Detection of Rubella Antibodies by Hemagglutination Inhibition, Indirect Fluorescent-Antibody Test, and Enzyme-Linked Immunosorbent Assay

MINAS V. ZARTARIAN,* GARY FRIEDLY, ELLENA M. PETERSON, AND LUIS M. DE LA MAZA

Microbiology Division, Department of Pathology, University of California Irvine Medical Center, Orange, California 92668

Received 17 April 1981/Accepted 25 June 1981

Using hemagglutination inhibition (HAI) as a reference method, 292 (40 non-immune, 252 immune) human serum samples were tested by indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA) for immune status and quantitation of rubella antibodies. The overall agreement with HAI for immune status was 99.7% (291/292) with IFA and 98.6% (288/292) with ELISA. Two specimens (0.7%, 2/292), negative by HAI, were equivocal by ELISA. Initially a 6.5% (19/292) overall disagreement was obtained for immune status evaluation between HAI and IFA, which was reduced to 0.3% (1/292) upon repeat testing. All of these samples were near the immune/nonimmune cutoff point (95 samples), reflecting an initial disagreement of 20% (19/95) in this category (HAI titers ≤ 1:20). Likewise, an initial overall disagreement of 4.5% (13/292) was obtained between HAI and ELISA which was reduced to 0.7% (2/292) upon repeated testing. Eleven of the 13 samples were near the immune/nonimmune cutoff point, reflecting an initial disagreement of 11.6% (11/95) with sera having an HAI antibody titer of ≤1:20. Quantitation of rubella antibodies by IFA showed an overall correlation with HAI of 86.6% within ≤twofold titer and 99.3% within ≤fourfold titer. In testing the ability of ELISA to quantitate antibody, a correlation coefficient (r) of 0.996 was obtained by plotting the measured average optical density (405 nm) of ELISA against the corresponding log of HAI titer. Both IFA and ELISA showed good correlation with HAI for immune status evaluation and for quantitation of rubella antibodies. Technically the HAI was the most cumbersome to perform, whereas IFA was the least technically demanding. Originally, 308 samples were tested; 16 samples (5.2%) could not be evaluated by IFA because of a high level of nonspecific fluorescence. The strict requirement of controlling the temperature range (23 to 24°C) during substrate hydrolysis proved to be a problem with the ELISA test in our laboratory.

Hemagglutination inhibition (HAI) is currently the most widely accepted method for the diagnosis of rubella virus infection and for evaluation of immune status (4). However, the HAI test is time consuming to perform, certain inherent variables can affect its accuracy, and its interpretation is somewhat subjective (8, 12). The need for a more objective, less laborious assay has resulted in the development of the indirect fluorescent-antibody test (IFA) and enzyme-linked immunosorbent assay (ELISA). These methodologies have been adapted for use in the clinical laboratory and are now available in kit form.

In the conventional rubella IFA test developed by Brown et al., serum dilutions were added to a cover-slip culture of a cell line that was chronically infected with rubella virus (3). After a series of incubations and washings, the endpoint titer was determined by examining each culture microscopically. An automated version of the IFA test (FIAX) was recently marketed by International Diagnostic Technology, Santa Clara, Calif. Evaluations by Brody et al. and Cremer et al. showed good correlation between FIAX and HAI, when the HAI titer was ≥1:64, but only fair agreement at lower HAI titers (2, 6).

The ELISA test for rubella was first used by Voller et al., who found an overall discrepancy rate of 3.7% as compared with various HAI antibody levels (15). The ELISA method has been used in other viral assays, and preliminary data indicate that it could be used for the diagnosis...
of most viral infections (9). An almost linear relationship between viral antibody titers and ELISA optical density was demonstrated by several investigators (7, 13–15). ELISA has been found to be objective, sensitive, specific, and more economical than current methodologies.

In this study we compared rubella immune status evaluation by IFA, ELISA, and HAI. Titers obtained by HAI were compared to those obtained by IFA, and the ELISA optical density was correlated with the HAI titers.

**MATERIALS AND METHODS**

**Specimens.** A total of 308 serum specimens submitted to the Microbiology Laboratory at the University of California at Irvine Medical Center were tested by the three methods. The serum samples were obtained from the prenatal clinic, nursing personnel undergoing preemployment physicals, and marriage certificate applicants. Patients suspected of having current infection with rubella virus were not included. The sera were tested by HAI first, and then stored at −40°C for no longer than 6 months until subsequent testing by HAI and ELISA.

**HAI.** The RUBA-tect kit (Abbott Laboratories, North Chicago, Ill.) used in the HAI study employs the heparin-MnCl₂ serum treatment method. The HAI test was performed in accordance with the manufacturer’s instructions except that incubation with 50% Duracyte suspension in the serum treatment procedure was extended from 1 h to overnight incubation at 2 to 8°C. Twofold dilutions from 1:10 to 1:1,280 were prepared in "V"-bottom microtiter plates. To these dilutions, 4 hemagglutination units of rubella antigen was added. After 1 h of incubation at 2 to 8°C, indicator cells (0.4% Duracyte) were delivered to all wells. After 60 to 90 min of incubation at 2 to 8°C, the results were read using a reader mirror. The titer was reported as the highest dilution showing a complete inhibition pattern (sharp compact button = no agglutination). A serum control for each specimen, cell control, and three control sera (high positive, low positive, negative) were included with each run. The working dilution of antigen (4 hemagglutination units) was checked in triplicate by back titration. An antibody titer of ≥1:10 was considered immune, whereas <1:10 was considered nonimmune.

**IFA.** Test kits supplied by International Diagnostic Technology were used in the IFA study as outlined in the package insert. Dual-surface StiQ samplers provide a solid-phase rubella antigen on side one and a blank on side two. All incubations were carried out at room temperature on a horizontal shaker supplied by International Diagnostic Technology. The StiQ samplers were immersed in a 1:40 dilution of serum and incubated for 30 min. Next, the samplers were washed briefly and immersed in fluorescein-labeled goat anti-human immunoglobulin. After a final wash, the samplers were read by a fluorometer (International Diagnostic Technology). The fluorescence readings were transmitted to a microcomputer (International Diagnostic Technology) capable of subtracting the background from the test reading to evaluate net fluores-

**of rubella agglutination.** The highest titer was assigned to the continuous FIAX titers (8, 9, 10, etc.) to accommodate the twofold nature of the HAI titers (1:10, 1:20, 1:40, etc.) (Table 1). A difference greater than a fourfold titer between HAI and IFA was considered a disagreement and treated in the same manner as a disagreement for immune status.

**ELISA.** The Rubelisa kit (M. A. Bioproducts, Walkersville, Md.) was used in the ELISA study. Strips, each equipped with 10 joined cuvettes, were furnished by the manufacturer as the solid-phase component. Even-numbered cuvettes were coated with rubella antigen whereas the odd-numbered cuvettes contained background antigen. The background antigen was made up of noninfected cells and served as the blank, subtracting from the tests all color due to nonspecific binding. A 1:50 dilution of the serum was delivered to a consecutive pair of cuvettes (blank, test). The cuvettes were incubated at 23 to 24°C in a moist chamber for 2 h. After washing with buffer, alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G (IgG) was added and incubated for 2 h at 23 to 24°C. The cuvettes were then thoroughly washed with buffer. After removal of the buffer, the enzyme-substrate reaction was started by the addition of the substrate, p-nitrophenyl phosphate. This step was performed on the Gilford PR-50 dispenser which is a part of a semiautomated assembly designed for ELISA assays. All fractions were read at exactly 45 min after the addition of p-nitrophenyl phosphate, at
405 nm on the PR-50 spectrophotometer. The PR-50 automatically subtracts the blank from the test and prints the net average optical density (ΔOD). A net ΔOD of ≤0.15 reflects no immunity, whereas a ΔOD of ≥0.18 reflects immunity. A ΔOD in the range 0.16 to 0.17 is inconclusive (equivocal).

Data analysis. Any disagreement with immune status was followed with a repeat run by each method. If the disagreement persisted, then it was noted as a discrepancy and counted as such; if results in agreement were obtained, the method which converted was counted for an error on the first run only, and the results were considered to be in agreement. Therefore, after the second run, if the reference method (HAI) duplicated its original result, this was considered to be the true status of the specimen, regardless of FIAX and ELISA findings. On the other hand, if the reference method failed to reproduce itself, the “accurate” status of that specimen was then decided upon by statistical analysis. The result (immune or nonimmune) which was obtained a majority of the time by all methods tested (≥4 times out of 6) was considered to represent the true status.

RESULTS

HAI versus IFA. Sixteen low-antibody (FIAX titer ≤ 16) specimens (5.2%, 16/308) were considered unsuitable for comparison due to high nonspecific background in the IFA test. With the remaining 292 sera, an agreement of 99.7% (291/292) was obtained for immune status between HAI and IFA (Table 2). Initially, 19 samples were in disagreement. After repeat runs, 18 of the 19 came to an agreement. Nine of the 18 sera were initially read as immune by IFA, all with an “assigned titer” of 10, and were in disagreement with HAI on the first run, but were shown to be nonimmune, both systems in agreement (<1:10) after the repeat runs (Table 3). Eight of 18 sera initially read as nonimmune by HAI were shown to be immune, agreeing with the HAI results after repeat testing (Table 3).

These data show that, at low antibody levels near the immune cutoff point (≤1:20), IFA was in error on the first run 9.5% (9/95) of the time with nonimmune sera. Similarly, HAI failed on the first run 8.4% (8/95) of the time with immune sera and 1.1% (1/95) with nonimmune sera, accounting for a total of 9.5% error on the first run (Table 4). Only one specimen persisted to show contradictory results for rubella immunity, with the IFA titer at 15 (immune) and the HAI at <1:10 (nonimmune). The repeat runs with this specimen gave similar results (Table 3).

A correlation of 86.6% (253/292) was established between HAI and IFA titers at an agreement level of within ±twofold titer and 99.3% (290/292) correlation within ±foursfold titer (Table 5). Ten immune sera were in disagreement for their antibody titer (>fourfold) when assayed by both methods initially. This large difference in titers was corrected for 9 of 10 specimens with the repeat runs. With three of these sera, both IFA and HAI were in error initially; with the remaining six specimens, HAI was in error on the first run with three and IFA was in error with the other three. This reflected a first run error of 3.0% (6/197) with both IFA and HAI for specimens with antibody levels of >1:20 (Table 4). The one remaining discrepancy was a low titer according to IFA (first run, 39; second run, 40) and a much higher level with HAI (first and second runs, 1:320). The background control fluorescence by the IFA for this specimen was high, near the maximum limit of acceptability, which could have affected the sensitivity even at this high antibody level.

HAI versus ELISA. A 98.6% (288/292) agreement was shown for rubella immune status between ELISA and HAI (Table 2). No errors on the first run were obtained by ELISA when testing sera with ≤1:20 HAI antibody level.

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Sera with HAI antibody for rubella virus</th>
<th>Agreement (%)</th>
<th>Disagreement (%)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>252</td>
<td>99.7</td>
<td>0.3</td>
<td>97.5</td>
<td>100</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>0</td>
<td>99.7</td>
<td>0.3</td>
<td>97.5</td>
<td>100</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td>98.6</td>
<td>0.7</td>
<td>95</td>
<td>99.2</td>
</tr>
</tbody>
</table>

This table represents the results of the 292 samples including the final data on the 23 samples that had to be retested.

Total number of specimens with ≥1:10 HAI antibody titer.

Total number of specimens with <1:10 HAI antibody titer.
Aside from the nine initial HAI errors with the low antibody sera already accounted for, four other specimens were initially in disagreement; after repeat testing, two of these four were resolved (Table 3). Both changes occurred with ELISA reflecting a first run failure of 1.0% (2/197) within the category of >1:20 HAI antibody level (Table 4). Two of the four were repeatedly read as nonimmune by ELISA, whereas HAI reported them twice immune (1:10, 1:20) (Table 3). Two equivocal results were obtained by ELISA on two nonimmune sera by HAI. These were not considered to be in disagreement since equivocal was interpreted as neither immune nor nonimmune. The ΔOD with its standard deviation for each HAI titer was calculated and plotted on semilog graph paper (Fig. 1). The corresponding coefficient of correlation by linear regression was 0.996, slope was 0.90, and intercept was 0.023. A 10 to 23% range for standard deviations was obtained (Fig. 1).

**Cost and time analysis.** Variables such as turnover time, technologist time, reagent and equipment cost and shelf life concerning all three kits (HAI/Rubatect, IFA/FIAx, ELISA/Rubella) are summarized in Table 6.

**DISCUSSION**

The results of the comparison study indicated that there was good agreement between the HAI, IFA, and ELISA methods for detecting rubella antibodies. The specificities of IFA and ELISA in reference to HAI when evaluated at the nonimmune antibody level (≤1:10) were 97.5 and 95%, respectively. The sensitivities of both methods (IFA, ELISA) were 100 and 99.2%, respectively.

This study included ninety-five sera with ≤1:20 HAI antibody titer. In this category, 18 errors on the first run, involving immune status, occurred. Nine of those errors were with HAI, and the other nine were with IFA, whereas zero errors on the first run occurred with ELISA.

Other investigators reported that the majority of disagreements between HAI and other methods occurs with sera containing a low level of antibody (5, 6). It is known that beta-lipoproteins, if not completely removed during the serum treatment procedure, will cause nonspecific

---

**Table 3. Immune status of specimens in duplicate runs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Method</th>
<th>1st run</th>
<th>2nd run</th>
<th>Assigned immune status</th>
<th>No. of specimens in each group</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HAI</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>9</td>
<td>IFA in error on the 1st run</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>+</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HAI</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>HAI in error on the 1st run</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HAI</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>HAI in error on the 1st run</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HAI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>ELISA in error on the 1st run</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>−</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HAI</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>IFA discrepant results</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HAI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>ELISA discrepant results</td>
</tr>
<tr>
<td></td>
<td>IFA</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Table 4. Index of error on the first run**

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Rubella Ab ≤1:20 (95 specimens)</th>
<th>Rubella Ab &gt;1:20 (197 specimens)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N^b</td>
<td>E'</td>
</tr>
<tr>
<td>HAI</td>
<td>9</td>
<td>9.5</td>
</tr>
<tr>
<td>IFA</td>
<td>9</td>
<td>9.5</td>
</tr>
<tr>
<td>ELISA</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Ab, Antibody.  
^b Number of specimens in error on the first run.  
^c Percent error on the first run [(N/95) × 10^9].  
^d Percent error on the first run [(N/197) × 10^9].
1:8
nine
initial
less than that of
be
experienced with
accuracy
of
titer
contribute
to
during
Rubelisa
6
63
6
3.5
FIAX
6
37.0 (17/46)
65
38.5 (25/65)
80
29.0 (18/62)
40
62
25.6 (10/39)
10
16
56.3 (9/16)
<10
40
97.5 (39/40)
HAI titer
<table>
<thead>
<tr>
<th>% IFA titers within</th>
<th>% IFA titers within</th>
<th>% IFA titers with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ twofold</td>
<td>≤ fourfold</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a N, Number of specimens.

Table 6. Cost analysis based on 50 determinations and shelf life of reagent

<table>
<thead>
<tr>
<th>Kit</th>
<th>Turnover time (h)</th>
<th>Technologist time (h)</th>
<th>Reagent cost ($)</th>
<th>Total cost* (reagent + labor) ($)</th>
<th>Additional equipment cost ($)</th>
<th>Shelf life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubatext (HAI)</td>
<td>6</td>
<td>4</td>
<td>56.00</td>
<td>100.00</td>
<td>None</td>
<td>6 mo</td>
</tr>
<tr>
<td>FIAx (IFA)</td>
<td>3.5</td>
<td>2 (1)</td>
<td>77.50</td>
<td>99.50</td>
<td>8,350 (7,150)</td>
<td>3 wk to</td>
</tr>
<tr>
<td>Rubelisa (ELISA)</td>
<td>6</td>
<td>3 (2)</td>
<td>57.50</td>
<td>90.50</td>
<td>1,525 (12,000)</td>
<td>4 mo</td>
</tr>
</tbody>
</table>

* Number in parentheses indicates time or extra cost needed accordingly when additional optional equipment was used (not necessary to perform test).

Based on technologist's hourly rate of $11.00.
FIAX-fluorometer, shaker (minimal additional equipment necessary to perform test).
Microcomputer, diluter-pipettor.
ELISA reader (minimal additional equipment necessary to perform test).
PR-50 (processor-printer-dispenser).

FIG. 1. ELISA ΔOD correlation with HAI titer.

inhibition resulting in false positives. On the other hand, specific antibody is sometimes removed during the serum treatment reducing the antibody titer (8). These problems, combined with the complexity of the HAI method, may contribute to the reproducibility failures experienced with the test at low antibody levels. The accuracy of the IFA when used to test sera in the 1:8 to 1:32 titer range has been reported to be less than that of HAI (6). In our study, the nine initial errors concerning immune status that occurred with the IFA test were all false positives in the titer range of 8 to 13. If the cutoff level of immunity in the IFA test was raised to 15, with the introduction of an equivocal zone of 8 to 14, this could improve the reliability of reporting accurate IFA results at low antibody levels. Since any detectable titer is believed to protect against subsequent rubella infection, it is essential for a particular assay to perform reliably and accurately in order to identify susceptible individuals with low antibody levels (10). Our results suggest that when a titer of ≤1:20 is obtained by either the IFA or HAI methods, it may not be possible to definitely establish immune status unless repeat testing is done. In our laboratory, where the FIAx is used to determine rubella-immune status, all specimens with FIAx titers of 8 to 14 are retested for a second time (10%). If the results are <8 on the second run, the specimens are reported as non-immune; if the first results are duplicated (≥8) they are signed out as immune. For testing specimens with high background, we use the HAI as the alternate method.

With specimens having >1:20 HAI antibody
titer, there was a 1% disagreement of ELISA from the first run (2/197), both false negatives that converted to positive on the repeat run. Two immune sera with HAI antibody titers of ≤1:20 gave false-negative results with ELISA, accounting for 2.1% (2/95) discrepancy within the low antibody category which is equivalent to a 0.7% (2/292) disagreement overall. Other investigators have also reported false negatives and false positives with ELISA. However, some sera, negative by HAI but positive by ELISA, have been substantiated to contain low antibody levels to rubella not detectable by HAI (1, 11, 13).

A prerequisite for the serological diagnosis of infectious diseases is that the test employed accurately measures all levels of antibody. We showed good correlation for antibody titers between IFA and HAI. Further testing of paired sera (acute, convalescent) is a prerequisite for confirming the diagnostic capability of FIAX.

We have demonstrated good quantitative correlation between ELISA and HAI (r = 0.996). However, due to the large standard deviations obtained with ELISA at each HAI titer, we do not recommend the reporting of antibody titer for ELISA from the correlation curve of ELISA ΔOD versus log of HAI titer.

Serum treatment to remove nonspecific inhibitors required by HAI is not necessary in the IFA or ELISA protocols. Technically, the HAI is the most cumbersome to perform, especially when large numbers of sera are to be tested, whereas FIAX is the least technically demanding. However, 16 samples originally included in the study (5.2%) could not be accurately evaluated by IFA because of a high level of nonspecific binding on the blank side of the StIQ sampler used in the FIAX test.

The strict requirement of controlling the temperature range (23 to 24°C) for a prolonged period of time (4.75 h) was a problem with the Rubelisa test for the last 45 min of incubation when the enzymatic substrate hydrolysis occurred. An incubator capable of maintaining a 23 to 24°C temperature range regardless of higher room temperature seems to be a possible solution to this problem.

In our laboratory, where we also employ the Rubetect, 11.4% of the samples exhibit nonspecific agglutination and consequently have to be retreated a second time. This problem is completely eliminated in the ELISA test. However, when ΔOD is in the equivocal range (2/292, 0.7%), the laboratory must use alternate methods before a decision of immunity can be reached.

LITERATURE CITED

Downloaded from http://jcm.asm.org/ on July 6, 2017 by guest