Nested reverse transcription polymerase chain reaction for the detection of rubella virus in clinical specimens


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Background & objectives: Rubella virus (RV) is one of the leading causes of childhood blindness in India. In this study we applied an optimized nested reverse transcription polymerase chain reaction (nRT-PCR) to detect RV in clinical specimens.

Methods: nRT-PCR was optimized using total RNA extracted from standard strain of RV using nested sets of primers specific for E1 open reading frame. nRT-PCR was applied onto 30 lens aspirates and corresponding peripheral blood leucocytes of 30 infants with congenital (29)/developmental (01) cataract. Serology for anti-RV IgG and IgM antibodies was done. RV isolation was attempted using Vero and SIRC cell cultures.

Results: Optimized nRT-PCR was specific for RV and sensitive to detect 10 fg of RV RNA. Among 30 patients, nRT-PCR detected presence of RV in lens aspirates of 6 (20%) and 4 corresponding leucocytes. RV was isolated from 3 (10%) lens aspirates (nRT-PCR positive) of the 30 patients. Sera of these 6 patients showed presence of anti-RV IgG and IgM in one, only anti-RV IgG in 3 others and none in the other two. Of the remaining 24 patients, anti-RV IgG was detected in 3 and no anti-RV IgM antibodies in others.

Interpretation & conclusion: Findings of our study showed that the nRT-PCR was a more sensitive and rapid technique to detect RV from lens aspirates compared to conventional methods of virus isolation and serology.

Key words: Congenital cataract - congenital rubella syndrome - lens aspirates - RT-PCR - rubella virus

Rubella virus (RV) is a non-arthropod borne RNA virus and is the sole member of the genus Rubivirus in the family Togaviridae. Rubella infection is generally mild with few complications, but when acquired by pregnant woman during the first trimester of pregnancy, the virus is transmitted to the foetus with 90 per cent chances of development of congenital malformations in the newborn. Earlier studies have shown that 10 to 40 per cent of congenital cataract was due to RV
associated cataract in India and these were based on serology, results of which are difficult to interpret\textsuperscript{1-5}. Ocular consequences of congenital rubella syndrome (CRS) may clinically develop during or after neonatal period\textsuperscript{6}. Since RV has been known to be shed for up to 1 to 3 yr in clinical specimens such as the cataractous lens, a positive virus culture in such specimens from neonates is diagnostic of congenital infection\textsuperscript{7}. We had earlier studied the association of congenital cataract with RV based on virus isolation\textsuperscript{8}. RV replicates in a wide range of cell lines producing subtle and slow developing cytopathic effect often difficult to visualize because of low multiplicities of infection and is detected by immunofluorescence or RT-PCR\textsuperscript{9}. A variety of PCR assays have been described for detecting RV genome directly from clinical samples\textsuperscript{10-15}. In order to improvise the sensitivity of the detection rate, for the association of the virus to congenital cataract, an optimized nested RT-PCR was applied to detect RV in lens aspirates and peripheral blood samples of infants with congenital or developmental cataract.

**Material & Methods**

**Patients & specimens**: In this study over a period of 10 months (1\textsuperscript{st} March to 31\textsuperscript{st} December 2005) at the tertiary care ophthalmic hospital, Sankara Nethralaya, Chennai, lens aspirates and peripheral blood leukocytes obtained from 30 infants (< 1 yr of age) undergoing therapeutic lensectomy for congenital (29)/developmental (1) cataract were investigated for the association of RV infection following informed consent from the parents to use the material for research purpose and approved by the Institutional Research and ethics committee. The lens aspirate specimen was collected as described earlier\textsuperscript{8}. The peripheral blood sample (2-3 ml) of the patients was also collected prior to the surgery for determining the presence of RV in blood samples by the conventional cell culture method\textsuperscript{7} and molecular method of nRT-PCR.

**Serology**: Serum samples from all the 30 patients were collected at varying periods of 2 days to 3 months prior to surgery and tested for the presence of anti-RV IgG and IgM antibodies by ELISA using BIO ELISA procured from BIOKIT, Barcelona, Spain, as per the instructions provided in the manual.

**Isolation of rubella virus**: Virus isolation was attempted by standard conventional test tube culture method\textsuperscript{7} using Vero cells (supplied by National Facility for Animal Tissue Culture, NFATCC, Pune, India) and Statens Serum Institute Rabbit Corneal Epithelial Cell line (SIRC) cells (provided by Dr Savithri Sharma, L.V. Prasad Eye Institute, Hyderabad)\textsuperscript{8}.

**Preparation of RNA**: The lens aspirate specimens were extracted using Qiagen Viral RNA extraction kit procured from Qiagen, Hilden, Germany, as per the instructions provided in the manual. Extraction of RNA from the leucocytes was done using the standard guanidium thiocyanate protocol\textsuperscript{16}.

**Nested reverse transcription polymerase chain reaction (nRT-PCR)**: Rubella virus cDNA was generated using the one step RT-PCR kit (Qiagen, Germany). The reaction was performed in 50μl volumes as per the manufacturer’s instructions. The primer sequences for the I and II round of nRT-PCR are shown in the Table\textsuperscript{13}. The enzyme mix facilitates both reverse transcription and polymerase chain reaction. The annealing temperature was optimized to 61°C as against 60°C in the original report\textsuperscript{12}. Amplification of 143 bp produced in nested PCR indicated the presence of RV specific RNA. The sensitivity of the primers were determined by diluting the initial cell culture harvest 10-fold in Dulbeccos Minimum Essential Medium (DMEM), following which the RNA extraction was performed from each of the 10 log dilutions and nRT-PCR was set up for each. The specificity of the primers was determined by performing nRT-PCR using the RNA extracted from the following that commonly cause
ocular infections and human DNA from peripheral blood leucocytes from healthy blood donors. The bacterial RNAs tested include *Staphylococcus aureus* (ATCC 25293, American Type Culture Collection, USA), *Pseudomonas aeruginosa* (ATCC 7853, American Type Culture Collection, USA), *Mycobacterium tuberculosis* (H37Rv, Tuberculosis Research Centre, Chennai) and *Chlamydia trachomatis* serotype A (ATCC VR 517B). The virus RNAs tested include - Adenovirus serotype 2 (ATCC 846-VR, National Institute of Allergy and Infectious Diseases, USA), Herpes simplex virus-1 (ATCC 733 VR, Chemicon, USA), Cytomegalovirus (AD169, provided by Dr Sridharan, Christian Medical College, Vellore), Varicella zoster virus (Oka Vaccine strain - Varilix vaccine, Smithkline Becham, Belgium) and ECHO11 (provided by Dr Nalini Ramamoorthy, King Institute, Chennai). Fungus RNAs includes *Candida albicans* (ATCC, American Type Culture Collection, Ranbaxy, New Delhi) and *Aspergillus flavus* (laboratory isolate).

**DNA sequencing:** The amplified products were confirmed for specificity by performing DNA sequencing. Fifty µl of the 185 bp first round amplified products of nRT-PCR of three RV isolates (only these three were chosen since they were isolated in cell cultures out of the six nRT-PCR positive specimens) were electrophoresed on a 2 per cent agarose gel and visualized under the UV transilluminator. The DNA was eluted from the agarose gel using the Mini Gel elution kit from Eppendorf, USA, in a total of 30µl elution volume and cycle sequencing was set up using 2µl of the eluted product. The cycle-sequenced product was loaded into the DNA sequencer machine 3100 Avant, ABI Prism, USA, and sequenced.

**Results**

**Virus isolation:** From among the 30 patients, RV was isolated from 3 lens aspirates in SIRC and one of them also in Vero cell lines. The virus was not isolated from any of the peripheral blood leucocytes of these patients.

**Specificity, sensitivity of nRT-PCR:** The optimized nRT-PCR for E1 gene was specific for RV genome since there was no amplification of RNAs from infective agents other than RV included (Fig. 1). The detection limit of the primer set was 10 fg of RV RNA equivalent to 10 viral particles (Fig. 2). On application of nRT-PCR on clinical specimens from 30 patients, consisting of 30 each of lens aspirates and peripheral blood samples, RV was detected in 6 of 30 (20%) lens aspirates and 4 of the corresponding peripheral blood leucocytes (13%). The results of the nRT-PCR on some of the lens aspirate specimens are shown in Fig. 3.

**Serology findings:** The results of the serology of the six nRT-PCR positive patients showed the presence of anti-RV IgG and IgM in one, only anti-RV IgG in three others and none in the other two. Among the remaining 24 patients (of patients negative for RV genome in their clinical specimens by nRT-PCR) anti-RV antibodies were not present except in three in whom anti-RV IgG alone was detected.

**DNA sequencing of the 185bp products of nRT-PCR obtained from the RV isolates:** The results of the DNA sequencing of 185bp 1st round products of nRT-PCR of three RV isolates from lens aspirates of three patients who were serologically negative was submitted to BLAST search tool of NCBI, USA to

**Table.** Primer sequences used in nested reverse transcription polymerase chain reaction (nRT-PCR) targeting the E1 gene of Rubella virus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences</th>
<th>Amplified product</th>
</tr>
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<tbody>
<tr>
<td>R1</td>
<td>5’ CAA CAC GCC GCA CGG ACA AC 3’</td>
<td>185 bp</td>
</tr>
<tr>
<td>R2</td>
<td>5’ CCA CAA GCC GCC GCG AGC AGT CA 3’</td>
<td>143 bp</td>
</tr>
<tr>
<td>R3</td>
<td>5’ CTC GAG GTC CAG GTC CTG CC 3’</td>
<td>143 bp</td>
</tr>
<tr>
<td>R4</td>
<td>5’ GAA TGG CGT TGG CAA ACC GG 3’</td>
<td>143 bp</td>
</tr>
</tbody>
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**Source:** Ref. 13
Fig. 1. Agarose gel electrophoretogram showing the specificity of the primers used in the nested reverse transcription polymerase chain reaction (nRT-PCR) targeting the E1 gene of rubella virus: Lane NC represents the negative (buffer) control; Lane NC1 represents the first round negative control; Lane 1 represents *Staphylococcus aureus* amplified product; Lane 2 represents *Mycobacterium tuberculosis* amplified product; Lane 3 represents *Chlamydia trachomatis* amplified product; Lane 4 represents Varicella zoster virus amplified product; Lane 5 represents cytomegalovirus amplified product; Lane 6 represents adenovirus type 2 amplified product; Lane 7 represents herpes simplex virus - 2 amplified product; Lane 8 represents *Candida albicans* amplified product; Lane 9 represents *Aspergillus flavus* amplified product; Lane 10 represents ECHO -11 amplified product; Lane P represents positive (Rubella virus RNA) control; Lane M represents the molecular weight marker (ΦX 174 DNA/Hinf I digest).

*Note:* The RNA was extracted from these organisms and cDNA was amplified using the nested sets of primers targeting the E1 gene of Rubella virus.

Fig. 2. Agarose gel electrophoretogram showing the sensitivity of the primers used in the nested reverse transcription polymerase chain reaction (nRT-PCR) targeting the E1 gene of rubella virus: Lane NC represents the negative (buffer) control; Lane NC1 represents the first round negative control; Lane 1 represents the neat RNA of rubella virus amplified product; Lane 2-14 represents 10 log dilutions of rubella virus RNA amplified product; Lane M represents the molecular weight marker (ΦX 174 DNA/Hinf I digest).

Fig. 3. Agarose gel electrophoretogram showing the application of the optimized nRT-PCR targeting the E1 gene of rubella virus on the lens aspirate specimens: Lane NC represents the negative (buffer) control; Lane NC1 represents the first round negative control; Lane 1-13 represents lens aspirate specimens; Lane 9, 11 represents lens aspirate positive specimens; Lane P represents positive (Rubella virus RNA) control; Lane M represents the molecular weight marker (ΦX 174 DNA/Hinf I digest).
calculate the percentage homology with the standard strains of RV (851006 K-VR 9529) and 92 per cent homology was found with that of the type species of RV.

Discussion

The results of the present nRT-PCR based study on lens aspirates indicated association of RV with congenital cataract in 20 per cent patients of less than one year age, which was higher than our earlier study based only on virus isolation. The detection rate by nRT-PCR directly on lens aspirates was 50 per cent more compared to virus isolation as RV was isolated by conventional cell culture methods in only three lens aspirates compared to six positives by nRT-PCR. Among the two cell lines, isolation rate was more in SIRC than in Vero. Bosma et al. applied RT-PCR on lens aspirates stored in liquid nitrogen and reported detection rate of 53.3 per cent compared to 20 per cent in our study in which fresh lens aspirates were used. The nRT-PCR detected RV genome in six lens aspirates including the three culture positive specimens and no false positive results were obtained. The isolates of RV were confirmed by direct DNA sequencing of the first round amplified product. Our results were comparable with that of another study from south India, where ELISA on the salivary secretions was used. Angara reported 21 per cent of RV associated congenital cataract in north India. These results may not be comparable because of the differences in the laboratory techniques. Although serological tests are supportive tests, direct detection of the viral RNA in clinical specimens by RT-PCR or by virus isolation is the ideal choice for association of RV to congenital cataract. The whole procedure of RNA extraction along with the nRT-PCR was completed within seven hours as against 7-14 days in case of virus isolation. Moreover, nRT-PCR was more sensitive compared to conventional virus isolation method. Virus isolation needs expensive maintenance of cell cultures, and is technically more demanding with results being available only after 3-4 wk. Failure to isolate the virus as happened in three of our nRT-PCR positive specimens could be attributed to low viral load in these specimens which was confirmed by the detection of the RV genome amplified product only in the second round. Use of nRT-PCR on lens aspirates appeared to be of more value than on the peripheral blood leucocytes. Serology showed minimal role for such investigations. In conclusion, the nRT-PCR was found to be a rapid, sensitive and specific technique for the detection of RV in the clinical specimens for congenital cataract patients compared to conventional methods of virus isolation and serology.

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References


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