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► **To cite this version:**

Yan Feng, Sabine Santibanez, Hazel Appleton, Yiyu Lu, Li Jin. Application of new assays for rapid confirmation and genotyping of isolates of rubella virus. *Journal of Medical Virology*, 2010, 83 (1), pp.170. 10.1002/jmv.21941 . hal-00599786

**HAL Id: hal-00599786**

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Submitted on 11 Jun 2011

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### Application of new assays for rapid confirmation and genotyping of isolates of rubella virus

Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-10-1807.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	28-Jul-2010
Complete List of Authors:	Feng, Yan; Centre for Disease Control and Prevention of Zhejiang Province; Centre for Infections, Health Protection Agency Centre for Infections, Health Protection Agency Santibanez, Sabine; Robert Koch-Institut Appleton, Hazel; Centre for Infections, Health protection Agency Lu, Yiyu; Centre for Disease Control and Prevention of Zhejiang Province Jin, Li; Health protection Agency, Virus Reference Department
Keywords:	rubella virus isolates, PCR assays, genotyping

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Manuscripts

JMV-10-1807

**Application of new assays for rapid confirmation and genotyping of isolates of rubella virus**

**Yan Feng<sup>1,2</sup>, Sabine Santibanez<sup>3</sup>, Hazel Appleton<sup>2</sup>, Yiyu Lu<sup>1\*</sup>, Li Jin<sup>2\*</sup>**

<sup>1</sup>Institute of Viral Diseases, Centre for Disease Control and Prevention of Zhejiang Province, Hangzhou, China

<sup>2</sup>Virus Reference Department, Centre for Infections, Health Protection Agency, London, UK

<sup>3</sup>National Reference Centre for Measles, Mumps and Rubella, Department of Viral Infections, Robert Koch-Institut, Berlin, Germany

\*Correspondence to

Dr. Li Jin, Virus Reference Department, Centre for Infections, Health Protection Agency, 61Colindale Avenue, London NW9 5EQ, UK. E-mail: [li.jin@hpa.org.uk](mailto:li.jin@hpa.org.uk)

Dr. Yiyu Lu, Institute of Viral Diseases, Centre for Disease Control and Prevention of Zhejiang Province, 630 Xin Cheng Road, Hangzhou 310051, China.

E-mail: [luyiyuzjh@yahoo.com.cn](mailto:luyiyuzjh@yahoo.com.cn)

**Abstract**

Rubella virus (RV) isolation is recommended by the WHO Measles and Rubella Labnet for studying the aetiology and epidemiology of rubella. However, the absence of cytopathologic effects (CPE) in many of the cell lines used commonly makes it difficult to confirm RV growth. In this study, two assays amplifying RV cDNA were developed and validated in order to confirm and genotype RV isolates after cell culture. A SYBR Green I-based real-time PCR (Rtime-SGE317) was established for initial rapid detection of RV in Vero cells and a nested PCR (PCR-E860) was used for amplifying further the 739nt window of the E1 gene for identification of RV genotype as recommended by the WHO. Sensitivities of the two assays were evaluated using eight RV isolates, two from infants with the congenital rubella syndrome (CRS) and six from patients with acute rubella. All the isolates had cycle threshold (Ct) values <37 after the third passage, which is recommended as the cut-off for confirmation of a viable RV isolate. Phylogenetic analysis based on the 739nt window generated by the PCR-E860 showed that the eight RV isolates belonged to genotypes 1E, 1G and 2B. The Rtime-SGE317 assay can be carried out in local public health laboratories, which would extend the molecular surveillance of rubella and contribute to the WHO goal of eradicating rubella worldwide.

**KEY WORDS:** rubella virus isolates; PCR assays; genotyping

## 1 INTRODUCTION

2 Rubella is a mild, sub-clinical disease with few complications, but infection  
3 during early pregnancy can lead to severe birth defects known as congenital  
4 rubella syndrome (CRS) [Best and Enders, 2007; WHO,2000]. Since the  
5 introduction of rubella vaccination, the incidence of rubella has been reduced  
6 greatly and CRS is very rare in countries, such as the United Kingdom and the  
7 United States of America, which have established rubella vaccination  
8 programmes [WHO, 2000]. In 2007, 975 suspected cases of CRS were reported  
9 from 34 countries in the western hemisphere where CRS surveillance has been  
10 established [WHO, 2008]. The World Health Organization (WHO) has estimated  
11 that more than 100,000 cases of CRS occur in developing countries every year  
12 [WHO, 2000]; this represents a considerable social and economic burden.

13 Rubella is regarded as another target for eradication in countries where  
14 transmission of measles virus has already been interrupted [WHO, 2000].

15  
16 Rubella virus (RV), the sole member of the genus Rubivirus of the family  
17 Togaviridae, is a positive single stranded RNA virus [Chantler et al., 2001]. The  
18 viral genome encodes five proteins, two non-structural proteins (P90 and P150)  
19 and three structural proteins (E1, E2 and the capsid protein). Major neutralizing  
20 and haemagglutinating epitopes and T cell recognized sites are located in the  
21 structural proteins [Chantler et al., 2001; Cordoba et al., 2000]. The high G+C  
22 content, of up to 65%, is the most significant characteristic of the viral genome.,

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24 Clinical diagnosis of rubella is unreliable and therefore laboratory confirmation is  
25 necessary, particularly for the diagnosis of rubella-like illness during pregnancy.

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3 1 Serological diagnosis, by the detection of rubella-specific immunoglobulin M  
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5 2 (IgM) in a single serum or oral fluid sample, is the common method for  
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7 3 confirming rubella cases, and has proved a good indicator for demonstrating  
8  
9 4 recently acquired infection [Banatvala and Brown, 2004]. Prenatal diagnosis of  
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11 5 rubella infection based on rubella specific IgM alone is unreliable until at least 22  
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13 6 weeks gestation [Enders and Jonatha, 1987].  
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20 8 Virus isolation is a conventional laboratory method, and important for monitoring  
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22 9 virus excretion in CRS infants. Although virus isolation is time-consuming,  
23  
24 10 labour-intensive and not particularly sensitive, it is still recommended by the  
25  
26 11 WHO for studying the aetiology and epidemiology of rubella [WHO, 2007]. RV  
27  
28 12 can be cultured in a variety of cell lines, such as RK13, SIRC, BHK and Vero  
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30 13 (Vero/SLAM) cells, but serial propagation can be established only in a limited  
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32 14 number of cell lines [Figueiredo et al., 2004]. Previous studies have shown that  
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34 15 specific CPE may appear in RK13 cells under controlled conditions, or in RK13  
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36 16 and SIRC cells after one or two passages in Vero cells [Figueiredo et al., 2000;  
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38 17 Best et al., 2005].  
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46 19 The immunofluorescence assay (IFA) is a conventional method for confirming  
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48 20 rubella growth, but it is time-consuming, relies on the operator's interpretation  
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50 21 and requires a fluorescence microscope. Recently, the reverse-transcriptase  
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52 22 polymerase chain reaction (RT-PCR) followed by hybridisation assays, the  
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54 23 replicons-based method and the indirect immunocolorimetric assay (ICA) have  
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56 24 been developed to detect rubella virus infected cells [Zhu, et al. 2007; Chen, et  
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58 25 al. 2007].  
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6 2 Genetic characterization of virus isolates has relied on nested RT-PCR assays to  
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8 3 generate the 739nt region of the E1 gene that is required for genotyping in  
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10 4 accordance with the recommendations of the WHO [WHO, 2005]. In this study, a  
11  
12 5 SYBR Green I-based real-time PCR assay (Rtime-SGE317) was developed,  
13  
14 6 initially at Health Protection Agency in the UK (WHO Rubella Strain Bank), and  
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16 7 applied subsequently to rapid confirmation of RV isolates by testing samples of  
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18 8 tissue culture fluid from Vero cells at the Centre for Disease Control and  
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20 9 Prevention of Zhejiang Province, China. Compared with other methods, namely  
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22 10 IFA and electron microscopy (EM), the Rtime-SGE317 can be carried out easily  
23  
24 11 at local public health laboratories. The nested PCR (PCR-E860), developed  
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26 12 initially at the Robert Koch-Institut, was used both at the Health Protection  
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28 13 Agency and the Centre for Disease Control and Prevention of Zhejiang Province  
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30 14 to generate the required E1-739nt region from eight confirmed RV isolates for  
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32 15 genotyping. By combining the two assays, RV isolates could be confirmed and  
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34 16 genotyped very quickly. These two tests offer the opportunity of extending the  
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36 17 molecular surveillance of rubella and contributing to the WHO goal of eradicating  
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38 18 rubella worldwide.  
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## **MATERIALS AND METHODS**

### **Specimens**

Eight RV isolates were investigated in the study. Two (H1 and U6) were isolated from nasal and throat swab specimens of two Sudanese infants with serologically confirmed CRS [Omer et al., 2010]. These two isolates were also used for comparing the PCR assays developed in this study with other detection methods, EM and IFA (Table II). The other six (ZJ1~ZJ6) were isolated originally at the Centre for Disease Control and Prevention of Zhejiang Province, China between 2005 and 2008 from throat swab specimens collected from patients with acute rubella confirmed serologically.

### **Virus isolation and blind passages**

Vero cells were seeded at a concentration of  $5 \times 10^5$  cells/ml in glass tubes. Growth medium (GM) was Dulbecco's MEM (DMEM) containing 5% foetal bovine serum (FBS), 50µg/ml gentamicin and an additional 200mM L-glutamine. Cells were incubated at 37°C. Once a monolayer of cells had formed, the GM was removed and the cells inoculated with 100µl clinical specimen and left for one hour at 37°C. 0.9 ml maintenance medium (MM) containing 2% FBS was then added to the monolayer and the tubes were incubated stationary at 37°C. The cells were observed daily. After seven days the tubes were frozen at -80°C for one to two hours. After thawing, the cells were scraped into the culture medium, thoroughly mixed and then centrifuged at 200 x g for 10min. Supernatant and cells were harvested separately as the 1<sup>st</sup> passage. 100µl cells and supernatant were passaged separately into fresh Vero cells to the 5<sup>th</sup> passage.



## RNA extraction and reverse transcription

Total RNA was extracted from 100 $\mu$ l tissue culture fluid supernatant/cells using the MagNA Pure extractor (Roche, UK). In a 50 $\mu$ l reaction mixture, a 20 $\mu$ l sample of RNA was reverse transcribed to cDNA using random primers as described previously [Cooray et al., 2006; Vyse and Jin, 2002].

Alternatively, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany). 200 $\mu$ l tissue culture fluid mixture was extracted and eluted into 50 $\mu$ l RNase-free water. cDNA was generated using M-MLV Reverse transcriptase (Invitrogen or Promega) and either the specific primer Ru1r for the PCR-E860 or random primers for both the nested PCR-E860 and Rtime-SGE317. The 10 $\mu$ l reaction mixture contained 2.5 $\mu$ l sample RNA.

At the Robert Koch-Institut, total RNA was extracted from 140 $\mu$ l tissue culture fluid using the QIAamp Viral RNA Mini kit (Qiagen, Germany) and was eluted into 60 $\mu$ l RNase-free water. A 2.5 $\mu$ l sample RNA was used to generate cDNA using the specific primer Ru1r.

## SYBR Green I-based real-time PCR assay for rubella virus (Rtime-SGE317)

The primer pair of E1.7 and E1.8, which is used routinely as a part of the nested PCR-E317 assay at the Health Protection Agency and as described previously [Jin and Thomas, 2007], was adapted. The primer concentration was optimised at a range of 0.5-1.0 $\mu$ M. The reaction with a final concentration 1.0 $\mu$ M gave the best result for both sensitivity and specificity (results not shown). The assay was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Germany) on a

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4 1 LightCycler 2.0 (Roche, Switzerland). Each 20 $\mu$ l reaction mixture contained 10 $\mu$ l  
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6 2 of 2x SYBR Green I buffer, 0.2 $\mu$ l of each primer (100pmol/ $\mu$ l), 7.6 $\mu$ l dH<sub>2</sub>O and  
7  
8 3 2 $\mu$ l cDNA or plasmid DNA. The T<sub>m</sub> of the specific product amplified was 89 $^{\circ}$ C  
9  
10 4 according to the melting curve, and therefore a fourth step of incubating at 82 $^{\circ}$ C  
11  
12 5 for 5 sec was added to eliminate the effect of non-specific amplification. The  
13  
14 6 optimised amplification conditions are shown in Table I .  
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21 8 Sensitivity of the Rtime-SGE317 was evaluated using serial diluted plasmid DNA  
22  
23 9 of the E1 gene containing both the window 739nt and the target of the  
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25 10 Rtime-SGE317, and the random primer generated cDNA of the Thomas strain of  
26  
27 11 rubella (used routinely as a positive control at the Health Protection Agency).  
28  
29 12 The plasmid DNA used as standards for quantitation was diluted serially from 10<sup>6</sup>  
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31 13 copies/ $\mu$ l to 10 copies/ $\mu$ l. Standard curves were plotted with related copy  
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33 14 numbers, and the reproducibility of the assay for RV genome detection was  
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35 15 quantified in triplicate using tissue culture fluid samples of four RV isolates ZJ1-4  
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37 16 (Table II).  
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#### 18 **Nested PCR-E860 for rubella virus (PCR-E860)**

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47 19 The nested PCR assay (PCR-E860) was developed initially at the Robert  
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49 20 Koch-Institut. The primers for the first round, Ru1r (5'-CTG CCG GGT CTC CGA  
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51 21 CAC AGC) and Ru2f (5'-GAC ACC GTG ATG AGC GTG TTC), and for the  
52  
53 22 nested round, Ru3r (5'-GTG GTG TGT GTG CCA TAC AC) and Ru4f (5'-CTT  
54  
55 23 GCT AGC TAC GTC CAG CAC) generate a 860nt fragment of RV E1 gene  
56  
57 24 (nt8687-9546, Genbank accession number L78917), covering the 739nt window  
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59 25 (E1 nt8731-9469) as recommended by the WHO for genotyping. In a total  
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1 volume of 25 $\mu$ l per reaction, 2.5 $\mu$ l (Robert Koch-Institut, Centre for Disease  
2 Control and Prevention of Zhejiang Province, China) or 10 $\mu$ l (Health Protection  
3 Agency) cDNA was added as the template for the first round PCR. 2 $\mu$ L (Robert  
4 Koch-Institut and Centre for Disease Control and Prevention of Zhejiang  
5 Province, China) or 4 $\mu$ l (Health Protection Agency) of the first round amplicon  
6 was used for the nested PCR-E860 assay. The PCR amplification conditions for  
7 both first and second rounds are shown in Table I .

### 9 **Sequencing for RV genotyping**

10 The products of Rtime-SGE317 and the nested PCR-E860 were purified using  
11 the MinElute PCR Purification Kit (Qiagen, Germany) and then sequenced by  
12 commercial companies (Invitrogen Com Ltd, Shanghai, China; MWG, Germany).  
13 The primers E1.7 and E1.8 for the amplicons of the Rtime-SGE317 and primers  
14 Ru3r and Ru4f for the amplicons of the nested PCR-E860 were used for  
15 sequencing. The phylogenetic trees were constructed using DNASTar software  
16 on the basis of either the 280nt or 739nt sequences.

### 18 **Indirect Immunofluorescence assay (IFA)**

19 The medium was removed from infected Vero cells and the cells were washed  
20 gently with PBS. The PBS was removed and 0.25%Trypsin-EDTA was added so  
21 as to just cover the cell monolayer and left for 30 seconds. After a low speed spin,  
22 the cells were re-suspended in PBS to the required cell density. The cells were  
23 fixed on slides at -20 $^{\circ}$ C for 20 min with methanol and acetone (1:1), then 1:100  
24 dilution mouse monoclonal antibodies containing anti-rubella capsid protein C  
25 (provided by CDC, USA) and anti-structural glycoprotein E1 (gifted by Dr. D

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1 Samuel, MicroImmune, UK) was added and incubated at 37°C for 20min. After  
2 washing with PBS and deionised water, 1:20 diluted FITC-goat anti-mouse  
3 IgG+M+A antibody (Invitrogen) was applied for 20min at 37 °C. Negative and  
4 positive control slides were prepared and observed at the same time according  
5 to the WHO manual [WHO, 2007].  
6

### 7 **Electron microscopy (EM)**

8 Rubella is a fragile virus morphologically. Therefore, cultures were fixed prior to  
9 processing, by adding Carson's buffered formaldehyde (Bios Europe Ltd) to give  
10 a final concentration of 1.5% (v/v) formaldehyde in the culture medium, and  
11 stored at 4oC until examined. Cells were scraped into the medium and the  
12 mixture of cells and tissue culture fluid was centrifuged at 48000 x g for 45min.  
13 The supernatant was discarded and the pellet re-suspended in distilled water.  
14 The suspension was treated in an ultrasonic bath to break up clumps of cells. A  
15 drop of suspension was stained with phosphotungstic acid (PTA) pH6.8 and  
16 placed on a formvar-carbon coated EM grid. Grids were examined in a Philips  
17 420 transmission electron microscope fitted with an AMT XR60 digital imaging  
18 system.  
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## 1 RESULTS

### 2 **Specificity, sensitivity and reproducibility of the Rtime-SGE317**

3 The Rtime-SGE317 was developed to replace the probe-based real-time PCR  
4 assay used previously [Jin and Thomas, 2007]. The new assay had the  
5 advantage of being rapid and specific for confirmation of RV isolates. Even  
6 though the primer pair gave consistent results with different RV strains,  
7 specificity of the assay was evaluated further with measles virus and B19 virus,  
8 which cause similar symptoms to rubella. Measles cDNA and B19 DNA were  
9 tested by Rtime-SGE317 and all samples were negative with no cross-reactions  
10 (results are not shown). The time taken to perform the Rtime-SGE317 assay was  
11 2.5 hours less than for the previous assays (Table I ). Sensitivity of the  
12 Rtime-SGE317 was also compared with nested PCR-E317 and nested  
13 PCR-E860. The Rtime-SGE317 detected 200 copies/reaction of plasmid DNA  
14 (Table I ).

15  
16 The tissue culture fluid samples from four Chinese strains (ZJ1-4) were tested in  
17 triplicate to evaluate the reproducibility of the assay. Table II showed that the Ct  
18 values differed by less than one within each sample, whereas the deviation  
19 based on the copy numbers ranged from 4.6 to 18.3 times, e.g. strain ZJ1 and  
20 ZJ3 (indicated in italic in Table II ). Correlation between Ct values and copy  
21 numbers was plotted using the data collected from all samples and RV plasmid  
22 DNA tested in this study. The samples with Ct value  $\geq 40$  were defined as  
23 negative, though high copy numbers up to  $\sim 10,000$  copies were estimated  
24 based on the standards (Figure 1). Figure 1 shows that distribution of copy  
25 numbers was much wider when Ct values were  $>20$  or copy numbers  $<10^6$ ,

1 which indicated that Ct value was more stable and reliable for estimation of viral  
2 load in cell cultures.

#### 3 4 **Comparison of the methods for confirmation of RV isolates**

5 The tissue culture fluids collected from the first to fifth passage of two RV  
6 isolates from CRS patients (H1 and U6) were tested by three methods, IFA, EM  
7 and the Rtime-SGE317 (Table II). Clinical specimens from the two cases of CRS  
8 were inoculated and passaged up to four rounds (the harvest from the last one  
9 named as the 5<sup>th</sup> passage, P5). Specimens were incubated in Vero cells for  
10 7days each passage, but specific CPE was not clearly apparent. The infected  
11 cells collected from the 2<sup>nd</sup> to 5<sup>th</sup> passage were tested by IFA and EM. Weak  
12 fluorescence was observed in the 2<sup>nd</sup> passage. Fluorescence became stronger  
13 with passage affecting about 30% cells in the 3<sup>rd</sup> passage and 50% in the 5<sup>th</sup>  
14 passage.

15  
16 RV particles were not observed by EM in the 2<sup>nd</sup> passage, but small groups of  
17 particles were seen in the 3<sup>rd</sup> passage, and large clumps of virus particles were  
18 found in the 4<sup>th</sup> and 5<sup>th</sup> passage (Figure 2). Rubella virus is usually strongly cell  
19 associated and the viral particles observed were all attached to cell debris.

20  
21 Supernatant and cells of RV isolates were each tested quantitatively using the  
22 Rtime-SGE317. RV genome was detected from the 1<sup>st</sup> passage of sample U6  
23 and the 3<sup>rd</sup> passage of sample H1. Results for both Ct value and copy number  
24 are shown in Table II. The copy numbers continually increased with each  
25 subsequent passage and were higher for the cells than the supernatant. The

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1 viral load in sample U6 was much greater than in sample H1 in every passage,  
2 which corresponds with the EM observations (Table II).

3

#### 4 **Application of the Rtime-SGE317 for confirmation and the nested** 5 **PCR-E860 for genotyping of RV isolates**

6 RV isolates were obtained at the Centre for Disease Control and Prevention of  
7 Zhejiang Province by inoculating throat swab specimens into Vero cells. Four  
8 isolates were passaged four times (P1 to P5) and two twice only (P1 to P3). The  
9 presence of RV in each passage was confirmed by detecting the RV genome  
10 using the Rtime-SGE317. The six isolates were confirmed as the Ct values were  
11 <35.0 after three blind passages.

12

13 In order to determine the genotypes of the six Chinese isolates, the E1-739nt  
14 window was amplified using the new nested PCR-E860 assay. The amplicons  
15 were purified and sequenced with primers Ru3r and Ru4f. These sequences,  
16 together with reference strains recommended by the WHO, were used to  
17 construct a phylogenetic tree (Figure 3). These results were in agreement with  
18 those based on the 280nt products generated by Rtime-SGE317 (Results not  
19 shown).

20

21 Based on the 739nt window of the E1 sequences (Figure 3), five isolates were  
22 assigned to genotype 1E. Isolates RVi/Zhejiang.CHN/15.05 (ZJ-1) and  
23 RVi/Zhejiang.CHN/17.05 (ZJ-2) originating from two outbreaks in Quzhou and  
24 Lishui city within two weeks of each other in 2005 were 100% identical.  
25 RVi/Zhejiang.CHN/15.07/1 (ZJ-3) and RVi/Zhejiang.CHN/15.07/2 (ZJ-4)

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4 1 obtained from an outbreak in 2007 were also identical in sequence to each other,  
5  
6 2 but differed by 14nt from the 2005 isolates. RVi/Zhejiang.CHN/22.08 (ZJ-5)  
7  
8 3 isolated in Hangzhou city in 2008, was more closely related to the genotype 1E  
9  
10 4 strain detected in Hong Kong in 2008 (RVs/HongKong.CHN/27.08-1E, GenBank  
11  
12 5 accession no. FJ436377). The only 2B strain RVi/Zhejiang.CHN/23.08 (ZJ-6)  
13  
14 6 was isolated in Lishui city in 2008, and is close (4nt differences) to the Hong  
15  
16 7 Kong genotype 2B isolate, RVs/HongKong.CHN/49.08-2B (GenBank accession  
17  
18 8 no.FJ656218).  
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For Peer Review



## 1 DISCUSSION

2 Rubella virus replicates in a variety of continuous cell lines. RK13 and SIRC cells  
3 have been used because specific CPE is readily observed. However, these cells  
4 do not generate high titre virus [Figueiredo, et al., 2000, Rajasundari, et al.,  
5 2008]. The Vero/SLAM cell line is currently recommended by the WHO for  
6 measles and rubella isolation in the Global Measles/Rubella Laboratory Network  
7 [WHO,2007]. Viral CPE is not visible in Vero/SLAM cells in most cases, even  
8 after several passages. A previous study has shown that RV can replicate rapidly  
9 and reach higher titres in Vero cells because they do not produce interferon  
10 [Figueiredo, et al., 2000]. Therefore, the Vero cell line was used throughout this  
11 study. Similar to Vero/SLAM cells, specific CPE was not visible in Vero cells  
12 normally. However, it was visible when inoculated with a viral concentration of  
13  $10^5$  TCID<sub>50</sub> or higher [Guido et al., 1989]; or when the tissue culture fluid  
14 samples of each passage harvested in the first attempt were inoculated again  
15 simultaneously into fresh cells and observed in parallel which was carried out in  
16 the study (result not shown). It can be difficult still to differentiate between the  
17 CPE produced in infected cells and normal dead cells. Determination of RV  
18 growth after cell culture based on CPE observation alone is unreliable and  
19 subjective. Therefore, a simple method for confirmation of RV presence is  
20 required.

21  
22 In this study, the Rtime-SGE317 assay was shown to be simple, rapid and  
23 specific for confirmation of RV isolation (Table I ). It has been reported  
24 previously that the primer pair of E1.7 and E1.8 is specific and sensitive when  
25 used in a block-based nested PCR (PCR-E317) [Jin and Thomas, 2007]. The

1 fourth step of incubating at 82 °C for 5sec was added to increase the specificity. A  
2 melting curve analysis for every test is strongly recommended as it is a  
3 non-probe based real-time PCR assay [Robert et al., 2009]. Even though the  
4 sensitivity was 100-fold lower than the nested PCR-E317, the Rtime-SGE317 is  
5 one tenth of the cost and four times faster, and therefore suitable for rapid  
6 detection of RV in large numbers of samples. The Rtime-SGE317 is less  
7 sensitive compared to other real-time assays for rubella confirmation developed  
8 previously in the UK or China [Jin and Thomas, 2007; Zhao et al., 2006]. This  
9 could be due to its non-probe based method and the size of product. The  
10 advantage of Rtime-SGE317 is that the product can be assessed quantitatively  
11 and sequenced directly for confirmation and preliminary genetic classification.  
12 Using plasmid DNA as standards, the viral load can be quantified based on copy  
13 numbers. The copy numbers of viral load of two Sudanese isolates were higher  
14 in cells than that in supernatant. Electron microscopy has confirmed that RV is  
15 strongly cell associated. However, this does not mean that concentrated cells  
16 are necessarily more suitable, as the remaining lysed cells may affect viral  
17 propagation in fresh cells.

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19 The study results show that the Ct value is more reliable than viral genome copy  
20 number for estimation of viral load. The large deviation between copy numbers  
21 may be caused by several factors, such as errors occurring during the manual  
22 dilution and concentration measurement of the plasmid standards. In addition,  
23 risk of contamination is greater using plasmid DNA. This suggests that plasmid  
24 DNA as standard may not be necessary when RV isolates are confirmed by  
25 Rtime-SGE317, so avoiding the risk of contamination and reducing the cost and

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3 1 work load at local or sub-national laboratories. By testing a serial dilution of RV  
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5 2 cDNA, it was shown that confirmation of a rubella isolate could be based on the  
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7 3 viral load when the Ct value was <37 and the amplicon is large enough for  
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9 4 sequencing the 280nt of the E1 gene. If the Ct value is between 37 and 40, the  
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11 5 shape of the amplification curve should be carefully checked to ensure that the  
12  
13 6 sample is a real isolate. Samples with Ct value >40 could be passaged further,  
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15 7 but there is no point in continuing beyond the fifth passage as numbers of  
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17 8 defective virus particles may increase. Products with Ct value <30 may be  
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19 9 directly sequenced without purification.  
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27 11 Two other methods, IFA and EM, were compared with Rtime-SGE317 using the  
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29 12 two CRS isolates. IFA is a routine assay used at the Health Protection Agency  
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31 13 and is recommended by WHO. IFA indicates viable virus in cells by green  
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33 14 florescence. It is a classic assay, but requires fluorescence microscope and  
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35 15 experienced personnel to use it. The whole IFA process takes at least four hours  
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37 16 and a further PCR assay to generate the RV genome from confirmed strains for  
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39 17 genotyping. The recently developed indirect immunocolorimetric assay (ICA)  
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41 18 has the same sensitivity as IFA, and RV infected cells can be detected as  
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43 19 blue-staining spots on monolayers by the naked eye [Zhu et al., 2007; Chen et  
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45 20 al., 2007]. However, it was less sensitive than diagnostic PCR and standardised  
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47 21 high quality colorimetric reagents are not available commercially. Electron  
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49 22 microscopy requires highly experienced staff, is time consuming and is not  
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51 23 particularly sensitive. Although it can give an indication of the morphological  
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53 24 state of the viral particles, it is not suitable for rapid testing of large numbers of  
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55 25 samples.  
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3 1 Results of phylogenetic analysis of the six Chinese RV strains supported the  
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5 2 previous suggestion that the 280nt of E1 region can be used for preliminary  
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7 3 genetic classification [WHO, 2005], especially when the 739nt window is not  
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9 4 detectable. Based on the E1-739nt window, five of the six Chinese RV strains  
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11 5 belonged to genotype 1E and one to 2B, and the two strains isolated in 2008  
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13 6 (ZJ5-6) were close to two Hong Kong 1E and 2B isolates respectively. However,  
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15 7 there was no information available to link the viral transmissions. The results  
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17 8 indicated two genotypes of RV strains were co-circulating and genotype 1E  
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19 9 could have been the dominant genotype in Zhejiang province between 2005 and  
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21 10 2008. The preliminary identified RV isolates could finally be confirmed  
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23 11 definitively by sequencing the product of the nested PCR-E860 assay, the  
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25 12 window E1-739nt, as recommended by WHO for RV genotyping [WHO, 2005].  
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34 14 In addition to rubella IgM and IgG tests, tissue culture for RV isolation is routinely  
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36 15 carried out in most of provincial laboratories and some municipal CDC  
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38 16 laboratories in China as well as in many other countries in the WHO LabNet for  
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40 17 Measles and Rubella. However, tissue culture fluid specimens have to be sent to  
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42 18 a national/regional laboratory for confirmation of RV growth because most  
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44 19 sub-national laboratories do not have a fluorescence microscope for IFA or the  
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46 20 reagents for the ICA [WHO, 2007; Zhu et al., 2007; Chen et al., 2007]. Results of  
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48 21 RV confirmation and genotyping from the national laboratory are usually  
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50 22 reported back several months later. In addition, transportation of such tissue  
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52 23 culture fluid specimens has become more complicated and costly because of  
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54 24 biological safety regulations. RV tissue culture fluid specimens cannot be sent  
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56 25 away in time for confirmation and genotyping, and RV isolation is no longer  
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1 performed at some of the sub-national laboratories, which compromises rubella  
2 surveillance.  
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4 Most of the sub-national laboratories in China are well equipped with real-time  
5 PCR instruments and staff are well trained in performing real-time PCR assays.  
6 Therefore, the Rtime-SGE317 is a viable alternative method when considering  
7 costs, time available and technical skills. Since the products of Rtime-SGE317  
8 can be directly sequenced, confirmation and preliminary genetic classification of  
9 RV strains can be completed in the sub-national laboratories before sending to  
10 the National Reference Laboratory. The remaining 50 throat swab specimens,  
11 collected from 2005 to 2008 in Zhejiang province, were cultured for RV in Vero  
12 cells, but were not tested locally by IFA or ICA nor sent away for confirmation.  
13 These will be investigated further using the two new assays. Application of the  
14 assays would improve rubella surveillance locally and speed up the  
15 epidemiological reporting to national/regional Centres for Disease Control and  
16 Prevention.  
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## 1 **ACKNOWLEDGMENT**

2 The authors are grateful to Ruth Parry, Yvonne Suehong and Brain Megson  
3 (Health Protection Agency) for their technical assistance, to Omer Adam (Sudan)  
4 for submitting his RV isolates to WHO measles and Rubella strain Bank at  
5 Health Protection Agency, and to Dr. Kevin E Brown for his critical comments  
6 and support of this study. This work was supported by the Key Lab of  
7 Emergency Detection for Public Health of Zhejiang Province, China and the  
8 National Nature Science Foundation of China (project 30771903).

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## 1 **Legends for Figures**

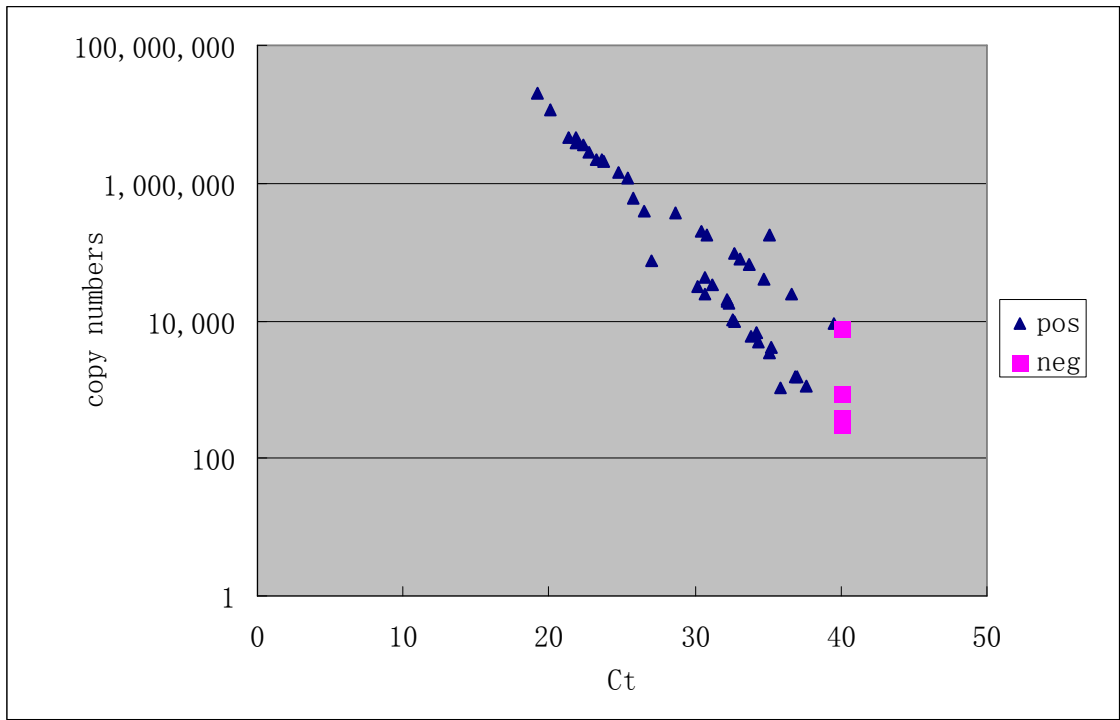
2 Figure 1. Correlation between Ct and copy number when detecting RV isolates  
3 by the Rtime-SGE317.

4  
5 Figure. 2. Electron micrograph of rubella virus strain U6 isolated in Vero cells.  
6 Virus particles can be seen closely associated with the cellular debris.  
7 Negatively stained with phosphotungstic acid; Bar represents 100nm.

8  
9 Figure 3. Genetic relationship amongst the eight RV isolates (in bold) and the  
10 reference strains from Genbank based on the 739nt of the E1 gene sequences.  
11 The phylogenetic tree was constructed using Clustal W method of the MegAlign  
12 program (DNASTar x.0 software). Eight RV isolates in this study were bolded and  
13 the reference strains downloaded from GenBank were indicated with genotype  
14 at the end of strain name.

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1 Figure 1

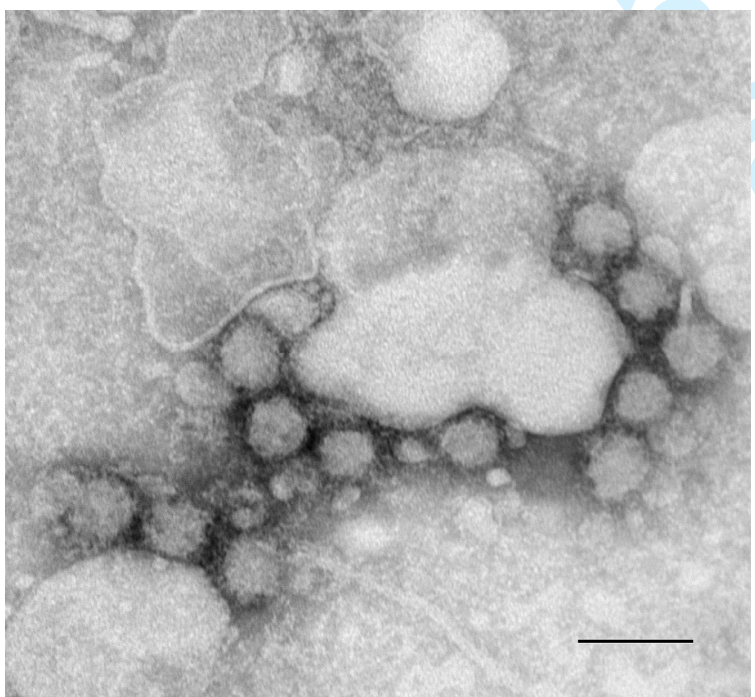


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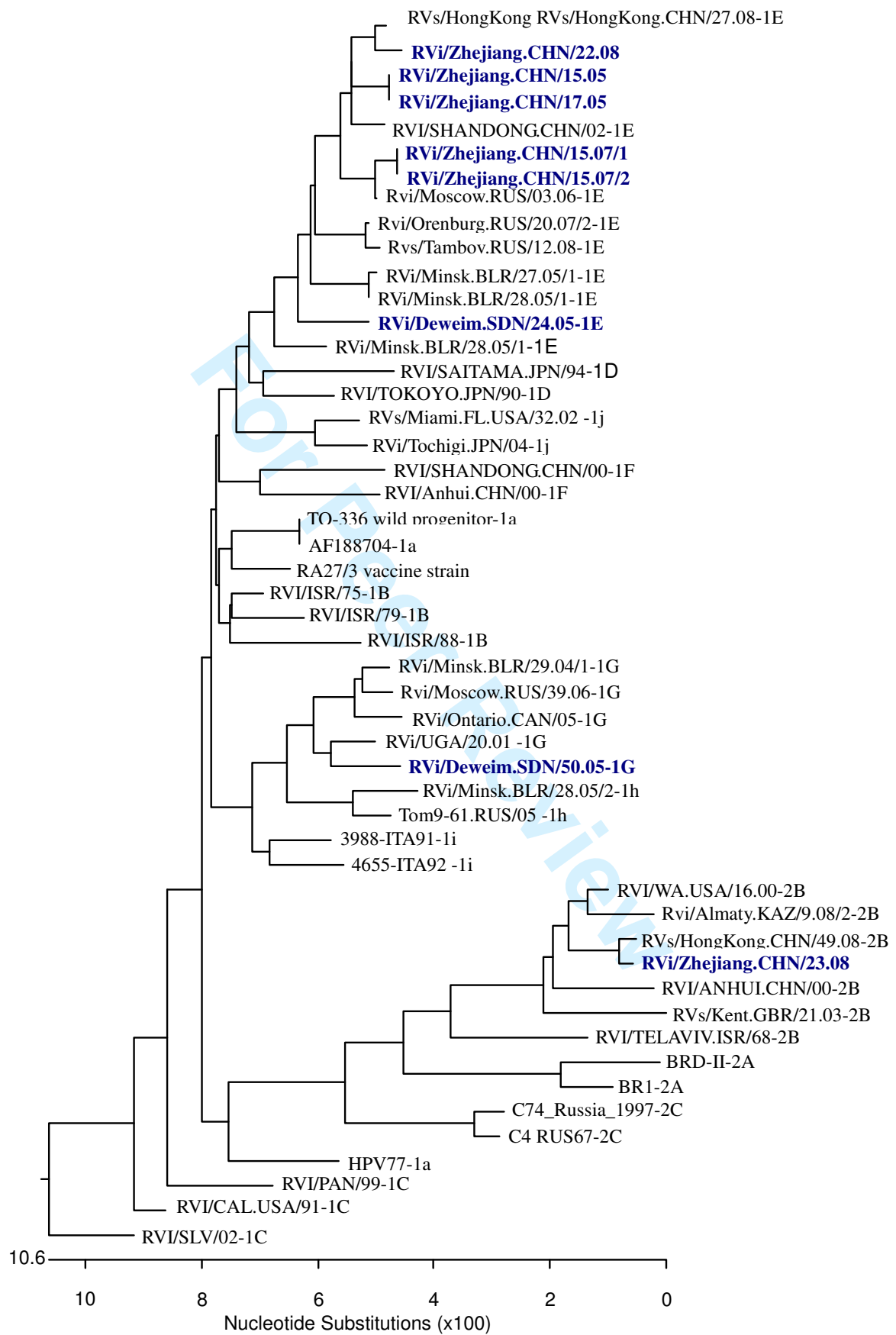
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5 Figure 2



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Figure 3



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**Table I**  
**Comparison among the Rtime-SGE317, the Nested PCR-E860 and the Nested PCR-E317 assays**

Method		Rtime-SGE317	Nested PCR-E860	Nested PCR-E317
Item				
Primer pair (100pmol/ul)		E1.7 and E1.8	Ru1r and Ru2f Ru3r and Ru4f	E1.5 and E1.6 E1.7 and E1.8
Programme		50°C 2min; 95°C15min 94°C 15sec 55°C 20sec 72°C 20sec 82°C 5sec Single 95°C 0sec 65°C 1min 95°C 0sec Cont 40°C 30sec <div style="display: flex; align-items: center; margin-left: 100px;"> <div style="font-size: 2em; margin-right: 5px;">}</div> <div>45Cycles</div> </div> <div style="display: flex; align-items: center; margin-left: 100px; margin-top: 10px;"> <div style="font-size: 2em; margin-right: 5px;">}</div> <div>Melting</div> </div>	96°C 1 min 94°C 30 sec 55°C 30 sec 68°C 90 sec 68°C 5 min <div style="display: flex; align-items: center; margin-left: 100px; margin-top: 10px;"> <div style="font-size: 2em; margin-right: 5px;">}</div> <div>35 Cycles</div> </div>	Nested PCR [Jin and Thomas, 2007]
Sensitivity	Plasmid DNA	200 copies/reaction	Not done	2 copies/reaction
	Virus (Thomas) cDNA dilutions	10 <sup>-3</sup>	10 <sup>-2</sup>	10 <sup>-4</sup>
Time for the process		1.5h	>4h	>4h
Suggested application		Confirmation of RV isolates	Genotyping of RV isolates	Confirmation of clinical samples

**Table II**  
**Confirmation and quantitative detection of RV isolates in Vero cell culture**

Sample (Type)	Method		1 <sup>st</sup> passage (inoculation)	2 <sup>nd</sup> passage	3 <sup>rd</sup> passage	4 <sup>th</sup> passage	5 <sup>th</sup> passage	Strain name GB accession No.
H1 (NS)	IFA		Not tested	Pos (2 <sup>a</sup> )	Pos (30%)	Pos (40%)	Pos (60%)	RVi/Deweim.SD N/24.05[1E]CRS  FJ775000
	EM		Not tested	Not detected	occasional single particles and small groups	some large groups	some large groups	
	Rtime-SG E317 (Copy no./Ct)	Sup	Neg	Neg	4,000/35.2 24,400/36.6	Not tested 67,100/33.7	17,900/32.4 80,800/33.1	
		Cells	Neg	Neg 9,020/39.5	6,850/34.2 91,900/32.8	Not tested 180,000/30.8	43,200/30.7 199,000/30.5	
U6 (TS)	IFA		Not tested	Pos (4 <sup>a</sup> )	Pos (30%)	Pos (40%)	Pos (50%)	RVi/Deweim.SDN /50.05[1G]CRS  FJ774999
	EM		Not tested	Not detected	Single particles, pairs and small groups	several groups	Mix of more large and few small groups	
	Rtime-SG E317 (Copy no./Ct)	Sup	19,300/32.2 Not tested	394,000/26.5 369,000/28.7	2,230,000/23.3 1,440,000/24.7	Not tested 2,150,000/23.6	3,590,000/22.4 2,020,000/23.7	
		Cells	33,400/31.2 Not tested	606,000/25.7 1,160,000/25.3	4,700,000/21.9 4,510,000/21.4	Not tested 2,830,000/22.7	19,700,000/19.2 3,800,000/21.9	

ZJ1 (TS)	Rtime-SG E317 (Copy no./Ct)	Mix	Not tested	1500~11900/ 26.6~27.0	5800~32400/ 24.7~25.4	3200~19800/ 25.6~26.3	10000~46200/ 23.9~24.8	RVi/Zhejiang.CH N/15.05 [1E] GQ374567
ZJ2 (TS)	Rtime-SG E317 (Copy no./Ct)	Mix	Not tested	1600~12100/ 26.5~26.7	3900~23500/ 25.3~25.9	3100~20900/ 25.6~26.3	4900~29900/ 25.0~25.7	RVi/Zhejiang.CH N/17.05 [1E] GQ374568
ZJ3 (TS)	Rtime-SG E317 (Copy no./Ct)	Mix	Not tested	430~4500/ 28.5~28.8	300~2610/ 29.0~29.5	82~1500/ 30.5~30.9	190~2470/ 29.6~30.3	RVi/Zhejiang.CH N/15.07/1[1E] GQ374569
ZJ4 (TS)	Rtime-SG E317 (Copy no./Ct)	Mix	Not tested	120~1280/ 30.3~30.8	230~2480/ 29.4~29.6	200~2590/ 29.5~29.6	110~1160/ 30.6~31.0	RVi/Zhejiang.CH N/15.07/2[1E] GQ374570
ZJ5 (TS)	Rtime-SG E317 (Copy no./Ct)	Mix	Not tested	1220/31.5	1270/31.4	Not tested	Not tested	RVi/Zhejiang.CH N/22.08 [1E] GQ374571
ZJ6 (TS)	Rtime-SG E 317 (Copy no./Ct)	Mix	Not tested	306/33.7	934/31.9	Not tested	Not tested	RVi/Zhejiang.CH N/23.08 [2B] GQ374572

a: Number of positive cells observed;

Sup: supernatant; Mix: mixture of supernatant and cells; Neg: negative; Pos: positive; NS: Nasal swab; TS: Throat swab

Numbers before or after "~" indicate the range of Copy no./Ct for three triple on each sample.

Copy no./Ct of sample H1 and U6 were generated twice: Data in the upper row were quantified with water-diluted RV plasmid DNA. Otherwise were quantified with cell cDNA diluted- RV plasmid DNA.