



A Comparison of the Analytical level of agreement of Nine Treponemal Assays for Syphilis and Possible Implications for Screening Algorithms

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Title page**A Comparison of the Analytical level of agreement of Nine Treponemal Assays for Syphilis and Possible Implications for Screening Algorithms****Corresponding author:**

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Keywords: Comparison analytical agreement of syphilis treponemal assays

Summary:

This study describes the effect of the comparison of eight qualitative and quantitative treponemal syphilis assays.

Cover Letter

Dear Sirs,

We are including for your consideration, the manuscript entitled “A Comparison of the Analytical level of agreement of Nine Treponemal Assays for Syphilis and Possible Implications for Screening Algorithms.” This work describes the effect that occurs when eight treponemal syphilis assays are evaluated both qualitatively and quantitatively to demonstrate the variability on analytical sensitivities and to determine the feasibility in selecting the proper assay for a reverse algorithm system.

Competing Interests

One of the functions of the Laboratory Reference and Research Branch (LRRB) of the Division of STD Prevention of the National Center for HIV/Aids, Viral Hepatitis, STD and TB Prevention of the Centers of Disease Control and Prevention is to evaluate public domain procedures to determine its diagnostic value in proving or disproving a particular claim.

Sources of support

There are no sources of support for this study that require acknowledgement.

Duplication

Please note that, there is no related paper or publications in reference to this work that describes the effect of the analytical level of agreement of nine treponemal assays for syphilis serology and its possible implications in selecting the proper assays for the reverse screening algorithm.

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Funding:

The Centers for Disease Control and Prevention provided financial support for this study in the form of allowing the principal investigator and CDC coauthors to devote time and effort in conducting this study.

Conflict of interests: There is no conflict of interest related to this study.

Ethics approval: This study was performed on serum samples supplied by the Georgia Department of Health laboratories from which all patient identifiers had been removed. Protocol for studies using these sera were reviewed by institutional review board of the CDC (protocol no 2018) and determined to be exempt from further review.

Contributors:

DLC was the project microbiologist, developed the test protocol, design the test parameters and functional components of the test.

ARC assisted in the developing of the test protocol, designed the test parameters and was the corresponding author.

HAJ performed the evaluation of the assays.

YT assisted in the evaluation of the assays.

KK assisted in performing the evaluation.

YFF assisted in performing the evaluation.

AAZ assisted in the statistic evaluation of the data.

All authors contributed to the write up and critically reviewed the manuscript and approved the final draft.

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Abstract

Objective: The serological diagnosis of syphilis requires the detection of two distinct antibodies, the nontreponemal and treponemal. CDC recommends screening first with a nontreponemal test such as (RPR/VDRL), and then confirming those results with one of several treponemal tests (FTA-ABS, EIA, CIA, TP-PA or POC). Due to the high volume of samples processed by some laboratories using automated systems, the screening with treponemal assays and confirming with nontreponemal tests is becoming the established norm. The purpose of this study was to evaluate eight treponemal assays using TP-PA as the predicate assay.

Methods: Two hundred ninety stored serum samples were tested qualitatively according to the manufacturer's directions.

Results: Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-ABS 94.5% - 100%, Trep-Sure 100% - 98.9%, Bioelisa 100% - 98.9%, INNO-LIA 99.1% - 99.4%, Bioline 100% - 98.9%, Captia IgG 100% - 97.2%, Trep-ID 100%-100%, and LIAISON 100% - 99.4%. In order to properly evaluate the performance of these assays, the analytical sensitivity was determined by endpoint titration of serial dilutions of the reactive serum samples in normal sera. The median endpoint titer varied from 1:4 for FTA-ABS to 1:512 for Trep-Sure.

Conclusions: The performance of the treponemal serological assays was comparable when using medium and high titer sera. However, the varying performance on specimen dilutions suggest that there may be differences in sensitivity with low titer sera that are more prevalent in primary and late syphilis cases.

Introduction

Syphilis is a sexually transmitted disease caused by the bacterium *Treponema pallidum*. The clinical diagnosis is difficult due to the complexity of manifestations which requires the aid of serological interpretation. The serological diagnosis of syphilis depends upon the detection of two distinct antibodies, the nontreponemal or heterophile antibodies (reagin) directed against cardiolipin released from damaged host cells and from the treponemes themselves. These antibodies can also be present in other diseases and human conditions such as (Lupus, malaria, HIV, IV drug users, etc.) (1). The presence of nontreponemal antibodies is indicative of active infection, thus a reduction in titer can suggest a successful antibiotic therapy, and a significant increase can indicate a possible relapse or reinfection (2). The treponemal antibodies are primarily directed against specific lipoprotein antigens

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3 31 of the bacterium such as 15-, 17-, and 47-kDa. Even after treatment or time, these treponemal
4 32 antibodies usually remain present for life. A positive treponemal test cannot distinguish between
5 33 active, treated, and old cases of syphilis.
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10 34 The traditional algorithm is to screen with a nontreponemal assay such as VDRL or RPR and those
11 35 serum samples found reactive are then confirmed using a treponemal test. With the introduction of
12 36 automated systems which are appropriate for high output volumes, the reverse algorithm is gaining
13 37 acceptability and its usefulness is arguable justified (6). However, the adoption of this reverse
14 38 algorithm has increased the number of discrepant results between the screening and confirmatory
15 39 tests (4, 5). One reason the traditional algorithm was established was to avoid the detection of
16 40 previously treated cases, especially in low prevalence settings where the majority of sera will test
17 41 negative and not require further evaluation, unless a patient has been reinfected or in rare
18 42 circumstances, their non-treponemal antibody titers remain serofast. With the application of the
19 43 reverse algorithm even in low prevalence settings all previously treated cases will likely be detected.
20 44 When the screening treponemal test is positive and the confirmatory nontreponemal test is negative,
21 45 the CDC guidelines recommend using a second treponemal test to validate the results of the
22 46 screening test. The selection of a second confirmatory test introduces the possibility of having an
23 47 analytically less sensitive test which would suggest a false positive screening or a less sensitive
24 48 second confirmatory test. Then it is important to determine if all available treponemal assays are
25 49 comparable in analytical sensitivity to avoid uncertainty in their selection (3).
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43 44 45 46 47 48 49 50 51 **Methods**

52 Two hundred ninety stored serum samples randomly selected from our serum bank were used for
53 this study. Originally the serum samples were obtained from the Georgia Public Health Laboratory
54 with all identifiers removed and under a CDC IRB approval. Due to the prevalence of high lipid
55 contents of the sera, the samples were treated with Cleanascite HC (PureBiotech, Middlesex, NJ) (7).
56 The patterns of reactivity of the test panel were determined at CDC by a quantitative RPR (Becton
57 Dickinson, Baltimore, MD) and a qualitative TP-PA (Fujirebio Diagnostics, Inc., Malvern, PA). All
58 serum samples were divided into aliquots and frozen at -20°C so that the number of freeze-thaw
59 cycles was consistent for each sera tested. Eight of the treponemal assays: FTA-ABS; (Zeus
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Scientific, Raritan, NJ), LIAISON Treponema Assay (DiaSorin, Inc., Stillwater, MN), SD Bioline Syphilis 3.0 Rapid Point of Care (POC) test (Standard Diagnostics, Inc., Korea), INNO-LIA immunoblot (Innogenetics, Gent, Belgium), Bioelisa (BioKit, Barcelona, Spain) and the Captia IgG, Trep-ID, and Trep-Sure (Trinity Biotech, Jamestown, NY) were tested qualitatively, then quantitatively with reactive samples. In the quantitative test, two-fold serial dilutions (from 1:2 to 1:16,384) were prepared using normal nonreactive human plasma converted to serum (8) as a diluent. All dilutions were treated as neat sera and tested with the nine different assays according to the manufacturer's package insert. The test was performed blind, the EIA results were collated and compared to the TP-PA used as the predicate for the qualitative test.

Statistical Methods.

The endpoint titer data was converted to dilution data from the exponential scale (titer) to a linear scale (dilution) by taking the logarithm of the endpoint titer, i.e., $\log_2(\text{titer}) = \text{dilution}$. This transformation reduces the variance within the test and between the tests. A generalized linear model was fitted to these data by the Generalized Estimating Equations (GEE) method to test for the differences between the mean dilutions of these tests. Adjustments for multiple comparisons between the mean dilutions of these tests were made by Tukey-Kramer method. A p-value of <0.05 was used as a significance level for the differences. All statistical analyses were conducted using SAS, version 9.3 (SAS Institute, Cary, North Carolina).

Results

All treponemal syphilis assays were performed according to the manufacturer's direction circulars using two hundred ninety stored serum samples of which 109 were reactive and 181 nonreactive for TP-PA. Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-ABS 94.5% - 100%, INNO-LIA 99.1% - 99.4%, LIAISON 100% - 99.4%. Trep-Sure 100% - 98.9%, Bioelisa 100% - 98.9%, Bioline 100% - 98.9%, Captia IgG 100% - 97.2%, Trep-ID 100%-100%. These results indicated that most of the assays were comparable to TP-PA when the serum samples were tested undiluted. However, FTA-ABS failed to detect six TP-PA reactive samples and INNO-LIA one, while the other four assays missed none. One hundred and nine stored serum samples

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3 89 reactive by TP-PA were serially diluted in nonreactive human plasma converted to serum (8), with
4 the purpose of demonstrating the endpoint titer of each sample with the nine different assays. With a
5 simple comparison the endpoint titers indicated that Trep-Sure had a median value of 1:512,
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7 91 Bioelisa 1:128, LIAISON 1:64, Trep-ID 1:64, Bioline 1:32, INNO-LIA 1:16, TP-PA 1:16, CAPTIA
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9 92 IgG 1:8 and FTA-ABS 1:4 (Table 2).
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13 94 Of the 109 TP-PA reactive sera, 95(85%) were nonreactive for the confirmatory RPR test. Fourteen
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15 95 samples were reactive for both TP-PA and RPR. All other assays were able to detect treponemal
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17 96 antibody in those undiluted sera that were reactive by the TP-PA. However, five sera found to be
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19 97 reactive by the CAPTIA IgG test and one by the BIOLINE were found to be non-reactive by all
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21 98 other tests suggesting false positives. All other sera found to be non-reactive by the TP-PA but
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23 99 reactive by another test could be considered as true positives since the sera were found to be reactive
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25 100 by at least two other tests (see Table 1). If the TP-PA would have been selected as the screening test
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27 101 for the reverse algorithm then eight samples reactive by the other assays would have been missed
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29 102 resulting in a false serological result.

30 103 Mean dilutions and their standard errors of eight quantitative treponemal assays and the TP-PA
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32 104 estimated by the generalized linear model are shown in Table 2. There were significant ($p < 0.0001$)
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34 105 differences between the mean dilutions of all tests, except between TP-PA and INNO-LIA ($p = 1.000$)
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36 106 and Trep-ID and LIASON ($p = 1.000$). This analysis was repeated after subtracting the FTA-ABS
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38 107 titer from the titer of the corresponding test. In this analysis also there was no significant difference
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40 108 between TP-PA and INNO-LIA ($p = 1.000$) and Trep-ID and LIASON ($p = 1.000$), and the differences
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42 109 among the rest of the pairs were significant ($p < 0.0001$). (Table 2)
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45 111 **Discussion:**

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47 112 The reverse sequence syphilis screening algorithm is being used because it takes advantage of
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49 113 automated systems. However, the selection of these assays to screen serum samples with suspected
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51 114 cases of syphilis presents problems that were not clearly anticipated (6). In cases where the
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53 115 treponemal test is positive and the confirmatory RPR test is negative, the CDC recommends that a
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55 116 second treponemal test be used to determine the serological validity of the screening test (5). If the
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57 117 second treponemal test is negative, then it is inconclusive whether the first screening test is a false
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3 118 positive or a more sensitive test. This situation presents a dilemma for physicians and
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5 119 understandably they will be asking for clarification as to whether it is or not a case of syphilis in
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7 120 asymptomatic situations. Consequently, it would be advisable for the laboratorian to consider the
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9 121 selection of more sensitive screening and confirmatory treponemal assays to avoid having discrepant
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11 122 results. This is not an easy task because of the different assay platforms presently used for screening
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13 123 in the reverse algorithm system ranging from FTA-ABS, EIA, CIA, TP-PA and POC. In this study
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15 124 an attempt was made to determine the analytical level of agreement of the assays with the purpose
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17 125 of better understanding the abilities of the assays for detection of low level antibodies. When the
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19 126 nine assays were tested quantitatively, the FTA-ABS was the treponemal test with the lowest
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21 127 median endpoint titer (1:4). In contrast, Trep-Sure had a median endpoint titer of (1:512) (Table2).
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23 128 Since the FTA-ABS was the least reactive, its reevaluation should be considered.

24 129 Further studies with documented clinical samples are necessary to demonstrate the correlation
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26 130 between the antibody titer and the stage of the disease. With these comparison results we then could
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28 131 infer that there are marked differences between the analytical sensitivities of these assays especially
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30 132 with low titer sera such as those found in primary or latent cases. The variability of results with the
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32 133 different assays may be due in part to the composition of their systems. Those assays using the
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34 134 double antigen or sandwich configuration such as Trep-Sure, Bioelisa, Trep-ID and LIAISON where
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36 135 the recombinant antigen such as Tp15, 17, 47 KD or combination of them are both attached to the
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38 136 ELISA plate or latex particle and are directly conjugated to horse radish peroxidase (HRP) or
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40 137 chemiluminescence (CIA). This configuration tends to be more sensitive because they are able to
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42 138 detect both IgG and IgM antibodies in the sera of patients with syphilis. In addition, the detection of
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44 139 IgM antibodies amplifies the signal due to its multiple binding sites. On the other hand, normally
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46 140 those assays using the indirect or anti-human system such as Captia (IgG) and FTA-ABS are limited
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48 141 in that they only detect one single immunoglobulin.

49 142 When selecting the reverse algorithm system for syphilis diagnostic serology and in cases when the
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51 143 treponemal test is reactive and the RPR nonreactive, it would be advisable to consider the selection
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53 144 of more analytically sensitive screening and confirmatory treponemal assays preferably those based
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55 145 on the double antigen or sandwich method, such as Trep-Sure, Bioelisa, Trep-ID and LIASON to
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57 146 avoid the necessity of using a less sensitive second confirmatory treponemal assay.

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TABLE 1. Percent concordance of eight qualitative syphilis treponemal test compared to the predicate TP-PA

TP-PA Predicate samples	Total no of samples	FTA-ABS		INNOLIA		Liaison		Trep-Sure		Bioelisa		Bioline		Captia (IgG)		Trep-ID	
		R ^a	NR ^b	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR
Reactive	109	103	6(94.4)	108	1(99.1)	109	0(100)	109	0(100)	109	0(100)	109	0(100)	109	0(100)	109	0(100)
Nonreactive	181	0	181(100)	1	180(99.4)	1	180(99.4)	2	179(98.9)	2	179(98.9)	2	179(98.9)	5	176(97.2)	0	181(100)

^a R, reactive

^b NR, nonreactive

TABLE 2. Mean Dilutions (Quantitative end point titers) for nine treponemal syphilis assays of TP-PA reactive sera

Test	Median Titer	Estimates of Mean Dilutions and 95% Confidence limits				Mean Dilution difference from FTA-ABS	
		Mean Dilution	Std Error	Lower 95% CL	Upper 95% CL	Mean Difference	Std Error
FTA-ABS*	4	1.9266	0.1655	1.6022	2.2510	n/a	n/a
Captia IgG*	8	3.1560	0.1552	2.8517	3.4602	1.229	0.082
TP-PA**	16	4.0734	0.1624	3.7551	4.3917	2.147	0.102
INNO-LIA*	16	4.0917	0.1927	3.7141	4.4694	2.165	0.123
Bioline**	64	5.6606	0.1886	5.2910	6.0301	3.734	0.135
Trep-ID**	64	6.2752	0.1906	5.9016	6.6489	4.349	0.123
LIAISON**	128	6.3119	0.1603	5.9977	6.6262	4.385	0.105
Bioelisa**	128	7.1560	0.1789	6.8053	7.5066	5.229	0.112
Trep-Sure**	512	8.8899	0.1747	8.5475	9.2323	6.963	0.1182

* IgG only

** IgG/IgM detection



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Primary Subject Heading:	Infectious diseases
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1 Abstract

2 **Objective:** The serological diagnosis of syphilis requires the detection of two distinct antibodies, the
3 nontreponemal and treponemal. CDC recommends screening first with a nontreponemal test such as
4 (RPR/VDRL), and then confirming those results with one of several treponemal tests (FTA-ABS,
5 EIA, CIA, TP-PA or POC). Due to the high volume of samples processed by some laboratories
6 using automated systems, the screening with treponemal assays and confirming with nontreponemal
7 tests is becoming the established norm. The purpose of this study was to evaluate eight treponemal
8 assays using TP-PA as the predicate assay.

9 **Methods:** Two hundred ninety stored serum samples were tested qualitatively according to the
10 manufacturer's directions.

11 **Results:** Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-
12 ABS 94.5% and 100%, Trep-Sure 100% and 98.9%, Bioelisa 100% and 98.9%, INNO-LIA 99.1% and
13 99.4%, Bioline 100% and 98.9%, Captia IgG 100% and 97.2%, Trcp-ID 100% and 100%, LIAISON
14 100% and 99.4% respectively. In order to properly evaluate the performance of these assays, the analytical
15 sensitivity was determined by endpoint titration of serial dilutions of the reactive serum samples in normal
16 sera. The median endpoint titer varied from 1:4 for FTA-ABS to 1:512 for Trep-Sure.

17 **Conclusions:** The performance of the treponemal serological assays was comparable when using
18 medium and high titer sera. However, the varying performance on specimen dilutions suggest
19 that there may be differences in sensitivity with low titer sera that are more prevalent in primary and
20 late syphilis cases.

21 Introduction

22 Syphilis is a sexually transmitted disease caused by the bacterium *Treponema pallidum*. The clinical
23 diagnosis is difficult due to the complexity of manifestations which requires the aid of serological
24 interpretation. The serological diagnosis of syphilis depends upon the detection of two distinct
25 antibodies, the nontreponemal or heterophile antibodies (reagin) directed against cardiolipin released
26 from damaged host cells and from the treponemes themselves. These antibodies can also be present
27 in other diseases and human conditions such as (Lupus, malaria, HIV, IV drug users, etc.) (1). The
28 presence of nontreponemal antibodies is indicative of active infection, thus a reduction in titer can
29 suggest a successful antibiotic therapy, and a significant increase can indicate a possible relapse or
30 reinfection (2). The treponemal antibodies are primarily directed against specific lipoprotein antigens

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3 31 of the bacterium such as 15,17, and 47-kDa. Even after treatment or time, these treponemal
4 32 antibodies usually remain present for life. A positive treponemal test cannot distinguish between
5 33 active, treated, and old cases of syphilis.

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9 34 The traditional algorithm is to screen with a nontreponemal assay such as VDRL or RPR and those
10 35 serum samples found reactive are then confirmed using a treponemal test. With the introduction of
11 36 automated systems which are appropriate for high output volumes, the reverse algorithm is gaining
12 37 acceptability and its usefulness is arguable justified (6). However, the adoption of this reverse
13 38 algorithm has increased the number of discrepant results between the screening and confirmatory
14 39 tests (4, 5). One reason the traditional algorithm was established was to avoid the detection of
15 40 previously treated cases, especially in *low* prevalence settings where the majority of sera will test
16 41 negative and not require further evaluation, unless a patient has been reinfected or in rare
17 42 circumstances, their non-treponemal antibody titers remain serofast. With the application of the
18 43 reverse algorithm even in low prevalence settings all previously treated cases will likely be detected.
19 44 When the screening treponemal test is positive and the confirmatory nontreponemal test is negative,
20 45 the CDC guidelines recommend using a second treponemal test to validate the results of the
21 46 screening test. The selection of a second confirmatory test introduces the possibility of having an
22 47 analytically less sensitive test which would suggest a false positive screening or a less sensitive
23 48 second confirmatory test. Then it is important to determine if all available treponemal assays are
24 49 comparable in analytical sensitivity to avoid uncertainty in their selection (3).

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40 41 51 **Methods**

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44 52 Two hundred ninety stored serum samples randomly selected from our serum bank were used for
45 53 this study. Originally the serum samples were obtained from the Georgia Public Health Laboratory
46 54 with all identifiers removed and under a CDC IRB approval. Due to the prevalence of high lipid
47 55 contents of the sera, the samples were treated with Cleanascite HC (PureRiotech, Middlesex, NJ) (7).
48 56 The patterns of reactivity of the test panel were determined at CDC by a quantitative RPR (Becton
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50 58 serum samples were divided into aliquots and frozen at -20°C so that the number of freeze-thaw
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60 Scientific, Raritan, NJ), LIAISON Treponema Assay (DiaSorin, Inc., Stillwater, MN), SD Bioline
61 Syphilis 3.0 Rapid Point of Care (POC) test (Standard Diagnostics, Inc., Korea), INNO-LIA
62 immunoblot (Innogenetics, Gent, Belgium), Bioelisa (BioKit, Barcelona, Spain) and the Captia
63 IgG, Trep-ID. and Trep-Surc (Trinity Biotech, Jamestown, NY) were tested qualitatively, then
64 quantitatively with reactive samples. In the quantitative test, two-fold serial dilutions (from 1:2 to
65 1:16,384) were prepared using normal nonreactive human plasma converted to serum (8) as a
66 diluent. All dilutions were treated as neat sera and tested with the nine different assays according to
67 the manufacturer's package insert. The test was performed blind, the EIA results were collated and
68 compared to the TP-PA used as the predicate for the qualitative test.

70 **Statistical Methods.**

71 The endpoint titer data was converted to dilution data from the exponential scale (titer) to a linear
72 scale (dilution) by taking the logarithm of the endpoint titer, i.e., $\log_2(\text{titer}) = \text{dilution}$. This
73 transformation reduces the variance within the test and between the tests. A generalized linear model
74 was fitted to these data by the Generalized Estimating Equations (GEE) method to test for the
75 differences between the mean dilutions of these tests. Adjustments for multiple comparisons
76 between the mean dilutions of these tests were made by Tukey-Kramer method. A p-value of <0.05
77 was used as a significance level for the differences. All statistical analyses were conducted using
78 SAS, version 9.3 (SAS Institute, Cary, North Carolina).

80 **Results**

81 All treponemal syphilis assays were performed according to the manufacturer's direction circulars
82 using two hundred ninety stored serum samples of which 109 were reactive and 181 nonreactive for
83 TP-PA. Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-ABS
84 94.5% and 100%, INNO-LIA 99.1% and 99.4%, LIAISON 100% and 99.4%. Trep-Sure 100% and
85 98.9%,
86 Bioelisa 100% and 98.9%, Bioline 100% and 98.9%, Captia IgG 100% and 97.2%, Trep-ID 100% and
87 100% respectively.

86 These results indicated that most of the assays were comparable to TP-PA when the serum samples
87 were tested undiluted. However, FTA-ABS failed to detect six TP-PA reactive samples and INNO-

88 LIA one, while the other four assays missed none. Of the 109 TP-PA reactive sera, 95(85%) were
1 nonreactive for the confirmatory RPR test. Fourteen samples were reactive for both TP-PA and RPR. All
2 other assays were able to detect treponemal antibody in those undiluted sera that were reactive by the TP-
3 PA. However, five sera found to be reactive by the Captia IgG test and one by the BIOLINE were found to
4 be non-reactive by all other tests suggesting false positives. All other sera found to be non-reactive by the
5 TP-PA but reactive by another test could be considered as true positives since the sera were found to be
6 reactive by at least two other tests (see Table 1). If the TP-PA would have been selected as the screening test
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10 101 for the reverse algorithm then eight samples reactive by the other assays would have been missed
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12 102 resulting in a false serological result.

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14 One hundred and nine stored serum samples reactive by TP-PA were serially diluted in nonreactive
15 human plasma converted to serum (8), with the purpose of demonstrating the endpoint titer of each sample
16 with the nine different assays. With a simple comparison the endpoint titers indicated that Trep-Sure had a
17 median value of 1:512, Bioelisa 1:128, LIAISON 1:64, Trep-ID 1:64, Bioline 1:32, INNO-LIA 1:16, TP-PA
18 1:16, Captia IgG 1:8 and FTA-ABS 1:4 (Table 2).
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24 103 Mean dilutions and their standard errors of eight quantitative treponemal assays and the TP-PA
25 104 estimated by the generalized linear model are shown in Table 2. There were significant ($p < 0.0001$)
26 105 differences between the mean dilutions of all tests, except between TP-PA and INNO-LIA ($p = 1.000$)
27 106 and Trep-ID and LIASON ($p = 1.000$). This analysis was repeated after subtracting the FTA-ABS
28 107 titer from the titer of the corresponding test. In this analysis also there was no significant difference
29 108 between TP-PA and INNO-LIA ($p = 1.000$) and Trep-ID and LIASON ($p = 1.000$), and the differences
30 109 among the rest of the pairs were significant ($p < 0.0001$). (Table 2)
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37 38 111 **Discussion:**

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41 112 The reverse sequence syphilis screening algorithm is being used because it takes advantage of
42 113 automated systems. However the selection of these assays to screen serum samples with suspected
43 114 cases of syphilis presents problems that were not clearly anticipated (6). In cases where the
44 115 treponemal test is positive and the confirmatory RPR test is negative, the CDC recommends that a
45 116 second treponemal test be used to determine the serological validity of the screening test (5). If the
46 117 second treponemal test is negative, then it is inconclusive whether the first screening test is a false
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3 118 positive or a more sensitive test. This situation presents a dilemma for physicians and
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5 119 understandably they will be asking for clarification as to whether it is or not a case of syphilis in
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7 120 asymptomatic situations. Consequently, it would be advisable for the laboratorian to consider the
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11 122 results. This is not an easy task because of the different assay platforms presently used for screening
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13 123 in the reverse algorithm system ranging from FTA-ABS, EIA, CIA, TP-PA and POC. In this study
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19 126 nine assays were tested quantitatively, the FTA-ABS was the treponemal test with the lowest
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21 127 median endpoint titer (1:4). In contrast, Trep-Sure had a median endpoint titer of (1:512) (Table 2).
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23 128 Since the FTA-ABS was the least reactive, its reevaluation should be considered.

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25 129 Further studies with documented clinical samples are necessary to demonstrate the correlation
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27 130 between the antibody titer and the stage of the disease. With these comparison results we then could
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29 131 infer that there are marked differences between the analytical sensitivities of these assays especially
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31 132 with low titer sera such as those found in primary or latent cases. The variability of results with the
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33 133 different assays may be due in part to the composition of their systems. Those assays using the
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35 134 double antigen or sandwich configuration such as Trep-Sure, Bioelisa, Trep-ID and LIAISON where
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37 135 the recombinant antigen such as Tp15, 17, 47 KD or combination of them are both attached to the
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39 136 ELISA plate or latex particle and are directly conjugated to horse radish peroxidase (HRP) or
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55 144 of more analytically sensitive screening and confirmatory treponemal assays preferably those based
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57 145 on the double antigen or sandwich method, such as Trep-Sure, Bioelisa, Trep-ID and LIASON to
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59 146 avoid the necessity of using a less sensitive second confirmatory treponemal assay.

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TABLE 1. Percent concordance of eight qualitative syphilis treponemal test compared to the predicate TP-PA

TP-PA Predicate	Total no of samples	FTA-ABS		INNOLIA		Liaison		Trep-Sure		Bioelisa		Bioline		Captia (IgG)		Trep-ID	
		R ^a	NR ^b	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR
Reactive	109	103	6(94.4%)	108	1(99.1%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)
Nonreactive	181	0	181(100%)	1	180(99.4%)	1	180(99.4%)	2	179(98.9%)	2	179(98.9%)	2	179(98.9%)	5	176(97.2%)	0	181(100%)

^aR, reactive

^bNR, nonreactive

188 TABLE 2. Mean Dilutions (Quantitative end point titers) for nine treponemal syphilis assays of TP-PA reactive sera
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Test	Median Titer	Estimates of Mean Dilutions and 95% Confidence limits				Mean Dilution difference from FTA-ABS	
		Mean Dilution	Std Error	Lower	Upper	Mean Difference	Std Error
				95% CL	95% CL		
FTA-ABS*	4	1.9266	0.1655	1.6022	2.2510	n/a	n/a
Captia IgG*	8	3.1560	0.1552	2.8517	3.4602	1.229	0.082
TP-PA**	16	4.0734	0.1624	3.7551	4.3917	2.147	0.102
INNO-LIA*	16	4.0917	0.1927	3.7141	4.4694	2.165	0.123
Bioline**	64	5.6606	0.1886	5.2910	6.0301	3.734	0.135
Trep-ID**	64	6.2752	0.1906	5.9016	6.6489	4.349	0.123
LIAISON**	128	6.3119	0.1603	5.9977	6.6262	4.385	0.105
Bioelisa**	128	7.1560	0.1789	6.8053	7.5066	5.229	0.112
Trep-Sure**	512	8.8899	0.1747	8.5475	9.2323	6.963	0.1182

191 * IgG only

192 ** IgG/IgM detection

1 Abstract

2 **Objective:** The serological diagnosis of syphilis requires the detection of two distinct antibodies, the
3 nontreponemal and treponemal. CDC recommends screening first with a nontreponemal test such as
4 (RPR/VDRL), and then confirming those results with one of several treponemal tests (FTA-ABS,
5 EIA, CIA, TP-PA or POC). Due to the high volume of samples processed by some laboratories
6 using automated systems, the screening with treponemal assays and confirming with nontreponemal
7 tests is becoming the established norm. The purpose of this study was to evaluate eight treponemal
8 assays using TP-PA as the predicate assay.

9 **Methods:** Two hundred ninety stored serum samples were tested qualitatively according to the
10 manufacturer's directions.

11 **Results:** Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-
12 ABS 94.5% and 100%, Trep-Sure 100% and 98.9%, Bioelisa 100% and 98.9%, INNO-LIA 99.1% and
13 99.4%, Bioline 100% and 98.9%. Captia IgG 100% and 97.2%, Trcp-ID 100% and 100%, LIAISON
14 100% and 99.4% respectively. In order to properly evaluate the performance of these assays, the analytical
15 sensitivity was determined by endpoint titration of serial dilutions of the reactive serum samples in normal
16 sera. The median endpoint titer varied from 1:4 for FTA-ABS to 1:512 for Trep-Sure.

17 **Conclusions:** The performance of the treponemal serological assays was comparable when using
18 medium and high titer sera. However, the varying performance on specimen dilutions suggest
19 that there may be differences in sensitivity with low titer sera that are more prevalent in primary and
20 late syphilis cases.

21 Introduction

22 Syphilis is a sexually transmitted disease caused by the bacterium *Treponema pallidum*. The clinical
23 diagnosis is difficult due to the complexity of manifestations which requires the aid of serological
24 interpretation. The serological diagnosis of syphilis depends upon the detection of two distinct
25 antibodies, the nontreponemal or heterophile antibodies (reagin) directed against cardiolipin released
26 from damaged host cells and from the treponemes themselves. These antibodies can also be present
27 in other diseases and human conditions such as (Lupus, malaria, HIV, IV drug users, etc.) (1). The
28 presence of nontreponemal antibodies is indicative of active infection, thus a reduction in titer can
29 suggest a successful antibiotic therapy, and a significant increase can indicate a possible relapse or
30 reinfection (2). The treponemal antibodies are primarily directed against specific lipoprotein antigens

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3 31 of the bacterium such as 15,17, and 47-kDa. Even after treatment or time, these treponemal
4 32 antibodies usually remain present for life. A positive treponemal test cannot distinguish between
5 33 active, treated, and old cases of syphilis.

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9 34 The traditional algorithm is to screen with a nontreponemal assay such as VDRL or RPR and those
10 35 serum samples found reactive are then confirmed using a treponemal test. With the introduction of
11 36 automated systems which are appropriate for high output volumes, the reverse algorithm is gaining
12 37 acceptability and its usefulness is arguable justified (6). However, the adoption of this reverse
13 38 algorithm has increased the number of discrepant results between the screening and confirmatory
14 39 tests (4, 5). One reason the traditional algorithm was established was to avoid the detection of
15 40 previously treated cases, especially in *low* prevalence settings where the majority of sera will test
16 41 negative and not require further evaluation, unless a patient has been reinfected or in rare
17 42 circumstances, their non-treponemal antibody titers remain serofast. With the application of the
18 43 reverse algorithm even in low prevalence settings all previously treated cases will likely be detected.
19 44 When the screening treponemal test is positive and the confirmatory nontreponemal test is negative,
20 45 the CDC guidelines recommend using a second treponemal test to validate the results of the
21 46 screening test. The selection of a second confirmatory test introduces the possibility of having an
22 47 analytically less sensitive test which would suggest a false positive screening or a less sensitive
23 48 second confirmatory test. Then it is important to determine if all available treponemal assays are
24 49 comparable in analytical sensitivity to avoid uncertainty in their selection (3).

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40 41 51 **Methods**

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44 52 Two hundred ninety stored serum samples randomly selected from our serum bank were used for
45 53 this study. Originally the serum samples were obtained from the Georgia Public Health Laboratory
46 54 with all identifiers removed and under a CDC IRB approval. Due to the prevalence of high lipid
47 55 contents of the sera, the samples were treated with Cleanascite HC (PureRiotech, Middlesex, NJ) (7).
48 56 The patterns of reactivity of the test panel were determined at CDC by a quantitative RPR (Becton
49 57 Dickinson, Baltimore, MD) and a qualitative TP-PA (Fujirebio Diagnostics, Inc., Malvern, PA). All
50 58 serum samples were divided into aliquots and frozen at -20°C so that the number of freeze-thaw
51 59 cycles was consistent for each sera tested. Eight of the treponemal assays: FTA-ABS; (Zeus

Scientific, Raritan, NJ), LIAISON Treponema Assay (DiaSorin, Inc., Stillwater, MN), SD Bioline Syphilis 3.0 Rapid Point of Care (POC) test (Standard Diagnostics, Inc., Korea), INNO-LIA immunoblot (Innogenetics, Gent, Belgium), Bioelisa (BioKit, Barcelona, Spain) and the Captia IgG, Trep-ID. and Trep-Sure (Trinity Biotech, Jamestown, NY) were tested qualitatively, then quantitatively with reactive samples. In the quantitative test, two-fold serial dilutions (from 1:2 to 1:16,384) were prepared using normal nonreactive human plasma converted to serum (8) as a diluent. All dilutions were treated as neat sera and tested with the nine different assays according to the manufacturer's package insert. The test was performed blind, the EIA results were collated and compared to the TP-PA used as the predicate for the qualitative test.

Statistical Methods.

The endpoint titer data was converted to dilution data from the exponential scale (titer) to a linear scale (dilution) by taking the logarithm of the endpoint titer, i.e., $\log_2(\text{titer}) = \text{dilution}$. This transformation reduces the variance within the test and between the tests. A generalized linear model was fitted to these data by the Generalized Estimating Equations (GEE) method to test for the differences between the mean dilutions of these tests. Adjustments for multiple comparisons between the mean dilutions of these tests were made by Tukey-Kramer method. A p-value of <0.05 was used as a significance level for the differences. All statistical analyses were conducted using SAS, version 9.3 (SAS Institute, Cary, North Carolina).

Results

All treponemal syphilis assays were performed according to the manufacturer's direction circulars using two hundred ninety stored serum samples of which 109 were reactive and 181 nonreactive for TP-PA. Concordance with specimens tested as reactive or nonreactive using TP-PA was: FTA-ABS 94.5% and 100%, INNO-LIA 99.1% and 99.4%, LIAISON 100% and 99.4%. Trep-Sure 100% and 98.9%, Bioelisa 100% and 98.9%, Bioline 100% and 98.9%, Captia IgG 100% and 97.2%, Trep-ID 100% and 100% respectively.

These results indicated that most of the assays were comparable to TP-PA when the serum samples were tested undiluted. However, FTA-ABS failed to detect six TP-PA reactive samples and INNO-

LIA one, while the other four assays missed none. Of the 109 TP-PA reactive sera, 95(85%) were nonreactive for the confirmatory RPR test. Fourteen samples were reactive for both TP-PA and RPR. All other assays were able to detect treponemal antibody in those undiluted sera that were reactive by the TP-PA. However, five sera found to be reactive by the Captia IgG test and one by the BIOLINE were found to be non-reactive by all other tests suggesting false positives. All other sera found to be non-reactive by the TP-PA but reactive by another test could be considered as true positives since the sera were found to be reactive by at least two other tests (see Table 1). If the TP-PA would have been selected as the screening test for the reverse algorithm then eight samples reactive by the other assays would have been missed resulting in a false serological result.

One hundred and nine stored serum samples reactive by TP-PA were serially diluted in nonreactive human plasma converted to serum (8), with the purpose of demonstrating the endpoint titer of each sample with the nine different assays. With a simple comparison the endpoint titers indicated that Trep-Sure had a median value of 1:512, Bioelisa 1:128, LIAISON 1:64, Trep-ID 1:64, Bioline 1:32, INNO-LIA 1:16, TP-PA 1:16, Captia IgG 1:8 and FTA-ABS 1:4 (Table 2).

Mean dilutions and their standard errors of eight quantitative treponemal assays and the TP-PA estimated by the generalized linear model are shown in Table 2. There were significant ($p < 0.0001$) differences between the mean dilutions of all tests, except between TP-PA and INNO-LIA ($p = 1.000$) and Trep-ID and LIASON ($p = 1.000$). This analysis was repeated after subtracting the FTA-ABS titer from the titer of the corresponding test. In this analysis also there was no significant difference between TP-PA and INNO-LIA ($p = 1.000$) and Trep-ID and LIASON ($p = 1.000$), and the differences among the rest of the pairs were significant ($p < 0.0001$). (Table 2)

111 Discussion:

112 The reverse sequence syphilis screening algorithm is being used because it takes advantage of
113 automated systems. However the selection of these assays to screen serum samples with suspected
114 cases of syphilis presents problems that were not clearly anticipated (6). In cases where the
115 treponemal test is positive and the confirmatory RPR test is negative, the CDC recommends that a
116 second treponemal test be used to determine the serological validity of the screening test (5). If the
117 second treponemal test is negative, then it is inconclusive whether the first screening test is a false

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TABLE 1. Percent concordance of eight qualitative syphilis treponemal test compared to the predicate TP-PA

TP-PA Predicate	Total no of samples	FTA-ABS		INNOLIA		Liaison		Trep-Sure		Bioelisa		Bioline		Captia (IgG)		Trep-ID	
		R ^a	NR ^b	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR	R	NR
Reactive	109	103	6(94.4%)	108	1(99.1%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)	109	0(100%)
Nonreactive	181	0	181(100%)	1	180(99.4%)	1	180(99.4%)	2	179(98.9%)	2	179(98.9%)	2	179(98.9%)	5	176(97.2%)	0	181(100%)

^a R, reactive

^b NR, nonreactive

188 TABLE 2. Mean Dilutions (Quantitative end point titers) for nine treponemal syphilis assays of TP-PA reactive sera
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Test	Median Titer	Estimates of Mean Dilutions and 95% Confidence limits				Mean Dilution difference from FTA-ABS	
		Mean Dilution	Std Error	Lower 95% CL	Upper 95% CL	Mean Difference	Std Error
FTA-ABS*	4	1.9266	0.1655	1.6022	2.2510	n/a	n/a
Captia IgG*	8	3.1560	0.1552	2.8517	3.4602	1.229	0.082
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Trep-Sure**	512	8.8899	0.1747	8.5475	9.2323	6.963	0.1182

191 * IgG only

192 ** IgG/IgM detection

Correction

Castro A, Jost H, Cox D, *et al.* A comparison of the analytical level of agreement of nine treponemal assays for syphilis and possible implications for screening algorithms. *BMJ Open* 2013;3:e003347. The order of the authors was published incorrectly in this paper. The correct order is: Heather Jost, Arnold Castro, David Cox, Yetunde Fakile, Susan Kikkert, Ye Tun, Akbar Zaidi, Mahin Park.

BMJ Open 2013;3:e003347. doi:10.1136/bmjopen-2013-003347corr1