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Comparison of Hemagglutination Inhibition Test and Enzyme-Linked Immunosorbent Assay for Determining Antibody to Rubella Virus

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The hemagglutination inhibition test (HAI) and the enzyme-linked immunosorbent assay (ELISA) for detecting antibody to rubella virus were compared by testing 25 sets of paired sera taken before and after infection and 10 sets of sera taken during acute and convalescent stages of the disease and by screening 700 serum samples from the Collaborative Perinatal Project, NIH/NINCDS. The tests were found to be comparable in their ability to detect positive and negative sera, rises in titers, and seroconversions. When a purified antigen and carefully prepared reagents were used, ELISA was found to be as accurate and reliable as HAI. ELISA required no pretreatment of serum, could easily be automated, and was less time-consuming than HAI.

Widespread rubella vaccination in the United States has reduced the incidence of rubella infection considerably, but small outbreaks continue to occur. For children and adults the disease is relatively benign, but for the developing fetus carried by an infected woman the results can be serious. The accurate determination of antibody levels to rubella virus, therefore, is of importance not only in screening for immune status, but also in confirming recent infections.

The hemagglutination inhibition test (HAI) is currently the most commonly used technique for determination of rubella antibody titer. This test is time- and labor-consuming. Satisfactory performance requires careful absorption of nonspecific inhibitors, and the method is not easily automated. Reports from the Centers for Disease Control indicate that there is considerable laboratory to laboratory variation, both in the detection of antibody and in the determination of titer by HAI (13-15). The enzyme-linked immunosorbent assay (ELISA), which has been shown to offer good sensitivity, ease and speed of performance, and adaptability to semiautomation, has been suggested as an alternative method for rubella serology. We report in this paper a comparison of HAI and ELISA using sera from a large perinatal study.

MATERIALS AND METHODS

Specimens. Sera for these tests were selected from a long-range study of over 6,000 women who were pregnant during the 1964 epidemic of rubella in the United States and were included in the Collaborative Perinatal Project, NIH/NINCDS. Studies of these women and their children have been reported previously (12). The sera were stored at −20°C before use. Test sera included samples from 25 patients before and after natural rubella infection during pregnancy and from 10 patients during the acute and convalescent stages of rubella infection. Randomly selected sera from 500 pregnant women were used for overall comparison, and 200 samples selected because of negative or low HAI rubella titers were also tested.

HAI. The HAI procedure has been described in detail previously (9, 11). Briefly, the test used kaolin absorption of the sera, newborn chick erythrocytes, and Flow HAI (Flow Laboratories, McLean, Va.) antigen (Gilchrist strain in Vero cells) in microtiter plates. The starting serum dilution was 1:4. The samples used had previously been tested by HAI, but all were retested for this study.

ELISA. (i) Antigen. HPV-77 rubella virus grown in Vero cells was purified on a sucrose gradient by Preston Dorsett. The method of purification and concentration has been previously described (4). The optimum working dilution, 1:200, was determined by block titration, using known positive and negative sera and a pretested conjugate. The dilution chosen corresponded to approximately four antigenic units on the basis of endpoint titration of known positive serum in cuvettes sensitized with serial twofold dilutions of antigen. Antigens were stored at −70°C for up to 6 months before use with no loss in activity.

(ii) Conjugates. Commercially available (Cappell Laboratories, Cochranville, Pa.) goat anti-human immunoglobulin G (heavy and light chain) was purified and conjugated to alkaline phosphatase by a method previously described (6).

(iii) Assay. Performance of the test and quantitation of the results has been described elsewhere (6).

Briefly, disposable polystyrene microcuvettes (Finnpipette Labsystems, Helsinki, Finland) were sensitized with rubella antigen diluted in phosphate-buffered saline, pH 7.4. After overnight incubation at +4°C, they were washed with phosphate-buffered saline plus 0.05% Tween 20 and then distilled water. Serum samples diluted 1:50, 1:500, and 1:5,000 in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% ethylenediaminetetraacetic acid were added to the sensitized cuvette. After 90 min of incubation at 37°C, the cuvette was washed as above and alkaline phosphatase conjugate was added. The test was incubated for 60 min at 37°C and washed, and the substrate (*p*-nitrophenyl phosphate; Sigma Chemical Co., St. Louis, Mo.) in diethanolamine buffer (pH 9.8) was added. After 30 min at 37°C the reaction was stopped with 1.5 M NaOH. The intensity of the reaction (color change) was measured with a nine-channel spectrophotometer (model FP9, Finnpipette Labsystems) attached to a programmable calculator.

Positive and negative standard sera were included with the test samples. Each day a standard reference curve was prepared by plotting absorbance readings of serial half-log dilutions of positive and negative standard sera (Fig. 1). This curve was used to determine the relative antibody activity of the test samples. The linear part of the S-shaped curve thus obtained represented the optically effective absorbance range. Test samples gave a similar and parallel curve. The difference in logs between the two curves in the optically effective absorbance range was the ED. Samples with lower activity than the reference serum had a negative ED; those with higher activity had a positive ED. To simplify the expression, an arbitrary zero titer of log −2.8, corresponding to the average ED value of the known negative serum, was used. Positive ED values were then derived by subtracting this value from all EDs determined from the curve. A programmable Hewlett-Packard 9815A calculator was interfaced with the ELISA reader and automatically made these determinations, giving both optical density and ED readings.

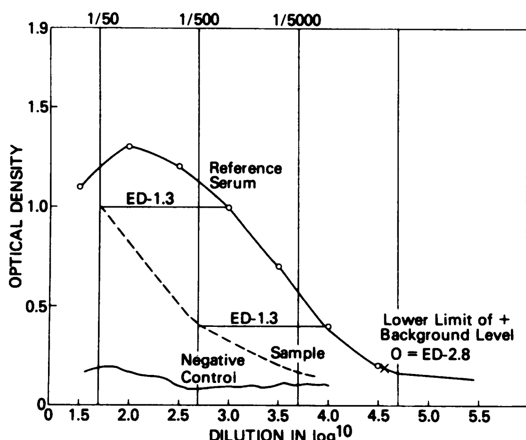

FIG. 1. *Quantitation of rubella antibody. A standard curve was obtained by plotting the optical density readings of a serial 0.5-log dilution of known positive serum. Samples were tested at dilutions of 1/50, 1/500, and 1/5,000 corresponding to log −1.7, −2.7, and −3.7, respectively. The difference in logs between a sample and the standard is the ED. In these tests the difference in logs between the curve of the positive standard and the negative standard was found to be −2.8. Positive ED values were derived by equating log −2.8 to zero and subtracting it from all derived ED values. Thus, an ED of −1.3 becomes 1.2 [log −1.3 −log −2.8) = log 1.2], or in the case of a reading higher than the positive standard such as ED + 0.2, the corrected ED is 3.0 [+0.2 − (−2.8) = 3.0].*

Three dilutions of each test sample were run because sera with very low or high antibody levels frequently gave absorbance readings which were outside of the linear part of the reference curve at one or two of the dilutions.

Sucrose gradient density centrifugation. Sixteen low-antibody-level samples giving divergent results by the two tests were retested after centrifugation on a sucrose gradient.

The serum fractionation procedure on a sucrose gradient as outlined in the Center for Disease Control procedural guide was used (8). A total of 0.5 ml of serum was layered on a 10 to 50% sucrose gradient (pH 7.2) and centrifuged at 32,000 rpm for 18 h in a Spinco ultracentrifuge rotor (SW50.1). Ten 0.5-ml fractions were collected from the bottom of the tube and tested by single radial diffusion to determine those fractions containing immunoglobulin G and M components. HAI and ELISA determinations were repeated after the immunoglobulin fractions were pooled, concentrated to the original volume, and dialyzed against phosphate-buffered saline to remove the sucrose.

RESULTS

Results of HAI and ELISA for 25 paired sera obtained before and after known rubella infections are presented in Table 1. All preinfection sera were negative by HAI, whereas 9 of 25 samples gave minimal ELISA readings ranging from ED 0.3 to ED 0.6. Postinfection sera all gave strongly positive readings by both tests. Significant seroconversions were observed with all paired sera by both HAI and ELISA. The low-level ELISA readings with the preinfection sera indicated that an ED of 0.6 was not protective and probably did not indicate the presence of true antibody. To avoid these "false positive" readings, we selected an ED twice that obtained with any of the known preinfection sera and used this as the threshold value for protective antibody (ED 1.2).

In Table 2 the results of HAI and ELISA with sera obtained from 10 patients during the acute and convalescent phases of rubella infections are presented. The first sample was taken while the rash was apparent, and the second was taken 16 to 30 days later. According to Leinikki and Passila (6), a change of 0.2 in ED value represents

TABLE 1. *Comparison of HAI and ELISA results on sera of pregnant women before and after onset of clinical rubella infection*

Patient no.	ELISA ED		HAI titer		Time (wk)[a]	
	Pre	Post	Pre	Post	Pre	Post
1	0	3.1	<4	256	10	23
2	0	3.0	<4	128	7	18
3	0.6	3.0	<4	128	4	23
4	0.6	3.3	<4	512	11	13
5	0	3.0	<4	512	5	29
6	0	2.8	<4	256	5	15
7	0	3.0	<4	256	3	20
8	0.6	2.9	<4	128	5	30
9	0	3.0	<4	128	9	9
10	0	3.0	<4	256	2	18
11	0	2.5	<4	128	16	9
12	0.4	2.4	<4	256	10	3
13	0	2.4	<4	128	20	5
14	0	2.8	<4	256	8	28
15	0	2.8	<4	256	8	16
16	0	3.0	<4	256	2	22
17	0	3.1	<4	512	10	10
18	0	3.2	<4	256	2	9
19	0.4	3.2	<4	512	10	10
20	0.5	3.2	<4	256	26	9
21	0.5	2.5	<4	128	18	7
22	0	2.3	<4	64	4	12
23	0	2.5	<4	256	10	8
24	0.6	2.4	<4	64	13	9
25	0.3	2.5	<4	128	13	13

[a] Time serum was taken relative to clinical onset of rubella.

TABLE 2. *Comparison of HAI and ELISA on acute and convalescent rubella serum*

Patient no.	ELISA ED		HAI Titer[a]	
	Acute[b]	Convalescent[c]	Acute[b]	Convalescent[c]
1	1.0	3.0	32	512
2	1.0	2.0	32	128
3	1.0	1.9	16	64
4	0.4	2.0	4	64
5	0.9	2.6	16	256
6	1.5	2.2	128	256
7	1.2	3.0	8	512
8	1.5	2.7	32	256
9	1.9	3.0	128	512
10	1.1	2.1	8	128

[a] Reciprocal of serum dilution.
[b] Drawn during rash.
[c] Drawn 16 to 34 days later (average, 19 days).

a significant change in antibody level. All paired sera showed significant increases in antibody by both HAI (≥4-fold increase in titer) and ELISA (≥0.2 increase in ED value).

Comparisons of HAI titers with ELISA ED values for sera from 500 randomly selected pregnant women are presented in Fig. 2. There was close agreement in the identification of positive and negative sera with the tw tests. The distribution of results with low-level titers suggested that the greater sensitivity of ELISA might assist in the problem of detecting low rubella antibody levels. Samples from the perinatal study of 200 sera which had low or negative rubella HAI titers were selected and tested by HAI and ELISA. A comparison of the results is presented

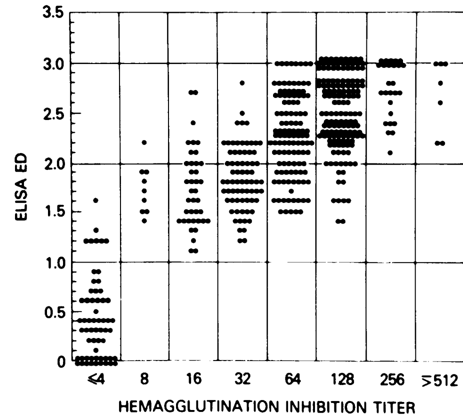

FIG. 2. *Correlation between rubella HAI titers and ELISA ED values for sera from 500 randomly selected pregnant women.*

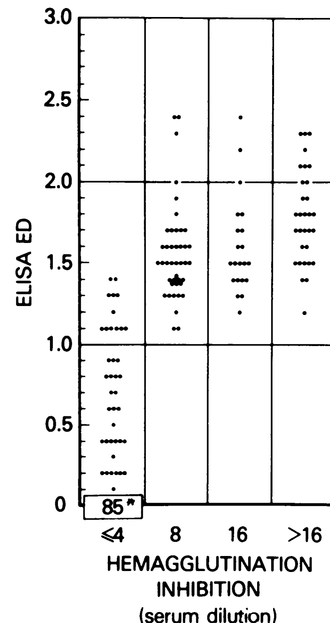

FIG. 3. *Comparison of low rubella titers in pregnant women by HAI and ELISA. Of 200 samples tested, six could be false positive and two could be false negative by ELISA. Eighty-five samples were ≤4 by HAI and had ELISA ED readings of 0.*

in Fig. 3. Again, there was close agreement on the determination of positive or negative sera.

Of the 700 sera tested, 183 had an HAI titer of ≤4. Of these, 80 gave ED readings ranging from 0.2 to 1.6. Sixteen samples from the 80 were subjected to centrifugation on a sucrose gradient. After retesting there was very little change in either HAI titer or ELISA ED value.

The data indicated that the threshold ED of 1.2 was quite comparable to an HAI titer of 8, the level which is generally accepted, on the basis of clinical evidence, as the lower limit of antibody which will render an individual immune. Using these thresholds (HAI ≥ 8 and ED ≥ 1.2), the sensitivity and specificity of ELISA relative to HAI could be determined. Of the total 700 sera in the two groups, 517 were positive by HAI. Of these 517, 514 were positive by ELISA, giving a sensitivity of 99.4%. By HAI, 183 samples were negative, and of these, 170 were negative by ELISA, yielding a specificity of 92.8%.

DISCUSSION

The data presented show that HAI and ELISA were comparable for demonstrating seroconversions, rises in titer, and the presence or absence of antibody to rubella virus. We have obtained a good correlation between an HAI titer of ≥8 and ELISA ED values of ≥1.2 and currently use these as our threshold levels. The problem of low-level rubella antibody was not clarified. Samples with low positive ELISA and low positive or negative HAI were retested by both methods after sucrose gradient centrifugation. Results of the tests were not changed by the purification procedure. The clinical significance of this low-level activity in predicting susceptibility to infection and the possibility of teratogenic effects of rubella require further studies based on both laboratory and clinical data. Monitoring serological responses such as rises in specific antirubella immunoglobulins G and M after vaccination or exposure to wild-type rubella in persons with low-level ELISA or HAI antibody levels or with both should be studied. Well-documented serology in conjunction with studies of rubella during pregnancy and its effect on the product of that pregnancy could be assured if, as has been suggested, sera tested for rubella are held for 1 year (10). With such sera not only could we document whether infection had occurred, but also it may eventually be possible to establish whether damage to the fetus occurs only with seroconversions (from negative to positive) or also with a rise in titer. Without these clinical studies we hesitate to predict susceptibility to rubella on the basis of positive ELISA and negative HAI. A recent report (1) comparing HAI, ELISA, and lymphocyte transformation and suggesting that ELISA may be a better indicator of prior rubella infection or vaccination than HAI also requires further clinical documentation.

In general, serum specimens with higher HAI titers had higher ED values. There was, however, considerable overlap and no clear correlation could be made between individual ED values and HAI serum titers, probably because the two tests are not dependent upon exactly the same antibody attachments. If more specific titer information is required, ELISA endpoint determinations on serial serum dilutions may be of more value.

Although the rubella ELISA has been described in the literature many times (2–5, 7, 15), no standardized method has yet emerged. Standard high-positive, low-positive, and negative sera should be included as controls in any test. Use of national or international reference standards in all variations of the test would permit more meaningful evaluation and reporting of results.

In our laboratory, the ELISA, performed by experienced technicians with highly purified antigen and carefully prepared reagents, gave satisfactory results and was particularly well adapted to screening large numbers of sera. No pretreatment of serum is necessary and the test is rapid, sensitive, and specific. The system can be adapted to endpoint titrations and can be easily automated and adapted to a wide variety of antigens. With increasing availability of reliable reagents and reading equipment, ELISA could be the method of choice for many laboratories.

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