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One-step treponemal *vs.* non-treponemal antibody antenatal screening, and syphilis serodiagnosis using the traditional algorithm against the reverse algorithm in a high HIV prevalence setting

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ABSTRACT:

Objective: No single serology test on its own can confirm a syphilis diagnosis. We assessed the utility of a reactive treponemal test (TT) against a non-treponemal test (NTT), each as a sole indicator of infection warranting immediate treatment and the performances of the traditional algorithm (TA) *vs.* reverse algorithm (RA) in the serodiagnosis of syphilis in a high human immunodeficiency virus (HIV) prevalence setting.

Patients and Methods: A rapid plasma reagin (RPR)-NTT and an immunochromatographic (IC)-TT were tested in parallel for the presence of syphilis antibodies in fresh baseline sera of pregnant women living with/without HIV (WLWH/WLWoH) ≥20 weeks gestational age. Reactive samples were confirmed using either the TA or RA. Indeterminate results were resolved using a second but different IC-TT, which detected different spirochaete antigen preparations from those present in the first one-step syphilis screening (1-SSS)-IC-TT.

Results: 1,208 pregnant women were enrolled in the study. 600 were WLWoH, whilst 608 were WLWH. 1-SSS-RPR-NTT detected early syphilis at 28 (2.3%) seroprevalence, while 25 (2.1%) were further confirmed positive by the IC-TT. Re-testing of the three indeterminate samples using the second but different IC-TT confirmed two more cases, slightly increasing the seroprevalence to 2.2%. One WLWoH result remained indeterminate. Using 1-SSS-IC-TT first, early/past syphilis cases were 53 (4.4%), and 25 (2.1%) were further confirmed active infections using the RPR-NTT. Of the 28/53 samples reactive on 1-SSS-IC-TT but negative on RPR-NTT, 20 (1.7%) tested positive on the second but different IC-TT, indicating potentially past infections. However, 8 cases remained inconclusive, with 5 being from WLWoH. Twenty-eight (28) of the 53 1-SSS-IC-TT positives, presenting with past syphilis infections or inconclusive test results, were immediately treated together with the 25 active cases appropriately requiring treatment, resulting in these 28 women receiving unnecessary interventions (overtreatment). This was in contrast with the 1-SSS-RPR-NTT first approach, which resulted in only 1 case of overtreatment. Potential false-positivity rates were 8/1,208 (0.7%) and 1/1,208 (0.08%), respectively. Sero-diagnosis of syphilis, as determined by the RA and TA, was both at 25/1,208 (2.1%), showing a 100% agreement.

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— ***Conclusions:*** 1-SSS-RPR-NTT detected more active cases at a quarter of the cost of 1-SSS-IC-TT. 1-SSS-IC-TT often resulted in antibiotic over-treatments in patients presenting with previous infections, posing a public health threat of antimicrobial resistance. Sero-diagnostic performance was comparable between TA and RA. Interestingly, inconclusive results were relatively common in WLWoH.

— ***Keywords:*** Treponemal and non-treponemal antibody screening, Pregnant women living with and without HIV, Traditional algorithm, Reverse algorithm, False seropositivity rate, Over-treatment rates, Resource-limited setting.

— ***List of Abbreviations:*** ART – Antiretroviral therapy; IC – Immunochomatographic; NTT – Non treponemal test; POC – Point of care; STIs: – Sexually transmitted infections; 1-SSSs – One-step syphilis screenings; RPR – Rapid plasma reagin; TT – Treponemal test; UZBCS: – University of Zimbabwe Birth Cohort Study; WHO: – World Health Organisation.

INTRODUCTION

Direct tests for the detection of *Treponema pallidum,* such as dark-field microscopy and fluorescent antibody tests, remain expensive and not readily available. Thus, routine syphilis antibody screening is mainly done using either a non-treponemal test (NTT) or a treponemal test (TT). The confirmation of the presence of the syphilis antibody is done using either the traditional algorithm (TA) or reverse algorithm (RA) approach, as no single serology test on its own can confirm a diagnosis[1]. In TA, a reactive NTT is confirmed with a TT, whilst in RA, a positive TT is followed by an NTT in sero-diagnosis[2].

Ordinarily, the first antibody screening test in the TA is an NTT called rapid plasma reagin (RPR), a one-step syphilis screening (1-SSS-RPR-NTT). RPR is a precipitation test that detects reagin, a mixture of IgM and IgG antibodies normally present in the sera of syphilis-exposed individuals. Reagin is produced as part of the host immune response to a lipoidal mixture of lecithin (phosphatidylcholine and phosphatidylethanolamine), cardiolipin and cholesterol released from damaged or dying host cells, including lipids from the cell membrane of the spirochaete during early syphilis infection[3]. Seroconversion occurs around six weeks following infection, with antibodies reaching peak levels in 1-2 years in the absence of any treatment[4].

On the other hand, in RA, the first syphilis antibody screening test is a 1-SSS-IC-TT, and reactive samples are confirmed using an NTT. TTs make use of whole cell lysates of *T. pallidum* or a mixture of recombinant treponemal antigens to detect antibodies against specific spirochaete cellular components. Lateral flow or flow-through point of care (POC) testing targets are treponemal recombinant proteins: TpN47, TpN17, TpN15, and TmpA, normally coated as lines on nitrocellulose membrane[5]. TTs test positive approximately five weeks following infection; hence, it may be useful in the detection of early primary syphilis often missed by NTTs[6]. However, treponemal antibodies remain detectable for life even after successful treatment; thus, the distinction between active and past infections remains a challenge that often results in over-treatment among patients presenting with past infections[7].

According to the College of American Pathologists' 2015 report[8], 80% of laboratories use the TA, and the other 20% use the RA. However, proficiency testing results in developed countries showed that the use of RA has been on the increase at about 36% from the previous prevalence rate of 20%[8].

As the numbers of syphilis cases continue to increase globally, better and simpler testing strategies that accurately detect the presence of spirochaete are urgently needed. This is essential because there is no universally accepted gold standard for syphilis serodiagnosis. In resource-limited settings, the standard of care in pregnancy is mainly an on-site single syphilis screening at the first antenatal booking. The single reactive test result is often indicative of infection warranting immediate treatment, yet the performances and cost-effectiveness of the TA *vs.* RA in the serodiagnosis of syphilis remain poorly described in these resource-poor settings where HIV prevalence is also high.

We assessed the utility of a single reactive TT against an NTT and the performances of the TA *vs.* RA in the serodiagnosis of syphilis in pregnant WLWH and WLWoH.

We aimed to:

- Compare the detection rates of syphilis antibodies and the potential over-treatment rates late in pregnancy using 1-SSS–RPR-NTT *vs.* 1-SSS-IC-TT.
- Determine the rates of potentially false positive results using the TA and RA approaches in the serodiagnosis of syphilis.
- Determine the performance and cost-effectiveness of the TA and RA approaches in the serodiagnosis of syphilis.

PATIENTS AND METHODS

Study Setting

This was a baseline assessment of maternal syphilis antibodies in the University of Zimbabwe College of Health Sciences Birth Cohort Study (UZ-CHS

Birth Cohort Study, NCT04087239), which aimed to investigate the role of maternal HIV status and coinfections with syphilis, including hepatitis B virus and cytomegalovirus on pregnancy outcomes and infant health[9]. The UZ-CHS Birth Cohort Study recruited 608 pregnant women living with HIV (WLWH) on life-long antiretroviral therapy and 600 women living without HIV (WLWoH) from 4 primary health centres in the southwestern high-density residential areas of Harare, Zimbabwe[9].

Zimbabwe follows the World Health Organisation sexually transmitted infections (STIs) guidelines[10] that recommend screening of all pregnant women for HIV and syphilis at their first antenatal care visit. A single on-site syphilis screening is the current standard of care using a TT combined with HIV POCTs (Abbott Bioline HIV/Syphilis Duo, Yongin-si, Republic of Korea). A reactive TT is currently used as the sole indicator of syphilis infection requiring immediate treatment to mitigate adverse pregnancy outcomes.

Study Design

This was a baseline determination of the presence of syphilis antibodies and assessment of the performances of TT and NTT testing in a prospective observational birth cohort study designed to investigate the role of maternal HIV status, including coinfections on pregnancy outcomes as well as infant health.

The study was conducted at primary health centers and polyclinics in the high-density suburbs of Harare, specifically in Kuwadzana, Rujeko in Dzivaresekwa, Budiriro and Glenview. It included pregnant women at least 20 weeks gestational age.

Inclusion and Exclusion Criteria

The inclusion criteria included being ≥15 years old at enrolment and being a woman planning to deliver at any of the above-selected four study sites.

Exclusion criteria were the presence of mental health disorders that made the participant unable to provide informed consent and/or comply with study procedures as previously described[9].

Ethical Approval and Consent to Participate

The study complied with the ethical principles of the Declaration of Helsinki and was conducted in compliance with the International Council for Harmonization of Good Clinical and Laboratory Practice guidelines and local regulatory requirements. Ethical approval was obtained from the Joint Research Ethics Committee of the University of Zimbabwe Faculty of Medicine and Health Sciences and The Parirenyatwa Group of Hospitals (JREC/81/15) and the Medical Research Council of Zimbabwe (MRCZ/A/1968). Literacy is nearly universal in Zimbabwe, and all potential participants were able to read and comprehend the informed consent form. All study participants provided written informed consent.

Syphilis Screening Tests

A carbon antigen RPR-NTT and a solid phase IC-TT were run in parallel on fresh sera from pregnant women ≥20 weeks gestational age.

Anti-NTT Screening Using RPR

The RPR carbon antigen NTT test kit (Fortress Diagnostics Limited, Antrim, Northern Ireland) consists of particulate carbon suspensions coated with lipid complexes that flocculate in the presence of serum reagin. Black clumps or visible agglutinations denote the presence of reagin in reactive samples. A reactive test is indicative of a current or active infection. However, a negative test result does not necessarily preclude the possibility of *T. pallidum* infection. This is a semi-quantitative test with results reported as non-reactive, weakly reactive, or reactive depending on the presence and sizes of the agglutination clumps visible to the naked eye; hence, results interpretation may be subjective. In addition, this method requires a mechanical rotor to mix the serum/plasma sample with the carbon particles. Reactive RPR-NTT samples were confirmed using the IC-TT, the TA approach.

Anti-TT screening using SD Bioline Syphilis 3.0

A solid phase qualitative assay, the IC-TT test kit [Standard Diagnostics (SD) Bioline Syphilis 3.0 (06FK10)] (Gyeonggi-do, Republic of Korea) was used to detect antibody isotypes against *T. pallidum*. The kit consists of a pre-coated membrane strip coated with recombinant *T. pallidum* antigens (0.7±0.14 µg) on the test line site. The control line contains 0.075±0.15 µg of goat anti-*T. pallidum*. The maternal serum sample diluent and the recombinant *T. pallidum* antigens-colloidal gold conjugate move chromatographically to the test region, forming a visible line as the antigen-antibody-antigen gold particle complex forms. A visible line in the test region denotes a positive result for the detection of IgG/IgA/IgM *T. pallidum*-specific antibodies. The absence of the line/colour on the test line implies a negative test result. According to the manufacturer, the relative sensitivity and relative specificity are 99.3% and 99.5%, respectively. A reactive TT is indicative of a current and/or past syphilis infection(s). However, a negative test result does not necessarily preclude the possibility of *T. pallidum* infection. An RPR-NTT, RA approach confirmed samples reactive on IC-TT-SD Bioline 3.0.

Further Evaluation of Indeterminate Results

Indeterminate results were resolved through the use of a second but different TT-Hightop. The TT-Hightop (Hightop Biotech Co. Ltd, Qingdao City, China, www.hightopqd.com) uses different spirochaete antigens from those of the initial IC-TT-SD Bioline 3.0. TT-Hightop syphilis test uses a double antigen sandwich method to detect *T. pallidum* antibodies. The test line is coated with *T. pallidum* antigen 2. Like the first IC-TT-SD Bioline 3.0 test kit,

the control is also coated with a goat anti-mouse IgG antibody. When the concentration of *T. pallidum* antibody in the test sample is greater than or equal to the minimum detection limit, the antibody forms an "antibody-labelled coupling protein *T. pallidum* antigen-1 complex" with the labelled conjugate protein *T. pallidum* antigen-1 complex, which is captured by the *T. pallidum* antigen 2 pre-coated on the nitrocellulose membrane, resulting in a line if the sample is reactive. No test line appears if the *T. pallidum* antibodies are absent or if the sample antibody concentration is lower than the detection limit.

Interpretations of Syphilis Test Results

Any reactive RPR-NTT or IC-TT-SD Bioline 3.0 results were considered positive for syphilis antibodies. If the initial NTT or TT were non-reactive, no further testing was done, but results were considered true seronegative if both tests were seronegative.

If both the RPR-NTT and IC-TT-SD Bioline 3.0 were positive, this confirmed active or early syphilis infection, provided there was no recent treatment. Two different reactive TTs, either IC-TT-SD Bioline 3.0 or TT-Hightop and a non-reactive NTT, also confirmed active syphilis[11]. More results interpretations used with respect to TA and RA approaches are shown in Table 1 and Table 2.

Statistical Analysis

Data were entered and managed using Research Electronic Data Capture (REDCap© v. 8.0, 2020; Vanderbilt University, TN, USA). Quality assurance on the accuracy of data entry included independent double entries and verification in case of discrepancies. Descriptive summary statistics were calculated for outcome variables of interest using frequencies and percentages for categorical variables.

RESULTS

Utility of 1-SSSs–RPR-NTT

Of the 1,208 pregnant women tested for syphilis antibodies, 608 were WLWH, mainly on Tenofovir/Lamivudine/Efavirenz, while 600 were WLWoH. 1-SSSs–RPR-NTT resulted in a seroprevalence of 2.3% (28/1,208). From these, 25 (2.1%) were further confirmed positive by the IC-TT-SD Bioline, TA approach. However, follow-up testing of the three indeterminate results using the second but different TT-Hightop confirmed two more positive cases. Thus, these two cases were potentially early infections missed by the confirmatory IC-TT-SD Bioline 3.0, slightly increasing the serodiagnosis rate to 2.2% (27/1,208). The only remaining inconclusive test result, a potentially false positive sample with respect to the initial RPR-NTT, was from a WLWoH.

Utility of 1-SSS-IC-TT-SD Bioline

1-SSS–IC-TT-SD Bioline resulted in a 4.4% (53/1,208) sero-positivity rate. Of these, 2.1% (25/1,208) (95% CI 1.3-2.9) were again further confirmed positive by the RPR-NTT with the RA approach. Of the 28 IC-TT-SD Bioline positives that initially tested negative on the confirmatory RPR-NTT, 20 further tested positive on the second TT-Hightop. These reactive 1.7% (20/1,208) were potentially past infections, with the remaining eight (five were WLWoH) testing negative on the second TT-Hightop, hence inconclusive. These eight were potentially false positives with 1-SSS–IC-TT-SD Bioline 3.0.

Concordance of TA and RA

Interestingly, syphilis diagnosis using the RA and TA approaches had 100% agreement at 25/1,208:2.1%, 95% CI [1.3-2.9] (Figure 1).

Over-Treatment Rates

The Ministry of Health and Child Care recommends a single rapid test screening with IC-TT-SD Bioline 3.0 followed by immediate treatment. Twenty-eight (28) of the 53 1-SSS-IC-TT positives, presenting with past syphilis infection or inconclusive test results, were immediately treated together with the 25 active cases appropriately requiring treatment, resulting in the 28 women receiving unnecessary interventions (overtreatment).

This was in contrast with the RPR-NTT approach, which resulted in only one case of overtreatment (Figure 1). Consequently, the IC-TT-SD Bioline approach would increase treatment costs and require more follow-ups, as detailed in Figure 2.

Table 1. Interpretations of syphilis test results – the TA approach.

RPR-NTT as first test	IC-TT-SD Bioline 3.0 second test	TT-Hightop; (tie breaker), third test	Results interpretation
Reactive	Reactive	No further testing	Active /early infection
Non-reactive	Non-reactive	No further testing	No evidence of infection
Reactive	Non-reactive	Non-reactive	Syphilis infection unlikely, RPR-NTT biological false positive likely
Non-reactive	Reactive	Reactive	Potentially early acute infection (missed by RPR-NTT), false negative RPR-NTT

Rapid plasma regain, RPR; Non treponemal test, NTT; Immunochomatographic, IC.

Table 2. Interpretations of syphilis test results – the RA approach.

IC-TT-SD Bioline 3.0 as first test	RPR-NTT second testt	TT-Hightop; (tie breaker), third test	Results interpretation
Reactive	Reactive	No further testing	Active or past infection
Non-reactive	Non-reactive	No further testing	No evidence of infection
Reactive	Non-reactive	Reactive	Past or potentially early active infection
Reactive	Non-reactive	Non-reactive	Potentially false positive IC-TT-SD Bioline 3.0
Non-reactive	Reactive	Reactive	Potentially early infection or false negative IC-TT-SD Bioline 3.0.

Rapid plasma regain, RPR; Non treponemal test, NTT; Immunochomatographic, IC.

DISCUSSION

We report the results on the utility and cost-effectiveness of screening for syphilis antibodies using 1-SSS–IC-TT-SD Bioline 3.0, and NTT-RPR late in pregnancy in a high HIV prevalence setting. The reactive samples were confirmed using the RA and TA approaches. The following were the main findings:

– 1-SSS-RPR-NTT resulted in a syphilis antibody seroprevalence of 2.3%, while 2.1% were confirmed positive by the TA approach. Re-testing the three indeterminate samples on a second but different TT-Hightop confirmed two more positives, slightly increasing the sero-diagnosis from 2.1% to 2.2%, and 0.08% false positivity rate concerning 1-SSS-RPR-NTT.

– 1-SSS–IC-TT resulted in a syphilis antibody seroprevalence of 4.4%, and 2.1% were confirmed positive by the RA, with potentially past infections at 1.7%. Re-testing of indeterminate results using a second but different TT-Hightop resulted in potentially false seropositivity rates of 0.7%.

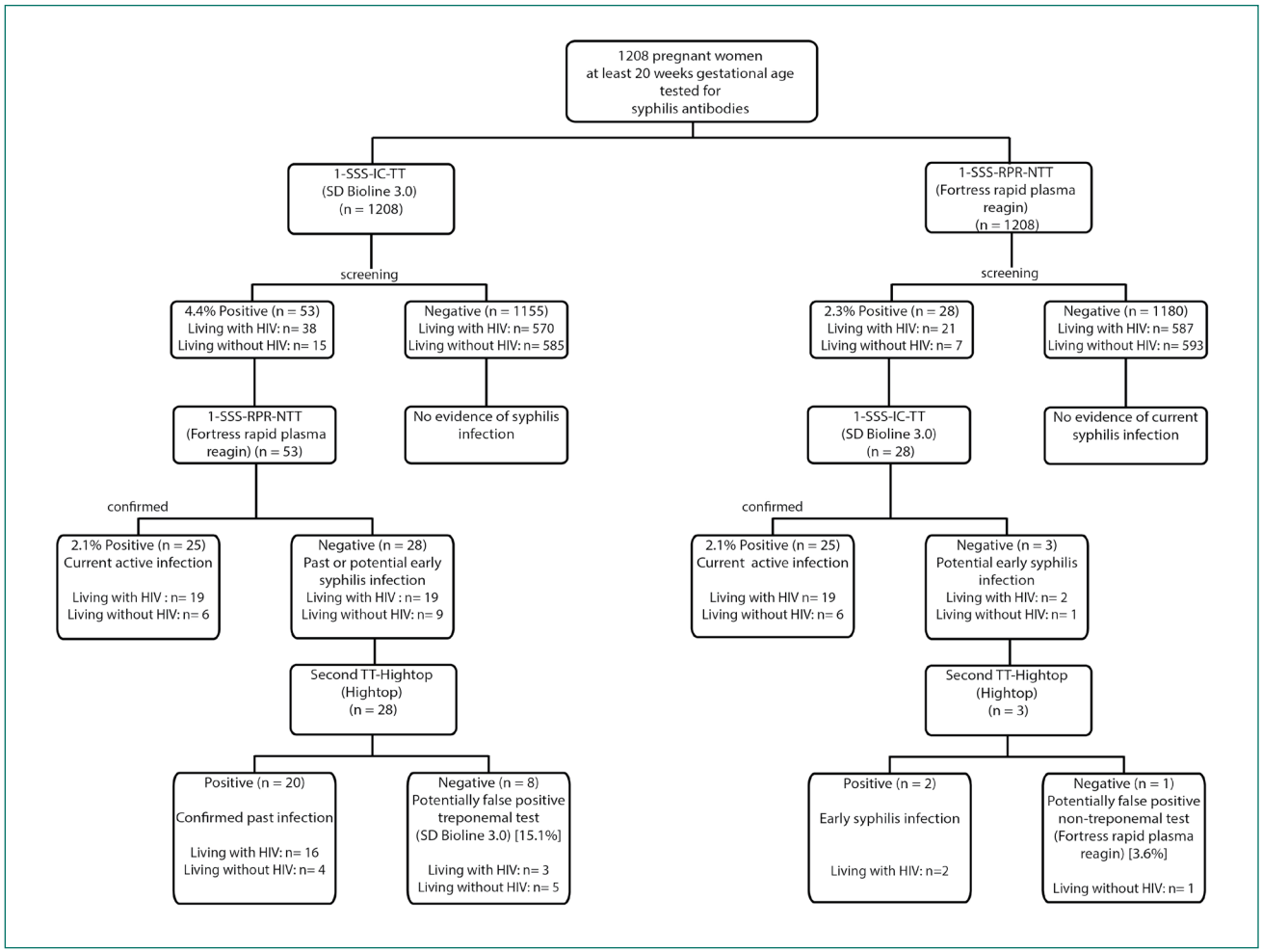

Figure 1. Flow diagram for one-step syphilis screening and confirmation of results using Reverse algorithm and Traditional algorithm.

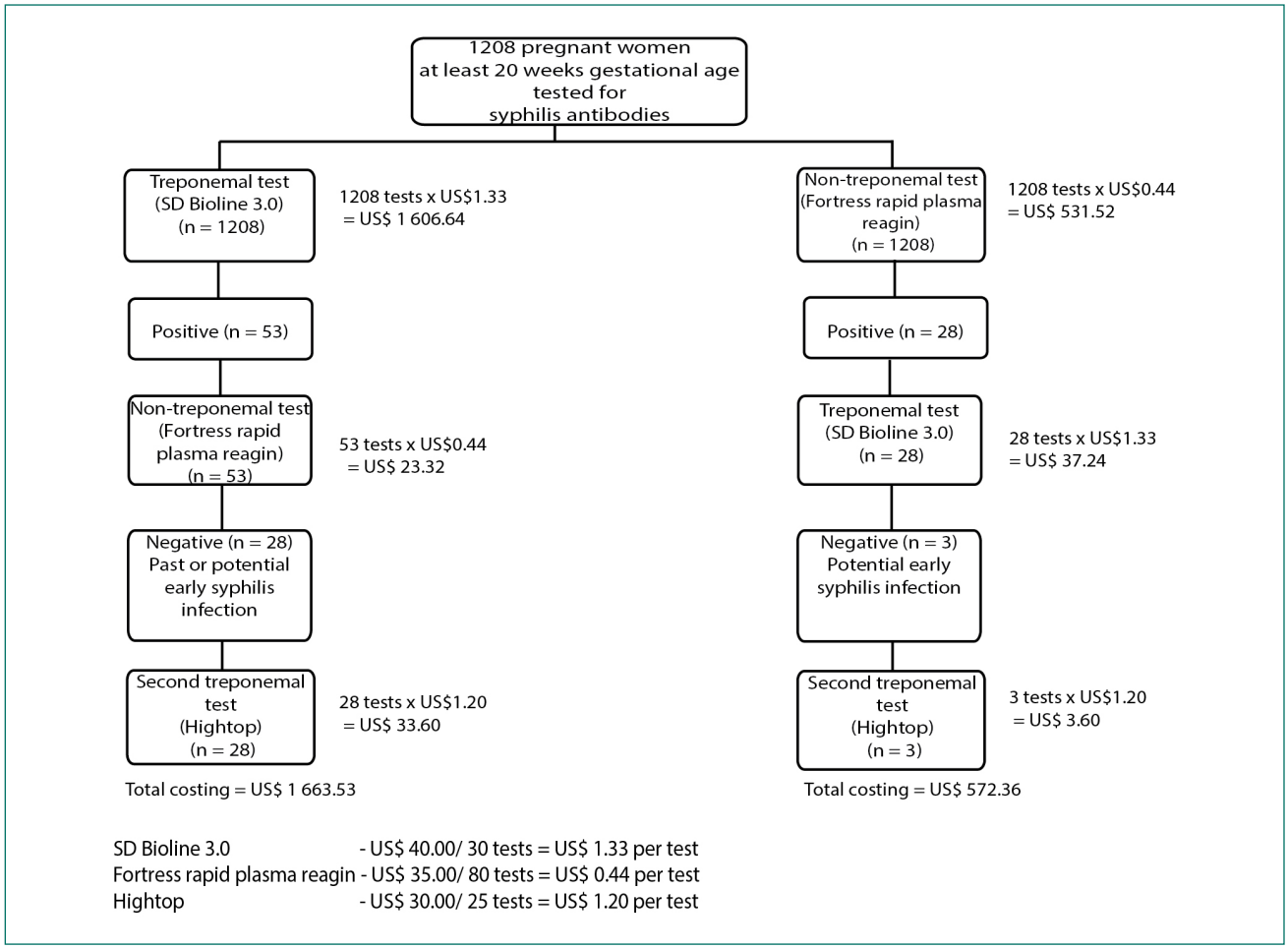

Figure 2. Comparative Zimbabwean costs of Standard Diagnostics (SD) Bioline 3.0 as a first test (reverse algorithm approach) *vs.* rapid plasma reagin as a first test (the traditional algorithm) in United States dollars. The reverse algorithm would be more than four times more expensive in terms of the actual costs of the test kits, treatment, and the required patient follow-up expenses in cases of inconclusive test results.

– RA and TA showed similar serodiagnosis performance, with a 100% syphilis concordance rate. Both had a seroprevalence of 2.1%.

Overall, the RA approach was four times more expensive than the TA in terms of the actual costs per test, treatment for reactive TT and/or NTT, and the required patient follow-up expenses in cases of inconclusive test results.

Tests Performance

We observed a similar seropositivity rate of 2.1% for both the RA and TA diagnostic approaches. However, further follow-up testing using a second TT-Hightop increased the seroprevalence to 2.2% using the TA approach, which seemed more sensitive and specific.

Inconclusive results were observed at 0.08% for the TA approach, where the first screening test was RPR-NTT. This observation was consistent with the findings by Unemo et al[12] that showed RPR detected syphilis cases with high sensitivity and specificity. In immunocompetent individuals, syphilis antibody titers decline following successful treatment and become non-reactive within six months, with 20% remaining in the serofast status, persistently reactive but at low titres[13]. However, with NTT-RPR, the prozone phenomenon remains a concern as it may cause false negative results, underscoring the importance of confirming NTT screening test results with more specific TTs.

Other studies[14] showed the incidence of false positive RPR results in the population living with HIV to be significantly higher compared to that of individuals without HIV. This was difficult to compare with our study results as only one case of potentially false positive RPR was observed. In spite of being simple and cheap, limitations of TA as first-line syphilis screening with NTT-RPR include low specificity and sensitivity, including the challenge to automate. In addition, the interpretation of the results is subjective.

Over-treatment rates were higher using 1-SSS-IC-TT, but these immunochromatographic methods have the advantage that they can use whole blood (obtained by finger-prick) that does not necessarily require special equipment such as a refrigerator or centrifuge to separate plasma or serum. In addition, automated TT

platforms with high throughput are now readily available, and since they are not flocculation assays, false negative results due to the prozone effect do not occur. Previous studies[15,16] reported a false seropositivity rate of IC-TT-SD Bioline 3.0 of 0.2-0.8% in non-pregnant adults. This range is consistent with the 0.7% potentially false positives observed in our study. Other previous studies[17] have shown that most (65%) pregnant women with discordant screening results (treponemal multiplex flow immunoassay IgG+/rapid plasma reagin) but with a non-reactive confirmatory *Treponema pallidum*-particle agglutination assay were more likely indicative of a false-positive reaction.

Combined TT and NTT point of care test (POCT) in an HIV clinic in Botswana has been shown to diagnose active syphilis more accurately and, at the same time, avoid over-treatment[18]. This has an influence on the standard clinical practice as POCTs dual NTT/TT or modifying the current dual TT/HIV-IC POCTs tests currently in use in our setting to triple NTT/TT/HIV IC-POCTs tests would greatly reduce the over-treatment rates in an effort to curb the antibacterial resistance challenge. Interestingly, in our study, inconclusive results were relatively more common in WLWoH

The strengths of our study include a large number of pregnant women, of whom ~50% were WLWH, and ~50% were WLWoH, all sampled concurrently from the same community. However, the distinction between primary and secondary syphilis among women was not possible. In addition, syphilis antibody titers were not measured.

This paper summarizes syphilis in simple language to connect with a broader audience, given its ongoing global health threat. If untreated, it can cause serious health issues, yet unnecessary treatment or over-treatment may result in the development of antimicrobial resistance. It is essential to educate the public on this important health issue.

CONCLUSIONS

Despite being relatively more labor-intensive, 1-SSS–RPR-NTT was cheaper, detected more syphilis cases, and was associated with lower over-treatment rates compared to the 1-SSS–IC-TT. Sero-diagnosis performance was comparable between TA and RA. Interestingly, the inconclusive results were relatively common in WLWoH, and this observation warrants further investigations. From time to time, syphilis algorithm evaluations remain essential for laboratory managers and policymakers to make informed decisions on the best algorithm to choose depending on their settings and/or financial situations. As the numbers of syphilis cases continue to increase globally, better tests that accurately detect the presence of spirochaete are urgently needed to reduce over-treatment rates to curb the current public health threat of antimicrobial resistance.

Conflict of Interest:
ll authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Approval:
The protocol was reviewed and approved by the Joint Research Ethics Committee of the University of Zimbabwe and Parirenyatwa Group of Hospitals (JREC) (reference number JREC/81/15 in 2015), and the Medical Research Council of Zimbabwe (reference number MRCZ/A/1968). UZBCS complied with the ethical principles of the Declaration of Helsinki of 1975, later revised in 2013. It was conducted in compliance with the International Council for Harmonisation of Good Clinical and Laboratory Practice guidelines and local regulatory requirements.

Informed Consent:
All potential participants were able to read and comprehend the informed consent form. All study participants provided written informed consent.

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Authors' Contributions:
The study was conceived by KD and designed by LRM and KD. PTM, AJM, HM and PN were responsible for data collection and entry. KD and LRM were involved in the interpretation of findings and the writing of the manuscript. KD wrote the first draft. All authors were involved in manuscript revisions and approved the final draft.

Data Availability:
Data sharing does not apply to this article as no datasets were generated or analyzed during the current study.

Availability of Data and Materials:
The datasets obtained during this study will be available upon reasonable request to the corresponding author.

AI Disclosure:
No artificial intelligence (AI) or assisted technologies were used at any stage of this study or the write-up.

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