MATERIALS AND METHODS

Studied sample

A sample of 673 sera were assayed by both, Rubella-UME and HIA: 498 sera (group 1) were routinely submitted to our Virology Department for testing of the immune status againts Rubella virus and 175 sera (group 2) were from healthy blood donors. Any serum showing discrepant results was repeated in both test systems. The results of the re-testing were considered final. Paired sera from 127 patients clinically suspected as natural Rubella infection were also assayed.

Seventeen additional serum samples received in our laboratory for determination of the immune status to Rubella virus were used to compare Rubella-UME and a Difco-Rubella-Cube and to test the UME reproducibility.

HIA

This test was performed as described by Schmidt and Lennette (1970). Sera were treated with kaolin and goose red cells. Inhibition hemagglutination antibody titers (IHA) ≥1/10 were considered as an indication of immunity. Fourfold seroconversion or greater in a paired serum was considered a criterium of recent infection.

Antigen

Rubella virus (kindly offered by Dr Najera, Majadahonda Institute, Spain) was cultured in Vero cells mantained in Medium 199 with 2% newborn calf serum and antibiotics. When cytopathogenic effect (CPE) was maximum, usually after 5 or 6 days postinoculation, fluid supernatant was harvested and virus purified as described by Vejtorp (1986). Briefly, antigen was concentrated by ultrafiltration using an XM300 membrane. Concentrated antigen was purified on a sucrose gradient (15-70%, W/V) for 2 hours at 25000 rpm in an Hitachi ultracentrifuge. A thin, well defined layer could be distinguished. Fractions were tested in a Rubella-IgG UME at 1:100 dilution with positive and negative reference sera. The P/N ratios were calculated. Fractions with the highest values of P/N were pooled and used as antigen for Rubella-UME.

Rubella-UME

An indirect 10 μl UME was performed.

Plates (PVC, SUMA) were coated overnigth at 4°C with the appropriate antigen dilution (1:150) in carbonate-bicarbonate buffer pH 9.6. On the following day, the antigen-sensitized plates were rinsed once with PBS + Tween 20 (0.05%) pH 7.2-7.4 in a plate washer. 20 μl of blocking solution (PBS + Tween 0.05%) with 0.1% of bovine albumin fraction V and 5% of sacharose was added to each well. After 1 hour at 37°C, solution, was aspirated and plates were left at 37°C for 30 min.

The test sera and controls were diluted at 1:40 in standard IHA trays by adding 5 μl of serum to 195 μl of serum diluent (buffer: Tris 15mM pH 7.8, 0.05% Tween 20, 5% sheep serum). Ten microliters of each diluted serum was added to two wells in the plate. The plate was then incubated at 37°C during 30 minutes. After that, the plate was washed 4 times with washing solution (buffer: Tris 15 mM pH 7.8, 0.05% Tween 20).

Later on, each well was filled with 10 μl sheep anti-human IgG conjugated to alkaline phosphatase at 1:1000 dilution and incubated at 37°C for 30 minutes. The plates were washed once again as above and 10 μl of substrate (4 metyllumbelliferyl phosphate) were added to each well at 0.13 mg/ml in diethanolamine buffer pH 9.8. 4 metyllumbelliferona was used as fluorescent reference solution (Koch Ligth Limited, England).

After 30 min at room temperature, reaction was read on a SUMA spectrofluorimeter (121 B model).

Intra-Assay controls included one positive serum (≥1/64) and one negative serum (< 1/10) as previously tested by HIA. All sera were tested in duplicate in each assay.

The following formula was applied to spectrofluorimetric reading:

$$E = F\text{-}B / P\text{-}B$$

where E represents the index value, F represents the average of fluorescence of the tested serum, B represents blank fluorescence reading and P represents the fluorescence reading of positive serum control. Any sample with and E≥0.210 was considered as positive for the presence of rubella IgG antibodies.

In order to assess the serum titer by Rubella-UME, sera were diluted from 1:40 to 1:320. The greatest serum dilution with an E≥0.210 was considered the serum's titer by Rubella-UME. Seroconversion of more than 4 fold in a paired serum was considered a criterion of recent infection by Rubella-UME.

Difco Rubella-Cube for IgG detection

The commercial Difco Rubella-Cube, for the qualitative detection of rubella virus specific IgG, was used for the comparison with Rubella-UME. 17 untreated serum samples with IH antibody titers from < 1/10 to 1/160 were diluted 1/5 and applied onto the Rubella-Cube membrane. All washing steps were carried out according to the manufacturer's instructions. Results were read visually. The development of an uniform blue dot in the center of the membrane surface was considered a positive test for the presence of rubella specific IgG antibody in the specimen. The absence of an uniform blue dot was considered negative for Rubella spacific IgG antibody.

Statistical Analysis

Sensitivity, specificity and percent of agreement with the reference method were calculated according to Grimer *et al.* (1981).

RESULTS

The reagent concentrations and conditions of the reaction in the UME test were selected by cross board titration method. Those giving the best ratio with the positive and negative reference sera were used in the assay (figure 1).

Determination of cutoff value

Twenty sera with IH antibody titers of < 1/10 to 1/20 were assayed by Rubella-UME. An immune status cutoff value of 0.210 (index value) was predetermined and applied in subsequent experiments.

Comparison of HIA and Rubella-UME:

The results of the immune status comparison are presented in table 1. From the first study group of sera, 241 were positive and 247 were negative by both methods. The results were different for 10 sera (9 were positive by Rubella-UME and negative by the

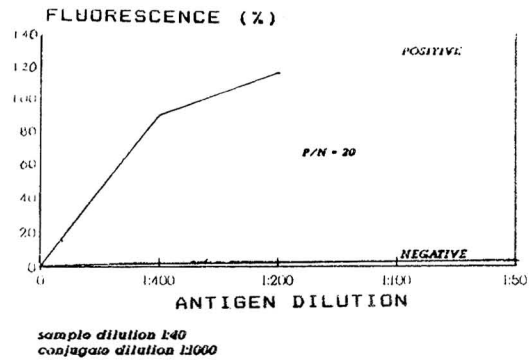

Fig. 1. Assay conditions of UME Rubella. The antigen dilution y 1:150 was chosen for coating the ultramicroplates.

alternative assay and 1 was negative by Rubella-UME but was positive by HIA). Therefore, the sensitivity, specificity and coincidence of Rubella-UME with regards to HIA was of 99.5, 96.4 and 97.9% respectively.

In the second group of sera a similar of coincidence was found (97.1%) with disagreement in the results of 5 sera. Three of them were negative by HIA and positive by Rubella-UME and the index value varied from 0.249 to 0.295 (Table 1).

Table 1
Comparison between detection of rubella antibodies by HIA and Rubella-UME.

Group	No. sera	Sensitivity (%)	Specificity (%)	Coincidence (%)
1	498	99.5	96.4	97.9
2	175	98.6	89.6	97.1
Groups 1+2	673	99.2	95.7	97.7

When all sera were studied as a single group, values of 99.2, 95.7 and 97.7% were obtained for sensitivity, specificity and coincidence of Rubella-UME with respect to HIA.

Comparison of HIA and Rubella-UME to determine seroconversion:

From the 127 paired sera from patients clinically diagnosed as Rubella, serial dilutions were done to each serum and tested by Rubella-UME and HIA. Antibody titer was calculated in each serum. Results were coincident in 100% of sera. 10 paired sera showed seroconversion by HIA and Rubella-UME. Table 2 presents the antibody titer by HIA, Rubella-UME and the index value at the reading dilution. As described by others authors (Boteler *et al.*, 1984), critical ratios are interpreted as highly indicative of an active infection. Critical ratios were calculated for each paired serum. Values =1.79 included 95% of all paired sera without seroconversion neither by Rubella-UME or HIA.

Table 2
Comparison of antibody Rubella titer by HIA and UME-Rubella in paired sera of clinically suspected patients.

Case	IH titer*	UME titer*	Index value**	Critical Ratio
1	<10\20	<40\80	0.005\0.38	76
2	<10\40	<40\80	0.040\0.39	10
3	<10\20	<40\160	0.090\0.54	6
4	<10\40	<40\160	0.030\0.61	20
5	<10\80	<40\>320	0.100\0.90	9
6	<10\160	<40\>320	0.050\0.75	15
7	<10\160	<40\>320	0.120\0.88	7
8	160\10	320\<40	0.830\0.02	0.02
9	<10\40	<40\160	0.040\0.49	12
10	20\160	40\160	0.080\0.65	8

(*) Reciprocal of titer in acute serum\convalescent serum
(**) Index value at 1/40 dilution

Comparison of Rubella-UME with Difco Rubella-CUBE

Seventeen sera were processed by both tests in 5 assays: 10 were positive by Rubella-UME and Difco Rubella-Cube and 7 were negative. Correlation was of 100%. Table 3 shows the antibody titer of these sera by HIA, Rubella-UME and the qualitative results of Difco Rubella-Cube. The coefficients of variability (CV) obtained form each serum sample in the UME is also included.

DISCUSSION

The detection of humoral antibodies is the only feasible mean which is available to clinical laboratories for assesing immune status to Rubella virus (Freeman *et al.*, 1983). Similarly, the need for full serological investigation of pregnant women who present a rash, or who report contact with Rubella is relevant (Miller, 1989). Besides, detection of the increase of the IgG Rubella antibodies is used in the verification of an acute infection. HIA is still recognized as the reference test for Rubella immune status determination (Wielaard *et al.*, 1987), however, alternative assays for the detection of antirubella antibodies have been developed. Several tests have become available which meet the demand for

Table 3
Comparison of Rubella-UME index value, Difco Rubella-Cube results and HIA titers.

No. of sera	IH titer*	UME titer*	(Index value)	CV (%)	Difco
2267	160	>320	0.81	2.4	+
2264	80	>320	0.34	5.5	+
2123	40	80	0.23	9.0	+
2318	20	160	0.23	8.3	+
2242	20	40	0.26	7.0	+
2205	40	160	0.36	3.1	+
2137-1	<10	<40	0.06	8.9	-
2137-2	20	80	0.21	4.3	+
2202-1	<10	<40	0.04	5.2	-
2202-2	40	80	0.32	8.2	+
2310	<10	<40	0.13	6.8	-
2334-1	<10	<40	0.08	7.5	-
2334-2	<10	<40	0.08	6.4	-
2196	20	80	0.22	3.8	-
2199	<10	<40	0.09	5.9	-
2314	80	160	0.31	8.2	+
2238	<10	<40	0.19	4.5	-

* Reciprocal of the antibody titer.

simpler and faster procedures requiring less 'hands on time'. However, some of them give only qualitative results, require individual manipulation for each serum and evaluated vissually. These latter aspects make them unsuitable for automation and hence, less attractive for large scale screening of Rubella antibodies. Passive hemagglutination and latex agglutination tests are among these (Wielaerd *et al.*, 1987).

Reports indicate a good agreement of enzyme-immunosorbent assay with the HIA (Enders, 1985). We therefore developed a simple, easy and cheap test based on the enzyme-immunosorbent assay principle.

Rubella-UME for IgG antibody detection is simple. It is performed in 90 minutes, requires only 10 μl of the sera and reagents to be tested and the results can be read spectrofluorimetrically. This makes the test an attractive candidate for large scale Rubella screening.

The data presented on sensitivity (99.2%), specificity (95.7%) and coincidence (97.7%) illustrate that Rubella-UME assay is substantially equivalent to HIA for determining immune status to Rubella virus.

Several groups of investigators (Kleeman *et al.*, 1983) have suggested that the newer more sensitive assay methods are capable of detecting low levels of Rubella antibodies which escape detection by HIA. The latter measures only antibodies to the Rubella hemagglutinin where as at least theoretically, Rubella-UME could detect any IgG antibody specific to Rubella virus (Hancock *et al.*, 1986).

Twelve sera were found positive by Rubella-UME and it is possible that low antibody levels could be detected by this system. Available data continue to support the presumption that any antibody level that is measured by a licensed assay and is above the standard positive cut-off value for it can be considered as an evidence of immunity (MMWR, 1990).

The clinical evaluation of Rubella-UME employed in the study of 127 paired sera showed that it correlated very well with the HIA and demonstrated that Rubella-UME was at least as sensitive as HIA in detecting a rise in antibody to Rubella virus. Therefore, Rubella-UME could be used not only for the screening of immune status but for serological verification in suspected Rubella patients.

Critical ratios for ELISA kits are interpreted as highly indicative of an active infection if values are more than 1.47 (Boteler *et al.*, 1984). These values are equivalent to a fourfold or greater increase in HI antibody titer. When adapted for Rubella-UME, critical ratios included 95% of the paired negative sera allowing the use of this parameter as indicator, of a recent Rubella infection.

The 100% of coincidence between Rubella-UME and Difco Rubella-Cube (a commercial system with a very good previous evaluation) and the very low CV values observed for all the sera included in the UME indicates that the small volume of the serum sample and the reagents used in this test (10 μl) do not involve a possibility of or error by manipulation.

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