Comparative Evaluation of Five Serological Methods for the Diagnosis of Sporotrichosis

SHARON O. BLUMER, LEO KAUFMAN, WILLIAM KAPLAN, DAVID W. MCLAUGHLIN, AND DOROTHY E. KRAFT

Center for Disease Control, Department of Health, Education, and Welfare, Atlanta, Georgia 30333

Received for publication 8 March 1973

The diagnosis of sporotrichosis can be time consuming. Serological procedures could facilitate the rapid and accurate diagnosis of this disease. A slide latex agglutination (SLA) test for sporotrichosis was developed and compared with the tube agglutination (TA), complement fixation (CF), and immunodiffusion (ID) tests in the serological study of 80 proven human cases of sporotrichosis representing the cutaneous, subcutaneous, and extracutaneous forms of the disease. In addition, the indirect fluorescent antibody (IFA) technique was applied to 61 case sera. In the SLA test, latex particles sensitized with culture filtrate antigens from the yeast form of Sporothrix schenckii (B 959) detected 94% of the cases, as compared to 96% of the cases detected by the TA test, 68% by the CF test, and 56% by the ID test. The IFA test detected 90% of the 61 cases. The SLA and ID tests were specific, showing no reactions with sera from 86 persons with no disease or with diseases other than sporotrichosis. Because of its sensitivity, specificity, ease of performance, and ability to provide results in 5 min, the SLA test is highly recommended for routine use in the clinical laboratory.

The classic type of localized cutaneous-subcutaneous sporotrichosis, in which a patient has ulcerative lesions on an extremity with an associated linear chain of nodules along the lymphatics, can often be recognized by an astute clinician and can be unequivocally diagnosed by culture or fluorescent antibody techniques. In the disseminated cutaneous-subcutaneous type of the disease, a primary lesion is often absent, and hard moveable subcutaneous nodules may be scattered over the entire body. Clinically, this form of sporotrichosis is not easily recognized and must be differentiated from other mycotic infections as well as from syphilis, tularemia, glanders, pyogenic lesions, and tuberculosis. Extracutaneous sporotrichosis involving joints, bones, lungs, and other organs presents an even more difficult diagnostic problem due to the absence of skin lesions, nodules, or any other ready source for obtaining Sporothrix schenckii. The lack of distinct clinical features for the extracutaneous or systemic form of sporotrichosis emphasizes the need for specific and sensitive serological methods to be used in diagnosis.

The present study was devised to select and or develop one or more rapid and accurate serological methods for the diagnosis of sporotrichosis. Over a period of 3 years, test procedures, namely, slide latex agglutination (SLA), tube agglutination (TA), complement fixation (CF), and immunodiffusion (ID) tests, were evaluated in parallel with sera from 80 patients with proven cases of sporotrichosis, 77 persons with bacterial, parasitic, or heterologous mycotic infections, and 9 normal persons. In addition, the value of the indirect fluorescent antibody (IFA) test for the serodiagnosis of sporotrichosis was also investigated. The efficacy of these tests as diagnostic tools is discussed.

MATERIALS AND METHODS

Serum. Sera evaluated in this study were from 80 patients with culturally proven, clinically defined cases of sporotrichosis, 9 apparently healthy persons, and 77 individuals with culturally proven actinomycosis, blastomycosis, cryptococcosis, coccidioidomycosis, histoplasmosis, paracoccidioidomycosis, syphilis, tuberculosis, tularemia, amoebic abscess, or leishmaniasis. All sera were preserved with merthiolate (1:10,000) and stored at -20 C.

Reference antiserum. Reference serum was produced in rabbits by intravenous injection of methanol-killed S. schenckii strain B 959. Antigens were prepared from 1-week-old cultures grown in flasks containing brain-heart-infusion broth (Difco) maintained at 37 C and shaken at 150 to 160 rpm. The
fungal elements, predominantly yeast cells, were suspended in saline containing merthiolate and adjusted to the density of a no. 5 McFarland standard. Injections of 1.0, 2.0, and 2.0 ml were given on 3 consecutive days. A rest period of 5 days was followed by a second series of intravenous injections. Rabbits were exsanguinated 10 days after the last injection in the second series. These reference anti-\textit{S. schenckii} sera had TA titers of 1:320 to 1:1,280 and demonstrated one to three precipitin bands when tested with the homologous antigen. Merthiolate (1:10,000) was added as a preservative, and sera were stored at -20 C.

**SLA test.** The yeast form of \textit{S. schenckii} strain B 959 was grown as described above. The shaken broth cultures were placed in a 62 to 65 C water bath for 2.5 h. The heat-killed cultures were then allowed to stand at room temperature for 48 h before the yeast cells were removed by filtration through a Whatman no. 41 filter paper. Merthiolate was added (1:5,000) as a preservative, and the filtrates were stored at 4 C. Sera were initially diluted 1:4 with glycine-buffered saline (pH 8.4) containing 0.1% bovine serum albumin and inactivated at 56 C for 30 min before testing. A spectrophotometrically standardized (1) suspension of 0.8- \mu m polystyrene latex particles was sensitized by mixing with an equal volume of the optimal dilution of filtrate antigens. The optimal quantity of filtrate was the highest dilution which produced a clear 2+ agglutination with the highest reactive dilution of rabbit reference serum in a box titration. The SLA test was performed by adding 0.04 ml of the inactivated (56 C/30 min) serum to 0.02 ml of latex-sensitized particles and rotating the reactants at 150 rpm for 5 min. The antibody titer was recorded as the highest dilution of serum showing 2+ agglutination, i.e., small but definite clumps with the background slightly cloudy.

**TA test.** \textit{S. schenckii} cells were grown and harvested by filtration as described above. The cells were washed twice in merthiolated saline, filtered through four layers of gauze, and adjusted to a 1:100 dilution by using a Hopkins tube and centrifuging at 650 \times g for 30 min. Box titrations against rabbit reference serum determined the optimal dilution of this 1:100 stock suspension. The test was performed essentially as reported previously by Norden (5) except that 0.85% NaCl containing merthiolate (1:10,000) was used as the diluent, and the initial dilution of serum was 1:8.

**CF test.** CF tests were performed by the standardized Center for Disease Control Laboratory Branch complement fixation test (10). The antigen showing the greatest sensitivity was an acetone-precipitated, 10-times concentrated mycelial \textit{S. schenckii} culture filtrate. Mycelial fragments of \textit{S. schenckii} (B 959) were inoculated into flasks containing brain-heart infusion broth and incubated at 25 C for 14 days while rotating at 150 to 160 rpm. Merthiolate was added to a concentration of 1:5,000, and the flask was allowed to stand at 25 C for 48 h. After the mycelium was removed by filtration, two volumes of cold acetone were added slowly to the filtrate. The mixture was kept at 4 C for 24 h, after which the precipitate was separated by centrifugation in the cold at 10,000 rpm for 20 min. The precipitate was dissolved in a volume of sterile, distilled water equal to one-tenth of the original filtrate volume. Merthiolate was added as a preservative, and the 10-times concentrated antigen was stored at 4 C. The optimal dilution for this concentrated antigen was determined by box titration against the rabbit anti-\textit{S. schenckii} reference serum.

**ID test.** Agar gel double diffusion test antigen was prepared by acetone precipitation of the yeast broth filtrate as described for the SLA test. The precipitate was obtained and treated in the same manner as that described for the CF test. Optimal dilution for the ID antigen was determined by box titration against the rabbit reference serum. The agar medium and matrix used have been described (2). Sporotrichosis or heterologous human case sera and rabbit \textit{S. schenckii} serum were placed in peripheral wells and preincubated for 30 min at 25 C. Optimally diluted antigen was placed in the center well, and the reactants were incubated in a moist chamber at 25 C for 48 h.

**IFA test.** The antigen used in the TA tests was employed as the antigen in the IFA procedure. Preliminary tests with rabbit reference serum showed that best results were obtained with a 1:20 dilution of the stock suspension of whole \textit{S. schenckii} yeast-form cells. Smears of the antigen were prepared by placing one drop of the suspension on a clear glass slide. The smears were allowed to air-dry and were then heat-fixed. Three drops of a 1:32 dilution of test serum in phosphate-buffered saline, pH 7.2, were placed over the fixed cells with a Pasteur pipette. Three drops of a 1:32 dilution of serum from a normal individual, that served as a control, were placed on a replicate smear. Dilutions of 1:32 were selected because preliminary studies indicated that sera from patients with diseases other than sporotrichosis diluted 1:16 or less frequently cross-reacted with the IFA antigen. The preparations were incubated in a moist chamber for 30 min at 37 C. The slides were then rinsed for 10 min in phosphate-buffered saline, pH 7.2, and for 5 min in distilled water and then allowed to air-dry.

Three drops of fluorescein-labeled goat antihuman globulins, diluted 1:4 with phosphate-buffered saline, pH 7.2, were then applied to the test and to the control preparations. Box titrations against reference sera from three human cases of sporotrichosis determined the optimal dilution of the conjugated antiglobulins. The preparations were then incubated for 30 min at 37 C in a moist chamber and rinsed in phosphate-buffered saline and in distilled water, as previously described. After rinsing, the slides were allowed to air-dry and were then mounted in phosphate-buffered glycerol, pH 7.2.

The stained preparations were examined with a Reichert microscope. A Reichert Fluorex unit, fitted with an HBO 200 high-pressure mercury vapor lamp, served as a light source. An interference exciter filter developed by the American Optical Co. (7) was used in combination with a Schott GG-9 ocular filter. Cell wall staining, ranging in intensity from 1 to 4+, was considered a positive reaction.

**RESULTS**

The relative sensitivity of the SLA, TA, CF, and ID tests in the diagnosis of sporotrichosis
was determined with serum specimens from 80 patients with culturally proven cases (Table 1). Of the 80 cases, 55 were the localized cutaneous or subcutaneous type. The results indicate that the TA test was the most sensitive of the four procedures for detecting localized sporotrichosis. It detected 53 of the 55 cases (96%), followed closely by the SLA test with 51 cases positive (93%). The CF and ID tests were much less sensitive, reacting positively in 25 (47%) and 23 (42%) cases, respectively. Sera from 12 of the 55 cases were anticomplementary (AC) in the CF test and could not be titered. TA and CF titers ranged from the initial dilution of 1:8 to 1:512, whereas SLA test titers ranged from the starting 1:4 dilution to 1:64. Of the 23 cases sera containing precipitins, 10 demonstrated a single precipitin band in the ID test and 13 showed two precipitin bands. There was no consistent correlation between the number of precipitin bands and the titer obtained in either the TA, SLA, or CF tests.

Five of the 80 patients suffered with disseminated subcutaneous or nodular sporotrichosis. Specimens from all five were positive by both the TA and the SLA tests. Titers obtained with the TA test ranged from 1:16 to 1:256, whereas those for the SLA test ranged from 1:8 to 1:128. One specimen was negative by both the CF and ID tests, and another was AC in the CF procedure (Table 1).

Twenty of the sera came from patients with pulmonary, articular, osseous, or meningeal sporotrichosis. Nineteen of these extracutaneous case sera (95%) were detected with the TA or SLA tests and 17 were detected with the CF (89%) or ID (85%) tests (Table 1). Five of the systemic cases had concomitant cutaneous infection. TA titers for the systemic cases ranged from 1:8 to 1:256; titers obtained with the SLA test varied from 1:8 to 1:512. Some sera were negative by the CF test, but others showed titers as high as 1:128. One to two bands were present in cases positive by ID. There was apparently no correlation between the type of tissues involved and the number of precipitin bands or the height of the titer in the TA, SLA, or CF tests.

In the 80 cases, the TA test was positive in 77 or 96% (Table 1); the SLA test showed a sensitivity of 94%, and the CF and ID tests were reactive in 68 and 55% of the cases, respectively. Neither the CF nor the ID tests detected antibodies in sera from patients negative by the SLA and TA tests. The combined use of the TA and SLA procedures resulted in positive serologies in 78 of the 80 cases (98%). The high number of AC specimens as well as the comparative insensitivity of the CF test indicated that this procedure was of less value either alone or in combination with any of the other tests. Similarly, the low sensitivity of the ID test indicated that it did not have value as a serological test even when combined with the TA or SLA techniques.

In 23 of the 80 cases studied, two or more sera, covering periods of 1 to 16 months, were received. In only five of these cases did fourfold or greater titer changes occur which could be considered prognostically significant. Two of these five cases were of the localized cutaneous type, one was of the nodular, disseminated subcutaneous form, and two were of the extracutaneous type—one involving bone and skin, the other involving the lungs.

The results of the IFA tests are summarized in Table 2. Sera from 61 cases were tested by this procedure, and 90% were positive. All sera from patients with extracutaneous disease were positive. Sera from 85% of the 48 patients with localized cutaneous-subcutaneous sporotrichosis were positive.

The specificity of the five procedures was determined by testing sera from patients with various bacterial, fungal, and parasitic infections as well as sera from apparently healthy individuals. The data presented in Table 3 reveal that all the tests, with the exception of the IFA procedure, demonstrated a high degree of specificity. When TA or SLA tests were performed on undiluted heterologous sera, a greater number of positive reactions occurred. The nonspecific reactions were not limited to any particular disease category. This observation prompted

### Table 1. Comparative sensitivity of four serological tests for sporotrichosis

| Clinical form of sporotrichosis | No. of cases | No. of positive cases by test |
|---------------------------------|--------------|-------------------------------|
|                                 |              | CF   | ID   | SLA  | TA   |
| Localized cutaneous-subcutaneous | 55           | 25*  | 23   | 51   | 53   |
| Disseminated cutaneous-subcutaneous | 5            | 3*   | 4    | 5    | 5    |
| Pulmonary                       | 8            | 7    | 7    | 7    | 7    |
| Articular                      | 6            | 5    | 5    | 6    | 6    |
| Osseous                        | 2            | 2    | 2    | 2    | 2    |
| Articular and osseous          | 2            | 2    | 2    | 2    | 2    |
| Articular and pulmonary        | 1            | 1    | 1    | 1    | 1    |
| Meningeal                      | 1            | AC   | 0    | 1    | 1    |
| **Totals**                     | **80**       | **45** | **44** | **75** | **77** |
| **Percent positive by test**   |              | **68*** | **55** | **94** | **96** |

* Twelve sera AC.
* One serum AC.
* Calculation based on total of 67 non-AC sera tested.
our choice of 1:8 and 1:4 starting dilutions for the TA and SLA procedures, respectively. With these initial dilutions, minimal false-positive reactions occurred with the TA test and none occurred with the SLA test. Two sera from patients with leishmaniasis showed positive TA reactions at titers of 1:8 and 1:16, respectively. As Table 3 shows, four specimens formed precipitin bands in the ID test but none were considered positive, since they were not identical with the bands produced by the rabbit S. schenckii reference sera. No attempt was made to characterize these bands.

Of the sera from the 64 patients with diseases other than sporotrichosis, seven (11%) were positive by IFA. The positive sera were from one patient with amoebic abscess, one with blastomycosis, one with coccidioidomycosis, two with cryptococcosis, one with histoplasmosis, and one with tularemia.

**DISCUSSION**

The first TA and CF tests for sporotrichosis were reported in 1910 (11), and a diagnostic precipitin test that employed a polysaccharide antigen was first described in 1947 (6). More recently there has been a re-evaluation of the more standard serological techniques as well as attempts to develop new tests for the serodiagnosis of sporotrichosis.

Our findings that the agglutination-type tests, TA and SLA, are more satisfactory and reliable than either the CF or ID test are in agreement with those of numerous investigators (3, 4, 5, 9), who report that CF and ID tests, with a variety of S. schenckii antigens, were less sensitive. In one recent study (S. McMillen and E. R. Laverty, Bacteriol. Proc., p. 115, 1969), reactions to a gel precipitin test were positive in 12 of 14 cases, whereas reactions to the CF test were positive in only 8 of 14 cases. However, 11 sera from cases lacking cultural or clinical evidence of sporotrichosis were also positive with the gel precipitin test.

Similarly, in another study (3) positive ID reactions were reported in 20 of 125 (16%) persons with no disease or with fungal disease other than sporotrichosis. These reactions might have been more accurately interpreted if precipitin-positive reference sera had been used in each test. These same investigators, by using a 1000 yeast filtrate, found the sera of only three of nine patients with cutaneous sporotrichosis to be reactive in the CF test. However, the sera from all nine patients with extracutaneous disease were positive by CF testing. In our experience with the CF test and a 10,000 mycelial antigen filtrate, sera from 25 of 55 cases of cutaneous sporotrichosis were positive, and sera from 20 of 25 cases of extracutaneous disease were positive, or 45 and 80%, respectively.

The TA and newly developed SLA tests showed greater sensitivity for these same groups of sera. Regardless of the clinical form of sporotrichosis, 93% or more of the sera reacted in either procedure.

The sensitivity of the IFA test approached that of the agglutination-type tests. The sensitivity of this test for all the clinical forms of sporotrichosis was 90%. The maximal sensitivity was for the extracutaneous forms.

Nonspecific reactions were minimal with all the tests studied (Table 3). Of the five tests studied, however, only the SLA and ID tests demonstrated a specificity of 100%.

Reports on the development of new serological techniques for the diagnosis of sporotrichosis

**Table 2. Sensitivity of the IFA test for sporotrichosis**

| Clinical form of sporotrichosis | No. of cases | No. positive |
|---------------------------------|--------------|-------------|
| Localized cutaneous-subcutaneous | 48           | 41          |
| Disseminated cutaneous-subcutaneous | 4            | 4           |
| Pulmonary                       | 4            | 4           |
| Articular                       | 3            | 3           |
| Osseous                         | 1            | 1           |
| Meningeal                       | 1            | 1           |
| Totals                          | 61           | 54          |
| Percent positive                | 90           |             |

**Table 3. Reactivity of five sporotrichosis serological tests with sera from normal individuals and patients with diseases other than sporotrichosis**

| Clinical diagnosis             | No. of case sera positive/ no. tested |
|--------------------------------|---------------------------------------|
|                                | CF         | ID         | IFA        | SLA        | TA        |
| Actinomycosis                  | 0/5        | 0/5        | 0/6        | 0/7        | 0/7       |
| Amoebic abscess                | 1/1        | 0/1*       | 0/1        | 0/1        | 0/1       |
| Blastomycosis                  | 0/12       | 0/12       | 0/12       | 0/10       | 0/10      |
| Coccidioidomycosis             | 1/5        | 0/5*       | 1/5        | 0/4        | 0/4       |
| Cryptococcosis                 | 0/6        | 0/6        | 0/5        | 0/7        | 0/7       |
| Histoplasmosis                 | 0/7        | 0/7        | 0/7        | 0/7        | 0/7       |
| Leishmaniasis                  | 0/1        | 0/7        | 0/4        | 0/10       | 2/10      |
| Paracoccidioidomycosis         | 0/7        | 0/7*       | 0/7        | 0/7        | 0/7       |
| Syphilis                       | 0/2        | 0/2        | 0/2        | 0/5        | 0/5       |
| Tuberculosis                   | 0/2        | 0/5        | 0/5        | 0/5        | 0/5       |
| Tularemia                      | 0/4        | 0/4*       | 0/8        | 0/8        | 0/8       |
| No disease                     | 0/9        | 0/9        | 0/9        | 0/9        | 0/9       |
| Totals                         | 2/61       | 0/69       | 7/64       | 0/86       | 2/86      |

*One serum produced a band that was nonidentical to the rabbit reference control bands.
have been few. One group of investigators (8) recently evaluated the immuno-cyto-adherence (ICA) test in detecting antibody in experimentally infected guinea pigs. They found that the ICA test was sensitive and of value as a procedure for the detection of an immunological response in sporotrichosis. However, the ICA technique is too complicated to be useful in most clinical and diagnostic laboratories. Other investigators (4) described a tube latex agglutination test. They used a suspension of 1.3-μm latex particles sensitized with S. schenckii yeast culture filtrate antigens, and the test required 16 to 18 h. This latex tube test appeared sensitive in reactions with hyperimmune rabbit sera, but data were not presented for the reactivity of this test with human case sera. Furthermore, no comparisons were made with the yeast agglutination, precipitation, or CF tests.

Of the five serological techniques evaluated for the diagnosis of sporotrichosis, the TA and SLA procedures apparently have the greatest potential for diagnostic application. We prefer the SLA test because it is highly sensitive and specific. It is relatively easy to perform and provides results within minutes. These qualities are extremely important for the small clinic or hospital laboratory, where use of a battery of tests or more complicated procedures is not feasible. The sensitized latex particles are easily prepared and remain stable at 3 to 6 C for 2 years or more.

LITERATURE CITED

1. Bloomfield, N., M. A. Gordon, and D. F. Elmendorf, Jr. 1963. Detection of Cryptococcus neoformans antigen in body fluids by latex particle agglutination. Proc. Soc. Exp. Biol. Med. 114:64–67.
2. Busey, J. F., and P. F. Hinton. 1965. Precipitins in blastomycosis. Amer. Rev. Resp. Dis. 92:637–639.
3. Jones, R. D., G. A. Sarosi, J. D. Parker, R. J. Weeks, and F. E. Tooh. 1969. The complement fixation test in extracutaneous sporotrichosis. Ann. Intern. Med. 81:913–918.
4. Karlin, J. V., and H. S. Nielsen, Jr. 1970. Serologic aspects of sporotrichosis. J. Infect. Dis. 121:316–327.
5. Norden, A. 1951. Sporotrichosis. Clinical and laboratory features and a serologic study in experimental animals and humans. Acta Pathol. Microbiol. Scand. Suppl. 89:1–119.
6. Ochoa, A. G., and E. S. Figueroa. 1947. Polisacaridos del Sporotrichum schenckii. Datos inmunologicos: in-tradermoreacción en el diagnosticó de la esporotricosis. Rev. Inst. Salubr. Enferm. Trop. 8:148–153.
7. Richards, O. W., and A. A. Waters. 1967. A new interference exciter filter for fluorescence microscopy of fluorescein tagged substances. Stain Technol. 42:320–321.
8. Roberts, G. D., and H. W. Larsh. 1971. A study of the immuno-cyto-adherence test in the serology of experimental sporotrichosis. J. Infect. Dis. 124:264–269.
9. Roberts, G. D., and H. W. Larsh. 1971. The serologic diagnosis of extracutaneous sporotrichosis. Amer. J. Clin. Pathol. 56:597–600.
10. U.S. Public Health Service. 1965. Standardized diagnostic complement fixation method and adaptation to micro test. U.S. Public Health Service Publication 1228.
11. Widal, F., P. Abrami, E. Joltrain, E. Brissaud, and A. Weill. 1910. Serodiagnostic mycosique. Applications au diagnostic de la sporotrichose et de l’actinomycose. Les coagglutinatons et cofixations mycosiques. Ann. Inst. Pasteur 24:1–33.