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Establishment and Application of a TaqMan Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction Assay for *Rubella Virus* RNA

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Abstract The aim of this study was to establish and apply a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) for *rubella virus* (*RV*) RNA. First, the primer and TaqMan probe concentrations, as well as reaction temperatures were optimized to establish an efficient real-time quantitative RT-PCR assay for *RV* RNA. Next, an *RV*-specific PCR amplicon was made as an external standard to estimate the linearity, amplification efficiency, analytical sensitivity and reproducibility of the real time quantitative assay. Finally, the assay was applied to quantify *RV* RNA in clinical samples for rubella diagnosis. The *RV*-specific PCR amplicon was prepared for evaluation of the assay at 503 bp, and its original concentration was 2.75×10^9 copies/μl. The real time quantitative assay was shown to have good linearity (R^2=0.9920), high amplification efficiency (E=1.91), high sensitivity (275 copies/ml), and high reproducibility (variation coefficient range, from 1.25% to 3.58%). Compared with the gold standard, the specificity and sensitivity of the assay in clinical samples was 96.4% and 86.4%, respectively. Therefore, the established quantitative RT-PCR method is a simple, rapid, less-labored, quantitative, highly specific and sensitive assay for *RV* RNA.

Key words *rubella virus*; PCR amplicon; real-time fluorescence quantitative RT-PCR

Rubella virus (*RV*) is a positive-sense RNA genome virus within the family *Togaviridae*. *RV* can cause a disease known as rubella. Primary infection occurs in the upper respiratory mucosa or the nasopharyngeal lymphoid tissue, followed by a systemic infection. Infection with *RV* can cause quite severe complications such as subacute sclerosing panencephalitis (SSPE), which may lead to death. More importantly, if rubella occurs during the first trimester of pregnancy, there is a 90% risk of congenital malformations in the fetus [1]. Persistent fetal infection may result in a series of birth defects known as the congenital rubella syndrome (CRS) [2]. Therefore prevention of CRS is the main goal of rubella immunization, which can be achieved by using the live attenuated rubella vaccine for women of childbearing age [3]. At present, there is a trend of decrease in the use of measles-mump-rubella (MMR) vaccine, which means rubella could spread again with serious potential hazards for susceptible pregnant women. This is of particular concern in areas where vaccine coverage has remained low or where there are significant numbers of immigrants from countries without rubella vaccination programs [4].

Currently, laboratory diagnostics of *RV* are mainly based on serological techniques. Enzyme-linked immunosorbent assay (ELISA) for detecting specific immunoglobulin M (IgM) to the *rubella virus* is used widely for diagnosis of rubella. But anti-rubella virus IgM could only be detected at least 5 days after rubella onset. Moreover, false-positive IgM results may occur due to interference by rheumatoid factor, parvoviral infections, or some other viral agents such as Epstein-Barr virus and measles virus [5,6]. This issue of false positive results is especially important when

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detecting suspected rubella in pregnant women because of the risk of CRS, so a more specific diagnostic assay should be used in such situations [7]. Real-time fluorescence quantitative reverse transcription-polymerase chain reaction (RT-PCR) has recently been widely accepted as a valuable technique for the specific detection and quantification of RNA targets [8–10].

From March to May in 2006, there was a rubella outbreak in Shandong Province, China. In this study we have established a quantitative RT-PCR assay for *RV* RNA to diagnose rubella infection as early as possible in order to reduce the risk of the infection of pregnant women. We prepared an *RV*-specific PCR amplicon as an external standard to analytically assess the assay.

Materials and Methods

Gold standard for rubella diagnosis, rubella cases and clinical samples

According to the Diagnostic Criteria and Principles of Management for Rubella (GB17009-1997) advanced and approved by the Ministry of Health of China, the presence of IgM antibody in a serum sample, accompanied by the symptoms such as fever and red maculopapular rash which begins on the face and spreads through the whole body, serves as gold standard for rubella diagnosis.

There were a total of 22 rubella patients hospitalized in Qilu hospital during the outbreak. Clinically, the patients exhibited prodromal symptoms such as fever and malaise followed by a red maculopapular rash, which started on the face and then progressed down to the whole body. Posterior auricular lymphadenopathy was characteristic. The *RV* infection was confirmed serologically (IgM antibody in serum sample was positive). Among the 22 rubella patients, 12 cases were male and 10 were female. The average age was 20 years old (from 2 to 34 years). Nasopharyngeal swabs were collected from rubella cases within a week after confirmed diagnosis with permission. Twenty-eight healthy persons were sampled as the control with permission. The collected nasopharyngeal swabs were stored in individual transport glass tubes at –20 ºC. All nasopharyngeal swabs collected from 22 rubella patients and 28 healthy persons were carried out in a single run by the established quantitative RT-PCR assay.

Total RNA isolation, quantification of total RNA and cDNA synthesis

One milliliter of sterilized 0.9% sodium chloride was added to each transport glass tube containing the nasopharyngeal swabs. The submersed nasopharyngeal swabs were well shaken and squeezed. All liquid samples in glass tubes were transferred to the centrifugation tubes, and centrifugated at 10,000 *g* for 5 min. The total RNA was then extracted from the pellets using UNIQ-10 centrifugal column total RNA extraction and purification kit (Sangon, Shanghai, China). Extracted total RNA was finally eluted in 50 μl of RNase-free water and stored at –20 ºC. Quantification of total RNA was carried out by measurement of A_{260} using an RS232C BioPhotometer (Eppendorf, Hamburg, Germany). The range of concentrations was from 1.0 to 2.6 μg/ml. The range of amounts of all RNA templates was from 0.05 to 0.13 μg. cDNA was synthesized by reverse transcription (RT). RT mixture was composed of 5.0 μl of RNA template, 4.0 μl of 5×RT buffer, 1.0 μl of AMV reverse transcriptase XL (5 U/μl; TaKaRa, Dalian, China), 4.0 μl of dNTP mixture (each 2.5 mM), 0.5 μl of RNase inhibitor (40 U/μl), 1.0 μl of Random 9 Primer (50 μM), and 4.5 μl of sterilized DEPC water added to a final volume of 20 μl. RT mixture was incubated at 30 ºC for 10 min, 42 ºC for 60 min, 99 ºC for 5 min, 5 ºC for 5 min in a T-1 Thermal Cycler (Biometra, Göttingen, Germany).

Preparation of the *RV* RNA standard

To evaluate the real-time quantitative RT-PCR, we prepared a *RV*-specific PCR amplicon as a standard for the assay in the laboratory. Primers were designed from the highly conserved region of the *RV JR23 E1* gene (GenBank accession No. DQ255946) isolated by the laboratory [11]. The forward primer was 5′-ATGCGTCCG-CTTTGAGTC-3′, and the reverse primer was 5′-TATGTC-CGTGCGGCGTGTTAG-3′. Primers were chemically synthesized by Invitrogen (Shanghai, China). The *RV* amplicon was made by conventional PCR using E1-containing plasmid pBluscriptII SK-E1 constructed by the laboratory [12] as a template in the T-1 Thermal Cycler. The PCR mixtures were composed of 1.0 μl of each primer (25 μM), 0.5 μl of *Ex-Taq* (5 U/μl) (TaKaRa), 5 μl of 10×*LA* PCR buffer II (Mg^{2+} free), 5 μl of $MgCl_2$ (25 mM), 8 μl of dNTP mixtures (each 2.5 mM), 10 μl of pBluscriptII SK-E1 template, and 19.5 μl of the distilled water added to a final volume of 50 μl. The *RV* amplicon cDNA was amplified by conventional PCR for 35 cycles at 93 ºC for 45 s, 55 ºC for 1 min and 72 ºC for 3 min. The final extension step was conducted for 5 min at 72 ºC. Plasmid pET30a(+) DNA was used as a negative control. The PCR products were analyzed by 1% agarose gel electrophoresis. Then, a significant amount of the amplicon, produced by many

PCR reactions was purified by an EZNA spin gel extraction kit (Omega, Doraville, USA). Purified *RV* PCR amplicon was quantified by the *RV* fluorescence PCR diagnostic kit (Daan Gene Diagnosis Inc., Guangzhou, China) on ABI Prism 7300 (Applied Biosystems, Foster City, USA).

A real time quantitative RT-PCR assay for *RV* RNA

The same primers used in the conventional PCR were also used in the real time quantitative RT-PCR. TaqMan probe was designed near the forward primer between the forward and reverse primer [13,14]. The sequence of the probe was 5′-GATTGTGGACGGCGGCT-3′. TaqMan probe was chemically synthesized by Invitrogen. Ten microliters of sample cDNA was added to PCR mixture. PCR mixture was composed of 1.0 μl of each primer (25 μM), 1.0 μl of TaqMan probe (25 μM), 0.5 μl of *Ex-Taq* (5 U/μl), 5 μl of 10×*LA* PCR buffer II (Mg^{2+} free), 5 μl of $MgCl_2$ (25 mM), 8 μl of dNTP mixtures (each 2.5 mM), and 18.5 μl of the distilled water added to a final volume of 50 μl. Real time quantitative RT-PCR was performed on ABI Prism 7300 for 30 cycles at 93 ºC for denaturation for 45 s, 55 ºC for annealing for 1 min and 72 ºC for extension for 3 min. For quantification, a series of ten-fold dilutions from 1×10^7 to 1×10^2 of *RV* amplicon standard (prepared as described above) and negative control (distilled water) were run alongside on ABI Prism 7300.

Statistical analysis

Linear regression was used to analyze the data obtained from the standard curve of quantitative RT-PCR in this research. The assessing parameters including amplification efficiency, specificity, sensitivity and reproducibility were analyzed by SPSS 10.0 software (SPSS Inc., Chicago, USA).

Results

RV gene specific PCR amplicon standard

An *RV* gene specific PCR amplicon standard was prepared in the laboratory. The *RV* amplicon was made by conventional PCR. In the electrophoresis gel, the *RV* amplicon of proper size (503 bp) was acquired as shown in **Fig. 1**. Then the amplicon produced by many PCR reactions was purified and quantified. The purified *RV* PCR amplicon was measured in triplicate on ABI Prism 7300. The average concentration was 2.75×10^9 copies/μl ($2.70–2.80\times10^9$ copies/μl). The above-quantified *RV* stock amplicon solution and a dilution of 2.75×10^9 copies/ml used throughout the study as an external standard were then both kept at –20 ºC.

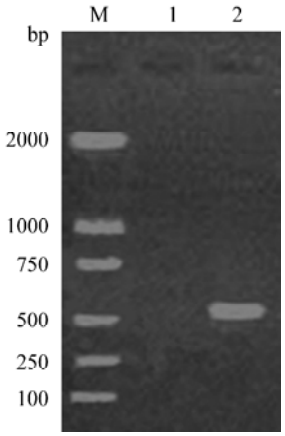

Fig. 1 RV amplicon standard was electrophoresed in 1.0% agarose gel

M, DNA marker DL2000 ladder; 1, negative control (distilled water); 2, expected size of 503 bp.

Linearity, amplification efficiency and detection limit of the TaqMan real-time quantitative RT-PCR assay

First, the primers and TaqMan probe concentrations, as well as reaction temperatures were optimized to establish an efficient real-time quantitative RT-PCR assay for *RV* RNA. The TaqMan quantitative assay was carried out on a series of ten-fold dilutions from 1×10^7 to 1×10^2 of RV amplicon standard prepared as described above in duplicate. The dynamic amplification curve (**Fig. 2**) and the standard curve (**Fig. 3**) were generated automatically. The standard curve showed the linear relationship between the log of the concentration of target RNA and the cycle threshold (Ct) value. The correlation coefficient of the standard curve was 0.9960, indicating a precise log-linear relationship between the concentration of target RNA and the Ct value. The regression equation was as **Equation 1**:

$$y=-3.578x+29.448 \quad (1)$$

Based on conventional quantitative RT-PCR theory [15, 16], the regression coefficient and the PCR amplification efficiency are related according to **Equation 2**:

$$b=-(1/\lg E) \quad (2)$$

where E is efficiency of PCR reaction, b is regression coefficient (slope of the standard curve). According to the equation, the PCR amplification efficiency calculated was 1.91. The detection limit of the assay was determined

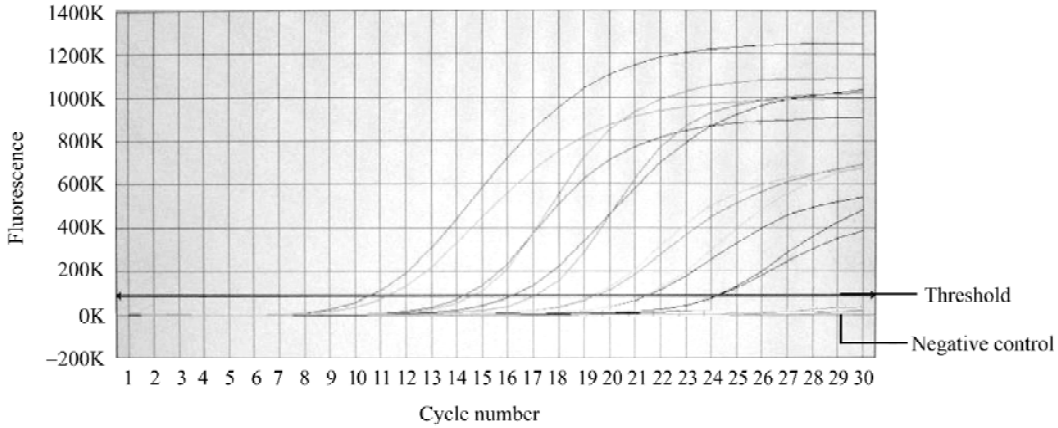

Fig. 2 A rubella quantitative RT-PCR assay performed on serial dilutions from 1×10^7 to 1×10^2 of *RV* amplicon standard
The curves from left to right correspond to 2.75×10^7, 2.75×10^6, 2.75×10^5, 2.75×10^4, 2.75×10^3, 2.75×10^2 copies/ml, respectively.

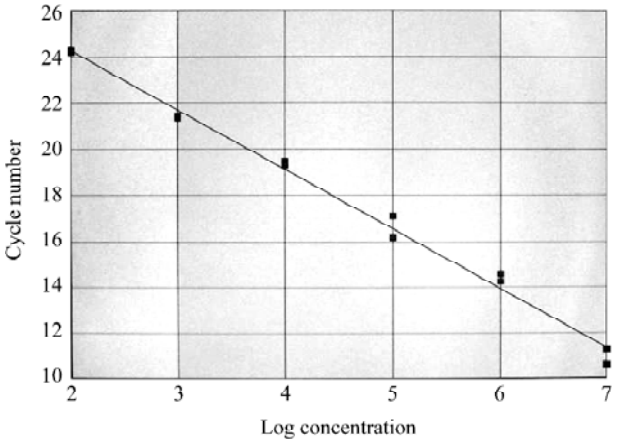

Fig. 3 The linear relationship between log concentration and Ct values for a series of dilutions from 1×10^7 to 1×10^2 of RV amplicon standard (R^2=0.9920)
Each dilution of RV amplicon standard was assayed in duplicate by quantitative RT-PCR.

by performing a series of dilutions of *RV* amplicon standard for five repeats. The detection limit of the quantitative RT-PCR assay was 275 copies/ml (**Table 1**).

The reproducibility of the quantitative RT-PCR assay

The quantitative RT-PCR assay was performed on a series of dilutions from 1×10^7 to 1×10^2 of *RV* amplicon standard. Each dilution was performed three times to examine its reproducibility. The variation coefficient range was from 1.25% to 3.58% (**Table 2**).

Application of the real-time quantitative RT-PCR assay

The real-time quantitative RT-PCR assay was applied to detect *RV* RNA in nasopharyngeal swab specimens collected from 22 rubella cases. The quantification of *RV* RNA showed that 19 cases were positive and three cases were falsely negative (**Fig. 4**). Viral loads of the 19 positive cases ranged from 6.50×10^3 to 2.04×10^8 copies/μg of the RNA sample. Among the 28 nasopharyngeal swab specimens collected from healthy persons in the control group, one case was found falsely positive. Therefore the specificity and sensitivity of TaqMan quantitative assay was 96.4% and 86.4% respectively, compared with the gold standard (**Table 3**).

It was probable that the nasopharyngeal swab specimens from the three rubella cases were negative due to the inhibition of the RT-PCR inhibitors. The inhibitors of the RT-PCR negatively affect PCR amplification efficiency during template preparation. Common inhibitors include various materials such as components of body fluids, organic and phenolic compounds, DNA fragmentation [17],

Table 1 Detection limit of the TaqMan *RV* quantitative assay using a series of dilutions of RV amplicon standard

RV amplicon standard (copies/ml)	Number of positive samples/ number of tested sample	Positive (%)
575	5/5	100%
500	5/5	100%
425	5/5	100%
350	5/5	100%
275	5/5	100%
200	1/5	20%
125	0/5	0%

Table 2 The reproducibility of the quantitative RT-PCR for detection of RV

Concentration of *RV* amplicon standards (copies/ml)	Values of Ct[a]			Mean	SD[b]	CV[c] (%)
	1	2	3			
2.75×10^7	11.98	11.96	11.03	11.66	0.417	3.58
2.75×10^6	14.05	14.99	14.04	14.36	0.420	2.92
2.75×10^5	17.02	17.01	17.99	17.34	0.434	2.51
2.75×10^4	19.99	19.26	19.55	19.60	0.260	1.33
2.75×10^3	21.22	21.68	21.82	21.57	0.270	1.25
2.75×10^2	24.97	24.08	24.05	24.37	0.403	1.66

Ct[a]: cycle threshold; SD[b]: standard deviation; CV[c]: coefficient of variation.

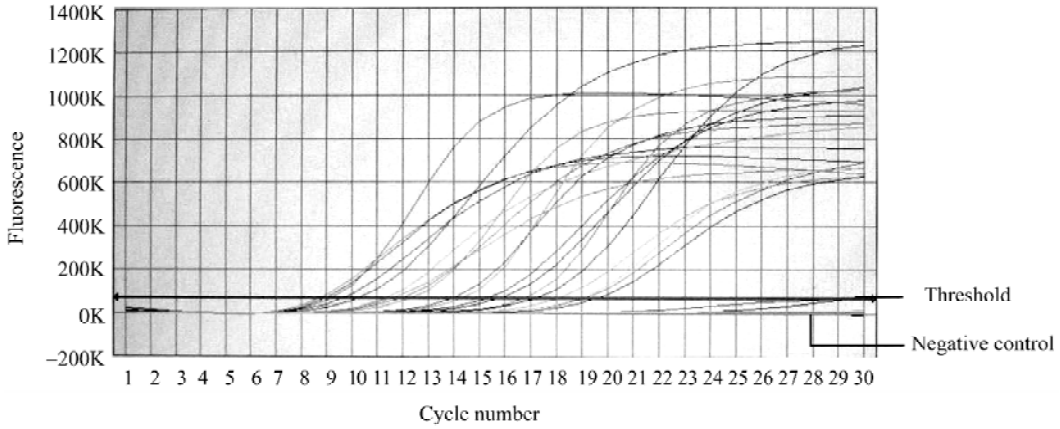

Fig. 4 Fluorescence curves in 22 cases of nasopharyngeal swabs from rubella patients

Table 3 The specificity and sensitivity of the real-time quantitative RT-PCR assay for *RV* RNA

Assay		Gold standard		Sum total
		+	–	
qRT-PCR	+	19	1	20
	–	3	27	30
Sum total		22	28	50

and anticoagulant heparin [18]. In addition, laboratory plastics have been identified as a potential source of PCR inhibitor [19]. Currently, the use of internal controls in every specimen allows false-negative results due to the PCR inhibition to be tightly monitored [20].

Discussion

The selection of a diagnostic test relies on multiple parameters including sensitivity, specificity, cost, ease-of-use, turn-around time, availability and adaptability to automation [7]. When a monolayer cell culture assay has been used to detect *RV* in clinical samples, there is wasted effort in preparing excess monolayer cell cultures. Moreover, monolayer cell culture assay has potential cross-infection from one specimen to another [21]. Real time PCR is carried out in a closed system without PCR post treatment (no gel analysis is needed), so the risk of contamination is substantially reduced. The real-time quantitative RT-PCR assay outperforms previous clinical diagnostics not only because of its speed, lower contamination risk, and ability for quantification but also because of its higher sensitivity and specificity for the RNA determination of clinical specimens [22].

In this study, primers and TaqMan probes were designed, and PCR conditions were defined for efficient amplification and quantitation of *RV* RNA. An *RV* amplicon standard of a 503-bp highly conserved sequence from *RV E1* gene was made by conventional PCR to estimate analytically the quantitative RT-PCR assay. The quantitative RT-PCR assay established in this study was shown to have

a good linearity (R^2=0.9920), a high PCR amplification efficiency (1.91), a high sensitivity (275 copies/ml), and a good reproducibility (variation coefficient range from 1. 25% to 3.58%). In this study, we used a relatively stable *RV* amplicon cDNA as an external standard. A positive RNA standard sample allows the RT part of the reaction to be controlled in each TaqMan run, so an RNA standard would be more desirable. Compared with the gold standard, the specificity and sensitivity of the assay for detection of the *RV* RNA in clinical samples were 96.4% and 86.4%, respectively, which were close to those of the quantitative RT-PCR for detection of other RNA virus in clinical specimens [22]. Therefore this assay is a technically simple, less time-consuming, less handling, highly specific and highly sensitive method for quantification of *RV* RNA. This study confirmed that real time TaqMan quantitative RT-PCR is one of the most appealing techniques in the field of basic research and molecular laboratory diagnosis for quantification of nucleic acids [23].

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