Comparison of the Latex Agglutination Test with the Hemagglutination Inhibition Test, Enzyme-Linked Immunosorbent Assay, and Neutralization Test for Detection of Antibodies to Rubella Virus

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The ability of a rapid, latex agglutination test to diagnose rubella infection and to measure immune status was evaluated by comparison with the hemagglutination-inhibition (HAI) test, enzyme-linked immunosorbent assay (ELISA), and the neutralization (NT) test. The latex agglutination test accurately detected serological conversions in 74 pairs of sera representing 21 natural infections and 53 immunizations. The antibody levels of 276 sera from the general population were determined by latex agglutination, HAI, and ELISA. The correlation coefficients between the titers obtained by HAI and latex agglutination and by ELISA and latex agglutination were statistically significant. Results on 12 sera did not agree when measured by the three tests. These sera were included among the 196 specimens tested by NT. The correlation coefficient between NT and latex agglutination titers was statistically significant. There was one serum positive by latex agglutination but negative by NT, and five sera were negative by latex agglutination but had titers of 4 to 8 in the NT. The relative sensitivity of detecting antibody was greater by latex agglutination than by HAI. An additional 49 sera containing residual nonspecific hemagglutinin inhibitors were evaluated by latex agglutination and NT. The untreated sera showed no false positive reactions, and 36 of 39 NT positive sera were positive in the latex agglutination test.

The hemagglutination-inhibition (HAI) test is currently the most widely accepted method for both serological diagnosis of rubella infection and determination of immune status. However, the HAI test is time-consuming, labor intensive, and difficult to standardize between laboratories (10). For example, the average values from annual proficiency testing between 1971 and 1979 indicated 21.1 to 37.2% of HAI results from U.S. laboratories fell outside the acceptable variation range (23). An additional problem encountered in the HAI test is that 5 to 10% of treated test sera display residual nonspecific inhibitors which cause abnormal patterns of reactivity and make the results difficult to interpret (3, 19, 28).

Several other antibody assays have recently been developed and tested including: radioimmunoassay (18), passive hemagglutination (1), single radial hemolysis in gel (4, 5), indirect immunofluorescence test (4, 6), and enzyme-linked immunosorbent assay (ELISA; 25, 26). These tests are rapid, simple, and usually require no pretreatment of sera. Numerous reports

(2, 4, 6-8, 19, 24, 28) indicate generally good correlation between these tests and the HAI.

A latex agglutination test for the detection of antibodies to rubella is now available in the form of a kit (Rubascan; Hynson, Westcott and Dunning, Baltimore, Md.). Limited preliminary tests indicated that this test reliably detected antibody to rubella virus after an 8-min incubation period (4). In the present study, we compared the latex agglutination test with ELISA, HAI, and neutralization (NT) tests for detecting antibodies to rubella after immunization and after natural infection.

MATERIALS AND METHODS

Sera. To evaluate the ability of the latex agglutination test to detect seroconversion, paired sera from 74 individuals were tested. Twenty-one of these were military recruits who experienced natural infection. The remainder were school children and young adults immunized with either RA27/3, HPV₇₇DE₅, or Cende-hill vaccine. The ability of the latex agglutination test to determine immune status was examined with 276

sera sent to this laboratory for titration of antibody to rubella virus.

HAI test. The dextran sulfate-calcium chloride system was used to remove nonspecific inhibitors before testing the sera with human type O erythrocytes by the methods of Liebhaber (16). A limited number of sera had been previously tested by HAI in the course of other investigations. Comparative testing with reference sera supplied by the Connecticut State Health Department Laboratory indicated that this procedure regularly yielded titers in complete agreement with reference HAI titers.

NT test. We used the semimicro-NT method previously described (20). Briefly, dilutions of heat-inactivated (56°C for 30 min) sera were incubated for 12 to 14 h at 4°C with 50–50% tissue culture infective doses of wild-type rubella virus strain Therien in the presence of 2% guinea pig complement. Each serum-virus mixture was then inoculated into four wells of a 24-well cell culture plate containing just confluent Vero cells. The cultures were refed with medium containing 1% heat-inactivated kaolin-treated fetal bovine sera and incubated at 37°C for 11 days. End-point titers were determined by viewing the cytopathic effect and confirmed by testing fluid from wells for hemagglutinating activity. Titers determined in this NT assay correlated with HAI titers (20).

ELISA. The method for growing and purifying rubella virus was basically as outlined previously (17). However, before sucrose gradient ultracentrifugation, the virus was concentrated by membrane filtration (Pellicon cassette; Millipore Corp., Bedford, Mass.). Those fractions from the gradient which showed peak hemagglutinating activity were pooled and used as antigens. A negative control antigen was prepared in an identical manner from mock-infected cells disrupted by one freeze-thaw cycle.

The optimum dilution of all reagents used in the ELISA was determined by checkerboard titrations. A volume of 0.1 ml per well was used for all incubations. The washing procedure between each step consisted of five successive washes with 0.2 ml of phosphate-buffered saline with 0.05% Tween 20 using an automatic washing apparatus (Miniwash; Dynatech Laboratories, Inc., Alexandria, Va.). Test sera were diluted in twofold steps starting at 1:50 in phosphate-buffered saline-Tween 20 containing 0.1% bovine serum albumin and 50 µg of dextran sulfate per ml (analytical grade; ICN, Cleveland, Ohio). Anti-human immunoglobulin G, conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was diluted in the same buffer.

The ELISA followed previously established methodology (27). The rubella antigen (and the negative control antigen adjusted to an equal protein concentration) was diluted in 0.06 M carbonate coating buffer (pH 9.6) (27), then added to 96-well polystyrene plates (substrate flat bottom; Dynatech), and incubated for 14 h at 4°C. The antigen-coated plates were washed, and dilutions of test sera were added to duplicate wells. The plates were incubated 1 h at 37°C and washed, and enzyme-conjugated anti-human serum was added and incubated 1 h at 37°C. After another wash, the substrate (1 mg of *p*-nitrophenyl phosphate per ml of diethanolamine buffer [pH 9.8] [27]) was added and incubated 30 min at 37°C. The reaction was stopped by the addition of 0.1 ml of 3 M NaOH, and

absorbance values were read at a wavelength of 405 nm in a microplate spectrophotometer (Multiskan; Flow Laboratories, Inc., Rockville, Md.).

In reading the results, a specific activity was calculated for each serum dilution by subtracting the average optical density in duplicate wells coated with negative control antigen from the optical density of the wells coated with rubella antigen. A series of at least five antibody-negative sera were also included in each assay, and a dilution of serum was considered positive only if it yielded a specific activity which was 2.5 standard deviations greater than the mean specific activity of these negative sera.

In comparative studies, we found that antibody titers using this ELISA method were generally two- to fourfold higher than those obtained when testing dilutions of sera with two commercially available kits.

Latex agglutination test. The procedure followed was that recommended in the Rubascan latex agglutination kit. The test involves placing 0.025 ml of untreated serum on an imprinted circle on a Teflon-coated card. An applicator was used to spread the serum to cover the encircled area. One drop (approximately 0.015 ml) of rubella virus-coated latex beads was added to the serum. The card was placed in a humid chamber on a rotating shaker (100 rpm) at room temperature for 8 min, and agglutination was then read with the aid of a high-intensity incandescent lamp. The kit provided positive and negative control sera to be included on each card. To determine end-point titrations, twofold dilutions of sera were made with dilution buffer contained in the kit. The antibody titer was recorded as the reciprocal of that dilution which when mixed with a drop of latex beads produced agglutination. Therefore, a serum yielding agglutination only in the undiluted state was recorded as a titer of 1.

During this study, we encountered a small percentage of antibody-positive sera which yielded a weak agglutination when tested undiluted, but showed strong agglutination upon dilution. Consequently, all sera were initially tested both undiluted and diluted 1:4.

Complement fixation test. The complement fixation test was performed as previously described (11).

RESULTS

Detection of seroconversion. Paired sera from 21 persons with natural infection and from 53 who had been immunized with various vaccines were tested in ELISA, latex agglutination, and HAI tests. Postinfection or postvaccination sera were collected 30 to 60 days after infection or immunization. The latex agglutination test accurately detected seroconversions in all pairs of sera (Table 1).

Determination of immune status. Sera from 276 individuals submitted to our laboratory for determination of immune status were tested by HAI, ELISA, and latex agglutination. Figure 1 shows the titers obtained in the HAI and latex agglutination tests. The overall agreement between the tests was 97.5% (269 of 276 sera); the Spearman rank coefficient of antibody containing sera was $r = 0.662$, indicating that the

TABLE 1. Evaluation of the latex agglutination test for detecting seroconversion after rubella immunization and infection

Rubella infection	No. of pairs of sera	Mean titer (range) in postimmunization or infection sera ^a		
		ELISA	Latex agglutination	HAI
RA27/3	33	1,600 (400–3,200)	64 (16–256)	64 (16–256)
Cendehill	11	1,600 (400–6,400)	64 (32–256)	128 (64–128)
HPV ₇₇ DE ₅	9	800 (400–3,200)	32 (16–64)	32 (16–64)
Naturally acquired	21	3,200 (400–12,800)	128 (16–256)	128 (64–512)

^a All preexposure sera were negative in each test.

relationship between titers was statistically significant ($P < 0.001$). Figure 2 presents a comparison of titers from the ELISA and the latex agglutination test. The overall agreement was 98.6% (272 of 276 sera); the correlation coefficient was $r = 0.639$, and it was significant ($P < 0.001$).

Table 2 gives results on 12 sera which showed a disparity when tested for antibody in the experiments depicted in Fig. 1 and 2. Although no sera were positive by HAI and negative in the latex agglutination test, eight were negative by HAI and positive in the latex agglutination test (Fig. 1). No sera were negative by ELISA and positive in the latex agglutination test, but four were ELISA positive and latex agglutination (and HAI) negative (Fig. 2). To resolve the

antibody status of these eight possibly false positive and four possibly false negative results, we retested these sera along with 184 of the 276 specimens for which adequate sera for analysis by the NT test was available.

A comparison of the titers obtained in the latex agglutination test and the NT test is shown in Fig. 3. Overall agreement was 97.5% (191 of 196); the correlation coefficient was $r = 0.593$, and the relationship between titers of antibody containing sera was statistically significant ($P < 0.001$).

Table 2 shows that seven of the eight sera negative by HAI but positive in the latex agglutination test were positive by NT with titers from 8 to 32. The one false positive result was with a serum that agglutinated only at an undiluted concentration, and was positive in the ELISA at 1:100. Three of the four sera positive in the ELISA but negative by latex agglutination test (Table 2) had NT titers from 4 to 8. We detected two sera with NT titers of 4 and 8 which were negative in all other tests.

Relative test sensitivities. Of the 196 sera tested by NT, 111 were positive (NT titer, ≥ 4) and were used to determine the relative sensitivities of the HAI, ELISA, and latex agglutination tests. This group of 111 sera included 28 with low levels of antibody, i.e., ≤ 8 by the standard short-incubation HAI method. The sensitivity of the HAI was 91% (101 of 111), and that of the latex agglutination was 95.5% (106 of 111) with one false positive result. The sensitivity of the ELISA was 98.2% (109 of 111) with two false positive results, both titring 100.

Measurement of complement-fixing antibodies. To ascertain if the latex agglutination test was measuring complement-fixing antibodies, we used a previously assayed series of sera collected at 2- to 3-year intervals for 7 years from 12 individuals immunized with RA27/3 vaccine. The initial postimmunization complement fixation antibody titer disappeared, but the antibody measured by latex agglutination remained positive through 7 years and correlated with the titer determined by HAI and ELISA (data not shown).

FIG. 1. Comparison of titers of antibody to rubella virus determined in 276 sera tested with the HAI and latex agglutination tests.

FIG. 2. Comparison of titers of antibody to rubella virus determined in 276 sera tested with the ELISA and latex agglutination test.

Evaluation of sera containing inhibitors of the HAI test. Despite various treatments to remove nonspecific inhibitors which interfere with the HAI test, 5 to 10% of sera still show nonspecific inhibition (3, 19, 28) and require additional treatment. We selected 49 such sera and tested them without treatment in the NT and latex agglutination tests. Thirty-nine were positive (titer, ≥ 4) in the NT test, and 36 of these 39 were positive in the latex agglutination test. There were no false positive sera in the agglutination test, and the three false negative sera titrated only 4 to 8 by the NT test and 100 to 200 by ELISA.

DISCUSSION

This study confirmed the sensitivity and reliability of the latex agglutination test for the serological diagnosis of rubella infection and for the assessment of immune status. The test is particularly valuable, since it is rapid, requires no special technical training or equipment, and all reagents are contained in the kit. Furthermore, when sera containing nonspecific inhibitors in the HAI test were tested without pretreatment in the latex agglutination test, the results were comparable to NT test results. In preliminary studies, we have observed that hemolyzed samples, sera which have undergone numerous freeze-thaw cycles, and blood reconstituted after being dried on filter paper yielded results comparable to those found with fresh sera in latex agglutination and ELISA tests.

The sensitivity of the latex agglutination test

TABLE 2. Sera showing a disparity in results when tested by latex agglutination, ELISA, HAI, and NT tests

No. of sera	Detection of antibody			
	HAI	Latex agglutination	ELISA	NT
7	—	+	+	+
1	—	+	+	—
3	—	—	+	+
1	—	—	+	—
2	—	—	—	+

appears to be greater than the HAI, and perhaps this reflects the ability to examine undiluted sera containing minimal amounts of antibody. This advantage was important in our sample of 111 NT-positive sera, since 28 of them contained low levels of antibody (≤ 8 in HAI). An HAI procedure using overnight incubation has been shown to be more sensitive. Although we have used the NT test as a reference assay, it should be noted that this assay is complex and could yield variable results with sera containing low amounts of antibody.

There was one positive serum in the latex agglutination test and two in the ELISA (one of which was latex agglutination positive) which were negative by NT. The problem of false positive reactions has been encountered in previous studies using ELISA (7, 19, 21). Although the methodology was modified in attempts to reduce these problems (2, 7, 8, 14, 24, 29), it was not always possible to determine whether the results were false positives or whether the ELISA was more sensitive than the HAI test used as reference (7, 21). Best et al. (1) have shown the ELISA to be more sensitive than HAI; similarly 10 of 12 of the ELISA positive-HAI negative sera reported above were NT positive.

It is possible that the ELISA and latex agglutination false-positive results represent detection of rubella-specific nonneutralizing antibody, since it has been shown that a small percentage of persons who acquire rubella infections naturally, and some vaccinated with HPV₇₇DE₅, develop HAI but not NT antibody (9, 15, 22). However, with regard to immune status, these sera must be judged as false positives.

Preliminary studies indicated that the temporal development of antibodies measurable by latex agglutination, ELISA, and HAI parallel one another. Latex agglutination, HAI, and ELISA antibodies appeared between days 14 and 28 postimmunization with RA27/3 and between days 3 and 5 postonset of illness caused by natural rubella infection. These positive reactions in early postexposure sera could represent

FIG. 3. Comparison of titers of antibody to rubella virus determined in 196 sera tested with the latex agglutination and NT tests.

detection of immunoglobulin M and G antibodies, since immunoglobulin M fractions that we have prepared by absorption of sera with protein A (13) followed by fractionation on an ion-exchange column (12) react positively in the latex agglutination test.

We encountered six high-titered sera which showed weaker agglutination when tested undiluted than when tested diluted. These sera always yielded a typical strong agglutination pattern when tested at 1:2 and higher dilutions. This prozonelike phenomenon was accentuated if during the performance of the test one did not first spread the sera to fill the imprinted circle on the Teflon card before adding latex beads. It would be desirable to retest at a 1:2 or 1:4 dilution any sera which show equivocal or weak agglutination in a test of undiluted sera.

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