Characterization of Treponema pallidum Particle Agglutination Assay-Negative Sera following Screening by Treponemal Total Antibody Enzyme Immunoassays

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Following a laboratory audit, a significant number of Treponema pallidum particle agglutination assay (TPPA)-negative sera were identified when TPPA was used as a confirmatory assay of syphilis enzyme immunoassay (EIA) screening-reactive sera (SSRS). Sera giving such discrepant results were further characterized to assess their significance. A panel of 226 sera was tested by the Abbott Murex ICE Syphilis EIA and then by the Newmarket Syphilis EIA II. TPPA testing was performed on 223 sera. Further testing by the Venereal Disease Research Laboratory (VDRL) test, the Mercia Syphilis IgM EIA, the fluorescent treponemal antibody (FTA-ABS) assay, and INNO-LIA immunoblotting was undertaken in discrepant cases. One hundred eighty-seven of 223 (83.8%) SSRS were TPPA reactive, while 26 (11.6%) sera which were reactive in both the ICE and Newmarket EIAs were nonreactive by TPPA. The majority (68%) of the TPPA-discrepant sera were from HIV-positive patients and did not represent early acute cases, based on previous or follow-up samples, which were available for 22/26 samples. FTA-ABS testing was performed on 24 of these sera; 14 (58.3%) were FTA-ABS positive, and 10 (41.7%) were FTA-ABS negative. Twenty-one of these 26 sera were tested by INNO-LIA, and an additional 4 FTA-ABS-negative samples were positive. In this study, significant numbers (18/26) of SSRS- and TPPA-negative sera were shown by further FTA-ABS and LIA (line immunoblot assay) testing to be positive. The reason why certain sera are negative by TPPA but reactive by treponemal EIA and other syphilis confirmatory assays is not clear, and these initial findings should be further explored.

Treponema pallidum hemagglutination assay (TPHA), introduced during the 1960s, has been shown (17, 19) to be highly sensitive and specific at detecting treponemal antibodies and is still used by many laboratories. A modification of the TPHA is the Treponema pallidum particle agglutination assay (TPPA), which has been shown (1) to perform as well as the hemagglutination assay.

In recent years, a number of highly sensitive and specific enzyme immunoassays (EIAs) (7) have become available, and some of these can simultaneously detect syphilis IgG and IgM, thus shortening the seronegative window following infection. Two such assays are the Abbott Murex ICE Syphilis EIA (1) and the Newmarket Laboratories Syphilis EIA II (18). United Kingdom guidelines have proposed (9, 10) that either an EIA alone or a combination of VDRL/rapid plasma reagin (RPR) tests and TPPA/TPHA can be used for syphilis screening. Furthermore, specimens that are reactive on screening require confirmatory testing with a different treponemal test that has a sensitivity equal to that used for screening and, ideally, that has greater specificity. The fluorescent treponemal antibody (FTA-ABS) test has been widely used as a confirmatory test; however, treponemal Western blot/immunoblot assays (5), which have been shown to perform as well as the FTA-ABS test, have proved an attractive alternative because of their reported high sensitivity and specificity combined with their simplicity.

The HPA Birmingham West Midlands Public Health Laboratory acts as a confirmatory syphilis testing center for the West Midlands of England. The aim of this evaluation was to optimize confirmatory testing of referred syphilis screening-reactive sera (SSRS).

MATERIALS AND METHODS

Serum samples and syphilis confirmatory testing serology. Archive serum samples with a volume of at least 300 μl which had been stored at ~20°C and sent to our laboratory for syphilis confirmatory serology between November 2006 and January 2007 were selected for this study. In all, 226 samples were identified. According to the laboratory syphilis confirmatory testing protocol, the samples were initially tested by the Abbott Murex ICE Syphilis EIA, TPPA, and the VDRL test, and most were tested by the Mercia Syphilis IgM EIA. For comparison, all 226 samples were tested on the Newmarket Syphilis EIA II IgG/IgM. Twenty-six samples which gave discrepant results were tested by the Zeus Scientific FTA-ABS assay. A further 21 of the 26 samples which tested negative by TPPA but positive by the Newmarket Syphilis EIA II and Abbott Murex ICE Syphilis EIA were tested on the INNO-LIA syphilis score line immunoblot assay.

Screening and confirmatory assays used in the evaluation. All the tests were performed and interpreted in accordance with the manufacturers’ instructions delineated in the kit inserts.

The Abbott Murex ICE Syphilis EIA (Abbott Murex, Dartford, United Kingdom) is an enzyme immunoassay for the detection of T. pallidum-specific (Tp15, Tp17, and Tp47) antibodies. The Serodia TPPA (Fujirebio Inc., Tokyo, Japan) uses gelatin particle carriers sensitized with purified T. pallidum (Nichols strain). The test is based on the principle of sensitized particles being agglutinated by T. pallidum-specific antibodies present in the serum or plasma. The test can be performed in a qualitative
TABLE 1. Serological profiles of samples which tested negative by the Abbott Murex ICE Syphilis EIA*

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Serological result by indicated assay</th>
<th>Serodia TPPA (titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>546</td>
<td>Negative</td>
<td>Positive (1:320)</td>
</tr>
<tr>
<td>751</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>768</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>172</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>282</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>826</td>
<td>Negative</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Samples were referred to the Birmingham HPA laboratory for confirmatory syphilis serology.
* Positive with an optical density cutoff of less than 2, i.e., a low-positive result which is close to the cutoff.

or quantitative manner. All positive and/or indeterminate reactions in the qualitative test were confirmed in a quantitative test.

The Abbott Murex VDRL carbon antigen test (Abbott Murex, Dartford, United Kingdom) is a rapid plasma reagin (circulating antibodies directed against tissue components) test for the detection and titration of reagin in human serum or plasma. The test uses tissue cardiolipin in a colloidal suspension as a nonspecific syphilis antigen. Microparticulate carbon enhances the visual distinction between positive and negative reactions as clearly visible clumps of black particles when the serum or plasma is mixed with the carbon antigen on a reaction card.

The Mercia Syphilis IgM EIA (Microgen Bioproducts, Ltd., Cambridge, United Kingdom) is an IgM antibody capture enzyme immunoassay for the detection of T. pallidum-specific IgM antibodies in human serum.

The FTA-ABS indirect fluorescent antibody (IFA) test system (Zeiss Scientific Inc., Carlsbad, CA) is a confirmatory test procedure designed to confirm positive nontreponemal screen reagin test results for syphilis. It uses fixed nonviable T. pallidum (Nichols strain) cells on a slide as a substrate (antigen). Preabsorption of patient sera removes group treponemal antibodies. The treated serum is layered onto an antigen-coated slide, and specific treponemal antibodies, if present, form an antigen-antibody complex which persists after a wash step. Goat anti-human immunoglobulin labeled with fluorescein isothiocyanate (FITC) is then added, and finally the substrate cells are examined under a fluorescent microscope. Intensity of staining is graded on a scale of 1+ to 4+ or as negative (no fluorescence).

The Newmarket Syphilis EIA II (Lab 21 Healthcare, Cambridge, United Kingdom) is an enzyme immunoassay for the detection of T. pallidum-specific (TpN15, TpN17, and TpN47) antibodies.

The INNO-LIA syphilis score assay (Innogenetics N.V., Ghent, Belgium) is based on the enzyme immunoassay principle in which three recombinant proteins (TpN47, ToN17, and TpN15) and one synthetic peptide (Tempo) are coated as discrete lines onto a nylon strip with plastic backing. The test sample is incubated in a test trough together with the multiple-antigen-coated test strip. Specific T. pallidum, if present, will bind to the individual antigen in the strip. A goat anti-human IgG labeled with alkaline phosphates is added and will bind to antigen-antibody complexes. A dark brown line forms, proportionate to the amount of specific antibodies, after incubation with the substrate nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylophosphate (BCIP).

RESULTS

A panel of 226 sera submitted to the HPA Birmingham West Midlands Public Health Laboratory for syphilis reference serology was assembled for this study. All sera submitted were tested by the Abbott Murex ICE Syphilis EIA: 220/226 (97.3%) were reactive, and 6/226 (2.6%) tested negative. The serological profiles of the sera which tested negative by the ICE assay are shown in Table 1. Five of the ICE-negative sera were positive when tested by the Newmarket EIA, and one also had a positive TPPA test result with a titer of 1:320. All six ICE-negative sera were tested by the VDRL test, and all were negative; four were tested by the Mercia Syphilis IgM EIA, and all were negative for treponemal IgM. All 226 sera were also tested by the Newmarket Syphilis EIA II. Six sera were negative in the Newmarket EIA, yet five were positive in the ICE assay. TPPA, VDRL tests, and Mercia Syphilis IgM EIA of these sera were all negative. The serological profiles of the sera which tested negative by the Newmarket EIA are shown in Table 2.

Results of TPPA testing were available for 223 sera, and 187/223 (83.8%) were classified as TPPA reactive. There were 26 (11.6%) sera which were reactive in both ICE and Newmarket EIA but nonreactive by TPPA. The serological profiles of these sera are shown in Table 3. FTA-ABS testing was performed on 24 of these sera: 14 (58.3%) were FTA-ABS positive, and 10 (41.7%) were FTA-ABS negative. Of the 22 LIA (line immunoblot assay) results, 10 (45.5%) were positive, 2 (9%) indeterminate, and 10 (45.5%) negative. There were 7 (excluding the 2 indeterminate results) discordant results between the FTA-ABS and LIA, with 3 negative FTA-ABS results testing positive and 4 positive FTA-ABS results testing negative on the LIA.

It would be reasonable to classify the 18/26 (69%) TPPA-negative but EIA-positive and FTA-ABS- and/or LIA-positive sera as true treponemal antibody seropositives, and therefore the TPPA results would be regarded as false negative. Clinical data were not available for this study; however, it was possible to look up the test histories for the individuals who tested TPPA negative and EIA positive. Previous positive treponemal serology was identified for 21 (80.7%) of the TPPA-negative individuals, and subsequent negative treponemal serology was identified for one (3.7%) individual when followed up 4 months later. For four (15.3%) individuals, no test history was available.

DISCUSSION

Currently published guidelines (9, 13), including the United Kingdom National Standard Operating Procedure (10), recommend treponemal EIAs for screening for syphilis. If a treponemal EIA is used for screening, an alternative treponemal test, such as TPPA, should be used for confirmatory testing. A recent audit (3) documented that 57% of primary diagnostic laboratories in England and Wales performed only a single screening assay for syphilis diagnosis.
TABLE 3. Serological profiles of 26 samples which tested negative by TPPA but positive by the Newmarket Syphilis EIA II and Abbott Murex ICE syphilis EIA

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Newmarket Syphilis EIA II</th>
<th>Murex ICE syphilis EIA</th>
<th>VDRL test</th>
<th>Mercia syphilis IgM EIA</th>
<th>FTA-ABS</th>
<th>INNO-LIA (TpN15/TpN17/TpN47/TmpA)</th>
<th>HIV status</th>
<th>Previous and subsequent syphilis serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>441</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>-/+/+/-; Pos</td>
<td>Neg</td>
<td>EIA pos 6 mo previously</td>
</tr>
<tr>
<td>924</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/+/-; Pos</td>
<td>Pos</td>
<td>VDRL/TPPA pos 7 mo previously</td>
</tr>
<tr>
<td>170</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>No data</td>
<td>-/+/+/-; Pos</td>
<td>Neg</td>
<td>TPPA pos 5 mo previously and 9 mo later</td>
</tr>
<tr>
<td>260</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Neg</td>
<td>Pos</td>
<td>EIA always pos; FTA pos once 4 mo previously</td>
</tr>
<tr>
<td>318</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Neg</td>
<td>Pos</td>
<td>No previous/ subsequent samples</td>
</tr>
<tr>
<td>587</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Ind^c</td>
<td>Pos</td>
<td>TPPA pos 4 mo previously</td>
</tr>
<tr>
<td>634</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Ind</td>
<td>Pos</td>
<td>EIA/FTA pos 1 mo previously; TPPA pos 11 mo later</td>
</tr>
<tr>
<td>692</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>No data</td>
<td>-/+/-/-; Neg</td>
<td>NKd</td>
<td>No previous samples; 1 mo later VDRL pos (neat); FTA neg</td>
</tr>
<tr>
<td>718</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>-/+/-/-; Pos</td>
<td>Pos</td>
<td>TPPA pos 6 mo previously and 7 mo later</td>
</tr>
<tr>
<td>735</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>No data</td>
<td>-/+/-/-; Neg</td>
<td>Pos</td>
<td>EIA pos 1 mo previously</td>
</tr>
<tr>
<td>761</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>No data</td>
<td>-/+/-/-; Pos</td>
<td>Pos</td>
<td>Evidence of acute syphilis 3 yr earlier; EIA always pos, occasional TPPA pos</td>
</tr>
<tr>
<td>765</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Only subsequent samples for 2 yr; EIA pos, occasional TPPA and FTA pos</td>
</tr>
<tr>
<td>834</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>-/+/-/-; Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>EIA mostly pos over 3 yr; FTA occasionally pos; never any VDRL/TPPA pos</td>
</tr>
<tr>
<td>902</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>-/+/-/-; Neg</td>
<td>Neg</td>
<td>No previous/ subsequent samples</td>
</tr>
<tr>
<td>519</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>No data</td>
<td>-/+/-/-; Pos</td>
<td>Neg</td>
<td>No previous/ subsequent samples</td>
</tr>
</tbody>
</table>

^a Individual antibody results indicated by plus or minus are followed by the overall serology result. Pos, positive; Neg, negative.

b Positive with an optical density cutoff of less than 2, i.e., a low-positive result which is close to the cutoff.

c EQ, equivocal.

d NK, not known.

e Ind, indeterminate.

with financial or staff/skill resources cited as the reason for the inability to undertake additional testing. An audit performed in our laboratory showed that a significant number of sera screened by ICE were TPPA negative. The sera were then screened by the Newmarket EIA to determine if there was any problem with the ICE assay. We found the ICE and Newmarket EIAs to perform similarly, which is not unexpected, as both these EIAs use recombinant treponemal antigens (TpN15, TpN17, and TpN47) and detect both IgG and IgM. Similar performances of ICE and Newmarket EIAs have been reported previously (7). In view of our findings of dual syphilis EIA reactivities, TPPA-negative sera need further confirmatory testing.

It is highly desirable that a suitable confirmatory test should have, at least, sensitivity and specificity equivalent to those of the screening assay. Manavi and colleagues (16) have suggested that, in the absence of a specific treponemal IgM EIA, a TPPA test should be performed whenever there...
is clinical suspicion of primary infection, as the ICE EIA is less sensitive than the TPPA in primary infection. In this study, 26 (11.6%) sera which were reactive in both the ICE and Newmarket EIA were nonreactive by the TPPA. Four of the TPPA-negative sera gave reactive results in the Mer-
cia treponemal IgM EIA; however, there is no clinical data to identify the clinical stage of infection. Previous or sub-
sequent serological profiling available in 3 of the 4 cases suggests that the IgM reactivities are most likely false-pos-
itive reactions (Table 3).

Traditionally, the FTA-ABS test is regarded as the “gold standard” for confirmatory syphilis serology (2, 20). Reser-
vations have been expressed (6) that when sensitive trepo-
nemal EIAs are used for screening, the FTA-ABS test may fail to confirm the screening reactivity. Additionally, it has been reported (21) that the FTA-ABS test is less sensitive (94.5%) than the TPPA (99.4%) or ICE EIA (99.4%). We can make no comment on these reports, as our study does not address these issues. We applied the FTA-ABS test to
further characterize the TPPA-negative, dual-EIA-reactive sera and found 14/24 (58.3%) to be FTA-ABS positive (Ta-
ble 3). It is possible that the 10 sera which were nonreactive in the FTA-ABS test were false negatives, perhaps due to
lack of sensitivity or an operator error in identifying specific
fluorescence. Alternatively, the FTA-ABS-positive sera may be false positives, as for unknown reasons the FTA-ABS test has been reported to give false-positive results when used as a
screening assay (14). Autoimmune disorders such as sys-
temic lupus erythematosus and rheumatoid disease can also
lead to false positivity in the FTA-ABS test (15). Cross-
reacting antibodies produced following infection by other
spirochetes, for example, Borrelia burgdorferi (12), may also
produce FTA-ABS false positivity. In view of the fact that
the FTA-ABS results have been generated for dual trepo-
nemal recombinant antigen EIA-positive sera, we do not
consider the aforementioned causes of false-positive reac-
tivity to apply to our findings. Slightly fewer (45.5%) of the
dual-EIA-positive, TPPA-negative sera were reactive by
INNO-LIA (8), which can also be used as a confirmatory
assay.

HIV infection (4, 11) may lead to false-positive or -neg-
ative syphilis serology, and in our study, 63.6% (14/22) of
the patients with discrepant serology results were HIV pos-
itive. The majority of the patients had evidence of previous
or subsequent positive syphilis serology, and the specific
syphilis antibody levels were low and close to the cutoff,
which could explain the transient and discrepant nature of
TPPA and FTA test results (Table 3). Low-level antibody to
certain syphilis antigens (TpN47, TpN17, TpN15, and
TnpA) could also explain the 8 discrepant FTA-ABS and
LIA results, especially since the LIA was performed after
many freeze-thaw cycles and prolonged storage. It is impor-
tant to note, however, that in the majority of follow-up
samples, the EIA reactivity persisted, and EIA testing was
least associated with occasional nonreactive or reactive re-
sults, as has been found with TPPA and in some cases with
the FTA-ABS test (Table 3).

This study highlights the fact that TPPA failed to confirm
16.2% of the SSRs, although the clinical significance re-
mains questionable. A shortcoming is the lack of clinical
data; however, much can be inferred from the previous or
repeat serological profiles in 24 of the 26 discrepant cases. It
is likely that most of the TPPA-nonreactive, EIA-reactive
cases were either old or treated cases of mostly HIV-positive
patients. Aberrant results in laboratory tests for syphilis are
well known to occur in HIV-infected individuals (15, 20).
We would, therefore, not advocate that syphilis confirma-
tion algorithms change but rather highlight the fact that in
repeatedly screened populations, such as HIV-positive indi-
viduals, discrepancies between treponemal EIA and TPPA
results are quite prevalent. This seems to be a function of
very low levels of syphilis-specific antibodies, and in such
instances clarity is needed as to how to confirm the initial
EIA-reactive result. Confirmation by a second EIA or im-
munoblot assay may be useful. Additional studies are
planned to determine the most appropriate confirmatory
testing strategy.

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ness Park, Newmarket, United Kingdom, for the provision of Syphilis
EIA II kits free of charge.

REFERENCES

Serodia Treponema pallidum particle agglutination, the Murex Syphilis ICE
and the Enzywell TP tests for serodiagnosis of syphilis. Int. J. STD AIDS
16:294–298.
fluorescent treponemal antibody absorption test for detection of antibodies
(immunoglobulins G and M) to Treponema pallidum in serologic diagnose
3. Amin, A. K., R. J. Manuel, C. A. Ison, R. Woodham, M. Shemko, H. Maguire,
J. Giraudon, J. Forde, and S. H. Gillespie. 2009. Audit of laboratory diag-
4. Augenbraun, M. H., J. A. DeHovitz, J. Feldman, L. Clarke, S. Landesman,
and H. M. Minkoff. 1994. Biological false-positive syphilis test results for
women infected with human immunodeficiency virus. Clin. Infect. Dis. 19:
1040–1044.
Evaluation of a Treponema pallidum Western immunoblot assay as a confir-
by enzyme immunoassay and variation in fluorescent treponemal antibody
serological assays for the detection of syphilis infection. Eur. J. Clin. Micro-
F. Hulstaert, and M. Zein. 2000. Validation of the INNO-LIA Syphilis kit as
a confirmatory assay for Treponema pallidum antibodies. J. Clin. Micro-
Standard Method VSOP 44, issue 1. Health Protection Agency, London,
negative secondary syphilis in a patient infected with the human immuno-
1987. Sero-

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