Evolution of *Treponema pallidum* Hemagglutination Assay among Varying Titers of the Venereal Disease Research Laboratory Test

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**Abstract**

**Background and Objective:** Syphilis, besides being a significant cause of perinatal morbidity and mortality, is a substantial cause of adult morbidity. A discordant serological result can present a diagnostic challenge; hence, a fundamental knowledge about the diagnostic limitations or interpretation of these assays becomes imperative for the clinicians to avoid management dilemma. The study was proposed to see the usefulness and correlation of *Treponema pallidum* hemagglutination assay (TPHA) with varying titers of Venereal Disease Research Laboratory (VDRL) test. **Materials and Methods:** Over a period of 2 years, 22,351 sera were subjected to screening for syphilis by VDRL test. TPHA test was performed for confirmation in 243 of the total sera. **Results:** VDRL reactivity was seen in 0.77% of the tested sera. TPHA positivity was 58.85% among the sera tested. Calculated sensitivity, specificity, positive predictive value, and negative predictive value of VDRL against TPHA were 87.41%, 52%, 72.25%, and 74.29%, respectively. TPHA positivity was found to be 100% and 55% in VDRL reactive cases with titers ≥32 and <8, respectively. **Conclusion:** Screening and diagnostic serological tests for syphilis should be reviewed in routine by the treating physician in the light of clinical presentation and the history of infection and treatment.

**Key Words:** Syphilis, treponema pallidum hemagglutination assay, venereal disease research laboratory

**Introduction**

Syphilis, besides being a significant cause of perinatal morbidity and mortality, is a substantial cause of adult morbidity by increasing the risk of transmission of human immunodeficiency virus (HIV) or other sexually transmitted diseases (STDs). Untreated syphilis is a contagious systemic disease featuring sequential clinical stages added to the years of latency. The infection, caused by the bacteria *Treponema pallidum*, is endemic in several parts of the world and continues to remain a public health concern.[1] Syphilis, a genital ulcerative disease, results in overwhelming multiorgan involvement with irreversible sequelae if not properly treated. Serological tests continue to remain the mainstay of diagnosis for syphilis, despite the fact that one among the first of these tests was developed almost 1000 years ago.[2] Noncultivability in vitro and limited availability of direct visualization techniques or molecular assays could be responsible for the same.[3] Nontreponemal tests such as the Venereal Disease Research Laboratory (VDRL) test use lipoidal antigens (cardiolipin, lecithin, and cholesterol) that flocculate with immunoglobulin M or G (IgM and IgG). Seroconversion occurs from 21 days of exposure till about up to 6 weeks after infection.[4] These nontreponemal tests have the advantages of being inexpensive, simple, and suitable for mass screening and the baseline titer can be used to follow-up the treatment response. Confirmation by a treponemal test, however, is required as the sensitivities and specificities of nontreponemal tests vary with the stages of infection or the prevalence of biological false positivity in the population.[5,6] False negativity due to prozone phenomenon, subjective interpretation, and unsuitability for automation are few other limitations of these tests. The *T. pallidum*...
hemagglutination assay (TPHA), using sensitized sheep erythrocytes coated with T. pallidum (Nichols strain), is a microhemagglutination assay for IgM and IgG antibodies. Treponemal tests such as TPHA, having lower sensitivities in primary syphilis, remain positive despite treatment and give false positive results uncommonly. Positive treponemal enzyme immunoassay tests along with negative nontreponemal test results reflect higher false positivity of treponemal tests. Several factors such as history of syphilis in the past, the stage of the infection, baseline titers, the immune status of the patient, or the treatment taken may influence the rate of fall of titer. Serological tests, providing indirect evidence of infection, may be reactive in the absence of clinical, historical, or epidemiologic evidence of syphilis. A combination of treponemal and nontreponemal tests has been recommended by the WHO since 1982 for the screening and diagnosis of syphilis. The traditional US Centers for Disease Control and Prevention approach of screening and confirming by a nontreponemal and a treponemal assay, respectively, was later followed by a reverse sequence algorithm, and the latest recommendation by the European Centre for Disease Prevention and Control suggests screening and confirming by two different treponemal assays. Several new tests are being deployed and testing algorithms are being modified. In the absence of a reliable gold standard test for diagnosis, a discordant serological result can present a diagnostic challenge; hence, a fundamental knowledge about the diagnostic limitations or interpretation of these assays becomes imperative for the clinicians to avoid management dilemma, especially in routine screening of low-risk population. The present study was proposed to see the usefulness and correlation of TPHA with varying titers of VDRL.

Materials and Methods

This study was carried out over a period of 2 years at the Immunology Laboratory, Microbiology Department, University College of Medical Sciences and Guru Teg Bahadur Hospital, a tertiary care hospital in north India. A total of 22,351 patients were referred to this center for VDRL testing during the study period. Of these, 1316 were from the various indoor departments and 21,035 were from outpatient departments (OPDs) of the same hospital. Among the samples tested positive by at least one of the two tests of VDRL or TPHA, 89 were from the STD clinic, followed by 45 from the obstetrics and gynecology department, 40 from the antiretroviral therapy (ART) clinic, 7 from the surgery department, 6 from the medicine department, 2 from the dermatology department, and 1 each from the orthopedics department and the Neonatal Intensive Care Unit. The serum was separated and stored at 4°C till further processing.

All the 22,351 sera were subjected to screening for syphilis by VDRL test (Trepolipin kit of Tulip Diagnostics Pvt. Ltd., India). The qualitative and quantitative VDRL tests were performed as per the manufacturer’s instructions. These were based on the principle that after syphilis infection, host develops nontreponemal antilipoidal antibodies in response to the release of lipoidal material from damaged host cells in addition to the antibodies produced against T. pallidum. These nontreponemal antibodies are traditionally referred to as reagins which react with cardiolipin to give a nontreponemal flocculation reaction.

TPHA was performed on all the sera demonstrating reactivity with VDRL test and in some other cases where the treating physician desired for the TPHA test result. A total of 243 sera were tested for TPHA (IMMUTREP TPHA kit of Omega Diagnostics Ltd., Scotland, United Kingdom). This kit used the T. pallidum sensitized formalized tanned fowl erythrocytes; unsensitized formalized tanned fowl erythrocytes; diluent; and control sera. On mixing the diluted positive samples with sensitized erythrocytes, antibody to the sensitizing antigen led to agglutination of cells. Agglutination of cells signified a positive reaction. In the absence of antibody, cells settled down to form a compact button in the well which constituted a negative reaction. This kit had a reported sensitivity of 98.5% and a specificity of 99.6%. Reproducibility of IMMUTREP TPHA had been reported to be 100% (±1 doubling dilution).

In this study, cases were categorized under the following headings: syphilis-negative (negative by both screening [VDRL] and confirmatory tests [TPHA]); biological false positives (positive by screening test but negative by confirmatory test); probable past syphilis infection (positive by confirmatory test but negative by screening test); and probable active syphilis infection (positive by both screening and confirmatory tests).

Data were entered into the Microsoft Excel sheet. Sensitivity, specificity, negative predictive value, and positive predictive value of VDRL against TPHA (reference test) were calculated.

Results

In this study, the age of the patients who tested positive by at least one of the two tests, VDRL and TPHA ranged from a neonate to the eldest being 64 year old. Figure 1 demonstrates the demographic profile of these patients. Nearly 91.62% of the patients belonged to 15–45 years of age group, with the majority (59.69%) belonging to 15–30 years of age group, followed by 31.94% in 30–45 years of age group. A total of 99 (51.83%) of these patients were male. Majority of the cases who tested positive by at least one of the tests of VDRL or TPHA
were from the STD clinic. Majority of the male patients in these groups were referred from the STD clinic whereas majority of the female patients were referred from the antenatal clinic.

Of the 22,351 serum samples tested during the study, 173 (0.77%) serum samples were reactive by VDRL test. TPHA was performed in 243 serum samples and it was positive in 143 (58.85%) of the sera tested. Calculated sensitivity, specificity, positive predictive value, and negative predictive value of VDRL against TPHA were 87.41%, 52%, 72.25%, and 74.29%, respectively [Table 1].

Table 2 shows the frequency distribution of cases who tested positive by at least one of the two tests of VDRL or TPHA on the basis of gender. About 65.45% of these patients had a probable active syphilis infection, followed by biological false positive cases. The highest number of cases with probable active syphilis infection was referred from the STD clinic, followed by ART clinic and antenatal clinic.

Table 3 and Figure 2 demonstrate the distribution of TPHA positivity according to VDRL titer in VDRL reactive cases. TPHA positivity was found to be 100% in VDRL reactive cases, with a titer of 32 or more. A total of 100 VDRL reactive cases had a titer <8 and TPHA percentage positivity was 55% in these cases. Seventy-three VDRL reactive cases had a titer ≥8 and TPHA percentage positivity was 95.89% in these cases. In antenatal cases, the positivity of TPHA was 100% in VDRL reactive cases with a VDRL titer of 8 or more [Figure 3]. Figures 4 and 5 demonstrate the frequency distribution of TPHA positive and negative cases according to VDRL titer in VDRL reactive cases of antenatal, ART, and STD clinics, respectively.

Discussion

Ours is a tertiary care hospital in north India that caters to patients from east Delhi and the adjoining states. VDRL reactivity was found to be 0.77% among 22,351 patients referred to this center for the test during the study, and TPHA positivity was found to be 58.85% among the 243 samples tested. A study by Bala et al. from the same region on the assessment of treponemal tests in nontreponemal nonreactive cases reported similar findings with 1% reactivity by VDRL. The same study reported 4.3% positivity by TPHA in VDRL nonreactive sera.[11] In our study of 243 samples in which TPHA was done, 18 (25.7%) of 70 samples that were VDRL nonreactive turned out to be TPHA positive. As mentioned earlier, TPHA was not done in VDRL nonreactive cases in our setup unless the treating physician felt the need to do so. A study done in the same geographical region from a tertiary care hospital to see the prevalence of syphilis among OPD patients reported 18.5% and 3.06% positivity of VDRL and TPHA done in 2543 samples tested during one year, respectively. The same study reported TPHA positivity of 16.5% among VDRL reactive sera.[12] Another study reported 8% and 16.6% positivity by rapid plasma reagin (RPR) and TPHA among 150 HIV seroreactive patients at an Integrated Counseling and Testing Center, respectively.[13] Several factors could be responsible for the discrepancy in the prevalence, such as, variable awareness about counseling, testing, and treatment for syphilis including other STDs post-HIV pandemic.
era. Adequate supervision with correct performance of laboratory tests also was a crucial determinant. We presumed that patients referred to our testing center, a state-level STI clinic, were adequately counseled regarding previous history of syphilis treatment or other factors that could result in false positivity or negativity. Moreover, our testing center maintained an External Quality Assurance Scheme (EQAS) program with the Regional STD Teaching, Training and Research Center.

Of the VDRL reactive sera, low titer (<1:8) was found in 100/173 (57.8%) cases which was lower than that reported by a recent Indian study. TPHA could detect 55% of these low-titer (<1:8) VDRL-positive cases.

In a study done to compare VDRL, RPR, and TPHA in 450 patients tested for the evidence of syphilis, of 53 VDRL reactive sera, VDRL was able to detect low titers more frequently than RPR in six cases. The same study reported a biological false positive (BFP) of 1.1%. A similar study mentioned above also reported a BFP and probable active infection of 0.2% and 0.8%, respectively. In our study too, BFP and probable active infection were found in 0.21% and 0.56%, respectively.

Antenatal serological screening for syphilis has been recognized as an effective tool to reduce the devastating consequences of congenital syphilis. Therefore, early serological screening for syphilis in pregnant women is of paramount importance for commencing an early adequate treatment. Among 42 antenatal VDRL reactive sera, low titer (<1:8) was found in 32/42 (76.2%) cases.

### Table 3: Variation of *Treponema pallidum* hemagglutination positivity with Venereal Disease Research Laboratory titer among Venereal Disease Research Laboratory reactive cases (n=173)

| VDRL titer | TPHA positivity (%) |
|------------|---------------------|
| 1:1        | 37.50               |
| 1:2        | 57.14               |
| 1:4        | 69.70               |
| 1:8        | 92.86               |
| 1:16       | 90                  |
| 1:≥32      | 100                 |

VDRL: Venereal Disease Research Laboratory, TPHA: *Treponema pallidum* hemagglutination assay

Figure 2: Distribution of *Treponema pallidum* hemagglutination assay positivity according to Venereal Disease Research Laboratory titer in Venereal Disease Research Laboratory reactive cases (n=173)

Figure 3: Distribution of *Treponema pallidum* hemagglutination assay positivity in antenatal cases according to Venereal Disease Research Laboratory titer in Venereal Disease Research Laboratory reactive cases (n=42)

Figure 4: Distribution of *Treponema pallidum* hemagglutination assay positivity according to Venereal Disease Research Laboratory titer in Venereal Disease Research Laboratory reactive cases of antenatal, antiretroviral therapy, and sexually transmitted disease clinics (n=116)

Figure 5: Frequency distribution of *Treponema pallidum* hemagglutination assay negativity according to Venereal Disease Research Laboratory titer in Venereal Disease Research Laboratory reactive cases of antenatal, antiretroviral therapy, and sexually transmitted disease clinics.
and TPHA detected in 11/32 (34.4%) of these low-titer (<1:8) VDRL-positive cases. Another study in the same region had reported 71.4% (10/14) detection by TPHA among low-titer (<1:8) VDRL-positive cases, higher than our finding.[14] In the present study, 23.8% antenatal attendees had VDRL reactive high-titer (≥1:8) sera and no BFP was observed in this group.[16] Among STD clinic attendees, VDRL reactive low-titer (<1:8) sera was observed in 43/78 (55.1%) and TPHA could detect in 60.5% of these low-titer cases whereas another study reported low-titer (<1:8) VDRL reactive sera in 20/26 (76.9%) and TPHA positivity in 90% of VDRL reactive low-titer sera.[14]

Considering the screening algorithm, it becomes significant to assess the usefulness of the treponemal test in use among various titers of VDRL ranging from low to high. A study by Bala et al. on the usefulness of TPHA as an aid in the diagnosis of syphilis in cases having VDRL titer <1:8 reported BFP ranging between 0.1 and 0.3% and that 86.8% of weak reactive cases (titer <1:8) by VDRL were TPHA positive.[14] In our study, 55% of the weak reactive VDRL samples were TPHA positive. Of the 243 cases tested for both VDRL and TPHA in our study, 18 (7.41%) were TPHA positive but VDRL negative suggesting the possibilities of past treatment for syphilis, late or latent syphilis.[11]

Conclusion
Serology, especially in the late stage, has continued to remain the cornerstone of diagnosis in syphilis. The enigmatic nature of this pathogen along with its interaction with HIV infection has further emphasized the need for better understanding of the currently used tests. Screening and diagnostic serological tests for syphilis should be reviewed in routine by the treating physician in the light of clinical presentation and the history of infection and treatment. There being no direct test of cure for syphilis, the most relevant marker of treatment response in the current scenario would be to correctly define the case clinically and to continue serological follow-up.

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Conflicts of interest
There are no conflicts of interest.

References
1. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the global burden of disease study 2010. Lancet 2012;380:2095-128.
2. Wasserman A, Neisser A, Bruck C. Eine serodiagnostische reaktion bei syphilis. Dtsch Med Wochenschr 1906;32:745-6.
3. Seña AC, White BL, Sparkling PF. Novel Treponema pallidum serologic tests: A paradigm shift in syphilis screening for the 21st century. Clin Infect Dis 2010;51:700-8.
4. Ballard RC. Genital ulcer adenopathy syndrome. In: Holmes KK, Sparling PF, Stamm WE, Piot P, Wasserheit J, Corey L, et al., editors. Sexually Transmitted Diseases. 4th ed. New York: McGraw Hill; 2008. p. 1201-8.
5. van der Sluis JJ. Laboratory techniques in the diagnosis of syphilis: A review. Genitourin Med 1992;68:413-9.
6. Larsen SA, Pope V, Johnson RE, Kennedy EJ Jr. A Manual of Tests For Syphilis. Washington, DC: American Public Health Association; 1998.
7. Larsen SA, Hambie EA, Pettit DE, Perryman MW, Kraus SJ. Specificity, sensitivity, and reproducibility among the fluorescent treponemal antibody-absorption test, the microhemagglutination assay for Treponema pallidum antibodies, and the hemagglutination treponemal test for syphilis. J Clin Microbiol 1981;14:441-5.
8. Centers for Disease Control and Prevention (CDC). Syphilis testing algorithms using treponemal tests for initial screening – Four laboratories, New York city, 2005-2006. MMWR Morb Mortal Wkly Rep 2008;57:872-5.
9. Janier M, Hegyi V, Dupin N, Unemo M, Típlica GS, Potočnik M, et al. 2014 European guideline on the management of syphilis. J Eur Acad Dermatol Venereol 2014;28:1581-93.
10. Sommese L, Paoillo R, Sabia C, Costa D, De Pascale MR, Iannone C, et al. Syphilis detection: Evaluation of serological screening and pilot reverse confirmatory assay algorithm in blood donors. Int J STD AIDS 2016;27:644-9.
11. Bala M, Singh V, Muralidhar S, Ramesh V. Assessment of reactivity of three treponemal tests in non-treponemal non-reactive cases from sexually transmitted diseases clinic, antenatal clinic, integrated counselling and testing centre, other different outdoor patient departments/indoor patients of a tertiary care centre and peripheral health clinic attendees. Indian J Med Microbiol 2013;31:275-9.
12. Kalyan R, Singh M, Singh AK, Agarwal J. Prevalence of syphilis at a tertiary care setup of Northern India: A hospital based study. Indian J Public Health Res Dev 2013;4:153-7.
13. Chopdekar KA, Patil SS, Joshi A, Chowdhary A. Serodiagnosis of syphilis in HIV Sero-reactive Patients. Indian J Basic Appl Med Res 2014;3:108-10.
14. Dheepa D. A comparative analysis of VDRL, RPR, Immunrepid TPHA and instachk TP. Univ J Pre Para Clin Sci 2016;2:2455-879.
15. Tankhiwale SS, Naikwade SR. Seroprevalence of syphilis and biologically false positive cases in a tertiary care center. Indian J Dermatol Venereol Leprol 2014;80:340-1.
16. Bala M, Toor A, Malhotra M, Kakran M, Muralidhar S, Ramesh V, et al. Evaluation of the usefulness of Treponema pallidum hemagglutination test in the diagnosis of syphilis in weak reactive venereal disease research laboratory sera. Indian J Sex Transm Dis 2012;33:102-6.