East African Medical Journal Vol. 98 No. 9 September 2021
COMPARISON OF SEROLOGICAL AND MOLECULAR *TREPONEMA PALLIDUM* TESTS IN HIV PATIENTS IN KENYA
James Wachira Wambugu, Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology ,P.O Box 62000-00200 Nairobi , Michael Kahato, Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology P.O Box 62000-00200 Nairobi, James Kigera, School of Medicine, University of Nairobi P.O Box 30197 ,GPO, Nairobi.

Corresponding author: James Wachira Wambugu, Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology. P.O Box 62000-00200 Nairobi,Email: wachirajames726@gmail.com

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J. W. Wambugu, M. Kahato and J. Kigera

ABSTRACT

***Background*: Co-infection of HIV and Syphilis has profound impact in a patient. Syphilis infection is caused by *Treponema pallidum*. Two category of test methods are used; Nontreponemal test, e.g., RPR (rapid plasma regain) and Treponemal test such as TPHA (*Treponema Pallidum* Hemagglutination Assay). The current gold standard in Kenya/Nyeri is TPHA. Polymerase chain reaction (PCR) has higher sensitivity and specificity than serological assays.**

***Objective*: To evaluate the test performance of serological methods (VDRL, RPR and TPHA) in syphilis testing using PCR as the gold standard among HIV patients visiting Nyeri county referral hospital.**

***Design*: Cross-sectional study**

***Setting*: Patients routinely attending the CCC clinic for antiretroviral treatment.**

***Subjects*: 177 HIV patients receiving ARVs**

***Main outcome measures*: Prevalence of syphilis, Test performances.**

Interventions: Since TPHA performance has been shown to agree strongly with PCR, patient samples should be tested using TPHA to save time and cost with necessarily starting with VDRL or RPR

***Results*: The mean age of the study patients was 48.3 years with the majority 60.5% being female. The prevalence of syphilis was 18.6% by PCR. The sensitivity, specificity and kappa of tests were: RPR: 100%, 76.4% and kappa (0.546); VDRL: 100%, 55.6% and kappa (0.317); TPHA: 100%, 94.4% and kappa (0.864) and combination of RPR/VDRL and TPHA: 100%, 54.2%, and kappa (0.306).**

***Conclusion*: The results show that neither RPR nor VDRL assay could be recommended as a stand-alone or as a confirmatory assay. The results of TPHA were the most concordant with those of PCR.**

INTRODUCTION

Infection with syphilis is caused by the bacterium *T. pallidum* with *pallidum* as its subspecies .[1] There has been variation of prevalence in terms of syphilis/HIV co infection based on geographical, study population and time period. [2] Syphilis incidences (primary/secondary) has been rising yearly on a global scale due to HIV

pandemic.[3] In Kenya for instance, syphilis infection prevalence stood at 6.4% among HIV infected individuals whereas in HIV negative persons the prevalence was lower (1.6%).[4]

Syphilis diagnosis is made through immunological/serologic testing.[5] There are two types of serological screening tests. Test based on cardiolipin components are VDRL (Venereal Disease Research Laboratory) and RPR (rapid plasma regain). The treponemal tests for specific antibodies are TPHA (Treponema *pallidum* haemagglutination assay), TPPA (*Treponema. pallidum* particle agglutination assay), FTA (fluorescent based treponemal antibody test) and EIA (enzyme immunoassay) that test for Immunoglobulin G and/or Immunoglobulin M.[6] Their sensitivities ranges from 70 to 90%.[7] This is important because some cases are missed therefore proper treatment is not started on time.

PCR-DNA presents a promising alternative for *T. pallidum* detection. [8, 9] These tests are highly specific to treponemes that are pathogenic: Specificity at 95 to 97% with a sensitivity of 91 to 95%. [8, 9] This study evaluated the performance (sensitivity, specificity, predictive values and kappa statistics) of three serological tests (RPR, VDRL and TPHA) and a molecular based method to detect syphilis among HIV patients attending Comprehensive Care Centre (CCC) at the Nyeri County Referral Hospital (NCRH).

METHODS

Ethical considerations: The study approval was obtained from Kenyatta National Hospital and University of Nairobi (KNH-UON) Ethics and Research Committee (Ref: KNH-ERC/A/419).

Consent: Consent was obtained in written form for all the study respondents after being informed

Data Management: All patient information and biological samples were given unique number for identification. Inputting of data into the electronic databases was only linked with a unique number in files with safe password. A twofold entry system was preserved for the data. All records for paper research were conserved in a password protected, secured filing cabinet situated in a controlled access area at the research center. Entry of data, cleaning and validation were performed in order to achieve a clean data

Study design and study population: This study was a cross-sectional analysis carried out between November 2019 to May 2020 among consenting patients routinely attending the Nyeri County Referral Hospital (NCRH) Comprehensive Care Centre (CCC) for HIV treatment and care. Subjects were selected in this study after meeting the following criteria: (i) HIV positive individual aged above 18 years, (ii) be on ARV management for at least 6 months, (iii) had viral load results for HIV available at 6 months.

Sample size: Using the Borderer's (1996)[10] formula based on specificity $=Z^2 1-\alpha/2$ X Sp X (1−Sp) / L^2 X (1-P); S_P = 0.95, $z_{1-\alpha/2}$ = z = 1.96 for 95% confidence level (two-tailed), L = 0.05 and P = 0.064 syphilis prevalence of amongst HIV positive individuals in Kenya. [4] Including a 30% of participants to account for sample getting lost or rejected, a total of 193 HIV patients meeting inclusion criteria were consented and enrolled in this study.

Data collection: A structured interview was employed to gather patient information (including demographic characteristics, clinical history and ART type among others). After the interviews, the blood samples collection was carried out by a phlebotomist and put into EDTA (ethylene-diaminetetraacetic acid) Vacutainers. The blood samples were centrifuged (HETTICH, Model EBA 280 Serial No. 71451) and the collected plasma stored at -30^{0C} until shipped to the laboratory for further, testing.

Laboratory procedures:

RPR test

The RPR was done using the ASI card for RPR in testing for syphilis (Arlington Scientific, Inc., Springville, UT USA) following instructions from the manufacturer. The appearance of aggregates in the central part of the test card circle was an indicative of a reactive result ranging from weak to marked or intense. A grey appearance that was smooth on the test card circle was regarded as negative results. The results were reported only as positive or negative, irrespective of the grade of reactivity.

VDRL test

VDRL was done using Syphilis Ab Rapid Test strip (Laborex IVD Italiano SRL-Italy) according to the manufacturer's instruction. Briefly, this is an immunochromatographic assay which involve qualitative detection of antibodies in the serum. The positive result was shown by two distinct lines, one line in area indicated Control and another line in the Test area. Negative result was indicated by a single line appearing in the Control (C) area and lack of red or pink line appearing in the test (T) region.

TPHA test

The TPHA was done using the TPHA test kit by ASI according to givens instructions by the manufacturers. Using the U-well micro titer-plates TPHA-0004, the results were expressed using the agglutination intensity (- to 4+). All samples showing a positive or weakly positive result (+/- to 4) were retested using the quantitative procedure.

Analysis by PCR

T. pallidum DNA detection using PCR was done following the test method outlined by Palmer et al., (2003).[9] Firstly, Extraction of *T. pallidum* DNA from 1 ml of plasma with EDTA as the anticoagulant was achieved using a mini blood Kit called QiaAmp DNA (Qiagen, Inc., Valencia, CA, USA) with strict adherence to manufacturer's instructions. Thermal cycling conditions and primers outlined by Palmer et al., (2003)[9] was used in this study. This method involved amplification of a 260bp region of the 47 kDa integral membrane lipoprotein gene with the use of these primers KO4 5'CAGAGCCATCAGCCCTTTTCA and KO3A 5'GAAGTTTGTCCCAGTTGCGGTT. Every reaction had 0.5 mM of KO3A and KO4 primers, 16 PCR buffer (50 mMKCl, 20 mM TRIS-HCl pH 8.4), 1.5 mMMgCl2, 0.2 mMdNTPs, and 1.25 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Paisley, UK). A negative control (25 mls of distilled water) sample was included in each PCR. Also, a positive control specimen (distilled water with 100 pg DNA for *T. pallidum*) and an inhibition control for every sample (25 ml sample spiked with 100 pg DNA for *T. pallidum*) were included. Amplification was executed on a Perkin Elmer 9700 utilizing and setting the conditions for thermal cycling in this way: 95^0C for two minutes, followed by 35 cycles of 95^0C for twenty seconds, 62^0C for twenty seconds, and 72^0C for twenty seconds. Electrophoresis was used to visualize PCR using a 2% agarose gel.

Statistical Analysis: We used descriptive statistics i.e. standard deviation, mean and frequency in percentage to describe the patients' attributes and laboratory parameter. Performance of the test (specificity, sensitivity, predictive values and kappa statistics) was analyzed as follows: Specificity = Number of true negatives (TN)/sum of TN and the number of false positives (FP). Sensitivity was calculated as the number of true positive (TP) divided by addition of the TP number and false negative (FN) number. The positive predictive value (PPV) = TP / sum FP +TP; while the negative predictive value (NPV) = TN / TN + FN. 95% confidence interval for the test performance were also calculated. Cohen's kappa coefficient (k) analyses was used to test the agreement of tests against a reference standard. STATA version 13 (StataCorp LP,

College Station, TX, USA) using $P \leq 0.05$ as significance level was used for all statistical analysis.

RESULTS

Baseline characteristics

One hundred and seventy-seven patients with all the required data were analyzed. The study patients mean age in years was 48.3 (SD 11.07) with most being female aged 51 years and above and married. Of the male patients recruited, only 4 (5.5%) were not circumcised while among the female patients all 107 (100%) were not pregnant. Majority of the patients had been living with HIV for eight years or less and were taking the currently recommended ART regimen combination of Tenofovir/Lamivudine/Dolutegravir or Adefovir (TDF + 3TC + DTG/AFV) having changed their previous ART regimen type due to optimization. Majority of the patients 92.1% had undetectable viral load with some 7.9% having HIV viral load above 1000 copies/ml (indication of failing treatment). There was 1.1% of the patient with a pervious syphilis positive result (Table 1).

Table 1
Baseline Characteristics of the Study Population

Variable	Frequency	Percent
Gender		
Female	107	60.5
Male	70	39.5
Age Group		
20-30	10	5.7
31- 40	34	19.2
41 - 50	62	35
51 - 60	51	28.8
≥61	20	11.3
Marital status		
Divorced/separated	27	15.3
Married	104	59.1
Single (never married)	21	11.9
Widowed	24	13.6
Duration with HIV		
≤ 4	43	24.3
4 to 8	43	24.3
8 to 12	42	23.7
12 to 16	39	22
≥ 16	10	5.7
Previous STI infection		
Yes	16	9.1
No	161	90.9
Specific previous STI infection		
Gonorrhea	13	81.3
Gonorrhea and Trichomoniasis	1	6.3
Syphilis	2	12.4
Any other Medical conditions		
No	153	87.9
Yes	21	12.1
Presence of chancre /lesion		
Yes	3	1.7
No	174	98.3
Previous blood transfusion		
Yes	6	3.4
No	169	96.6
Current ARV Regimen		
AZT + 3TC + ETV	13	7.3
TDF + 3TC + DTG/AFV	164	92.7
Changed ART regimen		
Yes	157	88.7
No	20	11.3
Reasons for ART regimen change		
Clinical failure	4	2.5
Optimization	143	90.5
Virological failure	11	7
Viral load (Copies/ml)		
1000	14	7.9
Undetectable	163	92.1
Previous syphilis test		
Yes	77	43.2
No	100	56.8

AZT - Zidovudine; 3TC - Lamivudine; ETV -Entecavir; TDF - Tenofovir
DTG - Dolutegravir; AFV - Adefovir; mL - milliliter
STI - Sexually transmitted infection; ≥ - Greater than or equal to
≤ - Less than or equal to

Prevalence of syphilis among the study population
The prevalence of syphilis varied based on the test method including 97(54.8%) by VDRL, 67(37.9%) by RPR, 41(23.3%) by TPHA and 33(18.6%) by PCR (Figure 1).

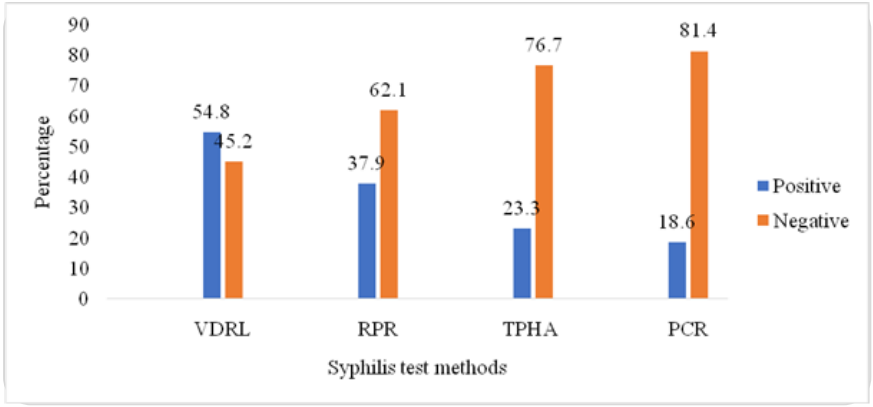

Figure 1. The prevalence of syphilis by test methods

Performance of syphilis Diagnostic Methods using PCR as the gold standard

Results concordant with those of PCR score were obtained in 143/177 (80.8%; 95% CI 74.4% – 85.9%) patients by RPR, 113/177 (63.8%; 95% CI 56.5% – 70.6%) by VDR and 169/177 (84.2%; 95% CI 78.1% – 88.8%) by TPHA. The kappa of tests showed the level of agreement between RPR and PCR was kappa (0.546 - moderate agreement); kappa (0.317 - fair agreement) by VDRL and PCR and kappa (0.864 – near perfect agreement) between TPHA and PCR (Table 2).

Using PCR test outcomes as the standard, sensitivities of the serological tests were: RPR criteria (100%; 95% CI 89.6% - 100%) of the 33 true syphilis positive results by PCR, VDRL 33 (100%; 95% CI 89.6% - 100% of the 33 true syphilis positive results by PCR and 33 (100%; 95% CI 89.6% - 100%) by TPHA of the 33 true positive samples by PCR.

The specificities of each test were: 76.4% (95% CI 68.8% - 82.6%) out of the 14 true negative samples by PCR, 55.6% (95% CI 47.4% – 63.4%) by VDRL and 94.4% (95% CI 89.4% – 97.2%) by TPHA test out of 144 true negative results by PCR. (Table 2).

The positive predictive values (PPV) for the three methods were as follows; 33(34.1%) by VDRL out of 97 by PCR, followed by 33(49.3%) by RPR out of 67 by PCR and 33(80.5%) by TPHA out of 41 by PCR. The negative predictive values (NPV) for all the three tests RPR, VDRL and TPHA were 100% each against PCR as the gold standard (Table 2).

Using PCR as the gold standard we combined the results of all the three tests RPR/VDRL and TPHA and compared the performance against PCR. The test concordance of this was111/177 (62.7%; 95% CI 55.4% – 69.5%) with 0.306 as kappa indicating fair agreement. This combination sensitivity did not offer any improvement compared to the individual tests 100% (95% CI 89.6% – 100%) and specificity dropped to 54.2% (95% CI 46.1% – 62.1%). The NPV of this combination was not different compared to individual tests at 100% and the PPV dropped to 34.1% (Table 2).

Performance of syphilis Diagnostic Methods using TPHA as the gold standard

Using TPHA as our standard (Gold), the performance of these tests in terms of their sensitivity, specificity and kappa were: RPR: 97.6%, 80.1% and kappa (0.636 - substantial agreement); VDRL: 97.6%, 58.1 and kappa (0.377 - fair agreement); and RPR/VDRL combined: 97.6%, 57.4% and kappa (0.369 - fair agreement). The use of TPHA as the gold standard lowers the sensitivity of RPR 100% to 97.6% and that of VDRL from 100% to 97.6% but improves the specificity of RPR 76.4% to 80.1% and that of VDRL from 55.6% to 58.1%. Considering all the TPHA weakly reactive samples as negative improved both the sensitivity and specificity of TPHA against PCR as gold standard each to 100%. On the other hand, when all the TPHA weakly reactive samples are considered as negative lowers both the specificity and sensitivity of VDRL and RPR with TPHA being the gold standard (Table 2).

Table 2

Performance of RPR, VDRL, TPHA and combined RPR/VDRL/TPHA tests against PCR or TPHA results as the gold standard

PCR (Gold standrad)

Test	N	Concordant results (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	NPV (%) 95% CI	PPV (%) 95% CI	Kappa	Agreement
RPR	177	80.8(74.4 - 85.9)	100(89.6 - 100)	76.4(68.8 - 82.6)	100(96.6 - 100)	49.3(37.7 - 60.9)	0.546	Moderate
VDRL	177	63.8(56.5- 70.6)	100(89.6 - 100)	55.6(47.4 - 63.4)	100(95.4 - 100)	34.1(25.4 - 43.9)	0.317	Fair
TPHA	177	95.5(91.3 - 97.7)	100(89.6 - 100)	94.4(89.4 - 97.2)	100(97.3 - 100)	80.5(66.1 - 89.8)	0.864	Near perfect
RPR/VDRL/TPHA	177	62.7(55.4 - 69.5)	100(89.6 - 100)	54.2(46.1 - 62.1)	100(95.3 - 100)	34.1(25.4 - 43.9)	0.306	Fair

TPHA (Gold standrad)

Test	N	Concordant results (%) 95% CI	Sensitivity (%) 95% CI	Specificity (%) 95% CI	NPV (%) 95% CI	PPV (%) 95% CI	Kappa	Agreement
RPR	177	84.2(78.1 - 88.8)	97.6(87.4 - 99.6)	80.1(72.7 - 86)	99.1(95 - 99.8)	59.7(47.7 - 70.6)	0.636	Substantial
VDRL	177	67.8(60.6 - 74.2)	97.6(87.4 - 99.6)	58.1(49.7 - 66)	98.8(93.3 - 99.8)	41.2(32 - 51.2)	0.377	Fair
RPR/VDRL	177	66.7(59.4 - 73.2)	97.6(87.4 - 99.6)	57.4(49 - 65.4)	98.7(93.2 - 99.8)	40.8(31.6 - 50.7)	0.369	Fair

N - number; % - Percentage; CI - Confidence Interval; NPV -Negative Predictive Value; PPV - Positive Predictive Value

DISCUSSION

The goal of this study was to determine the utmost accurate test or combination of tests. Serum testing for syphilis was carried out in population with a higher prevalence of syphilis infection among HIV infected individuals receiving treatment. The prevalence of syphilis in this population by PCR was 18.6% which was higher than previous studies in Kenya including a prevalence of 9.6% among high-risk fishermen in Kenya. [11] The assessed commonness and occurrence of syphilis differed significantly by locality or country. Although, countries with low-and middle income , syphilis prevalence has decreased among the ordinary population, the infection still remains an major issue in a number of high-risk sub-populations, like female sex workers and their male customers with a prevalence rates of 21% in Uganda [13], 21% in South Africa; [12] and 8.9% in Sudan.[14]

Serological assay testing therefore is the most common means to test and diagnose syphilis in symptomatic or asymptomatic individuals.

In this study, we assessed the test performance of syphilis diagnostic methods beginning with a non-treponemal test (NTT) that is affirmed with a treponemal test (TT) using PCR as the gold standard. [15] The analytic performance of a test or the precision of a test to separate between seropositive and seronegative samples using PCR as the gold standard also measured by kappa of tests showed that; TPHA (treponemal based test) was higher in general sensitivity to detect syphilis among this population contrasted with the two non-treponemal test (RPR and VDRL) as calculated by their sensitivities and specificities (TPHA 100%, 94.4%; RPR 100%, 76.4% and VDRL 100%, 55.6%) respectively. Because PCR is generally unavailable in Kenya, the use of TPHA as the best quality standard lowers RPR and VDRL sensitivities from 100% to 97.6% each but improves the specificity of RPR 76.4% to 80.1% and that of VDRL from 55.6% to 58.1%. Previous reports have indicated differing sensitivity for syphilis for VDRL/RPR and TPHA/TPPA ranging between 70 to 80%. [7, 9] Studies have thrown caution on the performance of NTTs whose performance among early cases and recently treated diseases which can be missed inferable from their inferior sensitivity. [16] Moreover, bogus negative NTT results can emerge from the prozone impact. [16]

PCR-DNA presents a promising alternative for *T. pallidum* detection. [8, 9] These tests are highly specific to treponemes that are pathogenic, Specificity at 95 to 97%. Specimens from the mouth and rectum when analyzed can have a sensitivity of 91 to 95% and can detect as low as one to sixty-five microorganisms. [8, 9] PCR method utilizing distinct regions of the DNA polymerase I gene of *T. pallidum* end up being very valuable in detection of treponemes in numerous clinical specimens (such as amniotic fluid, genital ulcer swabs, blood, and CSF samples). With a reported cutoff of 1 to 65 microrganisms, this advanced innovation has demonstrated to be sensitive (with a sensitivity of >90%) and specific (specificity of >95%). [8]

Limitations

This study has two major limits. The main restriction is that the subjects were from a solitary locale of Kenya. Tests may perform contrastingly in different geographic zones. [17] Without broader testing, it is preposterous to generalize the result of this study to other populaces in other locations. Second, the use of PCR as gold standard was based on its performance based on samples collected from genital ulcer specimens. The influence of blood samples as source of *T. pallidum* DNA polymerase I gene of used during PCR cannot be ruled out. The utilization of PCR method using distinct regions of the *Treponema pallidum* DNA polymerase I gene detection of treponemes in numerous clinical

specimens (such as CSF, genital ulcer, blood and amniotic fluid), weakens this limitation.

CONCLUSION

Given these impediments, a sensible end that can be drawn from this data is that in this geographically characterized, high syphilis prevalence populace, neither RPR nor VDRL methods could be suggested as either an independent test or as a confirmatory test. TPHA results were the most concordant with those of PCR. Further research will be required to analyze biomarkers that can more accurately recognize active syphilis requiring treatment, past or treated infections, and distinguish patients who have gotten re-infected. Utilizing current serological tools, most syphilis patients remain serofast after treatment in some instances. This makes their management complicated. [16]

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