Haemagglutination-inhibition test for the detection of rubella antibody*

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SUMMARY

Between 1969 and 1972 three quality control studies were set up to investigate the variation in results that was occurring between and within laboratories performing routine tests for the diagnosis of rubella infection. No attempt was made to standardize the test in these studies, and a wide range in titres of sera was reported. The aims of the present studies were:

(i) to investigate in greater detail whether results were more reproducible between laboratories if test sera were compared with control sera of known potency and the results given in international units of activity, and

(ii) to ascertain whether results between laboratories would be more reproducible if a standard test procedure was used.

Eleven laboratories participated in testing 38 sera on three separate occasions by a prescribed standard technique and by that used routinely in each laboratory. Eight of the 38 sera consisted of four pairs of duplicate samples.

Analysis of results of the study showed that the reproducibility between laboratories was substantially improved when the test sera were compared with a control serum of known potency and when a standard test procedure was used.

Variation in results between laboratories was least when a control serum of low rather than high potency was used. Variation within laboratories can be reduced by increasing the number of times the control and test sera are tested.

Since the rubella antibody content of the British Standard anti-rubella serum is expressed in international units, the potency of the control and results of test sera should also be expressed in such units.

INTRODUCTION

For many years the rubella haemagglutination-inhibition (HI) test has been the main serological test used for the diagnosis of rubella infection and for the detection of antibody in screening tests before rubella vaccination. To confirm a recent infection with rubella virus, it has always been accepted that it is essential that two sera, collected at relevant times, are tested together. Under these circumstances the absolute values for antibody content are of less importance than the demonstration of a significant rise in titre. Occasionally problems of interpretation have arisen when sera have been submitted to different laboratories. If techniques of different sensitivity are used, apparent differences in titre will be recorded. Between 1969 and 1972 three quality control studies were set up by the Standards Laboratory, Central Public Health Laboratory, Colindale, to find out the variation in results that was occurring between and within laboratories (unpublished observations of the Public Health Laboratory Service). No attempt to standardize the test was made in any of these studies. In the first two a positive control serum was included in all tests and results were reported in titres. In the third the Standards Laboratory provided a standard serum which had been assayed against the British standard anti-rubella serum (Clarke *et al.* 1975) and given a potency in international units, so that results were reported in units.

Although in each of these studies between 80 and 90 % of all results for each serum were found at the median titre or two-fold above or below it, a wide range in titres of each serum was reported.

The primary aims of the present study were:

(i) to investigate in greater detail whether results were more reproducible between laboratories if test sera were compared with control sera of known potency and the results given in international units of activity, and

(ii) to ascertain whether more reproducible results were obtained from laboratory to laboratory if a standard test procedure was used.

DESIGN OF THE STUDY

On three separate occasions each of 11 laboratories tested 38 sera in two tests set up in parallel: in one test a prescribed standard technique (described below) was used and in the other the technique used routinely in the laboratory, details of which were reported with the results. Unknown to the participants, eight of the 38 sera consisted of four pairs of duplicate samples. The standard technique required that the test should be compared with two control sera, each control serum being tested in triplicate. All but one participant adopted this procedure for their routine method – laboratory 9 tested each control serum only once. The testing of all sera was carried out on one day and repeated on two further occasions with an interval of at least a week between each by all laboratories except laboratory 2 which tested only twice.

The standard technique

Materials used

The following materials and reagents were despatched on the same day from the Standards Laboratory, to the participating laboratories; Standards Laboratory haemagglutinin (HA), dextrose-gelatine-veronal buffer (DGV) (Clarke & Casals, 1958) to which 0·2 % bovine albumin (DGVA) was added by the laboratory, 25 % suspensions of kaolin (Flow Laboratories, U.K.), 38 human sera of which 36 contained rubella antibodies, and two positive control sera made from pools of human sera.

The sera were sent wet, preserved with 0·08 % sodium azide and were stored at or below − 20 °C by the laboratories; although the tests were performed on separate occasions, the sera were stored as single samples and then thawed at 37 °C and refrozen as necessary. Red blood cells (RBCs), from day-old chicks or pigeons were obtained by the individual participants and used when not more than 5 days old. Each laboratory used whichever of the micro (0·025 ml) or the macro (0·1 ml) volume techniques it used routinely. Cooke micro-plates (Sterilin Ltd) were supplied to those laboratories using the micro-technique.

The standard test

HA was reconstituted with distilled water and left overnight at 4 °C. It was then titrated and adjusted by dilution in DGVA to contain four complete units per unit volume for use in the test on the same day. Non-specific inhibitors of haemagglutination were removed from the sera by mixing 0·1 ml of serum with 0·3 ml of DGV and 0·6 ml of 25 % kaolin. This mixture was shaken well, kept for 1 h at room temperature and then centrifuged to sediment the kaolin. When day-old chick RBCs were used in the test, absorption with RBCs was not performed unless agglutinins were found to interfere with the HI test. When pigeon cells were used non-specific RBC agglutinins were removed from all sera by adding 0·05 ml of 30 % washed cells to the treated serum and incubating overnight at 4 °C. The sera were then centrifuged and the supernatant decanted. For use in the test proper, washed chick or pigeon cells were resuspended in DGVA to a concentration of 0·4 %. Rubella HI titrations were performed on the same day as the kaolin pretreatment when chick cells were used and the day after this treatment when absorption was done with pigeon RBCs.

Two-fold dilutions of kaolin-treated serum from 1/10 to 1/2560 were made in DGVA. Test sera were titrated once; control sera were titrated three times, in different plates, from the master 1/10 dilution. One volume of HA (4 units) was added to one volume of each serum dilution and the mixture was left for 1 h at room temperature. One volume of 0·4 % red cells was then added to each dilution. The plates were shaken and allowed to settle at 4 °C for 2–3 h or overnight. The 50 % end point was then read.

Results of all titrations, of both test and control sera, were recorded as titres and reported to the Epidemiology Research Laboratory, Colindale, where they were converted to international units and analysed.

Calibration of the control sera

In a separate study the two control sera were assayed repeatedly by the standard technique against the British Standard anti-rubella serum (69/60), which contains 360 i.u./ampoule (Clarke *et al.* 1975).

The potencies of the two control sera were found to be 187 and 297 i.u./ml.

Method of calculating the potencies in units/ml of the test sera

The titre of each of the test sera was converted to an estimate of potency by comparison with the corresponding titres of the control sera as follows:

Potency of test serum = titre of test serum × geometric mean

(potency of control/titre of control).

A detailed description of how to perform this conversion is given in the Appendix.

Statistical methods

The reproducibility of the test depends on two major sources of variation, that which arises between different laboratories and that which arises within individual laboratories. This can be represented by writing

Observed result = overall mean (or 'true') result + laboratory effect
+ error within laboratory.

The important difference between these two sources of variation is that the within laboratory error is generally random while the between laboratory variation is such that any one laboratory will tend to be consistently high or low by the same fixed amount.

These sources of variation can be further subdivided as follows:

Laboratory effect = effect removed by use of control sera + effect
removed by use of the standard technique + remaining laboratory effect.

Error within laboratory = day to day variation
(largely reduced by use of control sera) + basic error of the test.

In order to choose the strategy for routine testing that would give the best reproducibility the relative contributions of these various sources of error were examined.

The test results were expressed as logarithms to the base 2 since the data were recorded in terms of doubling dilutions. The total variation observed in these results was separated into the two independent components attributable to variation between and within laboratories using a random effects model with analysis of variance (Huitson, 1966).

Direct comparison of the variation between laboratories found with different methods was complicated by the lack of independence of the results for the alternative methods, which invalidated the use of the usual variance ratio test. Instead the mean results for each laboratory by each method were calculated. The number of results was large enough for the variation of these means to be approximately equal to the variation between laboratories. For any two methods these variances were then compared as for a paired sample (Armitage, 1971).

Table 1. *95% confidence intervals for a single result, expressed in terms of two-fold differences, according to method*

	Between laboratories*	Within laboratories†	Total‡
		Source of variation	
Units			
Standard technique	± 0·32	± 0·91	± 0·97
Routine technique	± 0·82	± 0·98	± 1·28
Titres			
Standard technique	± 1·84	± 0·98	± 2·09
Routine technique	± 1·47	± 0·85	± 1·70

* Assumes no within laboratories variation.
† Assumes no between laboratories variation.
‡ Includes both between and within laboratories variation.

RESULTS

The components of variation between and within laboratories are shown in Table 1. It can be seen from this Table that the variation within laboratories was about the same for all the methods but that the variation between laboratories was substantially less for the standard technique with conversion to units than for any of the other three methods.

As an overall criterion for comparing the results from different laboratories, the geometric means of all the results for the 38 sera for each laboratory are shown in Table 2. There was no significant difference between the standard and routine techniques in the spread of titre results between laboratories. There was a decrease in the spread of the results between laboratories when the titres were converted to units. This was highly significant ($P < 0.001$) for the standard technique but not quite significant ($0.05 < P < 0.1$) for the routine technique. The spread of the potency results between laboratories was significantly ($P < 0.02$) less for the standard than for the routine technique.

Effect on reproducibility of differences in test techniques

The results were examined for the effect of the known differences between the standard and routine techniques. One of the major differences was the agent used to remove non-specific inhibitors.

Where laboratories used kaolin in their routine and standard techniques there was no significant difference between the two techniques in the spread of the potency results between these laboratories. The potency results in these laboratories were significantly ($P < 0.01$) higher than in those laboratories using manganous chloride/heparin in their routine technique. This can be seen in Table 2. Other differences, e.g. fresh or commercial RBC suspensions, micro or macro method, between the standard and routine techniques did not appear to have an effect but they were too varied to investigate in detail.

Table 2. *Geometric means of all results for the test sera*

Laboratory	Geometric mean titre		Geometric mean potency	
	Standard technique	Routine technique	Standard technique	Routine technique
1	88·7	70·1*	158·4	150·6*
2	307·1	76·5*	213·2	163·0*
3	231·8	28·8†	164·3	129·6†
4	57·1	82·0*	165·0	170·8*
5	82·2	99·8*	155·6	130·0*
6	42·1	41·2†	189·5	96·4†
7	176·3	215·5*	170·1	168·2*
8	65·9	69·3†	137·2	113·6†
9	200·4	88·9†	185·9	72·9†
10	101·1	119·5*	167·2	151·3*
11	60·5	71·7*	161·8	203·3*
Overall geometric mean	105·4	77·3	168·8	135·9

* Kaolin used in the removal of non-specific inhibitors in the routine technique.
† Manganous chloride/heparin used in the removal of non-specific inhibitors in the routine technique.

Table 3. *95 % confidence intervals for a single potency result using a standard technique, expressed in terms of two-fold differences, according to the number of repetitions of the control and the test sera*

No. of repetitions		Source of variation		
Test serum	Control serum	Between laboratories*	Within laboratories†	Total‡
1	1	± 0·32	± 1·20	± 1·24
	2	± 0·32	± 1·04	± 1·08
	3	± 0·32	± 0·98	± 1·03
	4	± 0·32	± 0·95	± 1·00
	5	± 0·32	± 0·93	± 0·98
	6	± 0·32	± 0·91	± 0·97
	7	± 0·32	± 0·90	± 0·96
	8	± 0·32	± 0·90	± 0·95
	9	± 0·32	± 0·89	± 0·95
	10	± 0·32	± 0·89	± 0·94
2	1	± 0·32	± 1·04	± 1·08
	2	± 0·32	± 0·85	± 0·90
	3	± 0·32	± 0·77	± 0·83
	4	± 0·32	± 0·73	± 0·80
	5	± 0·32	± 0·71	± 0·77
	6	± 0·32	± 0·69	± 0·76
	7	± 0·32	± 0·68	± 0·75
	8	± 0·32	± 0·67	± 0·74
	9	± 0·32	± 0·66	± 0·73
	10	± 0·32	± 0·65	± 0·73

* Assumes no within laboratories variation.
† Assumes no between laboratories variation.
‡ Includes both between and within laboratories variation.

Table 4. 95% *confidence intervals for a single potency result using a standard technique, expressed in terms of two-fold differences, according to type of control used*

Control sera	Geometric mean potency	Source of variation		
		Between laboratories*	Within laboratories†	Total‡
Low	64	± 0·27	± 0·97	± 1·00
Medium (a)	315	± 0·64	± 1·45	± 1·58
Medium (b)	390	± 0·71	± 0·98	± 1·21
High	938	± 1·00	± 1·34	± 1·67

* Assumes no within laboratories variation.
† Assumes no between laboratories variation.
‡ Includes both between and within laboratories variation.

Effect on reproducibility of the number of repetitions made

When using the standard technique with conversion to units the remaining variation between laboratories, although substantially reduced, was found to be still significant ($P < 0.001$). This can be reduced only by further standardization of the reagents and procedures used. The variation within laboratories can however be decreased by increasing the number of times the control or the test sera are titrated. Table 3 shows the effect on the reproducibility of varying the number of times the control and the test sera are tested. It can be seen that the total variation steadily decreases as the number of repeated tests of the control serum increases, but that the improvement with each additional repetition steadily diminishes. It can also be seen that two repetitions of each test serum substantially reduces the total variation.

Effect of potency of the control serum on reproducibility

Eight of the 38 sera consisted of four pairs of duplicates. The reproducibility of the standard technique results of the other 30 test sera was investigated using each of these pairs as controls in turn. Table 4 gives the geometric mean potencies of these four pairs and shows the resulting variation.

It can be seen that the variation between laboratories increased with the potency of the control serum. This variation was significantly less when using the low potency control serum than when using the medium (a) ($P < 0.05$), medium (b) ($P < 0.01$) or the high ($P < 0.001$) potency control sera. The variation within laboratories was also least for the low potency control serum.

DISCUSSION

This study showed that the reproducibility between laboratories was substantially improved when the test sera were compared with a control serum of known potency. Since the rubella antibody content of the British standard anti-rubella serum is expressed in international units, the potency of control sera and results of test sera should also be expressed in such units.

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A further improvement in reproducibility between laboratories was achieved when a standard test procedure was used. One important factor in standardizing this test is to decide upon the use of either kaolin or manganous chloride/heparin for the removal of non-specific inhibitors. In this study it was decided to use kaolin in the standard method as seven of the 11 laboratories were already using kaolin in their routine technique and it has been preferred by others (Blom & Haukenes, 1974; Haukenes & Blom, 1975). For the standard technique used here, the kaolin suspension, the micro-plates and the diluent, normally prepared or purchased locally, were also supplied by the Standards Laboratory along with the haemagglutinin. It would not be practical to standardize routinely to this extent. A control serum of low potency should be used since the variation between laboratories was shown to increase with the potency of the control serum. A serum with a potency of about 100 i.u. would be suitable.

With the standard technique used most of the variation in results is due to variation within laboratories. This can be reduced by increasing the number of times the control and test sera are tested. Since it is not practical to examine all test sera more than once a reasonable strategy would be to test these once and the control serum three or more times. Using this strategy and the standard technique with conversion to units means that on average 95% of the results would lie within a two-fold difference of the true result.

In conclusion we recommend that laboratories use an agreed method for titrating HI antibody and use units for recording potency of rubella sera so that results of different laboratories will be comparable. Nevertheless, it is still essential that sera collected from a patient with suspected rubella are examined in a single laboratory.

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APPENDIX

Method of converting titres to units using a control serum

To convert titres to units by reference to a control serum of known potency the following simple procedure may be adopted.

(i) For each reading of the control serum find the corresponding number of doubling dilutions using Table A and take the arithmetic mean, C, of these. For example, if three tests of the control serum give titres of 1/40, 1/40, 1/80 the corresponding numbers of doubling dilutions are 2, 2, 3 and so $C = 2\cdot3$.

(ii) Find the number of doubling dilutions, T, corresponding to the titre of the test serum. If a serum is tested more than once take the average. Calculate $D = T - C$.

In the above example, if the titre of the test serum is 1/160 then $T = 4$ and $D = 1\cdot7$.

(iii) Refer to Table B and determine the multiplicative factor M corresponding to D. E.g. for $D = 1\cdot7$, $M = 3\cdot25$. Then calculate potency of test serum $= M \times$ potency of control serum.

When there are a large number of test sera it is most efficient to calculate the unitage corresponding to each of the possible titres and to construct a simple table to use for converting the test sera titres to unitages. Table C is such a table for the above example assuming that the potency of the control serum is 100 i.u.

The above procedure can be used for any series of doubling dilutions, the appropriate Table A being constructed with 0 corresponding to the most concentrated dilution.

Table A. *Number of doubling dilutions corresponding to each titre*

Titre ...	< 1/10	1/10	1/20	1/40	1/80	1/160
No. of doubling dilutions	−1	0	1	2	3	4

Titre ...	1/320	1/640	1/1280	1/2560	> 1/2560
No. of doubling dilutions	5	6	7	8	9

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Table B. *The multiplicative factor, M ($=2^D$), used in the formula, 'potency of test serum $= M \times$ potency of control serum' for a range of values of D, the number of doubling dilutions of the test serum minus the number of doubling dilutions of the control serum*

D	M	D	M	D	M	D	M	D	M	D	M	D	M	D	M	D	M	D	M	D	M	D	M
−4·0	0·06	−3·0	0·13	−2·0	0·25	−1·0	0·50	0·0	1·00	1·0	2·00	2·0	4·00	3·0	8·00	4·0	16·00	5·0	32·00	6·0	64·00	7·0	128·00
−3·9	0·07	−2·9	0·13	−1·9	0·27	−0·9	0·54	0·1	1·07	1·1	2·14	2·1	4·29	3·1	8·57	4·1	17·15	5·1	34·30	6·1	68·59	7·1	137·19
−3·8	0·07	−2·8	0·14	−1·8	0·29	−0·8	0·57	0·2	1·15	1·2	2·30	2·2	4·59	3·2	9·19	4·2	18·38	5·2	36·76	6·2	73·52	7·2	147·03
−3·7	0·08	−2·7	0·15	−1·7	0·31	−0·7	0·62	0·3	1·23	1·3	2·46	2·3	4·92	3·3	9·85	4·3	19·70	5·3	39·40	6·3	78·79	7·3	157·59
−3·6	0·08	−2·6	0·16	−1·6	0·33	−0·6	0·66	0·4	1·32	1·4	2·64	2·4	5·28	3·4	10·56	4·4	21·11	5·4	42·22	6·4	84·45	7·4	168·90
−3·5	0·09	−2·5	0·18	−1·5	0·35	−0·5	0·71	0·5	1·41	1·5	2·83	2·5	5·66	3·5	11·31	4·5	22·63	5·5	45·25	6·5	90·51	7·5	181·02
−3·4	0·09	−2·4	0·19	−1·4	0·38	−0·4	0·76	0·6	1·52	1·6	3·03	2·6	6·06	3·6	12·13	4·6	24·25	5·6	48·50	6·6	97·01	7·6	194·01
−3·3	0·10	−2·3	0·20	−1·3	0·41	−0·3	0·81	0·7	1·62	1·7	3·25	2·7	6·50	3·7	13·00	4·7	25·99	5·7	51·98	6·7	103·97	7·7	207·94
−3·2	0·11	−2·2	0·22	−1·2	0·44	−0·2	0·87	0·8	1·74	1·8	3·48	2·8	6·96	3·8	13·93	4·8	27·86	5·8	55·72	6·8	111·43	7·8	222·86
−3·1	0·12	−2·1	0·23	−1·1	0·47	−0·1	0·93	0·9	1·87	1·9	3·73	2·9	7·46	3·9	14·93	4·9	29·86	5·9	59·71	6·9	119·43	7·9	238·86

Table C. *An example conversion table of titres to potencies for a control serum with a potency of 100 i.u. and titres of 1/40, 1/40 and 1/80*

Titre ...	< 1/10	1/10	1/20	1/40	1/80	1/160
Unitage	10	20	41	81	162	325
Titre ...	1/320	1/640	1/1280	1/2560	> 1/2560	
Unitage	650	1300	2599	5198	10397	