Original Article

# Comparative study of Treponemal and non-Treponemal test for screening of blood donated at a blood center

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**Abstract:**

The non-Treponemal tests such as Rapid Plasma Reagin test (RPR) or the Venereal Disease Reference Laboratory test are the most commonly used test for screening of syphilis in the blood centers in India. Now, with the availability of Enzyme-linked immunosorbent assay (ELISA) and Immunochromatographic assays in the market, we decided to evaluate these assays in comparison with Treponema pallidum Haemagglutination Assay (TPHA) which was considered as a gold standard for this study. A total of 8 685 samples of voluntary blood donors were tested on Trepolisa 3.0 and then the initially reactive samples were retested in duplicate on the same assay as well as on Omega Pathozyme, RPR, RAPHA (Rapid Anti-Treponema pallidum Assay), and TPHA. Of the 158 initially reactive samples, 104 were repeatedly reactive on the same assay, 85 were reactive with RPR, 77 were reactive with RAPHA, 60 were reactive on Omega, and 53 were confirmed reactive on TPHA. 48 (56.4%) of the results on RPR were biological false positive, while 21.9% of results were false negative on RPR. We evaluated that Omega Pathozyme was quite in agreement with TPHA as compared with Trepolisa 3.0, RAPHA, and RPR. We concluded that Omega Pathozyme (ELISA) can be considered as a suitable test for screening of syphilis in a blood center.

**Key words:**

Enzyme immunoassay, hemagglutination, particle agglutination, syphilis

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## Introduction

Screening of blood for syphilis is mandatory for issuing safe blood. Antibodies to syphilis infection become detectable 3 to 4 weeks after exposure and may remain detectable for long periods after the treatment. Two group of antibodies are formed viz. one reactive with the non-Treponemal antigen used in Venereal Disease Reference Laboratory (VDRL) test and Rapid Plasma Reagin test (RPR) and other reacting with specific antigens of *Treponema pallidum*. Antibody to non-Treponemal antigens is found in active disease and the levels subside after successful treatment, while Treponema-specific antibodies persist for a long time after the infection has been successfully treated. The serological tests most commonly used to screen for the disease are the non-Treponemal and Treponemal tests. The non-Treponemal tests such as RPR or the VDRL test measure the hosts response to non-Treponemal antigens such as cardiolipin and lecithin released from the damaged host cells, as well as lipoprotein-like material released from the Treponema.[1-3] These non-Treponemal tests are generally considered to be sensitive in early syphilis, but their disadvantages being false-positive reaction,[4-6] false-negative reactions due to the prozone phenomenon,[7,8] and lack of sensitivity in the late stage of infection.[9,10]

The treponemal tests such as the *Treponema pallidum* Haemagglutination Assay (TPHA) and micro-haemagglutination assay for *Treponema pallidum* have high sensitivity for all the stages of disease other than very early primary syphilis.[2] These tests detect human serum/plasma antibodies to *T. pallidum* by means of an indirect hemagglutination method.

Recently, several enzyme immunoassays, some of which are based on specific *T. pallidum* recombinant antigen,[10-12] have been developed and evaluated as Treponemal test for syphilis.[3,13-17] The advantages of ELISAs are the capacity to process large number of samples and ability to have a print out of the objective spectrophotometric readings, while the TPHA, TPPA (*Treponema pallidum* particle agglutination assays), and FTA-ABS (Fluorescent Treponemal Antibody absorption) are subjective assays[2,18] because they rely on the skill and training of the individual reader which is variable and depends upon the person reading the test. The TPI (*Treponema pallidum* Immobilization) test and FTA-ABS utilize pathogenic *T. pallidum* as the antigen, but these tests present some difficulties for routine serodiagnosis. The TPI requires living pathogenic *T. pallidum*, while FTA-ABS test requires a fluorescence microscope.[2] In addition, both these tests require a high level of specialist expertise.

Recently, immunochromatographic Treponemal

assays are also available wherein the assay uses immunoassay principles on a nitrocellulose strip for the detection of syphilis antibody in human blood.

## Aim and Objective

The aim of this study was to evaluate the suitability of commercially available non-Treponemal and Treponemal tests like RPR, Omega Pathozyme, Qualpro Diagnostics Trepolisa 3.0, and RAPHA (Rapid Anti-*Treponema pallidum* Assay) in comparison with that of TPHA for either screening or confirmation.

## Material and Methods

A total of 8 685 samples of blood collected from the voluntary blood donors during the year 2008-2009 were subjected to syphilis testing by Trepolisa 3.0 (Batch no.25008, 25009, 25010, 25013) and then the aliquots of sera which were reactive on Trepolisa 3.0 were stored at –70$^0$C and at a later date they were run on RPR (Batch no.7016779, 701703), Omega Pathozyme (7018448, 7019011, 7019595, 7020285, 7020784, 7018110), RAPHA (2007111603, 2007122905, 2008072519), and TPHA (7015629, 043058, 7021043)

Trepolisa 3.0 – 3$^{rd}$ generation ELISA and Omega pathozyme syphilis competition were performed according to the manufacturer's instructions. Trepolisa 3.0 uses two negative controls and two positive controls, while the Omega pathozyme uses two negative controls, two positive controls, and two low positive controls; besides the above mentioned controls, in-house controls were also used for both the assays.

The Trepolisa 3.0 – detects total antibodies, i.e., IgG, IgM, IgA, etc., to *T. pallidum*. It is a double antigen sandwich ELISA using recombinant Treponemal antigen while the Omega Pathozyme is competitive EIA for the detection of total antibodies to *T. pallidum* but primarily IgG and IgM and therefore both have sensitivity to all disease stages.

In Omega Pathozyme, purified antigens derived from *T. pallidum* are bound to the surface of microtitration wells. Undiluted test sera are applied followed by anti *T. pallidum* antibody conjugated to horseradish peroxidase (HRP). Specific antibodies to *T. pallidum* in the test sera and the conjugate compete to bind to the antigens in the wells. Unbound material is then washed away. On addition of the substrate-stabilized $3_1$ $3^1$m 5.5[1] Tetramethylbenzidine, a color will develop only in those wells in which enzyme is present. This indicates the absence of human anti-*T. pallidum* antibody and is therefore a negative result. The enzyme reaction is stopped by addition of dilute 0.2 M sulfuric acid and then the absorbance is measured at 450 nm. Any result with an optical density less than the cut off is considered positive. The intensity of yellow color produced after stopping the reaction is inversely proportional to *T. pallidum* antibodies in the sample.

In Trepolisa 3.0, the microwell strips are coated with recombinant 47 kd and 17 kd antigens. The same antigens are conjugated to HRP. Samples along with positive and negative controls are added in the coated wells and incubated simultaneously with antigen HRP conjugate. The wells are washed to remove unbound components. Captured antibodies are detected by adding substrate. The reaction is stopped after specified time with dilute HCL acid and the absorbance is determined for each well at 450 nm. Any sample having absorbance more than the cut off is considered reactive.

The RPR test was performed qualitatively; results were recorded as positive and negative with respect to control sera.

The TPHA test was also performed qualitatively, wherein an even layer of agglutination of cells in a round bottom microtiter plate was interpreted as positive reaction, while a compact button is interpreted as a negative reaction. Agglutination in the control cell well together with the test cell well indicates the presence of nonspecific agglutination in the sample, thereby making the test invalid. The TPHA detects human serum antibodies to *T. pallidum* by means of an indirect hemagglutination method using preserved avian erythrocytes coated with the antigenic components of *T. pallidum* (Nichol's strain). These test cells agglutinate in presence of specific antibodies to *T. pallidum* and show characteristic patterns in microtiter plates. Any nonspecific reaction occurring can be detected by control cells which are avian erythrocytes not coated with *T. pallidum* antigens. Antibodies to nonpathogenic treponemes are absorbed by an extract of Reiters Treponema which is included in the cell suspension.

The immunochromatographic assays (RAPHA) use a double antigen sandwich principle for detection of syphilis antibody in human serum. A recombinant syphilis antigen is immobilized on the test band region and an antibody to syphilis is immobilized on the control band region of the nitrocellulose membrane. Another Syphilis antigen coupled with the colloidal gold particles is dried on a conjugate pad. The specimen reacts with the colored conjugate (antigen–colloidal-gold conjugate); the mixture then migrates chromatographically along the membrane by the capillary action. If the specimen contains Syphilis antibody, the recombinant antigen is immobilized on the membrane with the capture antigen-antibody-colloidal gold complex and form a colored test band on the membrane, indicating a positive result and absence of test band suggests a negative result.

The sensitivity and specificity were also calculated. Sensitivity of a test is defined as its ability to detect truly infected individuals as also its ability to detect very small amounts of analyte. The following formula was used to calculate the sensitivity.

True positive and True negatives were calculated in comparison with TPHA which was considered as gold standard.

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives + False Negatives}} \times 100$$

Specificity is defined as the ability of an assay to correctly identify all the uninfected individuals, that is, there should be no false positives. The following formula was used to calculate the specificity of the assays in this study.

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True negative + false positives}} \times 100$$

## Results

Of the 8,685 samples tested on Trepolisa 3.0, 158 initially reactive

samples on TREPOLISA 3.0 were studied. Of 158 initially reactive samples, 104 samples were repeatedly reactive on the same assay, 85 were reactive with RPR, and 77 were reactive with RAPHA, while 60 samples were reactive on Omega pathozyme and 53 on TPHA. The results of comparison of the Trepolisa 3.0 vs Omega pathozyme, RPR, RAPHA, and TPHA are given in Table 1. TPHA test was considered as a gold standard while comparing the results with other assays. For example, 52 True positive of Trepolisa 3.0 were interpreted as true positive when compared with 53 positive of TPHA.

Table 2 shows that the sensitivity of Trepolisa is 96.29% as against that of 96.15% by Omega Pathozyme and both of them are on par with TPHA, i.e., 100%. The difference between the two is not significant. The other Kits sensitivities were significantly less as compared with TPHA, Trepolisa, and Omega Pathozyme.

About specificity, Omega Pathozyme is 90.56% which is nearer to TPHA, i.e., 100%, but difference was insignificant. In other kits, specificities were significantly lower as compared with TPHA and Omega Pathozone.

## Discussion

As is the practice, most of the blood centers carry out the screening for syphilis by the RPR method; similarly, at our Blood Center also, RPR was being used, but with the introduction of two different ELISAs in the market viz. Omega Pathozyme and Trepolisa 3.0, we decided to carry out a comparative study of the Treponemal and non-Treponemal tests and assess the suitability of a screening test to be adopted. Biological false-positive reactions is one of the drawbacks of non-Treponemal tests which occur in those patients whose serum give a positive cardiolipin antigen test but negative Treponemal antigen tests.[3,7,8,19] In the present study, it was found that 48 (56.4%) of the results were biological false positives on RPR. [Refer Table 3] Acute or transient false-positive non-Treponemal reaginic test reaction may occur whenever there is a strong immunological stimulus,[8,20-22] e.g., acute bacterial or viral infection, vaccination, and HIV infection.

**Table 1: Comparison of reactivity expressed on various test**

| Results | Trepolisa 3.0 No. % (N = 158) | | Omega Pathozyme No. % (N = 158) | | RPR No. % (N = 158) | | RAPHA No. % (N = 158) | | TPHA No. % (N = 158) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Reactive | *104 | 65.8 | 061 | 38.6 | 085 | 53.8 | 077 | 48.7 | 053 | 33.5 |
| Non-reactive | 054 | 34.2 | 097 | 61.4 | 073 | 46.2 | 081 | 51.3 | 105 | 66.5 |

By Chi – square Test, *$P$<0.05 Significant
This table shows that 65.8% of the cases showed reactive result by Trepolisa 3.0 test which was significantly more as compared with 38.6% by Omega Pathozyme, 53.8% by RPR, 48.7% by RAPHA, and 33.5% by TPHA test.

**Table 3: Comparison of false reactivity on various assays**

| Results | Trepolisa 3.0 No. % (N = 54) | | Omega Pathozyme No. % (N = 12) | | RPR No. % (N = 64) | | RAPHA No. % (N = 40) | |
|---|---|---|---|---|---|---|---|---|
| False Positive | 052 | 96.3 | 010 | 83.3 | 048 | 75.0 | 032 | 80.0 |
| False Negative | 002 | 03.7 | 002 | 16.7 | 016 | 25.0 | 008 | 20.0 |

Young *et al*.,[14] reported that between 8 to 10% of patients with various stages of infection gave a false-negative results in VDRL test due to the prozone phenomenon. In the present study also, as observed in Tables 3 and 4, we found that 16 (21.9%) of the samples were false negative on RPR. Since false-negative results with VDRL and RPR are due to the prozone phenomenon, such test should not be used alone to screen for various stages of infection. Therefore, screening with a specific anti-Treponemal antibody such as TPHA, TPPA, or ELISA is a practical option rather than carrying out serum dilutions.[5,9,14] There are no definite policy guidelines from FDA as to the protocol of syphilis testing in India, but in Europe.[2,3,8,23] Treponemal tests are recommended for screening of blood donors along with confirmation by a second Treponemal test of a different format. In USA, non-Treponemal tests such as RPR are recommended for screening and then confirmed with standard Treponemal tests such as FTA-ABS test or TPHA, both of which are based on extracts of Nichols strain to detect antibodies directed against Treponemal components.

In the present study, we found one particular donor's sample that was Non-reactive with RPR and both the ELISA but reactive on TPHA, the donor refused to come for counseling or further testing. This could be an isolated case of syphilis that has been treated.

We evaluated that the Omega pathozyme was quite in agreement with TPHA, except in two particular cases where in it was Non-reactive on Omega but reactive on TPHA. We were unable to recall the donor as they were unwilling to come back for a recheck. However, there is a reference of study conducted[24] wherein they had contacted positive patients and interviewed them to look for clinical symptoms or history of syphilis. It was not possible for us to contact the donors with reactive results as there was no policy decision taken on their follow-up; however, in future, we do intend to have a confidential interview and look for symptoms or history of syphilis in order to be able to clinically correlate.

Several tests using ELISA format have been developed, but the only other alternative ELISA available in the market at present was TREPOLISA 3.0 and in our study, we observed that it had more false positive and was also less specific as compared with Omega pathozyme [Table 2] and we observed that the results of Omega pathozyme were comparable with TPHA. As observed in Table 1, it was found that of 158 samples that were reactive on TREPOLISA,

**Table 2: Comparison of sensitivity and specificity of various assays**

| Name of the kit | Sensitivity (%) | Specificity (%) |
|---|---|---|
| TPHA | *100.0 | *100.0 |
| TREPOLISA 3. | *96.29 | 50.00 |
| OMEGA PATHOZYME | *96.15 | *90.56 |
| RPR (GLAXO) | 70.00 | 54.28 |
| RAPHA | 84.90 | 69.52 |

By Chi – Square Test, *$P$<0.05 Significant

**Table 4: Comparison of true reactivity on various assays**

| Results | Trepolisa 3.0 No. % (N = 104) | | Omega Pathozyme No. % (N = 146) | | RPR No. % (N = 94) | | RAPHA No. % (N = 118) | |
|---|---|---|---|---|---|---|---|---|
| True Positive | 052 | 50.0 | 051 | 34.9 | 037 | 39.4 | 045 | 38.1 |
| True Negative | 052 | 50.0 | 095 | 65.1 | 057 | 60.6 | 073 | 61.9 |

only 60 were reactive on Omega pathozyme and 53 were reactive with TPHA, while 85 were reactive on RPR and 77 were reactive on RAPHA. The specificity of Omega Pathozyme was much better as compared with Trepolisa 3 [Table 2].

Several other ELISAs[24] which have been developed and are not available in the market at present such as Murex Syphilis ICE which has three recombinant Treponemal antigens and detect both IgG and IgM have been reported by various studies to be most sensitive Treponemal test with a very high specificity as well. The Enzywell TP is a new rapid ELISA which uses two recombinant antigens and also detects IgG, IgM and can be performed approximately in 1 hr time. In the study conducted by Aktas *et al*.,[24] it was observed that agreements of TPHA with Serodia TPPA, the Murex Syphilis ICE, and the Enzywell TP tests were 96.7%, 100%, and 99.1%, respectively, and they concluded that the Serodia TPPA, Murex Syphilis ICE, and Enzywell as an appropriate substitute for screening and serodiagnosis of syphilis.

## Conclusion

It can be concluded that given the high sensitivity and specificity of Omega pathozyme, it can be considered as a suitable screening test as ELISAs are ideally suitable for the blood center wherein large number of samples are being processed with an added advantage of an objective reading in the form of printout. The only disadvantage being the amount of time taken to carry out the testing, special precautions that need to be taken in carrying out all the steps, like dispensing appropriate volume of sample diluents and serum into the wells of the microtiter plate, dispensing controls, addition of conjugate, substrate and stop solution, washing, incubation, and the optical readings.

However, considering that Enzyme Immunoassays with better sensitivity and specificity are available in market, they could still be easily a preferred choice for screening and confirmation of blood for Syphilis in a blood center.

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