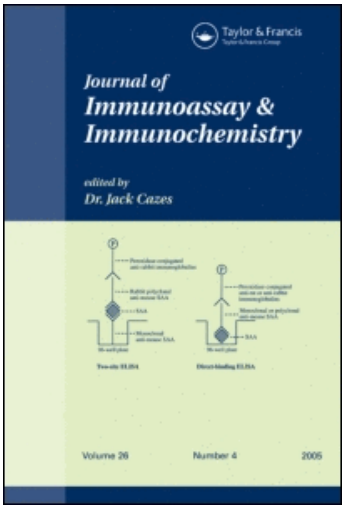

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Serological Diagnosis of Syphilis: Enzyme-Linked Immunosorbent Assay to Measure Antibodies to Individual Recombinant *Treponema pallidum* Antigens

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Abstract: We standardized an indirect ELISA for measurement of serum antibody levels to four individual treponemal recombinant proteins that have been commonly used in a number of commercial EIAs, mostly as a mixture of antigens. When tested with 127 syphilis-negative and 37 secondary syphilis sera, ELISA O.D.s obtained for each of the four antigens clearly distinguished between these two groups of samples. Sensitivity and specificity of 100% was obtained with the current set of samples. Further evaluations with sera from different stages of syphilis can help to define the applications of this ELISA test for each of the four antigens studied.

Keywords: Syphilis, Recombinant antigens, EIA

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INTRODUCTION

Despite advances in molecular diagnostic methods, and except in early primary infection, serology is still the most useful diagnostic approach applicable for almost all phases of syphilis infection.[1] A common testing algorithm for the serological diagnosis of syphilis involves a two-step approach of first screening with relatively inexpensive non-treponemal tests such as VDRL or RPR followed by a confirmatory test that uses treponemal antigen to confirm a positive screening test result.[2] However, this approach is not without limitations, e.g., non-treponemal tests are non-specific that detect anti-cardiolipin antibodies and therefore, may give rise to false positive results in a number of conditions that activate the immune system, such as auto-immune diseases, pregnancy, etc.[3] Besides potential specificity issues, cardiolipin tests like VDRL or RPR are also insensitive for detection of early primary syphilis, late stage infection or neurosyphilis.[4]

With the advent of recombinant DNA technology, and genome sequencing projects,[5] recombinant *Treponema pallidum* protein antigens are being widely used in enzyme immunoassays (EIAs).[6,7] Although such tests are more sensitive and can detect syphilis in all stages of infection, they cannot distinguish between previously treated infections and current active infections which require treatment. Whether quantitative measurements of antibody levels can differentiate between past and active infections has not been examined in detail. In addition, a number of EIAs use a mixture of recombinant *T. pallidum* proteins as antigens,[8] which, therefore, measure total antibody response to a number of antigens. A positive response will not reveal which antigen(s) is or are responsible for most of the activities. Furthermore, different stages of infection may elicit antibodies to different *T. pallidum* antigens.[9,10] Therefore, there is no general agreement as to which antigen or antigens are most suitable for the detection of treponemal-specific antibodies in the different stages of the infection. We have proposed that further studies with individual recombinant *T. pallidum* proteins as antigens in EIA are required in order to have a better understanding on the dynamics and kinetics of the serological response to treponemal antigens during syphilis.[11] In this study, we establish a quantitative indirect ELISA method that measures human serum antibody responses to four individual *T. pallidum* recombinant protein antigens.

EXPERIMENTAL

Serum Samples

One hundred and twenty-seven syphilis-negative and 12 syphilis-positive sera were selected from our collection of archived samples. Based on a consensus result derived from a battery of conventional screening and both conventional

and newer confirmation tests (including VDRL, RPR, FTA-ABS, TP-PA, and INNO-LIA) as described before,[11] sera were classified as syphilis-negative (both screening and confirmatory tests were negative), and syphilis-positive (both screening and confirmatory tests were positive and RPR titer of $>1:8$).

In addition, 25 sera from presumably secondary syphilis cases (defined by a RPR titer of $\geq 1:256$ and positive MHA-TP and FTA-ABS results) were provided by the Ontario Provincial Public Health Laboratory.

Also, commercially available syphilis-positive control (lot number BM124734) and syphilis-negative control (lot number BM121707) serum samples were obtained from BioMedical Resources, Oceanside, California.

Syphilis Serology

Samples were tested with the following syphilis serology tests according to the manufacturers' instructions: (1) RPR test (Pulse Scientific, Burlington, Ontario); (2) VDRL test (VDRL-Cardiolipin-Antigen, Difco, Sparks, Maryland); (3) Serodia-TPPA test (Fujirebio, Inc., Malvern, PA); (4) Virgo FTA-ABS test (Hemagen Diagnostics, Columbia, MD); or Zeus FTA-ABS IFA test (Somagen, Edmonton, AB); and (5) Innogenetics INNO-LIA (Phoenix Bio-Tech Corp., Mississauga, ON).

Indirect ELISA to Measure Antibodies to Individual Recombinant *T. pallidum* Proteins

An in-house recombinant *T. pallidum* antigen based ELISA using TpN15, TpN17, TpN44.5 (TmpA), and TpN47 proteins (Alto Bio Reagents Ltd., Dublin, Ireland), individually, as antigens in the assay, was developed using methods described below. Antigen coating optimization was done using serial dilutions of each antigen to coat wells of a Nunc MaxiSorp 96-well flat bottom Immuno microtiter plate (Nagle Nunc International, Rochester, N.Y.) for a checker-board titration with a known positive control serum (BM124734). Serum samples were tested at a single dilution, which was chosen based on a pilot study of titrating 12 syphilis positive and 35 syphilis negative sera samples. The optimal dilution of enzyme conjugated secondary antibody was also determined by checker-board titrations. Other procedures used in the assay were based on our in-house standard operating procedures.

After optimization, the assay format is as follows: briefly, 100 μL of each individual recombinant protein, TpN15, TpN44.5, TpN47 (at a concentration of 4 μg/ml in PBS), and TpN17 (at a concentration of 2 μg/ml in PBS) were coated onto wells of a microtiter plate at 4°C overnight. Wells were then washed three times with 0.9% saline with 0.05% Tween 20 (saline-Tween), and excess binding sites were blocked by the addition of 300 μL of 2% (w/v)

bovine serum albumin (BSA) in PBS to each well for incubation at 37°C for 90 minutes. After 3 washes with saline-Tween, antigen coated wells along with 'no antigen' control wells were incubated with 100 μL per well of either test serum samples (diluted 1:400 in 2% BSA-PBS) or reactive, weak reactive and non-reactive control serum samples (diluted 1:500 or 1:1,000 in 2% BSA-PBS based on prior titration of the sera) for incubation at 37°C for 90 minutes. Plates were then washed four times with saline-Tween, and incubated with 100 μL per well of a 1:2,000 dilution of horseradish peroxidase conjugated goat anti-human IgG $F(ab')_2$ fragment-specific antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 37°C for 90 minutes to detect all classes of antibodies. After a final washing step of 4 times with saline-Tween, 100 μL per well of a 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate solution (Roche Diagnostic, Laval, Quebec) was added for colour development at room temperature on a shaker for 30 minutes and plates were read at 405 nm.

Statistical Analysis of Data

The ELISA data were analysed by the Wald-Wolfowitz runs test and the Receiver Operating Characteristic (ROC) test using SPSS 15.0.[12]

RESULTS

Titration of Syphilis Control-Positive and Negative Sera Against Individual *T. pallidum* Recombinant Proteins

Figure 1 shows the ELISA titrations of positive (BM124734) and negative (BM121707) control sera towards each of the four recombinant *T. pallidum* antigens.

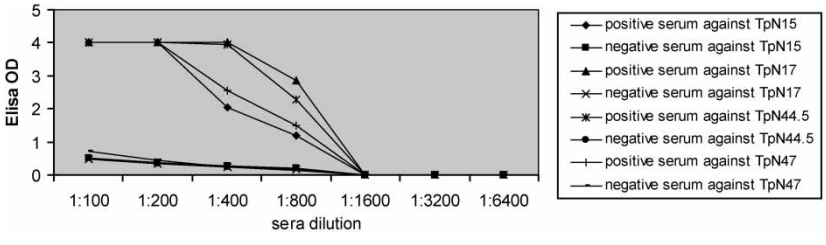

Figure 1. Titration of syphilis positive control serum and syphilis negative control serum against individual *Treponema pallidum* recombinant proteins: TpN15, TpN17, TpN44.5, and TpN47.

Based on these titrations, all subsequent tests included as controls a 1:500 (for TpN44.5 and TpN47 antigens) or 1:1,000 dilution (for TpN15 and TpN17 antigens) of the positive control sample.

Reproducibility of the Assay

The positive control (BM124734) and the negative control (BM121707) were repeatedly tested on 43 separate occasions against TpN47, TpN44.5 (at a serum dilution of 1:500), TpN17, and TpN15 (at a serum dilution of 1:1,000). The mean ELISA O.D.s, SDs, and CVs obtained are presented in Table 1. This in-house ELISA test gave coefficients of variation of 14, 12, 15, and 16%, respectively, when the positive control was tested against TpN47, TpN44.5, TpN17, and TpN15.

ELISA Reactivities of Syphilis-Positive and Syphilis-Negative Patients' Sera Against the Four Recombinant *T. pallidum* Antigens

One hundred and twenty-seven syphilis negative and 37 positive sera were tested at a single dilution (1:400) against each of the four *T. pallidum* recombinant proteins: TpN47, TpN44.5, TpN17, and TpN15. This single dilution of 1:400 was chosen based on a pilot study involving titrations of 12 syphilis-positive and 35 syphilis-negative samples. The distribution of the ELISA O.D. values obtained, together with their mean and the range of mean $+/-3$ SDs for each of the four antigens with the syphilis-positive and syphilis-negative sera are summarized in Table 2.

ELISA O.D. readings provided by the syphilis-negative sera against all four recombinant *T. pallidum* antigens followed a normal distribution; however, ELISA O.D. readings provided by the syphilis-positive samples

Table 1. Reproducibility of an in-house indirect ELISA for measurement of antibodies to individual recombinant *Treponema pallidum* protein antigens (TpN47, TpN44.5, TpN17, and TpN15)

	TpN47	TpN44.5	TpN17	TpN15
Positive control serum				
Mean ELISA O.D.	1.273	2.271	3.319	1.362
Standard deviation	0.177	0.266	0.505	0.214
Coefficient of variations	13.9%	11.7%	15.2%	15.7%
Negative control serum				
Mean ELISA O.D.	0.186	0.111	0.155	0.167
Standard deviation	0.033	0.014	0.034	0.034
Coefficient of variations	17.9%	12.7%	21.7%	20.1%

Table 2. ELISA measurements of serum antibodies to four individual *Treponema pallidum* recombinant protein antigens in 127 syphilis negative and 37 syphilis positive samples

	Treponema pallidum recombinant protein antigens			
Data	TpN47	TpN44.5	TpN17	TpN15
Syphilis-negative subjects:				
ELISA O.D. range	0.08–0.317	0.053–0.267	0.043–0.443	0.088 – 0.654
Mean[a] ELISA O.D.	0.173	0.111	0.212	0.315
Standard deviation (SD)	0.051	0.037	0.076	0.120
Mean + 3SD	0.320	0.220	0.440	0.679
Syphilis-positive subjects:				
ELISA O.D. range	0.894–4.000	1.061–4.000	1.557–4.000	1.164–4.000
Mean[a] ELISA O.D.	3.290	3.243	3.863	3.287
Standard deviation (SD)	0.946	1.029	0.521	0.918
Mean – 3SD	0.452	0.157	2.301	0.534

[a]Mean of duplicate determinations.

for all four antigens were dominated by many extremely high values. A Wald-Wolfowitz runs test on the two sets of data (ELISA O.D. readings from syphilis positive sera versus syphilis negative sera) gave p values of <0.001 for all four antigens.

Establishment of Cut-Off Values for Positive and Negative Results

To find ELISA O.D. readings as cut-offs for positive and negative tests, a ROC test was performed based on data from our set of samples. To achieve maximum sensitivity of 100% and specificity of 100%, the ROC test assigned the following ELISA O.D. readings as cut-offs for TpN15, TpN17, TpN44.5, and TpN47: 0.909, 1.0, 0.648, and 0.606, respectively.

DISCUSSION

While the two-step approach of screening with a non-treponemal test followed by confirmation with a treponemal assay is still the acceptable method for the serological diagnosis of syphilis in North America, some centers in North America as well as Europe and Australia have adopted using treponemal tests, such as TP-PA or EIA, for the screening of syphilis. This approach eliminates several potential problems associated with non-treponemal tests, such as, prozone phenomenon, biological false positives, and low sensitivity in detection of very early and latent syphilis.

We have recently examined one syphilis EIA, and identified several potential pitfalls.[11] For example, like many other commercial syphilis EIAs, multiple recombinant treponemal proteins are used together as antigens which may confound the results of serological testing as normal subjects and patients in different phases of syphilis infection may have different serological profiles towards the many *T. pallidum* antigens. Another common problem in commercial EIAs is the lack of "no antigen" control wells to control for the non-specific binding of primary (patient) antibody to plastic.[11] Furthermore, specificity of many commercial EIAs were evaluated with sera from normal blood donors,[6,13] and performance of such assays may not stand up to close scrutiny when they are applied on clinical samples from patients with various underlying conditions. It is with this background that we proposed syphilis serology with EIA should use individual *T. pallidum* antigen instead of a mixture of antigens. With the microtiter plate format, it is relatively easy to measure quantitative serological response to a number of defined antigens.

The proteome of *T. pallidum* contains over 1,000 proteins suggesting a very complicated antigenic structure.[14] Western immunoblot experiments with sera from syphilitic patients have consistently identified four proteins: TpN47, TpN44.5, TpN17, and TpN15.[10,15,16] Although western immunoblot has proven as a useful diagnostic tool in the past,[17,18] the cumbersome nature of the test, the non-quantitative results, subjective reading as well as potential denaturation of the antigens to reveal cross-reactive epitopes suggest possible improvements.

In this study, we standardize an indirect ELISA for measurement of serum antibody levels to four individual treponemal recombinant proteins, that have been commonly used in a number of commercial EIAs, mostly as a mixture of antigens.[8]

Results of our study with sera from secondary syphilis cases and non-infected subjects showed that serological responses to these four treponemal antigens gave clear distinction between these two groups of subjects. The Wald-Wolfowitz runs test gave p values of all <0.001 when applied to each of these four antigens. Using the ROC test, ELISA O.D. cut-offs can be set to provide 100% sensitivity and specificity. However, further adjustments to the cut-off values are possible if a large sample of syphilis positive sera are tested, including sera from primary syphilis cases. Furthermore, if sera from patients with different stages of syphilis (primary, secondary, and tertiary syphilis, as well as successfully treated past syphilis) can be applied to this test, it may be possible to establish cut-off values to differentiate between active from past infections as well as for diagnosing latent syphilis.

Because these four recombinant *T. pallidum* antigens are used individually on separate wells of an ELISA well, it is equally possible to find certain antigen may be more suitable for defining certain stages of disease. For example, even though the ranges as well as the mean ELISA O.D.s given by the infected subjects to each of the four antigens are similar, it

appears a wider margin of separation between the two groups of subjects may be possible if serum antibody responses are measured against TpN17 and TpN47 as opposed to TpN44.5 and TpN15. Therefore, we are currently evaluating this in-house indirect ELISA test against a panel of other conventional and newer serological tests using sera from syphilis patients at different stages of infection.

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