Multicenter Evaluation of a 1-h Enzyme-Linked Immunosorbent Assay for Rubella Serology

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A 1-h enzyme-linked immunosorbent assay (Rubestat) was developed for rubella virus immunoglobulin G detection. The assay used phenolphthalein monophosphate as the substrate, which, when developed, can easily be read visually. Rubestat compared very favorably to hemagglutination inhibition and commercial enzyme-linked immunosorbent assays in its ability to determine immune status. Rubestat demonstrated >97% specificity, sensitivity, and accuracy as compared with other methodologies at 10 different laboratories. The Rubestat index values were precise, with coefficients of variation for intra- and interassay variation of less than 10%. Mean index values had a linear correlation with hemagglutination inhibition titers ($r^2 > 0.97$). A population distribution of index values illustrated two distinct bell-shaped curves representing the positive and negative populations. Studies of acute and convalescent serum pairs showed Rubestat to be as accurate as hemagglutination inhibition in determining seroconversion.

The detection of antibody to rubella virus was first accomplished by neutralization (13, 22) and later by indirect immunofluorescence (2), complement fixation (15), and hemagglutination inhibition (HAI) (17). Historically, the HAI test has been the method of choice in rubella serology. In 1975, Voller and Bidwell (21) described an enzyme-linked immunosorbent assay (ELISA) for measuring rubella-specific antibody. Voller and Bidwell demonstrated that ELISA had several advantages over HAI, whereas it retained sensitivity and specificity comparable to HAI. Since the original observation, a number of other authors have described a variety of ELISA procedures with different enzyme conjugates and different solid phases (6, 8–11, 20). These procedures have had a number of limitations, including the time required to perform the test, usually 2 to 5 h, the requirement for equipment, and the need for specialized technicians. This paper presents the results of a multicenter study in which a standardized ELISA test with a 1-h protocol was evaluated relative to both standard HAI methods and commercially available enzyme immunoassay kits.

MATERIALS AND METHODS

Clinical sera. A sampling of 2,727 sera was assayed by both Rubestat and HAI or commercial ELISA at 10 different laboratories. The samples were sera routinely submitted to each center for immune status testing for rubella virus. The age and sex of the individuals were not available. Any sera showing discrepant results were retested in both test systems, during a routine run. The results of the retesting were considered final. Acute- and convalescent-phase serum pairs from 18 patients with natural rubella infection and 28 patients who received rubella vaccine were assayed at center 11, St. Luke’s-Roosevelt Hospital Center.

HAI and RUBAZYME tests. Four different HAI methods were performed in this study. Centers 1 and 2 utilized human O erythrocytes (RBCs) with heparin-MnCl2 as a serum pretreatment (Ortho), whereas centers 3, 4, and 11 used the same pretreatment but used chick RBCs (12). Center 5 used dextran sulfate-CaCl2 pretreatment and human O RBCs (3).

Kaolin pretreatment and human O RBCs were used by center 6. HAI titers of ≥1:8 were considered to indicate immunity, whereas HAI titers of <1:8 were considered to indicate lack of immunity. At centers 7 to 10, the Rubazyme assay (Abbot Laboratories, North Chicago Ill.) was performed according to the instructions of the manufacturer.

Antigen. The rubella virus (Gilchrist strain) was cultured in roller bottles of mycoplasma-free BHK-21 cells, maintained with Eagle minimal essential medium supplemented with 2 mM l-glutamine per ml and 50 µg of gentamicin (Whittaker M. A. Bioproducts) per ml. The antigen was extracted with alkaline buffers as previously described (14). The antigen was further purified by ultracentrifugation and was ether-treated for 1 h. After purification, the antigen was stored at ~70°C.

ELISA. The ELISA assay was performed in Removawell microtiter plates, (Dynatech Laboratories, Inc., Alexandria, Va.). Optimal antigen concentration was determined by block titration of antigen and conjugate. The appropriate antigen dilution was coated on the plates by using carbonate buffer as described by Voller and Bidwell (21). The assay was carried out by the procedure that follows, with incubations performed at room temperature (20°C to 25°C) and constant mixing on a microshaker. (i) The antigen-sensitized plates were rinsed twice with PBS-Tween 20 buffer by filling all wells and immediately emptying them. The wells were refilled and allowed to soak for 5 min and then emptied and dried on paper towels. (ii) The test serum and controls were prediluted in standard HAI trays by adding 10 µl of serum to 200 µl of serum diluent. Of each prediluted serum, 100 µl was added to a well in the reaction plate. The plate was incubated on a mixer for 15 min. (iii) The plate was washed as in step (i). (iv) Each well was filled with 100 µl of alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G (IgG). (v) After a 15-min incubation on a mixer, the plate was washed as in step (i). (vi) The wells were then filled with 100 µl of the substrate, phenolphthalein monophosphate in diethanolamine buffer (pH 9.8). (vii) After mixing for 15 min the reaction was stopped with 200 µl of 0.1 N NaOH. The pink-colored reaction was read spectrophotometrically on a Dynatech reader at 550 nm. (viii) Assay controls included

* Corresponding author.
TABLE 1. Rubestat performance data compared with that of HAI or Rubazyme

<table>
<thead>
<tr>
<th>Center (method)</th>
<th>Pretreatment type of RBCs</th>
<th>No. of sera</th>
<th>Rubestat performance (%) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitivity</td>
</tr>
<tr>
<td>(HAI) 1</td>
<td>Heparin-MnCl₂; human O RBCs</td>
<td>490</td>
<td>100</td>
</tr>
<tr>
<td>(HAI) 2</td>
<td>Heparin-MnCl₂; human O RBCs</td>
<td>137</td>
<td>99.2</td>
</tr>
<tr>
<td>(HAI) 3</td>
<td>Heparin-MnCl₂; chick RBCs</td>
<td>107</td>
<td>100</td>
</tr>
<tr>
<td>(HAI) 4</td>
<td>Heparin-MnCl₂; chick RBCs</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>(HAI) 5</td>
<td>Dextran sulfate-CaCl₂; human O RBCs</td>
<td>536</td>
<td>99.2</td>
</tr>
<tr>
<td>(HAI) 6</td>
<td>Kaolin; human O RBCs</td>
<td>646</td>
<td>97.2</td>
</tr>
<tr>
<td>(RUBAZYME) 7</td>
<td></td>
<td>110</td>
<td>98.9</td>
</tr>
<tr>
<td>(RUBAZYME) 8</td>
<td></td>
<td>173</td>
<td>100</td>
</tr>
<tr>
<td>(RUBAZYME) 9</td>
<td></td>
<td>259</td>
<td>99.6</td>
</tr>
<tr>
<td>(RUBAZYME) 10</td>
<td></td>
<td>180</td>
<td>100</td>
</tr>
</tbody>
</table>

* As percentage of HAI or Rubazyme performance.

one high titrated serum (>1:64), one negative serum (<1:8), and one low titrated serum (1:8). The control sera were assayed in triplicate in each assay. Each patient serum optical density (O.D.) was divided by the mean O.D. of the immune status serum to obtain an index value. Index values of ≥1.00 were considered positive, whereas sera with values of <1.00 were considered negative. Critical ratios for detection of a rise in antibody were determined by dividing the index for the convalescent serum by the index of the acute serum.

RESULTS

Determination of immune status cutoff value. A panel of 30 sera with HAI titers of 1:8 was assayed by Rubestat. An immune status cutoff value with an O.D. of 0.10 was determined, which was equal to that of the sera that reacted most weakly in the assay. A control serum was compounded with low positive sera and negative sera to equal this cutoff value of 0.10. The immune status control serum was run in triplicate on each subsequent assay to determine the immune status of each patient sample.

Immune status comparison. The results of the immune status comparison at each center are presented in Table 1. Sensitivity, specificity, and accuracy were obtained by considering HAI and Rubazyme values to be correct. The degrees of sensitivity and specificity varied from center to center but exceeded 96% in every case. A total of 2,772 sera were tested at the 10 centers by Rubestat and HAI or Rubazyme. Of these sera, 2,312 were positive on both the Rubestat and HAI or Rubazyme assays, and 388 were negative by both Rubestat and the alternate assay. Of the total sera tested, 22 were negative by Rubestat and positive by the alternate assay, and 5 sera were positive by Rubestat that were negative by the alternate assay; therefore, the overall sensitivity, specificity, and accuracy of the Rubestat assay was 99.1, 98.7, and 99.0, respectively. Of the 22 HAI positive, Rubestat negative sera, 15 were found at one center (6). Of these sera, eight were submitted to the Centers for Disease Control for confirmation and were found to be HAI negative, in agreement with Rubestat.

Precision. The precision of the Rubestat assay was determined by testing five different sera eight times on each of 2 different days. A Rubestat index was determined for each sample tested. The index values for each day were used to determine the intra-assay precision. The interassay precision was determined by using the index values from both days. The mean index values, standard deviations, and coefficients of variation are presented in Table 2. The coefficients of variation indicated that the Rubestat assay was reproducible both in one assay and between assays.

Correlation of Rubestat index values with HAI titers. The mean index values and standard errors for sera each HAI titer at three different centers are shown in Tables 3, 4, and 5. The index values were compared with the natural logs of the HAI titers, by standard linear regression analysis. The mean index value increased with increasing HAI titer in a linear fashion.

Comparison of Rubestat index values with Rubazyme index values. Rubestat index values (102) were compared with

TABLE 2. Intra- and interassay precision values for Rubestat index values

<table>
<thead>
<tr>
<th>Serum</th>
<th>Intra-assay (day 1)</th>
<th>Intra-assay (day 2)</th>
<th>Interassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x (SD) CV (%)</td>
<td>x (SD) CV (%)</td>
<td>x (SD) CV %</td>
</tr>
<tr>
<td>1</td>
<td>5.10 (0.277) 5.43</td>
<td>5.35 (0.409) 7.65</td>
<td>5.22 (0.362) 6.94</td>
</tr>
<tr>
<td>2</td>
<td>7.01 (0.273) 3.89</td>
<td>7.00 (0.621) 8.87</td>
<td>7.01 (0.463) 6.61</td>
</tr>
<tr>
<td>3</td>
<td>3.29 (0.201) 6.88</td>
<td>2.98 (0.175) 5.87</td>
<td>2.95 (0.185) 6.27</td>
</tr>
<tr>
<td>4</td>
<td>7.57 (0.292) 3.86</td>
<td>8.69 (0.439) 5.05</td>
<td>8.13 (0.685) 8.43</td>
</tr>
<tr>
<td>5</td>
<td>1.34 (0.064) 4.78</td>
<td>1.35 (0.132) 9.78</td>
<td>1.35 (0.100) 7.41</td>
</tr>
</tbody>
</table>

* Each sample was tested eight times on 2 days. x, Mean index value; CV, coefficient of variation.

TABLE 3. Comparison of mean Rubestat index values and HAI titers obtained at center 1

<table>
<thead>
<tr>
<th>HAI titer</th>
<th>No. of sera</th>
<th>Mean Rubestat index value (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8</td>
<td>80</td>
<td>0.34 (0.019)</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>1.40 (0.083)</td>
</tr>
<tr>
<td>16</td>
<td>59</td>
<td>2.12 (0.094)</td>
</tr>
<tr>
<td>32</td>
<td>124</td>
<td>3.50 (0.107)</td>
</tr>
<tr>
<td>64</td>
<td>114</td>
<td>4.89 (0.139)</td>
</tr>
<tr>
<td>128</td>
<td>64</td>
<td>5.90 (0.268)</td>
</tr>
<tr>
<td>256</td>
<td>26</td>
<td>7.00 (0.392)</td>
</tr>
<tr>
<td>512</td>
<td>5</td>
<td>8.93 (1.670)</td>
</tr>
</tbody>
</table>

* Coefficient of determination, 0.99.

TABLE 4. Comparison of mean Rubestat index values and HAI titers obtained at center 5

<table>
<thead>
<tr>
<th>HAI titer</th>
<th>No. of sera</th>
<th>Mean Rubestat index value (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8</td>
<td>42</td>
<td>0.34 (0.026)</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>1.50 (0.233)</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>1.82 (0.117)</td>
</tr>
<tr>
<td>32</td>
<td>15</td>
<td>2.60 (0.277)</td>
</tr>
<tr>
<td>64</td>
<td>110</td>
<td>3.94 (0.112)</td>
</tr>
<tr>
<td>128</td>
<td>148</td>
<td>5.37 (0.131)</td>
</tr>
<tr>
<td>256</td>
<td>99</td>
<td>6.45 (0.177)</td>
</tr>
<tr>
<td>512</td>
<td>42</td>
<td>7.77 (0.321)</td>
</tr>
</tbody>
</table>

* Coefficient of determination, 0.98.
Rubazyme index values by standard linear regression analysis. The values were obtained from center 7. A correlation coefficient of 0.884 was obtained with a slope of 2.21 and y-intercept of 0.77. The Rubazyme average of the positive population was 2.09, whereas the Rubestat average was 3.85. Index values at the cutoff of 1.00 were very close. However, as the index values increased, the Rubestat values became larger than the Rubazyme values. The Rubazyme values for more strongly reactive sera plateaued, thus making it difficult to estimate antibody differences in these sera.

Population distribution of Rubestat index values. The distribution of Rubestat index values for a general population is illustrated in Fig. 1. Index values for 536 sera from center 5 were plotted relative to the frequency of each index value. Approximate best-fit curves were drawn to enclose the data points. Two bell-shaped curves were evident, one for the positive population and one for the negative population. There was also a sharp delineation between the two populations.

Quantitation ability of Rubestat. Rubestat index values were determined for serial twofold dilutions of six positive sera. The index values were linear with the log₂ of dilution as analyzed by linear regression (Table 6), thus allowing for linear quantitation from a single serum dilution. As previously described (1, 9-11), critical ratios for ELISA kits produced by Whittaker M. A. Bioproducts are interpreted as highly indicative of an active infection if the values are ≥1.47. These values are equivalent to a fourfold or greater increase in HAI titer. Critical ratios are presented in Table 7 for 18 patients with natural rubella virus infection. In every case, there was a fourfold increase in HAI titer and a critical ratio of ≥1.47. In three cases the acute sera were HAI positive and Rubestat negative. All three sera were shown to be IgM positive by sucrose density gradient separation. Critical ratios for 28 patients who received rubella virus vaccine are included in Table 8. In all cases the critical ratio was ≥1.47. In one instance the Rubestat was positive for the convalescent serum, and the HAI was negative. This serum was not retested by another method.

### DISCUSSION

The data presented on sensitivity, specificity, and accuracy illustrate that the Rubestat assay is substantially equivalent to HAI for determining immune status to rubella virus. The data from each individual center fluctuated in terms of sensitivity and specificity. This was probably due in part to the use of different methods for the HAI testing at the centers, thus making one HAI test more sensitive than another. Differences in HAI assays from center to center have been reported in Centers for Disease Control surveys (18) and College of American Pathologists surveys (16). Therefore, it is impossible to standardize an assay to obtain 100% correlation at all laboratories. It has been reported that ELISA assays can be standardized to be more sensitive than HAI (4, 7). However, there are not enough data illustrating that low levels of antibody, undetectable by HAI, are actually protective against rubella infection. Until more clinical information becomes available regarding these patients, the Rubestat assay will yield results that are comparable to those obtained by the HAI method recommended by the Centers for Disease Control (12). As could be expected, the sensitivity, specificity, and accuracy of Rubestat and Rubazyme were essentially equal.

Mean Rubestat index values were shown to increase linearly with HAI titers; however, the values were quite varied for each titer. This phenomenon has been reported for rubella (10, 19, 23) and for measles virus serology (1), due to the different populations of antibodies measured by the assays. The HAI measures only antibody to rubella antigen that hemagglutinates, whereas theoretically the ELISA would detect any IgG antibody with specificity to rubella virus. The Rubestat index values were shown to correlate with Rubazyme index values; however, the sera that reacted strongly in the Rubazyme assay plateaued in O.D. This agrees with a previous report that sera with HAI titers of >64 were difficult to discriminate in the Rubazyme assay (C. Kroff, G. J. Haller, and J. A. Franco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C115, p. 330).

The population distribution curve of index values simulated a normal population of patients. Two bell-shaped curves were shown, signifying a normal distribution of values for the positive and negative populations. There was a clear-cut separation between the two populations, demonstrating that the assay delineates positive sera from negative sera. As with any assay, there will be a small percentage of sera near the immune cutoff level, for which it will be more difficult to determine immune status.

### Table 5. Comparison of mean Rubestat index values and HAI titers obtained at center 3*

<table>
<thead>
<tr>
<th>HAI titer</th>
<th>No. of sera</th>
<th>Mean Rubestat index value (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8</td>
<td>38</td>
<td>0.37 (0.036)</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>1.60 (0.364)</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>2.33 (0.206)</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>3.44 (0.421)</td>
</tr>
<tr>
<td>64</td>
<td>18</td>
<td>3.84 (0.278)</td>
</tr>
<tr>
<td>128</td>
<td>7</td>
<td>4.08 (0.653)</td>
</tr>
<tr>
<td>256</td>
<td>2</td>
<td>5.23 (2.45)</td>
</tr>
<tr>
<td>512</td>
<td>2</td>
<td>7.25 (1.96)</td>
</tr>
</tbody>
</table>

* Coefficient of determination, 0.98.

### Table 6. Rubestat index values for serial twofold dilutions of positive sera*

<table>
<thead>
<tr>
<th>Serum</th>
<th>Index value for dilution:</th>
<th>1:1</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>(\rho^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>6.36</td>
<td>4.27</td>
<td>2.91</td>
<td>1.82</td>
<td>1.00</td>
<td>0.967</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6.82</td>
<td>4.73</td>
<td>3.36</td>
<td>2.18</td>
<td>1.18</td>
<td>0.978</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>8.73</td>
<td>6.18</td>
<td>4.36</td>
<td>2.73</td>
<td>1.35</td>
<td>0.981</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>10.63</td>
<td>7.38</td>
<td>4.88</td>
<td>3.50</td>
<td>1.25</td>
<td>0.978</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>10.13</td>
<td>7.13</td>
<td>4.50</td>
<td>2.63</td>
<td>1.25</td>
<td>0.977</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>10.13</td>
<td>8.13</td>
<td>5.75</td>
<td>3.75</td>
<td>1.75</td>
<td>0.996</td>
</tr>
</tbody>
</table>

* \(\rho^2\), Coefficient of determination. Linear regression compared Rubestat index values to log₂ of dilution.
### TABLE 7. Critical ratios for acute and convalescent sera from patients with natural rubella infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample timing</th>
<th>HAI titer</th>
<th>Rubestat index</th>
<th>Critical ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Day 2 after rash</td>
<td>1:8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.294</td>
<td>12.62</td>
</tr>
<tr>
<td></td>
<td>Day 12 after rash</td>
<td>1:128</td>
<td>3.71</td>
<td>10.2</td>
</tr>
<tr>
<td>2</td>
<td>Day 1 of rash</td>
<td>&lt;1:8</td>
<td>0.353</td>
<td>6.35</td>
</tr>
<tr>
<td></td>
<td>Day 7 of rash</td>
<td>1:64</td>
<td>2.24</td>
<td>1.94</td>
</tr>
<tr>
<td>3</td>
<td>Day 3 of rash</td>
<td>1:32</td>
<td>1.41</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>Day 8 of rash</td>
<td>1:128</td>
<td>2.74</td>
<td>1.88</td>
</tr>
<tr>
<td>4</td>
<td>Day 1 of rash</td>
<td>&lt;1:8</td>
<td>0.353</td>
<td>10.42</td>
</tr>
<tr>
<td></td>
<td>Day 9 of rash</td>
<td>1:512</td>
<td>3.68</td>
<td>1.94</td>
</tr>
<tr>
<td>5</td>
<td>Day 1 of rash</td>
<td>&lt;1:8</td>
<td>0.50</td>
<td>8.48</td>
</tr>
<tr>
<td></td>
<td>Day 11 of rash</td>
<td>1:512</td>
<td>4.24</td>
<td>1.74</td>
</tr>
<tr>
<td>6</td>
<td>Day 1 of rash</td>
<td>&lt;1:8</td>
<td>0.559</td>
<td>4.94</td>
</tr>
<tr>
<td></td>
<td>Day 22 of rash</td>
<td>1:128</td>
<td>2.76</td>
<td>1.88</td>
</tr>
<tr>
<td>7</td>
<td>Day 3 of rash</td>
<td>1:8</td>
<td>1.71</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>Day 12 of rash</td>
<td>1:128</td>
<td>3.24</td>
<td>1.88</td>
</tr>
<tr>
<td>8</td>
<td>Day 2 after rash</td>
<td>1:8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.935</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>Day 10 after rash</td>
<td>1:128</td>
<td>3.74</td>
<td>1.88</td>
</tr>
<tr>
<td>9</td>
<td>Day 1 after rash</td>
<td>&lt;1:8</td>
<td>0.645</td>
<td>6.45</td>
</tr>
<tr>
<td></td>
<td>Day 12 after rash</td>
<td>1:256</td>
<td>4.16</td>
<td>1.74</td>
</tr>
<tr>
<td>10</td>
<td>Day 3 after rash</td>
<td>1:16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Day 9 after rash</td>
<td>1:128</td>
<td>3.48</td>
<td>1.88</td>
</tr>
<tr>
<td>11</td>
<td>Day 1 after rash</td>
<td>&lt;1:8</td>
<td>0.613</td>
<td>5.21</td>
</tr>
<tr>
<td></td>
<td>Day 8 after rash</td>
<td>1:128</td>
<td>3.19</td>
<td>1.88</td>
</tr>
<tr>
<td>12</td>
<td>Day 2 after rash</td>
<td>1:8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.710</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>Day 6 after rash</td>
<td>1:64</td>
<td>2.84</td>
<td>1.74</td>
</tr>
<tr>
<td>13</td>
<td>Day 4 after rash</td>
<td>1:32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>Day 12 after rash</td>
<td>1:256</td>
<td>4.52</td>
<td>1.88</td>
</tr>
<tr>
<td>14</td>
<td>Day 2 after rash</td>
<td>1:8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>Day 6 after rash</td>
<td>1:64</td>
<td>2.55</td>
<td>1.74</td>
</tr>
<tr>
<td>15</td>
<td>Day 3 after rash</td>
<td>1:16</td>
<td>2.26</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Day 10 after rash</td>
<td>1:128</td>
<td>3.94</td>
<td>1.74</td>
</tr>
<tr>
<td>16</td>
<td>Day 1 after rash</td>
<td>&lt;1:8</td>
<td>0.548</td>
<td>4.71</td>
</tr>
<tr>
<td></td>
<td>Day 5 after rash</td>
<td>1:64</td>
<td>2.58</td>
<td>1.88</td>
</tr>
<tr>
<td>17</td>
<td>Day 3 after rash</td>
<td>1:16</td>
<td>2.19</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>Day 6 after rash</td>
<td>1:64</td>
<td>3.25</td>
<td>1.88</td>
</tr>
<tr>
<td>18</td>
<td>Day 2 after rash</td>
<td>1:16</td>
<td>1.19</td>
<td>3.34</td>
</tr>
<tr>
<td></td>
<td>Day 9 after rash</td>
<td>1:128</td>
<td>3.97</td>
<td>1.88</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rubella IgM positive by sucrose density gradient.

The data for the acute- and convalescent-phase serum pairs from rubella infection and vaccination demonstrated that Rubestat was at least as sensitive as HAI in detecting a rise in antibody to rubella virus. There is evidence that Rubestat may be more sensitive than HAI for detecting rises in antibody, because in one vaccination case the convalescent serum was not positive by HAI. This serum was positive and did show a rise in antibody when diagnosed by Rubestat. Three acute sera from early natural infections were HAI positive and Rubestat negative. Since these sera

### TABLE 8. Critical ratios for acute and convalescent sera from patients receiving rubella vaccination

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample timing&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HAI titer</th>
<th>Rubestat index</th>
<th>Critical ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.677</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td>10 wks after vac</td>
<td>1:64</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.387</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td>2 mo after vac</td>
<td>1:32</td>
<td>2.26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.935</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>3 mo after vac</td>
<td>1:16</td>
<td>2.39</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.677</td>
<td>4.71</td>
</tr>
<tr>
<td></td>
<td>6 mo after vac</td>
<td>1:64</td>
<td>3.19</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.903</td>
<td>2.46</td>
</tr>
<tr>
<td></td>
<td>1 yr after vac</td>
<td>1:8</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.516</td>
<td>3.06</td>
</tr>
<tr>
<td></td>
<td>2 mo after vac</td>
<td>&lt;1:8</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.387</td>
<td>5.01</td>
</tr>
<tr>
<td></td>
<td>3 yr after vac</td>
<td>1:16</td>
<td>1.94</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.742</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>3 mo after vac</td>
<td>1:32</td>
<td>3.19</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.323</td>
<td>6.29</td>
</tr>
<tr>
<td></td>
<td>4 yr after vac</td>
<td>1:16</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
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<td>Before vac</td>
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<td>5.42</td>
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<tr>
<td></td>
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<td>1:32</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.645</td>
<td>3.26</td>
</tr>
<tr>
<td></td>
<td>4 mo after vac</td>
<td>1:8</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.903</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>4 mo after vac</td>
<td>1:16</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.516</td>
<td>4.13</td>
</tr>
<tr>
<td></td>
<td>2 mo after vac</td>
<td>1:16</td>
<td>2.13</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.645</td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td>18 mo after vac</td>
<td>1:32</td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.354</td>
<td>6.93</td>
</tr>
<tr>
<td></td>
<td>6 mo after vac</td>
<td>1:16</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.839</td>
<td>3.08</td>
</tr>
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<td></td>
<td>2 yr after vac</td>
<td>1:64</td>
<td>2.58</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.824</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>3 mo after vac</td>
<td>1:64</td>
<td>2.59</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.500</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>2 mo after vac</td>
<td>1:32</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.618</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>4 mo after vac</td>
<td>1:16</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.588</td>
<td>4.45</td>
</tr>
<tr>
<td></td>
<td>3 mo after vac</td>
<td>1:32</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.441</td>
<td>4.80</td>
</tr>
<tr>
<td></td>
<td>3 mo after vac</td>
<td>1:16</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Before vac</td>
<td>&lt;1:8</td>
<td>0.50</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>1 yr after vac</td>
<td>1:16</td>
<td>1.74</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Rubella IgM positive by sucrose density gradient.
were shown to have specific IgM antibody by sucrose density gradient separation, they would be expected to be positive by HAI. The conjugate in the Rubestat assay has specificity against only IgG; therefore, the assay will not detect antibody in sera with only IgM antibodies. Cremer et al. reported that primary serological diagnosis was improved by using ELISA assays that have specificities for only IgG (5). Since HAI measures IgG and IgM, paired sera could have stationary titers due to decreasing IgM and increasing IgG. An ELISA assay for IgG will show an increase in antibody because the decreasing IgM will not be measured.

The Rubestat assay was easy to perform, allowing up to 600 determinations to be performed in 1 day. The immune status control sera provided a simple method to calibrate for variation in laboratory temperature and other parameters that affected enzyme kinetics. The phenolphthalein monophosphate substrate, when developed, had a very distinct color, making discrimination of positive and negative sera easy. Only very weakly reactive sera needed to be read spectrophotometrically for confirmation of immune status.

In summary, the Rubestat assay is a viable alternative to the labor-intensive HAI assay and other more-time-consuming ELISA assays.

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LITERATURE CITED