Assessment of IgM enzyme immunoassay and IgG avidity assay for distinguishing between primary and secondary immune response to rubella vaccine

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Abstract

The primary test for the laboratory confirmation of rubella is IgM serology. It is important to distinguish IgM reactivity caused by primary infection from that caused by reinfection or persistence, especially in pregnant women; as termination of pregnancy is considered when primary rubella is diagnosed during the first trimester.

In this study, the performance of rubella IgM enzyme immunoassay (IgM-EIA) and rubella IgG avidity assay were compared using well-defined panels of sera from persons vaccinated against rubella and commercial rubella IgM and IgG enzyme immunoassay kits (Dade Behring, Marburg, Germany).

The sensitivity and specificity of rubella IgM-EIA were found to be 77.4 and 97.9%, respectively, while the results for rubella IgG avidity assay were 100 and 100%. IgG avidity assay showed higher positive and negative predictive values than the IgM-EIA (100 and 100% compared to 96.9 and 82.9%).

In conclusion, the rubella IgG avidity assay is more sensitive and specific than IgM-EIA for differential detection of primary rubella infection from rubella reinfection.

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1. Introduction

Rubella is a viral disease that results in a transient maculopapular rash, lymphadenopathy and low-grade fever (Best and Banatvala, 2000; CDC, 2001). It is generally a mild illness, and serious complications are rare. However, if acquired during the first trimester of pregnancy, there is a 90% risk of congenital malformations in the fetus. Prevention of congenital rubella syndrome is the main goal of rubella immunization and can be achieved by universal immunization of children and immunization of susceptible women of childbearing age using the live attenuated rubella vaccine (CDC, 2001). There is a significant burden of disease globally in both developed (where there is no rubella vaccination program) and developing countries as a result of congenital rubella syndrome, and the World Health Organization recommends that countries should include rubella into their immunization programs if possible (Cutts et al., 1997; Robertson et al., 1997; WHO, 2000a, 2000b). Economic studies indicate that rubella immunization programs are beneficial economically in both developed and developing countries (Hinman et al., 2002).

Reinfection with rubella is usually subclinical and occurs most commonly among pregnant women who experienced close and prolonged contact with rubella virus (Best et al., 1998). Evidence of reinfection would be accepted if a patient who had pre-existing rubella antibodies showed a signif-
The diagnosis of acute rubella infection, other viral and some microbial infections rely often on the serological detection of immunoglobulin M (IgM) antibodies, but the available techniques have serious pitfalls that may lead to erroneous interpretation (Bodeus et al., 1998; Korhonen et al., 1999). The differential assay of rubella high avidity and low avidity IgG responses. Different values of the cut-off points were reported; in some studies avidity index <30% were regarded as low avidity while avidity index >50% were interpreted as high avidity IgG responses (Thomas et al., 1992; Thomas and Morgan-Capner, 1993). In another study the avidity index higher than 60% and lower than 40% was considered as high avidity and low avidity IgG, respectively (Bottiger and Jensen, 1997), whereas in another study, the cut-off point of avidity was reported equal to 55% (Gutierrez et al., 1999). These differences may arise as the result of using small numbers of defined sera as low and high avidity panels to calculate cut-off point. In this study, the ability of rubella IgM enzyme immunoassay and rubella IgG avidity assay to differentiate between primary immune response (infection) and secondary immune response (reinfection) to rubella vaccine were evaluated, using well-defined panels of sera from rubella vaccines with and without previous immunity against rubella virus. These panels consisted of relatively large numbers of sera for precise calculation of avidity cut-off point.

2. Materials and methods

2.1. Commercial enzyme immunoassays

The commercial rubella-specific IgM and IgG enzyme immunoassays were the enzygnost anti-rubella virus IgM and IgG (Dade Behring, Marburg, Germany). All assay protocols, cut-offs and result interpretations were carried out according to the manufacturer’s instructions.

2.2. Hemagglutination inhibition assay

The hemagglutination inhibition assays were carried out according to the standard rubella hemagglutination inhibition assay instruction described by Best (Best and O’Shea, 1995) with kaolin absorption of sera for the removal of non-specific inhibitors, and pigeon erythrocytes, using four units of antigen (Dade Behring, Marburg, Germany).

2.3. Serum samples from vaccines

Five hundred paired sera from measles–rubella vaccinated people were collected as part of the measles–rubella immunity assay Program in Iran during measles–rubella mass campaign in December 2003, and stored at −80 °C. First samples had been collected before vaccination and the second samples were collected one month after vaccination. The second serum samples were classified to primary and secondary anti-rubella antibody response panels according to their immune status for rubella virus before vaccination that revealed by testing of their first serum samples for rubella-specific antibodies.
2.4. Primary anti-rubella antibody response (primary rubella infection) panel

Two hundred and sixteen serum samples were selected from measles–rubella vaccinated people, which did not contain any anti-rubella antibodies before vaccination. Their first serum samples were tested for anti-rubella IgM and IgG using above-mentioned EIA kits, which indicated that they did not have any anti-rubella antibodies. First samples of all sera also were tested by hemagglutination inhibition assay, and they were all negative for rubella antibody.

2.5. Secondary anti-rubella antibody response (rubella reinfection) panel

Two hundred and eighty-four serum samples were selected from measles–rubella vaccinated people, who had anti-rubella IgG before vaccination. Their first serum samples were tested for anti-rubella IgM and IgG using above-mentioned EIA kits, and they had anti-rubella IgG antibody. First samples of all sera also were tested by hemagglutination inhibition assay, and they were all positive for rubella antibody.

2.6. Rubella panel

Paired sera from acute rubella cases were collected as part of the measles–rubella surveillance program in Iran. Cases were confirmed as rubella when their rubella IgM-EIAs gave positive results; or if IgG seroconversion or >4-fold rise in IgG titer was detected between acute and convalescent paired sera by hemagglutination inhibition assay, and they were all negative for rubella antibody.

2.7. Anti-rubella IgM assay

All sera from above panels were carried out to anti-rubella IgM assay using the above-mentioned kits. Two local (“in-house” preparations) weak and strong positive IgM standards were included as external control in every ELISA run.

2.8. Determining optimal serum dilution for rubella IgG avidity assay

In preliminary experiment a set of 20 sera, was compared using different dilutions of sera for avidity assay (methods described below). Each sample was tested at six dilutions (1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200), and each dilution at four replicates. The highest reproducibility rates (99%) for nearly all cases were found at 1:200 dilutions; therefore, all sera were tested at that dilution.

2.9. Anti-rubella IgG avidity measurement

All the above-mentioned sera were also tested by the anti-rubella IgG avidity assay. The avidity of IgG for rubella virus was measured by a protein-denaturing enzyme immunoassay where the antibodies were first allowed to bind to the rubella virus antigen, followed by elution by buffer with and without 35 mM diethyl amine (Thomas and Morgan-Capner, 1991; Gutierrez et al., 1999). Each sample was tested at two replicates and single serum dilution (1:200) was applied to each of two replicates. After incubation for 1 h, test plates were washed four times, and then one replicate of each two replicates was soaked for 5 min in washing buffer and other remaining replicate for 5 min in washing buffer containing 35 mM diethyl amine. Fresh buffers were applied and this step was carried out twice more. The plates were then washed four times with washing buffer. Then test was continued according kit’s procedure. Remaining specific antibody was then detected, and an avidity index (AI) was calculated as follows:

\[
AI(\%) = \frac{OD\text{ wells soaked with 35 mM DEA} - OD\text{ wells soaked with wash buffer}}{OD\text{ wells soaked with wash buffer}} \times 100
\]

2.10. Statistical methods

All avidity index values obtained by testing of both primary infection and reinfection panels were analyzed by Classification and Regression Tree (CART) and Roc Curve statistical methods (Lewis, 2000) using SPLUS and SPSS softwares. Then assessing parameters including sensitivity, specificity and predictive values in differential diagnosis of rubella primary infection and reinfection for both rubella IgM enzyme immunoassay and rubella IgG avidity assay were calculated. For each parameter, 95% confidence limits were calculated. To compare two proportions the Chi-square test was used to determine the statistical significance between the parameters of the two measurements. The \( p \)-values corresponding to \( p < 0.05 \) indicated a statistically significant difference between the two assays.

2.11. Calculation of the avidity cut-off point

All avidity index values obtained from testing of both primary rubella infection and reinfection panels were analyzed by Classification and Regression Tree (CART) and Roc Curve statistic methods. Both panels’ sera were reclassified based on avidity index values to low and high avidity groups by Classification and Regression Tree (CART) method. Sera with avidity index values >53% were classified as samples containing low avidity rubella IgG and sera with avidity index values >53% were classified as samples containing high avidity rubella IgG. Classification carried out without any misclassification error rate (i.e. all cases of primary infection panel sera are regarded as low avidity and all reinfection panel sera are determined as high avidity). Similar results obtained
by Roc Curve analysis. This analysis revealed that the highest sensitivity and specificity of rubella-specific IgG avidity assay were achieved only at cut-off point equal to 53%.

3. Results

3.1. Assessment of rubella IgG avidity assay and rubella IgM-EIA sensitivity

Sensitivity of the rubella IgG avidity assay and rubella IgM-EIA were determined using primary anti-rubella antibody response (primary infection) panel and the results are presented in Tables 1 and 2. As shown in Table 1, rubella-specific IgM was detected in 167 (77.4%) serum samples out of 216 serum samples from primary rubella infection, while rubella IgM-EIA failed to detect any rubella-specific IgM in 49 (22.6%) cases of rubella primary infection panel. However, at avidity cut-off point equal to 53%, rubella IgG avidity assay detected all primary rubella infection panel sera as low avidity rubella IgG response. The sensitivity of both tests, ranges from 77.4% for rubella IgM-EIA to 100% for rubella IgG avidity assay, and the difference between these two assays is statistically significant. The failure of IgM-EIA to detect rubella IgM in a proportion of cases may arise from diminishing rubella-specific IgM from patients serum sample when their sera were collected, but these results confirms previously reported findings (Tipples et al., 2004). These results indicated that, IgM-EIA has low sensitivity for detection of current or recent rubella infection and rubella IgG avidity assay is more sensitive than rubella IgM-EIA for detection of rubella primary infection.

3.2. Assessment of rubella IgG avidity assay and rubella IgM-EIA specificity

The rubella IgG avidity assay and rubella IgM-EIA specificity assessments were carried out using the secondary anti-rubella antibody response (rubella reinfection) panel and the results are shown in Tables 1 and 2. As shown in Table 1, rubella-specific IgM was detected in six cases (2.1%) of rubella reinfection panel sera, but 278 (97.9%) remaining cases were negative for rubella-specific IgM, while high avidity anti-rubella IgG was detected in all cases of rubella reinfection. The specificity of both tests, ranging from 97.8% for rubella IgM-EIA to 100% for rubella IgG avidity assay, and the difference between these two assays is not statistically significant.

3.3. Primary infection and reinfection among naturally acquired rubella

According to a calculated avidity cut-off point value, high avidity rubella IgG were detected in 26 cases out of 100 rubella panel sera (minimum avidity index, AI = 71%), while in remaining 74 serum samples low avidity rubella IgG were determined (maximum avidity index, AI = 28%). Among high avidity cases, which regarded as rubella reinfection, 23 cases (88%) had a documented history of measles, mumps and rubella (MMR) vaccination at 15 months of age and they were all above 14 years old. Their symptomatic rubella reinfection may have been resulted from secondary failure of the vaccine (i.e. diminishing of vaccine-induced immunity during a period of at least 13 years). In three other high avidity cases, the rubella vaccination status or history of rubella infection were unknown; their symptomatic rubella reinfection may have been resulted from diminished immunity against rubella induced by disremembered vaccine or rubella infection during early ages. All low avidity cases that are regarded as rubella primary infection, were from non-vaccinated people and they were all <10 years old. These findings indicate that the rubella IgG avidity assay is also competent to distinguish primary infection from reinfection in naturally acquired rubella infection.

Table 1

Distribution of rubella IgG avidity assay and rubella IgM-EIA results for the rubella primary infection and secondary reinfection panels

<table>
<thead>
<tr>
<th>Panels</th>
<th>Rubella IgM-EIA</th>
<th>Rubella IgG avidity assay</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>LA</td>
</tr>
<tr>
<td></td>
<td>#</td>
<td>%</td>
<td>#</td>
</tr>
<tr>
<td>Rubella primary infection</td>
<td>167</td>
<td>77.4</td>
<td>49</td>
</tr>
<tr>
<td>Rubella reinfection</td>
<td>6</td>
<td>2.1</td>
<td>278</td>
</tr>
</tbody>
</table>

* Total number of both panels = 500.

* Low avidity.

* High avidity.

Table 2

Relative sensitivity, specificity, predictive values of rubella IgM-EIA and rubella IgG avidity assay

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubella IgG avidity</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Rubella IgM-EIA</td>
<td>77.4 (71.8, 83)</td>
<td>97.9 (96, 99.6)</td>
<td>96.9 (94.3, 99.5)</td>
<td>82.9 (80.6, 85.2)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are 95% confidence intervals.
4. Discussion

The detection of IgM antibodies in serum samples by enzyme immunoassay is used commonly for diagnosing acute rubella infections (Best et al., 2002). The rubella-specific IgM antibodies are usually detected within 4 days after the onset of rash and for 4–8 weeks thereafter (Best and Banatvala, 2000). However, when IgM is not detected, a recent infection cannot be ruled out absolutely, since the extent of IgM antibody responses varies with each individual and some patients may not produce sufficient amounts of IgM antibodies to be detected, although they respond with detectable IgG antibodies (Inouye et al., 1984). Furthermore, high concentration of specific IgM may also be found in sera from proven cases of rubella reinfection (Thomas et al., 1992). Non-specific reactivity in IgM assays may also occur and persistent specific IgM reactivity has been detected in the absence of primary rubella infection or rubella reinfection (Thomas et al., 1992). This issue of false positive and negative rubella IgM results is especially important when investigating suspected rubella in pregnant women because of the risk of congenital rubella syndrome and the associated critical clinical management decisions, so additional diagnostic tests should be used in such situations (Best et al., 2002; Enders et al., 1985; Health Canada, 2002; Tipples et al., 2004). Regarding the above limitations of IgM-EIA, additional confirmatory tests are therefore required. Other laboratory tests such as reverse transcription polymerase chain reaction, IgG serology on paired acute and convalescent sera, and antibody avidity testing may also be used for rubella laboratory diagnosis and confirmatory testing (Akingbade et al., 2003; Best and Banatvala, 2000; Best et al., 2002; CDC, 2001; Health Canada, 2002; Rousseau and Hedman, 1988; Thomas et al., 1992; Tipples et al., 2004).

Rubella reinfection occurs and has been reported following both natural and vaccine-induced immunity (Thomas et al., 1995). Reinfection following immunization to rubella is mostly without consequences and presents a minimal risk for the fetus (Aboudy et al., 1997). The risk of intrauterine transmission of virus associated with maternal reinfection is extremely small, since maternal immunity (vaccine or naturally induced) usually protects against intrauterine infections (Aboudy et al., 1997). Only very few cases of congenital rubella syndrome after maternal reinfection have been documented (Thomas et al., 1995). Classically rubella reinfection is proved by increase in rubella-specific IgG titer in paired sera in the absence of rubella IgM, but it is documented frequently that specific rubella IgM antibody can be detected in rubella reinfection cases (Bottiger and Jensen, 1997). The avidity of IgG antibodies test seems to differentiate primary rubella infection from past infection and reinfection in a single serum sample (Inouye et al., 1984). An increase of rubella IgG antibody avidity was seen to occur during the first 3 months after the onset of rash (Aboudy et al., 1997; Bottiger and Jensen, 1997). Measurement of rubella IgG avidity is a good supplemental test for cases with rubella IgM reactivity to confirm or exclude a recent rubella infection or reinfection.

The most critical point of IgG avidity assay is precise calculation of the cut-off point for differentiating low avidity IgG from high avidity IgG. Therefore, it was essential that we have had panels with certain immune status before vaccination. In this study, all samples of both panels were composed of paired (before/post-measles–rubella vaccine) sera obtained from ages between 5–25-year-old populations in the context of measles–rubella immunity assay in Iran. Therefore, the anti-rubella IgG status of all cases before measles–rubella vaccination, were known. In the present study, a large number of these samples were tested by IgG avidity assay, and the results were analyzed by two statistical methods (Classification and Regression Tree and Roc-curve). Avidity index equal to 53% was determined as cut-off point by either of two methods. At this cut-off point, the most desired sensitivity and specificity (100 and 100%) were achieved to IgG avidity assay.

Rubella IgM specific antibody was detected in 77.4% of primary infection panel sera, but in 22.6%. IgM-EIA provided negative results. On the other hand, in these cases IgM-EIA could not demonstrate primary infection. In one study, it was shown that the percentage of rubella-specific IgM positive sera decreased from 100% at 15–28 days after the onset of infection through 71, 28 and 9% at 1–2, 2–3 and 3–4 months, respectively. After four months, all became negative for rubella-specific IgM antibody. However, low avidity specific IgG was detected in all of sera taken at 3 months. At 3–4 months 91% and at 5–7 months 21% of sera still showed low avidity (Thomas et al., 1992). In another study, the time course of maturation of rubella IgG avidity after acute infection was demonstrated. In above-mentioned study, well-characterized serial samples from 15 patients with acute rubella were tested and followed up to 5 months after the onset of rash. A high avidity index (>60%) was not observed until 13 weeks after infection (Bottiger and Jensen, 1997). Therefore, the differential assay of high avidity and low avidity IgG can be used as an alternative or a complementary test to the IgM antibody assay and is gaining popularity as a diagnostic method for the assessment of the time of infection (Korhonen et al., 1999).

Our results revealed that IgM could be detected in 2.1% of rubella reinfection panel cases that have high avidity IgG. These cases displayed a reinfection by rubella vaccine strain. As mentioned above rubella-specific IgM antibody can be detected in both primary infection and reinfection, thus the specificity of IgM-EIA decreases to differential diagnosis of these cases. It is essential that correct diagnosis of primary rubella can be achieved for the management of the pregnant women with a recent rash or contact with a rubelliform rash illness (Inouye et al., 1984). With the introduction and widespread use of the rubella vaccine, it is likely that, with time, relatively fewer cases of rubella infection during pregnancy will be primary infection and more will be rubella reinfection (Aboudy et al., 1997; Thomas et al., 1995). Therefore,
et al., 1992).

In this study, rubella IgM-EIA was compared with rubella IgG avidity assay. Results showed that sensitivity of IgM-EIA for diagnosing of primary rubella infection is 77.4% and IgM-EIA could not detect all the primary infection. However, IgM-EIA has appropriate specificity (97.9%) and it can detect non-primary rubella cases. Similar results have demonstrated in other studies. Previously the sensitivity and specificity of seven commercial rubella-specific IgM kits were assessed and it was shown that sensitivity of the most kits was within the ranges of 66.4–78.9% (median 73.9%). Specificity of these kits estimated to be between ranges of 85.6 and 96.1% (median 92.6%). In above-mentioned study, sensitivity and specificity of IgM-EIA for detection of rubella infection had shown by Behring’s indirect EIA kit to be 75.9 and 98.7%, respectively (Tipples et al., 2004). Another study has evaluated the sensitivity and specificity of 15 commercial rubella-specific IgM kits. Specificity of these kits was found to be in the range of 82–98% and their sensitivity was 63–92%. In above-mentioned study sensitivity and specificity of Behring’s kit has been reported to be 79 and 96%, respectively. In this study, a higher value of sensitivity is attributed to IgM capture EIA kits (Hudson and Morgan-Capner, 1996).

Our study shows that positive predictive value (PPV) and negative predictive value (NPV) of IgM-EIA are 96.9 and 82.9%, respectively. When the prevalence of rubella is low, such as in countries with high rubella vaccination cover-
age, the positive predictive value of IgM testing decreases such that there is a significant risk of false positive results, and additional confirming tests are therefore required (Best et al., 2002; Tipples et al., 2004). The measurement of rubella-specific IgG avidity is a specific and sensitive method for the serological diagnosis of recent primary infection and provides the distinction between primary infection and reinfection possible (Gutierrez et al., 1999).

Testing the natural rubella panel sera revealed that, the avidity cut-off point, which obtained from primary rubella infection and reinfection panels are applicable to naturally infected patients. Regarding calculated cut-off point of avidity, IgG avidity assay can detect all patients who had documented history of rubella vaccination, as rubella reinfection, and illness of non-vaccinated people are diagnosed as primary rubella infection.

Rubella IgG avidity assay is also useful in congenital rubella syndrome cases. Without appropriately timed specimen collection, serological confirmation of congenital rubella infection may be a problem. Rubella-specific IgM has been detected in all confirmed cases until the age of 3 months, in 86% of infants aged 3–6 months, and in 62% of infants aged between 1 year and 18 months, but rarely in children over 18 months of age (Best and Banatvala, 2000). Studies had revealed that the maturation of the immune response to rubella virus is abnormally slow in congenital rubella cases both in terms of the isotype switching and especially the development of high avidity specific IgG1 (Thomas et al., 1993). Thus, avidity studies can be useful for serological diagnosis of congenital rubella even after disappearance of specific IgM, because of low avidity IgG can be detected up to the age of 3 years (Best and Banatvala, 2000; Herne et al., 1997).

With the introduction and widespread use of the rubella vaccine, it is likely that with time, relatively fewer cases of rubella infection during pregnancy will be primary infections and more will be rubella reinfection. Therefore, it will be more appropriate that rubella IgG avidity assay be used as an alternative or complementary assay to testing rubella suspected cases especially in pregnant women, because of this the test is likely more precise and sensitive than rubella IgM-EIA and can distinguish primary rubella infection from rubella reinfection.

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