Serodiagnosis of Syphilis: Antibodies to Recombinant Tp0453, Tp92, and Gpd Proteins Are Sensitive and Specific Indicators of Infection by Treponema pallidum

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Syphilis serodiagnosis relies on a combination of nonspecific screening tests (antilipoidal antibodies) and Treponema pallidum-specific tests (anti-T. pallidum antibodies). We studied a group of six recombinant T. pallidum antigens for their sensitivities and specificities with sera from individuals with syphilis (n = 43), relapsing fever (n = 8), Lyme disease (n = 8), and leptospirosis (n = 9) and from uninfected individuals (n = 15). Three recombinant proteins, Tp0155, Tp0483, and Tp0751, demonstrated sensitivity values that ranged from 28 to 42%. In contrast, three other recombinant proteins exhibited the following sensitivity and specificity values: Tp0453, 100% sensitivity and 100% specificity; Tp92 (Tp0326), 98% sensitivity and 97% specificity; and Gpd (Tp0257), 91% sensitivity and 93% specificity. Tp0453, Tp92, and Gpd also were recognized by sera from individuals with early primary syphilis that were nonreactive with the antitrepotinal Veneral Disease Research Laboratory test. The reactivities of syphilis patient sera with Tp0453, Tp92, and Gpd were proportional to the titers of these sera with the treponemal test MHA-TP (microhemagglutination assay for T. pallidum). Thus, the recombinant T. pallidum antigens Tp0453, Tp92, and Gpd show promise as diagnostic antigens in the enzyme-linked immunosorbent assay-based assay.

Syphilis is a sexually transmitted disease caused by infection with the spirochete bacterium Treponema pallidum subsp. pallidum. Primary syphilis is characterized by a chancre, typically containing large numbers of spirochetes. Secondary syphilis presents as a generalized rash in which each characteristic lesion contains a high bacterial burden. Upon resolution of the secondary stage of syphilis, the disease enters a latent, asymptomatic phase. This stage of syphilis can typically be divided into an early latent phase (estimated onset of infection within the last 2 years) and a late latent phase (estimated duration of infection of >2 years). In the preantibiotic era, approximately 30% of those infected progressed to tertiary syphilis, where manifestations included neuropsychiatric illness, syphilitic gummas, and bone and cardiovascular involvement.

Syphilis diagnosis during the early primary stage can be accomplished by dark-field microscopy of primary chancre samples for the presence of spirochetes. Following the resolution of the primary chancre and in clinics lacking dark-field microscopy, the mainstay of syphilis diagnosis is a variety of serologic tests. The most common screening tests are the rapid plasma reagin and Venereal Disease Research Laboratory (VDRL) tests, both of which test for the presence of antilipoidal antibodies. Because neither of these tests assays for syphilis-specific antibodies, there are problems associated with both their specificity and their sensitivity. In early primary disease antilipoidal antibodies may not have developed, and in late syphilis (late latent and tertiary) up to 30% of individuals may lack antilipoidal antibodies (27). In addition, because a variety of conditions (e.g., lupus and increased age) lead to antilipoidal antibodies and false-positive results, a confirmatory test is often required. Confirmatory tests include FTA-Abs (fluorescent treponemal antibody absorption test), MHA-TP (microhemagglutination assay for T. pallidum), and TPHA (T. pallidum hemagglutination assay), which use crude T. pallidum antigens (12); tests using whole T. pallidum antigen extracts; and a variety of T. pallidum recombinant protein tests (6–9, 12, 16, 18–23, 27–30, 32).

There are several reasons to evaluate additional recombinant antigens for use in serologic testing for syphilis. First, recombinant protein tests are not widely used and there is no general agreement as to which protein antigens are best for sensitivity and specificity. Second, the whole genome of T. pallidum strain Nichols has been elucidated and new protein-encoding open reading frames (ORFs) are available for testing (5). Finally, surface-exposed proteins may be superior in sensitivity to the proteins currently used because of their immediate exposure to the immune system.

The purpose of this study was to screen recombinant proteins for sensitivity by using sera collected from individuals with syphilis. Recombinant proteins selected for screening included Gpd (Tp0257) (3, 26) and Tp92 (Tp0326) (4), which were previously identified from a serologic screen as being reactive with syphilis patient sera, and proteins predicted by computer analyses of the genome to be putative outer membrane proteins. Since other spirochetal diseases such as Lyme disease, relapsing fever, and leptospirosis would be expected to have antigens most similar to T. pallidum, we used sera col-

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Selected from individuals with these infections to test the specificity of the recombinant antigens for leptospirosis serodiagnostics.

**MATERIALS AND METHODS**

Reagents and serum samples. Human syphilis patient sera were obtained from the collection of the Ludwig Boltzmann Institute for Dermato-Venerological Serodiagnostic, Vienna, Austria. The sera were tested for syphilis serology titers by the VDRL (Dade Behring, Marburg, Germany) and the MHA-TP (Fujirebio, Tokyo, Japan) tests (22). The criteria used to establish the stage of syphilis were as follows: primary, typical chancre present; secondary, generalized typical rash and positive syphilis serology test; early latent, positive syphilis serology tests and a positive immunoglobulin M test to T. pallidum antigens (Moria Syphilis M enzyme-linked immunosorbent assay [ELISA]; Microgen Bioproducts, Camberley, United Kingdom) with no history of treatment and no clinical manifestations; and neurosyphilis, reactive cerebrospinal fluid (CSF) values plus TPHA index values greater than 70 (15). The TPHA index value was equal to the CSF MHA-TP divided by the albumin quotient (CSF albumin divided by serum albumin) and also divided by 1,000. Syphilis stage diagnoses were confirmed by dermatologists and/or neurologists. None of the subjects had been treated for syphilis at the time when the serum was obtained.

Sera from individuals with relapsing fever were obtained from the collection of Rocky Mountain Laboratories, Hamilton, Mont., and from the Centers for Disease Control and Prevention, Fort Collins, Colo. The criteria for relapsing fever diagnosis were compatible clinical history, exposure to tick-borne relapsing fever in eastern Washington or northern Idaho, and high seropositivity (greater than 1:2048) in the Western blot for Borrelia hermsii HS1. Lyme disease patient sera were obtained from the Centers for Disease Control and Prevention collection (Fort Collins, Colo.). The criteria for the diagnosis of Lyme disease were residience in an area of endemicity, clinical manifestations consistent with Lyme disease, and more than four reactive bands by Western blot analysis of Borrelia burgdorferi. Sera from individuals with severe leptospirosis were obtained from the collection of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) from individuals identified in Salvador in the convalescent phase of leptospirosis with clinical histories consistent with leptospirosis. These individuals had laboratory-confirmed diagnoses according to microaglutination test criteria of a fourfold rise in agglutination titers or a reciprocal agglutination titer greater than 1:800. Convalescent-phase sera were obtained 14 to 28 days after hospitalization of the collection of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) from individuals with severe leptospirosis.

**Preparation of recombinant proteins.** ORFs encoding computer-predicted putative outer membrane proteins were PCR amplified from T. pallidum subsp. pallidum (Nichols strain) genomic DNA with primers designed from the coding sequence of each gene (Table 1). Proteins were expressed without the N-terminal signal sequence, with the exception of Gpd (Tp0257), where the signal sequence was retained (Table 2). Where possible, the full-length ORFs were expressed. However, Tp0155 and Tp0751 were toxic to Escherichia coli when expressed as full-length proteins, and as a result N-terminal fragments of these proteins were instead expressed (Table 2). PCR products representing the portion of the ORF encoding the amino acids listed in Table 2 were ligated in frame with expression plasmids. All but one were expressed using the pRSET T7 expression plasmid in the E. coli expression strain BL21(DE3)pLysS (Invitrogen, Carlsbad, Calif.). Tp0326 (Tp92) was expressed in the pBAD TOPO TA expression plasmid in the E. coli strain TOP10 (Invitrogen).

Expression and purification of the resulting N-terminal six-histidine-tagged recombinant proteins were performed as previously described (4). The expressed proteins were renatured by dialysis based upon the renaturation protocol described by Qi et al. (17), with the dialysis modification described by Zhang et al. (31). This procedure has been shown to produce recombinant proteins that most closely resemble native T. pallidum proteins (31). Briefly, 0.5% Zwittergent 3-12 (Calbiochem, San Diego, Calif.) was added to the expressed proteins prior to dialysis against 100 mM Tris (pH 8.0)-200 mM NaCl-10 mM EDTA. Protein quantitation of each of the recombinant proteins was performed with the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

**ELISAs.** Ninety-six-well plates (Maxisorp F9; Costar) were coated overnight at 4°C with 50 μl of the recombinant T. pallidum proteins per well in phosphate-buffered saline (PBS), pH 7.4, with 0.1% sodium dodecyl sulfate at concentrations of 2 μg/ml for Gpd, Tp92, and Tp0483 and 4 μg/ml for Tp0155, Tp0483, and Tp0751. Plates were blocked at room temperature for 2 h with 1× PBS-4% milk. Human sera were diluted 1:200 (Gpd assays) or 1:100 (all other assays) in dilution buffer (1× PBS-4% milk-0.2% Triton X-100). In preliminary studies using serial dilutions (data not shown), a dilution of 1:100 was found to provide optimal sensitivity and specificity for all antigens except Gpd, which was optimal at a 1:200 dilution. The diluted sera were adsorbed overnight at 4°C with a 0.5% (vol/vol) lysate of E. coli expressing an irrelevant Trpansomos cruzi recombinant protein (SA85-1.1) in pRSET (10). This adsorption step was omitted from the sera tested for reactivity to Gpd since preliminary experiments with Gpd showed that this step had no effect on background reactivity. Samples were spun at 4°C at 12,000 × g for 10 min, and 50 μl of each serum was added to triplicate wells.
and incubated for 1 h at room temperature. After washing, 50 μl of a 1:3,000 dilution of goat anti-human (gamma specific) F(ab')2 peroxidase (Sigma-Aldrich, St. Louis, Mo.) was applied and incubated at room temperature for 1 h. Plates were developed for 30 min at room temperature with 100 μl of tetramethylbenzidine-H2O2 substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) per well, and the absorbance at 600 nm was measured.

**Statistics.** The cutoff absorbance values for assigning negative or positive results for each recombinant antigen were defined as the mean plus two times the standard deviation of the absorbance of the uninfected sera. The cutoff values were calculated from the values obtained from the 15 uninfected control sera for Gpd, Tp92, and Tp0453 or from the values from four separate ELISAs of 15 pooled uninfected control sera for Tp0155, Tp0483, and Tp0751. The cutoff values for the recombinant protein ELISAs were as follows: 0.0723 for Tp0155, 0.0667 for Gpd (Tp0257), 0.0576 for Tp92 (Tp0326), 0.0476 for Tp0453, 0.0517 for Tp0483, and 0.0431 for Tp0751. The negative sera were defined as those that yielded absorbance values less than or equal to the cutoff, while the positive sera were defined as those that gave absorbance values greater than this value. Differences between groups were measured by chi-square analysis, and significance was set as \( P < 0.05 \).

**RESULTS**

*T. pallidum* recombinant proteins. Six recombinant *T. pallidum* proteins were tested for their reactivities with sera from individuals with syphilis. Gpd and Tp92 had previously been reported to be strongly immunoreactive with *T. pallidum*-infected rabbit sera. The ORFs Tp0453, Tp0155, Tp0483, and Tp0751 were predicted from the *T. pallidum* subsp. *pallidum* Nichols genome as having a greater than 69% likelihood (Table 2) of encoding outer membrane proteins. These ORFs were selected since outer membrane proteins may be recognized by antibodies earlier in infection and may stimulate persistent immune responses late in infection.

**Sensitivities of the *T. pallidum* recombinant proteins in detecting sera from individuals with syphilis.** The recombinant proteins varied greatly in their recognition by antibodies in sera from individuals with syphilis as judged by ELISA (Fig. 1). Tp92, Tp0453, and Gpd were recognized by almost all of the sera from individuals with syphilis, but many of the syphilis patient sera failed to recognize Tp0155, Tp0483, and Tp0751. The same general pattern of reactivity was seen with sera from individuals with primary, secondary, and early latent forms of syphilis (Fig. 1). Tp0453 was recognized by all of the syphilis patient sera (n = 43). Reactivity to Tp92 and Gpd was nearly as complete, with only 1 and 4 of 43 sera, respectively, failing to show reactivity above background. By chi-square analysis, the difference in sensitivity between Tp0453 and Tp92 was not significant but the difference in sensitivity between Tp0453 and Gpd was significant (\( P < 0.05 \)). Significantly fewer syphilis patient sera reacted with Tp0155 (12 of 43), Tp0483 (18 of 43), and Tp0751 (18 of 43). The absorbance values of sera reactive against Tp0155, Tp0483, and Tp0751 were significantly lower than the values obtained with Tp92, Gpd, or Tp0453 (Fig. 1). These results suggest that the *T. pallidum* proteins Tp02, Tp0453, and Gpd elicit higher antibody levels in syphilis infection and further suggest that Tp0453 and Tp92 are the most sensitive antigens tested in this study.

**Specificity of immunoreactivity to the *T. pallidum* recombinant proteins.** Sera collected from uninfected individuals (n = 15) and individuals with other spirochetal diseases, specifically leptospirosis (n = 9), relapsing fever (n = 8), and Lyme disease (n = 8), were used to determine the specificity of immunoreactivity to Tp92, Tp0453, and Gpd. As shown in Fig. 2, none of the uninfected controls had a significant reactivity to Tp92 or Tp0453, and of the individuals with spirochetal diseases, only one relapsing fever patient serum sample had a marginally positive response to Tp92 (optical density = 0.104, mean plus two times the standard deviation of uninfected controls = 0.0576). More false-positive reactions were demonstrated with Gpd, with one serum from each of the uninfected individual and Lyme disease and relapsing fever patient samples showing absorbance values which were above the cutoff for negativity. Thus, the sensitivities and specificities of these recombinant protein antigens were as follows: Tp0453, 100% sensitivity and 100% specificity; Tp92, 98% sensitivity and 97% specificity; and Gpd, 91% sensitivity and 93% specificity.

**Comparison of immunoreactivity with the *T. pallidum* recombinant proteins with that for present syphilis diagnostic tests.** The VDRL lipoidal antigen test is often used to screen
sera for syphilis seroreactivity. The ELISA-based assay to detect reactivity to Tp0453 was more sensitive than the VDRL test in detecting syphilis seropositivity (Fig. 3). Four serum samples that had a negative VDRL result demonstrated reactivity against recombinant Tp0453 (Fig. 3); these sera were from individuals with early primary syphilis. In general, the absorbance values from the Tp0453, Tp92, and Gpd ELISAs did not correlate well with the VDRL titer (Fig. 3), reflecting the nontreponemal nature of the VDRL assay. In contrast, the immunoreactivity observed with Tp0453, Tp92, and Gpd ELISAs correlated well with the MHA-TP titer (Fig. 4), which is based on crude T. pallidum antigen preparations and represents a T. pallidum-specific serodiagnostic test.

**DISCUSSION**

Recombinant antigens have shown promise for syphilis serodiagnosis. A variety of T. pallidum proteins have been tested including TpN44.5 (TmpA, Tp0768), TpN15 (Tp0171), TpN17 (Tp0435), and TpN47 (Tp0574) (20, 29, 30, 32). These antigens
are sometimes used in combination in commercial tests. These tests have often been shown to identify individuals with active syphilis as well as those who have been treated successfully. From screening many hundreds of serum samples, it has been determined that the sensitivity and specificity of tests employing some of these antigens can be as high as 99.7% (7, 20, 29, 30, 32). However, not all those with early syphilis are detected with the use of these antigens (27), and more sensitive and specific recombinant *T. pallidum* proteins would be useful for syphilis seroscreening.

In this report, we have tested a variety of additional recombinant proteins for their potential suitability as antigens for the serodiagnosis of syphilis. We demonstrate that the putative outer membrane protein Tp0453 has excellent sensitivity for sera from individuals with early syphilis. In addition, the lack of reactivity of this protein with sera from 15 uninfected individuals and 25 individuals with other spirochetal infections, including leptospirosis, Lyme disease, and relapsing fever, demonstrates its specificity.

With the group of sera tested, Tp0453 appeared to have an advantage in sensitivity and specificity compared with Tp92 and Gpd. More extensive testing with larger numbers of sera collected from uninfected individuals and individuals with syphilis will need to be performed to demonstrate if Tp0453 is superior to Tp92, as the slight differences in observed sensitivity and specificity did not reach statistical significance. To define specificity, some reports have screened hundreds or thousands of blood donors for reactivity with commercially available recombinant *T. pallidum* antigen tests (7, 20, 30, 32). These screens have identified donor sera that were reactive with *T. pallidum* recombinant antigens and were also positive with syphilis-specific serologic tests. These sera were probably from individuals who were infected with *T. pallidum*, but since clinical histories are not available interpretation of these studies is difficult. Nonetheless, before the Tp0453 and Tp92 antigens can be definitively demonstrated to be helpful in serodiagnosis, larger numbers of sera will need to be tested by a high-throughput commercial serological assay.

Using recombinant *T. pallidum* antigens to test for syphilis seroreactivity has advantages over lipoidal antigen-based and crude *T. pallidum* antigen tests. Lipoidal antigen-based screening misses up to 30% of sera from individuals with very early and late syphilis (27). In the group of sera that we tested, there were four individuals with early primary syphilis who had no reactivity in the VDRL lipoidal antigen-based screening test, yet had good reactivity with Tp0453 and Tp92 antigens (Fig. 3). These individuals were also positive with the crude *T. pallidum* antigen test MHA-TP. Overall, the MHA-TP results correlated well with the Tp0453 and Tp92 results. However, there is a significant advantage in preparation of recombinant antigens over preparation of crude *T. pallidum* antigen. Recombinant *T. pallidum* antigens can be produced economically and in large quantities in *in vitro* *E. coli* culture, but crude *T. pallidum* antigens must be extracted from treponemes grown within the rabbit animal model. In addition, there is the potential for false-positive reactions with antigens present in the crude *T. pallidum* antigen extracts from rabbit tissues.

Computer analyses predict that Gpd is a lipoprotein (26), and [*14C*]palmitate labeling studies performed by Shevchenko et al. confirm that the protein is lipid modified (25). Similar to Gpd, many of the recombinant antigens used to date for syphilis serodiagnosis are lipoproteins, namely, TpN44.5, TpN15, TpN17, and TpN47. Lipoprotein antigens stimulate a strong immune response which is thought to be due to the ability of lipoproteins to activate antigen-presenting cells through Toll-like 2 receptors (1, 2). Though some of these lipoprotein antigens were originally believed to reside on the surface of the bacterium, these antigens are now thought to be concealed in the periplasm of intact *T. pallidum* (24). Presumably the antibody response to the internal lipoprotein antigens is first stimulated during the widespread phagocytosis and destruction of *T. pallidum* that occur during the clearance stages of early syphilis (14). Though the lipoprotein Gpd showed better sen-
sitivity than did the nonlipoprotein antigens Tp0155, Tp0483, and Tp0751, 4 of 43 (9%) syphilis patient sera, each of which was from individuals with early primary infection, failed to react with Gpd. By contrast, 100% of syphilis patient sera reacted with Tp0453 and 98% reacted with Tp92. In the early stage of infection prior to destruction of spirochetes, antigens that are surface exposed would be visible to the immune system in intact organisms and therefore would have an advantage over internal antigens. By computer models, Tp0453 and Tp92 are predicted to reside in the outer membrane and thus may be surface exposed. This may explain the superior ability of Tp0453 and Tp92 compared to that of Gpd in the detection of antibodies in individuals with early syphilis. Further, after initial clearance in early syphilis, small numbers of presumably intact treponemes remain and may cause late manifestations of disease. Antigens that are surface exposed would have a theoretical advantage in that they are continuously exposed to the immune system and continually stimulate robust antibody responses. This hypothesis was not tested in this study, and future studies should include testing Tp0453 and Tp92 for reactivity with sera from individuals with late syphilis and low antibody responses to T. pallidum.

Tp92 and Gpd are proteins that have homologues in a number of gram-negative bacteria (3, 4, 26). This may explain the reactivity of sera from some individuals with nonsyphilis spirochetal infections to these molecules. Tp0453, which has no sequence conservation of the Treponema pallidum subspecies pallidum glycerophosphodiester phosphodiesterase. FEMS Microbiol. Lett. 2612–2623.


