Comparison of Four Methods for Confirming Rubella Virus Infection Using Throat Swabs

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Running title: Four Methods to Confirm Rubella Virus Infection

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Abstract:

Laboratory tests are essential for confirming sporadic cases and outbreaks of rubella. Detection of rubella virus is often necessary to confirm rubella cases and to identify specimens to be used to characterize wild-type rubella viruses. The sensitivities of four methods for detecting rubella virus infection using throat swabs, which had been collected in Henan and Anhui provinces in China, were evaluated. These methods were RT-PCR followed by Southern hybridization using RNA extracted directly from clinical specimens, virus growth in tissue culture followed by virus detection by RT-PCR, low-background immunofluorescence in infected tissue culture cells using monoclonal antibodies to the structural proteins of rubella virus, and a replicon-based method of detecting infectious virus. Among these four methods, the direct RT-PCR followed by hybridization was the most sensitive method; the replicon-based method was the least difficult to perform.

Keywords: Rubella virus detection, Immunofluorescence, RT-PCR, replicons.
Introduction:

Rubella is a mild rash disease with few complications. However, rubella virus infection early in pregnancy often causes death of the fetus and, if the fetus survives, causes congenital defects in about 90% of the newborns. The major defects include deafness, cataracts, and heart disorders, which are collectively known as congenital rubella syndrome (CRS) (12).

Although elimination of rubella and CRS has been achieved in United States with an effective vaccination program, in countries without an immunization program or without a good program, rubella is still endemic (27, 11), and explosive outbreaks may occur (9, 13). It was estimated in 2003 that more than 100,000 infants were born with CRS each year worldwide (16). In general, a large proportion of unimmunized populations in rubella endemic areas are infected and become immune before puberty. Nevertheless, approximately 3-23% of adults remain susceptible in various countries and areas (15, 17, 4, 8).

Routine rubella vaccination is not included in the national immunization program in China, although rubella vaccine is available in certain urban areas. A seroprevalence study in 1993-95 of 2610 women aged 16-30 years in 5 provinces in China found that only 83.6% were immune to rubella. Thus, CRS is still a public health concern in China (27). An estimate of the incidence of CRS in China in 2005 was at least 20,000 cases per year (14). Several large rubella outbreaks have been reported in different regions since 1987, including Shandong province and Hangzhou city (1987) (24, 22), Shanghai city (1993-1994; nearly 60,000 cases) and Beijing city (1994; over 18,000 cases) (27). Examples of recently reported outbreaks include Guangxi province (2000; more than
Two different rubella vaccines are currently available in China. The BRDII attenuated vaccine strain of rubella virus (Tian Tan Biological Products Corporation, Beijing) was introduced in 1994 in some parts of China including the city of Shanghai and Shandong province. In addition, imported MMR vaccine containing the rubella virus vaccine strain RA27/3 (Merck and Co., Inc., Whitehouse Station, N.J.) has been available since 1996 in large cities (26, 21). Since the mid-1990s, despite large outbreaks, the incidence of rubella in China has dramatically decreased (6). Unfortunately, rubella vaccine has not yet been introduced into the Chinese national immunization program and the disease is still epidemic in rural areas.

In order to achieve the goal of measles elimination, because rubella is often the final diagnosis of suspected measles cases, the World Health Organization (WHO) has decided to include laboratory testing for rubella in the measles surveillance system and has established the global measles and rubella laboratory network. Approximately 30-50% of suspected measles cases turn out to be rubella cases (16).

A common laboratory confirmation of rubella cases is to detect rubella-specific IgM in sera from suspected cases. In postnatal rubella infections, IgM is often not detectable until several days after the rash appears. False negative results may be obtained if only a single serum taken near the day of rash is collected, the most likely day for serum collection. However, virus is usually present in the throat or nasopharynx from a few days before until about 5 days after the onset of rash, making virus detection a possible accessory test for diagnosis (2). Furthermore, detection of rubella virus in
clinical specimens is necessary for important control activities such as molecular epidemiology.

Rubella virus is the sole member of the Rubivirus genus in the Togaviridae family. The genome of rubella virus is a single-strand RNA of positive polarity. It contains a 5’ proximal open reading frame (ORF) that encodes nonstructural proteins (NSP), which are responsible for viral genome replication and a 3’ proximal ORF, which encodes 3 structural proteins, C, E2 and E1(10). In some studies, reporter genes such as green fluorescent protein (GFP) and chloramphenicol acetyltransferase (CAT) were used to replace all or part of the 3’-proximal ORF (18). Such modified rubella RNAs (replicons) can still replicate inside cultured cells, but are not infectious because they lack some or all of the structural proteins necessary for assembly of virions. Expression of the capsid protein (C) has been shown to enhance the replication of replicons (5).

Several laboratory techniques were used in the present work to detect rubella virus in clinical specimens, including RT-PCR using rubella virus RNA extracted from the clinical specimen followed by Southern hybridization (RT-PCR + hybridization), RT-PCR using RNA recovered from infected tissue culture (Culture + RT-PCR), a low-background immunofluorescent assay (IFA) to detect viral proteins in infected tissue culture cells, and a replicon-based method to detect infectious virus (replicon cells). Culture + RT-PCR is well established for detecting infectious virus in clinical samples. The immunofluorescent assay for rubella virus infected cells used here was implemented using monolayer culture and monoclonal antibodies to the rubella proteins, since both were necessary to reduce background; low background is essential for good specificity in IFA detection of rubella virus infected cells. The sensitive RT-PCR + hybridization and
the replicon-based diagnosis are newly developed methods for detecting rubella virus.

Work showing a proof of concept, that replicons can be used for detection of rubella virus infected cells, has been reported (19). For the current study, a rubella replicon cell line was established with BHK cells expressing C lacking the first 8 amino acids and using a rubella virus replicon capable of expressing GFP. All 4 methods were compared using 22 throat swabs from clinically diagnosed rubella patients from Henan and Anhui provinces.
MATERIALS AND METHODS:

Clinical specimens. Twenty-two throat swabs specimens were collected from clinically suspected rubella cases in China (Table 1). Thirteen specimens were collected from an outbreak in 2001 and 2002 in Henan province, and 9 were collected from a single outbreak in 2000 and 2001 in Anhui province.

IgM testing. Indirect rubella IgM kits used in Henan province were purchased from Hangzhou Everlong Biochemical Products Company (Hangzhou city, Zhejiang province, China), and indirect IgM kits used in Anhui province were from Hainan Chemical Products Company (Haikou city, Hainan province, China). All of the serologic tests for rubella IgM reported here were done by the provincial laboratory staffs. All IgM results reported here were confirmed at the National Institute for Viral Disease Control and Prevention using an indirect test kit (Dade Behring, Mannheim, Germany).

RT-PCR. Viral RNA from throat swabs was extracted using Tri-Reagent LS, for liquid samples (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer’s protocol. The RT-PCR was performed using Superscript II One-Step RT-PCR (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s recommendation, to amplify a 185-nt region in the E1 coding region (nt 8807-nt 8991) using a forward primer (RV11; 5’ CAA CAC GCC GCA CGG ACA AC 3’) and a reverse primer (RV12; 5’ CCA CAA GCC GCG AGC AGT CA 3’). After the reverse transcription step for 30 min at 55°C and denaturation for 5 min at 95°C, the reactions were incubated for 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. These primers and cycling conditions were based on a reported RT-PCR system which gave a DNA product from rubella virus and not from 16 other RNA viruses (3). The system
used here gave no product when tested with Sindbis virus infected cells. Sindbis virus is
an Alphavirus in the Togaviridae family related to rubella virus. Negative controls (5
reactions containing water instead of template) were included with each set of clinical
specimens. If any control was contaminated, all results from the run were discarded.
The positive control was RNA from a rubella virus laboratory strain (Therien). The PCR
products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.
Hybridization of RT-PCR products. To perform Southern hybridization, DNA in the
agarose gel was denatured using 1.5 M NaCl, 0.5 M NaOH followed by neutralization
using 1.5 M NaCl, 0.5 M Tris (pH 7.5) and the DNA was transferred to a nylon
membrane by standard blotting techniques. Hybridization was done using agarose gel-
purified, DIG labeled probe, in DIG Easy Hyb for 3 hrs at 44˚C (Roche Molecular
Biochemicals, Mannheim, Germany). The 143-bp DNA-probe was made by RT-PCR
using a forward primer (RV13; 5’ CTC GAG GTC CAG GTC CYG CC 3’) and a reverse
primer (RV14; 5’ GAA TGG CGT TGG CAA ACC GG 3’), DIG-labeled dUTPs (Roche
Molecular Biochemicals, Mannheim, Germany), and Therien RNA. These primers were
part of a nested set reported previously (3). Detection of the PCR products after
hybridization was done using HRP-conjugated anti-DIG antibody followed by
chemiluminescence with detection of emitted light on film. The RT-PCR + hybridization
described here gave no signal with Sindbis virus infected cells.

Culture + RT-PCR protocol. Vero cells in 35 mm plates were inoculated with material
from throat swabs according to standard methods (2). Briefly, cells inoculated with
clinical material were incubated at 35˚C for 7 days. The culture media was harvested and
used to inoculate fresh cells for 2 additional 7-day passages. Total RNA was extracted
from the cells at each passage of the culture using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Detection of rubella-specific RNA by RT-PCR was done as described above, without hybridization. Negative results after each of 3 passages was required for a specimen to be considered negative.

Mock-infected Vero cells were always carried through this protocol with each set of clinical specimens as a negative control. The positive control was RNA from Therien infected Vero cells which were cultured separately from clinical specimens to avoid contamination during multiple passages in tissue culture.

IFA protocol. IFA was done using cells grown in a monolayer and inoculated with supernatant from the first culture passage. At 3-days post-inoculation, cells were fixed with 2% paraformaldehyde on ice and permeablized with 100% methanol. The presence of virus was determined by detection of viral structural proteins in cells using rubella specific mouse monoclonal antibodies to the E1(MAb 1-6), E2 (MAb 26-24) and C (2-36) proteins (Viral Antigens; Memphis, TN) and AlexaFluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes; Portland, OR). The cell nuclei were counterstained with propidium iodide and the result was examined by fluorescence microscope. The use of this low-background IFA protocol was necessary since rubella virus replicates to relatively low levels compared to other viruses (e.g. measles virus). Since this assay was done with the supernatant from the first passage, this method is considered to be detection of the presence of virus at the second passage of the clinical specimen.

Replicon-based detection of infectious rubella virus protocol. A cell line was established using BHK cells expressing C*, which has two stop codons and an extra G
residue immediately after the initiation codon for C, using pCI-Neo (Promega, Madison, WI, USA); a second start codon is presumably used resulting in C* lacking the N-terminal 8 amino acids (5). Both replicons and rubella virus replicate better in cells C* than in BHK cells (M-H Chen, unpublished results).

The rubella virus replicon in the C* expressing BHK cell line contained duplicated rubella virus intergenic regions (RUBdsPAC/GFP\_400). Intergenic regions are thought to contain the promoter for rubella virus subgenomic RNA synthesis; this replicon also contained the NSP coding region and 3' terminal 400 nucleotides of the rubella genome. A puromycin-resistance gene (PAC) was inserted after the first subgenomic promoter and the gene encoding the green fluorescent protein (GFP) was inserted after the second subgenomic promoter. Replicons are not packaged in BHK cells expressing C* due to the lack of E1 and E2 proteins.

The presence of helper virus (in this case, virus from the clinical samples) was determined by transferring the supernatant from the replicon cell culture (C\_*-RUBdsPAC/GFP\_400) which has been inoculated with clinical specimen onto uninfected Vero cells. If infectious virus was present in the clinical specimen, then replicons were packaged, transferred to the uninfected Vero cells, and the expression of reporter gene (GFP gene) was visible in these Vero cells.

To detect infectious virus in the clinical specimens, clinical specimens were inoculated onto (C\_*-RUBdsPAC/GFP\_400) replicon cells and incubated for 5-7 days. The replicon cultures were centrifuged and the supernatants were transferred to uninfected Vero cells. At about 24-48 hr post-infection, if infectious virus was present in clinical
samples, expression of the GFP gene could be observed in the Vero cells with a fluorescence microscope. The negative control in these assays was mock infected cells which always gave no fluorescence. Sindbis virus infected cells were repeatedly tested with this assay and never gave any fluorescent cells. Because replicon-based detection of rubella virus requires additional passage in Vero cells to examine GFP expression, replicon results are considered to be at the second passage of virus in tissue culture.
RESULTS:

Twenty-two throat swab specimens were analyzed in this study (Table 1). The 22 samples were from Henan and Anhui provinces, all were clinically diagnosed as rubella and 17 were laboratory confirmed by IgM. Test results using the four methods are summarized in table 2. By RT-PCR alone, 68% of specimens (15/22) tested positive. The sensitivity of direct RT-PCR was much improved after hybridization; 6 more samples became positive (91% of the clinical cases) including two specimens with negative serological results (Table 1).

Approximately 77% (17/22) of clinically defined rubella cases were positive using any of the three techniques using culture. Using RT-PCR + hybridization as the standard, 81% (17/21) of the positive specimens were identified using culture techniques. The results from all three culture-based methods agreed with one another. None of the 22 specimens was negative by the Culture + RT-PCR method after the first passage and positive after the second or third passage (Table 1).

Representative results from the direct RT-PCR assay before (Figure 1, panel A) and after Southern hybridization (Figure 1, panel B), from the IFA assay (Figure 2, panel A) and from the replicon-based assay (Figure 2, panel B) are shown. In the current study, negative specimens such as RVTS35 had no fluorescent cells and the positive specimens such as RVTS20 had many fluorescent cells. However, since the background in the replicon-based assay is low, even a single fluorescent cell would have been considered a positive result.

Description of results for selected, individual specimens. Among the 13 specimens from Henan province, although 3 of them were IgM negative (RVTS17, RVTS19,
RVTS23) and 2 of them (RVTS 24 and RVTS 25) were not tested for IgM, 10 were positive by direct RT-PCR without Southern hybridization and all (13/13) were positive after hybridization (Table 1). The three methods that required tissue culture gave the same positive and negative specimens; four that were negative by culture + RT-PCR (RVTS17, RVTS22, RVTS23, and RVTS24), were also negative by the IFA and replicon-based tests. Thus, compared with RT-PCR + hybridization, these culture-based methods each detected about 69% (9/13) of the positive specimens from Henan. Among the three specimens which were RT-PCR negative before Southern hybridization, RVTS17, RVTS22, and RVTS29, only one was positive using the three viral culture techniques (RVTS29), suggesting that RT-PCR + hybridization is more sensitive than culture at detecting rubella cases.

Two patients (RVTS 17 and RVTS 23) were IgM negative, negative by techniques which include culture, but RT-PCR + hybridization positive one day after rash onset. Many rubella cases are IgM negative and infectious virus positive at one day after rash (2). Thus, it is reasonable that these specimens initially, contained infectious virus which was inactivated during collection or transport. RVTS19 was positive by both direct RT-PCR and the 3 methods that include tissue culture. The RVTS19 serum was IgM negative, although it was collected at 5 days after rash, when most rubella cases should be IgM positive. Thus, the IgM negative result for this patient is suspect.

Five of 9 specimens from Anhui province were positive by direct RT-PCR and 8 of 9 were positive after Southern hybridization. All 8 positive specimens were also positive using the 3 methods that required infectious virus for a positive result.
after rash and was serologically positive. It is possible that the IgM result for this
specimen is incorrect or that degradation of virus and viral RNA in the specimen
occurred after collection.

Viral isolates were obtained from both provinces by using the culture methods
described; the isolates were characterized further for molecular epidemiologic purposes
(data not shown).
DISCUSSION:

Laboratory techniques which detect rubella virus RNA or infectious rubella virions are important for supporting rubella control programs. Testing specimens for rubella RNA or infectious virus can be used to confirm IgM serologically positive cases or, since as many as 50% of rubella cases are IgM negative on the day of rash, as the only laboratory data confirming a rubella case (2, 7). Confirmation of a positive serologic result is especially important when the number of rubella cases is low, such as when a country is on the verge of rubella elimination, since false IgM positives are expected to make up a large portion of suspected rubella cases under these circumstances.

Furthermore, identification of specimens which contain rubella RNA or infectious virus, allows viral RNA from these specimens to be further characterized for molecular epidemiologic purposes, which can be an important component of rubella control programs (28).

In China, 331 sub-national laboratories were established and a laboratory surveillance network was started in 2000. Considerable testing for rubella occurs in the surveillance network, partly because measles and rubella are frequently difficult to distinguish clinically. Thus, it is necessary for the network to establish sensitive, specific, and, above all, economical methods for rubella diagnosis. Samples from rural areas are sent to provincial laboratories, where basic testing by serologic methods and/or tissue culture methods are completed; positive samples are then transferred to the national laboratory at China CDC for confirmation. Once the samples are confirmed by additional IgM testing or more advanced tests such as RT-PCR, the national laboratory is required to return the results to each provincial lab. One important means of confirming rubella virus
infection is detection of rubella RNA or infectious virus using throat swabs.

Only about 40% of rubella cases are IgM positive on the day of rash, rising to about 100% IgM positive by about 4 days post rash (2). However, about 90% of rubella patients are positive on the day of rash for virus in the throat by culture; this percentage of positive patients declines rapidly in the first week after rash. Therefore, 2 types of specimens are good to collect if unforeseen difficulties result in degradation of one or the other type of specimen (e.g. poor storage of sera). As the results of this study show, both sera and throat swab collection in the first few days after rash is prudent.

Comparing the four methods used in this study, the direct RT-PCR + hybridization correlated best with clinical diagnosis of rubella. Because this method does not require recovery of infectious virus from clinical specimens, it can be done in about 2 days. Since high sensitivity of the RT-PCR was increased by the addition of the hybridization step rather than a nested PCR, contamination by PCR products which often occurs with the nested RT-PCR technique, was minimized. However, this technique is a complex procedure and requires good molecular reagents.

Compared with direct RT-PCR + hybridization, the other three techniques, which used tissue culture methods to recover infectious virus, were less sensitive. This may be primarily due to the loss of infectivity of viruses during transport. The culture + RT-PCR and IFA methods are rather complex procedures, require good molecular reagents, and are time consuming, requiring 3 weeks to complete all passages. Protocols for both IFA and the replicon-based method both require a fluorescent microscope. Among the techniques requiring tissue culture, the replicon-based method is particularly simple to do, requiring only tissue culture and microscopic techniques.
Previous descriptions of the use of GFP expressing replicons for detection of rubella virus are cumbersome for a number of reasons, including the need to transfet previously infected Vero cells with replicon RNA (19). The technique used here relies on a stable cell line expressing a replicon and only requires standard virus passage and fluorescent microscopic techniques for detection of infections virus (i.e. no RNA synthesis, no transfection, etc.). The stable cell method has been shown to be as accurate as the transfection-dependent replicon-based detection method (M-H Chen, unpublished data).

In summary, the RT-PCR + hybridization protocol was the most sensitive method and allowed detection of rubella viruses in clinical samples directly. However, the other 3 methods also performed well, detecting most of the RT-PCR + hybridization positive specimens (81%). The 3 methods based on growth of virus in tissue culture have the advantage of isolation of viruses, which can then be characterized in more detailed (25, 28). The newly developed method utilizing GFP expressing replicons performed well and required no laboratory techniques except tissue culture and simple fluorescent microscopy. Thus, this technique is considered the least difficult method to perform and could be automated. The techniques described here require only equipment which is commonly available, unlike some other techniques (e.g. real time RT-PCR).
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vaccination strategy: contribution of a rubella susceptibility study of women of


FIGURE LEGENDS:

Figure 1. Representative results of RT-PCR + hybridization protocol. Panel A shows the RT-PCR products from 4 throat swabs after electrophoresis on agarose gel and staining with ethidium bromide and panel B shows results after hybridization. The sample in each lane is (1) Marker (2) Dig-marker (3) H2O (4) RVTS25 (5) RVTS36 (6) RVTS37 (7) RVTS38 (8) Empty (9) Positive control (rubella virus (Therien) RNA). Additional laboratory controls run with throat swabs were all negative (data not shown).

Figure 2. Representative results from IFA protocol and replicon protocol. Panel A: Vero cells infected with the supernatant from the first passage of clinical specimens were fixed at 3 days post-infection. The presence of virus was determined by detection of viral structural proteins, using rubella specific mouse monoclonal antibodies and AlexaFluor 488 conjugated secondary antibody (green). The nuclei were stained using propidium iodide (red) as a counterstain. Panel B: GFP expressed from replicons was detected in Vero cells only when the infectious virus was present in the specimen (RVTS20) but not in the absence of infectious virus (RVTS35).
Table 1. Epidemiologic information and laboratory results from clinical rubella cases.

<table>
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<th>Sample ID</th>
<th>Province</th>
<th>Day(s) after Rash when Specimen collected</th>
<th>Rubella IgM</th>
<th>City and year*</th>
<th>RT-PCR</th>
<th>RT-PCR+ Hybridization</th>
<th>RT-PCR</th>
<th>IFA</th>
<th>Replicon</th>
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<td>(-)</td>
<td>(+)</td>
<td>P1(+)</td>
<td>(+)</td>
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<td>(+)</td>
<td>Heifei, 2000</td>
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<td>P1(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

*P2 and P3 refer to passage number in Vero cell culture.

^Not available because patient was not tested for rubella virus specific IgM.

#Specimens from outbreaks in Henan (2001 and 2002) and Anhui (2000 and 2001)
Table 2. Comparison of sensitivity of techniques for detecting infectious rubella virus or viral RNA using specimens from Anhui and Henan.

<table>
<thead>
<tr>
<th>Technique</th>
<th>No. of patients with clinical rubella*</th>
<th>No. Positive sample</th>
<th>% Positive ^</th>
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<tbody>
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<td>RT-PCR</td>
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<td>15</td>
<td>68</td>
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<tr>
<td>RT-PCR + hybridization</td>
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<td>21</td>
<td>91</td>
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<td>IFA</td>
<td>22</td>
<td>17</td>
<td>77</td>
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<td>22</td>
<td>17</td>
<td>77</td>
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<tr>
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<td>22</td>
<td>17</td>
<td>77</td>
</tr>
<tr>
<td>Total after passage 3</td>
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<td>17</td>
<td>77</td>
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<tr>
<td>Replicon cells</td>
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<td>17</td>
<td>77</td>
</tr>
</tbody>
</table>

*See table 1 for results from individual specimens.

^Percent of clinical rubella cases which tested positive.

Three patients were rubella IgM negative and 2 patients were not tested for IgM.
Figure 2