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Detection of Immunoglobulins G and M to Rubella Virus by Time-Resolved Immunofluorometry

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We describe new methods for the detection of immunoglobulin G (IgG) and IgM rubella-specific antibodies in serum. The IgG assay was based on a solid-phase rubella antigen immobilization approach, and the IgM assay was based on the IgM capture assay principle. Both assays used biotinylated antibodies (anti-human IgG and antirubella monoclonal antibody, respectively). The tracer system was based on streptavidin labeled with a fluorescent europium chelate. The final measurements were done by using time-resolved fluorescence. Both assays were thoroughly evaluated with clinical samples and compared successfully with established techniques. We anticipate that these assays are suitable for routine clinical use.

Because of the teratogenic potential of rubella and the limited reliability of clinical diagnosis, rapid laboratory confirmation of rubella infection is often required. For patients who have recently had rubellalike illness, this may be achieved by demonstrating the presence of rubella-specific immunoglobulin M (IgM) in serum. These antibodies, however, persist for only short periods of time and are replaced by higher titers of rubella-specific IgG antibodies. The assay of rubella IgG antibodies in serum is an important test that is used to identify women at risk and as an aid in immunization programs.

Several methods have been proposed to detect IgG and IgM rubella antibodies in serum. The classical hemagglutination inhibition assays (6, 17–19) and the hemolysis-in-gel assay (3) have been largely replaced by rapid and simple immunological procedures which are based on the following principles. (i) For specific IgG detection, a solid phase is coated with the rubella antigen which binds the specific antibodies from the sample from the patient. The captured antibodies are then detected by reacting them with an anti-human IgG antibody labeled with a tracer such as a radioactive nuclide, an enzyme, or a fluorescent probe (5, 12, 16, 24, 26). (ii) For specific IgM detection, the so-called IgM capture assay is preferable because of its increased specificity (1, 2, 4, 10, 21; P. Morgan-Capner, J. Hodgson, K. Bellamy, and P. S. Gardner, Eur. Group Rapid Viral Diagn. Newsl. **7**:35, 1984). In this assay, an anti-human IgM antibody is immobilized on a solid phase to capture the IgM antibodies that are present in patient serum. Rubella antigen is then added, along with another rubella antigen-specific antibody which is labeled with a tracer system. The immunocomplex (anti-human IgM-IgM-rubella antigen-anti-rubella-antibody-label) that is formed on the solid phase can then be quantified accordingly.

Recently, a new detection method, time-resolved fluorescence in combination with fluorescent rare earth chelates (and especially europium chelates), has been introduced in the field of nonisotopic immunoassays. This technique has been reviewed recently (7, 14, 23) and applied to the immunoassay of, for example, a variety of protein hormones, steroid and thyroid hormones, drugs, and tumor markers. Time-resolved fluorescence immunoassays (TR-FIAs) have been designed in two different configurations. The DELFIA (Phamacia, Montreal, Quebec, Canada) system uses Eu^{3+} as the label (15). The FIAgen system CyberFluor Inc., Toronto, Ontario, Canada) uses the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) as the label (11). These two systems have recently been compared critically (7). The DELFIA system has already been used successfully for detecting antigens and antibodies in serum (13, 20, 22).

In this report, we describe the first application of the FIAgen design for detecting IgG and IgM antibodies to rubella virus in serum.

MATERIALS AND METHODS

Instrumentation. All solid-phase fluorescence measurements were done with the CyberFluor 615 immunoanalyzer (CyberFluor Inc.) This instrument uses a nitrogen laser for excitation and is capable of measuring time-resolved fluorescence on the bottom of white microtitration wells. Data reduction is done automatically. A description of the instrument can be found elsewhere (7).

Materials. Purified rubella antigen was purchased from Immunosearch (Toms River, N.J.). Monoclonal antibody to rubella antigen was purchased from Clonatec (Paris, France). Goat anti-human IgM and biotinylated goat anti-human IgG were obtained from Bio/Can Scientific (Mississauga, Ontario, Canada). All other proteins were purchased from Sigma Chemical Co. (St. Louis, Mo.). Europium(III) chloride hexahydrate was a product of Aldrich Chemical Co., Inc. (Milwaukee, Wis.). The europium chelating reagent BCPDA was synthesized as described previously (11). All other chemicals were purchased from Sigma or Fisher Scientific Co. (Unionville, Ontario, Canada).

Assays were done in white opaque 12-well microtitration strips (Microfluor; Dynatech Laboratories, Inc., Alexandria, Va.).

Tracer systems. For the rubella IgG assay, we used a tracer system consisting of streptavidin labeled with 14 ± 1 BCPDA molecules [SA-$(BCPDA)_{14}$] as described previously (8). For the rubella IgM assay, we used a tracer system

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consisting of streptavidin labeled with multiple BCPDA molecules through a carrier protein (thyroglobulin [TG]). The preparation of this streptavidin-based macromolecular complex $[SA(TG)_3(BCPDA)_{480}]$ has been described in detail elsewhere (9; R. C. Morton and E. P. Diamandis, submitted for publication). Both streptavidin preparations were diluted 50-fold just before use in tracer diluent (a 50 mM Tris hydrochloride buffer [pH 7.20] containing 40 g of bovine serum albumin, 9 g of NaCl, and 40 μmol of Eu^{3+} per liter).

Coating of microtitration strips. For the anti-rubella IgG assay, white microtitration wells were coated overnight with 50 μl of rubella antigen diluted in 0.1 M sodium carbonate (pH 9.6). The optimal dilution of rubella antigen stock solution used for coating had to be determined for each lot of stock. For the anti-rubella IgM assay, the strips were coated with 500 ng of goat anti-human IgM in 100 μl of the same coating buffer.

After the strips were coated with rubella antigen or anti-IgM, they were washed with a 10 mM potassium phosphate buffer (pH 7.4) and blocked for 2 h with 200 μl of the same buffer containing 10 g of bovine serum albumin and 1 g of sodium azide per liter.

Rubella standards and controls. Sera from single donors that were positive and negative for anti-rubella IgG were calibrated against the second international reference preparation (World Health Organization [Geneva, Switzerland], obtained from Statens Seruminstitut, Copenhagen, Denmark) and were used as standards for the IgG assay. Positive and negative controls for the IgM assay were purchased from International Enzyme Inc. (Fallbrook, Calif.).

Specimen predilution. Before the rubella IgG or IgM assay, all standards and patient serum samples were prediluted 100-fold (20 μl in 2 ml) in the assay buffer (50 mM Tris [pH 7.80] containing 9 g of NaCl, 5 g of bovine serum albumin, 0.5 g of bovine gamma globulin, and 0.1 ml of Tween 40 per liter).

Procedure for rubella IgG. One hundred microliters of prediluted standard or serum samples (in duplicates) was pipetted into the rubella antigen-coated wells and incubated for 1 h. The strips were then washed four times with the wash solution (0.5 g of Tween 20 and 9 g of NaCl per liter), and 100 μl of a 5-mg/liter biotinylated goat anti-human IgG solution (diluted in the assay buffer) was added. This was followed by a 30-min incubation and washing as described above. A total of 100 μl of a diluted tracer solution $[SA(BCPDA)_{14}]$ was then added and incubated for an additional 30 min. All incubations were carried out in a 37°C air oven. After washing, the strips were dried with a stream of cold air for 5 min. Surface fluorescence was measured on the CyberFluor 615 immunoanalyzer. The instrument has an automatic data reduction capability, and results are printed automatically along with the calibration curve as soon as the readings are complete (about 5 min for a 96-well plate). The final immunocomplex on the solid phase is shown diagrammatically in Fig. 1.

Procedure for rubella IgM. One hundred microliters of prediluted control and patient serum samples was pipetted into the anti-IgM-coated wells and incubated for 1 h at 37°C. The strips were then washed four times, and 100 μl of rubella antigen (diluted 10-fold in the assay buffer) was added. This was followed by overnight (12 to 16 h) incubation at room temperature and washing as described above. One hundred microliters of a biotinylated monoclonal rubella antibody solution was then added and incubated for 30 min at 37°C, followed by another washing cycle. One hundred microliters of a diluted tracer solution $[SA(TG)_3(BCPDA)_{480}]$ was then added, and the assay was completed exactly as described above for the rubella IgG assay.

The final immunocomplex on the solid phase is shown diagrammatically in Fig. 2.

Comparison methods. Rubella IgG was measured by using two enzyme immunoassay kits, the Rubazyme IgG assay from Abbott Diagnostics (Mississauga, Ontario, Canada) and the Rubenostika IgG assay (Organon Teknika, Scarborough, Ontario, Canada). A latex agglutination kit (Rubascan; Becton Dickinson, Mississauga, Ontario, Canada), was also used.

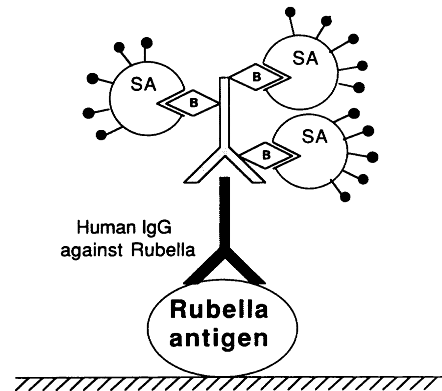

FIG. 1. Schematic representation of the final immunocomplex on the solid phase of the rubella IgG assay. The surface was coated with rubella antigen, to which the antirubella IgG in human serum (solid area) bound. Biotinylated antihuman IgG (shaded area) bound to this and was detected with streptavidin tracer (see text). Abbreviations and symbols: B, biotin; SA, streptavidin; —●, europium chelator BCPDA.

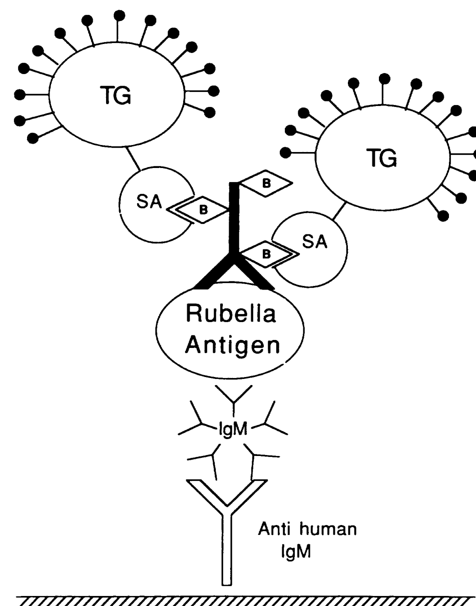

FIG. 2. Schematic representation of the final immunocomplex on the solid phase of the rubella IgM assay. The surface was coated with antihuman IgM (shaded symbols), which captured the IgM in human serum. Rubella antigen bound to the captured antirubella monoclonal antibody (solid area) which bound the streptavidin tracer (see text). Abbreviations and symbols: B, biotin; SA, streptavidin; TG, thyroglobulin; —●, europium chelator BCPDA.

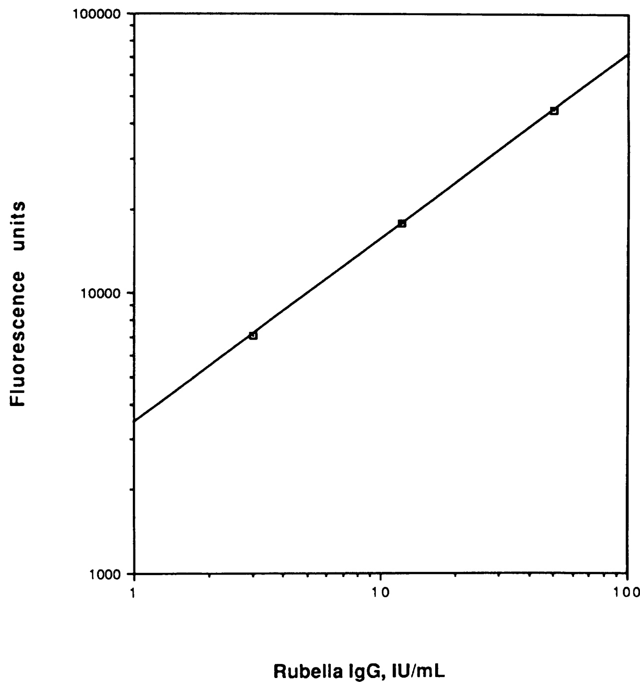

FIG. 3. A typical standard curve for the TR-FIA for rubella IgG. Three calibrators of 3, 12, and 50 IU/ml were used. The assay was carried out as described in the text.

Rubella IgM measurements were compared with two enzyme immunoassays, the Rubazyme IgM from Abbott Diagnostics and the Rubenostika IgM from Organon Teknika.

RESULTS AND DISCUSSION

Rubella IgG assay. (i) Optimization. The anti-rubella IgG assay was optimized for concentration of the coating antigen and for dilution of serum samples and incubation times. Strips were coated with antigen that was diluted from 5- to 200-fold. In a typical lot of antigens, the fluorescence for positive sera increased with an increasing concentration of antigen up to a dilution of approximately 10-fold. At this concentration, the ratio of fluorescence of positive and negative sera was maximum.

The assay was performed at various dilutions of serum in buffer. At a 100-fold dilution, sera with low titers (1/10 by latex agglutination) could be easily distinguished from negative sera, and sera with high (1/160) and very high (1/320) titers could be distinguished from each other.

Time courses were done to optimize the assay incubation times. Maximum binding of anti-rubella IgG to the coating antigen was achieved in 1 h, while binding of goat anti-human IgG to the rubella-specific IgG was complete in 30 min (data not shown).

(ii) Criteria for positive results. A typical dose-response curve for a rubella IgG assay is shown in Fig. 3. Three controls (a negative, a low positive, and a high positive sample) were standardized against the second international reference preparation 86 for anti-rubella serum. The test results were then expressed as international units of anti-rubella IgG per milliliter. The negative control was 3 IU/ml, the low positive control was 12 IU/ml, and the high positive control was 50 IU/ml. A result under 10 IU/ml was considered to be negative. A value of greater than 10 IU/ml reflects

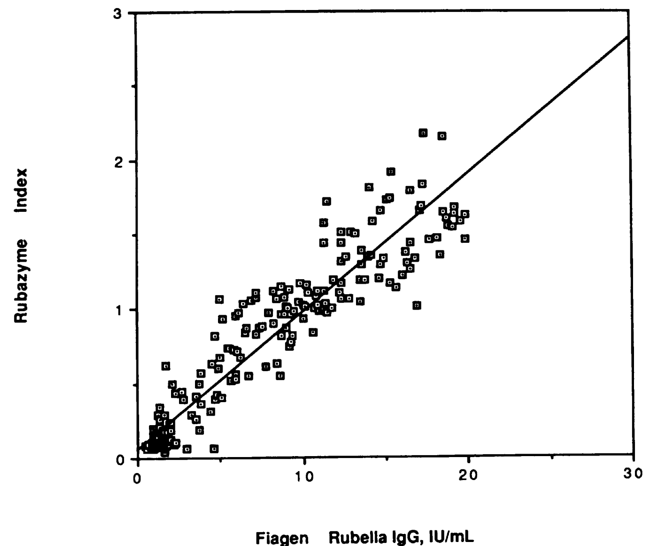

FIG. 4. Comparison of the FIAgen rubella IgG with the enzyme immunoassay Rubazyme IgG. The correlation line was calculated for those samples in which the IgG measured <20 IU/ml by FIAgen. The correlation coefficient was 0.94.

prior exposure to rubella infection or vaccination against rubella virus, and an antibody level of 15 to 25 IU/ml is generally considered to be protective against reinfection (25).

(iii) Comparison with other methods. In order to validate the results of the TR-FIA used in this study, it was compared with two commercial enzyme-linked immunoassay kits for anti-rubella IgG and with a latex agglutination assay.

A total of 427 samples were analyzed by the present method and the Rubazyme (Abbott) IgG assay (Fig. 4). Above 20 IU/ml, the enzyme-linked immunosorbent assay was insensitive to increasing antibody concentration, and the absorbance readings leveled off. Thus, the correlation coefficient was determined only for sera with concentrations of ≤20 IU/ml and was found to be 0.94. The diagnostic specificity and sensitivity of the present assay were found to be 96.8 and 94.0%, respectively, when the Rubazyme kit was taken as the reference. However, all false-positive and false-negative results were in the equivocal zone of both assays. Of 16 false-negative results, 11 were just below the cutoff concentration of 10 IU/ml (Table 1). The remaining five false-negative results measured 1.0 to 1.1 absorbance units. The cutoff in the Rubazyme assay was 1.0 absorbance units.

Ninety-four samples were analyzed by TR-FIA and by the

TABLE 1. Correlation of the TR-FIA with Rubazyme rubella IgG for 427 serum samples

TR-FIA result (IU/ml)	No. of samples in the Rubazyme assay that were:	
	Negative (index <1)	Positive (index >1)
Negative		
<8	142	5
8–10	12	11
Positive		
10–12	5	15
>12		237

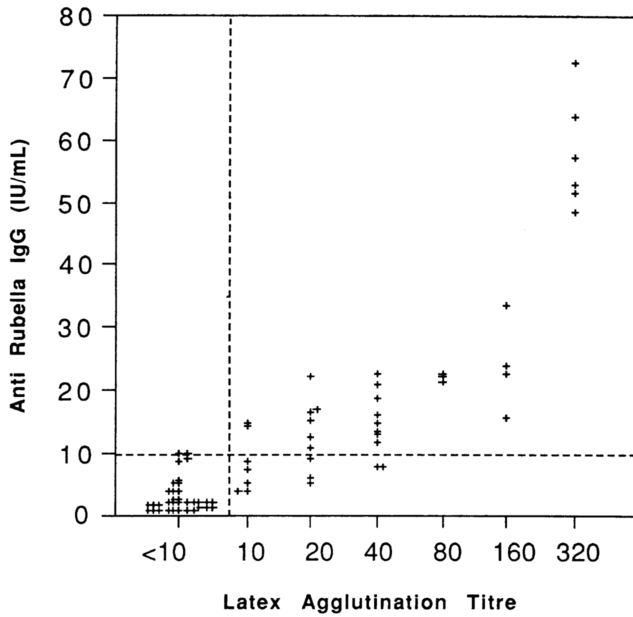

FIG. 5. Comparison of the FIAgen rubella IgG with a latex agglutination assay. The dotted lines show the demarcation values between sera that were positive and negative for rubella IgG.

Rubenostika (Organon) IgG assay, which also measured the antibody concentration in international units per milliliter. The correlation coefficient between the two assays was 0.95 in the concentration range of 0 to 80 IU/ml, and the regression equation was y = TR-FIA = 1.48 + 0.94 enzyme-linked immunosorbent assay x.

Seventy-four samples assayed by the method described in this report and by latex agglutination correlated well. These results are shown in Fig. 5. All samples with titers of <1/10 were negative by the fluorescence assay. All but two samples with titers of 1/10, which is the demarcation titer for immunity to rubella infection, were negative by the TR-FIA. All samples with titers of >1/10 except two, which had titers of 1/20 and 1/40, were found to be positive by TR-FIA. Two of the samples with titers of 1/10 which had results of <5 IU/ml and three of the four samples with positive latex agglutination titers but negative titers by TR-FIA were measured by using a commercial enzyme immunoassay kit. This assay confirmed the negative results.

We investigated the seroconversion in 28 paired serum specimens; 11 pairs were collected before and after patients were given the rubella vaccination, 13 pairs were collected from patients during the acute and convalescent stages of rubella infection, and 4 pairs were collected from patients in the acute and convalescent stage of a disease other than rubella. Seroconversion occurred if there was a fourfold increase in the antibody titer after exposure to rubella antigen. In the 11 paired serum samples taken before and after patients were vaccinated with rubella antigen, all the prevaccination antibody concentrations were under 7 IU/ml and all of the postvaccination concentrations were >24 IU/ml. In each case the antibody concentration ratio after: before vaccination was greater than 4. The results for sera taken from patients in the acute and convalescent stage of rubella infection were similar. In each case there was at least a fourfold increase in the concentration of antirubella antibody. In contrast, in the serum pairs collected from patients with nonrubella disease, the rubella antibody concentrations varied from weakly positive to positive in those with the acute stage of the disease and were unchanged in those in the convalescent stage of the disease. These results are shown in Fig. 6.

(iv) Assay performance characteristics. The linearity of the assay is demonstrated in Table 2. Four samples with positive titers of antirubella IgG were serially diluted with negative serum samples. These were diluted 1/100 with buffer and assayed. The correlation coefficient of the observed values to the concentration expected after dilution was 0.98.

The precision of the assay was determined. For within-assay precision, each of three serum specimens (a negative, a low positive, and a high positive specimen) was measured 12 times. The within-run coefficients of variation were 7.7, 6.4, and 4.7%, respectively. Four positive serum samples were analyzed in duplicate for seven or eight times in a 2-week period. The between-run precision varied from 3 to 7.5%. These results are summarized in Table 3.

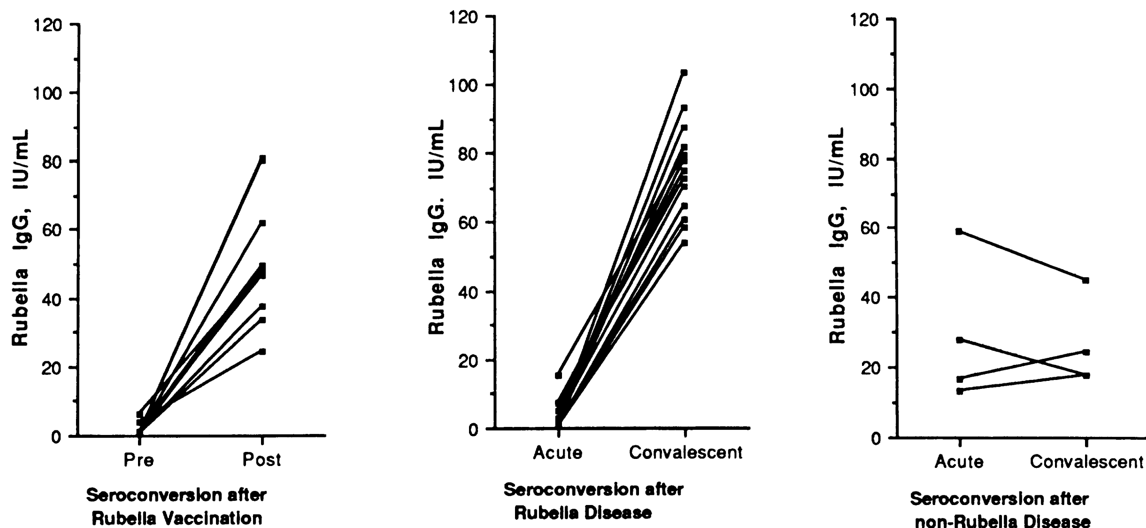

FIG. 6. Demonstration of seroconversion in paired serum samples after rubella vaccination or disease. IgG was measured by FIAgen as described in the text.

TABLE 2. Linearity of the rubella IgG assay[a]

Sample no.	Dilution	IU/ml	
		Expected	Observed
1	Undiluted		16.5
	2×	8.25	8.1
	4×	4.12	5.5
	8×	2.06	3.1
2	Undiluted		22.8
	2×	11.4	10.8
	4×	5.7	7.0
	8×	2.8	4.1
3	Undiluted		37.0
	2×	18.5	14.8
	4×	9.2	9.8
	8×	4.6	7.5
4	Undiluted		56.0
	2×	28.0	27.7
	4×	14.0	14.3
	8×	7.0	8.9

[a] Four samples were diluted with a serum sample that had a low concentration of anti-rubella IgG. All the samples were further diluted 100 times in buffer prior to analysis. The correlation coefficient of the observed (y) versus the expected (x) value was 0.98.

Rubella IgM assay. (i) Optimization. The antirubella IgM assay was optimized for sample dilution, antigen dilution, and incubation times. The assay was performed at various dilutions of patient serum. At a 100-fold dilution (the dilution used for the IgG assay), it was possible to clearly distinguish a negative and a low positive serum specimen; this was therefore chosen as the working dilution.

TABLE 3. Precision data for the IgG and IgM rubella antibody assays

Assay	Sample no.	Mean ± SD[a]	% CV[b]	n
Within run				
IgG	1	2.05 ± 0.16	7.7	12
	2	18.54 ± 1.20	6.4	12
	3	70.33 ± 2.10	4.7	12
IgM	1	0.21 ± 0.01	7.0	12
	2	0.67 ± 0.05	7.4	12
	3	1.09 ± 0.08	7.9	12
	4	2.23 ± 0.14	6.4	12
	5	5.39 ± 0.16	2.9	12
	6	5.43 ± 0.24	4.5	12
Between run				
IgG	1	17.19 ± 1.30	7.5	8
	2	26.63 ± 1.67	6.3	8
	3	43.44 ± 2.50	5.7	7
	4	58.44 ± 3.00	5.2	7
IgM	1	0.22 ± 0.02	8.8	12
	2	0.82 ± 0.07	8.8	12
	3	1.35 ± 0.09	6.7	8
	4	2.84 ± 0.21	7.4	9
	5	5.99 ± 0.51	8.5	11
	6	5.46 ± 0.51	9.3	11

[a] Values for IgG are international units per milliliter; for IgM, units are index arbitrary units, as defined in the text.
[b] CV, Coefficient of variation.

After IgM capture, rubella antigen which bound to the antirubella-specific IgM was added. It was found that a 10-fold antigen dilution was optimum. Binding of rubella antigen to captured IgM was very slow and required many hours (>4 h at room temperature). In order to complete the assay in a convenient manner, incubation at room temperature overnight was selected. Although antibody and antigen could be incubated simultaneously, sufficient antibody must be added to saturate all the rubella antigen for maximum sensitivity. Therefore, the antibody was added in a separate step after unbound antigen was washed away. Thirty-minute incubations at 37°C were found to be sufficient for both biotinylated antibody and streptavidin tracer binding (data not shown).

(ii) Criteria for positive results. Since no international standard is available for antirubella IgM, a standard curve could not be defined. Three controls were therefore used for the assay, as follows: control 1, an antirubella IgM negative specimen; control 2, a low positive specimen; and control 3, a high positive specimen. An IgM index was defined as the ratio of the fluorescence of a sample or control to the fluorescence of control 2. The low positive control therefore had an IgM index of 1.0.

A cutoff level between IgM-positive samples and IgM-negative samples must then be defined. On the basis of analysis of approximately 200 serum specimens from healthy people, a cutoff level was defined as the average IgM index of presumed rubella IgM-negative sera plus 5 standard deviations.

Based on the assay of 196 serum samples from apparently healthy donors, a cutoff index value of 0.32 was established which was equal to the mean IgM index of the 196 serum samples plus 5 standard deviations. The range of IgM indices for the 196 negative serum samples was 0.06 to 0.30, with a mean value of 0.12 ± 0.04. Serum samples whose IgM index is greater than the cutoff index value but less than 1.2 times the cutoff value are considered equivocal and should be retested.

(iii) Comparison with other methods. Forty-seven serum samples that were positive for rubella IgM were compared by the Rubazyme (Abbott) IgM assay and the assay described in this report. All 47 serum samples were found to be positive by both assays. The IgM indices varied from 0.74 to 7.43 (data not shown).

A total of 59 serum samples (including the 47 samples described above) were analyzed and compared with a second antirubella IgM enzyme immunoassay kit, the Rubenostika (Organon) IgM assay. The correlation is shown in Fig. 7. The dotted lines indicate the cutoff levels of each assay.

(iv) Assay performance characteristics. Rubella IgM was measured by an IgM capture assay in order to eliminate interference caused by high concentrations of antirubella IgG which could bind to antigen-coated plates, thereby preventing the binding of specific IgM. In the capture assay it was possible to have nonspecific binding of antirubella IgG to anti-IgM-coated plates, giving rise to false-positive results. Therefore, 100 serum samples that were positive for antirubella IgG were tested in the antirubella IgM assay. The specific IgG concentrations varied from 21.4 to 126.8 IU/ml; the IgM indices varied from 0.07 to 0.31.

There have also been reports of possible false-positive readings for rubella-specific IgM in sera containing Epstein-Barr virus antibodies or rheumatoid factor. Samples positive for Epstein-Barr virus antibodies and rheumatoid factor were therefore tested by the fluorescence rubella IgM assay. All such samples were negative (Table 4).

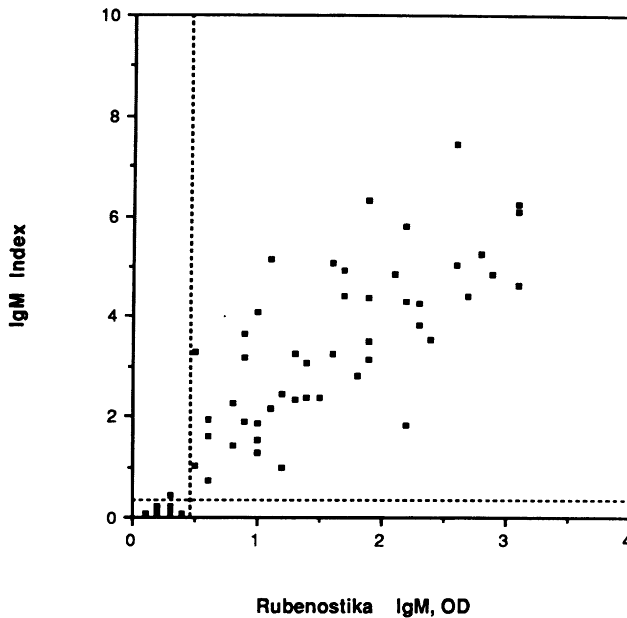

FIG. 7. Comparison of the FIAgen rubella IgM assay with the enzyme immunoassay Rubenostika for IgM. The dotted lines show the demarcation values between sera that were positive and negative for rubella IgM. OD, Optical density.

The precision of the IgM assay was determined. For within-assay precision, each of six serum samples (two negatives, two low positive and two high positive samples) was measured 12 times. The within-run precision (coefficients of variation) varied from 2.9 to 7.9%. The same six serum samples were analyzed in duplicate 8 to 12 times over a period of 2 weeks. The between-run precision varied from 6.7 to 9.3%. These results are summarized in Table 3.

These results demonstrate that the two antirubella immunoglobulin assays described here are sensitive. They are simple to perform and can accurately determine the immune status of a patient or confirm a diagnosis of rubella infection.

In recent years, solid-phase enzyme immunoassays have replaced the agglutination assays for rubella IgG and IgM. The TR-FIA described here retains the advantages of these solid-phase assays. The serum was untreated before analysis, although it was diluted. The assays were done in a 96-well microtitration plate format, which lent itself readily to the large batch sizes used in screening assays. In addition, TR-FIA had a very large range of linear response (greater than 3 orders of magnitude). Thus, the linear range of an assay is limited by the antibody system used and not by the detection system. It is proposed that these newly developed assays are suitable for routine use in clinical and microbiological laboratories.

TABLE 4. Interference studies in the antirubella IgM assay

Interfering substance	n[a]	Mean IgM index ± SD
Antirubella IgG	100	0.122 ± 0.039
Anti-Epstein-Barr virus	12	0.098 ± 0.019
Rheumatoid factor	9	0.110 ± 0.030

[a] Number of different samples tested, as further exemplified in the text.

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