# Single-Radial Hemolysis as a Cost-Effective Determinant of *Rubella* Antibody Status

JOHN M. FORGER III* AND ROBERT F. GILFILLAN

*Virology Laboratory, State Laboratory Institute, Massachusetts Department of Public Health, Boston, Massachusetts 02130*

Single-radial hemolysis was examined for sensitivity, reliability, and cost for determination of rubella antibody levels in the general population. Results obtained with single-radial hemolysis plates made in this laboratory, and those under development by a commercial manufacturer, were compared with those obtained by the hemagglutination inhibition method normally used for rubella antibody determinations. The results suggest that single-radial hemolysis is comparable to hemagglutination inhibition with respect to sensitivity and reliability; it is superior to hemagglutination inhibition in terms of ease of manipulation and economy of use.

Single-radial hemolysis (SRH) is a simple and inexpensive method for the determination in serum of the presence and amount of antibody to specific viral antigens. First described by Weiler et al. (9), this technique consists of suspending in agarose erythrocytes (RBC) coated with the antigens of interest. The antiserum under test is then added to a circular well in the solidified agarose. After a suitable period of time for antibody diffusion, complement (C′) is added; the combination with antibody and complement causes lysis of the antigen-sensitized cells. The result is a circular area of hemolysis around the well, contrasting sharply with the surrounding intact RBC and varying in size according to the amount of antibody present in the serum sample added (3, 5, 7).

Limited experiments have shown that SRH can be used to detect and measure antibody against a variety of viral antigens including rubella (2). The technique has been used extensively in seroepidemiological studies of influenza (7). It has not, however, received widespread attention as a diagnostic method for large-scale application to rubella.

The purpose of this study was, first, to assess the sensitivity, accuracy, and reliability of SRH plates prepared in this laboratory (with commercially available components) to plates under development and prepared by a commercial manufacturer. Second, this study was designed to compare the cost effectiveness of both laboratory and commercially prepared plates to the standardized hemagglutination inhibition (HI) test (6) for: (i) the laboratory diagnosis of rubella infection and (ii) the determination of immunity in candidates for vaccine. The present commu-

nication describes results obtained from this study and presents evidence to show that SRH compares favorably with the HI test in identifying recent rubella infections and candidates for vaccination.

## MATERIALS AND METHODS

**SRH procedure.** The SRH plates were made essentially according to the methods of Grillner and Strannegard (2). Lyophilized rubella antigen (Flow Laboratories, Bethesda, Md.) was reconstituted to 1 ml with distilled water; the hemagglutination titer was 1:256 by a standard test (6). This antigen was used to sensitize sheep RBC as follows: a 10% suspension (packed RBC volume [milliliters]/milliliter of antigen) of cells was made in reconstituted antigen and allowed to stand for 10 min at room temperature. After centrifugation at $6,000 \times g$ for 5 min, the sensitized cells were resuspended at $0°C$ in $5 \times 10^{-3}$ M sodium, 5,5′-diethyl barbiturate buffer, pH 7.3, containing 0.14 M NaCl, $5 \times 10^{-4}$ M $MgCl_2$, and $5 \times 10^{-5}$ M $CaCl_2$. Suspended cells were centrifuged as above and resuspended at a concentration of 10% (vol/vol) in the barbiturate buffer ($0°C$). Agarose (L'Industrie Biologic Francaise, S.A.) was dissolved at a concentration of 1.5% (wt/vol) by boiling in phosphate-buffered saline, pH 7.2, to which 0.1% (wt/vol) sodium azide had been added as a preservative. The molten agarose was then distributed into 5-ml glass tubes (2.7 ml/tube) and cooled for 45°C in a water bath. A 0.3-ml amount of the 10% RBC suspension was added quickly to each tube of agarose, and the contents of the tube were mixed vigorously and poured into empty diffusion plates (Hyland Laboratories, Costa Mesa, Calif; catalog no. 085-710) to cool. After solidification, wells 2 mm in diameter were punched in the RBC/agarose, and 0.02 ml of the test or control sera (heat inactivated at 56°C for 30 min) was added to each well. The plates were then allowed to stand for 18 to 24 h at 4°C. At the end of this time, 2.0 ml of guinea pig serum (a

source of C') diluted 1:10 in barbiturate buffer (above) was pipetted over the surface of the gel. Lyophilized/reconstituted guinea pig sera used in this study had complement activities of $280 \pm 30$ 50% hemolytic complement units/ml. The plates then stood for 3 to 6 h at 37°C; after removal of the excess C', diameters of the hemolytic zones were measured to the nearest 0.5 mm.

**Commercially prepared SRH plates.** For comparative studies, rubella hemagglutinin-sensitized sheep RBC SRH plates were obtained through the courtesy of Norman Finter, Wellcome Laboratories, Kent, England, along with supplies of strong and weak positive and negative control sera. Control and test sera were tested as described above.

**HI.** HI tests were carried out by a standardized method, using fresh human type O Rh(−) cells as indicator (6). Test sera were not heat inactivated. All test sera had been submitted to the laboratory for immunity tests or for serodiagnosis of rubella infection.

**RBC.** Sheep, chicken, and chick RBC were obtained from laboratory animals maintained at the State Laboratory Institute. Human type O Rh(−) cells (6) were obtained from the Red Cross Laboratories, Boston. Cells were collected aseptically and stored in Alsever solution (6) at 4°C. Sheep cells remained usable for 6 weeks after collection; chicken, chick, and human O cells could be stored for 3 weeks.

**Serum samples for SRH.** In experiments designed to determine the sensitivity and accuracy of the SRH method, serum samples were selected, grouped according to categories, and tested unblinded (Table 1; Fig. 1). After exploration and development of the SRH test presented here, samples were selected and tested blind by code (Tables 2 and 3).

## RESULTS

The optimal values of several variables were examined with a view toward balancing maximum economy of reagents with sensitivity and reliability. We studied final RBC concentration in the gel, the RBC/antigen ratio during sensitization, the antigen concentration during sensitization, and the minimum amount of complement needed for an interpretable result.

**Concentration of sheep RBC.** RBC are commonly used at a concentration of 1% in the gel. Since some economy can be realized by using fewer cells, we examined final concentrations of 0.83, 0.66, 0.50, 0.33, and 0.1%. Using triplicate determinations with several different antisera, we found no effect on the diameter of the hemolytic zones as a function of RBC concentration. The clarity of the zones, however, changed noticeably; as the red background decreased, it became increasingly difficult to discern hemolysis. Below 0.33% the contrast was too small to be suitable for routine diagnostic use. Thus, final RBC concentrations of greater than 0.5% (generally 1%) were used for the present study.

**Ratio of RBC to reconstituted antigen.** The RBC/antigen ratio, i.e., packed RBC volume (in milliliters) per milliliter of reconstituted antigen, was examined next. The ratios tested were 0.1/0.9 (as cited in Materials and Methods), 0.2/0.8, 0.3/0.7, 0.4/0.6, and 0.5/0.5. Three different antisera (one each of high, intermediate, and low titer) were tested in triplicate for each ratio. We observed that variation in the ratio had no effect on the measured hemolytic zone diameter. However, the opacity of the zone decreased as the amount of antigen per RBC decreased. The observable difference between hemolyzed and intact cells on the plates was unacceptable for routine use as ratios of 0.4/0.6 and 0.5/0.5. However, a ratio of 0.3/0.7 was suitable and represented a considerable savings in antigen, since 0.7 ml of antigen yielded 0.3 ml of usable sensitized cells, whereas at the 0.1/0.9 ratio (prominent in the literature and cited in Materials and Methods) a volume of 0.9 ml of antigen yielded only 0.1 ml of usable cells.

**Antigen concentration.** In a similar experiment, we varied the antigen concentration while maintaining the same final RBC percentage (by volume) during sensitization. The antigen was used at three different levels: (i) normal 1× reconstitution of the antigen (cf. Materials and Methods); (ii) a 10-fold dilution of the normal reconstitution; (iii) a 10-fold concentration of the usual amount (achieved by hydrating the antigen as usual, centrifuging the reconstituted antigen at 105,000 × g for 45 min, and redissolving the precipitate in supernatant fluid at 1/10 the starting volume).

Forty sera with HI titers of 1:8 were run at each antigen dilution. Diameters of lysis were measured, converted into area, and compared for each antigen concentration.

The mean area of the hemolytic zone did not vary significantly as a function of antigen level used during sensitization. Qualitatively, however, the contrast on the 0.1× plates was poor, zones of clearing were much more pronounced at 1×, and in the 10× plates the zones of hemolysis were extremely sharp and free of unlysed cells.

**Concentration of complement.** Experiments were also carried out to test the effect of varying the concentration of complement used. The following dilutions of guinea pig serum were used: undiluted, 1:5, 1:10, 1:20, and 1:40. In triplicate tests on two different antisera, representing high and low titers, the respective diameters of the hemolytic zones did not change as a function of C' concentration. The clarity of these zones, however, decreased with increasing C' dilution so that 1/10 was the highest dilution

which could be successfully employed for routine use.

**Comparison of Massachusetts Department of Public Health Virus Laboratory (VL) and commercial rubella SRH plates.** Having determined optimal values for several variables important in the preparation of SRH plates, it was then decided that a comparison between plates prepared in our laboratory and those available commercially would be in order. If this comparison proved favorable, considerable economic benefit would result.

For the comparison, serum samples were selected at random from specimens submitted to the State Laboratory Institute Virus Laboratory for determination of rubella antibody status: 287 sera were tested on VL plates, and 279 of these sera were tested on commercial (Wellcome [W]) plates.

Initially, the samples were grouped by HI titer and run on SRH plates. The resulting data were converted to areas and the mean and standard error of the mean were calculated for $n$ samples in each group as shown in Table 1. These data are also plotted in Fig. 1, which illustrates the linear relationships between the SRH areas and the logarithm of the HI titer; this linearity was seen in data from both sets of plates. The equations for these relationships assume the form: SRH (area) = $K$ log HI titer + $K'$, where $K$ is the slope of the line and $K'$ is the $y$ intercept.

Using the method of least squares, the respective equations of the lines in Fig. 1 were determined, along with the correlation coefficient ($r$). It is evident that the VL and W plates generate very similar equations; in both cases the lines are excellent representations of the respective data used to derive them ($r = 0.97$ and $0.98$ for the VL and W plates, respectively).

**Sensitivity of SRH versus HI.** The equations derived above suggest a general relation-
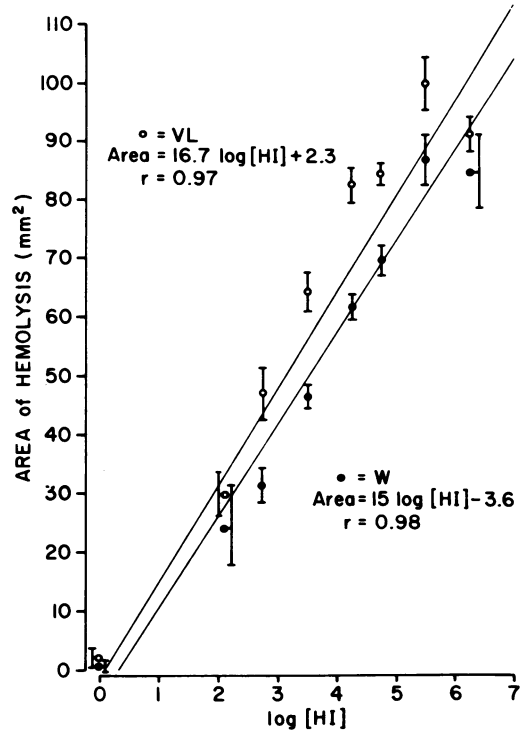


FIG. 1. *Relationship of the SRH area to the HI titer for VL and W plates.*

ship between SRH and HI in the case of rubella antiserum titers. They do not, however, reveal any systematic differences in the sensitivities of the respective methods. We thought it desirable to assess what (if any) proportion of sera chosen at random (including acute, convalescent, and negative sera) and tested blind would give negative results when screened by SRH versus those which were negative by HI.

Table 2 gives the results on 293 specimens tested by HI and SRH (both VL and W).

Two specimens found positive by HI contained no rubella antibody measurable by SRH. Both sera negative by SRH had HI antibody titers of 1:16 and were acute specimens from diagnostic pairs. Differences between HI and SRH reactivities may be due to false-positive HI tests or to lack of immunoglobulin M antibody activity in SRH (2, 8). The sera were not fractionated and tested for rubella-specific immunoglobulin M antibody (6). Results from VL and W plates were essentially the same.

We also compared the relative efficiency of SRH and HI in determining recent rubella infections. Paired (acute/convalescent) sera from 12 cases clinically diagnosed as rubella were tested by HI and on W plates (Table 3). In all

TABLE 1. *Comparison of hemolysis on VL and commercially prepared (W) SRH plates*

| HI titer | W | | VL | |
|---|---|---|---|---|
| | $n^a$ | Area (mm²)$^b$ | $n$ | Area (mm²) |
| <8 | 59 | 1.9 ± 1.1 | 44 | 2.1 ± 1.2 |
| 8 | 7 | 24.0 ± 6.7 | 40 | 29.4 ± 2.3 |
| 16 | 31 | 31.1 ± 3.1 | 27 | 47.3 ± 4.5 |
| 32 | 64 | 46.5 ± 2.0 | 60 | 64.6 ± 2.7 |
| 64 | 54 | 61.9 ± 2.1 | 58 | 83.0 ± 2.7 |
| 128 | 44 | 69.8 ± 2.4 | 39 | 84.8 ± 1.4 |
| 256 | 13 | 87.1 ± 4.1 | 12 | 101.8 ± 4.3 |
| 512 | 7 | 85.0 ± 6.8 | 7 | 91.7 ± 2.7 |

$^a$ Number of samples tested at each range of antibody level.

$^b$ Mean hemolytic area ± standard error of the mean.

TABLE 2. *Detection of antibody by SRH and HI*

| Method | Positive/total[a] | % Positive |
|---|---|---|
| HI | 252/293 | 86.0 |
| SRH | 250/293 | 85.3 |

[a] Total number with detectable antibody/total tested.

instances a fourfold increase in antibody against rubella was seen by HI. By use of the W equation from Fig. 1, we determined the HI equivalents of the SRH reading for each serum (Table 3). In all 12 pairs, fourfold or greater antibody titer rises were also found by SRH.

**Specificity.** The HI test is subject to misleading results caused by nonspecific agglutinins which may be present in the sample (6). When chick cells are used as indicators, the agglutinins are routinely removed by adsorption of the sera with chicken cells; if trypsinized human type O Rh(−) cells are used, prior absorption is not normally required (8). We tested 85 unadsorbed serum samples containing chick cell agglutinins (at titers of 1:8 or greater) by HI with human O Rh(−) cells and by SRH on VL plates. By both methods, 63 rubella-positive samples were found, and 22 negatives.

## DISCUSSION

An effort was made to study the SRH test in order to define those circumstances permitting maximum reliability and sensitivity with minimum expenditure of resources. The most expensive components of the test are blood cells, antigen, and C'. It was found that varying amounts and ratios of these reagents did not markedly affect the final values observed but did affect the ease of measurement. At present, the cost per test for the components can be as low as $0.16 (12 sera/plate) for an easily interpretable test compared to $1.15 for an HI test. All components for the HI test are based on bid prices from commercial sources.

Some preliminary tests in our laboratory suggest that SRH plates (both VL and W) have a shelf life of about 2 weeks. Thus, sufficient plates can be prepared and standardized in 1 day and stored to anticipate short-term needs.

The major advantage of this technique is that only one well in the plate and one simple measurement are required to titrate the antibody concentration of a serum. Once the plates are made, the technician need only add the sample, incubate, add C', and measure the hemolytic zone. Thus, many more tests can be completed than by the more cumbersome conventional dilution techniques for antibody titration.

Plates made in this laboratory compared favorably with plates under development by a

commercial source. For a large number of tests the effort in making the plates from starting materials is very small as compared to the effort of standard HI determinations. Thus, savings are realized from two sources: (i) the inherent efficiency of the SRH technique versus the HI procedure; (ii) the economy of using homemade plates as opposed to commercially produced ones. The disadvantage of SRH as compared to HI is that SRH requires 24 h for completion, whereas the HI test can be completed in 1 working day.

Two sera with HI titers of <1:8 produced low levels of hemolysis by SRH (Table 1). Whether reactivity in gel was a response to antibody missed by HI or is in fact an SRH false-positive reaction cannot be determined from the present experiments. Serum samples with HI titers of <1:8 may contain antibody in the range of 1:2 to 1:7 and not be detected by HI. It is not known whether antibody to rubella measured by SRH is the same as HI or neutralizing antibody. Klingeborn and Dinter (4) carried out experiments with equid herpesvirus which demonstrated that antibody mediating SRH was probably that responsible for neutralization.

Two sera (Table 2) were found to contain low levels of antibody by HI (1:8 and 1:16) but not by SRH. Differences between HI and SRH reac-

TABLE 3. *Diagnosis of recent rubella infection by SRH*

| Case no. | Acute/convalescent (a/c) serum specimen | Reciprocal HI titer | SRH area (mm²) | HI equivalent[a] |
|---|---|---|---|---|
| 1 | a | <8 | 0 | <8 |
|  | c | 128 | 88.0 | 300 |
| 2 | a | <16 | 0 | <8 |
|  | c | 256 | 71.5 | 120 |
| 3 | a | <8 | 0 | <8 |
|  | c | 256 | 96.8 | 500 |
| 4 | a | <8 | 0 | <8 |
|  | c | 64 | 49.7 | 40 |
| 5 | a | <8 | 0 | <8 |
|  | c | 64 | 56.5 | 50 |
| 6 | a | <8 | 0 | <8 |
|  | c | 128 | 43.2 | 30 |
| 7 | a | <16 | 0 | <8 |
|  | c | 512 | 106 | 1,000 |
| 8 | a | <8 | 0 | <8 |
|  | c | 128 | 79.5 | 220 |
| 9 | a | <8 | 0 | <8 |
|  | c | 32 | 43.2 | 30 |
| 10 | a | <8 | 0 | <8 |
|  | c | 64 | 71.5 | 120 |
| 11 | a | <16 | 0 | <8 |
|  | c | 128 | 79.5 | 220 |
| 12 | a | <8 | 0 | <8 |
|  | c | 256 | 88 | 300 |

[a] Determined graphically, using the W relationship of Fig. 1.

tivities may be due to nonspecific false-positive HI reactions or to immunoglobulin M antibody (8). Rubella immunoglobulin M antibody tests were not carried out on these sera. As a diagnostic tool, however, this is not a particular disadvantage since paired sera and seroconversion or fourfold rises in antibody are required.

In the development of new methodology for diagnostic investigation, it is necessary to have a simple and reliable means of relating the new technique to more established procedures in widespread use. The SRH technique is readily related to conventional HI titration by a simple linear function. Thus, results determined by SRH can be readily converted to the more familiar HI format if so desired.

## ACKNOWLEDGMENTS

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