A Single Radial Haemolysis Technique for the Measurement of Influenza Antibody

By S. M. RUSSELL*, D. McCAHON* AND A. S. BEARE†

* Virology Dept., Wellcome Research Laboratories, Langley Court, Beckenham, Kent and † M.R.C. Common Cold Unit, Harvard Hospital, Salisbury, Wiltshire, U.K.

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SUMMARY

A single radial haemolysis in gel technique has been developed for the detection and measurement of antibody to influenza haemagglutinin. The method combines the sensitivity of haemagglutination-inhibition with the accuracy of single radial diffusion. It is simple, quick, reproducible, does not require purified or concentrated virus, and is unaffected by non-specific inhibitors. The method is particularly suitable for the routine screening of large numbers of serum samples, and may have application also to viruses other than the influenza group.

INTRODUCTION

There are a number of methods at present available for the detection of antibodies with specificity for the haemagglutinin of influenza virus. Qualitative information can be obtained by gel immuno-double-diffusion tests with detergent-disrupted virus (Schild, 1970). Alternative quantitative methods are the haemagglutination-inhibition test (Hirst, 1942) and single radial diffusion (Schild, Henry-Aymard & Pereira, 1972).

Haemagglutination-inhibition (HAI) has the advantage of good sensitivity, but great care is necessary to achieve reproducible results. Other disadvantages are that sera must be treated to remove non-specific inhibitors before they can be tested (Robinson & Dowdle, 1969) and small differences in antibody titre cannot reliably be resolved. It is commonly accepted that differences in titre of less than 400% are not statistically significant.

The single radial diffusion (SRD) technique is much simpler to perform, and has greatly improved accuracy. It is, however, less sensitive than HAI (Schild et al. 1972). There is also the disadvantage that relatively large quantities of purified and concentrated virus must be used.

Single radial haemolysis in gel systems has been used by Weiler, Melletz & Breuninger-Peck (1965) to estimate the haemolytic activity of mouse IgG antibody and by Hiramoto et al. (1971) to measure mouse 19S anti-red blood cell antibody.

We describe here a single radial haemolysis in gel (SRH) technique for the measurement of anti-haemagglutinin antibody which combines as far as possible the advantages of SRD and HAI. The technique is simple and quick to use, can resolve small differences in antibody level and has a similar sensitivity to that of HAI.

METHODS

Viruses. Influenza viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. The following viruses were used: WRL 44, inhibitor-resistant recombinant (A/Okuda/57-A/England/42/72, H3N2) with the surface antigens of A/England/42/72. X31
Antisera. Ferret antisera were prepared by the intranasal inoculation of infected allantoic fluid. The animals were bled 14 or 21 days later. Rabbit antisera were raised by the intramuscular injection of highly purified virus in Freund's complete adjuvant on two occasions 14 days apart.

Some rabbit antisera, raised by the same method, were provided by Dr C. M. Brand.

Red blood cells. Chicken, sheep or bovine red blood cells were collected in anticoagulant (Alsever's medium) and stored at 4 °C for up to 4 weeks. Before use the cells were washed three times in 0.01 M-phosphate buffered saline, pH 7.2.

SRH technique

Coating of red blood cells with virus. Equal volumes of washed packed red cells and dilute potassium periodate (Analar) in saline were mixed and allowed to stand at room temperature for 10 min. The concentration of periodate used depends on the species from which the red blood cells are obtained. $5 \times 10^{-4}$ M-KIO$_4$ was found to be optimal for sheep erythrocytes, and $1.25 \times 10^{-3}$ M-KIO$_4$ for bovine erythrocytes.

Allantoic fluid containing influenza virus was then added, the volume being at least ten times that of the packed red cells, and the haemagglutination titre approx. 1000/o.25 ml. The quantity of virus was thus not less than $4 \times 10^8$ H.A.U./o.1 ml packed red blood cells. The mixture was allowed to stand at room temperature for 10 min, the coated cells then washed three times in barbitone buffered saline (Wellcome Reagents Ltd), and packed at 1500 g for 5 min. They were finally made up to a 50 %, v/v, suspension in barbitone buffered saline. This method is a modification of that described by Fazekas de St. Groth (1949).

Preparation of gels. Agarose (Indubiose A37, L'Industrie Biologique Francaise SA) was made up to give a final concentration of 1 % in barbitone buffered saline, pH 7.2, incorporating 0.1 % sodium azide. 0.1 ml of the 50 %, red blood cell suspension plus 0.1 ml of guinea pig serum (as a source of complement) was mixed with 2.8 ml of the agarose at 45 °C. The mixture was poured at once into an empty immuno-diffusion plate (Hyland Division, Travenol Labs Inc. U.S.A.) and allowed to set. Three mm diam. wells were then punched in the gel.

Measurement of antibody. Each well was filled with 10 μl of serum, and the gels stored in a moist box overnight at 4 °C to allow completion of diffusion. Incubation of the gels at 37 °C for 2 to 3 h then resulted in the formation of zones of lysis around those wells filled with sera having antibody to the virus coating the red blood cells.

It should be noted that the concentration of the complement is not critical. The complement does not affect the size of the zone produced, but merely serves to make it visible by lysing the red blood cells which have virus-antibody complexes at their surfaces.

For each zone, two diameters at right-angles were measured using a × 8 magnifier equipped with a graticule calibrated in 0.1 mm divisions (Matchless Machines Ltd, U.K.).

Gels kept as a permanent record were preserved by treatment with 5 % formalin in saline. Storage for many months at 4 °C is then possible without deterioration if the gels are kept in a moist atmosphere.

Haemagglutination-inhibition test. Before use all sera were treated with receptor destroying enzyme (RDE) (Phillips-Duphar, Holland) at the recommended dose. Haemagglutination-
Single radial haemolysis for influenza

Fig. 1. Influence of the concentration of virus adsorbed to erythrocytes on the zone size obtained with a ferret antiserum (anti A/HK/68). The concentrations shown are expressed as H.A.U. of X31 virus per 0.1 ml of packed sheep erythrocytes; 2000 H.A.U. (●—●), 4000 H.A.U. (●— ●), 8000 H.A.U. (●— ●), 16000 H.A.U. (●—●). Each point represents the mean of two estimations on the same plate.

inhibition tests were performed in plastic trays according to the technique described by the Expert Committee on Respiratory Virus Diseases (1959), except that serum-virus mixtures were kept for 1 h at room temperature before addition of the erythrocyte suspension.

RESULTS

Factors affecting sensitivity

Concentration of virus in the gel

This parameter is controlled both by the number of red cells added to each gel, and by the amount of virus which coats them.

It was found that as the concentration of coated red cells in the gel was reduced, so the zone size increased, but contrast between lysed and unlysed areas of gel was diminished. The concentration of red cells chosen for routine use was thus a compromise, and was fixed at 100 μl of a 50% suspension in 3 ml of agarose gel.

The susceptibility of the technique to variation introduced by changes in the concentration of the virus used to coat the red blood cells was also examined. 0.2 ml volumes of packed sheep erythrocytes were treated with 5 x 10^-4 m-potassium periodate, and then to each was added 4 ml of a dilution of allantoic fluid containing X31 virus. Gels were made, and tested with dilutions of ferret anti-A/Hong Kong/1/68 serum. Results are shown in Fig. 1.
Table 1. Variation in area of lysis within and between gels from the same batch*

<table>
<thead>
<tr>
<th>Area of lysis (mm²) calculated from mean diam.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel 1</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Mean†</td>
</tr>
<tr>
<td>Standard deviation</td>
</tr>
<tr>
<td>Coefficient of variation‡</td>
</tr>
</tbody>
</table>

* Gels contained bovine red blood cells coated with WRL 44 virus. Seven replicate wells in each gel filled with 10 µl rabbit Anti-A/England/42/72 serum diluted 1:20.
† Overall mean area = 78.24 mm².
‡ Overall coefficient of variation = 2.93%.

As expected, reduction in virus concentration increases the sensitivity of the technique. However, above 4000 H.A.U./0.1 ml. of packed sheep erythrocytes quite large changes in virus concentration have relatively little effect on zone size. It seems probable that at this concentration of virus, most of the available receptors on the red cell surface have been saturated with virus. Therefore, as long as the virus concentration was adjusted to approx. 40000 H.A.U./0.1 ml of packed erythrocytes, reproducible results could be achieved. The technique is thus much more tolerant of variation in this respect than is HAI.

**Diffusion time**

Since diffusion of an antiserum through a gel is time-dependent, zone size should be measured either at a fixed time after the start of diffusion, or when diffusion is substantially complete. In the experiments described, zones of lysis were measured after a minimum of 18 h. Increase of this time up to 72 h produced no significant increase in zone size.

**Volume of serum sample**

The size of zone produced depends on the absolute amount of antibody free to diffuse. Increase in the volume of serum will thus increase zone size. In the work described, 3 mm diam. wells were used. These would conveniently hold 10 µl of serum at a single filling and the technique was then capable of adequate sensitivity.

**Reproducibility**

Three levels of variation were investigated; that occurring within a gel, that between gels prepared at the same time, and that occurring between gels prepared on different days.

The first two levels of variation were examined simultaneously. Table 1 shows the results obtained by running seven replicates of a 1:20 dilution of a rabbit anti-A/England/42/72 serum in each of four gels containing bovine erythrocytes coated with WRL 44 virus. The variation in zone areas within each gel is less than that between gels, but both are of a low order.

Also of importance is the variation between gels made at different times. Information on the extent of this variability was obtained by testing dilutions of 2 rabbit and 2 ferret anti-A/Hong Kong/1/68 sera by SRH on each of three successive day. The same serum dilutions were used throughout, but sheep erythrocytes were freshly coated with X31 virus and new gels poured on each day. The results are shown in Table 2.

Again, the variation obtained on each day is low, and, as expected, is exceeded by the
Single radial haemolysis for influenza

Table 2. Variation in area of lysis between gels made on different days*

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>1:1</th>
<th>1:8</th>
<th>1:64</th>
<th>1:512</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferret A</td>
<td>96.93 ± 7.79†</td>
<td>50.87 ± 4.96</td>
<td>17.87 ± 1.75</td>
<td>0</td>
</tr>
<tr>
<td>Ferret B</td>
<td>92.97 ± 6.12</td>
<td>47.23 ± 3.49</td>
<td>17.15 ± 0.77</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit A</td>
<td>122.42 ± 10.23</td>
<td>84.87 ± 6.68</td>
<td>45.83 ± 4.79</td>
<td>15.23 ± 1.83</td>
</tr>
<tr>
<td>Rabbit B</td>
<td>151.71 ± 8.66</td>
<td>104.69 ± 3.48</td>
<td>70.54 ± 2.72</td>
<td>24.59 ± 3.23</td>
</tr>
</tbody>
</table>

* Gels contained sheep erythrocytes coated with X31 virus. Antisera were all anti-A/Hong Kong/1/68 sera.
† Overall mean area ± standard deviation of 3 to 4 replicate tests repeated on each of 3 days.

Fig. 2. Linear relationship between zone area and serum dilution. Gels contained sheep erythrocytes coated with X31 virus. Four sera prepared against the homologous virus were examined: ●, rabbit A; ○, rabbit B; ■ ferret A and B (identical results). Each point represents the mean of 3 estimations on the same plate with the appropriate standard deviation.

variation between days. Even in the latter case, however, overall standard deviations are low, and coefficients of variation do not exceed 13%.

Thus the coefficient of variation of results obtained with a single gel can be less than 2%, that between gels of the same batch, 3%, and that between batches, only 13%.

The results shown in Fig. 2 indicate that a straight-line relationship exists between the area of lysis and the logarithm of the antibody concentration. Statistical analysis has shown that the slope obtained with the rabbit sera differed slightly but consistently from those of the ferret sera. Within each species, the slopes were very similar. It is likely that this result reflects not a species difference, but the difference between convalescent and hyperimmune sera.
Fig. 3. Resolving ability of SRH technique. Closely spaced dilutions of a rabbit antiserum (anti-A/Eng/42/72) in normal rabbit serum were tested in gels containing bovine erythrocytes coated with WRL 44 virus. From left to right, top row 1:10, 1:12.5, 1:15, 1:20, bottom row 1:25, 1:30, 1:40, normal rabbit serum control.

Table 3. Resolving ability of SRH*  

<table>
<thead>
<tr>
<th>Rabbit Anti-A/England/42/72 serum dilution</th>
<th>Normal rabbit serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>1:12.5</td>
</tr>
<tr>
<td>Mean area† (mm²)</td>
<td>74.6</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0</td>
</tr>
</tbody>
</table>

* Closely spaced dilutions of the rabbit antiserum were made in normal rabbit serum and tested in gels containing bovine erythrocytes coated with WRL 44 virus.
† Areas are means of 4 replicates measured on 4 different plates.

Accuracy

The ability of SRH to distinguish reliably between sera of similar titre was examined. Close-space dilutions of rabbit anti-A/England/42/72 serum were made in normal rabbit serum (NRS). Thus, the only constituent to vary significantly between dilutions was the influenza-specific antibody. Each serum dilution was pipetted into one well in each of four replicate gels containing bovine erythrocytes coated with WRL 44 virus.

The appearance of the zones of lysis produced is shown in Fig. 3. Zone areas and standard deviations are presented in Table 3. This result clearly indicates the ability of the technique to discriminate between sera of similar titre. Also shown is the absence of any zone of lysis around the well containing NRS only, indicating that non-specific inhibitors do not produce false positive results.

The role of non-specific inhibitors in SRH was examined further as follows. Fourfold dilution steps of the rabbit anti-A/England/42/72 serum, starting at a 1:20 dilution, were made either in PBS or in normal rabbit serum. Thus, inhibitors and specific antibody were in one case diluted to the same degree, whilst, in the other, antibody only was diluted and the non-specific inhibitors remained at a high level. Both dilution series were then tested by SRH, with no attempt to remove the inhibitors, and by HAI, after treatment with receptor-destroying enzyme (RDE).

Table 4 displays the results of this experiment, and demonstrates that SRH is little affected by the presence of large concentrations of non-specific inhibitors, whilst HAI is badly affected, even after treatment of the serum with RDE. The superior resolution of SRH is also clearly evident and there is an indication of its sensitivity compared with the HAI technique.
Table 4. Insensitivity of SRH to non-specific inhibitors*

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Test</th>
<th>Serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:20</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>SRH area (mm²) ± SD</td>
<td>74.7 ± 3.46</td>
</tr>
<tr>
<td></td>
<td>HAI reciprocal titre</td>
<td>288</td>
</tr>
<tr>
<td>PBS</td>
<td>SRH area (mm²) ± SD</td>
<td>72.6 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>HAI reciprocal titre</td>
<td>576</td>
</tr>
</tbody>
</table>

* Rabbit anti-A/Eng/42/72 serum was diluted in parallel in normal rabbit serum and in PBS. The SRH gels contained bovine erythrocytes coated with WRL 44 virus and the areas shown are the means of 4 replicates in different gels with their standard deviations. For HAI the sera were first treated with RDE to remove inhibitors.

Table 5. Specificity of SRH*

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Virus</th>
<th>X31 (Hong Kong)</th>
<th>WRL 44 (A/Eng/42/72)</th>
<th>MRC 11 (A/PC/I/73)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferret</td>
<td>Anti-A/HK/1/68 serum</td>
<td>1:00 †</td>
<td>0.83</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-A/HK/1/68 serum 2</td>
<td>1:00</td>
<td>0.86</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-A/E/42/72 serum 1</td>
<td>0.71</td>
<td>1:00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti-A/E/42/72 serum 2</td>
<td>0.79</td>
<td>1:00</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Anti-A/PC/I/73 serum 1</td>
<td>0</td>
<td>0.87</td>
<td>1:00</td>
</tr>
<tr>
<td></td>
<td>Anti-A/PC/I/73 serum 2</td>
<td>0</td>
<td>1:00</td>
<td>1:00</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Anti-A/E/42/72 serum 1</td>
<td>0.70</td>
<td>1:00</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Anti-A/E/42/72 serum 2</td>
<td>0.46</td>
<td>1:00</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Anti-A/PC/I/73 serum 1</td>
<td>0</td>
<td>0.66</td>
<td>1:00</td>
</tr>
<tr>
<td></td>
<td>Anti-A/PC/I/73 serum 2</td>
<td>0</td>
<td>0.89</td>
<td>1:00</td>
</tr>
</tbody>
</table>

* Sera were tested at several dilutions in gels containing sheep erythrocytes coated with X31, WRL 44 or MRC 11 virus.
† Normalized values were calculated for each serum by dividing zone areas by the area obtained with the homologous virus.

Specificity

During the process of coating the red blood cells with influenza virus, allantoic fluid is mixed with the cells. It might be thought that egg proteins would, therefore, interfere with the specificity of the test, making it unsuitable for distinguishing between closely related strains of virus.

A number of antisera were tested against three viruses bearing closely related haemagglutinins: A/Hong Kong/1/68, A/England/42/72, and A/Port Chalmers/1/73. The data obtained are shown in Table 5. For each antiserum the zone areas produced with each virus were divided by the area obtained with the homologous virus. A direct comparison of the different sera could then be made.

Although some sera are clearly more specific than others, closely related viruses may easily be distinguished. Also noteworthy is the finding that some sera failed to form a zone if tested against a more distantly related virus. Thus, sera raised against A/Hong Kong/1/68 failed to react in gels containing A/Port Chalmers/1/73 virus. No problems resulting from the use of unpurified virus have therefore been encountered.

Sometimes, sera have been found to contain antibody to the red blood cells used. Some rabbit sera are troublesome in this respect when tested in gels containing sheep erythrocytes.
Fig. 4. Correlation between SRH zone area and HAI titre in human sera. 102 sera were examined in parallel in HAI and SRH using X31 virus as the antigen.

The problem seems to be caused by the presence of an IgM antibody directed against Forssman antigen. The very small zone of lysis produced in the gel rarely interferes with the test, but can be eliminated by absorbing the serum with sheep erythrocytes at 37 °C for 30 min prior to testing. Alternatively, the use of bovine red blood cells in the gel in place of sheep cells will eliminate the problem.

Measurement of antibody in human sera and nasal washings

Correlation of SRH zone area with HAI titre

102 human sera from normal volunteers were screened for antibody to A/Hong Kong/1/68 haemagglutinin by both the SRH and HAI techniques. Of the 99 sera having HAI titres $\geq 1/12$, 92 were positive by SRH. As Fig. 4 shows, there is a positive correlation between the results obtained with the two methods, the calculated correlation coefficient being 0.7. A number of sera had significant HAI titres but did not give positive results by SRH. Such a discrepancy would occur if these sera contained residual non-specific inhibitors after RDE treatment.

Similar parallel assays of sera by the two techniques, but using A/Port Chalmers/1/73 or A/England/42/72 viruses, gave correlation coefficients of 0.61 or 0.59 respectively. In view of the known limits of error of the two techniques, it is likely that much of the scatter observed is attributable to the HAI assay.
Single radial haemolysis for influenza

Detection of seroconversions

A total of 69 paired sera from volunteers before and after challenge with live influenza virus were screened by SRH, and independently by HAI using A/England/42/72 antigens. When using the SRH technique, seroconversion was arbitrarily defined as an increase in zone area of at least 10 mm\(^2\), this being a conservative value. Of the 30 seroconversions detected by HAI, 28 were also detected by SRH. A further four conversions not found by HAI were in addition detected by SRH. 87 sera from this trial had an HAI titre \( \geq 1/24 \), of which 74 produced visible zones by SRH. Thus 85% of sera positive by HAI were also detected by SRH. Again, the discrepancy is largely attributable to the effect on the HAI technique of residual non-specific inhibitors.

Antibody in nasal washings

Nasal washings were collected from volunteers after challenge with live virus. The washings were concentrated 50-fold using Minicon B15 Macrosolute Concentrators (Amicon Corp. U.S.A.), and then assayed for antibody to A/England/42/72 haemagglutinin directly by SRH, or by HAI after RDE treatment.

A total of 21 nasal washings were examined. Of these, 16 were negative by both techniques. Three samples had HAI titres of 9, of which one was confirmed as positive by SRH. One sample having a titre of 12, and one of 20, were both detected by SRH.

It is presumed that the antibody detected by SRH was of the IgG class, since IgA would not normally be expected to fix complement.

DISCUSSION

A large number of human sera from various vaccine trials have been screened by SRH. There is good agreement with the HAI data about the presence or absence of specific antibody to influenza virus strains. In addition, rises in antibody level after infection, as measured by HAI, have been confirmed by SRH.

The SRH technique appears particularly suited to the screening of serum samples since it is simple, rapid, accurate, and unaffected by non-specific inhibitors. Thus, small differences in antibody level can be reliably detected, and, since samples do not require RDE treatment before testing, results may be easily obtained within 24 h.

Although the source of virus for the coating of erythrocytes is unpurified allantoic fluid, the technique is capable of distinguishing between closely related strains of influenza. Similarly, sera having a high level of anti-egg protein activity do not produce zones of lysis in gels containing an irrelevant virus. No problems of non-specificity have therefore been encountered.

The choice of erythrocyte is significant in this connection. If chicken erythrocytes are used, then anti-egg protein antibodies will cause problems. Other avian red blood cells are more acceptable, but their shape and size result in the production of less sharply defined zones. Sheep erythrocytes are satisfactory for many purposes, but a few sera will be encountered which contain antibody to sheep erythrocytes, and particularly to the Forssman antigen they carry. Prior absorption of these sera with packed sheep red blood cells at 37 °C for 30 min is sufficient to remove this antibody. The use of bovine erythrocytes in the gel will circumvent the difficulty, but some strains of influenza virus attach less readily to these cells than do others.

Preliminary experiments suggest that anti-virus IgG is detectable by the system. Work is
in progress to determine what other classes of antibody can be detected by SRH. Modification of the technique to incorporate antiglobulin reagents should allow the detection of each class of virus-specific antibody separately.

Further work is also required to determine whether antibody with specificity for neuraminidase can be measured using SRH. The evidence so far available suggests that this is not the case with the technique as described. Modification of the method to detect anti-neuraminidase antibody may however prove possible.

There is no theoretical reason why SRH should not be applied to the detection of antibody elicited by viruses other than the influenza group. Its application is limited only by the ease with which virus antigen can be fixed onto erythrocytes.

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REFERENCES


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