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Automated Enhanced Latex Agglutination Assay for Rheumatoid Factors in Serum

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This improved assay of rheumatoid factors in serum, described here for use with the Baker "Encore" centrifugal analyzer, is efficient, with 250-sample throughput per hour; reproducible, with between-batch CV = 5% and within-batch CV = 2% (mid-assay range); and results correlate well (r = 0.9) with those by other methods. The method is fully quantitative and automated, involves no predilution steps, and can be adapted for use in a wide range of systems. It has a sensitivity of 96% and specificity of 80% in diagnosing rheumatoid arthritis.

Additional Keyphrases: reference interval · values for patients with various rheumatic diseases · diagnostic value, clinical sensitivity · centrifugal analyzer · latex slide, Waaler–Rose assay compared

Detection of rheumatoid factors (RF), autoantibodies directed against antigenic sites located in the Fc region of IgG (1), is important in the diagnosis of rheumatoid arthritis (RA) (2), and also in the prognosis of the disease, high concentrations being an indication of the future development of complications. Some investigations have suggested a correlation between the clinical activity of the disease and RF concentrations (3-5), but one of the reasons this has been difficult to show clearly is the poor precision of conventional assays.

Most laboratories measure RF by the Waaler–Rose assay (6, 7) and the latex slide test (2). At best these can only be considered semiquantitative, given the wide variation in titers obtained by different laboratories for analysis of the same sample. We have previously described the adaptation of enhanced latex immunoassay to automated analysis (8). We present here an improved RF assay and evidence of its usefulness in the diagnosis of RA.

Materials and Methods

Apparatus. We used an "Encore" centrifugal analyzer with its associated P1000 sampler (Baker Instruments Ltd., Windsor, U.K.), a Heraeus minifuge GL (VA Howe Ltd., London, U.K.), and a probe sonicator (Model A180G; Ultrasonic Ltd., Shipley, U.K.) in this study.

Reagents and standards. Latex "polybead" polystyrene microspheres (25 g/L suspension), 57 (SD 5) nm in diameter, were obtained from Polysciences Ltd., Northhampton, U.K.

Human IgG was obtained from Sigma Ltd., Dorset, U.K., as was bovine serum albumin, 220 g/L, in isotonic saline. RF standard material with a value of 250 int. units/mL, as assigned with use of the Bureau of Laboratories Provisional Reference Preparation for Rheumatoid Arthritis, was from BCL Ltd., Lewes, U.K. All other reagents were "Analal" grade, from BDH Ltd., Atherstone, U.K.

Samples. Blood was sampled from patients in rheumatology clinics at Selly Oak Hospital and from presumably healthy blood donors. Serum samples were obtained without preservative; stored at 4°C, and either analyzed within seven days or stored at −20°C until assay.

Preparation of stock latex reagent. This is essentially the same as for our previously published method for C-reactive protein (8), except that we added 10 mg of human IgG instead of the antibody. This amount of stock latex suffices for about 500 analyses.

Measurement of RF. Working latex reagent was prepared by diluting the stock reagent 11-fold with glycine-buffered isotonic saline, pH 8.2. We added 10 μL of sample, standard, or control to 220 μL of working reagent, using 20 μL of distilled water as the diluent to minimize carryover by the sample probe. The Encore analyzer mixed the solutions for 1.6 s; 4 s later a blank reading was taken at 340 nm, then at 2-s intervals between 90 and 100 s. The mean absorbance of these last five readings was calculated and corrected by subtracting the 4-s blank absorbance. Instrument settings other than the above timings and volumes are the same as in our previously published method for C-reactive protein (8) except that we used a spline curve fit and a temperature of 25°C. For RF controls we used patients' serum, pooled to give suitable concentrations of RF, then aliquoted and stored at −20°C. A working standard series was prepared by diluting the stock RF standard in phosphate-buffered...
isotonic saline, pH 7.4; these were prepared freshly for each day of use.

*Treatment of sera with heat-aggregated human IgG.* To confirm that the method was specific for RF, we mixed 90 μL of serum with 10 μL of heat-aggregated human IgG (300 g/L, dissolved in phosphate-buffered isotonic saline, pH 7.4, and heated at 65 °C for 30 min). The samples were then incubated at 37 °C for 60 min before assaying.

*Treatment of sera with dithiothreitol.* To ascertain if IgM RF was being measured, we mixed 90 μL of serum with 10 μL of a 50 mmol/L solution of dithiothreitol in phosphate-buffered isotonic saline, pH 7.4. The samples were then incubated at 37 °C for 60 min before assaying.

*Other methods.* For nephelometry of RF we used the "Turbox" protein analyzer and its associated RF kit (Unipath Ltd., Bedford, U.K.). In this method one measures the change in light scatter caused by the polyethylene glycol-enhanced reaction of RF with aggregated human IgG.

**Results**

Analytical Variables

*Calibration curve and sensitivity.* A spline curve fit was used to fit the data for RF concentration (x) vs absorbance change (y); it yielded a sigmoidal curve (Figure 1). The limit of detection, defined as 3 SD for the smallest concentration that was distinguishable from the blank, was 12.5 int. units/mL.

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 1. (Top) Kinetics of the reactions of RF standards during the assay; (bottom) the resulting standard curve. Numerals after curves refer to cuvette positions (Baker Encore graphics mode)

2 = 12.5, 3 = 50, 4 = 100, 5 = 150, 6 = 200, 7 = 250 int. units/mL.

**Precision.** Within-batch precision was calculated by assaying each of five different pooled sera 20 times. Between-batch precision was calculated by assaying five pooled sera in 20 runs during one working day, standardizing each rotor. Between-batch precision was calculated during one month on 13 separate days with use of nine different stock reagents. The results are shown in Table 1.

**Specificity of the assay.** The addition of heat-aggregated IgG to patients' samples with above-normal RF concentrations (53–480 int. units/mL, n = 40) decreased the assay result to within the normal range except for two sera, these being inhibited to 22% and 44% of the original values of 426 and 210 int. units/mL, respectively. The same sera, when treated with dithiothreitol, also showed a marked decrease in their ability to react with the coated latex. All but two sera (59 int. units/mL from 87 int. units/mL and 90 int. units/mL from 233 int. units/mL) had values that fell within the normal range, but still retained a weak agglutination ability. Treatment of 40 blood-donor sera with dithiothreitol had virtually no effect on the assay value, but treatment of the same sera with heat-aggregated IgG decreased the assay value to near the limit of sensitivity for the method. Assay of two sera evaluated by the ELISA technique, one predominantly IgG RF and the other IgA RF, gave only a very weak reaction in our system. A third sera, predominantly IgM RF, gave a strong reaction (all three sera were purchased from The Binding Site Ltd., University of Birmingham, U.K.).

**Reference interval.** We assayed 500 blood-donor sera by our method. The mean value was 39.2 int. units/mL, the SD 7.0. A reference interval of 25 to 53 int. units/mL was obtained. Patients attending rheumatology clinics who had negative results for the latex slide test (n = 436) also had their sera assayed by our method. A mean of 39.6 int. units/mL (SD 11.8) was obtained. This mirrored our blood-donor distribution closely, but with a slight skew to the right. Sera falling within this skew, treated with dithiothreitol and heat-aggregated IgG, behaved in a similar fashion to sera from RA patients with similar RF concentrations. Therefore the skew was probably ascribable to the reduced sensitivity of the latex slide test failing to detect some borderline positives (Figure 2).

**Correlation.** The equation for the correlation between results from nephelometry (y) or Waaler-Rose titers (x', and our method (x) was \( y = 0.98x + 34.3 \) \( (SE = 179.5, n = 66, r = 0.89) \), and \( y' = 0.98x = 220 \) \( (SE = 321.5; n = 46; r = 0.76) \), respectively, on RF concentrations ranging up to 3000 int. units/mL.

We also (Table 2) compared results by our method with

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<th>Table 1. Precision Data for the RF Assays</th>
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*Assayed during one working day. A Assayed during one month.
method mean results obtained for a three-year distribution of 15 sera from the U.K. External Quality Assessment Scheme for Autoimmune Serology and Special Immunochrometry.

Interference studies. Hemoglobin (5 g/L), bilirubin (330 \( \mu \)mol/L), or triglyceride (6.78 mmol/L) had no effect on results by our method. Denatured lipoproteins, such as may be present after freeze-thawing of sera, also gave no discernible interference.

Speed of analysis. Thirty samples could be processed on one transfer disk (including blank, standards, and controls). Results were ready within 5 min, including pipetting time; a throughput of over 250 samples per hour could be achieved routinely.

Prozone effect. All fluid-phase immunochemical assays are susceptible to nondetection of antigen excess. Although the roles were reversed in our assay—i.e., the concentration of antigen-coated latex was constant and the analyte, RF autoantibody, was the unknown variable—this "prozone effect" still presented a problem. However, under our reaction conditions the prozone effect was not encountered at concentrations less than about 1000 int. units/mL, which is the case for >99% of sera from rheumatoid patients. Above this concentration, the absorbance change at 100 s declines to less than that of the top standard. However, the kinetic curves for these high samples differ from that for the standards: the rates are much quicker at the start of the reaction, becoming zero or negative at the chosen end-point (100 s), compared with that for the standards. This made it simple to distinguish them from sera with lower concentrations of RF but with the same absorbance change (Figure 3).

Diagnostic value and clinical sensitivity for RA. We used our method to assay sera from 142 patients previously diagnosed as having RA. Of these, 113 sera gave values exceeding our reference interval, a clinical sensitivity of 80%. Only 19 of the 500 blood donors gave a positive result, a clinical specificity of 96%. Only 17 sera (12%) had concentrations exceeding the upper limit of the standard curve (250 int. units/mL) and required dilution.

Sera from patients with other rheumatic diseases—psoriatic arthritis, systemic lupus erythematosus, connective tissue disease, and polyarteritis nodosa—were also assayed by our method, 7% (n = 14), 29% (n = 10), 15% (n = 13), and 33% (n = 6), respectively, gave values above our reference interval. All but one of these positive samples were within 4 SD of our mean normal value—i.e., <67 int. units/mL—the exception being a polyarteritis nodosa serum with a value of 77 int. units/mL (Figure 4).

A separate group of 69 RA patients who were found to be positive for RF by the latex slide screen but subsequently gave an insignificant result (titer less than 1 in 64) by the Waaler–Rose assay were also assayed by our method. A mean RF concentration of 105 int. units/mL (SD = 68) was obtained and only one result (50 int. units/mL) was within the reference interval. One result for RF in this group, for a patient with a clinically active disease state, was 536 int. units/mL. Further examination of this specimen by ELISA showed the presence of both IgA and IgM RF.

Another group of 42 RA patients, who were found to have bone erosion and were classified as definite RA on x-ray, were assayed by our method and by latex slide test, followed by the Waaler–Rose test; 95% (n = 40), 93% (n = 39), and 29% (n = 12) of these patients were positive by these

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*The mean of methods involving latex-enhanced techniques. \( b \) Sheep sensitized agglutination titer. \( c \) Major commercial kit in the scheme (distributed in the U.K. by Mast Diagnostics Ltd., Bootle, U.K.). \( d \) Hemagglutination assays other than \( b \) and \( c \). \( r \) Result for this method vs each of the other method means.
Measurement of RF by use of coated latex particles is not new (2). However, we are among the first to describe its adaptation to a fully quantitative and automated system. Any latex reagent has two essential constituents: the latex particle and the immunoreactant (antigen or antibody). Latex particles function as inert spacers in the antigen–antibody lattice, increasing the size of the complexes formed. There is, however, a limit to complex size, above which there is no further increase in absorbance. This is referred to as the "light-scattering plateau," and it depends on such factors as the refractive index of the suspending medium, particle geometry, and the wavelength of the incident light (11). Clearly, particle size is of prime importance. We have used the smallest particle available (57 nm in diameter) so as to give the widest possible analytical range without dilution of the sample. Coating the particles with concentrations of immunoglobulin less than we selected (10 mg per milliliter of latex suspension) failed to flocculate the latex, and this prevented the centrifugation stage and subsequent change of buffer. Using higher concentrations of immunoglobulin not only increases the expense but also decreases the sensitivity of the assay. Possibly this is because the RF in the sample binds to the excess IgG on the particles without participating in the lattice formation.

C1q has been identified as a potential source of interference in agglutination assays (12). Heat treatment of samples at 56°C for 30 min is a procedure commonly used to remove this reported interference, but this introduces an additional manual stage, which may affect precision of the assay as well as making the procedure more laborious. All sera for RF analysis by our method had not been heat treated. Comparison of our method with nephelometry (r = 0.89) indicated that any difference in interference by C1q between the two methods is negligible. Moreover, the comparison of our results for nationally distributed control sera with other laboratory method means, where heat treatment was usual, was also good. Our normal reference interval, 25–53 int. units/mL, compares well with that quoted for heat-treated samples by the Beckman I.C.S. system (<60 int. units/mL) (13). This indicates that C1q interference in sera from non-rheumatoid patients does not contribute to a clinical sensitivity of 80%—and a clinical specificity of 96% supports this view.

Results of our experiments with heat-aggregated IgG indicate that we are measuring RF, although two sera retained an agglutination ability exceeding the top limit of normal. Values for all sera with pathological RF concentration were decreased after treatment with dithiothreitol, all but two to within the normal range. Two sera with abnormally high concentrations of IgG and IgA RF gave hardly any agglutination in our system. These facts indicate that we are measuring predominantly IgM RF in pathological specimens. However, treatment of blood-donor sera with dithiothreitol had little or no effect, all samples retaining a weak agglutination ability compared with the reagent blank, to give an absorbance change up to 30–40 mA. Conversely, addition of heat-aggregated IgG decreased the RF concentration to near the limit of sensitivity for the method. This suggests that the agglutination for normal samples is caused by a different reactant than that in RF sera, which is probably predominantly IgM RF. The results for normal samples treated with heat-aggregated IgG suggest in this case that the agglutinating agents could be IgG or IgA RF, or both. These findings are difficult to explain as yet and need further study.

Fig. 3. Reaction kinetics during 100 s showing the different characteristics of standards 2 = 12.5, 3 = 50, 4 = 100, 5 = 150, 6 = 200, 7 = 250 int. units/mL, and high-concentration samples (9 = 3000, 10 = 1500, 11 = 750 int. units/mL)

Discussion

Perhaps the most useful and most requested test in rheumatology is the RF assay. Most routine laboratories still measure RF by the latex fixation test and by the Waaler–Rose assay. It is not possible to quantify RF by these methods except by titer, which is recognized to be very inaccurate, with plus or minus one or two tubes representing the 95% confidence limits. Other attempts have been made to improve RF measurement by a wide variety of techniques, such as particle counting (9), nephelometry, enzyme immunoassay, and radioimmunoassay (for a review, see reference 10). Most of these improvements require specialized equipment or unusual technical expertise, or both. Our method has the advantage of being easily adaptable for use in a wide range of analyzers or for manual application in a simple spectrophotometer.

Fig. 4. Comparison of RF values in different rheumatic diseases Rheumatoid arthritis (RA), psoriatic arthritis (PA), systemic lupus erythematosus (SLE), connective tissue disease (CTD), and polyarteritis nodosa (PAN). SDs shown are with reference to values for the normal population.
Method developers have the problem of justifying their improvements, but at the same time the results they produce have to agree with existing "inferior" assays. This problem is exacerbated when the current most-popular assays are at best semiquantitative, as with the Waaler–Rose and latex titer assays for RF. We have attempted to overcome this problem by assaying nationally distributed quality control sera and comparing our results with other laboratory method means (Table 2), as well as "in-house" comparisons with nephelometry and Waaler–Rose assays. The correlations obtained compare favorably with those for other methods (9, 13, 14).

Use of the Waaler–Rose and latex tube titers to monitor disease activity in seropositive patients is clearly unsatisfactory, owing to their inherent semiquantitative nature. A sensitivity of plus or minus one to two tubes may represent an RF concentration of several hundred international units per milliliter, even with a titer around 1 in 1000 (15). This may have played a major role in the lack of good correlation between disease state and RF concentration in the past. Attempts to correlate disease activity with RF concentrations by more recent quantitative methods such as radioimmunoassay (3), ELISA (4, 5), and nephelometry (13) have met with some success. The clinical sensitivity and precision of the method described here both suggest that our assay is capable of monitoring these changes, and a clinical sensitivity (80%) and specificity (96%) for our technique compare favorably with other assays (16–18).

There is a clear need for the assay of RF to be quantitative and automated. Subjective decisions of positivity and negativity based on semiquantitative assays have no place when more reliable techniques are available. Automation of the assays can only increase the reproducibility of RF results, and lead to less labor-intensive and hence more inexpensive procedures.

In summary, this latex-enhanced immunoassay for RF is precise, specific, and automated, and is capable of dealing with large workloads (sample throughput, 250 per hour). It may also be adapted with little modification for use with other analyzers or for manual use with a simple spectrophotometer.

We thank Dr. A. Milford Ward (director of the UK External Quality Assessment Scheme for Autoimmune Serology and Special Immunochemo, Royal Hallamshire Hospital, Sheffield, U.K.) for control sera and permission to use data obtained from his scheme. We also thank Unipath Ltd., Bedford, U.K., for use of the "Turbox" protein analyzer and its associated RF kit for our in-house nephelometric comparison.

References