecular Biochemicals, Mannheim, Germany). The elution of each RNA extract had 50 µl in volume ($5\times10^3$ copy numbers). 25µl of the solutions, containing $2.5\times10^3$ copy numbers of virus, was used for cDNA synthesis using the first strand cDNA synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). 7µl of each synthesized cDNA was employed as unknown samples for qRT-PCR reaction.

**Evaluation of test samples using qRT-PCR**

The plasmid DNA containing Rubella Amplicon (pRA) was used to determine the primer specificities and to optimize real-time PCR reaction conditions by generating a standard curve. Briefly, ten-fold serial dilutions of pRA were made ranging from $2\times10^3$ to $2\times10^7$ copies. The instrumentation and general principles of the one step real-time PCR system have been described in details in the operator's manual (Applied Biosystems, Foster City, CA). PCR Amplification was carried out in 48 well plates with cohesive caps. The final reaction volume was 20µl consisting of 10µl SYBR Green, PCR master mix (Applied Biosystems, Foster City, CA), 400nM of each specific primer, and 7µl of cDNA template. For each run, standard plasmids (serial dilutions of pRA), test samples and no template were all assayed in duplicate. The reaction conditions were 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 56°C. All experiments were repeated at least twice for reproducibility.

## Results

**Preparation and cloning of Rubella Amplicon**

**RNA extraction and cDNA synthesis.** After RNA extraction from trivalent vaccine and

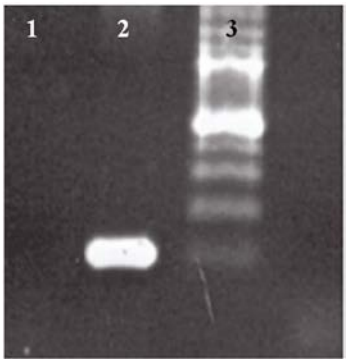


**Fig. 1.** PCR on Rubella cDNA constructed from trivalent MMR vaccine. Lane 1: Negative control (Distilled water as template), Lane 2: PCR product (109 bp), and Lane 3: 1 Kb DNA ladder (Fermentas, Lithuania).

cDNA synthesis, an RT-PCR was performed and the PCR product observed as a 109 bp band on a 1% agarose gel (Figure 1).

**Cloning of Rubella Amplicon.** After transforming bacteria with the ligation mixture, the bacteria were cultured on LB agar plate containing ampicillin. Only those colonies which grew on agar plate selected for colony PCR analysis.

Eight colonies were used as templates in each reaction (Figure 2).

The PCR products were then purified and sequenced and compaed with the whole gene sequence of Rubella in the GenBank (GenBank ID: FJ211588.1).

Finally, the cycle threshold (CT) values for the dilutions were plotted against concentrations. The slope of the linear graph is used to calculate the regression coefficient. Linear regression analysis was shown an excellent correlation coefficient ($R^2$=0.998) (Figure 3).

The RV copy numbers measured by the real time PCR assay ranged from $2\times10^7$ to $2\times10^3$ copies in samples (see C1-C5, Table 1). In addition, the unknown samples were found to be consisting of 693 and 658 copies of Rubella virus (C6 and C7, Table 1).

## Discussion

The selection of a diagnostic test depends on different parameters consisting of high sensitivity, high specificity, low price, simple procedure, less time consuming and adaptability to automation (14). qRT-PCR was

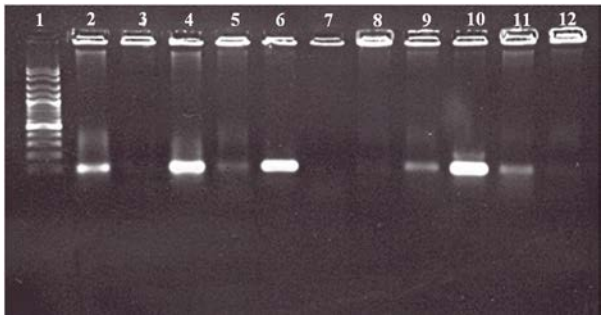


**Fig. 2.** Colony PCR analysis. Lane 1: 1 Kb DNA ladder (Fermentas, Lithuania), Lanes 2, 4, 5, 6, 9,10 and 11: Positive colonies (displayed an 109 bp band), and lanes 3, 7, 8, and 12: Negative colonies.

performed for virus titration would be expected to be a reliable and rapid diagnostic method in clinical applications, because it is known to be superior for detection of viral copies without virus isolation from samples. The qRT-PCR procedure has many advantages such as simplicity and rapidity, in comparison with virus isolation and plaque assays. Virus isolation requires lengthy and complex procedures including cell culture, which is not always successful, and is not used as an appropriate clinical laboratory diagnostic method. The genome amplification method

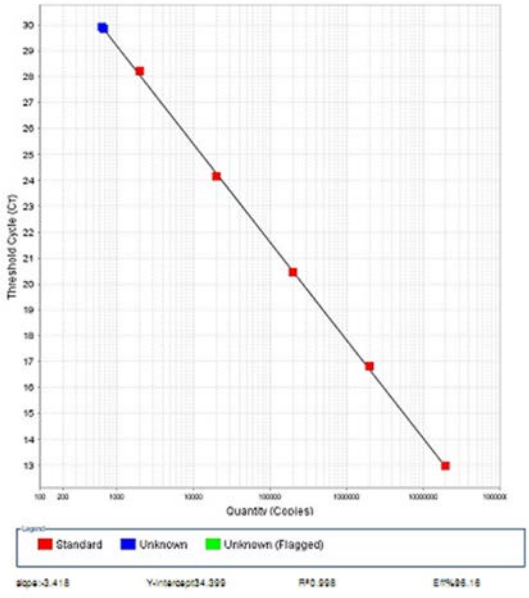


**Fig. 3.** The standard curve was depicted for $2\times10^7$ to $2\times10^3$ copies of pRA plasmids (red points). The copy number of two unknown samples could calculate by standard curve (blue points).

**Table 1:** The results of qRT-PCR for serially diluted of standards (C1-C5) and unknown samples (C6, C7). As seen, the unknown samples consist of 693 and 658 copies of virus.

| Sample | Target | Task | Quantity | CT | Tm1 |
|---|---|---|---|---|---|
| C1 | Target 1 | Standard | 20,000,000 | 12,9818 | 82.5679 |
| C2 | Target 1 | Standard | 2,000,000, | 16,8080 | 82.2697 |
| C3 | Target 1 | Standard | 200,000 | 20,4599 | 82.2697 |
| C4 | Target 1 | Standard | 20,000 | 24,1606 | 82.1206 |
| C5 | Target 1 | Standard | 2,000 | 28,2299 | 82.1206 |
| C6 | Target 1 | Unknown | 693,0246 | 29,8400 | 81.2261 |
| C7 | Target 1 | Unknown | 657,9828 | 29,9253 | 81.3752 |

always has the possibility of false positives due to cross-contamination (21), while, qRT-PCR is performed in a closed system without post treatment, therefore, the risk of any RNA contamination is substantially reduced. The qRT-PCR is not only accurate but is also faster than the old clinical diagnostic methods (22). There is also no risk of contamination in this method and its sensitivity and specificity for the RNA determination in clinical specimens is high enough for accurate quantification (23, 24). In this study, specific primers were designed, and PCR conditions were defined for efficient amplification and quantification of Rubella virus RNA. A standard amplicon of a highly conserved sequence of Rubella virus with 109bp was amplified using a conventional PCR protocol and confirmed by alignment with the sequence of Wistar RA27/3 RV strain successfully. The specific primers were reacted only with Rubella virus and the detection limitation was calculated as 700 copies in 20µl in each well of qRT-PCR plate. The qRT-PCR assay established in this study showed to have a good linearity ($R^2$=0.998), a high efficiency (96.18%), a high sensitivity (700 copies/20 µl). In conclusion, developed qRT-PCR in the current study is a simple, less time-consuming, and highly sensitive and specific. It also requires less manipulation for quantification of Rubella virus RNA. This study confirmed that our designed qRT-PCR experiment is a well appealing technique in the field of research and development (R&D) in biopharmaceutical industries as well as IPQC tests for vaccine manufacturing.

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The authors declare no conflict of interest.

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