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Rubella antibody measured by radial haemolysis. Characteristics and performance of a simple screening method for use in diagnostic laboratories*

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SUMMARY

A simple method for preparing radial haemolysis gels for rubella antibody screening is described. In use it gave clear zones of haemolysis when a standard serum was tested at dilutions down to 5·6 i.u./ml rubella antibody. In five laboratories 8404 sera were screened by the method and the results were read by comparing zones of haemolysis with that of a standard serum diluted to contain 15 i.u./ml antibody. A zone $\geqslant$ 15 i.u./ml, indicating immunity, was given by 7433 (88·4%) of the sera. No zone indicating susceptibility was seen with 748 (8·9%) sera. Small zones, $<$ 15 i.u./ml standard, were given by 189 (2·2%) sera, and in only 34 cases (0·4%) did non-specific haemolysis interfere with the test readings. Further testing of the radial haemolysis negative and low positive sera by the haemagglutination inhibition test gave rise to some discrepant results which are discussed.

* A report of a Public Health Laboratory Service Working Party. Other members were A. D. Macrae (Nottingham Public Health Laboratory), H. G. S. Murray (Central Public Health Laboratory, Colindale) and R. Pilsworth, (Chelmsford Public Health Laboratory).

INTRODUCTION

Radial haemolysis (RH) is now widely known as a method of detecting antibody to haemagglutinating viruses. Tests using several viruses including influenza (Russell, McCahon & Beare, 1975), mumps (Grillner & Blomberg, 1976), measles (Wesslen, 1978) and rubella (Skaug, Ørstavik & Ulstrup, 1975), have been described, and it is known that a number of other viral haemagglutinins will mediate this reaction. Particular interest has been shown in the application of the RH method to rubella, and several different formulations of rubella RH gels have been published. These do not differ in principle, but variations, for example in the red cells used (chick, pigeon, sheep, trypsinized human 'O', etc.) and in the way in which complement is added to the gels, make it difficult to decide which formulation best meets the needs of the diagnostic laboratory performing upwards of a hundred rubella screening tests one or more times per week.

This Working Party was asked to recommend a rubella RH method in anticipation of a national vaccination campaign beginning in 1979 that was expected to increase the demand for antibody screening tests. We were concerned to find a simple and economical method of making and using RH gels, based on readily available reagents for which there would be several commercial sources. It was considered that the gels should have a shelf life of at least a week so that they could be made in bulk and used daily from stock. We aimed to obtain results that could easily be read against a readily available control serum which should be put up on each gel at a dilution that would give a zone of haemolysis equivalent to a minimum immune titre (Bradstreet *et al.* 1978).

Preliminary work was carried out to define an RH method that would meet these criteria. It was found that sheep red cells were particularly suitable for rubella RH, as well as being readily available to diagnostic laboratories. They were not prone to non-specific lysis as a result of storage or the presence of complement incorporated in the gel. It was also found that the amount of haemagglutinin required to sensitize sheep cells for RH was no more than for the red cells of other species.

The use of complement was investigated. It was found that Richardson's preserved guinea-pig serum, as supplied wet or freeze-dried by several companies or prepared in the diagnostic laboratory, could be incorporated successfully into sheep cell RH gels without non-specific lysis of the red cells. The shelf life at 4 °C of these gels was at least one week.

The size of the gel and the number and distribution of wells to be cut was also considered. A square petri dish (10 × 10 cm) with 2 cm sides and a lid was chosen as the most suitable container. A template was drawn that allowed 63 wells to be cut (9 rows of 7) giving a well to well spacing of 13 mm. This was the maximum number that could be cut if overlapping reactions were generally to be avoided.

No formal costing of the RH test compared with the haemagglutination inhibition (HAI) test was made, but there was no obvious difference in the cost of reagents. Allowing for economies in working time, however, RH had a clear

advantage over the HAI test, particularly when large numbers of sera were to be screened.

This paper describes the RH method finally chosen by the Working Party, and records its performance when introduced into five routine laboratories.

MATERIALS AND REAGENTS

A total of 8404 sera were received in five laboratories for rubella antibody screening, the majority from ante-natal clinics.

Standard anti-rubella sera were supplied by the National Institute for Biological Standards and Control – 1st British Standard containing 360 i.u., and the PHLS Standards Laboratory for Serological Reagents and Control – PHLS 2/74 containing 180 i.u.

Sheep red blood cells were collected and stored in Alsever's solution. The other reagents used and found to be satisfactory for RH gels were:

Agarose (Indubiose 37; Koch-Light; Miles Laboratories; Sigma type II).

Complement (Don Whitley Scientific; Flow Laboratories; Tissue Culture Services; Wellcome Reagents).

Rubella haemagglutinin (Behring Diagnostic Reagents; Flow Laboratories; Wellcome Reagents).

Diluent. Complement fixation buffer (Oxoid BR 15 tablets).

METHODS

Radial haemolysis test

The following steps were taken to prepare one test and one control gel, sufficient to screen 60 sera.

(1) Two 15 ml quantities of 1% agarose in complement fixation buffer (CFB) were melted by steaming, and held at 44 °C in a water bath.

(2) 0·3 ml of a 15% suspension of sheep red cells in CFB, accurately determined by haematocrit or spectrophotometer was pipetted into each of two 30 ml capacity screw capped bottles (universals), one marked 'test' and the other 'control'.

(3) 0·3 ml of rubella haemagglutinin (titre 1/128, determined as described in PHLS Report, 1978) was mixed with the cells in the 'test' universal. For weaker or stronger haemagglutinins a proportionately larger or smaller volume was required to coat the red cells.

(4) After 30 min at room temperature both bottles were filled with CFB and centrifuged at 2000 rev/min for 10 min.

(5) The supernatants were discarded and the red cells resuspended in 0·5 ml CFB plus 0·06 mg benzylpenicillin and 0·10 mg streptomycin per ml (CFB-PS).

(6) 0·5 ml of undiluted Richardson's preserved complement was mixed with the cell suspension in the 'test' universal.

(7) The 'test' universal was placed in the water bath and one of the 15 ml agarose solutions was poured into it. The contents were then mixed and

immediately poured into a petri dish 100 mm × 100 mm (Sterilin Ltd) marked 'test' standing on a level surface.

(8) Steps 6 and 7 were repeated for the 'control' universal.

(9) The gels were left to set and then held at 4 °C in a humid box until used.

(10) At the time of use, wells 2–3 mm diameter (capacity 8–11 μl) were cut in the gels over a template and sucked out with a Pasteur pipette attached to a vacuum line and trap.

(11) Sera to be tested were heat inactivated for 20 min at 60 °C, i.e. for 20 min after it had been established by checks with a thermometer that the water all around the sera was at 60 °C.

(12) Each serum was added to the corresponding well in the 'test' and 'control' gel using a capillary tube. A control serum containing 15 i.u./ml of rubella antibody and a known negative serum were tested on each gel.

(13) The gels were placed in a humid box and incubated at 37 °C overnight.

(14) After incubation the gels were read without delay. The zones of haemolysis were trans-illuminated against a black background. Reactions were interpreted as follows:

(*a*) Test zone equal to or larger than that of the 15 i.u./ml control serum and that in the corresponding position on the control gel	Rubella antibody present ($\geqslant$ 15 i.u./ml) 'Immune'
(*b*) Test zone smaller than that of the 15 i.u./ml control serum but larger than in the corresponding position on the control gel	Low amount of rubella antibody ($<$ 15 i.u./ml) Regard as non-immune
(*c*) No test zone, or a test zone equal to or smaller than that of the corresponding position on the control gel	Rubella antibody not detected. 'Susceptible'

Haemagglutination inhibition test

The rubella HAI tests used in the five laboratories were closely similar to the standard PHLS method (PHLS report, 1978), but one laboratory used heparin/manganous chloride instead of kaolin to remove non-specific inhibitors of haemagglutinin.

RESULTS

Initial observations

The first British Standard anti-rubella serum was diluted in twofold steps in CFB from 360 to 1·4 i.u./ml and tested in triplicate by the RH method. A linear relationship was observed between the diameter of the zones of haemolysis and the unitage of the dilutions down to 5·6 i.u./ml (Table 1). Plate 1 illustrates the quality of this haemolysis and shows that no zones were clearly apparent with less than 5·6 i.u./ml rubella antibody.

From a group of sera being tested routinely for rubella HAI antibody 41 sera were taken for testing by RH. The HAI titres of the sera ranged from 1/10 to

Table 1. *Radial haemolysis of dilutions of the first British Standard serum*

Dilution	Unit equivalents	Radial haemolysis zone diameter (mm)		
Neat	360	12·0	12·0	12·0
1/2	180	11·5	11·5	11·5
1/4	90	10·0	10·0	10·0
1/8	45	8·5	8·5	8·5
1/16	22·5	7·5	7·5	7·5
1/32	11·3	6·0	6·0	6·0
1/64	5·6	5·0	5·0	5·0
1/128	2·8	neg	neg	neg
1/256	1·4	neg	neg	neg

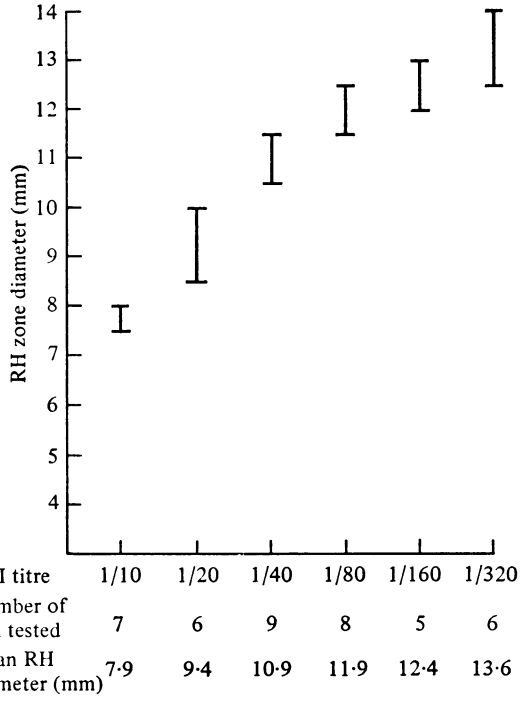

Fig. 1. Range and mean radial haemolysis zone diameters of 41 sera with HAI titres between 1/10 and 1/360.

1/320. The relationship between the HAI titres and the RH zone diameters of these sera is shown in Fig. 1.

During preliminary work it had been found that, after inactivation of specimens at 56 °C for 30 min, many sera gave small zones of haemolysis in the control gel, but that inactivation at 60 °C for 20 min greatly reduced this number. Table 2 shows the reactions in control gels of 360 unselected sera treated at 60 °C for 20 min. None gave reactions > 7 mm in diameter. It was also noted that where sera caused non-specific haemolysis in the control gel it usually appeared within a

Table 2. *Diameter (mm) of zones of haemolysis on the control gel. Tests on 360 sera inactivated at 60 °C for 20 min*

	No haemolysis	Zone diameter			
		4 mm	4–5 mm	5–6 mm	6–7 mm
Number	213	94	34	17	2
(%)	59·2	26·1	9·4	4·7	0·6

Table 3. *Zones of haemolysis of the 15 i.u./ml standard serum: result of tests in five laboratories*

	A	B	C	D	E
No. of observations ...	56	33	66	86	40
Range (mm)	6·5–9·0	7·5–8·7	7·1–9·0	7·7–10·0	6·5–8·5
Mean (mm)	8·28	8·16	7·90	9·00	7·38
Standard deviation	0·71	0·54	0·44	0·53	0·46

Table 4. *Results of radial haemolysis screening tests in five laboratories*

	A	B	C	D	E	Total
Number tested ...	1733	544	1839	2072	2216	8404
Zone ⩾ 15 i.u./ml standard	1474	506	1617	1840	1996	7433
Zone < 15 i.u./ml standard	31	2	23	105	28	189
No zone	224	32	178	123	191	748
Zones on test and control gels equal	4	4	21	4	1	34

few hours of incubation at 37 °C and was of a sharper quality than the specific lysis seen in the 'test' gels.

Use of the RH test in five laboratories

A total of 8404 sera were screened for rubella antibody by five diagnostic laboratories, A–E, and the results were recorded as described under Methods. All laboratories used a 1/12 dilution (15 i.u./ml) of the Standard anti-rubella serum, PHLS 2/74, as the positive control and measured the reaction of this on each gel. These observations are summarized in Table 3.

In Table 4 the screening results in the five laboratories are recorded. Overall 88 % of the sera screened were reported as immune. Four of the five laboratories measured the zone diameters of all sera tested. These results are collated in a histogram (Fig. 2).

Reactions in the control gel were recorded in 4 laboratories and it was found that only 33 specimens (0·5 %) gave a reaction ⩾ 7 mm diameter. In none of these cases was the reaction reported as smaller in the 'test' than in the control gel.

Specimens giving RH zones equal to or greater than the control serum were not tested further. Where possible each laboratory tested by HAI those sera found to

Table 5. *Results of further tests on specimens giving no reaction or a zone < 15 i.u./ml standard in radial haemolysis test*

	A	B	C	D	E
Number of sera examined	135	34	201	144	219
Agreement* between RH and HAI	119	34	185	110	212
Discrepancy between RH and HAI tests in original laboratory	16	0	16	34	7
Discrepancy between RH and HAI tests after testing in a reference laboratory	5	0	1	2	6

* HAI < 10, RH negative or HAI ⩾ 10, RH positive.

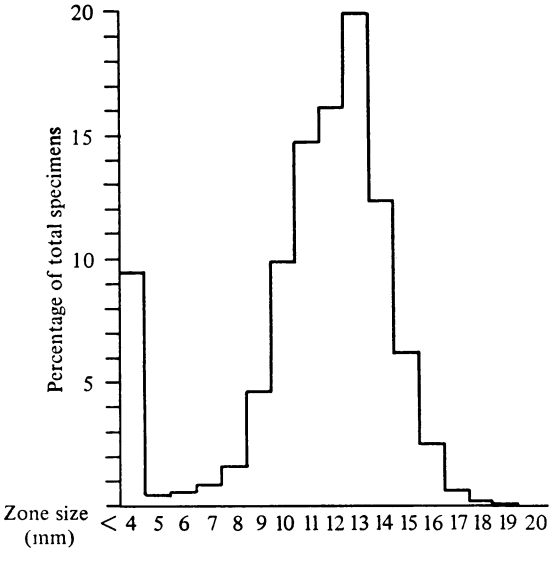

Fig. 2. Histogram of zone diameters of radial haemolysis reactions of 6188 sera tested in four laboratories, A, B, C, D.

be negative, or to give a zone size smaller than the 15 i.u./ml standard. Table 5 records the results on the specimens examined by HAI and it can be seen that in most cases results of the RH and HAI tests agreed. In laboratories C and D the discrepancies between the two tests were all of the form HAI ⩾ 10, RH no zone. Eleven of sixteen discrepancies in laboratory A and three of seven in laboratory E were of the same form. The other nine discrepant sera in these laboratories gave small zones of haemolysis by RH but were < 10 by HAI.

Where results disagreed in the original laboratory the specimens were sent to a reference laboratory to be re-tested and after that fourteen discrepancies remained. Eight specimens were HAI ⩾ 10, RH no zone. Two of these contained rubella IgM

antibody and the other six were negative by an indirect immunofluorescence antibody test (IFA) for rubella antibody carried out by Dr J. E. Cradock-Watson. The remaining six specimens were HAI < 10, but gave a small zone (< 15 i.u./ml standard zone) by RH and were positive by the IFA test.

DISCUSSION

This simple RH method was readily adopted by the staff in the five laboratories. Although many of them were unfamiliar with RH techniques, they reported few difficulties in preparing the gels. In routine use the RH test was simpler to perform and less time-consuming than the HAI test.

A quantitative assessment of rubella antibody can be made by measuring the diameter of a zone of haemolysis, but for screening purposes results could be rapidly read by a visual comparison of the zone size of a test serum with that of the standard anti-rubella serum. The strength of this standard was set at 25 i.u./ml following a survey by Bradstreet *et al.* (1978), but more recently they have revised this to 15 i.u./ml in the light of further experience (personal communication). The use of this standard simplifies the interpretation of the results as given in the Methods section. In practice the 15 i.u./ml standard gave mean zone diameters with a range 7·38–9·00 mm in the five laboratories (Table 3), and 96% of the test sera that gave a specific reaction in RH gels produced a zone of haemolysis equal to or greater than 9·00 mm (Fig. 2).

Some non-specific haemolysis was seen on the control gels but the zones were rarely of a size that would interfere with the reading of a positive test. Only 0·5% of the test sera gave zones of non-specific haemolysis of $\geqslant$ 7 mm diameter, none $\geqslant$ 9 mm. When the zones are of the same size on both test and control gels a distinction cannot be made between sera with low amounts of antibody and sera with none, but this occurred with only 34 of 8404 sera. Occasionally when gels were left at room temperature after incubation some non-specific zones gradually developed. To avoid this possibility gels should be read soon after removal from the incubator.

There were 73 discrepant results when RH rubella antibody negative and low positive sera were tested by HAI in the original laboratory (Table 5). After reference laboratory testing however, this number was reduced to 14. The results of the HAI tests done in the participating and the reference laboratories, indicates the inter-laboratory variability of the method. One of the explanations of this is that the sera from Laboratory D were contaminated with a Pseudomonas which can give rise to false positive results due to the failure of heparin/manganous chloride to remove all the non-specific inhibitors present (Bruce White & Tinnion, 1975). The reference laboratory found these sera were all HAI negative after absorption with kaolin.

Of the eight sera still giving HAI positive, RH negative results after reference laboratory testing six were negative by the IFA test; these HAI results were therefore likely to have been false positives. Two sera contained IgM rubella antibody and were found to have been collected in the acute phase of rubella at

which time RH reactions may be absent as the test will not detect antibody of the IgM (Strannegard, Grillner & Lindberg, 1975) or of the IgA (personal observation) class.

There were six discrepant results of the form RH gel positive, HAI negative. The zones of haemolysis of these were all $\leqslant$ 6 mm diameter and would have been interpreted as low amounts of antibody. This interpretation was supported by the IFA result which was positive in each case. As the RH gel test gave clear zones of haemolysis with the British Standard anti-rubella serum diluted to contain 5·6 i.u./ml (Table 1, Plate 1) an amount of antibody below the threshold detectable by the routine HAI method, it is perhaps surprising that there were not more RH gel positive, HAI negative results.

At low antibody titres therefore, the correlation between the two tests can be inexact as a result of variations in sensitivity between them and occasional difficulty in the reading of the results of one or other test. By using the zone size of the 15 i.u./ml standard as the lower limit representing immunity, discrepancies between the tests were avoided. Undoubtedly there will be sera containing low amounts of antibody that will be reported as susceptible ($< 4\%$, see Fig. 2), but incorrect reports of immunity should not occur with the RH test.

This study suggests that the HAI test gave some false positive results. There was no indication of erroneous positive reactions in the RH test, although the slight gain in sensitivity of this method gave rise to a few RH positive, HAI negative results. We conclude therefore, that, as the RH test is not only easier to perform but also less liable to incorrect interpretation, it should replace the HAI test as the method of choice for rubella antibody screening.

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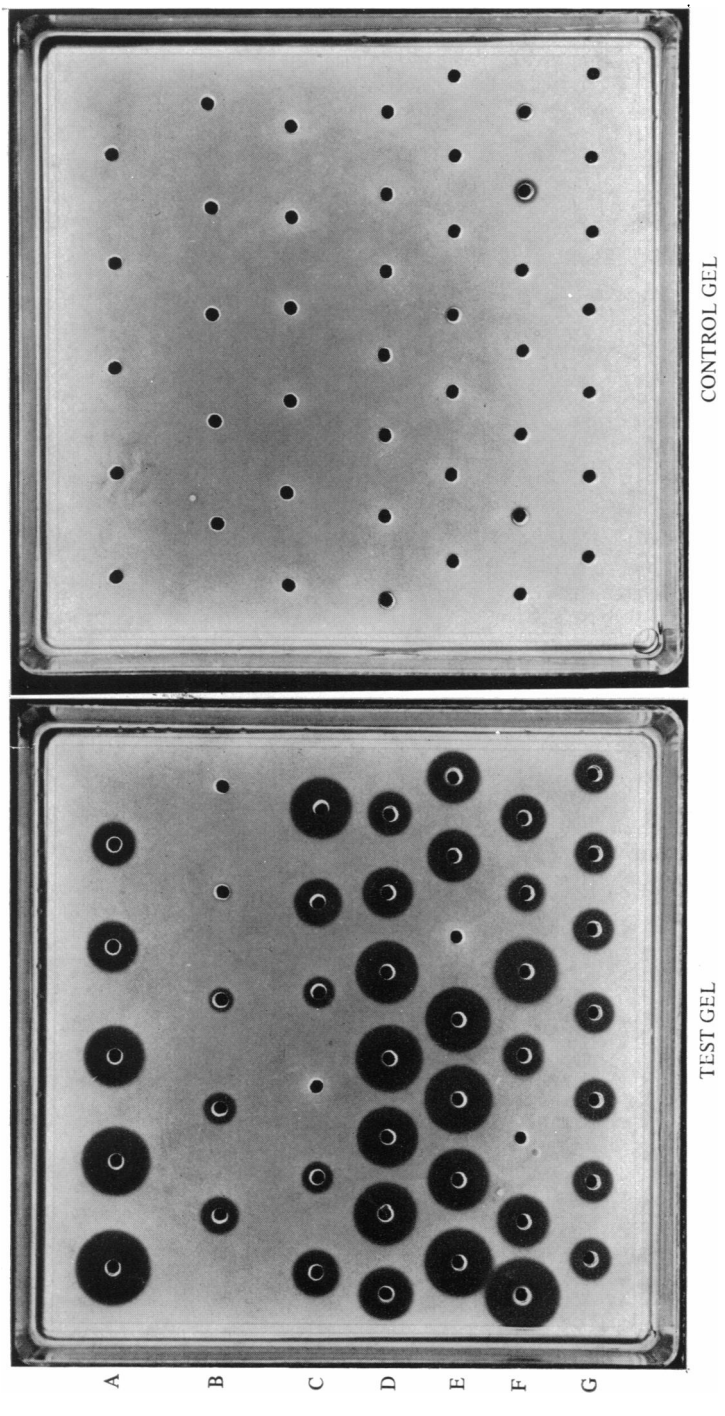
EXPLANATION OF PLATE

PLATE 1

Row A and B show successive two-fold dilutions of the first British standard rubella serum from 360 to 1·4 i.u./ml. Row C shows six specimens from a quality control distribution of sera. Their potency was 30, 7·5, nil, 7·5, 30, 100 i.u./ml. Rows D, E and F, contain specimens from a routine screening test and row G is seven replicates of the 15 i.u./ml dilution of PHLS 2/74 used to control these tests. Note that two specimens are without antibody and one, in row F, reacts less strongly than the control. Only one of the sera tested here reacted in the control gel (zone size 4 mm).


CONTROL GEL
TEST GEL
A
B
C
D
E
F
G