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Line Immunoassay and Enzyme-Linked Line Immunofiltration Assay for Simultaneous Detection of Antibody to Two Treponemal Antigens

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Two enzyme immunoassays, the line immunoassay (LIA) and the enzyme-linked line immunofiltration assay (ELLIFA), were studied for suitability in the serodiagnosis of syphilis. In both assays, antibody to treponemes was detected using the recombinant DNA derived treponemal protein TmpA and the purified axial filament derived from the Reiter treponeme. The antigens were applied in parallel lines onto nitrocellulose membranes. The sensitivity and specificity of both assays were compared with that of the *Treponema pallidum* hemagglutination assay (TPHA), the fluorescent treponemal antibody absorption test, and the axial filament and TmpA enzyme-linked immunosorbent assays. The sensitivity and specificity of the LIA and the ELLIFA were found to be comparable to that of the TPHA using serum samples from 65 untreated syphilitic patients, 95 patients treated for syphilis and 60 blood donors, except in the case of the LIA using axial filament. This latter test was slightly less sensitive in primary and early latent syphilis than the TPHA. In the LIA procedure, serum antibodies to two antigens could be detected simultaneously within two hours. This assay may be useful for fieldwork. In the ELLIFA procedure, antibodies to the two antigens could be detected simultaneously within 15 minutes. The ELLIFA procedure may provide a multiple antigen test with a very short assay operation time.

Recently, new methods based on enzyme immunoassay techniques have been developed for serological detection of syphilitic infection (1, 2). These methods not only have the advantage that the results can be easily read, they are also simple and rapid to execute. The recently developed *Treponema pallidum* enzyme-linked immunofiltration assay (TP-ELIFA) combines the high sensitivity and specificity of a test using *Treponema pallidum* as antigen with a short test duration of only 15 minutes (2). The specificity and sensitivity of enzyme immunoassays have been enhanced by the use of purified treponemal proteins obtained either from the cultivable *Treponema phagedenis* or *Escherichia coli* recombinants producing *Treponema pallidum* proteins (3–6). Furthermore, simultaneous detection of antibodies to antigens of different pathogenic microorganisms in one test procedure may simplify the screening of individuals at risk for multiple infectious diseases. This may be useful in cases where a particular sexually transmitted disease has been diagnosed: the patient may concurrently be suffering from other sexually transmitted infections that need attention. The recently described line immunoassay (LIA) in which multiple antigens are applied in parallel lines onto nitrocellulose strips permits the simultaneous detection of serum antibodies to multiple antigens (7). In the TP-ELIFA procedure, the simultaneous use of different antigens in combination with *Treponema pallidum* itself to detect more than one infectious disease is not recommended since the test sera must first be absorbed with a *Treponema phagedenis* derived absorbent to reduce false positive reactions (2). This absorbent may interfere with the detection of antibodies to other antigens. However, the application of purified or recombinant DNA derived antigens in the TP-ELIFA procedure circumvents the need for an absorbent and also permits simultaneous testing for infections other than syphilis (3, 6). In this study, the suitability of the purified *Treponema phagedenis* biotype Reiter derived axial filament (AF) and of the recombinant DNA derived *Treponema pallidum* membrane protein A (TmpA) for use in LIA and ELIFA procedures was assessed. In addition, the coating of antigens in parallel lines was evaluated for its suitability to deliver multiple treponemal antigens to one well in an ELIFA procedure. This new assay is designated enzyme-linked line immunofiltration assay (ELLIFA).

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Materials and Methods

Sera. A total of 160 syphilitic sera were obtained from 65 untreated syphilis patients and 95 patients treated for syphilis who attended the Sexually Transmitted Disease Clinic at the University Hospital in Rotterdam. The sera from untreated syphilitic patients were obtained from a collection stored at −20°C. The sera from treated syphilis patients were samples submitted to the National Institute of Public Health Laboratory of Bacteriology for syphilis serological tests. All samples were tested by the TPHA (Japan Lyophilization Company, Japan), the FTA-ABS test (8) and the VDRL test (8). The syphilis sera were classified according to the following criteria: a) no syphilitic disease: according to history and results of clinical and laboratory investigations no conclusive evidence of syphilitic infection; b) primary syphilis: localized skin and/or mucosal lesions alone or in combination with regional lymphadenopathy and demonstration of *Treponema pallidum* in the lesions or in the lymph node and/or reactive serological tests; c) secondary syphilis: generalized lesions of skin and/or mucosa alone or in combination with generalized lymphadenopathy and demonstration of *Treponema pallidum* in the lesions, and reactive serological tests; d) latent syphilis: no clinical signs of syphilis, history of past infection, positive serological tests; e) neurosyphilis: neurological disorders in combination with changes in the cerebrospinal fluid, positive TPHA and/or FTA test in combination with either a positive VDRL test or mononuclear cell count $> 5.0\,\mu l$, or IgG index $\geqslant 0.7$, or oligoclonal immunoglobulins; f) treated syphilis: diagnosed syphilis, treated according to current treatment regimens, with no evidence of reinfection; collection of serum samples six months to three years after treatment. Sixty control sera obtained from blood donors were tested using the TPHA only. None of these sera were positive in the TPHA.

Antigens. The TmpA antigen was obtained as follows. The construction of plasmid pRIT4661 encoding for overproduction of TmpA and concurrent TmpB antigens is described in brief. The fragment of plasmid pRIT4628 carrying the *tmpA* and *tmpB* genes (9) was inserted in the expression vector pPLc245 (10). To provoke the hyperexpression of TmpA with concurrent hyperexpression of TmpB, the leftward promoter of bacteriophage lambda, which is controlled by a thermosensitive repressor, was inserted in front of the *tmpA/tmpB* operon and the plasmid transformed into *Escherichia coli* K-12. The isolation and purification of the recombinant DNA-derived TmpA from *Escherichia coli* carrying plasmid pRIT4661 was done as described previously (6). It was shown in preliminary experiments that concentrations of sodium dodecyl sulfate (SDS) higher than 0.05% in the antigen preparation may hamper the non-electrophoretic attachment of proteins to nitrocellulose. To remove the SDS (0.1%) and the Coomassie Brillant Blue from these preparations, the TmpA was precipitated with a solution containing 5% triethylamine, 5% acetic acid and 10% acetone for 10 min and subsequently washed twice with acetone using the micromethod for removal of SDS from proteins (11). After the final centrifugation, the acetone was removed from the TmpA pellet by evaporation under vacuum. The protein was redissolved in Tris-buffer (0.01 M Tris.HCl pH 7.4 and 0.15 M NaCl) containing 0.05% SDS and was stored at −20°C until use. The 0.05% SDS stabilized TmpA in solution and did not interfere with the binding of TmpA to nitrocellulose.

To obtain the axial filament (AF) antigen, *Treponema phagedenis* biotype Reiter was cultured and the axial filament prepared and purified as described previously (12).

Coating of Antigen onto Nitrocellulose Sheets. The TmpA and AF antigens were diluted in phosphate-buffered saline (PBS, pH 7.0), and coated onto nitrocellulose sheets (BA 85, 0.45 μm; Schleicher and Schuell, FRG) in parallel lines 180 mm long (± 0.3 mm wide) as shown in Figure 1. For use in the LIA procedure coated nitrocellulose sheets were cut into 3 mm wide strips perpendicular to the antigen lines. Sheets to be used in the ELLIFA were cut into rectangles (70 × 40 mm). To determine the optimal concentration of TmpA and AF in both assays, various amounts of both antigens were mechanically applied onto nitrocellulose sheets. TmpA was diluted from a stock solution (1 mg/ml) and coated onto the nitrocellulose sheets in parallel lines at the final concentrations of 5.0, 2.5, 1.3 and 0.6 ng TmpA/mm line. The AF was diluted from a stock solution (5 mg/ml) and coated onto the nitrocellulose sheets in the same manner at the final concentrations of 5, 2.5, 1.3 and 0.6 ng AF/mm line. In addition, 1.3 ng human IgG/mm was coated onto the nitrocellulose sheets as internal control for the quality of the conjugates used in the test and as reference control for positive test results.

Line Immunoassay. Nitrocellulose strips were incubated for 2 h at room temperature with serum diluted 1:200 in a buffer consisting of 10 mM Tris-HCl of pH 7.4, 0.1 M $MgCl_2$, 0.5% Tween 20, 1% bovine serum albumin and 5% fetal calf serum (TMTB buffer). After rinsing three times (10 min each) in TMTB buffer, the strips were incubated for 2 h with 1 ml of horse radish peroxidase conjugated goat anti-human IgG (Tago, Denmark) which had been diluted 1:1000 in TMTB buffer. After two rinses of 10 min each with TMTB buffer and three rinses in Tris-buffer, the substrate solution containing 4-chloro-1-naphtol and hydrogen peroxide was added and the strips were incubated at room temperature in the dark for 30 min. After three rinses with distilled water the strips were dried and examined visually. Reactions with a lower colour intensity than that observed using the negative control serum were regarded as negative.

Enzyme-Linked Line Immunofiltration Assay. The assay was performed in a specially designed multi-hole filtration device as described previously (2). Precoated nitrocellulose sheets were inserted into the filtration device in such a way that two antigen lines were enclosed by each well of the filtration device. 0.2 ml phosphate-buffered saline containing 0.5% Tween (PBS-T) was added to each well and subsequently removed by vacuum (0.5 kg/cm^2). Two hundred μl of serum which had been diluted 1:50 in PBS-T were added to each well and filtered through the nitrocellulose sheet at a consistent flow rate obtained by calibrated suction for 5 min as described previously (2). After rinsing the wells with 0.2 ml PBS-T, 0.2 ml of horse radish peroxidase conjugated sheep anti-human IgG (Nordic, The Netherlands) which had been diluted 1:20,000 in PBS-T, was incubated for another 5 min by calibrated suction. The wells were rinsed and incubated with 0.2 ml of a substrate solution (0.6 mg/ml tetramethylbenzidine, 2 mg/ml dioctylsodiumsulfosuccinate and 0.015% hydrogen peroxide in 25% ethanol and citrate/phosphate buffer containing 5 mM citric acid, 10 mM Na2HPO4, pH 5.0) for 3 min. The colour intensity was examined visually.

Enzyme-Linked Immunosorbent Assay. The ELISA was performed with TmpA or AF antigen as has been described previously (6, 12).

Statistical Analysis. Sensitivity and specificity were calculated within their 95% confidence limits. The McNemar test was used for statistical evaluation.

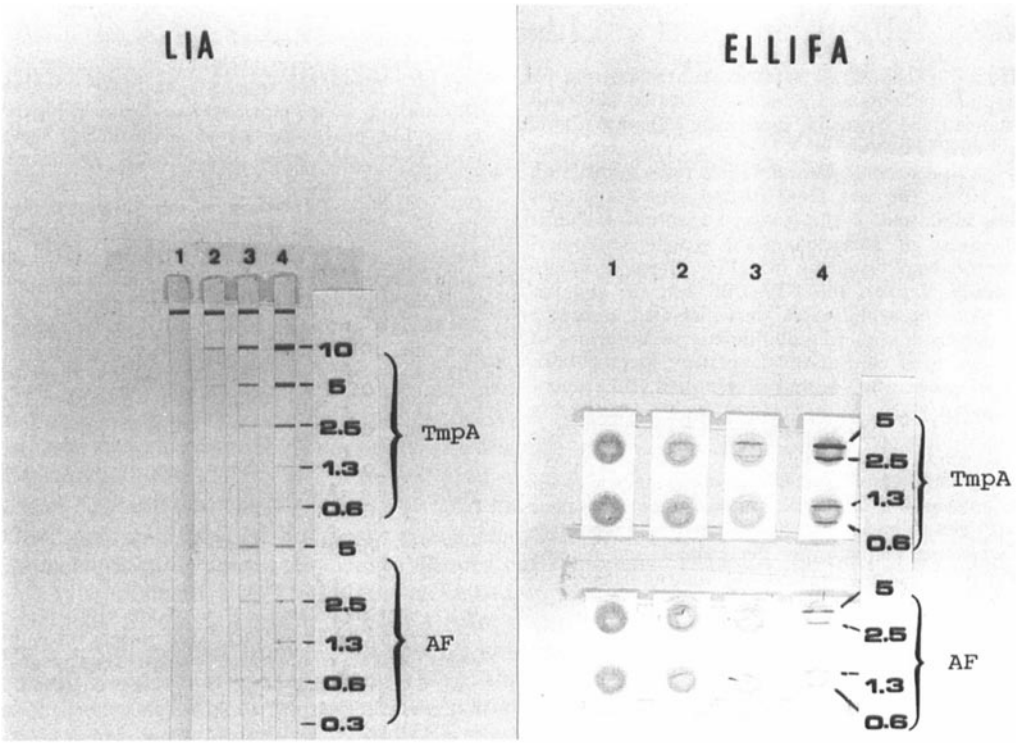

Figure 1: LIA and ELLIFA test strips using various concentrations of TmpA and AF with (1) a control (no serum), (2) a non-syphilitic serum, (3) a weakly reactive serum, and (4) a strongly reactive syphilitic serum. The upper line on the LIA strips represents the IgG control that was directly coated onto the nitrocellulose membrane.

Results

Optimal Antigen Concentration in LIA and ELLIFA. Figure 1 illustrates the test results of checkerboard titration of dilution buffer, a non-syphilitic serum and two syphilitic sera in the LIA and ELLIFA. To determine the optimum antigen concentration in the LIA procedure, the sensitivity and specificity of the LIA were measured at various concentrations of TmpA and AF antigens. The results presented in Table 1 show that the reactivity of primary, secondary and early latent syphilitic sera dropped substantially at lower concentrations of TmpA. At a concentration of 2.5ng TmpA/mm, 63 out of 65 sera from untreated patients with various stages of syphilis reacted positively, representing a sensitivity of 97%, whereas only 30 sera reacted positively at a concentration of 1.3ng TmpA/mm. The reactivity of the 60 blood donor sera declined substantially at a concentration lower than 5ng TmpA/mm. Only one donor serum reacted positively at a concentration of 2.5ng TmpA/mm, giving a specificity of 98%. Therefore, a concentration of 2.5ng TmpA/mm appeared to be optimal with respect to the sensitivity and specificity in the TmpA LIA. In Table 1 it can be seen that the reactivity of the sera from untreated patients with various stages of syphilis to AF at a concentration of 0.6ng AF/mm was significantly less (38/65) than to AF at a concentration of 1.3ng AF/mm (57/65). The blood donor sera appeared not to react at all at a concentration of 1.3ng AF/mm or less. Therefore, a concentration of 1.3ng AF/mm was considered optimal for use in the AF LIA. The optimal antigen concentrations of TmpA and AF were also determined for the ELLIFA. The ELLIFA procedure using TmpA was more sensitive at a concentration of 2.5ng/mm (61/65) than at a concentration of 1.3ng TmpA/mm (56/65) (Table 1). The specificity of the ELLIFA procedure using TmpA at a concentration of 2.5ng/mm (98%) and 1.3ng/mm (100%) did not differ significantly. Therefore, a concentration of 2.5ng/mm was considered optimal. Early syphilitic sera were slightly less reactive to AF at a concentration of 0.6ng/mm (58/65) than at a concentration of 1.3ng/mm (64/65). The specificity was 100% in both cases. Therefore, a concentration of 1.3ng AF/mm was considered optimal.

Table 1: Determination of optimal antigen concentration of LIA and ELLIFA.

Diagnosis	No. of sera	No. of reactive sera in LIA								No. of reactive sera in ELLIFA							
		TmpA (ng/mm)				AF (ng/mm)				TmpA (ng/mm)				AF (ng/mm)			
		5	2.5	1.3	0.6	5	2.5	1.3	0.6	5	2.5	1.3	0.6	5	2.5	1.3	0.6
Primary syphilis	16	15	14	6	5	15	15	12	7	16	13	10	6	16	15	15	14
Secondary syphilis	21	21	21	5	11	21	21	21	14	21	21	19	19	21	21	21	20
Early latent syphilis	28	28	28	19	16	28	28	24	17	28	27	27	25	28	28	28	24
No syphilis	60	9	1	0	0	31	9	0	0	12	1	0	0	11	2	0	0

Table 2: Results of serological tests for syphilis in serum samples from patients with untreated and treated syphilis and from blood donors.

Diagnosis	No. of sera	No. (%) of reactive sera							
		TmpA			Axial Filament			TPHA	FTA-ABS
		LIA	ELLIFA	ELISA	LIA	ELLIFA	ELISA		
Untreated syphilis									
Primary	16	14 (88)	13 (81)[b]	14 (88)	12 (75)[a]	15 (94)	12 (75)[b]	14 (88)[b]	16 (100)
Secondary	21	21 (100)	21 (100)	21 (100)	21 (100)	21 (100)	21 (100)	21 (100)	21 (100)
Early latent	28	28 (100)	27 (96)	28 (100)	24 (86)	28 (100)	27 (96)	28 (100)	28 (100)
Treated syphilis	95	83 (87)	90 (95)	80 (84)	ND	ND	ND	92 (97)	91 (96)
No syphilis	60	1 (2)	1 (2)	ND	0 (0)	0 (0)	ND	0 (0)	ND

ND = not done

[a] Of the 4 non-reactive sera: one serum was not reactive in TmpA and AF LIA, TmpA and ELLIFA, TmpA and AF ELISA; one was not reactive in TmpA LIA, AF ELISA and TPHA; one was not reactive in TmpA ELLIFA, TmpA and AF ELISA; one was not reactive in AF LIA only.

[b] The remaining non-reactive sera were not reactive in one test only.

Sensitivity and Specificity of LIA and ELLIFA. The reactivity of the syphilitic sera in the LIA and ELLIFA procedure using optimum TmpA and AF antigen concentrations as determined above, was compared to their reactivity in the TPHA, FTA-ABS test, TmpA ELISA and AF ELISA (Table 2). In primary syphilis, the sensitivity of the TmpA LIA (88%, 95% confidence limits (CL): 72–100%) and the TmpA ELLIFA (81%, CL: 62–100%) was comparable to that of the TPHA (88%, CL: 72–100%) and the TmpA ELISA (88%, CL: 72–100%). The reactivity of primary syphilitic sera in the AF ELLIFA (94%, CL: 82–100%) was slightly higher than in the AF LIA (75%, CL: 54–96%) or AF ELISA (75%, CL: 54–96%). Consequently, the sensitivity of the ELLIFA procedure using AF was comparable to that of the TPHA in primary syphilis. In secondary syphilis the sensitivity of all tests was 100%. In early latent syphilis, the sensitivity of the LIA and ELLIFA procedure using either TmpA or AF was comparable to that of the TPHA and ELISA except that the sensitivity of the AF LIA in early latent syphilis was lower. The reactivity of treated syphilis sera was determined for 95 sera using the TmpA antigen (Table 2). The reactivity of TmpA in the LIA (87%) and ELLIFA (95%) was comparable to its reactivity in the ELISA (84%). The specificity of both the LIA and ELLIFA using TmpA or AF was comparable to that of the TPHA and FTA-ABS test (Table 2).

Comparison of Sensitivity of LIA and ELLIFA. The difference in sensitivity between the LIA and ELLIFA procedure was studied by titration of a strongly, a moderately, and a weakly reactive syphilitic serum in both assays (Figure 2). The three sera were titrated against three different TmpA concentrations and two different AF concentrations. All three sera were reactive at a 2-fold to 4-fold higher titre in the ELLIFA than in the LIA when TmpA was used at the highest concentration in both the assays. At the other two TmpA

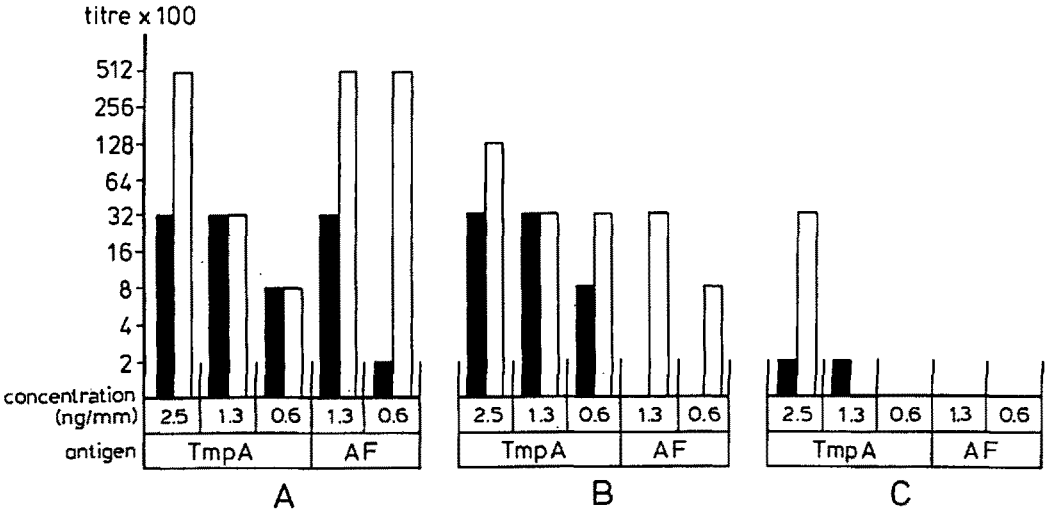

Figure 2: Comparison of the sensitivity of LIA and ELLIFA using three sera: (A) a strongly reactive serum, (B) a moderately reactive serum and (C) a weakly reactive serum. Shaded columns represent LIA test results and open columns ELLIFA test results.

concentrations (1.3 and 0.6ng/mm) the reactivity of the three sera in the ELLIFA was equal to or higher than in the LIA, except that the weakly reactive serum did not react to TmpA at a concentration of 1.3ng/mm in the ELLIFA. In this particular case, the LIA was slightly more sensitive than the ELLIFA procedure. The reactivity of both the strongly and the moderately reactive sera to AF was higher at concentrations of 1.3ng/mm and 0.6 ng/mm respectively in the ELLIFA than in the LIA. The weakly reactive serum did not react at all to AF in both the LIA and ELLIFA in this experiment.

Discussion

In this study the LIA and ELLIFA, enzyme immunoassays using TmpA and AF as antigens, were evaluated for use in the serodiagnosis of syphilis. The specificity of these assays did not differ significantly from that of the TPHA. However, in both the LIA and ELLIFA, a single serum from a blood donor reacted to TmpA antigen. A more extensive purification of TmpA reduced the non-specific reactivity of this serum to TmpA in the ELISA. Therefore, these aspecific reactions to TmpA may be due to contaminants derived from *Escherichia coli* in which TmpA was expressed. Although the use of AF in this study produced specific reactions with the limited number of sera tested, non-specific reactions may occur if larger numbers of sera are tested. Theoretically, non-specific reactions may be avoided by the use of an absorbent for the removal of antibodies reacting non-specifically. Since antibodies reactive to axial filament may cross-react with proteins from other non-pathogenic treponemes, non-specifically reacting antibodies will not be inhibited by an adsorbent. The sensitivity of the TmpA LIA, TmpA ELLIFA and AF LIA in the early stages of untreated syphilis was comparable to that of the TPHA. In contrast, the sensitivity of the AF LIA in primary syphilis and early latent syphilis appeared to be lower than that of the TPHA. However, using AF at a concentration higher than 1.3ng/mm the sensitivity increased substantially but the specificity decreased. This indicated that the AF concentration was critical. The ELLIFA was observed to be more sensitive than the LIA using different titres of sera from patients with different stages of syphilis. This may have been due to the filtration in ELLIFA in which the reactants not only react at the surface of the membrane but also with the antigens impregnated below the surface of the filter sheets. The higher sensitivity of the ELLIFA may be an advantage when this assay is used to detect antibodies to multiple antigens. Although in the LIA and ELLIFA the results can be read by the naked eye, it was observed in preliminary experiments that a transmission thin-layer-chromatography (TLC) scanner could easily be used to detect positive reactions in the LIA procedure. However, the TLC scanner was observed to be totally unsatisfactory for reading ELLIFA reactions. A reflection TLC scanner was found to be suitable for both ELLIFA and LIA. Preliminary results also showed that the LIA can be used as a simple multi-antigen test which simultaneously detects antibodies to human immunodeficiency virus, herpes simplex

virus, cytomegalovirus, varicella-zoster virus and mumps virus (7). In an outpatient clinic, the short operation time of the ELLIFA (15 min) would have the advantage that the test results are available at the same consultation. Moreover, the ELLIFA filtration device can be modified to test for up to 14 antigens simultaneously. Preliminary results indicated that up to 14 antigen lines can be accomodated in a single reaction field in the modified filtration device. Testing for multiple antigens has already been demonstrated using the LIA procedure (7). Multiple antigen testing needs the use of antigens that do not require an absorbent during serum incubation. Such an absorbent may influence the antibody binding to other antigens. For the serodiagnosis of syphilis, TmpA fulfils this requirement. Both the LIA and ELLIFA not only could be used in testing for multiple antigens in infectious diseases, but may also be useful in screening for allergic antigens, and serotyping or screening for monoclonal antibody producing hybridoma cell cultures.

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