JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1978, p. 148-152
0095-1137/78/0008-0148$02.00/0

Vol. 8, No. 2
Printed in U.S.A.

Counterimmunoelectrophoresis of Reiter Treponeme Axial Filaments as a Diagnostic Test for Syphilis

E. ELLEN NELL AND PAUL H. HARDY, JR.*

World Health Organization Collaborating Center for Reference and Research in Treponematoses, Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received for publication 9 June 1978

Purified axial filaments from the cultivable Reiter treponeme, previously shown to share an antigenic component with pathogenic *Treponema pallidum,* were evaluated as antigen in a diagnostic test for syphilis. Antibody to the filaments was revealed by counterimmunoelectrophoresis. Conditions that produced optimal results in the test were established. A total of 343 sera from normal individuals, biological false-positive reactors, and from patients in the different stages of syphilis were subjected to the test. The results indicate the test to be sensitive and highly specific for detecting treponemal antibodies in human syphilis.

During the past few decades, numerous tests utilizing treponemal antigens have been introduced for the diagnosis of syphilis. The first was the *Treponema pallidum* immobilization test (14), which proved to be specific for syphilis and other treponematoses. This test resolved, for the first time, the problem of distinguishing patients with syphilis from those individuals with other diseases that also generated Wassermann antibody. Despite its recognized value, the *T. pallidum* immobilization test has never been widely used because of its many technical difficulties. Nevertheless, it stimulated the search for an adequate but simpler substitute, and various tests utilizing nonpathogenic as well as pathogenic treponemes resulted.

Investigators' inability to culture pathogenic treponemes in vitro led D'Alessandro et al. to study the Reiter treponeme (alleged to be a nonpathogenic, cultivable strain of *T. pallidum*), and they reported that a protein component reacted with syphilitic serum (9). Later, D'Alessandro and Dardanoni (8) described the isolation and partial purification of this antigen and demonstrated its value in a complement fixation test to detect antibodies in human syphilis. Since that time, the Reiter protein complement fixation (RPCF) reaction has been used widely as a diagnostic test. False-positive reactions have been encountered in a small percentage of sera (4), and, as in all complement fixation tests, a few sera have given spontaneous nonspecific fixation (anti-complementary reactions). In an attempt to circumvent the latter difficulty, Bänffer (1) applied the counterimmunoelectrophoresis (CIE) procedure of Bussard (5) to detect antibodies against the Reiter protein in the sera of syphilitic patients. The antigenic reagent he used was a 10-fold concentrate of the RPCF antigen as prepared by DeBruijn and Bekker (10). The results reported from a small series of sera showed Reiter protein CIE to equal RPCF in detecting syphilitic infection, and further evaluation supported this finding (2). Subsequently, Bänffer et al. reported that there were fewer false-positive reactions in Reiter protein CIE than in RPCF (3).

We recently described a method for isolating and purifying axial filaments (flagella) from the Reiter treponeme (13). When these were studied by complement fixation reactions and gel diffusion precipitins with both rabbit and human syphilitic sera, they were shown to be immunologically closely related to the filaments of pathogenic treponemes. They presumably represent the Reiter protein antigen of D'Alessandro et al. (9). Since a single spirochetal antigen free from all other cellular components has not been available heretofore, the Reiter filaments seemed to offer a great advantage in developing a diagnostic serological test. To investigate this possibility, several immunological procedures were considered worthy of evaluation. CIE was the method chosen to study first because of its relative simplicity and quick results. Conditions necessary to detect the least amount of antibody and to prevent development of spurious precipitin lines have been explored and are here reported along with the serological results obtained.

MATERIALS AND METHODS

Antigen. Reiter treponeme filaments were prepared by the method previously described (13). Final

purification was accomplished either by the originally reported CsCl density gradient method or by column chromatography. In the latter procedure, a dense suspension of crude filaments suspended in 0.01 M phosphate buffer, pH 7.1, was entered on a column of diethylaminoethyl-cellulose (DE 52; Whatman, Inc., Clifton, N.J.) equilibrated with the same buffer. A linear gradient of 0 to 0.75 M NaCl in starting buffer was passed through the column, and the filaments were eluted at 0.44 M NaCl.

Filaments recovered by either method were assayed for purity by both physical and immunological means. Negatively stained preparations of dense suspensions (before sonic treatment) were examined by electron microscopy to assure the absence of contaminating particulate matter. The filaments, surrounded by an ice bath, were then subjected to sonic treatment with a Bronwill Biosonik IV sonic oscillator at 75% power capacity until the fragments were 20 to 400 nm long, as measured in electron micrographs. After this, they were assayed for immunological purity by immunodiffusion and CIE against standard sera (see reference 13). The products recovered by both methods were equal in all respects, including the protein concentration that gave optimal precipitin reactions in CIE.

Protein assay. The protein content of the antigen was determined by the Folin-Ciocalteau procedure as described by Chase and Williams (6). Crystalline bovine serum albumin was used as the standard.

Sera. The majority of the sera tested were from the Serologic Evaluation Research Assembly (SERA) (15) of 1956 to 1957, when 14 laboratories in the United States cooperated in evaluating the various tests for syphilis. At that time, sera from normal individuals, biological false-positives, and patients in all stages of the treponemal diseases were examined. Every serum was subjected to 38 tests, 18 with nontreponemal antigens, 13 with pathogenic *T. pallidum* antigens, and 7 with Reiter antigens, all of which were RPCF tests. In evaluating any new test, these well-documented sera are invaluable. Portions of sera remaining from the original survey had been stored at −20°C, and only well-sealed specimens with no evidence of drying during storage were tested. In addition, 7 established normal sera and 16 from early syphilitics diagnosed by positive dark field examination at the Johns Hopkins Hospital (Baltimore, Md.) were included.

Before testing, sera were heated at 56°C for 30 min or, if previously inactivated, for only 10 min.

Buffers. All chemicals were reagent grade or of the highest grade commercially available. Borax-borate, pH 8.6, and tris(hydroxymethyl)aminomethane-glycine, pH 9.2, were made by mixing 0.05 M solutions of each of the two component parts in the proportions necessary to give the desired pH. Barbital-HCl, pH 8.6, contained 7.94 g of sodium barbital and 115 ml of 0.1 N HCl per liter; it was 0.05 M. Barbital-borate-phosphate buffer, pH 8.6, had 14.5 g of sodium barbital, 3.1 g of boric acid, and 2.68 g of $Na_2HPO_4 \cdot 7H_2O$ per liter; it was 0.07, 0.05, and 0.01 M in terms of the respective components.

CIE. Microscope slides, 1 by 3 inches (2.5 by 7.6 cm), were covered with 2.5 ml of gel in the same buffer used to fill the reservoirs of the electrophoresis chamber. Four pairs of parallel wells 3 mm in diameter and 2.3 mm apart were cut in the agar. With a Hamilton microsyringe, 7.5 μl of the serum to be tested was placed in the anodal well, and the same volume of antigen was placed in the cathodal well. Electrophoresis was carried out in a chamber that accommodated eight slides, allowing 32 sera to be tested at once. Initially, paper wicks (Whatman no. 1) were used to make the gel-buffer contact, and electrophoresis was run for 2 h at 140 V. However, there was much less resistance with Telfa wicking (Kendall Co., Chicago, Ill.), and comparable results were obtained after electrophoresis for only 1 h at 80 V (a current of 5 mA per slide). Therefore, Telfa wicking and the latter conditions were used during most of this study.

Determination of EEO and protein mobility. Vitamin B_{12} was used as the neutral substance transported only by electroendosmosis (EEO). The mobility of a negatively charged protein was measured with bromophenol blue-labeled albumin. A mixture of the two substances was placed in a single well of a gel-covered slide, and after electrophoresis the distance that each component had migrated from the starting point was measured: EEO = cathodal migration distance of vitamin B_{12} from the well; apparent protein migration = anodal migration distance of albumin from the well; total protein migration = EEO + apparent protein migration; migration index = EEO/total protein migration.

Interpretation of results. Precipitin lines were most easily seen when the slide was placed over a viewing box with a black background and light impinging on the slide at an angle from a circular fluorescent bulb within the box. With the aid of a loupe (×4 magnification), weak lines were readily visible.

RESULTS

Reiter treponeme axial filaments and sera were subjected to electrophoresis in 1% agarose and various buffers (Table 1) to ascertain the agarose-buffer combination that gave the most satisfactory migrations of both reactants. Initially, borate-borax buffer, pH 8.6 (0.05 M; conductivity, 1.56 mmho/cm at 0°C), appeared to give excellent results. Straight precipitin lines developed rapidly near the midpoint between wells in a very clear gel. However, it was soon recognized that false (presumably nonimmune) precipitin lines formed with some known normal sera (7). Although these spurious precipitates could usually be distinguished from antibody filament precipitates by their curved shape, with the concave side near the serum well, the results were sometimes difficult to interpret. Bänffer et al. (2) had encountered similar reactions with some fresh sera, but not after these specimens had been frozen, thawed, and re-inactivated (heated at 56°C). Such treatment had no effect upon the false reactions we observed.

Electrophoresis with gels in tris(hydroxymethyl)aminomethane-glycine buffer, pH 9.2 (0.05 M; conductivity, 0.2 mmho/cm), also gave

TABLE 1. *EEO flow and electrophoretic migration in various buffer systems*[a]

Buffer	pH	mmho/cm at 0°C	Gel	EEO[b] (mm)	APM[c] (mm)	Total protein migration[d] (mm)	Migration index[e]
Boric acid-sodium borate	8.6	1.56	Agarose	5	7	12	0.42
Tris(hydroxymethyl)-aminomethane-glycine	9.2	0.2	Agarose	5	10	15	0.33
Tris(hydroxymethyl)-aminomethane-glycine + 0.025 M NaCl	9.2	1.66	Agarose	4	13	17	0.25
Barbital-HCl	8.6	1.56	Agarose	2	8	10	0.2
Barbital-boric acid-Na_2HPO_4	8.6	3.45	Agarose	3	8	11	0.27
Barbital-HCl	8.6	1.56	Purified agar	5	7	12	0.42

[a] A current of 140 V was applied for 2 h.
[b] EEO = cathodal migration distance of vitamin B_{12} from well.
[c] Apparent protein migration (APM) = anodal migration distance of albumin from well.
[d] Total protein migration = EEO + APM.
[e] Migration index = EEO/total protein migration.

false precipitin reactions. In addition, haloes of condensation formed around the serum well, making precipitins difficult to read. Increasing the ionic strength of the buffer with NaCl (conductivity, 1.66 mmho/cm) corrected the latter difficulty, but not the former.

The use of sodium barbital-HCl buffer, pH 8.6 (0.05 M; conductivity, 1.56 mmho/cm), eliminated the nonspecific precipitin lines observed with the former two buffers. However, several sera were negative which had given weak filament precipitates in the borate-boric acid buffer. A second barbital buffer (barbital-borax-PO_4) of higher ionic strength (conductivity, 3.45 mmho/cm; pH 8.6) was then tried, but sera with low concentrations of antibody still failed to give precipitin lines.

In further efforts to enhance the sensitivity of the reaction in this respect, various agarose concentrations between 0.5 and 1% in barbital-HCl were compared, but again there was no improvement. However, when agar preparations were investigated, 1% purified agar (Difco Laboratories, Detroit, Mich.) yielded results comparable to those of agarose in borate-boric acid buffer, without the nonspecific precipitin lines of the latter. The increased EEO with purified agar, equal to that with agarose in borate-boric acid buffer (Table 1), probably accounted for the similar high reactivities of the two systems. Use of the second barbital buffer mentioned above was not pursued, as a precipitate developed in the agar. Therefore, sodium barbital-HCl buffer, pH 8.6, with 1% purified agar was used for all subsequent studies.

Positive sera titrated against different concentrations of the axial filaments revealed that antigen concentration also affected the results of the test. The optimal antigen concentration proved to be 125 μg of protein per ml. At this protein level, strongly positive sera were reactive up to a twofold greater dilution than that required for a reaction with antigen twice as concentrated. In addition, some sera which were negative or produced a barely visible line with more antigen were unquestionably positive. At an antigen concentration below 125 μg/ml, weakly positive sera did not give visible precipitates.

Sera with high antibody titers that were subjected to electrophoresis with paper wicks at high voltage produced visible lines in 30 to 40 min, whereas 90 min or longer was required for visible reactions with some weak sera. In contrast, precipitin lines with high-titer sera were detected as early as 10 min when Telfa wicking and lower voltage were used. Regardless of the method used, an occasional serum was positive only if further diffusion was allowed. For this reason, all slides with negative results were stored in a moist chamber overnight before a final reading was made. Definition of very weak or questionable lines was increased by rinsing the slide in water and then immersing it in 2% tannic acid for 3 to 5 min.

When optimal conditions for the test had been established, sera were chosen at random for testing, and the diagnosis of each patient was not checked until after the results had been recorded. A total of 343 sera in different categories were tested. Confidence that the test was reproducible was established by testing each serum with two antigen preparations at different times. Duplicate results were obtained with 334 sera (97%) and are shown in Table 2. Of the 9 sera with discrepant results, 1 was in the treated secondary and 8 were in the treated late syphilis groups; each had given a very faint precipitin

TABLE 2. *CIE results of 138 nonsyphilitic and 196 syphilitic sera with Reiter axial filaments*[a]

Diagnosis	CIE		% Positive
	No. positive	No. negative	
Normal	0	106	0
Untreated primary syphilis	40	8	83
Treated primary syphilis	8	12	40
Untreated secondary syphilis	45	0	100
Treated secondary syphilis	5	4	55
Treated late syphilis	67	7	90
Biological false-positive (chronic)	0	32	0

[a] Reproducible results were obtained with each serum and two different axial filament preparations.

line on the occasion that it was positive. The data indicated that Reiter axial filaments in CIE behaved as a specific antigen in detecting treponemal antibodies. All sera from normal individuals and from chronic biological false-positive patients gave no reaction. In early untreated syphilis, sera from 83% of the primary and 100% of the secondary cases tested were positive. The number of positives (90%) in treated late syphilis was unexpectedly high.

DISCUSSION

In contrast to other available spirochetal antigens that have been used in developing serological tests for syphilis, Reiter treponeme axial filaments contain a single component that is reactive with syphilitic antibody. The characteristics of this antigen were described in a previous study (13), where it was shown that purified filaments are composed of two protein subunits, at least one of which is shared with the pathogenic treponemes. The specificity of any test cannot be questioned when a single antigen is used to detect the presence of antibody, and the data presented in this paper have established that Reiter filaments provide such an antigen. Although this antigen was isolated from a nonpathogenic treponeme, and, therefore, antibodies reacting with it are not necessarily specific for *T. pallidum*, the results presented here, together with numerous evaluations of the RPCF test, suggest that detectable antibody rarely, if ever, develops except in syphilis and the other treponematoses.

A rapid and sensitive test for detecting antitreponemal antibodies in human sera has been developed, using Reiter axial filaments as antigen in CIE. Numerous factors were found to be critical for achieving optimal results. Chopping the filaments into short fragments (20 to 400 nm) by prolonged sonic treatment was previously shown to be essential for ready migration through the gel. Both the buffer and the gelling agent contributed significantly to the results of the test. We found barbital-HCl buffer at pH 8.6, conductivity of 1.56 mmho/cm, far superior to any other buffer system tried. No spurious precipitin lines appeared, and all visible precipitates could be considered positive reactions. Using this buffer, the greatest sensitivity, i.e., the least amount of antibody required for a visible precipitate, was achieved when purified agar was the gel. The filament protein concentration of the antigen suspension was another factor important to the CIE reaction. In our preparations, 125 μg of protein per ml (approximately 1.0 μg per test) was optimal.

The results of detecting syphilitic antibody with Reiter axial filaments in the CIE test were shown to be very reliable. Sera from 343 patients were each tested at different times with two antigen preparations, and results with 97% of the sera were the same on both occasions. Although overnight incubation was required for maximal sensitivity, precipitin bands with most positive sera were readily visible at the end of the electrophoresis period. Therefore, the majority of results were achieved quickly, and all reactions were easily interpretated without bias. As the study sera were all from individuals whose statuses with respect to syphilis were well documented clinically and/or serologically, the specificity and sensitivity of the test, in an epidemiological sense, could be determined. Based on the negative results with all 138 sera from normal individuals and those with chronic biological false-positive reactions, the specificity in this series was 100%. Conversely, reactive tests with 85 of the 93 sera from untreated early syphilis gave a sensitivity of 91%.

Several factors contributed to the very high specificity and sensitivity achieved in this study. The highly purified filaments eliminated false reactions from antibodies cross-reacting with other treponemal components contaminating the antigenic reagent (4). The spurious, nonimmune precipitates (7) that occurred with some buffers were not observed with barbital-HCl. Like Ghinsberg et al. (12), we did not encounter nonspecific reactions preventable by freezing, thawing, and reheating serum before testing, as described by Bänffer et al. (2). This was not surprising, as most of our study samples had been repeatedly frozen and thawed in the past.

The use of a gel with high EEO was largely responsible for the marked sensitivity we found;

it eliminated the need for the preliminary electrophoresis of serum alone that Bänffer et al. (2) used to achieve the same end. The sensitivity was also increased by use of the optimal concentration of antigen (125 μg of protein per ml). This prevented negative results from soluble immune complexes, which we found with higher concentrations of antigen. The exceptionally high proportion of positive tests with sera from primary syphilitics (83%) and treated late infections (90%) suggested that the reaction was detecting unusually low levels of antibody.

CIE is the first immunological technique used to evaluate the purified axial filaments as antigen in a serological test for syphilis. Having proved to be both specific and sensitive in this test, this antigen will be applied to other techniques that can be more readily performed in a quantitative fashion and yet have equal or greater sensitivity. To date, the only other method tried has been complement fixation, but the comparatively large size of the filament fragments rendered them an insensitive antigen in this reaction. A quantitative hemagglutination test may be achieved by coupling filaments to erythrocytes. Also, the more recently described enzyme-linked immunosorbent assay (11) may offer the opportunity to use a method in which the final reaction step is dependent on color change and can be measured spectrophotometrically. These various procedures will be investigated, and their merits will be compared with those of CIE.

ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service research grant 5 RO1 AI02336 from the National Institute of Allergy and Infectious Diseases and by the World Health Organization.

LITERATURE CITED

1. **Bänffer, J. R. J.** 1972. Demonstration of precipitins against a treponemal antigen by counterimmunoelectrophoresis. Lancet i:996-997.
2. **Bänffer, J. R. J., S. Bissumbhar, and J. H. Bekker.** 1974. Evaluation of the counterimmunoelectrophoresis technique in syphilis serology. Br. J. Vener. Dis. **50**:101-103.
3. **Bänffer, J. R. J., S. Raghoenath, and A. M. Hulst.** 1975. Comparison of the counter-immunoelectrophoresis technique with the Reiter protein and three other serological tests as a first line test for syphilis. J. Clin. Microbiol. **2**:361-367.
4. **Bekker, J. H.** 1962. Limitations of the Reiter protein complement-fixation (RPCF) test. Br. J. Vener. Dis. **38**:131-133.
5. **Bussard, A.** 1959. Description d'une technique combinant simultanément l'électrophorèse et la précipitation immunologique dans un gel: l'électrosynérèse. Biochim. Biophys. Acta **34**:258-260.
6. **Chase, M. W., and C. A. Williams.** 1968. Chemical analyses. A. Protein analysis, p. 273-275. *In* C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunochemistry, vol. 2. Academic Press Inc., New York.
7. **Crowle, A. J.** 1973. Immunodiffusion, p. 174-179. Academic Press Inc., New York.
8. **D'Alessandro, G., and L. Dardanoni.** 1953. Isolation and purification of the protein antigen of the Reiter treponeme. A study of its serologic reactions. Am. J. Syph. Gonorrhea Vener. Dis. **37**:137-150.
9. **D'Alessandro, G., F. G. Oddo, R. Comes, and L. Dardanoni.** 1949. Sulla structure antigene del *T. pallidum*. Ricerche sullo stipite coltivable di Reiter. Riv. Ist. Sieroter. Ital. **24**:134-166.
10. **DeBruijn, J. H., and J. H. Bekker.** 1957. De toepassing van een eiwit-antigeen van *Treponema pallidum* (reiter-stam) bij de complementbindingsreactie. Ned. Tijdschr. Geneeskd. **101**:1615-1617.
11. **Engvall, E., and P. Perlmann.** 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry **8**:871-874.
12. **Ghinsberg, R., B. W. Grunbaum, and G. Blumstein.** 1977. Rapid detection of specific treponemal antibodies by counterimmunoelectrophoresis using an extract of *Treponema pallidum*. Isr. J. Med. Sci. **13**:557-560.
13. **Hardy, P. H., Jr., W. R. Fredericks, and E. E. Nell.** 1975. Isolation and antigenic characteristics of axial filaments from the Reiter treponeme. Infect. Immun. **11**:380-386.
14. **Nelson, R. A., and M. M. Mayer.** 1949. Immobilization of *Treponema pallidum in vitro* by antibody produced in syphilitic infection. J. Exp. Med. **89**:369-393.
15. **U.S. Department of Health, Education and Welfare.** 1957. Serology evaluation and research assembly. Public Health Service Publication no. 650. U. S. Government Printing Office, Washington, D.C.