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Dot-Immunogold Filtration Assay as a Screening Test for Syphilis

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A dot-immunogold filtration assay (DIGFA) for the rapid detection of reaginic antibody in the serum of syphilitic patients was developed. The assay was simple, rapid, and reproducible. The test completion time was 2 min, and the assay required no equipment. The positive dot was very obvious, and the results could easily be determined with the naked eye. A total of 350 serum samples were examined by DIGFA, the rapid plasma reagin test, and the fluorescent treponemal antibody-absorption test. The levels of agreement between DIGFA and the rapid reagin test and between DIGFA and the fluorescent treponemal antibody-absorption test were 100 and 98%, respectively. The results of clinical application indicated that DIGFA could be used as a routine screening test for syphilis.

There are two kinds of serologic tests for syphilis: the detection of specific antibodies, termed treponemal tests, and the detection of reagin (antilipid antibodies), termed nontreponemal tests (1). Treponemal tests such as the fluorescent treponemal antibody-absorption (FTA-ABS) test and the hemagglutination treponemal test for syphilis (HATTS) are highly specific. The FTA-ABS test requires an expensive fluorescence microscope, and both the FTA-ABS test and HATTS are tedious and time-consuming. Although nontreponemal tests are less specific, they are more simple to perform and are of greatest value when used as screening procedures and for following therapy. Many studies have shown that there is good agreement between treponemal and nontreponemal tests (2, 6). Because nontreponemal tests are simple, rapid, reproducible, and inexpensive, they can be performed in a variety of clinical laboratories and have been widely used. The nontreponemal test most frequently used today is the rapid plasma reagin (RPR) card test. As an agglutination reaction, more experience is required to determine the endpoint, especially when agglutination is lighter than that in an assay that uses a color reaction.

This report describes the development of a dot-immunogold filtration assay (DIGFA) for the detection of reaginic antibody in the sera of patients with syphilis.

Materials and methods. Three hundred serum samples from healthy individuals, 50 serum samples from patients with syphilis (34 with primary syphilis and 16 with late syphilis), and 60 serum samples from patients likely to be reagin positive because of a variety of human disorders (5) such as systemic lupus erythematosus, rheumatoid arthritis, and high lipid and high bilirubin levels (15 patients with each disorder) were collected and stored at −20°C until use.

Pooled serum from 10 patients with a positive result by the RPR test (reactive at a 1:1 dilution) and a positive result by the FTA-ABS test was used as a positive control, and pooled serum from 10 healthy subjects with negative results by both types of assays was used as a negative control.

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Cardiolipin antigen was derived from beef heart as described previously (3). The amount of purified cardiolipin antigen was determined as a dry weight measurement of the extracted material. Cholesterol was added to the coating antigens as a sensitizer (5). The coating antigen solution used was 50% alcohol (analytical grade) containing 2 mg of purified cardiolipin antigen, 2 mg of cholesterol, and 9 mg of NaCl (0.9%) per ml.

Nitrocellulose membranes (NCMs) with various pore sizes (0.25, 0.30, 0.45, and 0.60 μm) were purchased from Siqing Biochemistry Material Factory (Huangyan, Zhejiang, China). The NCM (pore size, 0.45 μm) was put in an 80% alcohol (analytical grade) solution for 10 min and was then allowed to air dry. One microliter of coating antigens was added to the NCM with a microinjector to form a small dot. Beside the dot, a stripe with pooled serum from healthy individuals diluted 1:5 was deposited and served as a reagent control. This NCM was incubated at 37°C for 5 min and was then immersed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) containing 2% bovine serum albumin (BSA) for 12 h. The NCM was then washed with 0.01 M PBS (pH 7.4). The NCM was dried at room temperature and was then stored in a small sealed plastic bag at 4°C.

A filtration device was manufactured according to our own design. It consisted of a small square plastic box (30 by 30 by 10 mm). A test hole 8 mm in diameter was cut into the center of the box cover. The box was filled with water-absorbing materials, and the NCM was laid on top of this material in such a manner that the antigen deposit and the control stripe would be visible in the opening. A small circle with filter paper enclosed in a funnel-like cap covered the opening and allowed for specimen loading (Fig. 1A).

Protein A was purchased from Shanghai Institute of Biological Products, Shanghai, China). The gold-labelled protein A conjugate was prepared by the method of Horisberger and Clerr (4) and before use was diluted 1:40 with 0.01 M PBS (pH 7.4) containing 2 g of BSA and 0.4 ml of Tween 20 per 100 ml.

The assay was performed at room temperature without incubation. The kit was taken from the refrigerator and was allowed to stand at room temperature for 10 min before use. Three drops (about 100 μl) of serum was placed into the funnel above the test opening and was allowed to adsorb completely. The funnel with its small filter was then removed. Four

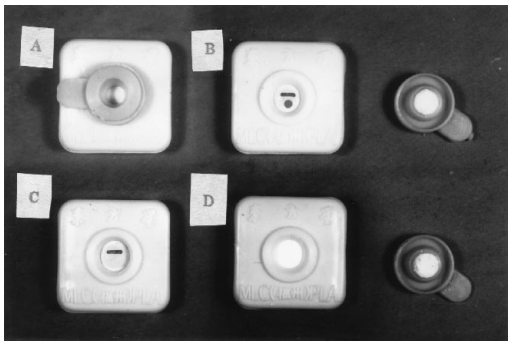

FIG. 1. (A) Unused kit. (B) Positive reaction for reaginic antibody. (C) Negative reaction for reaginic antibody. (D) The reagent was invalid.

drops (about 150 μl) of gold-labelled protein A conjugate was added to the opening and was allowed to absorb completely. Two drops of 0.01 M PBS (pH 7.4) was added and was allowed to absorb completely. The appearance of a red dot in the opening indicated a positive reaction for reaginic antibody (Fig. 1B), while a red line indicated a negative reaction (Fig. 1C). If neither the dot nor the stripe showed color, the test was considered invalid (Fig. 1D). If the titer of the reaginic antibody was desired, serum could be serially diluted (such as 1:2, 1:4, 1:8, and 1:16) with 0.01 M PBS (pH 7.4) and then tested by the method mentioned above. The results were reported in terms of the highest reactive dilution.

The RPR kits were purchased from Xiening Reagent Factory (Nanjing, Jiangsu, China). The FTA-ABS kits were obtained from the Venereal Disease Research Laboratory, Institute of Dermatology, Chinese Academy of Medical Sciences. (Nanjing, Jiangsu, China). The RPR and FTA-ABS tests were performed according to the manufacturers' instructions.

Results. To optimize the experimental conditions, initial experiments were performed to determine the most appropriate type of antigen dilution solution, NCM pore size, and concentrations of coating antigens and gold-labelled protein A conjugate. For these experiments pooled serum with positive results (reactive at a 1:1 dilution) by the RPR test and a positive results by the FTA-ABS test and pooled serum with negative results by both types of assays were used as controls. Because of the lipid antigens, alcohol was arbitrarily chosen as the coating antigen dilution solution. Coating antigens containing high concentrations of cholesterol (10 mg/ml) were initially used to coating the NCMs, but they gave poor results (chunky white dots); therefore, 2 mg of cholesterol per ml was chosen for use in subsequent studies. In order to determine the optimal conditions for the coating antigens, a constant amount of cardiolipin antigen (0.5, 1, 2, 4, 8, and 16 mg/ml) was added to the coating antigen. Different concentrations of gold-labelled protein A conjugate (dilution titers of 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160) were evaluated, and different pore sizes of NCM (0.25, 0.30, 0.45, and 0.60 μm) were tested. The results showed that the reactive signal (color of the red dot) would increase with an increase in the amount of cariolipin antigen in the coating antigen solution and a decrease in the dilution titer of the gold-labelled protein A conjugate for all pore sizes of NCM, but false-positive reactions occurred at concentrations in excess of 2 mg of cardiolipin antigen per ml or with a dilution titer of gold-labelled protein A of less than 1:40. The results also showed that if the pore size of NCM was smaller than 0.45 μm, the filtration rate would be slow. If the pore size was larger than 0.45 μm, the positive dot lacked sufficient color intensity. On the basis of these data, we chose standard conditions of 2 mg of cardiolipin antigen per ml and 2 mg of cholesterol per ml dissolved in 50% alcohol for coating of the NCMs, a 1:40 gold-labelled protein A conjugate, and 0.45-μm-pore-size NCMs for the assay.

We next examined the effects of different blocking times. The NCM was blocked with blocking solution for various times (4, 6, 8, 10, 12, and 24 h). The results showed that the cleanest background and the brightest dots indicating a positive result could be obtained after the NCM was blocked for 12 h.

To ensure that the test was specific, a cardiolipin antigen which was the same as that used as the coating antigen was added to 21 serum samples positive for reaginic antibody. After being completely mixed, the sera were centrifuged at 3,000 × *g* for 10 min. The supernatant of each serum sample was assayed by using the test system. The reactivity of each sample decreased or completely disappeared.

Sixty serum samples (15 from patients with systemic lupus erythematosus, 15 from patients with rheumatoid arthritis, 15 from patients with high serum lipid levels, and 15 from patients with high bilirubin levels) were assessed by DIGFA, and the results were all negative.

Thirty-four serum samples from patients with primary syphilis and 16 serum samples from patients with late syphilis were tested by DIGFA, and positive results were obtained for all samples.

One positive serum sample from an untreated patient was serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256) and was then assessed by both DIGFA and the RPR test. A dilution titer of 1:128 was noted by both assays.

Three serum samples that had positive titers of 1:1, 1:32, and 1:128, respectively, were serially diluted (from 1:1 to 1:256) and were tested by DIGFA daily for 10 days. Three parallel tests for every dilution titer of each sample were performed daily. Every outcome was the same as that of the first assay for each sample.

The kits were stored both at room temperature and at 4°C. Positive and negative control samples were assayed at 5- or 15-day intervals. The results showed that the kit was stable for at least 6 months when it was stored at 4°C and for 1 month when it was stored at room temperature.

A total of 350 serum samples were assayed by DIGFA, the RPR test, and the FTA-ABS test. Fifty samples were positive by DIGFA and the RPR test, and 48 samples were positive by the FTA-ABS test. DIGFA and RPR test results correlated

TABLE 1. Results of the three tests for syphilis

Diagnosis	No. of samples tested	No. of samples positive			No. of samples negative		
		DIGFA	RPR test	FTA-ABS test	DIGFA	RPR test	FTA-ABS test
Healthy individual	300	0	0	0	300	300	300
Primary syphilis	34	34	34	32	0	0	2
Late syphilis	16	16	16	16	0	0	0
Total	350	50	50	48	300	300	302

with a diagnosis of syphilis made on the basis of a clinical examination (Table 1).

Discussion. The preparation of high-quality DIGFA test kits is difficult. Our experience demonstrated that the pore size of NCM affected the filtration rate of serum. With smaller pore sizes, the filtration rate was slower. However, if the pore size was too large, less antigen would be adsorbed on the NCM, possibly leading to decreased sensitivity of the assay. We found that the optimal pore size of NCM was 0.45 μm.

Our investigation showed that NCM could adsorb lipid antigens. The fact that the reagin-cardiolipin complex would combine with protein A suggested that the reagin antibody was immunoglobulin G (IgG). Although the use of gold-labelled protein A conjugate to detect reaginic antibody limits isotype detection to that of IgG, while the RPR test detects IgM and IgG clinically except in patients with very early syphilis, reaginic IgG antibody always exists in the patient's serum. Therefore, use of DIGFA for the detection of reaginic antibody is clinically acceptable as suggested by the results of our experiments.

The present experimental results show that the results of DIGFA were in good agreement with those of the RPR test. DIGFA was simple, rapid, and reproducible. The test could be completed in 2 min without the need for equipment. The positive dot was very obvious and the result could easily be determined with the naked eye. The results of the clinical application showed that DIGFA could be used as a routine screening method for the presumptive diagnosis of syphilis.

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