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Dot-Blot ELISA for the Detection of IgM RF and IgA RF

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SUMMARY **Dot-blot ELISA was developed for the detection of IgM RF and IgA RF. Normal rabbit IgG (NRIgG), concentration 100 μg/ml, was used as the antigen for dotting on the 0.45 μm pore size nitrocellulose membrane. Serum, conjugate and substrate incubation conditions were at room temperature for 1 hour, 1 hour and 3 minutes, respectively. The membrane with NRIgG dot could be sotred for 6 weeks before use in the assay. Positive results of IgM RF, at the serum dilution 1:800, were found in 31/51 patients with either classical or definite rheumatoid arthritis and 3/68 normal healthy individuals. Positive IgA RF, at the serum dilution 1:100, was found in 27/51 of the former and none of the latter. Significant concordance with high agreement index was found between the results of the dot-blot ELISA developed and those obtained from ELISA performed in microtitre plate (Kappa ⩾ 0.78 for IgM RF and 0.83 for IgA RF, $p < 0.001$).**

Rheumatoid arthritis (RA), a disease with chronic nonsuppurative inflammation of the diarthrodal joints and a variety of extra-articular manifestations, has a worldwide distribution and afflicts all racial groups.[1] One of criteria for the diagnosis of rheumatoid arthritis, as revised by the American Rheumatism Association in 1987, is the presence of serum rheumatoid factors.[2] Rheumatoid factors (RF) are antibodies directed against Fc portion of IgG.[3] They have been found in all classes of immunoglobulins and can react with IgG of various species, e.g. human rabbit, horse.[4-6] With conventional (agglutination) tests for RF, positive tests with rabbit IgG have been found to be more specific for RA than those tests with human IgG.[7,8] The importance of RF isotypes identification has been reported. IgG RF and IgM RF have been reported to be associated with the severity of RA more than other RF isotypes.[9] Some investigators suggested that IgA RF were strongly associated with bone erosion of patients with RA.[10] However, with the agglutination test, the class of RF cannot be actually identified, although it has been claimed that RF detected by this method are mainly IgM RF. Various assays including radioimmunoassay and enzyme immunoassay enable measurement of the RF isotype.[3,5,11-13] Among these techniques, enzyme immunoassay is the one which can be performed without the need of expensive equipment.

In this study, we have developed a dot-blot ELISA for the detection of IgM RF and IgA RF and compared it to ELISA in which a microtitre plate has been used as the solid phase. The results suggest that dot-blot ELISA would be a useful tool for the study of RF isotypes.

MATERIALS AND METHODS

Sera

Fifty-one sera were obtained from patients with either classical or

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definite rheumatoid arthritis. The diagnosis was based on the criteria established by the American Rheumatism Association.[2] These rheumatoid arthritis patients had ages ranging from 11 to 65, the mean age being 41 years.

Sixty-eight sera from normal healthy individuals were obtained from blood donors at the Department of Transfusion Medicine, Faculty of Medicine, Siriraj Hospital, Bangkok, by the courtesy of Professor Dr. Dasnayanee Chandhanayingyong. These subjects had ages ranging from 19 to 58 years old, the mean age being 36 years.

Twenty-two sera obtained from rheumatoid arthritis patients, with positive rheumatoid factors (RF) as detected by latex agglutination test (Rheumafac® , ICL Scientific, California, USA) were pooled and used as pooled positive reference serum while those from 20 normal healthy individuals and negative for RF by latex agglutination test were pooled and used as pooled negative serum.

These sera were kept in aliquots at -20° C until use.

Dot-blot ELISA

Fifty microlitres of 100 μg/ml normal rabbit IgG (NRIgG) (Sigma Chemical Company, ST. Louis, M.O., USA) diluted in PBS pH 7.1 containing 0.02% NaN_3 was blotted on nitrocellulose membrane, 0.45 μm pore size (Schleicher & Schuell, Dassle, W. Germany), by using the Hybri-dot Manifold® (BRL, Life Technology Inc., USA). The membrane was left air dried, then washed 3 times with 0.01 M phosphate buffered saline, pH 7.1 containing 0.05% Tween 20 (PBST). Each area of the membrane with NRIgG dot was cut into a small round piece and incubated with 100 μl of a diluted serum sample. Each serum was diluted with PBST as serial two fold dilutions. The incubation was at room temperature for 1 hour. After washing 3 times with PBST, the membrane was incubated with 100 μl of the conjugate, which was either alkaline phosphatase labelled anti-IgM $F(ab)'_2$ or alkaline phosphatase labelled anti-IgA $F(ab)'_2$ (Sigma Chemical Company), diluted in PBST containing 0.5% (w/v) BSA (PBST-BSA). After incubation at room temperature for 1 hour, the membrane was washed with PBST and then incubated for 3 minutes with the substrate solution, which was a mixture at ratio 1:1 of 0.6% β-naphthyl phosphate and 0.05% *O*-dianisidine tetrazotized (Sigma Chemical Company), each of which was diluted in 0.05 M carbonate buffer, pH 9.8 containing 0.005 M $MgCl_2$. Excess substrate solution was washed out with distilled water. The colour developed was read visually.

In each experiment, a direct conjugate control (DCC) and reference positive control serum were also included. The PBST was used instead of a serum sample for the DCC dot. Thus, the DCC provided a control for the extent of non-specific conjugate binding to the membrane.

The results were interpreted by observing the highest dilution of tested specimens that still gave rise to the purple colour on the dot-blotted membrane, which could be distinguished clearly from the colour observed at the DCC dot.

Microplate ELISA

The indirect ELISA was used for the detection of RF in this study. Each well of Microelisa Immulon 1® plate (Dynatech, Verginia, USA) was coated with 100 μl of NRIgG diluted in 0.01 M PBS pH 7.1, containing 0.02% sodium azide, to a concentration of 5 μg/ml. The incubation at this step was at 37° C for 3 hours. After 3 washes with 0.85% sodium chloride solution containing 0.05% Tween 20 (NSST), the plate was tapped dry. One hundred microlitres of serial two fold diluted serum samples in PBST were then added into each well of the NRIgG coated plate and incubated at 4° C overnight. After the washing step, 100 μl of either anti-human IgM $F(ab)'_2$ alkaline phosphatase conjugate or anti-human IgA $F(ab)'_2$ alkaline phosphatase conjugate diluted in PBST containing 0.5% BSA was added into each well and incubated at 37° C for 3 hours. The excess conjugate was then washed out and the substrate, which was 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company) in 0.05 M carbonate buffer pH 9.8 containing 0.005 M $MgCl_2$, was added. The reaction was allowed to take place at 37° C for 1 hour and then stopped by the addition of 25 μl 3 N NaOH. The colour developed was read spectrophotometrically at 405 nm, with a Titertek Multiskan® (Flow Laboratories, GmbH, Bonn, West Germany).

In each plate, the wells of DCC and reference positive control serum were also included.

To evaluate the results, the mean absorbance value of DCC was always subtracted from the mean absorbance value of each tested sample. Such corrected absorbance value of tested samples was then used for the calculation of results. The level of RF present in each specimen was determined by reading the dilution of that specimen which gave the corrected absorbance value 0.2 unit above base line level of the semi-logarithmic curve, obtained by plotting the corrected absorbance values against corresponding reciprocal dilutions of that specimen. Such a dilution was then expressed as the titre of RF in each specimen tested.

RESULTS

Dot-blot ELISA for IgM RF and IgA RF determination

Fig. 1 demonstrates the dot blot ELISA for IgM RF detection, which can be seen that, with the use of NRIgG antigen at a concentration of 100 μg/ml, the bright purple colour

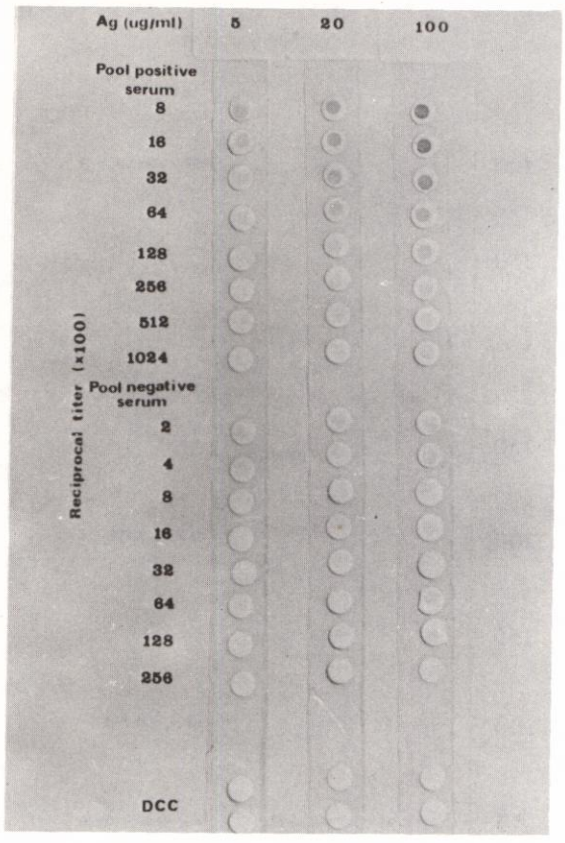

Fig. 1 Dot-blot ELISA for IgM RF determination, using various concentration of normal rabbit IgG antigen.

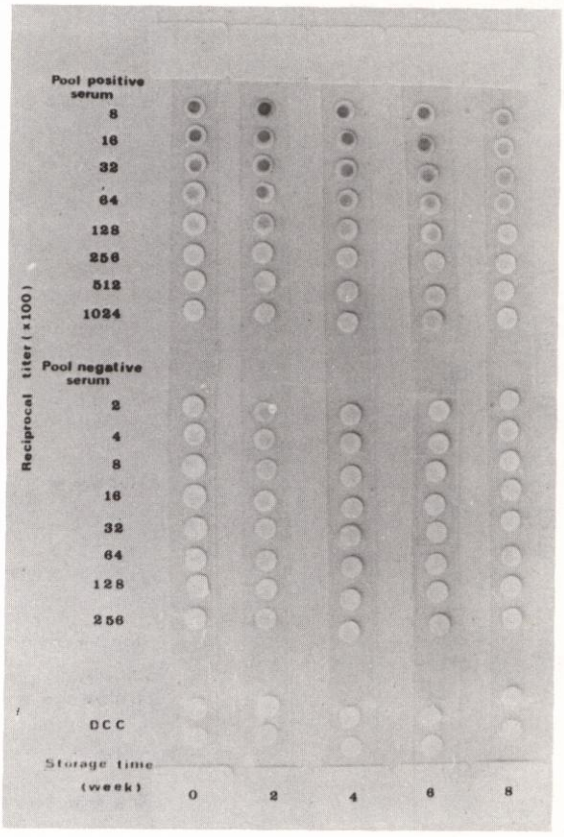

Fig. 2 Dot-blot ELISA for IgM RF determination performed with NRIgG dot-blotted nitrocellulose membrane stored for various period of time at 4°C.

reflecting positive results can be distinguished clearly from that with negative results. Dot-blot ELISA for IgA RF detection also gave the same appearance.

The nitrocellulose membrane with the dot-blot of normal rabbit IgG could be stored for up to 6 weeks, at 4°C in dry plastic box, before use in IgM RF and IgA RF detection. It can be seen from Fig. 2 that the colours developed in the tests, with membranes stored between 0 to 6 weeks, are not different from each other. However, the colour in positive tests faded with the use of the NRIgG dot-blotted membrane which had been stored for 8 weeks (Fig. 2).

Titres of IgM RF and IgA RF in sera of patients with rheumatoid arthritis and normal controls, as determined by dot-blot ELISA, are shown in Fig. 3. It can be seen that the IgM RF titres in rheumatoid arthritis patients vary from $\leqslant$ 1:400 to $\geqslant$ 1:12,000 while those in normal controls vary from $\leqslant$ 1:400 to 1:800. The mean and standard deviation (SD) of reciprocal IgM RF titre in normal controls were 300 and 144.553 respectively. For IgA RF, the titres ranging from $\leqslant$ 1:25 to $\geqslant$ 1:400 were found in the patient group while those from $\leqslant$ 1:25 to 1:50 were found in normal controls. The mean and SD of reciprocal IgA RF titres in the normal controls were 25.147 and 14.997 respectively.

When the cut-off levels for positive results were chosen to be at mean RF titre found in normal controls + 2SD of the titre, the cut-off levels for positive IgM RF and IgA RF were 1:600 and 1:60 respectively. When the cut-off level for positive results was chosen to be the lowest RF titre that was found in less than 5% of normal controls, then the cut-off level for IgM RF and IgA RF were 1:800 and 1:100, respectively. With either of these 2 criteria, positive IgM RF were found in 31/51 rheumatoid arthritis patients and 3/68 normal individuals while positive IgA RF were found in 27/51 of the former and 0/68 of the latter.

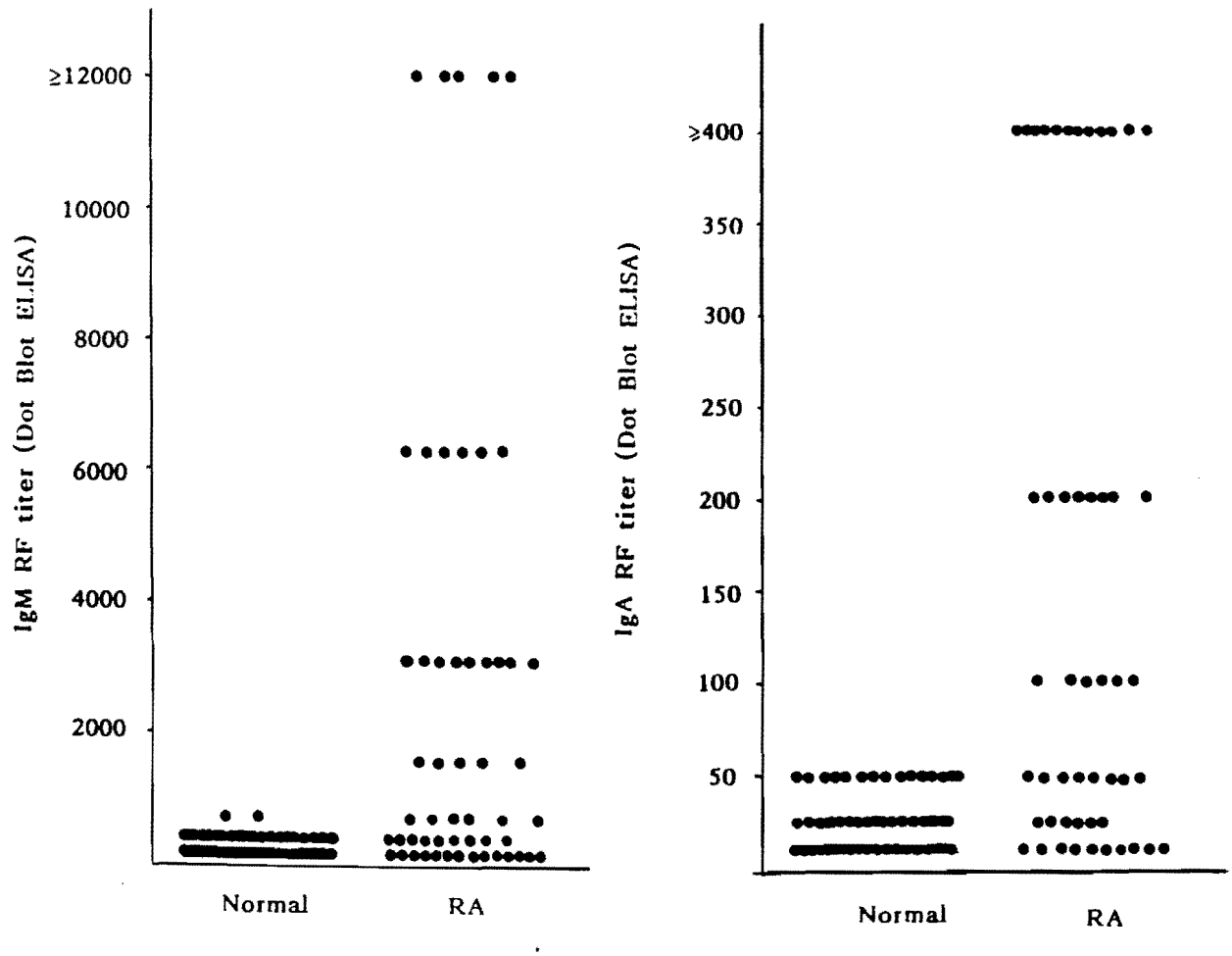

Fig. 3 IgM RF and IgA RF titres in sera of patients with rheumatoid arthritis and normal controls, as determined by dot-blot ELISA.

Microplate ELISA for IgM RF and IgA RF determination

The results of IgM RF and IgA RF determinations by microplate ELISA are shown in Fig. 4. It can be seen that IgM RF titres determined in the group of rheumatoid arthritis patients were in the range of $\leqslant$ 1:200 to $\geqslant$ 1:14,000 while those in the normal control group were between $\leqslant$ 1:200 to 1:2,800. The mean and SD of reciprocal IgM RF titres in the normal controls were 1,121.97 and 714.40, respectively. Titres of IgA RF in the patient group were found to be between $\leqslant$ 1:25 to $\geqslant$ 1:1,400 while those in normal controls were between $\leqslant$ 1:25 to 1:190. The mean and SD of reciprocal IgA RF titres in the normal controls were 72.706 and 52.253 respectively.

When the cut-off level for a positive result was chosen to be at mean RF titre found in normal control + 2 standard deviations of the titre, the cut-off levels for positive IgM RF and IgA RF were 1:2,600 and 1:180, respectively. When the cut-off level for positive IgM RF and IgARF were chosen to be the lowest titre which was found in less than 5% of normal controls, such a level was found to be 1:2,500 and 1:190, respectively. At the cut-off titre of 1:2,600, positive IgM RF were found in 25/51 rheumatoid arthritis patients and 1/68 normal controls. At the cut-off titre of 1:2,500, positive IgM was also found in 25/51 RA patients but in 2/68 normal controls. However, with any of the 2 cut-off levels, positive IgA RF were found in 25/51 of the former and 1/68 of the latter.

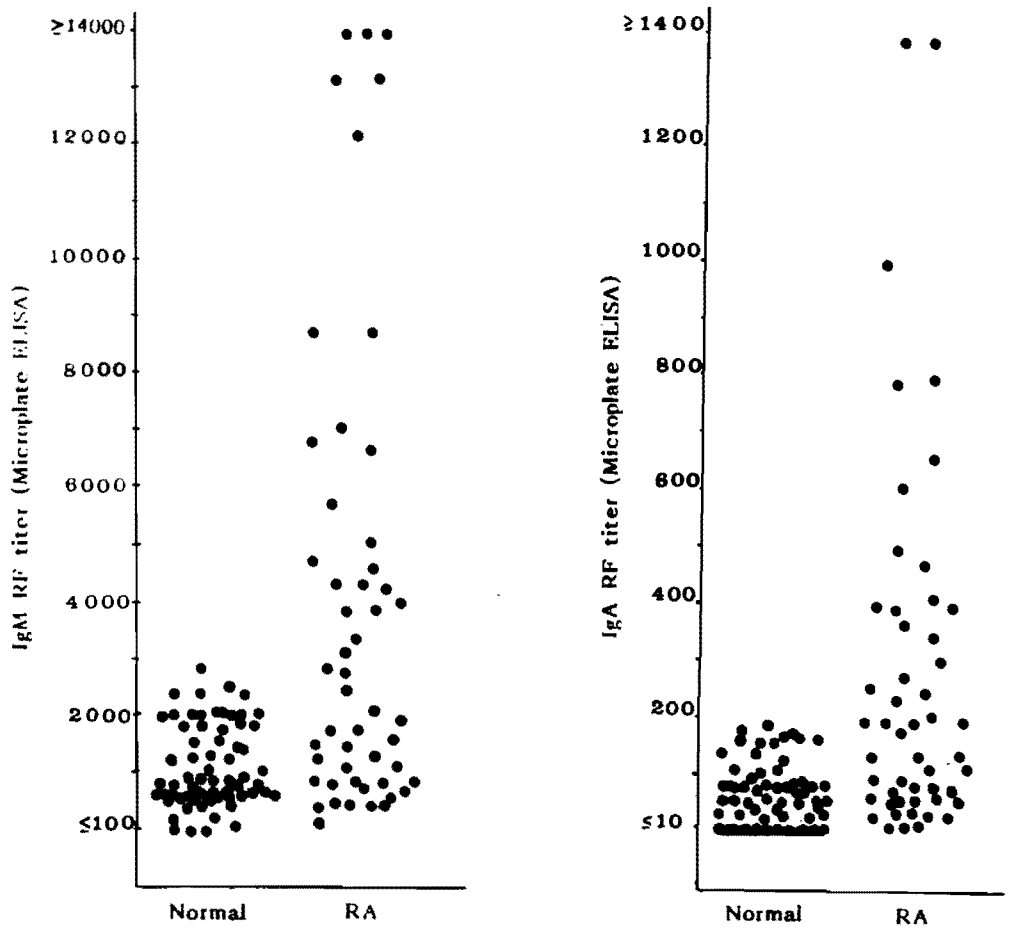

Fig. 4 IgM RF and IgA RF titres in sera of patients with rheumatoid arthritis and normal controls, as determined by micro-plate ELISA.

Table 1. Results of dot-blot ELISA and microplate ELISA for IgM RF detection, using the cut-off level or positive results at IgM RF titre ⩾ 1:800 in dot–blot ELISA and ⩾ 1:2,500 in microplate ELISA.

	Microplate ELISA positive	Microplate ELISA negative
Dot-blot ELISA positive	26	8
DotOblot ELISA negative	1	84

K= 0.803, $p < 0.001$

Comparison between results obtained from dot-blot ELISA and microplate ELISA

When the results of dot-blot ELISA for IgM RF and IgA RF determination were compared with those of microplate ELISA, it was found that msot of the results were in agreement, as shown in Tables 1, 2 and 3. By statistical analysis, it was found that the results obtained from the 2 procedures had significant concordance with a high agreement index (Kappa ⩾ 0.78, $p < 0.001$ for IgM RF and Kappa = 0.83, $p < 0.001$ for IgA RF determinatons).

Table 2. Results of dot-blot ELISA and microplate ELISA for IgM RF detection, using the cut-off level for positive result of IgM RF titre $\geq$ 1:600 in dot-blot ELISA and 1:2,600 in microplate ELISA

	Microplate ELISA positive	Microplate ELISA negative
Dot-blot ELISA positive	25	9
Dot-blot ELISA negative	1	34

K = 078, $p < 0.001$

Table 3. Results of dot-blot ELISA and microplate ELISA for IgA RF detection

	Microplate ELISA postive	Microplate ELISA negative
Dot-blot ELISA positive	23	4
Dot-blot ELISA negative	3	89

K=0.83, $p < 0.001$

DISCUSSION

One of the criteria for the diagnosis of rheumatoid arthritis (RA), as revised by the American Rheumatism Association in 1987, is the presence of RF in serum.[2] It has been reported that different RF isotypes may be associated with different conditions of RA.[9,10] In order to determine RF isotypes, various techniques have been used, including ELISA performed in microtitre palte.[3,5,11-14] In this study, we have developed a dot-blot ELISA for the determination of IgM RF and IgA RF. We have found that this variation of ELISA can be performed more rapidly than ELISA in which microtitre plates were used as the solid phase, in order to obtain the most sensitive results of each system. The results obtained from the dot-blot ELISA can be read visually with marked discrimination between positive and negative results. In addition, the results obtained from dot-blot ELISA also had significant concordance (Kappa $\geq$0.78, $p <$ 0.001) with those from microtitre plate ELISA, which were read spectrophotometrically. Furthermore, we also found that the nitrocellulose membrane, which contained the dot of normal rabbit IgG, could be stored for up to 6 weeks before use for RF determination. This makes dot-blot ELISA even more practical for use in RF isotype determination.

Although we have performed dot-blot ELISA as a semiquantitative test for IgM RF and IgA RF determinations, this procedure can be used as a qualitative test by performing the assay with a single dilution of serum samples by selecting the dilutions of 1:800 and 1:100 as the working dilutions for IgM RF and IgA RF detection respectively. This will allow dot-blot ELISA to be more suitable as a screening test for RF isotype detection. At these working dilutions of serum samples, positive IgM RF and IgA RF were found in 31/51 and 27/51 rheumatoid arthritis patients while they were found in 3/68 and 0/68 normal controls, respectively.

In conclusion, this study has shown that dot-blot ELISA can be used successfully for RF isotype determination and would be a useful tool in any investigation on RF isotypes.

ACKNOWLEDGEMENTS

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