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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 used widely 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 (TpN15, TpN17, and TpN47) 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

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TABLE 1. Serological profiles of samples which tested negative by the Abbott Murex ICE Syphilis EIA[a]

Specimen	Serological result by indicated assay		
	ICE syphilis EIA	Newmarket EIA	Serodia TPPA (titer)
546	Negative	Positive	Positive (1:320)
751	Negative	Positive	Negative
768	Negative	Negative	Negative
172	Negative	Positive[b]	Negative
282	Negative	Positive	Negative
826	Negative	Positive	Negative

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

TABLE 2. Serological profiles of samples which tested negative by the Newmarket Syphilis EIA II[a]

Specimen	Serological result by indicated assay		
	Newmarket EIA OD CO	ICE syphilis EIA OD CO	Serodia TPPA titer
842	Negative	Positive	Negative
768	Negative	Negative	Negative
160	Negative	Positive	Negative
457	Negative	Positive[b]	Negative
916	Negative	Positive	Negative
672	Negative	Positive[b]	Negative

[a] Samples were referred to the Birmingham HPA laboratory for confirmatory syphilis serology.
[b] 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 (Zeus Scientific, Inc., Raritan, NJ) is a confirmatory test procedure designed to confirm positive nontreponemal screen reagin tests 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-indolylphosphate (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 EIAs 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 EIAs 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

Specimen	Test result by indicated assay						HIV status	Previous and subsequent syphilis serology
	Newmarket Syphilis EIA II	Murex ICE syphilis EIA	VDRL test	Mercia syphilis IgM EIA	FTA-ABS	INNO-LIA (TpN47/TpN17/TpN15/TmpA)[a]		
441	Pos	Pos	Neg	Pos	Neg	−/+/±/±; Pos	Neg	EIA pos 6 mo previously
924	Pos	Pos	Neg	Neg	Neg	−/+/±/−; Pos	Pos	VDLR/TPPA pos 7 mo previously
170	Pos	Pos	Neg	Neg	No data	−/+/±/−; Pos	Neg	TPPA pos 5 mo previously and 9 mo later
260	Pos	Pos	Neg	Neg	Neg	−/±/−/−; Neg	Pos	EIA always pos; FTA pos once 4 mo previously
318	Pos[b]	Pos[b]	Neg	Neg	Neg	−/−/−/−; Neg	Pos	No previous/subsequent samples
587	Pos	Pos[b]	Neg	Neg	Neg	−/+/−/−; Ind[e]	Pos	TPPA pos 4 mo previously
634	Pos	Pos[b]	Neg	Neg	Neg	−/+/−/−; Ind	Pos	EIA/FTA pos 1 mo previously; TPPA pos 11 mo later
692	Pos	Pos	Neg	Neg	No data	−/±/−/−; Neg	NK[d]	No previous samples; 1 mo later VDRL pos (neat); FTA neg
718	Pos	Pos	Neg	Neg	Neg	±/+/±/−; Pos	Pos	TPPA pos 6 mo previously and 7 mo later
314	Pos	Pos[b]	Pos neat	Neg	Neg	−/±/−/−; Neg	NK	EIA pos 1 mo previously
391	Pos	Pos	Neg	Pos[b]	Neg	−/±/−/−; Neg	Neg	EIA and weak pos IgM only 1 mo later
735	Pos	Pos	Neg	Neg	Neg	No data	Pos	EIA and FTA pos 3 mo previously; EIA only pos for next 3 yr
180	Pos	Pos[b]	Neg	Neg	Pos	−/+/±/−; Pos	Pos	EIA pos and FTA mostly pos for 8 yr; occasional TPPA pos
182	Pos	Pos	Neg	Neg	Pos	±/+/+/−; Pos	Pos	EIA pos and FTA mostly pos for 5 yr; occasional TPPA pos
196	Pos[b]	Pos[b]	Neg	Pos	Pos	−/±/−/−; Neg	Neg	EIA/TPPA pos 1 mo previously; only EIA pos 2 mo later
477	Pos	Pos	Neg	Neg	Pos	−/±/±/−; Pos	Pos	EIA pos and FTA mostly pos for 9 yr; occasional TPPA/VDRL pos
761	Pos	Pos	Neg	Neg	Pos	No data	Pos	Evidence of acute syphilis 3 yr earlier; EIA always pos, occasional TPPA pos
765	Pos	Pos	Neg	Neg	Pos	−/±/−/−; Neg	Pos	Only subsequent samples for 2 yr; EIA pos, occasional TPPA and FTA pos
834	Pos	Pos[b]	Neg	Neg	Pos	−/±/−/−; Neg	Pos	EIA mostly pos over 3 yr; FTA occasionally pos; never any VDRL/TPPA pos
902	Pos	Pos	Neg	Neg	Pos	−/±/−/−; Neg	Neg	No previous/subsequent samples
040	Pos	Pos[b]	Neg	Neg	Pos	No data	NK	4 mo later, all serology neg
565	Pos	Pos[b]	Neg	Neg	Pos	No data	Pos	EIA/TPPA pos 2 mo previously; all serology neg 10 mo later
631	Pos	Pos	Neg	EQ[c]	Pos	−/+/±/−; Pos	Neg	EIA pos for previous 6.5 yr; FTA/TPPA occasionally neg
085	Pos	Pos	Neg	Neg	Pos	No data	Neg	No previous/subsequent samples
519	Pos	Pos[b]	Neg	Neg	Pos	±/+/−/−; Pos	Neg	EIA/FTA pos 2 mo previously and 1 mo later
694	Pos	Pos	Neg	Neg	Pos	±/+/±/−; Pos	NK	No previous/subsequent samples

[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 EIAs were nonreactive by the TPPA. Four of the TPPA-negative sera gave reactive results in the Mercia treponemal IgM EIA; however, there is no clinical data to identify the clinical stage of infection. Previous or subsequent serological profiling available in 3 of the 4 cases suggests that the IgM reactivities are most likely false-positive reactions (Table 3).

Traditionally, the FTA-ABS test is regarded as the "gold standard" for confirmatory syphilis serology (2, 20). Reservations have been expressed (6) that when sensitive treponemal 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 (Table 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 systemic 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 treponemal recombinant antigen EIA-positive sera, we do not consider the aforementioned causes of false-positive reactivity 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 -negative syphilis serology, and in our study, 63.6% (14/22) of the patients with discrepant serology results were HIV positive. 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 TmpA) 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 important 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 results, 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 remains 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 confirmation algorithms change but rather highlight the fact that in repeatedly screened populations, such as HIV-positive individuals, 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 immunoblot assay may be useful. Additional studies are planned to determine the most appropriate confirmatory testing strategy.

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