

## Antigenic Cross-Reactivity Between *Treponema pallidum* and Other Pathogenic Members of the Family *Spirochaetaceae*

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The antigenic cross-reactivity between *Treponema pallidum* and several pathogenic members of the family *Spirochaetaceae* was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting techniques. Blots of *T. pallidum* antigens were incubated with antiserum from rabbits infected or immunized with *T. pallidum*, *Treponema paraluisuniculi*, *Treponema hyodysenteriae* (strains B204 and T22), *Borrelia hermsii* serotype 7, or *Leptospira interrogans* serogroup Canicola. *T. pallidum* contained 22 antigenic molecules ranging from 85,000 to 12,000 daltons which were recognized by serum from rabbits infected with *T. pallidum*. Serum from rabbits infected with *T. paraluisuniculi* cross-reacted with 21 of these molecules and faintly reacted with a band at 15,000 daltons which was not recognized by anti-*T. pallidum* serum. Antisera directed against strains B204 and T22 of *T. hyodysenteriae* cross-reacted with 11 and 10 antigens of *T. pallidum*, respectively. *B. hermsii* and *L. interrogans* serogroup Canicola antisera detected 11 and 10 treponemal antigens, respectively. Many of the *T. pallidum* antigens detected by antisera against *T. hyodysenteriae*, *B. hermsii*, or *L. interrogans* serogroup Canicola have been previously identified as containing moieties also found on the nonpathogenic *Treponema phagedenis*, biotype Reiter, and may therefore represent group antigens common to members of the family *Spirochaetaceae*.

Members of the family *Spirochaetaceae* are slender, helically coiled bacteria that exhibit a characteristic corkscrew motility. They vary in length from 3 to 500  $\mu\text{m}$  and consist of an outer envelope and a protoplasmic cylinder, between which lie axial filaments (flagella) (31). Three genera of this family contain human pathogens. The genus *Treponema* includes organisms that cause chronic granulomatous diseases in humans: *Treponema pallidum* (venereal and endemic syphilis), *Treponema pertenue* (yaws), and *Treponema carateum* (pinta). Animal pathogens are *Treponema paraluisuniculi* (rabbit spirochetosis) and *Treponema hyodysenteriae* (swine dysentery). Members of two genera produce acute diseases in both humans and animals: *Borrelia* (relapsing fever) and *Leptospira* (leptospirosis, Weil's syndrome) (32). These genera have distinctive morphological features. *T. hyodysenteriae* is the only pathogenic treponeme that can be continuously cultivated in vitro; *Borrelia* and *Leptospira* species can also be cultivated on artificial media (16).

Immunological cross-reactivity has been reported among the treponemes and, to a lesser degree, between species of *Treponema* and *Borrelia*. Among the pathogenic treponemes, most comparisons have been made between *T. pallidum* and *T. pertenue*; several experiments have demonstrated variable degrees of cross-immunity based upon symptomatic reinfection to challenge with heterologous organisms (23, 26, 30, 37) and serological cross-reactivity (3, 16). The molecular basis for immunological cross-reactivity between *T. pallidum* and *T. pertenue* has also been described previously (4, 5, 34). Infection with *T. paraluisuniculi* confers less protection against challenge with *T. pallidum* than does *T. pertenue* (38). Graves demonstrated partial resistance to intradermal challenge with *T. pallidum* in rabbits infected with *T. paraluisuniculi*; the extent of protection was dependent upon the length of the immunizing

infection and the size of the challenge inoculum (9, 10). Khan demonstrated that serological cross-reactivity between *T. paraluisuniculi* and *T. pallidum* or *T. pertenue* paralleled the results of challenge experiments: immobilizing titers were 6- to 12-fold lower against heterologous organisms compared to homologous organisms (17). Little is known of the antigenic relationship between *T. hyodysenteriae* and *T. pallidum* except that immunization of rabbits with *T. hyodysenteriae* fails to provide any protection against challenge with *T. pallidum* (D. L. Harris, communicated by L. A. Joens, University of Arizona, Tucson).

Members of the genus *Borrelia* appear to have some antigenic cross-reactivity with the treponemes as determined by agglutination tests (29). *Borrelia*-infected mice produce antibody that reacts in the fluorescent treponemal antibody-absorbed (FTA-ABS) test; conversely, asymptomatic syphilitic infection in mice yields antibody that gives positive immunofluorescence against *Borrelia duttoni* (41). Absorption of sera with protein antigen from the nonpathogenic *Treponema phagedenis*, biotype Reiter, removes these cross-reactive antibodies. Plasma from guinea pigs infected with *Borrelia hispanica* reacts by immunofluorescence against *T. pallidum*; this reactivity can also be absorbed by using sonicated Reiter treponemes (2). In contrast, Coffey and Eveland were unable to show any serological cross-reactivity between the four major serotypes of *Borrelia hermsii* and *T. pallidum* by immunofluorescence of whole organisms (8).

Members of the genus *Leptospira* possess several morphological and biochemical characteristics that distinguish them from the other genera, including hooked or bent ends, aerobic metabolism, and distinctive lipid components (36). No published references of immunological cross-reactivity between *Treponema* and *Leptospira* could be found; however, occasional biological false-positive, nontreponemal tests for syphilis have been reported to occur in Weil's syndrome (33).

The present investigation was undertaken to examine more closely the nature of antigenic cross-reactivity among

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pathogenic members of the *Spirochaetaceae* with reference to *T. pallidum*. Antigens common to *T. pallidum* were identified by staining Western blots of separated *T. pallidum* polypeptides with antisera produced against *T. paraluiscuniculi*, two strains of *T. hyodysenteriae*, and *T. pallidum*, Nichols strain. Comparisons were also made with antisera to the more distant relatives, *B. hermsii* and *Leptospira interrogans*, serotype Canicola.

(This study was presented in part previously [S. A. Baker-Zander and S. A. Lukehart, Annu. Meet. Am. Soc. Microbiol. 1983, E31, p. 81]).

#### MATERIALS AND METHODS

**Animals.** Adult male New Zealand white rabbits were obtained from a local supplier (R & R Rabbitry, Stanwood, Wash.). Each rabbit was tested for evidence of *T. paraluiscuniculi* infection by the VDRL (Venereal Disease Research Laboratory) and the FTA-ABS tests. Only those rabbits with nonreactive serological tests as well as absence of clinical signs of infection were included in this study. All rabbits were housed individually at 19 to 20°C and were given antibiotic-free food and water.

**Source of spirochetes.** *T. pallidum*, Nichols strain, was propagated in rabbits by testicular passage as previously described (22). *B. hermsii* serotype 7 was provided by P. Perine, Uniformed Services University, Bethesda, Md.; *L. interrogans* serogroup Canicola (Moulton strain) was provided by R. C. Johnson, University of Minnesota, Minneapolis; and L. A. Joens, University of Arizona, Tuscon, provided Formalin-fixed *T. hyodysenteriae* B204. *T. paraluiscuniculi* infection in rabbits received from a local supplier was identified by the presence of symptoms or reactivity in the VDRL and FTA-ABS tests.

**Sera and antisera.** Nonimmune (normal) rabbit serum was obtained from four VDRL and FTA-ABS nonreactive rabbits and pooled. Rabbit anti-*T. pallidum* sera were obtained from four rabbits 3 to 4 months after intratesticular infection and pooled. Sera were collected and pooled from four rabbits with *T. paraluiscuniculi* infection as identified above or from rabbits which were infected by transfer of lymph nodes from the index cases (infection in the recipient animals was confirmed by development of darkfield positive lesions or seroconversion or both). These sera were provided by R. DiGiacomo, University of Washington, Seattle. Rabbit antisera to *T. hyodysenteriae* T22 and B204 were obtained from L. Joens. Preimmunization sera from these rabbits were nonreactive when tested against treponemal antigens by enzyme-linked immunosorbent assay. A pool of antiserum to *B. hermsii* was produced in our laboratory by intramuscular inoculation of two adult male rabbits with  $10^8$  *B. hermsii* serotype 7 cells emulsified in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The rabbits received booster injections at 3 weeks and were bled for serum two weeks later. Pooled rabbit antisera to *L. interrogans* serogroup Canicola (Moulton strain) were similarly prepared by using  $7.5 \times 10^8$  organisms for immunization. All rabbits used for antiserum production in our laboratory were VDRL and FTA-ABS nonreactive. Sera collected in this laboratory were obtained by cardiac puncture or from the medial ear artery and were allowed to clot at room temperature for 2 h in sterile glass tubes. Clotted blood was centrifuged at  $250 \times g$  for 10 min; the serum was withdrawn and samples were stored frozen at  $-20^\circ\text{C}$ .

**Serological tests.** The VDRL slide flocculation and FTA-ABS tests were performed as described in the *Manual of Tests for Syphilis* (7) with modifications (21). Antisera were

tested for reactivity against *T. pallidum*, *T. hyodysenteriae* (strain B204), *B. hermsii* (serotype 7), and *L. interrogans* serogroup Canicola (Moulton strain) by an immunofluorescence (IF) test. Approximately  $2 \times 10^5$  washed organisms were placed on slides, allowed to air dry, and fixed with 10% methanol for 20 s. Serial twofold dilutions of antisera were added to the slides and incubated for 30 min at 37°C. The slides were washed 3 times (5 min each) in phosphate-buffered saline. Fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (1:1,600) (Cappel Laboratories, West Chester, Pa.) diluted in phosphate-buffered saline-2% Tween 80 was then added to each slide and incubated for 30 min at 37°C. The slides were washed; mounting medium and cover slips were applied. Slides were examined on a Zeiss fluorescence microscope, and the degree of fluorescence was recorded on a 1 to 4<sup>+</sup> scale.

**Preparation of samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** *T. pallidum* antigens were prepared by the method of Lukehart et al. (20). Organisms were washed once in 10 mM Tris-hydrochloride (pH 7.5) and diluted in 10 mM Tris to a concentration of  $10^{10}$  treponemes per ml. The preparation was sonicated on ice for 6 min (20 KHz, 200 W) (Sonicator Ultrasonic Cell Disrupter, Ultrasonics, Inc., Plainview, N.Y.) and diluted 1:1 in 0.1 M Tris-hydrochloride (pH 6.8) containing 2% SDS, 2% 2-mercaptoethanol, and 20% glycerol. The antigen was boiled for 5 min, and bromphenol blue was added as a tracking dye.

**SDS-PAGE.** Approximately  $2 \times 10^8$  solubilized organisms (ca. 25  $\mu\text{g}$  of protein) were applied to each lane (5 mm wide) and electrophoresed on 12.5% polyacrylamide slab gels (1.5 mm thick by 12 cm long) in the discontinuous Tris-glycine system as described by Laemmli (18). Electrophoresis was continued until the dye front had migrated to within 1 cm of the bottom of the gel. Gels were fixed and stained with 0.25% Coomassie brilliant blue or were used for Western blotting. Protein standards for estimating molecular weights (14,400 to 92,500; Bio-Rad Laboratories, Richmond, Calif.) were also included; approximate molecular weights were determined by the method of Weber and Osborn (40).

**Western blots.** Electrophoretic transfer of proteins to 0.2- $\mu\text{m}$  nitrocellulose paper (Millipore Corp., New Bedford, Mass.) (Western blot) was performed by the technique of Towbin et al. (35), using a Trans Blot Cell (Bio-Rad). Our earlier blotting procedure (20) was modified (4); proteins were transferred at 5 to 7 V for 18 h at room temperature in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Efficacy of transfer was confirmed by staining of the transferred proteins and molecular weight markers with amido black (35). Blots were cut into individual lanes and stained as previously described (4) with antiserum (1:100 or 1:50 in 6 ml) and  $^{125}\text{I}$ -labeled staphylococcal protein A ( $^{125}\text{I}$ -protein A) (New England Nuclear Corp., Boston, Mass.) (ca.  $10^6$  cpm per lane) diluted in 0.5% bovine serum albumin-10 mM Tris-saline (pH 7.4) containing 0.2% Triton X-100. Blots were exposed for 18 h to Cronex MRF-32 film (Du Pont Co., Wilmington, Del.) at  $-70^\circ\text{C}$  with enhancing screens.  $^{125}\text{I}$ -protein A alone failed to bind treponemal antigens.

#### RESULTS

**Serological reactivity of antisera used for Western blotting.** Antisera used in this study were tested by IF against both homologous organisms (when available) and *T. pallidum* Nichols strain. All antisera reacted with *T. pallidum* or the homologous spirochete with 4<sup>+</sup> fluorescence. Twofold serial dilutions of each antiserum were made; the resulting titers

are reported in Table 1. The endpoint of the test was the highest dilution that still yielded 2<sup>+</sup> fluorescence. All antisera tested demonstrated IF titers of 1:1,024 or greater against the homologous spirochete. With the exception of the anti-*Leptospira* preparation, all antisera had IF titers of 1:512 or greater against *T. pallidum*. *L. interrogans* antiserum, however, had a high titer ( $\geq 1:2,048$ ) when tested against the homologous organism, but a titer of only 1:4 when tested against *T. pallidum*.

***T. pallidum* antigens in rabbit spirochetosis.** Western blots of *T. pallidum* Nichols strain were incubated with pooled *T. pallidum* or *T. paraluisuniculi* antiserum and <sup>125</sup>I-protein A. The resulting autoradiographs are shown in Fig. 1. Twenty molecules containing common antigenic determinants are revealed: major reactivity is seen at 80,000, 69,000, 48,000, 43,000, 41,000, 37,000, 35,000, 33,000, 30,000, and 14,000 daltons. Faint bands are also apparent with both antisera at 85,000, 75,000, 54,000, 37,500, 28,000, 24,500, 24,000, 19,000, 14,500, and 12,000 daltons. Two obvious differences are seen between the staining patterns of these two antisera, however. *T. paraluisuniculi* antiserum faintly stains a protein band at 15,000 daltons, whereas *T. pallidum* antiserum reveals molecules at 16,000 and 15,500 daltons. Also, *T. paraluisuniculi* antiserum stains the 80,000- and 12,000-dalton protein bands much less intensely than does *T. pallidum* antiserum.

***T. pallidum* antigens in swine dysentery.** Western blots of *T. pallidum* Nichols strain were incubated with antisera raised against *T. hyodysenteriae* B204 (a swine pathogen) and T22 (pathogenic for mice but not for swine) and <sup>125</sup>I-protein A. The resulting antigenic profiles are shown in Fig. 2. Both strains of *T. hyodysenteriae* have antigens in common with *T. pallidum* on molecules having molecular weights of 80,000, 69,000, 48,000, 43,000, 41,000, 37,000, 35,000, 33,000, and 30,000. Strain B204 antiserum also shows very minor reactivity with a band at 37,500 daltons. Additionally, strains B204 and T22 antisera show faint reactivity against molecules of *T. pallidum* with approximate molecular weights of 65,000 and 70,000, respectively. These two bands are not seen with pooled rabbit anti-*T. pallidum* serum.

**Antigens common to other pathogenic spirochetes.** Western blots of *T. pallidum* Nichols strain (Fig. 2) were also incubated with antisera against *L. interrogans* serogroup Canicola (Moulton strain) and *B. hermsii* serotype 7. Both antisera revealed antigenic identity with *T. pallidum* determinants on molecules of 80,000, 69,000, 48,000, 35,000, 33,000, and 30,000 daltons, with very faint reactivity at 41,000 and 28,000 daltons. In addition, *B. hermsii* antiserum showed reactivity with bands at 85,000 and 75,000 daltons, as well as faint reactivity with an 87,000-dalton molecule that

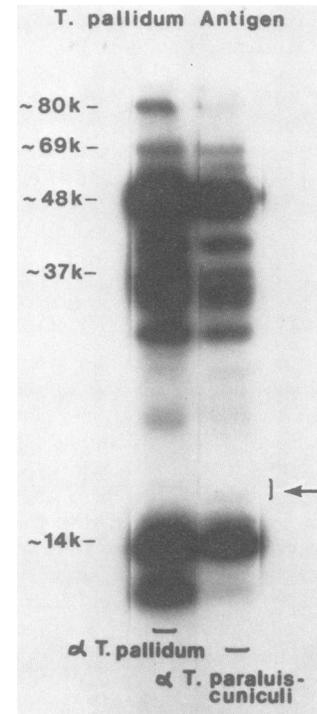


FIG. 1. Antigens of *T. pallidum* Nichols strain, identified by staining with pooled rabbit anti-*T. pallidum* serum (left lane) or pooled rabbit anti-*T. paraluisuniculi* serum (right lane) and <sup>125</sup>I-protein A. Arrow indicates the region of minor differences. Approximate molecular weights are shown.

is not detected with anti-*T. pallidum* sera. *L. interrogans* serogroup Canicola antiserum showed faint reactivity with the 43,000-dalton molecule and strong reactivity with the 37,000-dalton molecule. Faint, hazy reactivity was seen inconsistently with some of the lower-weight molecules when these antisera and the *T. hyodysenteriae* antisera were used. Distinct bands failed to appear, however, even after prolonged exposure to film.

## DISCUSSION

The molecular basis for antigenic cross-reactivity among members of the family *Spirochaetaceae* and within members of the genus *Treponema* was examined with reference to *T. pallidum*, using the techniques of SDS-PAGE and Western blotting. The taxonomic relationship of these organisms is currently based on morphological and biochemical criteria; each displays certain characteristic features of ultrastructure, locomotion, and oxygen requirement (36). We have shown that these organisms can induce antibodies that react with certain protein antigens of *T. pallidum*; a summary schematic of this cross-reactivity is shown in Fig. 3. The Western blot technique involving *T. pallidum* antiserum pooled from syphilitic rabbits detects 22 distinct antigenic bands. These include the 8 major antigens previously reported (20) as well as 14 additional antigenic bands now routinely seen after a modification of our blotting procedure as described earlier (4). The 22 antigenic molecules that we have identified correlate well with those reported by others who have used human syphilitic sera (13, 14, 39) and sera from experimentally infected rabbits (12) in the Western blot. Immunoprecipitation of radiolabeled *T. pallidum* with hu-

TABLE 1. Immunofluorescence titers of rabbit antisera used for Western blotting

Antiserum against	Titer <sup>a</sup>	
	Homologous	<i>T. pallidum</i> Nichols
<i>T. pallidum</i> Nichols	1:1,024	
<i>T. paraluisuniculi</i>	ND <sup>b</sup>	1:512
<i>T. hyodysenteriae</i> T22	ND	1:512
<i>T. hyodysenteriae</i> B204	$\geq 1:2,048$	$\geq 1:2,048$
<i>B. hermsii</i> serotype 7	$\geq 1:2,048$	1:512
<i>L. interrogans</i>	$\geq 1:2,048$	1:4
serogroup Canicola		

<sup>a</sup> The endpoint was highest dilution still yielding 2<sup>+</sup> fluorescence.

<sup>b</sup> ND, Not done; spirochete unavailable for IF testing.

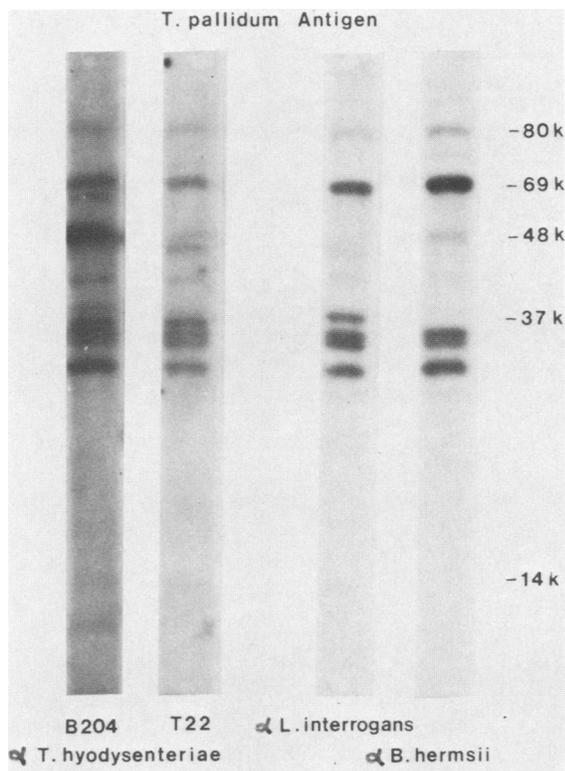


FIG. 2. Antigens of *T. pallidum* Nichols strain, identified by staining with rabbit antisera directed against *T. hyodysenteriae* (strains B204 and T22), *B. hermsii* (serotype 7), and *L. interrogans* (serogroup Canicola).

man syphilitic sera (5, 27) and with sera from experimentally infected rabbits (1) has also revealed numerous antigenic molecules in a similar molecular weight range, although some minor differences do occur.

Of the organisms examined in this study, *T. paraluisuniculi* contained the greatest number of antigenic molecules in common with *T. pallidum*; only subtle differences between the profiles of antigens seen with *T. paraluisuniculi* antiserum and *T. pallidum* antiserum could be discerned. Despite the similarity in antigenic profiles detected by this technique, it is evident that unique determinants probably exist inasmuch as infection with *T. paraluisuniculi* fails to fully protect rabbits against challenge with *T. pallidum* (9, 10, 37), fails to infect humans (11, 19), and produces clinical manifestations with distinctive features (15). Immobilization titers of sera from rabbits infected with either *T. pallidum* or *T. paraluisuniculi* are substantially lower when these antisera are tested against the heterologous, as opposed to the homologous, organism (17). Similar results are seen with agglutination tests (37). However, our pools of *T. pallidum* antisera and *T. paraluisuniculi* antisera both reacted with high titers by a modified fluorescence test in which *T. pallidum* was used as antigen; *T. paraluisuniculi* was unavailable for use in this test.

Comparisons of *T. pallidum* Nichols strain with two strains of the only cultivable pathogenic treponeme, *T. hyodysenteriae* (16), revealed the presence of common antigens on nine molecules, with faint reactivity to several additional molecules not detected with *T. pallidum* antisera. These patterns are significantly different from those seen with antisera to the noncultivable pathogens (*T. pertenuis* [4]

and *T. paraluisuniculi*), perhaps indicating a more distant relationship between *T. hyodysenteriae* and the other pathogenic treponemes. Indeed, the acute gastrointestinal illness caused by *T. hyodysenteriae* is quite different from the chronic, largely cutaneous diseases caused by other pathogenic treponemes. Genetic studies by Miao et al. failed to show DNA homology between *T. hyodysenteriae* or the cultivable nonpathogen *T. phagedenis* biotype Reiter and *T. pallidum* Nichols strain (24, 25), despite the large number of shared antigenic determinants detected in this study and others (6, 14, 20, 27, 28).

Coffey and Eveland (8) prepared antisera against four major serotypes of *B. hermsii* and were unable to show cross-reactivity with *T. pallidum* Nichols or the Reiter treponeme by IF. In contrast, our antisera raised against *B. hermsii* serotype 7 showed reactivity with 11 molecules of *T. pallidum* and was reactive against *T. pallidum* by IF. This may simply reflect differences in the titers of the two sera; Coffey's immunization regimen differed from ours and did not include the use of adjuvant. The presence of common antigens in *T. pallidum* and *Borrelia* species is supported by the ability of syphilitic human and rabbit sera to mildly agglutinate an HCl-extracted antigen of *Borrelia anserina*, a fowl pathogen (29). Further, plasma of guinea pigs infected with *B. hispanica* (3) and serum of mice infected with *B. duttoni* (41) reacted by IF against *T. pallidum*. This reactivity could be removed by absorption with the Reiter treponeme. It is possible that the cross-reactive antigens demonstrated here represent common antigens expressed by both pathogens and nonpathogens; indeed, a large number correspond to antigens previously identified on the Reiter treponeme (6, 14, 20, 27). The ability of extracts of Reiter treponemes to absorb antibody reactivity against *T. pallidum* from sera of *Borrelia*-infected animals (2, 41) supports this hypothesis.

Although antisera raised against *L. interrogans* serogroup Canicola detected the presence of 10 common antigens on molecules of *T. pallidum*, no previously published reports of cross-reactivity between *T. pallidum* and members of the genus *Leptospira* could be found. Despite a very high IF titer ( $\geq 1:2,048$ ) against the homologous organism, our rabbit anti-*Leptospira* serum demonstrated an IF titer of only 1:4 against *T. pallidum*. The extent of molecular cross-reactivity that we found may be explained by alteration or denaturation of antigenic molecules which occurs during SDS-PAGE and Western blotting techniques. Antigenic sites not accessible in an IF assay might thus be revealed, allowing the identification of common determinants heretofore unreported.

Antisera directed against *T. paraluisuniculi*, as well as *T. hyodysenteriae* strains and *B. hermsii*, showed faint reactivity in the Western blot against molecules of *T. pallidum* which were not detected by the use of antisera directed against *T. pallidum*. These results might be a reflection of different methods of antiserum production which might cause differences in avidity or affinity of antibodies within a given antiserum; some antisera were obtained from infected animals, whereas others were produced by artificial immunization. However, antiserum produced against *T. pallidum* in our laboratory by artificial immunization, using incomplete Freund adjuvant, binds the same profile of treponemal antigens in the Western blot as serum from actively infected rabbits. This suggests that the results described above reflect real differences between the organisms. They may indicate the existence of numerous antigenic determinants present on individual molecules, only a few of which are shared between *T. pallidum* and the other organisms. Alternatively,

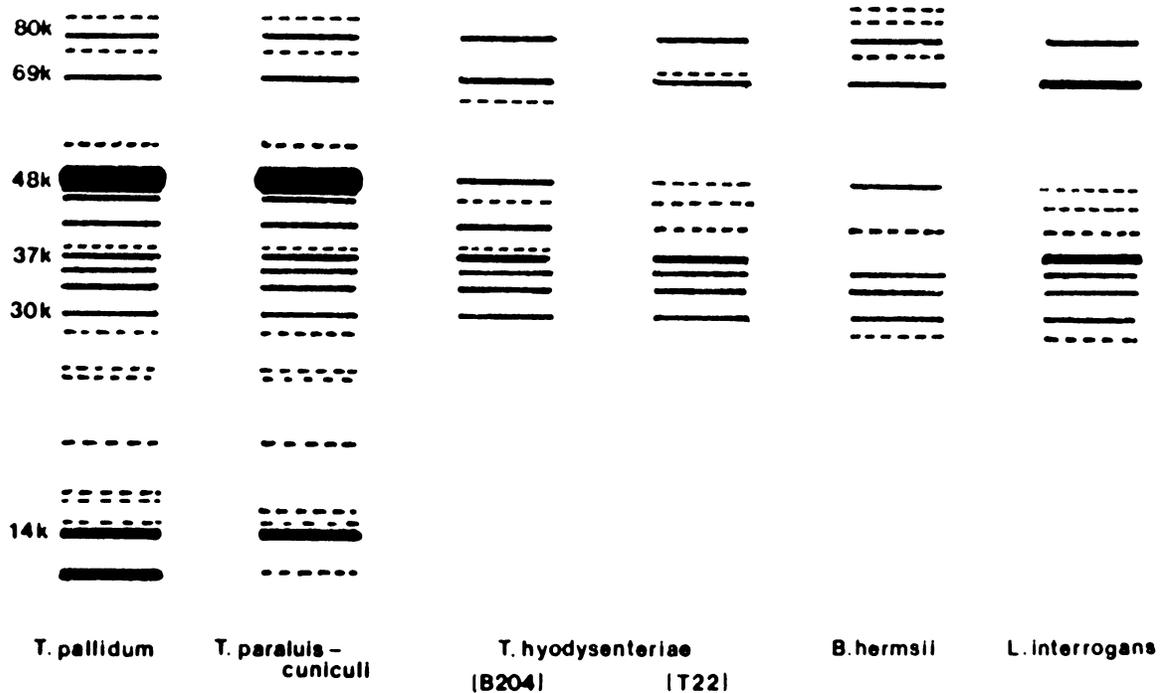


FIG. 3. Summary schematic depicting the SDS-solubilized antigens of *T. pallidum* Nichols strain revealed in the Western blot technique by antisera as indicated. Dotted lines indicate weakly reactive antigens; heavy lines indicate molecules with intense reactivity. Approximate molecular weights are shown.

moieties with common peptide sequences may occur on molecules present in each organism but may be exposed for immunological recognition in only one. Although this Western blot procedure only allows the examination of protein or protein-containing molecules that can withstand the conformational changes caused by SDS solubilization, and cannot distinguish between molecules which may comigrate at the same molecular weight, it remains one effective technique for evaluating bacterial antigens. Further studies, including serum absorption, two-dimensional gels, and peptide mapping, are required to dissect the biochemical and immunological nature of the shared and unique antigens of *T. pallidum*.

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