J. Hyg., Camb. (1982), **88**, 453
Printed in Great Britain

The detection of rubella-specific IgM by an immunosorbent assay with solid-phase attachment of red cells (SPARC)

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(*Received* 2 *January* 1982; *accepted* 10 *February* 1982)

SUMMARY

An immunosorbent assay using solid-phase attachment of red cells (SPARC) was used for the detection of rubella-specific IgM. The method is described and the results compared with those obtained by the IgM antibody capture radioimmunoassay (MACRIA). One hundred and ninety-nine sera were investigated for the presence of rubella-specific IgM and only one discrepant result occurred, namely a false positive obtained by MACRIA in a patient with infectious mononucleosis. Rheumatoid factor, heterophile antibody, and rubella-specific IgG did not interfere with the results obtained by the SPARC technique. Advantages of the SPARC technique include the ease and lack of expense of testing large numbers of sera, the small volume of sample required and the fact that pretreatment of serum is not necessary.

INTRODUCTION

The diagnosis of primary rubella infection is of particular importance during early pregnancy since, in many countries, termination of pregnancy has become an acceptable means of preventing the congenital rubella syndrome. Such a diagnosis is usually performed serologically, but often sera are received too long after the onset of the illness for a rise in total antibody titre to be demonstrated in paired sera. In such cases, the diagnosis of recent rubella is made by the detection of rubella-specific IgM, as these antibodies usually persist for only a limited period. The detection of rubella-specific IgM is also of importance in distinguishing between primary infection and reinfection, the latter being considered to present minimal risk to the foetus (Cradock-Watson *et al.* 1981).

Several techniques are currently in use for the demonstration of rubella-specific IgM. At present the most commonly used techniques depend on the physical separation of IgM from IgG by sucrose density gradient ultracentrifugation (Best, Banatvala & Watson, 1969) or gel filtration (Morgan-Capner, Davies & Pattison, 1980). Although these methods are simple, giving reliable results, they are time-consuming and the equipment required is expensive.

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The next generation of techniques were those based on the binding of rubella-specific IgM in sera by rubella antigen absorbed to a solid phase. The bound IgM can be detected by antibody to the Fc portion of immunoglobulin M (anti-μ) labelled with I^{125} (Meurman, Viljanen & Granfors, 1977) or with an enzyme (Leinikki *et al.* 1978). For such techniques to be reliable with adult sera, however, a source of purified rubella antigen is usually required. Measures are also required to prevent false positive results due to the presence of rheumatoid factor (Meurman & Ziola, 1978).

The latest generation of techniques are the immunosorbent assays in which the solid phase is coated with anti-μ. This principle has been applied to the detection of rubella-specific IgM by Mortimer *et al.* (1981) who detected any rubella-specific IgM, within the total IgM retained by the anti-μ, by sequential addition of rubella haemagglutinating antigen (HA) and I^{125}-labelled anti-rubella IgG. This M-antibody capture radioimmunoassay (MACRIA) is a sensitive technique which allows large numbers of sera to be tested, but does require the preparation of radio-labelled anti-rubella IgG. The use of such a label may pose problems in some laboratories, and the method has been adapted to use an enzyme labelled anti-rubella IgG (Kurtz & Malik, 1981).

M-antibody capture immunosorbent techniques using red blood cells as an indicator have been described for the detection of rubella-specific IgM. The first such technique was described by Krech & Wilhelm (1979). This technique has been followed by adaptations such as those published by van der Logt, van Loon & van der Veen (1981) and Goldwater & Banatvala (1981). These techniques appear to depend on the presence of precise concentrations of rubella HA and red cells within the system. The technique we describe here is similar but overcomes the problem of rubella HA and red cell concentrations, giving a more flexible system. Quality control of the technique is achieved by using previously established negative and low positive sera.

MATERIALS AND METHODS

Sera and controls

A total of 64 sera from patients with post-natal rubella were examined for the presence of rubella-specific IgM. Sixteen of these were convalescent sera from patients showing seroconversion by haemagglutination inhibition (HI) and 48 were single sera which had detectable rubella-specific IgM by gel filtration on Sephacryl S-300 and HI on the fractions obtained. One hundred and three sera were examined from patients with rubella-like symptoms or recent contact with a rubella-like illness who were rubella-specific IgM negative by gel filtration. A further 10 sera from patients giving a history of contact with a rubella-like illness and who had elevated HI titres ($>$ 800 international units of rubella antibody) were specially selected. None had detectable rubella-specific IgM by gel filtration.

To investigate possible interference due to the presence of rheumatoid factor, 10 sera, obtained from the Department of Immunology, King's College Hospital, containing high levels of rheumatoid factor, as determined by the latex agglu-

tination technique, were examined. All of these sera contained rubella-specific antibody determined by radial haemolysis (Kurtz *et al.* 1980).

Six sera from patients with infectious mononucleosis and six sera from individuals with a recent cytomegalovirus infection (Epstein-Barr or cytomegalovirus-specific IgM positive) were also examined. These sera were included as controls to determine whether the presence of specific IgM to other viruses interfered with the assay.

A rubella-specific IgM standard was prepared from a pool of strongly positive rubella-specific IgM sera and was designated 100 units. This was diluted in a pool of rubella antibody negative sera to 30, 10, 3, 1 and 0·3 u., correlating to the standards as described for MACRIA. A serum containing 3,200 i.u. of rubella-specific IgG antibody, but which did not contain rubella-specfic IgM by gel filtration, was used as the negative control.

Gel filtration

Pretreated serum was layered onto a column of Sephacryl S-300 as described by Morgan-Capner, Davies & Pattison (1980). HI tests were performed on the immunoglobulin-containing fractions eluted from the column using overnight rubella HA fixation. The presence of HI activity in the IgM-containing fractions indicated the presence of rubella-specific IgM.

MACRIA

The MACRIA method employed in this evaluation was adapted from that described by Mortimer *et al.* (1981). Polystyrene beads, 6·4 mm diameter, made by the Plastic Ball Co., Chicago, U.S.A. and supplied by Northumbria Biologicals Ltd, U.K., were used as the solid phase. The beads were coated with rabbit anti-μ (Dako, Copenhagen, Denmark, supplied by Mercia Brocades Ltd, Weybridge, Surrey, U.K.) by agitating them for 1 h at room temperature in a 1:500 dilution of anti-μ in N-HCl. The beads were then stored at 4 °C for at least 48 h before use. For use, the required number of beads were incubated for 3 h at room temperature in phosphate-buffered saline (PBS) containing 1% heat-inactivated rubella antibody negative cord serum. Test and control sera were diluted 1:40 in phosphate-buffered saline containing 0·05% Tween 20 (PBST) in duplicate in the wells of polystyrene plates (Abbott Ltd, Basingstoke, U.K.) and a coated bead added to each well. The trays were incubated at 37 °C for 4 h before washing each bead three times in PBST. Rubella antigen (200 μl), original HA titre 128 (supplied by the Public Health Laboratory Service, Colindale, U.K.), was diluted 1 in 10 in PBST, added to each bead and the trays incubated for 18 h at 4 °C. The beads were washed three times in PBST. To each bead was added 200 μl of I^{125}-labelled rabbit anti-rubella IgG (kindly supplied by R. Tedder, Middlesex Hospital Medical School, U.K. and P. Mortimer, Public Health Laboratory Service, U.K.), diluted in PBS containing 0·2% Tween 20, 10% heat-inactivated human rubella antibody negative serum and 20% heat-inactivated rabbit serum so that 200 μl gave a count of 100000/min. After incubation at 37 °C for 3 h the beads were washed four times

in PBST and the bound radioactivity determined by counting each bead for 10 min in a gamma counter (NE 1600, Nuclear Enterprises, Edinburgh, U.K.).

The control sera were included in each run and a standard calibration curve obtained by plotting the ratio of control serum counts to negative serum counts. The results of test sera were expressed as units of rubella-specific IgM by comparison of the ratio of the test serum counts to the negative serum counts with the standard calibration curve. Sera were considered to contain rubella-specific IgM if a ratio greater than the 3 u. control was obtained.

SPARC technique

The SPARC assay was a four-stage procedure. The first stage was the coating of polystyrene wells with rabbit anti-μ. A dilution of patient's serum was incubated in the coated wells so that a sample of the patient's total IgM was retained by the anti-μ coating. The third stage was an incubation with rubella HA to select only retained rubella-specific IgM. Finally day-old chick red cells were added as the indicator system. After each stage the plates were washed.

The various components of the system were evaluated using the 3 u. rubella-specific IgM positive control and a rubella-specific IgM negative control which had a high HI titre (3,200 i.u.). The use of carbodiimide (Nash, 1976) resulted in reproducible binding of the anti-μ to the polystyrene so that well-to-well variation was not a problem. Two rubella HA antigens were evaluated, that produced by Flow Laboratories, Irvine, U.K., proving to be superior in the SPARC technique to that produced by the Public Health Laboratory Service. After evaluation of the concentration of anti-μ, serum dilutions, rubella HA and chick red cell concentration, and of the incubation times, the following method was found to give the necessary sensitivity and specificity.

Ninety-six well polystyrene round-bottomed microtitre plates (Sterilin Ltd, Teddington, U.K.) were coated with a 1:500 dilution of rabbit anti-μ (Dako, Copenhagen, Denmark, supplied by Mercia Brocades Ltd, Weybridge, Surrey, U.K.). Equal volumes of 1:250 anti-μ in 0·05 M Tris-HCl, pH 7·4, 0·15 M sodium chloride and 0·03 % sodium azide (TSA) and carbodiimide (1 mg/ml) in TSA, were mixed and 100 μl added to each well. The plates were covered and left overnight at room temperature in a humid box before storing at 4 °C. Such plates could be stored for at least one month before use. For use, the anti-μ solution was tapped out and the plates washed twice with PBST.

Patients' sera and controls (3 u. positive control and negative control) were diluted from 1:80 to 1:20, 480 in PBST and 100 μl of each dilution added to each well in a row across the plate with 100 μl of the 1:80 dilution being duplicated in the final well to act as serum control. After a 2 h incubation at 37 °C the serum dilutions were tapped out and the plates washed twice with PBST and tapped dry.

A portion (25 μl) of rubella HA (Flow Laboratories, Irvine, U.K.) diluted to 12–16 HA u. in PBST was added to each test well and 25 μl PBST added to the serum control wells. After overnight incubation at 4 °C the rubella HA was tapped out, the plates immersed in PBS and excess allowed to drain by inversion on paper towels.

Table 1

	Rubella-specific IgM by SPARC	
	Present	Absent
Confirmed rubella	64	0
Remote rubella*	0	103
Rheumatoid factor and rubella antibody present	0	10
High rubella HI antibody present (> 800 u.)	0	10
CMV and EBV infections	0	12**

* Sera from patients with a rubella-like illness or recent contact with rubella who were specific IgM negative by gel filtration.

** Includes one serum from a case of infectious mononucleosis which was rubella-specific IgM positive by MACRIA but negative by gel filtration (see text).

Day-old chick cells (Tissue Culture Services, Slough, U.K.) were washed three times in dextrose gelatin veronal buffer (DGV) and resuspended in DGV tc give a final concentration of 0·125 %. A 25 μl portion of the red cell suspension was added to all wells and the cells distributed by tapping the plates gently for 20–30 s. The plates were left at 4 °C for 2 h, then removed to room temperature for a further 30 min before reading. The presence of detectable rubella-specific IgM was indicated by a haemagglutination-like pattern, whereas its absence was indicated by a button of cells.

RESULTS

Initial evaluation of the SPARC technique indicated that at lower serum dilutions (1:40) haemagglutination-like patterns could be observed with the negative control, but at a dilution of 1:80 this occurred infrequently. On testing the control positive serum series the 0·3 u. and 1 u. controls were negative by the SPARC technique (Plate). However, the 3 u. control was always positive at a dilution of 1:80 and occasionally up to a dilution of 1:320. The incorporation of the negative control and the 3 u. control on every plate ensured that adequate control was achieved. If either serum did not give its expected result, the test was repeated.

No discrepant results were obtained with the SPARC technique with the 64 positive and 103 negative diagnostic sera when compared with diagnosis achieved by HI and detection of rubella-specific IgM by gel filtration (Table 1). The 64 containing rubella-specific IgM were also positive by MACRIA (> 3 u.). A comparison of the titres obtained with the SPARC technique and the rubella-specific IgM content in units as determined by MACRIA is shown in Fig. 1. A correlation coefficient was computed taking the results for all 64 sera and assigning a value of 100 u. to values > 100 u. in MACRIA and a titre of 20480 to those > 20480 in SPARC. The result showed that there was a very good correlation

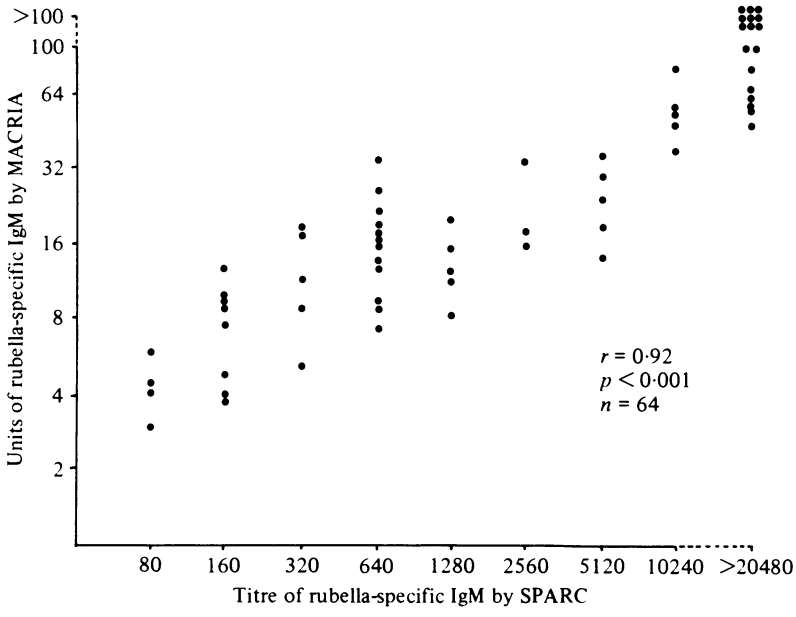

Fig. 1. Comparison of rubella-specific IgM titres of 64 sera determined by SPARC and the units of specific IgM determined by MACRIA. r, 0·92; $P < 0·001$; n, 64.

between the values obtained by both methods ($r = 0·92$, $P = < 0·001$). The 103 sera negative by the SPARC technique were also negative ($\leqslant 3$ u.) by MACRIA (Table 1).

There was no evidence of interference with the SPARC technique by high levels of rubella-specific IgG or by rheumatoid factor. In addition 10 early convalescent sera from EB or CMV infections were all negative. Nine of these sera were also negative by MACRIA. The exeption was a serum from a patient with infectious mononucleosis (heterophile antibody present; EBV IgM present) which gave a MACRIA result equivalent to 25 u. of rubella-specific IgM. This serum was negative by gel filtration and HI using Flow Laboratories rubella HA antigen. It was also negative by MACRIA ($< 0·3$ u.) if Flow Laboratories rubella HA antigen was used. Unfortunately, insufficient serum remained to perform gel filtration and use PHLS rubella HA antigen for the HI tests on the fractions obtained.

No haemagglutination-like patterns were ever seen in the control wells, indicating the absence, in the sera tested, of IgM directed against chick red cell surface components.

DISCUSSION

The SPARC technique described for the detection of rubella-specific IgM differs significantly from the methods described by Krech & Wilhelm (1979), van der Logt, van Loon & van der Veen (1981) and Goldwater & Banatvala (1981) in that the

rubella HA which had not been retained by rubella-specific IgM was gently washed from the wells before the addition of chick red cells. The above previously described methods depend on retained rubella-specific IgM removing rubella HA from the supernatant, to which red cells are added. Thus in the method of Krech & Wilhelm (1979) no haemagglutination occurs in the presence of rubella-specific IgM with the antigen having been removed from the fluid phase so that a button of cells occurs, but there being insufficient retained on the solid phase to result in a haemadsorption pattern. In the absence of rubella-specific IgM haemagglutination occurs with the rubella HA still being present in the fluid phase. In the method of van der Logt *et al.* (1981) the retained rubella-specific IgM binds rubella HA and then red cells so that a haemadsorption pattern occurs. In the absence of rubella-specific IgM haemagglutination occurs which, on centrifugation of the plates, becomes a button of cells with a serrated edge. The method of Goldwater & Banatvala (1981) results in haemagglutination-like (haemadsorption) pattern in the presence of rubella-specific IgM and a button of cells in its absence. This test requires that the rubella HA titre decrease during incubation so that no haemagglutination occurs if the rubella HA is not retained by rubella-specific IgM. In these methods, with the unattached rubella HA not being washed from the wells prior to the addition of red blood cells, it seems the amount of rubella HA (1–2 u.) used is critical. In the test described here the gentle washing from the wells of rubella HA prior to the addition of chick red cells ensures a greater flexibility in the amount of rubella HA used.

The method described by Denoyel, Gaspar & Peyramond (1981) entails simultaneous addition of red cells and rubella HA. A haemadsorption pattern occurs in the presence of rubella-specific IgM compared with haemagglutination in its absence. The SPARC technique has the advantage that the presence or absence of rubella-specific IgM is indicated by a haemagglutination-like pattern compared to a button of cells.

The SPARC technique reproducibly detected 3 u. of rubella-specific IgM but did not detect 1 u. or 0·3 u. One unit and 0·3 u. of rubella-specific IgM are not detectable by gel filtration and HI but are detectable by MACRIA. However, after nine months' use of the MACRIA in routine diagnosis of naturally acquired rubella it is apparent that some sera may give non-specific results up to 3 u. and therefore, in practice, this apparent lack of sensitivity may not be important. Sera are only considered positive for rubella-specific IgM if a result greater than the 3 u. control is obtained.

We would suggest that the rubella-specific IgM control sera as used by Mortimer *et al.* (1981) should be internationally accepted. Thus appropriate comparisons could be made into sensitivity and specificity of tests for rubella-specific IgM, including those becoming commercially available.

As in MACRIA and other antibody capture immunosorbent assays, including those previously described for rubella, the presence of rheumatoid factor did not result in false positives. This is a considerable advantage over those immunoassay techniques based on solid-phase absorption of rubella antigen. High levels of rubella-specific IgG did not result in false positive results with the SPARC technique, demonstrating the high specificity of the Dako anti-μ used.

No false positive results occurred with the SPARC technique in patients with EB and CMV infection. However, the finding of one infectious mononucleosis patient who gave a positive result equivalent to 25 u. with MACRIA is of great interest, particularly as it occurred when PHLS rubella HA antigen was used but not with Flow Laboratories HA antigen. The PHLS rubella HA antigen is prepared by Tween/ether extraction of baby hamster kidney (BHK) cells infected with the Judith strain of virus, whereas Flow Laboratories antigen is prepared by Tween/ether extraction of the supernatant of BHK cells infected with Gilchrist strain. This problem is under active investigation and will be reported more fully later.

The SPARC technique requires only small volumes of serum (5 μl) and no pretreatment is required. It requires no capital outlay for equipment and the materials used require no special preparation and are readily available. It would seem ideally suited for those laboratories which already perform the rubella HI test but do not have available the equipment required for many of the currently accepted techniques for determining the presence of rubella-specific IgM.

For evaluating numerous sera MACRIA was less time-consuming than the SPARC technique as the beads could be coated with anti-μ in a single container, not as individual wells as in the SPARC technique. More particularly in MACRIA, a single dilution of serum was assayed without the necessity of making dilution series of a serum and transferring to the microtitre plate as with the SPARC technique. MACRIA also proved more reliable in week-to-week diagnostic use, no assay runs being rejected because of incorrect results with the control sera, whereas in up to 5% of assay runs, for no easily discernible reason, the SPARC technique had to be repeated because of inappropriate results with the control sera.

The use of a more limited dilution series in SPARC and a sensitive assay for rubella-specific IgG such as radial haemolysis (Kurtz *et al.* 1980) would enable even a laboratory with limited resources to fully evaluate all sera submitted for the diagnosis of acute rubella, often without the requirement for follow-up sera.

The authors wish to thank Mrs Dorothy Blewer for secretarial assistance and Dr Kay Thompson for editorial assistance. The authors are grateful for the provision of research facilities in the Rayne Institute, King's College Hospital Medical School.

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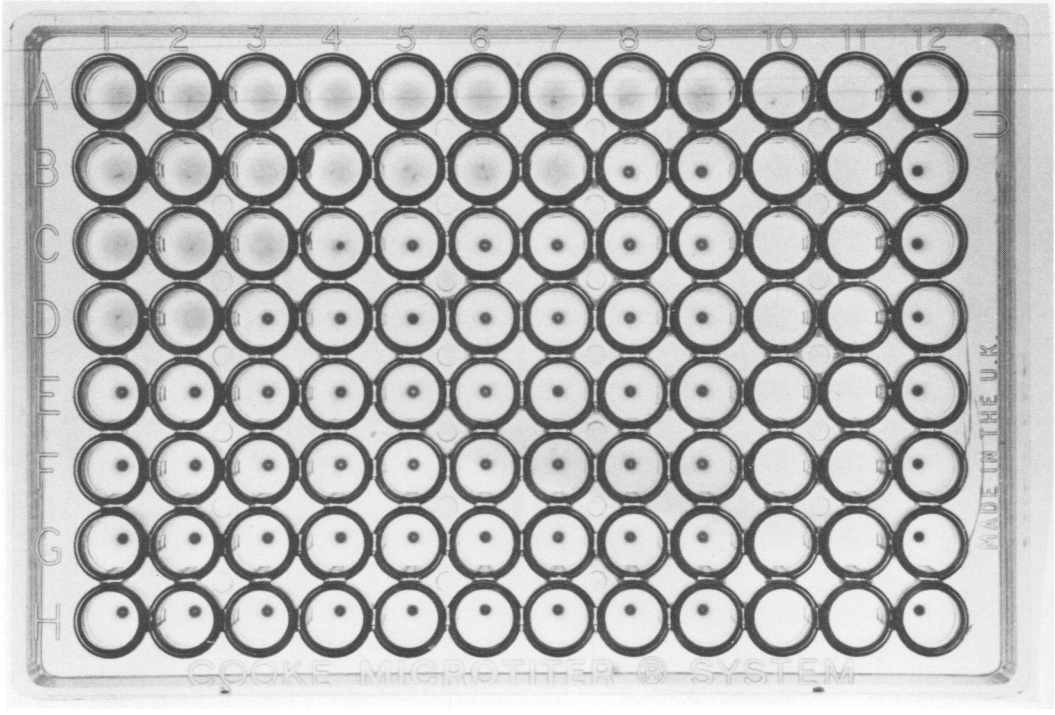
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EXPLANATION OF PLATE

Results of SPARC with the rubella-specific IgM control series. Row A, 100 u.; row B, 30 u.; row C, 10 u.; row D, 3 u.; row E, 1 u.; row F, 0·3 u.; row G, negative serum (HI 3200 i.u.); row H, negative serum (HI < 12 i.u.).