IS THIS THE TIME TO REPLACE NON-TREPONEMAL FLOCCULATION TESTS WITH A SUPERIOR TECHNIQUE FOR SYPHILIS DIAGNOSIS

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ABSTRACT

Syphilis is a sexually acquired infection, and is caused by the spiral shaped bacteria called *Treponema pallidum* subspecies *pallidum*. Non-treponemal and treponemal types of serological tests are the most routinely use for its diagnosis. In India, non-treponemal Rapid Plasma Reagin (RPR) or Venereal Disease Research Laboratory (VDRL) test is used for syphilis screening. Both are sensitive test, but require further confirmation of test result with a treponemal specific test as per traditional practice for syphilis diagnosis. These tests are still popular but the subjectivity in the result interpretation is the major obstacle. Hence, they are losing popularity. An attempt was made to develop a non-treponemal enzyme immunoassay (EIA) test. A machine record the result, hence result interpretation is easy. The non-treponemal cardiolipin antigen was attached to the microplate through a covalent linkage, and subsequently used in test development. Such antigen coated plate demonstrated a good coefficient of variation (CV%) of 2-7%, and that confirms its firm attachment to the microplate. A study with true syphilis positive and negative samples showed sensitivity and specificity of 92.4% and 97.2% respectively. Described test can be useful as an alternative to the non-treponemal flocculation tests. However, this new method needs to be improved further with the test sensitivity, and required a validation study with more number of samples.

Keywords: Syphilis, RPR, VDRL, Non-treponemal tests, Treponemal tests

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INTRODUCTION

Syphilis as a disease is known from many centuries. As described by Frith (2012), an Italian war of 1495 was the earlier documented evidence. Majorly, this disease is transmitted through sexual intercourse. However, the syphilis etiological agent, *Treponema pallidum* subspecies *pallidum* can also be transmitted from infected mother to her fetus congenitally and through blood transfusion as underscored by Goh (2005). An infection of syphilis advances through multiple stages. The appearance of ulcer on genital organ and other anatomical site is the characteristics of the primary syphilis. Typically, an ulcer is small and generally appears within three week after infection. It heals within 4-5 weeks after appearance without any treatment as illustrated by Avelleira et al. (2006). The ulcer is infectious and has many live bacteria in it. It can do disease transmission upon direct contact. Manifestation of skin rash on palms of hands, and soles of feet are the main sign of secondary syphilis and that follows after primary stage. All described symptoms heal spontaneously. However, Larsen et al. (1995) described that they may reemerge later if not adequately treated. Other signs that do appear include fever, muscle pain, impaired vision, loss of appetite and other generalized symptoms. These two symptomatic stages are followed by an asymptomatic stage called latent stage. It may last from 3 to 30 years. Untreated infection may advance further to the last stage called tertiary or late syphilis. Avelleira et al. (2006) mentioned that during late stage, syphilis bacteria could damage various tissues and organs.

Different techniques are used today for the diagnosis of syphilis. Animal infectivity tests, direct detection of bacteria from lesions through dark-field microscopy and direct fluorescent assay, serological tests, and molecular techniques are the principle methods. However, among all, serological tests are the most widely used method for syphilis diagnosis. They can be grouped into non-treponemal and treponemal types. The non-treponemal group includes Venereal Disease Research Laboratory (VDRL) slide test, Rapid Plasma Reagin (RPR) test, Unheated Serum Reagin (USR) test, and Toluidine Red Unheated Serum (TRUST) tests. They all in general use a combination of cardiolipin, lecithin and cholesterol in an optimized proportion and are able to detect IgM and IgG classes of non-treponemal antibody. This type of antibody is directed against the lipoidal material released from damaged host cells, and from the bacteria as described by Matthews et al. (1979). A treponemal group of test includes *T.pallidum* Hemagglutination assay (TPHA), *T.pallidum* Particle Agglutination Assay (TP-PA), Fluorescent Treponemal Antibody Adsorption (FTA-ABS) test, and *T.pallidum* specific Enzyme Immunoassay (EIA). All deals with the detection of treponemal bacteria specific antibody.

Screening of syphilis follows various algorithms. As per traditional screening algorithm proposed by Centers for Disease

Control (2008), a screening by a non-treponemal test and later confirmation with a treponemal specific test is the recommended approach. A non-treponemal test as per Larsen et al. (1995) may give false positive result in cases of other infections or conditions. Hence, a verification of its result is required with a treponemal specific test. The reverse screening algorithm is the opposite practice. It uses a treponemal specific test first for syphilis screening, and thereafter a non-treponemal test is used for confirmation as described by Lipinsky et al. (2012). Zhang et al. (2012) reported that the recent introduction of automated treponemal test has endorsed described reverse screening practice. However, this practice may give false positive test result in treated patients who had cured from the syphilis infection successfully as described by Binnicker et al. (2011). It might be very useful for screening a population with low syphilis incidence. In case of screening a high syphilis prevalence population, European centers for disease control and prevention (ECDC) recommended the use of two different treponemal tests for screening and confirmation as described by Tong et al. (2014).

A need for the better non-treponemal test has already been arisen. The subjectivity of a non-treponemal test adds difficulty in result interpretation and reporting. It is also very cumbersome when testing a huge number of samples. Certain studies were reported in the past that had claimed an equivalent test system to the traditional non-treponemal tests in performance. A VDRL EIA was developed, and reported by Pedersen et al. (1987). An ethanolic solution containing 0.03% w/v cardiolipin, 0.021% w/v lecithin, and 0.09% w/v cholesterol was evaporated on the microplate and used in EIA study. Their study claimed sensitivity and specificity of 96.6 % and 99.6% respectively for VDRL IgG EIA and, 94.9% and 99.5% respectively for VDRL IgM EIA. The potential of this test system to replace other conventional non-treponemal test was reported with the benefit of large scale sample evaluation. Later, White et al. (1989) reported another VDRL IgG EIA called, 'Visuwell reagin'. This test also employed cardiolipin, lecithin and cholesterol but had a different ratio than the one reported by Pedersen et al. Stoner et al. (1997) reported a new method that was able to detect IgG and IgM classes of antibody to VDRL antigen coated microplates. This test used a detector antibody coupled to red blood cells (RBC). This assay was also known as Capture-S system and showed sensitivity and specificity of 94% and 99.2% respectively.

Non-treponemal cardiolipin antigen is not soluble in water. Hence, all studies described above were made by dissolving it first in ethanol, and later dried on the surface of microplate. However, this method may results into poor physical adsorption of antigen to the microplate. It may not hold antigen strongly to the microplate. Castro et al. (2011) described a new method for non-treponemal antigen modification. Cardiolipin was oxidized, and after that was activated and used in

immunoassay development. Procedures for its coupling to different molecules were described by Castro et al. (2011). A similar method was studied in current research in which antigen was activated and subsequently attached to the microplate (Shukla et al., 2015). This procedure resulted into strong attachment of cardiolipin to the microplate through covalent linkage. An EIA system based on this concept was studied with various sample panels to validate test performance. Developed test showed promising results with the studied samples.

A non-treponemal cardiolipin EIA test can be very useful for syphilis diagnosis. It can have numerous advantages like potential for automation, large number of sample testing, easy result recording and interpretation as reported by White et al. (1989). In addition to that, such modern non-treponemal test can be used along with treponemal EIA to make syphilis screening quick and simple.

MATERIALS AND METHODS

Cardiolipin coated plates

Cardiolipin from bovine heart (Avanti Polar Lipids, USA) was oxidized as per the method described by Castro et al. (2011). A lyophilized oxidized cardiolipin preparation was provided for the study and evaluation purpose (CDC, USA). It was reconstituted to a 25 mg/ml concentration in distilled water and later activated with 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich, USA) and 10 mg of sulfo-NHS (Thermo Fisher Scientific, USA) chemicals. The solution was stirred for 1 hour at room temperature. The antigen stock was diluted to 0.125 mg/ml with 10 mM phosphate buffer, pH 5.6, and 0.1 ml/well volume was dispensed on a microplate. A special microplate with primary amine groups on it can be purchased commercially (BD pure coat amine, USA) as described by Shukla et al. (2015), or be prepared by coating poly-L-lysine (Sigma-Aldrich, USA) on high binding surface of microplate (Nunc, USA). Prepared cardiolipin coated plates were washed twice with phosphate buffered saline (PBS), and subsequently blocked with protein-free blocker (Pierce, USA) for 1 hour at room temperature. The solution was aspirated, microplates were dried, and sealed in foil pouches with desiccant. It can be stored at ambient temperature until use for testing as described by White et al. (1989).

Horseradish peroxidase (HRP) labeled antibody conjugates

Goat anti-human IgG (Fab')2 labeled HRP (Pierce, USA), and goat anti-human IgM (heavy chain) labeled HRP (Pierce, USA) conjugates were used in described EIA test. Each conjugate was diluted to a working dilution of 1:20,000 with 10% bovine serum albumin in PBS (BSA-PBS).

TMB-Substrate

A ready to use stabilized buffer solution comprise of hydrogen peroxide and 3,3',5,5'-tetramethyl benzidine (SeramunBlau-Slow, SeramunDiagnostica, Germany) was used in non-treponemal EIA

test. TMB as a chromogen is less toxic, produces low background, and high signal as reported by Deshpande (1996).

Sample panel

Arlington Scientific (USA) provided one thousand one hundred and forty six serum samples for assay validation study that were previously collected and stored frozen until use. After thawing, all were characterized by a non-treponemal RPR test (Arlington Scientific, USA) and a treponemal TPHA test (newBio, UK). Out of all studied samples, 475 were found as true syphilis positive (RPR reactive, TPHA positive), and 652 as true syphilis negative (RPR non-reactive, TPHA negative). Remaining 19 samples showed discrepant result between RPR and TPHA tests hence were excluded from study. Samples were tested with non-treponemal EIA test to validate test performance. A syphilis reference material from the National Institute of Biological Standards and Control (NIBSC, UK) was studied in developed EIA too to determine test sensitivity. This sample was serially diluted in normal human serum (RPR non-reactive, TPHA negative), and tested in developed test as per assay procedure described below.

Assay procedure

A testing method by Pedersen et al. (1987) was used as reference for the described study. However, the EIA testing procedure was modified during optimization study. Samples were diluted to 100 times with 10% BSA in PBS solution. A 100 µl of diluted sample was transferred on the cardiolipin coated plate followed by its incubation at room temperature for 45 minutes. Plate was washed for three times with PBS containing 0.05% of Tween-20 detergent. A conjugate mixture containing goat anti-human IgG (Fab')2 labeled HRP and goat anti-human IgM (heavy chain) labeled HRP was added at 100 µl per microwell volume and incubated further for 30 minutes at room temperature. Plate was washed again as described earlier with PBS and Tween-20. TMB-substrate reagent was added. The reaction was stopped after 20 minutes with 3M sulfuric acid. Obtained color was read at 450 nm wavelength using 620 nm filter as a reference.

Reproducibility study

A reproducibility study with non-treponemal EIA was performed by testing syphilis positive samples with different non-treponemal antibody titer. For intra-assay reproducibility study, six different true syphilis positive samples with RPR titer of 1, 2, 4, 8, 16, and 32 were selected. All were run twelve times in a single test run. For inter-assay reproducibility study, four true syphilis positive samples with RPR titer of 1, 4, 8 and 32 were selected. All samples were tested in duplicate each day, and the study was continued for 10 consecutive days.

Obtained data were used to determine coefficient of variation in percentage as described by Bastarache et al. (2011). It was calculated from the standard deviation and mean of the optical density

(OD) of tested samples by using following formula.

Coefficient of variation in percentage (CV %) = [Standard deviation / Mean] X 100

Data Analysis method

Obtained optical density of tested 1127 samples in non-treponemal cardiolipin EIA was used to do the data analysis. Jiang et al. (2013) showed a method for the assay cut-off calculation. It was determined from the mean and standard deviation of the tested true syphilis negative samples. Following formula was used to determine assay cut-off.

Cut-off = Mean + 2 (Standard deviation)

Calculated cut-off was used to normalize optical density of each sample. A sample optical density was divided by the calculated cut-off. Obtained value was used to classify a samples as positive (if> 1.1), negative (if < 0.9) or equivocal (if 0.9-1.1). Samples with equivocal status were re-tested to confirm their result.

RESULTS

Sample evaluation data

True syphilis positive and true syphilis negative samples were evaluated in non-treponemal cardiolipin EIA test as per the testing method described earlier. From the data of true syphilis negative samples, a mean OD of 0.092 and standard deviation of 0.054 was calculated in Microsoft excel worksheet. Hence, a calculated cutoff obtained was 0.201 as per the formula described earlier in data analysis method. Obtained sample data were normalized with calculated cut-off and were summarized in **Table-1**. Studied developed test showed a sensitivity and specificity of 92.4%, and 97.2 % respectively. It was obtained against the results of RPR and TPHA tests that were performed on all samples.

Reproducibility study data

The results of intra-assay, and inter-assay reproducibility study were described in **Table-2** and **Table-3** respectively along with sample data. Intra-assay reproducibility showed CV % of < 6%, whereas inter-assay reproducibility study showed CV% of < 8%.

Test sensitivity data with syphilis reference material

NIBSC syphilis reference material was studied in non-treponemal cardiolipin EIA. Obtained data were presented in **Table-4**. A graph was prepared by plotting antibody concentration (IU/ml) on x-axis Vs their optical density on y-axis. An R^2 value of 0.98 was obtained that showed a linear relation between sample antibody concentration, and their respective OD or signal in developed EIA test (**Figure-1**). Same dilutions were studied in RPR. It was determined that both tests showed an equal sensitivity of 0.3 IU/ml (**Table-4**).

DISCUSSION

Described study showed a new method for the non-treponemal antibody detection that has a potential to be useful as an alternative to non-treponemal flocculation

tests. Non-treponemal antigen was fixed to the microplate through covalent linkage. Hence, the developed test was expected to produce a consistent test result. This fact was confirmed through an intra-assay and inter-assay reproducibility study, and that showed an overall CV% of less than 8%.

Obtained serum samples from Arlington Scientific were characterized with RPR and TPHA tests to determine sample status. Both tests were used on all samples, and obtained findings were used as reference to validate non-treponemal cardiolipin EIA test performance. Total four hundred and seventy five true syphilis positive samples were studied. Developed test showed a sensitivity of 92.4%. It was found to be little less than the expected sensitivity of > 95%. All false negative samples with EIA showed a low RPR titer. Hence, it was assumed that non-treponemal EIA was not able to detect some syphilis samples with low titer. However, contrary to that, a study with NIBSC syphilis reference material showed that RPR and developed EIA test had an equivalent sensitivity of 0.3 IU/ml. Hence, it might be early to report that developed test is less sensitive. Tate et al. (2004) showed that sample quality could affect the test performance. Hence, further studies are required on test validation to draw conclusion on test sensitivity. Though, the described non-treponemal cardiolipin EIA has a scope for the further improvement with the test sensitivity. It can be done by using a superior conjugate system than the one used in described study. Current method used an optimized ratio of anti-human IgG (Fab')2 labeled HRP, and anti-human IgM (Heavy chain) labeled HRP conjugate. However, a test system based on double antigen sandwich assay principle could enhance the test sensitivity. Cardiolipin can be coupled to HRP enzyme and be employed in developed EIA system in place of used conjugate mixture. Quality of the detector molecule or conjugate can make a huge difference with test performance too. The specificity of the developed test was studied with six hundred and fifty two samples, and looked promising.

Non-treponemal RPR or VDRL has extensively been used for syphilis screening around the world. Easy test procedure, minimum equipment requirement, low test cost, and easily availability are the key advantages concluded by Larsen et al. (1995). All serves as the reason for their popularity so far. However, a non-treponemal test has some drawback too. Testing a huge number of samples is a cumbersome exercise. Subjectivity of test result is another major drawback. The introduction of automated treponemal test had solved both shortcomings, and hence it became instantly acceptable for syphilis diagnosis. Still non-treponemal tests are required for syphilis diagnosis. Ratnam (2005) reported that the presence of non-treponemal antibody indicates the active syphilis infection. In addition to that, it can be useful in monitoring treatment effectiveness. Hence, this type of test is still in use.

Described new technique can be useful as an alternative to the conventional non-treponemal tests. It has potential to

overcome the drawbacks associated with them. It may also be useful in monitoring treatment effectiveness. The testing protocol of EIA can be fed easily into automated system like Dynex DS2 (Gorton et al., 2015). Such automated system can be very useful to the blood banks where they need to screen a huge number of samples on a daily basis. Use of RPR or VDRL in such settings might not be realistic. Hence, automated version of non-treponemal test can be very useful. Described study showed a new approach for syphilis test development. However, it needs to be expand further with huge number of sample study. It also underlines the scope for improving test sensitivity so that it can be accepted for syphilis diagnosis.

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Table 1 Syphilis non-treponemal EIA test performance study

Sample category	n	Non-treponemal EIA		Performance Data
		Positive	Negative	
True syphilis positive	475	439	36	Sensitivity (%) 92.4[a]
True syphilis negative samples	652	18	634	Specificity (%) 97.2[a]

[a]Sensitivity and specificity were calculated by following formula (Lalkhen et al., 2008),
Sensitivity= [Test positive / (Test positive + False negative)] X 100
Specificity = [Test negative / (Test negative + False positive)] X 100

Table 2 Intra-assay reproducibility data

Sample ID	No. of repeats	RPR (Titer)	Mean OD[b]	SD[c]	CV%[d]
R3536	12	P(1)	0.249	0.014	5.5
R2067	12	P(2)	0.388	0.011	2.9
R3535	12	P(4)	0.657	0.019	2.8
R2183	12	P(8)	1.216	0.029	2.4
R2082	12	P(16)	1.995	0.04	2
R9624	12	P(32)	2.506	0.016	0.6

[b]OD= Optical density

[c]SD = Standard deviation

[d]CV%= Coefficient of variation in percentage

Table 3 Inter-assay reproducibility study

Sample ID	RPR (Titer)	Well	Day intervals										Mean. OD[b]	SD[c]	CV%[d]
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10			
			OD at 450 and 620 nm												
R3536	P(1)	A1	0.242	0.235	0.282	0.257	0.294	0.245	0.242	0.272	0.232	0.243	0.254	0.021	8.34
		B1	0.233	0.242	0.272	0.261	0.284	0.233	0.239	0.263	0.235	0.238	0.250	0.018	7.38
R3535	P(4)	C1	0.623	0.712	0.654	0.666	0.703	0.606	0.618	0.658	0.589	0.671	0.650	0.041	6.25
		D1	0.634	0.721	0.633	0.658	0.711	0.621	0.627	0.643	0.532	0.646	0.643	0.052	8.07
R2183	P(8)	E1	1.225	1.302	1.212	1.215	1.249	1.184	1.182	1.243	1.125	1.264	1.220	0.049	4.04
		F1	1.231	1.273	1.243	1.175	1.256	1.225	1.221	1.257	1.162	1.272	1.232	0.038	3.08
R9624	P(32)	G1	2.494	2.585	2.603	2.5	2.583	2.512	2.474	2.532	2.365	2.562	2.521	0.070	2.78
		H1	2.517	2.571	2.588	2.513	2.488	2.489	2.491	2.474	2.352	2.456	2.494	0.065	2.60

[b]OD= Optical density

[c]SD = Standard deviation

[d]CV%= Coefficient of variation in percentage

Table 4 NIBSC syphilis reference material data

Sample Dilution	Syphilitic antibody IU/ml	Non-treponemal EIA test			Semi-quantitative RPR
		OD[b]	OD/CO[ef]	Result	
Neat	3	1.927	8.88	P	P
1:2	1.5	0.94	4.33	P	P
1:4	0.75	0.768	3.54	P	P
1:8	0.38	0.372	1.71	P	P
1:16	0.19	0.233	1.07	E	N
1:32	0.09	0.143	0.66	N	N
1:64	0.05	0.104	0.48	N	N

[b]OD= Optical Density

[e]OD/CO = Optical density/cut-off

[f]Calculated assay cutoff was 0.217

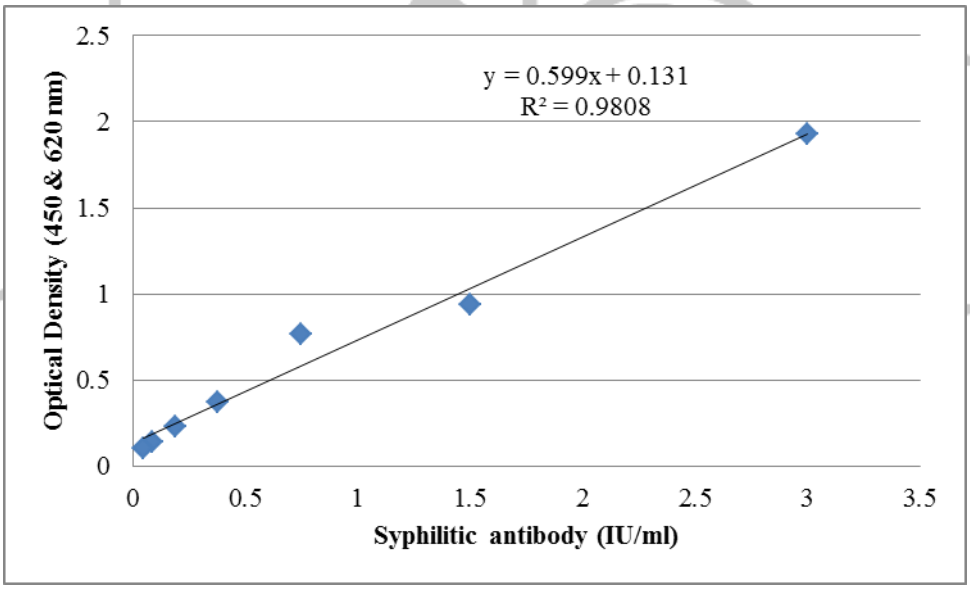

Figure 1 A graph of syphilitic antibody concentration (IU/ml) Vs optical density (450 & 620 nm). A liner relation was observed between NIBSC syphilitic antibody concentration and its respective optical density.

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