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Indirect Immunoperoxidase Staining of *Crithidia luciliae* for Detecting Anti-dsDNA: Comparison with Other Serodiagnostic Tests

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SUMMARY **The indirect immunoperoxidase (IIP) method was modified and improved for detecting anti-dsDNA. Using fetal calf serum to block the non-specific antibodies and staining with chromatic substrate solution at pH 6.2, the performance time is substantially decreased and the patterns of positive and negative staining are easily distinguished. IIP was compared with indirect hemagglutination (IHA), latex agglutination (LA) and the standard method indirect immunofluorescent technique (IFT). The sensitivities and specificities of the three methods were studied in 507 sera from various sources. Using IFT as the gold standard, sensitivities of IIP, IHA, LA were 98.18%, 88.18% and 50.91%, respectively, and the corresponding specificities were 99.5%, 98.24% and 100%. There was a good correlation between titers of IFT and IIP (Spearman correlation coefficient = 0.77, $p < 0.001$) with no significant difference between the titer of both tests (Wilcoxon Matched-Pairs Signed-Rank test, $p = 0.143$). These results indicate that the modified IIP method is both highly sensitive and specific and only needs a light microscope to perform it.**

Anti-deoxyribonucleic acid or anti-dsDNA is an important autoantibody which can be present in systemic rheumatic diseases.[1] It has been reported that anti-dsDNA is highly specific to systemic lupus erythematosus (SLE) and the level of circulating anti-dsDNA also correlated with severity of the disease. Sixty percent of the patients who had high reciprocal titer of anti-dsDNA have active SLE and eighty percent of those who had high level of anti-dsDNA eventually develop lupus nephritis.[2,3] The detection of circulating anti-dsDNA antibody in the sera of those patients is one of the criteria used in diagnosis of active SLE. The importance of examination of anti-dsDNA has led to the development of many serological tests such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence (IFT).[3] In IFT, the hemoflagellate protozoan, *Crithidia luciliae* is used as antigen substrate since its kinetoplast contains double stranded DNA. IFT has been used as the standard method for detection of anti-dsDNA due to its high sensitivity, specificity and the simplicity of the test.[4] However, this method still has some limitation because of the need for a fluorescent microscope. To obviate this problem, an immunoenzyme technique such as horseradish peroxidase was used for the detection of anti-dsDNA. The objective of this study was to modify IIP to be a simpler, rapid and accurate test for detecting anti-dsDNA and to compare this with others serological tests, indirect hemagglutination (IHA), latex agglutination (LA) and indirect immunofluorescent technique (IFT). The IFT was taken as the "gold standard ".

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MATERIALS AND METHODS

Samples

Serum samples were obtained from patients of Siriraj Hospital from January 1992- December 1994. They were divided into 3 groups as follows:

1. One hundred and ten sera from patients who had attended the Department of Dermatology and had been diagnosed as SLE (fulfilled the criteria of American Rheumatism Association for SLE) with positive anti-dsDNA by IFT.

2. One hundred and forty-five sera from patients with other diseases including 10 sera from patients with Rheumatoid Arthritis, 10 sera from patients with syphilis, 20 sera from patients with hepatitis B virus infection and 105 sera from patients who were suspected of having autoimmune diseases with ANA positive but anti-dsDNA negative by IFT.

The diagnosis of these diseases was made on the basis of clinical, laboratory and pathological evaluation.

3. Two hundred and fifty-two sera from healthy blood donors collected by random sampling method from the blood bank of Siriraj Hospital, Mahidol University.

All sera were kept at -70°C and tested using IFT, IIP, LA and IHA.

Preparation of antigen substrates for IFT and IIP

Crithidia luciliae were obtained from the Research Center, Faculty of Medicine Ramathibodi Hospital. The organisms were grown in bactotryptose medium, pH 7.4 as described by Aarden *et al.*[5] The suspension was washed 3 times with phosphate bufferred saline (PBS), pH 7.2. The concentration of the solution was adjusted to 40 organisms/ microscopic field at 400 x magnification. Then 5 µl of the solution was applied in each well of a teflon coated slide. The slides were air dried and fixed with 95% ethanol for 10 minutes. The prepared slides were kept at -20°C until use.

Indirect immunofluorescent technique (IFT)

Sera were diluted in four-fold dilution steps (from 1:16 to 1: 1,024) in phosphate bufferred saline, incubated on antigen slides at 37°C for 30 minutes, along with appropriate controls. Slides were then washed thrice in PBS and then overlaid with fluorescein conjugated anti-human globulins IgG, M, A (DAKO, Copenhagen, Denmark). After 30 minute incubation, slides were washed thrice in PBS and mounted with glycerol buffer. Slides were examined under high dry objective (40x) on a fluorescent microscope equipped with B9 (BP-490) exciter filter, B460 barrier filter and with an HBO 200 mercury burner, Olympus.

Modified indirect immunoperoxidase technique (IIP)

Antigen substrate slides were allowed to remain at room temperature. 1:50 fetal calf serum in 5% BSA was dropped in each well of the antigen slides and incubated for 10 minutes in a moist chamber at room temperature. Sera diluted four-fold dilution (from 1:10 to 1:640) were added to each well and incubated at room temperature for 30 minutes. The slides were washed twice (5 minutes/each) with PBS pH 7.2 and rinsed with distilled water. The peroxidase labeled goat anti-human IgG, M, A (DAKO, Copenhagen, Denmark) titer 1:160 (obtained from chequerboard titration) were added to each well and incubated at room temperature for 30 minutes. Diaminobenzidine tetrahydrochloride chromogen substrate (30 mg DAB + 100 ml DW + 10 mg sodium acetate trihydrate; pH 6.2+10 ml of 30% H_2O_2) was added into each well after washing. These were incubated for 5 minutes in the dark at room temperature. The slides were then washed, mounted with glycerol buffer, and examined under a light microscope.

Anti-dsDNA Quick Test (Human, Germany)

The test was performed according to the manufacturer's instruction. Briefly, 1 drop of undiluted serum was placed on a cell of the slide (positive and negative controls were separated) and one drop of the latex reagent was added to the patient specimen and the controls, then each well was mixed with an applicator stick. The slide was tilted back and forth slowly for two minutes and macroscopic agglutination was observed.

Indirect hemagglutination (Immunodiagnostic Center, Ramathibodi Hospital)

Samples were diluted with diluent provided in the test kit. Serial two-fold dilutions were made from 1:2 to 1:32 and then aliquoted 25 µl of each dilution into the microtiter plate. Twenty-five microlitres of sensitized cell (human O cell coated with double stranded DNA) was added into each well. In the control well, unsensitized cells (uncoated human O cell) were used. Plates were shaken to mix the contents of the wells, and the result of hemagglutination was read after in-

cubation for 1 hour in a moist chamber at room temperature. The cut-off titer at 1:2 was considered positive.

Statistical analysis

Correlations between IIP and IFT were compared by Spearman correlation coefficient test and Wilcoxon Matched-Pairs Signed-Rank test. The sensitivity, specificity and predictive values of various methods were performed using 2 x 2 table.

RESULTS

A total of 507 serum samples were assayed for anti-dsDNA with IIP, LA, IHA and standard method, IFT. Table 1 presents the sensitivity, specificity, positive predictive and negative predictive values in each test as compared with the standard method. IIP gave the highest sensitivity (98.18%). LA has the lowest sensitivity (50.91%) while all tests have high specificity (IIP, LA and IHA = 99.5%, 100% and 98.24%, respectively). Table 2 shows the positive results in the studied population groups with various examination methods. One serum sample from a hepatitis patient serum and 6 normal sera gave positive anti-dsDNA by IHA. Moreover, one serum from other autoimmune diseases and one normal serum were positive with IIP determination. LA showed no positive result in other population groups ex-

Table 1. Sensitivity, specificity and predictive values from various anti-dsDNA detection methods.

		LA		IHA		IIP	
		positive	negative	positive	negative	positive	negative
IFT	positive	56	54	97	13	106	4
	negative	0	397	7	390	2	395
Sensitivity		50.91%		88.18%		98.18%	
Specificity		100.0%		98.24%		99.50%	
Predictive of pos. value		100.0%		93.27%		98.18%	
Predictive of neg. value		88.03%		96.77%		99.50%	

Table 2. Anti-dsDNA positive rate determined by various examination methods in different disease categories.

Diseases	No.of sample tested	Anti-DNA positive by			
		LA	IHA	IFT	IIP
SLE	110	56	97	110	106
RA	10	-	-	-	-
Hepatitis	10	-	1	-	-
Syphilis	20	-	-	-	-
Other AI	105	-	-	-	1
Normal	252	-	6	-	1

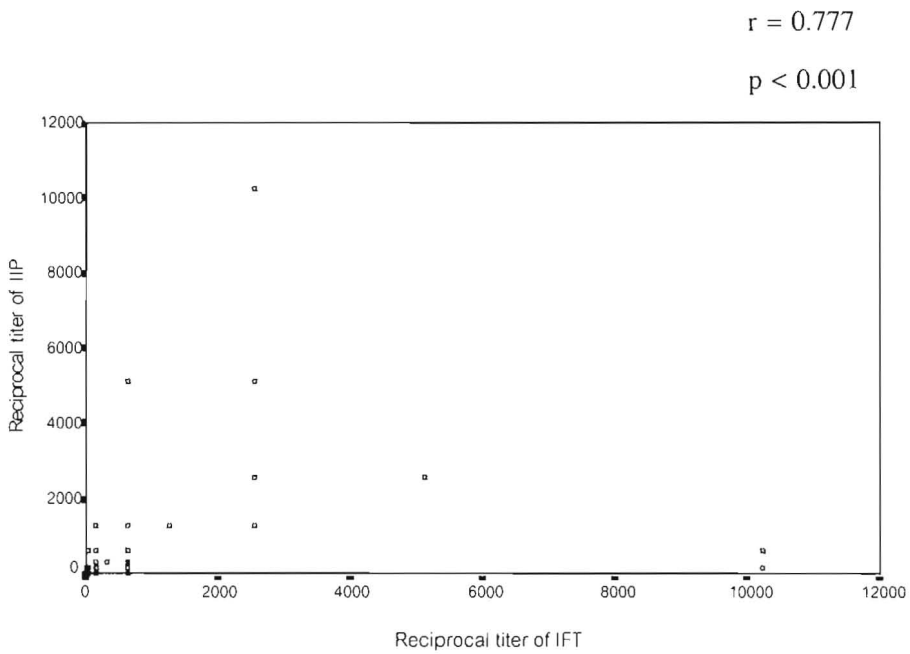

Fig. 1 Comparison between the reciprocal titers of 110 anti-dsDNA positive serum samples determined with IFT and IIP in SLE patients ($r = 0.777$; $p < 0.001$).

cept in SLE patients. Since IFT was used as the standard method, all positive results except in SLE was considered false positives. The correlation between the reciprocal titer of positive anti-dsDNA determined by IIP and IFT was compared by using the Spearman correlation coefficient. From Fig.1 it can be seen that there is a good correlation between the titers of both tests with a Spearman correlation coefficient of 0.77 ($p = 0.001$). In SLE patients with anti-dsDNA positive, 28 sera showed a higher titer with IFT than with IIP, whereas 49 sera revealed similar titers with both tests. However, there was no significant difference between the titer of the tests when Wilcoxon Matched-Pairs Signed-Rank test; ($p=0.143$) was used. Fig. 2 revealed the dark staining pattern of kinetoplast of *Crithidia luciliae* in anti-dsDNA positive serum which is clearly different from the staining pattern of negative serum.

DISCUSSION

Vladutiu *et al.*[6] have described the use of immunoperoxidase for detection of anti-dsDNA in SLE patients and reported that IIP assay was more sensitive than IFT. However, it takes more than two hours to perform the test. Lee *et al.*[7] also used immunoperoxidase assay for anti-dsDNA detection and used *Crithidia luciliae* as substrate. They reported the advantages of IIP assay over IFT. Both reports used 3,-3' diaminobenzidine tetrahydrochloride as a chromogen substrate and no blocking step to prevent non specific staining of the tests. Santivijai *et al.*[8] criticized the use of DAB as a chromogen substrate since the kinetoplast staining of positive and negative serum could not be clearly differentiated.

The present study describes the detection of anti-dsDNA by an immunoenzyme method using *C. luciliae* as substrate. IIP is shown to be another choice of assay for anti-dsDNA detection. One advantage of the IIP test is that it produces a permanently stained slide, making it easy to review the results. Moreover, no special equipment is required for the test. In contrast to IIP, IFT preparation is not permanent due to the fading of fluorescence. In IIP, peroxidase catalyses the oxidation of substrates by H_2O_2, resulting a highly insoluble colored

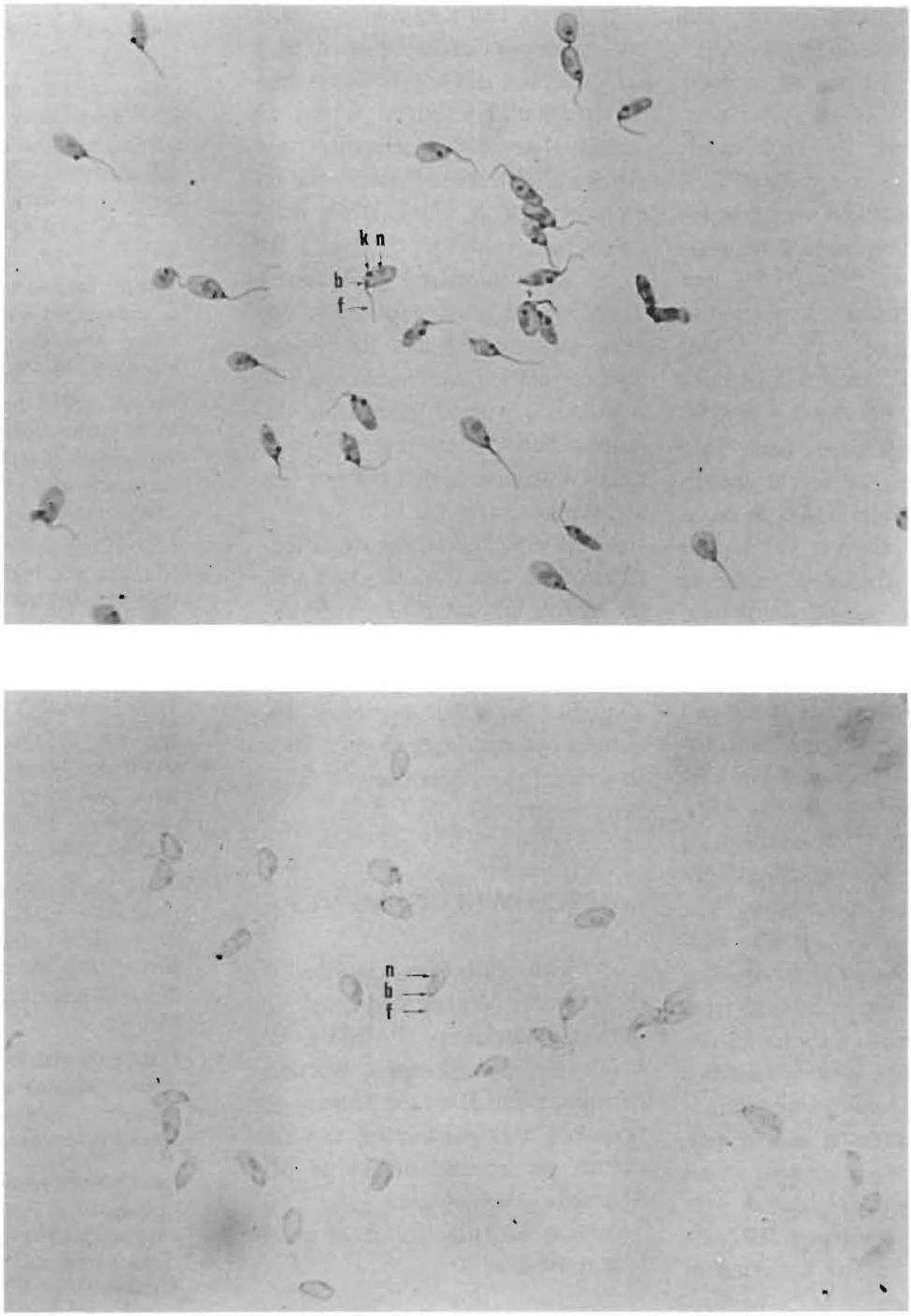

Fig. 2 Patterns of kinetoplast staining with IIP technique show anti-dsDNA positive (A) and anti-dsDNA negative (B); b = basal body, f = flagellum, k = kinetoplast, n = nucleus (magnification x 400).

product 'phenazine' at the Ag-Ab binding site which is visible on light microscopy.[9] Another advantage of IIP is that kinetoplast, nucleus and flagellum are easily distinguished.

Although IIP would seem to be a better choice of assay for anti-dsDNA detection, IIP took much longer to perform as compared with IFT. Another problem was that kinetoplasts, in the presence of positive and negative sera were not clearly differentiated, so we tried to modify and improve the test. Although many chromogen substrates can be used for horseradish peroxidase, some researchers have used Hanker yates reagent as chromogen substrate, we prefer DAB to Hanker yates since Hanker yates gave varying results when it was used as chromogen in immunoperoxidase technique.[9] Even when non-specific staining was prevented by blocking with non-immune serum (fetal calf serum), the result was still unsatisfactory. The pH of chromogen substrate solution is also another critical factor in IIP.[10,11] We then tried varying the pH of the chromogen substrate solution in our study. The results show the best staining pattern at pH 6.2. As a result, washing time could also be decreased from 30 minutes in each step to 10 minutes. Therefore, in this modified IIP method, the performance time is substantially decreased and the pattern of positive and negative serum staining is easily distinguished.

We also compared IIP with other methods for the detection of anti-dsDNA; namely latex agglutination and indirect hemagglutination tests. Sensitivities and specificities of these 3 methods were evaluated by comparing with the standard method, IFT. IIP is both highly sensitive and specific. Although LA was easy to perform it gave a very low sensitivity, so it is not recommended to be used as a screening test. IHA gave better sensitivity than LA but it is not as sensitive as IIP. Correlation and statistical difference between the reciprocal titer of anti-dsDNA positive serum tested by IFT and IIP were also compared and tested. There was a good correlation between titers of IFT and IIP (Spearman correlation coefficient = 0.777, $p < 0.001$) with no significant difference between the titers of both tests (Wilcoxon Matched-Pairs Signed-Ranks test, $p = 0.143$).

In conclusion, the modified IIP method can also be used for the routine laboratory detection of anti-dsDNA in hospitals where fluorescent microscopy is not available, but the test must be carried out with care at each step to prevent the occurrence of false positive results.

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REFERENCES

1. Ballou SP. Clinical aspects of anti-native DNA. In vitro 1988;1:19-24.
2. Chubick A, Southeimer RD, Gilliam JN. An appraisal of tests for native DNA antibodies in connective tissue diseases, Clinical usefulness of *Crithidia luciliae* assay. Ann Intern Med 1978; 89:168-92.
3. Ballou SP, Kushner I. Anti-native DNA detection by the *Crithidia luciliae* method. An improved guide to the diagnosis and clinical management of systemic lupus erythematosus. Arthritis Rheum 1979; 22:321-7.
4. Turgeon ML. Autoimmune Disorders: In Immunology and Serology in Laboratory Medicine, 2nd ed. New York Press 1996;341-66.
5. Aarden LA, Groot ER, Feltkamp TEW. Immunology of DNA III: *Crithidia luciliae*, a simple substrate for the detection of anti-dsDNA with the immunofluorescence technique. 1975; 254: 505-15.
6. Vladutiu AO, Pallumbo DA Asirwatham JE. Antibodies to native DNA: Detection by immunoperoxidase assay and determination of their immunoglobulin classes. Clin Chem 1979; 25:366-70.
7. Lee TK, Rothberger H. Immunoperoxidase assay for anti-dsDNA antibodies using *Crithidia luciliae* substrates. Scand J Rheumatol 1985;14:386-92.
8. Santivijai S, Rakthum S, Jaruchaimontri C *et al.* Detection of anti-deoxyribonucleic acid by indirect immunoperoxidase staining. Bull Fac Med Technol Mahidol Univ 1991; 15(2): 86-95.
9. Bourne JA. Handbook of immunoperoxidase staining methods. Immunochemistry laboratory, DAKO Cooperation, California, 1983
10. Sato. Rapid serologic diagnosis of tsutsugamushi disease employing the immunoperoxidase reaction with cultured Rickettsia. Clin Virol 1982; 8:428-9
11. Ripoche MA, Bernard OJ Bennet D. Nuclear labelling in immunoperoxidase studies of mouse tissue as detected by staining at acid pH. J Histochem 1979;27:841-5.