Problems in Determining Immune Status in Borderline Specimens in an Enzyme Immunoassay for Rubella Immunoglobulin G Antibody

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A total of 374 sera, found by enzyme-linked immunosorbent assay (Rubazyme; Abbott Laboratories, North Chicago, Ill.) to have borderline rubella antibody levels, were tested by hemagglutination inhibition. All sera had Rubazyme indexes in the range of 0.500 to 1.499. Rubazyme sensitivity was 59.0%, and specificity was 80.8%. The predictive value of Rubazyme-positive result was 91.4%, and that for a negative result was 36.4%. Immune and nonimmune results were significantly different between the two methods (P < 0.001). The same sera were retested with the Rubazyme test, with an inter-run agreement of 75.3%. A significant difference in Rubazyme indexes between runs (P < 0.001) was observed. An alternative method of testing specimens in the range close to the Rubazyme index cutoff value of 1.000 may be indicated.

The enzyme-linked immunosorbent assay (ELISA) for rubella immunoglobulin G (IgG) antibody has received wide acceptance as a method of screening sera for evidence of immunity to rubella. In our laboratory, the Rubazyme ELISA test (Abbott Laboratories, North Chicago, Ill.) has been used to test over 22,000 sera, primarily for the purpose of assisting in immunization decisions and for monitoring the level of rubella immunity in the New Mexico female population. The majority of these tests have yielded unequivocally positive (immune) or negative (nonimmune) results, but we have encountered problems with the reliability of data falling near the recommended cutoff value, that is, for specimens in what we call the borderline region. The purpose of this study was to define the sensitivity, specificity, and inter-run reproducibility of the Rubazyme test for specimens in this borderline area.

The Rubazyme test is a plastic bead ELISA method, using rubella virus antigen to capture patient rubella IgG and horseradish peroxidase-labeled goat anti-human IgG to form a sandwich on the bead surface. Bound enzyme is then detected by incubation with o-phenylenediamine · 2HCl substrate and subsequent spectrophotometric measurement of the end product at 492 nm. The serum of a patient is considered positive for rubella IgG antibody if its absorbance is equal to or greater than the mean of two low positive control values in the same test run; i.e., the Rubazyme index (RI) equals at least 1.000 (patient specimen optical density ÷ low positive control mean optical density). For a given test run to be considered valid, a concurrent high positive control serum must have an absorbance of at least 0.700, being at least 1.80 times as great as the absorbance of the low positive control mean.

We have been concerned to find in our day to day experience that even when all control criteria are met, specimens which first give negative test results (RI ≤ 0.999) sometimes, on subsequent retest, prove to be positive (RI ≥ 1.000), and vice versa. Thus, we must repeat tests on reference specimens and when interlaboratory discrepancies are brought to our attention. A certain amount of intra- and inter-run variability is to be expected, and some specimens which contain low antibody levels will of course cross over the 1.000 cutoff point. However, we have been concerned by the apparent frequency with which this happens and by the large variations in RI values observed. These findings have led to confusion and uncertainty in making practical, qualitative judgments about immunity or nonimmunity to rubella.

Here we report the results of a two-phase study of (i) the sensitivity, specificity, and predictive value, and (ii) the inter-run reproducibility of the Rubazyme method for sera with initial RI values falling between 0.500 and 1.499, referred to as borderline. In our laboratory, over 20% of all sera tested for rubella IgG fell in this range. The sera chosen for this study were routine specimens submitted to the New Mexico Scientific Laboratory Division over a 2-month period. Of 324 sera examined in this study, 162 were in the 0.500- to 0.999 range, and 162 were in the 1.000-to-1.499 range on initial testing. After the specimens were tested initially, they were frozen at −20°C for no longer than 2 months, thawed, and rerun in one batch. All specimens were tested according to the instructions of the manufacturer, using the same lot of reagents (lot 51-684HR).

In the first phase, Rubazyme results were compared qualitatively to hemagglutination inhibition (HI) results for the same sera. The HI test was performed according to the U.S. Public Health Service publication Rubella Hemagglutination-Inhibition Tests (4), using heparin-MnCl2 treatment for removal of nonspecific inhibitors and trypsinized human O cells. The starting serum dilution was 1:8. Individuals with titers of ≥8 were classified as immune. The results are shown in Table 1. The Centers for Disease Control has stated that "the presence of any level of HI antibody (≥1:8) indicates past rubella infection at some undetermined time and thus immunity to primary infection" (1, 2). Therefore, an HI result of ≥1:8 is the criterion against which other tests of rubella immunity are measured. For sera with initial Rubazyme test results falling in the borderline region, we found 59.0% sensitivity, 80.8% specificity, 91.4% predictive value for a positive test, and 36.4% predictive value for a negative test, using HI as a standard. The formulas used were as follows: sensitivity = [true positives/(true positives + false negatives)] × 100%; specificity = [true negatives/(true negatives + false positives)] × 100%; predictive value,

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positive = \{true positives/true positives + false positives\} \times 100\%; and predictive value, negative = \{true negatives/ (true negatives + false negatives)\} \times 100\%. True-positive and -negative results were based on HI. Agreement between the two methods was 63.9\%. These findings differ from those published by the manufacturer for sera in all RI ranges (1). Those data indicated 99.5% sensitivity, 98.8% specificity, 99.7% predictive value for a positive test, and 98.8% predictive value for a negative test. When compared qualitatively (positive and immune or negative and nonimmune), Rubazyme results for borderline sera were significantly different from HI results for the same sera ($X^2 = 35.8, P < 0.001$).

In the second phase, the 324 sera were tested again by the Rubazyme method, and the two trials were compared. The results are shown in Table 2. When paired RI values for Rubazyme trials one and two were compared by the twosided Student's $t$ test, they were found to be significantly different ($P < 0.001$). The standard deviation of intertrial differences was 0.454. Specifically, the differences lay mainly in the negative specimens (0.500 to 0.999); values were significantly different on retesting ($P < 0.001$). However, there was no significant difference between trials for positive specimens (1.000 to 1.499) (0.10 < $P < 0.20$). When the same data were expressed qualitatively, marked differences between test runs were still evident (Table 3). Interrun agreement was 75.3\% (244 of 324). The predictive value for a positive test in trial one with respect to trial two was 82.7\%, and the predictive value for a negative test was 67.9\%.

These findings confirmed our initial impression that RI values vary significantly for given specimens on subsequent retesting, at least for specimens close to the immune-nonimmune cutoff point. This wide variation accounts for changes in qualitative interpretation when specimens are retested. Variation may be equally great in specimens with higher and lower RI values (i.e., <0.500 and $\geq 1.500$), but such variation would go unnoticed if it did not affect immune status determination. The variation in RI values for the same specimen between runs was so great that there was only 75.3\% qualitative agreement in borderline specimens.

The comparison of the Rubazyme test with HI suggested that a single Rubazyme test may not be accurate for assessing immune status in borderline specimens, especially for negative sera. Qualitative agreement between the two methods was not high.

When extrapolated to rubella immunity screening programs, the present data have significant implications. For example, in our laboratory, we are currently testing about 15,000 specimens yearly for rubella antibody. Based on a review of past results, ca. 7.4\% (1,110) of these specimens in a given year would be predicted to have an RI between 0.500 and 0.999. With HI as a standard, 706 of these Rubazyme nonimmune results (64\%) would in fact be false negative. Also, when compared simply to a second Rubazyme test of the same specimens, 357 (32\%) of the negative results would be false. Most of the patients involved would receive unnecessary immunizations, with the accompanying economic waste and medical risks. The RI cutoff value of 1.000 is a conservative one, perhaps designed to avoid reporting of false-immune results. Nevertheless, too high a percentage of false-negative reports is equally unacceptable.

The problems revealed here are not peculiar to the Rubazyme method but, rather, are intrinsic in assigning a qualitative clinical interpretation (immune or nonimmune) to a quantitative ELISA result (RI) with no allowance for a range of indeterminate values. Other practical problems have been reported in the application of newer ELISA methods to rubella serology. Shekarchi and colleagues observed that sera with very low or high antibody levels frequently gave absorbance readings which were outside the linear part of their reference curve (5). Truant and co-workers found, in comparing HI with ELISA, that single specimens may not be quantitatively comparable (6). In another report, Kleeman and colleagues found that ELISA is more sensitive than HI, resulting in a large number of ELISA-positive, HI-negative sera which were found by absorption to be truly rubella specific (3).

### Table 1. Comparison of Rubazyme and HI tests for rubella immune status determination in borderline specimens

<table>
<thead>
<tr>
<th>Rubazyme test result</th>
<th>No. of sera with indicated test result (% of total)</th>
<th>Hemagglutination inhibition</th>
<th>Correlation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive(^a)</td>
<td>$(n = 251)$</td>
<td>Positive $(n = 73)$</td>
<td>148 (45.7) 14 (4.3) 63.9</td>
</tr>
<tr>
<td>Negative</td>
<td>$(n = 103)$</td>
<td>Negative $(n = 59)$</td>
<td>134 (41.4) 28 (8.6) 75.3</td>
</tr>
</tbody>
</table>

\(^a\) Titer, $\geq 18$.

\(^b\) RI, $\geq 1.000$.

### Table 2. Comparison of RI values on repeat testing of borderline specimens

<table>
<thead>
<tr>
<th>Sera (initial test value)</th>
<th>No. of sera tested(^d)</th>
<th>$\bar{d}$</th>
<th>$s$</th>
<th>Student's $t$ test statistic(^d)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (RI = 0.500–0.999)</td>
<td>162</td>
<td>-0.134</td>
<td>0.392</td>
<td>-4.351</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Positive (RI = 1.000–1.499)</td>
<td>162</td>
<td>-0.054</td>
<td>0.519</td>
<td>-1.324</td>
<td>0.10 &lt; $P$ &lt; 0.20</td>
</tr>
<tr>
<td>Both (RI = 0.500–1.499)</td>
<td>324</td>
<td>-0.098</td>
<td>0.454</td>
<td>-3.889</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^d\) Each tested in two trials.

\(^b\) Mean RI differences ($\bar{d}$) between trials for each sample (RI trial 1 – RI trial 2).

\(^c\) Sample standard deviation of RI differences ($s$) between trials for each sample.

\(^d\) $t = \bar{d}/SD$, where $SD = s/\sqrt{n}$. SD, Standard deviation.

### Table 3. Comparison of qualitative results for two separate rubazyme tests for Borderline specimens

<table>
<thead>
<tr>
<th>Rubazyme trial</th>
<th>No. of sera with indicated test result (% of total)</th>
<th>Correlation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubazyme trial 1</td>
<td>$(n = 186)$</td>
<td>Positive $(n = 110)$</td>
</tr>
<tr>
<td>Rubazyme trial 2</td>
<td>$(n = 138)$</td>
<td>Negative $(n = 110)$</td>
</tr>
</tbody>
</table>

\(^a\) RI, $\geq 1.000$. 
The potential of ELISA screening of rubella immunity is great, but there is a need to (i) define the results directly in clinical terms, independent of correlation to other methods, (ii) improve precision so that results are qualitatively reproducible, and (iii) define the range of indeterminate values and adopt a strategy for dealing with specimens in this range.

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LITERATURE CITED