Evaluation of Rubella Immune Status by Three Commercial Enzyme-Linked Immunosorbent Assays

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Three commercial indirect enzyme-linked immunosorbent assays (ELISAs) (Enzygnost-Rubella, RUBELISA, and ORTHO Rubella) were evaluated for the determination of immune status by testing 1,090 serum specimens, 410 of which were from nonimmune patients. In comparison with the standard reference technique, the hemagglutination inhibition (HAI) test, the sensitivities of ORTHO Rubella (100%) and Enzygnost-Rubella (99.26%) were excellent, whereas the sensitivity of RUBELISA (95.60%) was marginally lower because of the inability of this assay to detect antibody in 22% of the serum specimens with HAI titers of 10 and 11% of sera with HAI titers of 20. The specificity of all three systems was >97%. There was a linear correlation between mean ELISA values and increasing HAI titers ($r = 0.94$). Both ORTHO Rubella and Enzygnost-Rubella were shown to be suitable replacements for the HAI test, provided that an equivocal zone is incorporated in the ORTHO system and only unheated sera are used in the Enzygnost system.

Rubella is usually a relatively mild exanthematous illness in children or adults. Infection of women in early pregnancy may result in congenital abnormalities of the fetus. The control of rubella infection in Australia is being attempted by routine vaccination of schoolgirls aged 10 to 14 years and selected immunization of nonpregnant seronegative women of childbearing age (13). Accurate determination of the immune status of women of childbearing age and diagnosis of recent infection in pregnant women are of paramount importance.

The hemagglutination inhibition (HAI) test has been considered the standard procedure for diagnosis of rubella infection and evaluation of immune status (17). This test shows excellent correlation for protection to the fetus with the more cumbersome and technically difficult neutralization test (12), which some regard as the ultimate standard. However, the HAI test is time-consuming, the incomplete removal of nonspecific inhibitors and agglutinins (7) can diminish its accuracy, and standardization between laboratories is difficult. For these reasons, most laboratories recommend further testing for any pregnant woman with a titer of 10 or 20 who comes into contact with a case of rubella or who develops a rubella-like rash. On the other hand, as the HAI test has been in use for such a long time, many clinicians are familiar with the significance of the results of this test.

In recent years, solid-phase enzyme-linked immunosorbent assay (ELISA) methods have been advocated as replacements for the HAI test. ELISA methods have the advantage of the potential for automation by which a precise numerical readout which replaces visual interpretation and quantitation can be achieved on a single dilution of serum. Further, given the required reagents and equipment, these tests are simple and rapid to perform and have been shown to have precisions similar to that of HAI (8). In-house ELISAs are being progressively replaced by a number of commercial kits. Prestandardized reagents are available so that results can be meaningfully compared between laboratories. By linking microplate readers to microcomputers through comprehensive but flexible software packages, it is now possible to take advantage of the prodigious data-handling capacity of desk-top computers, thereby allowing a single operator to process more than 2,000 immunoassays per day. The computers save time in the analysis and reporting of results. Hence, an increasing number of laboratories without experience and expertise in rubella serology are using the relatively simple commercial kits for rubella antibody assay. All kits available in Australia are of the indirect type, employing rubella antigen attached to a solid phase. However, few in-depth independent evaluations of such kits have been carried out. This communication describes the evaluation of three commercial indirect ELISA systems and compares them with an in-house HAI test for the determination of immune status.

MATERIALS AND METHODS

Clinical specimens. The study materials consisted of serum specimens from 1,090 pregnant women (680 sera were from immune patients and 410 sera were from susceptible patients as determined by HAI). The sera tested were submitted mainly by hospitals, pathologists, and medical practitioners in New South Wales for routine antenatal testing. The sera were initially tested by HAI and then stored at −20°C for no longer than 10 months until subsequent testing by the three ELISA systems.

The range of serum samples studied was purposely skewed toward seronegative and low-positive levels, because the aim of an immunity screening test is to detect susceptible individuals. Furthermore, previous works in this laboratory (unpublished) and elsewhere (3, 18, 22) have shown that discrepancies tend to occur with sera containing low levels of HAI antibodies.

HAI. An in-house microtiter technique was used (5), with trypsinized human O cells at a concentration of 0.3% with HEPES (N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 6.2) containing 0.5% bovine albumin fraction V as the diluent and 4 U of commercially available rubella antigen (Behringwerke AG, Marburg, Federal Republic of Germany). Sera were pretreated with heparin-manganese chloride to remove nonspecific inhibitors. Con-
trol sera with titers of <10, 20, 80, and 320 were included in each run.

An antibody titer of <10 was considered nonimmune, whereas a titer of ≥10 was considered immune.

ELISA. Enzyme immunoassays were conducted on all serum specimens by three indirect ELISA systems for rubella immunoglobulin G (IgG) with commercial reagents. The systems used were RUBELISA (M.A. Bioproducts, Walkersville, Md.), Enzygnost-Rubella (Behringwerke) and ORTHO Rubella IgG ELISA Test System (Ortho Diagnostic Systems, Don Mills, Ontario, Canada).

All test systems used the principle of indirect ELISA and the procedures followed were those recommended by the manufacturers, except for one modification in the Enzygnost-Rubella system. This modification involved the stopping of the enzyme reaction at 20 to 25°C after a shorter time (usually 30 min) than that recommended by the manufacturer (45 min). When the reaction was stopped after 45 min, background (control antigen) absorbance values rose considerably, resulting in some false-negative results. In comparison, it was found in an earlier study (4) that the enzyme reaction in the Enzygnost-Rubella test needed to be stopped after 10 to 15 min.

There are some differences in the principal characteristics of the three ELISA systems. The solid-phase component in the RUBELISA and Enzygnost-Rubella systems consisted of antigen attached to microwells in removable strips in a plastic tray. The ORTHO Rubella system used a microdilution plate, but only the central 48 wells were coated with antigen, presumably to remove edge effects. (The latter system is now available in a removable strip format). Except for the ORTHO Rubella system, all systems employed a control antigen to test for nonspecific reactivity.

Serum dilutions were heat inactivated at 56°C for 30 min prior to testing by the ORTHO Rubella system. Unheated sera were used in the RUBELISA and Enzygnost-Rubella systems.

All systems used an anti-human IgG conjugate; this conjugate was polyclonal with the RUBELISA and Enzygnost-Rubella systems, but the ORTHO Rubella system used a monoclonal anti-human IgG (murine) highly specific for the Fc portion of the heavy chain of human IgG (10). The compositions of serum and conjugate diluents were not available from the manufacturer. Washing was performed with a processor (Behring ELISA Processor II; Behringwerke). The absorbances of the solutions in each well were measured directly in the plate with a vertically measuring photometer. For the Enzygnost-Rubella system, Behring ELISA Processor II was used, and for the RUBELISA and ORTHO Rubella systems, an automatic reader (MR 580 MicroELISA Auto Reader; Dynatech Laboratories, Inc., Alexandria, Va.) was employed.

Cost. The cost of reagents and materials for our in-house HAI test was approximately $0.40 per test. However, the use of commercial HAI kit sets can increase this cost to approximately $2.00, which is comparable to ELISA costs ($1.90, $2.30, and $2.95 for the ORTHO Rubella, Enzygnost-Rubella, and RUBELISA systems, respectively). (All costs are expressed in Australian dollars.)

The methods for determining the presence of rubella IgG appear in detail below (see Table 3, footnotes).

Discrepant and equivocal results. All patient serum specimens showing discrepant results were tested repeatedly until consistent results were obtained. Equivocal results were retested in duplicate.

Measurement of intrassay and interassay variations. The precision (coefficient of variation) was determined by testing commercial control sera with high- and low-positive ELISA values in each of the three ELISA systems. The intrassay precision was calculated by testing seven replicates of the two controls on each of three consecutive days by the three ELISA systems. For the determination of interassay precision, the two controls had 34, 28, and 30 runs in the RUBELISA, Enzygnost-Rubella, and ORTHO Rubella systems, respectively.

RESULTS

Evaluation of immune status. The comparison of the three ELISA methods with the HAI test for the determination of immune status in 1,090 antenatal patients is shown in Table 1. Performance characteristics were calculated with reference to HAI. The RUBELISA, Enzygnost-Rubella, and ORTHO Rubella systems showed sensitivities of 95.6, 99.26, and 100%, respectively, while their respective specificities were 99.76, 100, and 97.32%.

The Enzygnost-Rubella and ORTHO Rubella systems showed excellent overall agreement with the HAI test (99.54 and 98.99%, respectively), and similarly, the Enzygnost-Rubella and ORTHO Rubella systems demonstrated the

<table>
<thead>
<tr>
<th>Method and result</th>
<th>No. of specimens that give the indicated result with the HAI test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Agreement (%)</th>
<th>Predictive value (%) for the indicated result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUBELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>633</td>
<td>95.60</td>
<td>99.76</td>
<td>95.60</td>
<td>99.84</td>
</tr>
<tr>
<td>Negative</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td>89.69</td>
</tr>
<tr>
<td>Enzygnost-Rubella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>675</td>
<td>99.26</td>
<td>100</td>
<td>99.54</td>
<td>100</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>98.79</td>
</tr>
<tr>
<td>ORTHO Rubella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>680</td>
<td>100</td>
<td>97.32</td>
<td>98.99</td>
<td>98.41</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

* Performance characteristics (sensitivity, specificity, agreement, and predictive value) are in relation to the HAI test.

Percent sensitivity = 100 × ([total number of specimens positive to the HAI test] − [number of specimens negative to ELISA but positive to the HAI test])/([total number of specimens positive to the HAI test] × [total number of specimens negative to the HAI test]).

Percent specificity = 100 × ([total number of specimens positive to the HAI test] − [number of specimens positive to ELISA but negative to the HAI test])/([total number of specimens negative to ELISA but negative to the HAI test] × [total number of specimens negative to the HAI test]).
highest combined predictive values for positivity (100 and 98.41%, respectively) and negativity (98.79 and 100%, respectively).

Comparison of discordant ELISA results in relation to HAI titer. It is noteworthy that the only discrepancies occurred with patients having HAI titers of ≤20. Table 2 shows a breakdown of the discrepancies according to the HAI titer. Of 319 sera with HAI titers of 10 and 20, 47 (14.7%) showed an absence of prior exposure to rubella when tested by the RUBELISA system, whereas only 5 (1.6%) in this group showed no antibody when tested by the Enzygnost-Rubella system. It was significant that the disagreement was greater with sera having HAI titers of 10 than with those having HAI titers of 20. Of 101 sera with HAI titers of 10, 22 (21.8%) were nonreactive when tested by the RUBELISA system, whereas of 218 sera with HAI titers of 20, 25 (11.5%) were negative. In comparing the results obtained by the Enzygnost-Rubella system with HAI titers, 3 of 101 (3%) sera with HAI titers of 10 were negative while 2 of 218 (0.9%) sera with HAI titers of 20 were negative. The ORTHO Rubella system exhibited paramount sensitivity by detecting antibody in all the HAI low-titer positive sera. These results clearly demonstrate the differences in sensitivity of the three ELISA systems in the HAI low-titer range. In this range (Table 2), only the RUBELISA system showed a sensitivity significantly different from those of the Enzygnost-Rubella and ORTHO Rubella systems and the HAI test by the chi-square test (P < 0.001), but overall (Table 1), the three ELISAs and HAI showed no significant differences in sensitivity (P > 0.10).

The difference in specificity was less pronounced. Of 410 HAI seronegative samples, only 1 was repeatedly positive by the RUBELISA system (absorbance cutoff, 0.17; RUBELISA value, 0.18). Of 11 (2.68%) sera reactive in the ORTHO Rubella system, 10 were just positive in the range from 0.20 to 0.25 (the actual standardized ELISA values obtained were 0.20 [5 samples], 0.21 [2 samples], 0.23, 0.24, and 0.25) and 1 had a value of 0.30.

Comparison of HAI titers with ELISA values. The relationship between HAI titers and mean absorbance values (with standard error) obtained for each titer group when tested by each of the three ELISA systems is shown in Table 3. This comparison showed good correlation (r = 0.94) between HAI titers, after transformation to natural logs, and the corresponding ELISA mean absorbance values. All three ELISA absorbance values were found to increase proportionately with increasing HAI titers; thus, the antibody levels determined by the ELISAs reflect HAI titers.

Precision. The results showed a high degree of reproducibility for each ELISA system. For the RUBELISA system, the coefficients of variation were 8.1 and 9.1 for the high-positive and low-positive sera, respectively, for the intra-assay and 10.0 and 9.6, respectively, for the interassay; for the Enzygnost system, the coefficients of variation for the high-positive and low-positive sera were 5.6 and 5.9, respectively, for the intra-assay and 8.4 and 9.8, respectively, for the interassay. For the ORTHO Rubella system the corresponding coefficients of variation were 6.2 and 6.5, respectively, for the intra-assay and 9.8 and 9.2, respectively, for the interassay.

**DISCUSSION**

Although ELISA methods are potentially more sensitive than HAI tests, little is known about the clinical significance (i.e., the protective immunity) of low levels of antibody which may be detected in HAI-negative sera by ELISA (11). Therefore, rubella ELISA tests are calibrated to correlate with the currently accepted standard, the HAI test. This correlation has been the aim of the manufacturers of the three ELISA systems evaluated in the current study.

While not absolutely accurate in some hands (2), the HAI test is the best approximation for immunity at present, and in the

### TABLE 2. Discrepancies in three ELISA systems according to HAI titer

<table>
<thead>
<tr>
<th>HAI titer</th>
<th>No. of sera</th>
<th>RUBELISA</th>
<th>Enzygnost-Rubella</th>
<th>ORTHO Rubella</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>410</td>
<td>1 (0.24)</td>
<td>406 (99.76)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>101</td>
<td>79 (78.22)</td>
<td>22 (21.78)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>218</td>
<td>193 (88.53)</td>
<td>25 (11.47)</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 3. Correlation between mean absorbance values determined by the three ELISA systems for rubella IgG and HAI titers

<table>
<thead>
<tr>
<th>HAI titer</th>
<th>No. of sera</th>
<th>RUBELISA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enzygnost-Rubella&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ORTHO Rubella&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>410</td>
<td>0.08 (0.004)</td>
<td>0.05 (0.002)</td>
<td>0.12 (0.002)</td>
</tr>
<tr>
<td>10</td>
<td>101</td>
<td>0.29 (0.029)</td>
<td>0.38 (0.038)</td>
<td>0.47 (0.017)</td>
</tr>
<tr>
<td>20</td>
<td>218</td>
<td>0.32 (0.022)</td>
<td>0.45 (0.030)</td>
<td>0.52 (0.035)</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>0.35 (0.035)</td>
<td>0.55 (0.055)</td>
<td>0.62 (0.062)</td>
</tr>
<tr>
<td>80</td>
<td>105</td>
<td>0.39 (0.038)</td>
<td>0.80 (0.078)</td>
<td>0.75 (0.073)</td>
</tr>
<tr>
<td>160</td>
<td>85</td>
<td>0.42 (0.045)</td>
<td>0.81 (0.086)</td>
<td>0.93 (0.097)</td>
</tr>
<tr>
<td>320</td>
<td>47</td>
<td>0.48 (0.070)</td>
<td>1.34 (0.195)</td>
<td>1.15 (0.168)</td>
</tr>
<tr>
<td>640</td>
<td>20</td>
<td>0.55 (0.123)</td>
<td>1.38 (0.308)</td>
<td>1.23 (0.275)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The correlation coefficients for the correlation of absorbance values determined by ELISA and HAI titers are 0.94, 0.97, and 0.98 for the RUBELISA, Enzygnost-Rubella, and ORTHO Rubella systems, respectively.

<sup>b</sup> Absorbance values of ≥0.17 (obtained from a calibration curve) are equivalent to an HAI titer of ≥1:8.

<sup>c</sup> Values are expressed as net absorbances. Values of ≥0.2 are positive.

<sup>d</sup> Standardized values of ≥0.20 (obtained by multiplying absorbance values with a high-positive control ratio) indicate immune status.
our experience (unpublished), low HAI titers of 10 and 20 have shown complete agreement with single radial hemolysis (Rubazone; Scientific Measuring Instruments, Sydney, Australia) and the passive latex agglutination test (Virogen; Wampole Laboratories, N.J.), two tests which are unaffected by nonspecific inhibitors. It is well established that the presence of rubella antibody as detected by the HAI test accurately correlates with clinical protection of the fetus (8). The three ELISA systems showed good agreement with the HAI test and were found to be simple, rapid, and economical alternatives.

The only disagreement in sensitivity was observed with sera having HAI titers of 10 and 20. Whereas the ORTHO Rubella and Enzygnost-Rubella systems showed excellent sensitivities compared with the HAI test, the RUBELISA system was marginally less satisfactory. In some cases, it was apparent that the lack of sensitivity of the RUBELISA system may be attributed to an elevated absorbance value of the control antigen well relative to the absorbance value of the antigen well. The correlation curve of ELISA absorbance versus log of HAI titer (data not shown) had the most gradual slope for the RUBELISA system, reflecting the lower sensitivity of this system in comparison with the Enzygnost-Rubella and ORTHO Rubella systems, whose correlation curves had steeper slopes. However, because of individual serum absorbances scattering around the mean and overlapping multiple HAI values, the ELISA value of a single serum should not be used to report an equivalent HAI titer. Hence, ELISA results from clinical laboratories should be accompanied by an interpretative comment.

All three ELISA systems achieved excellent specificity. An explanation for the discrepant sera, which all had positive reactions very close to the absorbance cutoff point, may be that both the ORTHO Rubella and RUBELISA systems have had their cut-off points between immunity and susceptibility calibrated to correlate with an HAI test cutoff titer of 8, whereas our in-house HAI test has a marginally higher cutoff titer of 10. To minimize the occurrence of false low-positive results in the ORTHO Rubella system, one of two modifications could be incorporated. First, those sera with standardized ELISA values in the range from 0.20 to 0.25 could be regarded as equivocal and, second, the results must be confirmed by a different procedure. The instructions for the ORTHO Rubella system (Ortho Diagnostics Systems, Instructional booklet, 1986) draw attention to the difficulty of categorizing results close to the 0.20 criterion for immune status. Raising the cutoff point, thus increasing the proportion of false-negative results, is probably a less satisfactory alternative.

Another reason for the discrepancies in both sensitivity and specificity could be attributed to the difficulty in standardizing the HAI test between laboratories (2). Surveys by the Centers for Disease Control and the College of American Pathologists, as well as the Royal College of Pathologists of Australia, have confirmed these differences in HAI tests between laboratories (1). Therefore, only approximate evaluations can be made of different ELISA methods for rubella IgG by comparison with HAI tests (20). It may be that the HAI test used by M.A. Bioproducts for calibration of the RUBELISA system was less sensitive than our HAI test.

There have been few independent reports of the evaluation of commercial reagent sets for rubella IgG by micro-ELISA methods. The study reported herein is an in-depth and comprehensive comparative evaluation of three such reagent sets. Previous individual studies (3, 8, 18, 22) have shown that the RUBELISA system has a specificity of 99 to 100%, but sensitivity ranged from 91.4 to 67.7%, which was attributed, in the main, to the lack of detection of some sera with HAI titers of 10 and 20. All these published studies show that while specificity is excellent sensitivity is lacking. A new rapid ELISA that overcomes this lack of sensitivity with low-HAI-titer sera has been reported (1).

These three assays use markedly contrasting configurations (Table 3). Cutoff absorbance levels were fixed in the Enzygnost-Rubella system, compared with the floating threshold in others, which is more tolerant of interlaboratory variation. This assay also does not have a negative control serum, which may lead to a failure to detect inadequate washing. Control antigens have been used to detect nonspecific binding in the Enzygnost-Rubella and RUBELISA systems (6), whereas highly purified antigen, as used in the ORTHO Rubella system, bypasses the need for such controls (19, 21).

Heat-treated sera, used in concurrent complementary fixation tests or to inactivate infectious human immunodeficiency virus (16), were satisfactory substrates for the RUBELISA and ORTHO systems but not for the Enzygnost-Rubella system, as found with some other ELISA systems (14, 15). The heated sera show increased reactivity with control antigen wells, often above the cutoff, but the reason for this reactivity is not known (9; P. W. Robertson, personal communication).

In conclusion, on the basis of the present study, we would prefer to use either the Enzygnost-Rubella or the ORTHO Rubella system rather than the RUBELISA system instead of the time-consuming HAI test for the determination of immune status, provided that (i) sera for testing in the Enzygnost-Rubella system must not have been heat inactivated and (ii) for the ORTHO system, standardized ELISA values in the range from 0.20 to 0.25 should be regarded as equivocal unless confirmed by a different procedure.

LITERATURE CITED


