Yonsei Medical Journal
Vol. 32, No. 4, 1991

Detection of *Treponema pallidum* in Tissue: A Comparative Study of the Avidin-Biotin-Peroxidase Complex, Indirect Immunoperoxidase, FTA-ABS Complement Techniques and the Darkfield Method

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With 37 formalin-fixed, paraffin embedded specimens from the lesions of 30 patients with primary, secondary or gastric syphilis, we performed avidin-biotin-peroxidase complex (ABC), indirect immunoperoxidase (IIP) and FTA-ABS complement techniques. Darkfield examination was done in 17 skin lesions. The immunoperoxidase technique, especially the ABC technique, revealed higher reactivity than the FTA-ABS complement technique and darkfield examination in detecting Treponema pallidum in tissues. Furthermore, the ABC technique produced less intense nonspecific background staining than the IIP technique. Histologically, most of the treponemes were located in the upper dermis, epidermis and vessel walls in the order named, and rarely in the lower dermis of the syphilitic skin lesions.

Key Words: *Treponema pallidum*, immunoperoxidase and immunofluorescent techniques, darkfield, tissue

Confirming suspected syphilitic lesions depends entirely on detection of *Treponema pallidum*, because routine histopathology may only show nonspecific findings in primary, secondary and congenital syphilitic lesions, and serologic tests may be nonreactive in primary syphilis. For detection of *T. pallidum* in tissues, the darkfield examination or silver impregnation method have been used. However, because of the low sensitivity and specificity of these two tests, various tests using immunofluorescent technique have been employed (Yobs *et al.* 1964; Jue *et al.* 1967; Al-Samarrai *et al.* 1977). This technique has the following disadvantages: (1) low sensitivity when used on formalin-fixed, paraffin-embedded tissues, (2) gradual decrease of fluorescence with passing of time, and (3) necessity of a fluorescent microscope. However, the FTA-ABS complement technique, an improved immunofluorescent technique, is known to show relatively good results even when used in paraffin embedded tissues (Tateshita *et al.* 1980; Nakamura *et al.* 1983; Chung *et al.* 1987).

Immunoperoxidase techniques have been rapidly developed in various fields and are well known to show higher sensitivity and specificity than any other methods. Sternberger *et al.* (1970) developed the horseradish peroxidase-anti-horseradish peroxidase (PAP) technique and identified the treponemes in tissues. Beckett and Bigbee (1979) compared the results between the PAP and indirect immunoperoxidase (IIP) techniques in syphilitic lesions. The avidin-biotin-peroxidase complex (ABC) technique developed by Hsu *et al.* (1980) showed a far greater degree of sensitivity and specificity than any other immunoperoxidase techniques. With this technique, Jennings *et al.* (1983) successively identified the spirochetes in air-dried smears of dental plaque.

In this study, results of the ABC, the IIP, the FTA-ABS complement and the conventional darkfield methods in the detection of *T. pallidum* in tissues were compared. Histological distribution of treponemes was analyzed as well.

Received October 9, 1991
Accepted January 7, 1992

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MATERIALS AND METHODS

Tissue Specimens

Thirty seven formalin-fixed, paraffin embedded tissue specimens were obtained from 30 patients with syphilis confirmed by clinical history, physical examination, and serologic tests including VDRL, FTA-ABS, TPHA and 19s (IgM)-FTA tests in our department from 1978 to 1987. Of the specimens, five were from the lesions of primary syphilis, thirty one from the lesions of secondary syphilis, and one from gastric mucosa of suspected gastric syphilis. With these 37 tissue specimens, ABC, IIP and FTA-ABS complement techniques were employed. Darkfield examination was done in 3 primary and 14 secondary syphylitic lesions.

Immunoreagents

Biotinylated swine anti-rabbit immunoglobulin, peroxidase conjugated avidin-biotin complex, peroxidase conjugated rabbit anti-human immunoglobulin, and normal swine serum were obtained from Dakopatts (Accurate, NY). For the rabbit anti-*T. pallidum* immunoglobulin, serum obtained from the *T. pallidum* innoculated rabbits, which showed TPHA titers above 1 : 5, 120 was used. Normal rabbit serum was obtained from rabbits showing negative results in the TPHA. FITC-labeled anti-human C3c was obtained from the Behring Institute (Marburg, West Germany).

Immunoperoxidase staining

Sections with thickness of 4 μm were cut from the paraffin blocks, routinely processed through xylene and graded alcohols of 95% and 100%. Sections were treated with 3% H_2O_2 to eliminate the endogenous peroxidase activity and with 3% NH_4OH to break down aldehyde linkage formed by paraffin. Nonimmune sera were applied to the sections to reduce nonspecific background staining: normal swine serum in the ABC technique and normal rabbit serum in the IIP technique.

The immunostaining procedures of the ABC and IIP techniques are listed in Table 1. Each stage lasted 20 minutes and was followed by washing 10-minutes in phosphate buffered saline (PBS, pH 7.4). The application of diaminobenzidine-H_2O_2 (DAB-H_2O_2) for 10 minutes allowed visualization of the treponemes. It was made by adding 6 mg of 3,3-diaminobenzidine tetrahydrochloride in 10 ml of 0.05 M Tris buffer (pH 7.6) which contains 0.1 ml of 3% H_2O_2. The peroxidase conjugated avidin-biotin complex was made by mixing 10 μl of avidin with 10 μl of biotinylated horseradish peroxidase in 1 ml of 0.05 M Tris buffer (pH 7.6) 30 minutes before use. Method-specificity controls were performed by omitting the primary antibody, or replacing it with normal serum. All incubation steps were performed in room temperature.

FTA-ABS complement technique

Sections with thickness of 4 μm were cut from the paraffin blocks, routinely processed through xylene and graded alcohols of 95% and 100%, treated with 3% NH_4OH to break down aldehyde linkage formed by paraffin. The sections were subsequently treated with normal rabbit serum to reduce nonspecific background staining. The FTA-ABS complement technique of *T. pallidum* was performed in accordance with the previous method (Tateshita *et al.* 1980; Maestrone 1983; Nakamura

Table 1. Staining procedures of the ABC and IIP techniques

	ABC	IIP
Step 1	Rabbit anti-*T. Pallidum* Ig (TPHA 1 : 5,120) diluted 1 : 400	Syphilitic patient's serum (TPHA 1 : 5,120) diluted 1 : 50
Step 2	Biotinylated swine anti-rabbit immunoglobulin (1 : 200)	-
Step 3	Peroxidase conjugated avidin-biotin complex	Peroxidase conjugated rabbit antihuman immunoglobulin (1 : 50)
Step 4	Diaminobenzidine-H_2O_2 reaction	Diaminobenzidine-H_2O_2 reaction

ABC: avidin-biotin-peroxidase complex technique
IIP : indirect immunoperoxidase technique
Ig : immunoglobulin

et al. 1983) and includes the following steps: (1) 30-minute incubation with 1 : 5 dilution of syphilitic patient's serum (TPHA titer 1 : 5, 120), (2) 30-minute incubation with 1 : 5 dilution of normal human serum (complement addition), (3) 30-minute incubation with 1 : 5 dilution of FITC-labeled antihuman C3c, (4) mounting, and observation with the fluorescent microscope. All incubation steps were performed at 37°C and were followed by washing 10-minutes in phosphate buffered saline (PBS, pH 7.4).

RESULTS

The results of each detection method for *T. pallidum* in tissue are shown in Table 2. Immunoperoxidase techniques, especially the ABC technique, revealed a higher reactivity when compared with the FTA-ABS complement technique and darkfield examination. There was a tendency for higher reactivity in primary syphilitic lesions and condyloma lata than in the other types of syphilitic lesions. There were statistical differences between the results of the ABC and FTA-ABS complement techniques and also between the ABC technique and darkfied examination in secondary syphilitic lesions ($p<0.05$, χ^2-test) when the number of positive and negative lesions were compared. Tables 3 and 4 show the agreement ratios between each method. The agreement ratio between the ABC and IIP techniques was 89%, but the ratio between other methods were lower. Histologically, most of the treponemes were located in the upper dermis (Fig. 1), epidermis, and vessel walls in the order named, and rarely in the lower dermis of the syphilitic skin lesions. There was no remarkable difference in distribution of treponemes between the clinical stages

Table 2. Comparison of methods for the detection of *T. pallidum* in tissue

Clinical stage	No. positive/No. negative(%)			
	ABC	IIP	FAC	Darkfield
Primary	5/5(100)	5/5(100)	4/5(80)	3/3(100)
Secondary	28/31(94)[a]	27/31(87)	21/31(68)[b]	9/14(64)[c]
macule	14/15(94)	12/15(80)	10/15(67)	2/5(40)
papule	7/8(88)	7/8(88)	5/8(63)	2/3(67)
c. lata	8/8(100)	8/8(100)	6/8(75)	5/6(83)
Gastric	1/1(100)	1/1(100)	1/1(100)	Not done
Total	35/37(95)	33/37(89)	26/37(70)	12/17(71)

ABC: avidin-biotin-peroxidase complex techique
IIP: indirect immunoperoxidase technique
FAC: FTA-ABS complement technique
c. lata: condyloma lata
a, b, c: statistical differences between a and b, a and c ($p<0.05$, χ^2-test)

Table 3. Agreement ratios between the ABC technique and other detection methods for *T. pallidum* in tissue

ABC	IIP		FAC		Darkfield	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	32	3	26	9	12	4
Negative	1	1	0	2	0	1
% Agreement	89		76		76	

ABC: avidin-biotin-peroxidase complex technique
IIP: indirect immunoperoxidase technique
FAC: FTA-ABS complement technique

Fig. 1. *A treponeme seen in upper dermis of a chancre: FTA-ABS complement technique (× 1,000).*

Table 4. Agreement ratios between the IIP technique and other detection methods for *T. pallidum* in tissue

IIP	FAC		Darkfield	
	Positive	Negative	Positive	Negative
Positive	26	7	12	3
Negative	0	4	0	2
% Agreement	81		82	

IIP: indirect immunoperoxidase technique
FAC: FTA-ABS complement technique

Table 5. Distribution of *T. pallidum* in the 35 skin lesions positive in the ABC and/or IIP techniques

Clinical stage	No. distributed/No. positive(%)			
	Epidermis	Upper dermis	Lower dermis	Blood vessel
Primary	3/5(60)	5/5(100)	2/5(40)	4/5(80)
Secondary	24/30(80)	27/30(90)	15/30(50)	19/30(63)
macule	10/14(72)	11/14(79)	6/14(43)	8/14(57)
papule	6/8(75)	8/8(100)	4/8(50)	6/8(75)
c. lata	8/8(100)	8/8(100)	5/8(63)	5/8(63)
Total	27/35(77)	32/35(91)	17/35(49)	23/35(66)

ABC: avidin-biotin-peroxidase complex technique
IIP: indirect immunoperoxidase technique
c. lata: condyloma lata

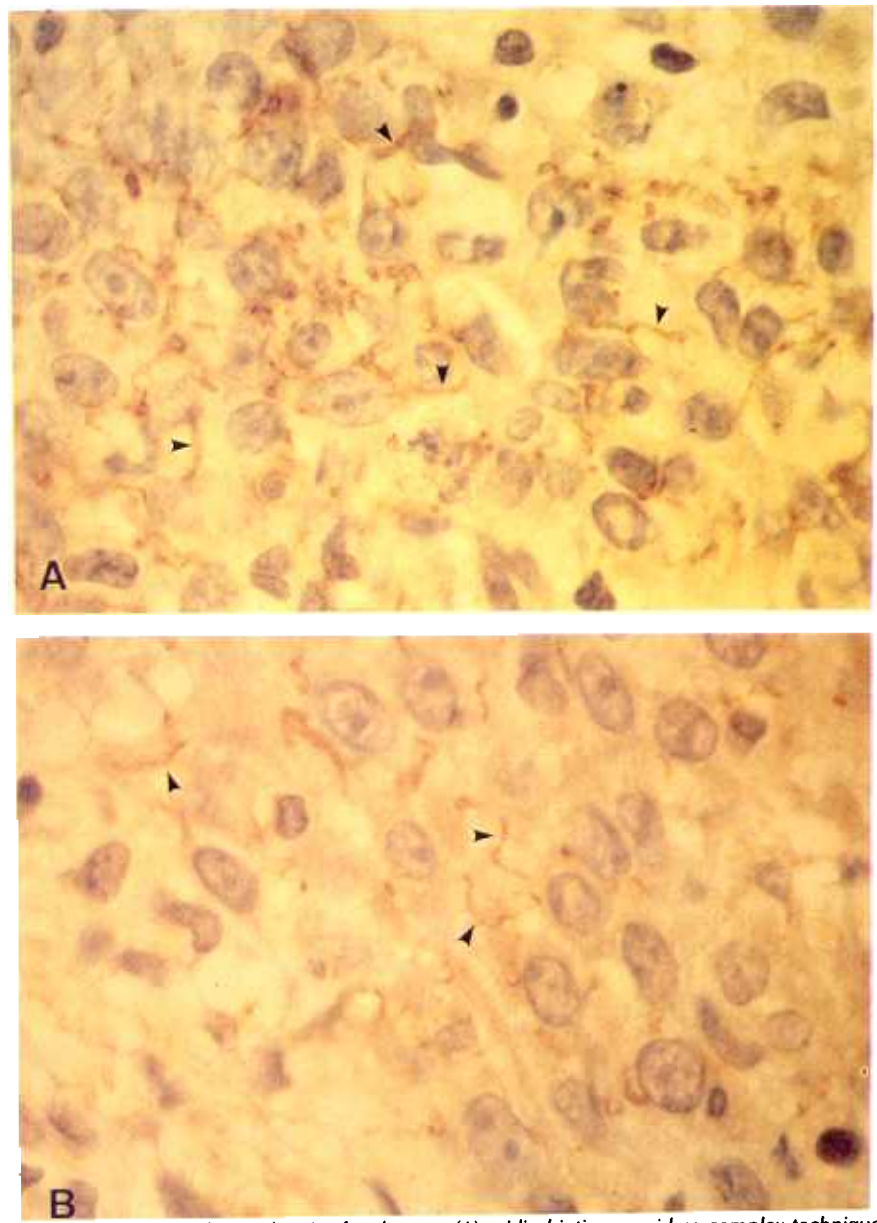

Fig. 2. *Numerous treponemes in the epidermis of a chancre: (A) avidin-biotin-peroxidase complex technique (× 1,000), (B) indirect immunoperoxidase technique (× 1,000).*

and types of syphilitic skin lesions (Table 5). Non-specific background staining was less intense in the ABC than in the IIP technique (Fig. 2).

DISCUSSION

Immunohistochemical technique is a valuable tool for both routine histopathology and research. Permanence of the reaction product and usefulness in fixed fissue sections, together with the facility for simultaneous pathological diagnosis, make the immunoperoxidase method the technique of choice in histopathology at the present time (Heyderman 1979).

Several immunoperoxidase staining techniques have been described. The PAP technique is the most commonly used method, not only because of its high sensitivity, but also because reliable reagents are available commercially (Sternberger *et al.* 1970). The IIP technique also shows fairly good sensitivity, but its use has been somewhat limited by background staining. However, Beckett and Bigbee (1979) compared the IIP and the PAP techniques for sensitivity and degree of nonspecific staining in syphilitic lesion, and reported the IIP technique to be better, based on the intensity of staining and the simplicity of the procedure.

The ABC technique is easy to perform, provides a higher sensitivity, and so is gaining general acceptance in diagnostic pathology. The high sensitivity of the ABC technique can be related to the formation of an avidin-biotin-peroxidase complex and multiple biotin molecules in association with the secondary antibody. Even though conjugated antibodies are used in this method, the strong affinity of avidin for biotin gives this method greater sensitivity than any other conjugated antibody techniques. The unlabeled antibody (PAP) and IIP techniques also yield satisfactory results, but it is well known that the staining intensity is less intense in these techniques than in the ABC technique. Another important advantage of the ABC technique is the relatively low background staining. This is attributable to the high dilutions of the secondary antibody as well as the avidin-biotin complex (Hsu *et al.* 1981).

Our study indicates that the immunoperoxidase technique is a more sensitive method than the FTA-ABS complement technique or darkfield examination for diagnosing suspected syphilitic lesions. Among the immunoperoxidase techniques, the ABC technique is considered a more useful and superior method than the IIP technique. The agreement ratio between the ABC and IIP technique was 89%. Also, nonspecific background staining was less intense in the ABC than in the IIP technique.

Nakamura *et al.* (1983) observed histologic distribution of *T. pallidum* in the syphilitic lesions using the immunofluorescent technique. According to their report, most treponemes were located around vessels in macular syphilitic lesions, and in the epidermis in papular syphilitic lesions. On the other hand, the report by Chung *et al.* (1987) using immunofluorescent technique, states that treponemes were distributed in the epidermis, dermoepidermal junction, papillary dermis and vessel walls in chancres, macular syphilids and condyloma lata lesions. In our study, most of the treponemes were located in the upper dermis, the epidermis and the vessel walls in the order named and rarely in the lower dermis of the syphilitic skin lesions. There was no remarkable difference in histologic distribution of treponemes between the clinical stages and types of syphilitic skin lesions.

There have been several reports on the methods for detection of treponemes in the gastric mucosal lesions using immunofluorescent techniques (Sachar *et al.* 1974; Butz *et al.* 1975; Chung *et al.* 1989). We have also demonstrated treponemes in the gastric mucosa of a suspected gastric syphilis using immunoperoxidase and immunofluorescent techniques. We regard these techniques as usefull confirmative methods for detecting treponemes in tissue or internal organs.

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