Diagnosis of foetal rubella virus infection by polymerase chain reaction

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We have used the polymerase chain reaction (PCR) to provide a very sensitive and unequivocal test for diagnosis of foetal rubella virus infection. RNA extracted from biopsy specimens (chorionic villi), placenta or products of conception was reverse-transcribed using a rubella virus-specific oligonucleotide primer and the cDNA was amplified by PCR. The specificity of the amplified fragment was confirmed by Southern blotting. Detection of rubella virus infection in five out of 41 clinical specimens examined by this approach was shown to be entirely consistent with clinical history and other methods of laboratory diagnosis in current use. The sensitivity of the test and the unequivocal nature of the results obtained could be invaluable in providing prenatal counselling following rubella virus infection during pregnancy.

Maternal viral infection during pregnancy frequently affects the foetus. The incidence of foetal infection and the risk of congenital deformities are particularly high after rubella (Miller et al., 1982). When foetal infection is suspected, a direct intrauterine diagnosis can provide a firm basis for prenatal counselling. This has now been made possible by the availability of foetal specimens which can be obtained with relatively low risk in suitably equipped and staffed obstetrics centres. Foetal rubella virus infection can be diagnosed by detection of specific IgM in foetal blood (Daffos et al., 1984; Enders, 1987; Ho-Terry et al., 1988). This approach is of limited application because the foetus does not usually develop IgM before weeks 18 to 20 of gestation and is therefore not suitable for diagnosing first trimester infections when the risk of foetal deformities is highest (Miller et al., 1982). Viral infection can also adversely affect the development of the foetal immunological system (Fuccillo et al., 1974).

An alternative to serological diagnosis is the detection of viral RNA in placental tissues [e.g. chorionic villous samples (CVS)] by hybridization with cloned rubella virus cDNA (Terry et al., 1986; Cradock-Watson et al., 1989). This method has advantages over serology in that it is not linked to foetal development and false positive results are rare. The sensitivity is of the order of 1 to 2 pg of viral RNA but for this amount specimens of at least 15 to 20 mg wet weight are required (Cradock-Watson et al., 1989). Approximately 30% of the CVS which we receive fall below this amount. It is clear that diagnosis by detection of RNA would be more efficient and reliable if viral sequences in the biopsy material could be amplified by the polymerase chain reaction (PCR) (Saiki et al., 1985).

Previously, we have obtained the complete nucleotide sequence of the region encoding the envelope protein (E1) of rubella virus strain Judith and have located the coding sequence for a major group of antigenic determinants between nucleotides 731 and 854 (Terry et al., 1988). The sequences flanking this region are highly conserved in various wild-type strains (Frey et al., 1986; Nakhasi et al., 1986; Clarke et al., 1987; Vidgren et al., 1987) and are likely to be present in most rubella virus-infected clinical specimens. Accordingly, specific primers designed to amplify this segment of the coding sequence by PCR were synthesized. The sequences were located in the conserved regions close to useful restriction sites and were as follows: R1, 5' AACTTCAGCCC-CAAGGGGCC 3' (complementary to nucleotides 1054 to 1073, numbered according to Frey et al., 1986), and R2, 5' CAACACGCAGCCGACAAC 3' (identical to nucleotides 651 to 670).

Cytoplasmic RNA was isolated (Maniatis et al., 1982) 24 h before peak virus release from Vero cells infected with RA27/3 rubella vaccine (Smith, Kline & French), RS (a wild-type virus isolated by Dr J. Cradock-Watson) or RJ (cloned from strain Judith) at an m.o.i. of 0-01 p.f.u./cell.

Clinical specimens were divided in two. One part was cocultivated with Vero cells for virus isolation. The remaining tissue was weighed, solubilized in 4 M guanidinium isothiocyanate at 60 °C and extracted with phenol–chloroform and chloroform at 60 °C. After precipitation with ethanol, the pellet was digested with

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proteinase K (200 μg/ml) in 0.1 M Tris-HCl pH 7.4, 50 mM-NaCl, 10 mM-EDTA and 0.2% SDS at 37 °C for 30 to 60 min (Maniatis et al., 1982). Following extraction with phenol–chloroform and chloroform at 60 °C, the nucleic acids were precipitated with ethanol, washed twice with 75% ethanol to ensure complete removal of SDS and resuspended in 300 μl of 10 mM-Tris–HCl, 1 mM-EDTA, pH 8.0 (TE) containing 100 units/ml of RNasin. The RNA was purified by selective elution from an anionic exchange resin immobilized in a micropipette tip using the protocol recommended by the manufacturer (Diagen). A Diagen tip 20 was equilibrated to binding conditions by washing with 400 mM-NaCl, 50 mM-MOPS, 15% ethanol, pH 7.0. RNA in 300 μl of TE with RNAsin (prepared as described above) was adjusted to 250 mM-NaCl and 35 mM-MOPS pH 7.0, and applied to the resin. After extensive washing of the resin with equilibration buffer to remove proteins, nucleotides and other impurities, RNA was eluted in 1.05 M-NaCl, 50 mM-MOPS, 2 M-urea, 15% ethanol, pH 7.0. DNA remained bound to the resin. RNA was precipitated with isopropanol at −20 °C. After washing twice with 75% ethanol, the RNA was taken up in sterile water at a rate of 5 μl per 10 mg tissue. Recovery of RNA from Diagen tips was approximately 70 to 80%. The final concentration of RNA obtained was 2 to 5 μg/μl.

Dot blot hybridization of RNA (Ho-Terry et al., 1988) and detection of rubella virus-specific IgM (Ho-Terry et al., 1984) were carried out as previously described. In addition, rubella virus-specific IgM was tested by two commercially available diagnostic kits: RubenZ M (Northumbria Biologicals) and Rubazyme M (Abbott Laboratories). Tissue specimens used in this investigation were from patients giving unequivocal and concordant serological results by all three tests for detection of IgM. Histochemical staining with specific antibody was carried out as described by Turner (1986) except that cell monolayers on plastic were used and were fixed with ethanol and air-dried.

Rubella virus-cDNA was synthesized by reverse transcription (RT). Two μl of RNA, extracted as above, was denatured in 10 μl of 10 mM-HEPES pH 6.9, 0.1 mM-EDTA and 1 μM R1 primer at 90 °C for 2 min. Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Pharmacia; 20 units in 10 μl of 100 mM-Tris–HCl pH 7.5 150 mM-KCl, 6 mM-MgCl₂, 20 mM-DTT, 1 mM each of dNTP, 20 units of RNasin) was added at room temperature and further incubation was at 37 °C for 90 min. The reaction was extracted with phenol–chloroform and chloroform and used directly as cDNA in the PCR without precipitation.

The PCR protocol used employs the heat-stable Taq polymerase (Saiki et al., 1988) and the final conditions were: 2.5 μl cDNA, 10 mM-Tris–HCl pH 8.3 (at 25 °C), 50 mM-KCl, 1.5 mM-MgCl₂, 0.01% gelatin, 50 pmol each of R1 and R2 primers and 1.25 units of Taq polymerase (Perkin-Elmer/Cetus) in a final volume of 50 μl. Initial denaturation was at 99 °C for 3.5 min. Enzyme was added at 70 °C. Amplification was usually 40 cycles of denaturation at 95 °C for 15 s followed by annealing/extension at 70 °C for 100 s. The final extension at 70 °C was for 8 min. Thermal cycling was carried out on an Intelligent Heating Block (Hybaid) and the times represent actual times at the given temperature.

Samples of PCR reactions were electrophoresed on a 2% agarose gel, blotted onto Zeta-Probe membrane (Bio-Rad) and probed with rubella virus-specific cDNA (comprising nucleotides 765 to 1018, numbered according to Frey et al., 1986), labelled by primer extension with [³²P]dCTP (Feinberg & Vogelstein, 1983). Hybridization was at 42 °C in 50% formamide for 16 h. The filter was washed at high stringency (68 °C in 0.1 x SSC and 0.1% SDS; 1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate) for 2 h and autoradiographed using an intensifying screen for 3 to 72 h.

The 423 bp rubella virus target sequence was detected by PCR using cDNA from Vero cells infected with the vaccine virus RA27/3, RS virus and RJ virus but not from uninfected control cells (Fig. 1) indicating that the primers chosen are capable of detecting at least three different rubella virus strains and should be useful in the amplification of rubella virus-specific nucleic acid sequences in clinical material. Titration of a preparation of total RNA from RA27/3-infected Vero cells by dot blot hybridization or by RT/PCR/Southern blot hybridization showed that detection of extracted rubella RNA by PCR is at least 1000-fold more sensitive (data not shown).

The protocol was assessed in clinical specimens obtained from patients with both unambiguous history with respect to rubella virus infection and unequivocal laboratory diagnosis (Table 1 and Fig. 1). CVS from five patients were available for examination. Of these, four (specimens 5 to 8) were negative. Specimens 5 and 6 were from patients with no clinical or laboratory evidence of rubella virus infection. Specimen 7 was obtained from a patient who was in contact with rubella virus 2 weeks before conception. Under these circumstances, infection of the products of conception is unlikely (Enders et al., 1988). Specimen 8 was from a patient with serological evidence of recent rubella virus infection but no evidence of foetal infection (absence of foetal IgM; Daffos et al., 1984). Rubella virus infection was detected only in specimen 9. This was from a patient with first trimester rubella and both maternal and foetal infection were demonstrable serologically. These results are in complete agreement with the results obtained in parallel by direct dot blot hybridization of the extracted RNA and virus isolation (Table 1).
Reverse transcriptase

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1 2 3 4 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

423 bp

Fig. 1. Southern blot analysis of PCR products. RNA samples were incubated in RT reactions with (+) or without (−) reverse transcriptase. The resulting cDNAs were subjected to PCR and amplified products were analysed by Southern blotting. RNA was from Vero cells infected with rubella vaccine RA27/3 (lanes 1), RS (lanes 2), RJ (lanes 3), uninfected Vero cells (lanes 4) and from clinical specimens 5 to 15 (lanes 5 to 15). The clinical history and laboratory analysis of specimens 5 to 15 is summarized in Table 1. A negative control PCR without template was included in every set of reactions (data not shown).

Table 1. A summary of clinical history and results of laboratory investigation of individual patients included in this study*

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Type of infection</th>
<th>Foetal (20-22 weeks) or infant</th>
<th>Laboratory diagnosis</th>
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<tbody>
<tr>
<td></td>
<td>Trimester</td>
<td>Maternal</td>
<td>Specimen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HY</td>
</tr>
<tr>
<td>5</td>
<td>Serologically confirmed CMV</td>
<td>1st</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>None (for karyotyping)</td>
<td>1st</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>Clinical rubella</td>
<td>Before pregnancy</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Clinical rubella</td>
<td>1st</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Clinical rubella</td>
<td>1st</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Vaccination</td>
<td>Before pregnancy</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Clinical rubella</td>
<td>2nd</td>
<td>+†</td>
</tr>
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<td>12</td>
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<td>14</td>
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<td>1st</td>
<td>+†</td>
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<tr>
<td>15</td>
<td>Clinical rubella</td>
<td>1st</td>
<td>+†</td>
</tr>
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</table>

* CVS, chorionic villous sample; POC, products of conception (full-term or post-termination); HY, RNA dot blot hybridization; RT/PCR/S, reverse transcription followed by polymerase chain reaction and Southern blotting; Ab, fluorescence microscopy† or histochemical staining with rubella virus-specific monoclonal antibodies; ND, not determined; NA, not applicable.
† Result obtained by another laboratory.

A total of 36 additional specimens were examined. No evidence of infection was obtained by RT/PCR/Southern blotting, in 30 term placentas from individuals with neither history nor laboratory evidence of rubella (data not shown). Rubella virus infection was not detected in specimen 10 from an individual vaccinated 2 weeks before conception or in specimen 11 (Table 1 and Fig. 1) from a patient with second trimester rubella, who went to
term and delivered an infant showing no evidence of intrauterine infection (absence of rubella virus-specific IgM in the infant). Positive detection of rubella virus infection was made in specimens 12 to 15. These were all from patients with first trimester infection who underwent therapeutic abortion, rubella virus being isolated from the aborted material. Since all of the terminations occurred before 18 weeks, serological examination of foetal blood was not appropriate.

The diagnosis of foetal infection involves invasive sampling and the amount of tissue available for laboratory analysis is usually limited. The results presented above show that analysis of RNA from biopsy specimens by RT/PCR/Southern hybridization is capable of providing an unequivocal result using these small specimens. In this system, the detection of rubella virus-specific nucleic acid is achieved by both target amplification (RT/PCR) and signal amplification (Southern blot with a $^{32}$P-labelled probe). A further advantage of the test is that it should be very robust. Rubella virus is extremely thermostable. At 37 °C the half-life for infectivity is estimated to be 70 min and this property probably accounts for the low frequency of isolation from clinical specimens. A large measure of protection against non-ideal handling of specimens is provided by the use of the PCR since the RNA target size for loss of infectivity (e.g. by RNase activity) is about 12000 bases (i.e. the intact genome) but for RT/PCR is only 423 bases with the primers used here. This could probably be reduced to only 100 bases by a different choice of primers.

Following amplification, the specificity of the PCR product can be monitored by the following methods.

(i) The size of the amplified fragment. In most cases the DNA band was directly visible by ethidium bromide staining. However, if host DNA was not removed from the specimen before RT or when the high cycle numbers routinely used here were employed in the PCR to maximize sensitivity, non-specific amplified products accumulated and further confirmatory tests were required. The use of a nested pair of primers in a second PCR designed to amplify further a shorter sequence within the originally amplified fragment might provide a higher immunity to amplification of non-specific sequences when the target sequence is present in the specimen at very low copy number, without resort to additional tests. However in view of the serious consequences of incorrect diagnosis we sought more direct confirmation in every case.

(ii) Southern blot hybridization with a rubella virus-specific probe which recognizes the target sequence but does not include the primer sequences.

(iii) Testing for the presence and position of a known BamHI restriction site in the amplified target sequence. This would shorten the procedure by 1 or 2 days if the PCR product is visible by ethidium bromide staining but might lead to false negative results if the restriction site is not present in some isolates.

(iv) The absence of the PCR product in identical reactions but with reverse transcriptase omitted. Together with reactions containing no template (by deliberate omission or by using known negative specimens) included in every RT/PCR run, this control guards against possible contamination of PCR reagents by target DNA derived from handling of plasmid clones or PCR products, and ensures that positive reactions result from amplification of an RNA target sequence. Alternatively, false positive results could also be minimized by u.v. irradiation before the addition of the cDNA (Sarkar & Sommer, 1990). Under our standard PCR conditions, Taq polymerase does not show detectable reverse transcriptase activity. Jones & Foulkes (1989) have shown recently such an activity associated with Taq polymerase, but only after prolonged incubation at 68 °C and not under routine PCR conditions. We have never obtained positive PCR results from known rubella virus-positive specimens unless reverse transcriptase from either avian myeloblastosis virus or MMuLV was present in the RT reaction.

Although rubella virus is antigenically stable, variants with different growth characteristics (Oxford, 1969; Gould & Butler, 1980) and with nucleic acid sequence variations (Frey et al., 1986; Nakhasi et al., 1986, 1989; Clarke et al., 1987) have been observed. The procedure described here has been used successfully to detect a vaccine strain, a wild-type strain and a laboratory-adapted virus strain in experimentally infected cells (Fig. 1). In addition, the technique has provided clear evidence of rubella virus infection in specimens, consistent with clinical history and results from alternative methods of laboratory diagnosis (Table 1) and is currently being used in our laboratory in preference to RNA dot blot hybridization to assess the status of cases where serological tests have given equivocal results. The diagnosis of intrauterine rubella virus infection carries grave consequences for the foetus. For this reason, we have adopted very elaborate procedures throughout our investigation to ensure accurate and unequivocal diagnosis of foetal infection. This is of paramount importance if the diagnosis is to be used for prenatal counselling.

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References


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