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Multiplex nested polymerase chain reaction for *Treponema pallidum* using blood is more sensitive than using serum

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ABSTRACT

BACKGROUND
Syphilis is a multistage disease transmitted primarily through sexual intercourse. Nowadays, the polymerase chain reaction (PCR) test for *Treponema pallidum* has been widely used and is expected to overcome problems in diagnostic tests for syphilis. The *Treponema pallidum* PCR is influenced by type of specimens, PCR methods and target genes. This study aimed to assess the use of blood and serum in multiplex nested PCR for *Treponema pallidum,* targeting the 23S rRNA.

METHODS
A cross-sectional study was conducted from April 2015 - April 2016. Sampling was carried out consecutively among patients with clinical features of secondary syphilis who came to Sexually Transmitted Disease (STD) clinics in Jakarta. All sera were also tested with Rapid Plasma Reagin (RPR) and *Treponema pallidum* Hemagglutination Assay (TPHA) assay, which was considered as the gold standard for this study. We determined the sensitivity and specificity of the multiplex nested PCR for *Treponema pallidum* using blood and serum.

RESULTS
PCR test was performed on 122 clinical specimens (61 blood and 61 serum). The positive results of PCR test on blood was 22.95% and serum was 6.56%, while the positive results of serology was 68.85%. The sensitivity of *Treponema pallidum* multiplex nested PCR on blood was 30.95% compared to serum 9.52% (p=0.006). PCR test on blood is able to detect 3.25 times higher than serum.

CONCLUSION
The use of blood has a higher proportion of positives compared to serum in *Treponema pallidum* multiplex nested PCR using 23S rRNA target gene.

Keywords: Secondary syphilis, *Treponema pallidum*, multiplex nested PCR test, blood, serum

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INTRODUCTION

Syphilis is one of the multistage diseases that are transmitted mainly through sexual intercourse. This disease shows diverse clinical manifestations, a protracted course, and serious complications. Syphilis constitutes a global health problem particularly in developing countries, including Indonesia.[1]

Since this disease was first known in the fifteenth to twentieth centuries, the prevalence of syphilis had already decreased, but in the last few decades, the incidence of early syphilis in various countries in America, Europe, and Asia was reported to be again on the increase.[1, 2] In Indonesia, based on a survey conducted by the Integrated Surveillance of Behavioral Biology program (*Surveilans Terpadu Biologi Perilaku*, STBP) in the years 2007 and 2011 on the population most at risk, there was an increase in the prevalence of syphilis.[3] This occurred particularly in the population most at risk, i.e. males who had sexual intercourse with males (MSM), from a prevalence of 4% in 2007 to 13% in 2011. The highest prevalence of syphilis was found in the transvestite population, i.e. a total of 28%.[3] According to data from the WHO in 2008, the prevalence of syphilis in Southeast Asian countries was 12.3 million, i.e. 6.2 millions in women and 6.1 millions in males. [4] In 2012, WHO estimated that there were 6 million new cases of syphilis each year in the whole world and most cases occurred in developing countries.[5]

The diagnosis of syphilis is established based on anamnesis, physical examination of the patient and accessory examination. The difficulty in diagnostic testing of syphilis is due to the fact that *Treponema pallidum* cannot be cultured in vitro.[6,7]

Microscopic examination may directly evaluate the presence of *Treponema pallidum,* but requires a special microscope or special staining, and cannot differentiate between *Treponema* species. At present, laboratory facilities for microscopic examination of syphilis are very limited. In addition, experienced laboratory technicians are required to evaluate *Treponema pallidum* by microscopy. Serologic tests are diagnostic tests for syphilis that are commonly used, including in Indonesia. The extremely sensitive nontreponemal tests are followed by the more specific treponemal confirmatory tests. However, there are several limitations in nontreponemal or treponemal tests, as they may show false positive, false negative, or dubious results.

At present, the polymerase chain reaction (PCR) for *Treponema pallidum* is widely used and is expected to be able to decrease the problems in diagnostic testing of syphilis. The published studies on the *Treponema pallidum* PCR test comprise conventional PCR, real-time PCR, nested PCR and multiplex PCR, with various target genes specific for *Treponema pallidum*, such as the target genes *PolA, tpp47*, and *bmp*.

At present, the PCR methods have become the diagnostic tests recommended by the CDC and the United Kingdom for detecting *Treponema pallidum*, in addition to microscopy.[8,9] The *Treponema pallidum* PCR test may use various types of clinical specimen depending on the course of the disease. The sensitivity and specificity of the molecular PCR test results are affected by the type of specimen, the PCR method, and the target gene used.[10,11]

Specimens that may be used for the *Treponema pallidum* PCR test in secondary syphilis are exudates from syphilitic lesions and blood. The sensitivity of PCR with smears from lesions of secondary syphilis reaches 80% and the specificity 98.6%.[12] The skin lesions in secondary syphilis are extremely infectious. However, not all patients with secondary syphilis show skin lesions; in addition, sample collection from skin lesions of patients with secondary syphilis is more difficult, invasive, and causes discomfort in the patients.

Blood is the specimen that may used for examination in all stages of syphilis.The number of *Treponema pallidum* in the blood is highest in

early syphilis.[13, 14] In addition to the complete set of blood components (whole blood), other blood components may also be used as specimen for the *Treponema pallidum* PCR, such as serum, plasma, and peripheral blood mononuclear cells (PBMC). Cruz et al.[13] found that blood is the best type of specimen for detecting *Treponema pallidum*.

Grange et al.[10] reported on the higher proportion of positives for blood as compared with serum, plasma, and PBMC for nested PCR testing in secondary syphilis. In contrast to that reported by Cruz et al.[13] and by Gayet-Ageron et al. [15] reported that serum was better than blood for detecting *Treponema pallidum* using PCR in secondary syphilis (47% and 36%).

The difference between the results of Cruz et al.[13] and Grange et al.[10] on the one hand and Gayet-Ageron et al.[15] on the other, is thought to be due to factors of the PCR test system used, such as difference in target gene and primer design.

The purpose of 23S rRNA with the multiplex nested PCR method is to detect two-point mutations causing *Treponema pallidum* resistance to azithromycin, one of the alternative drugs of choice for syphilis.[16] Therefore this study uses multiplex nested PCR to determine the better specimen between blood and serum for the application of this test.

METHODS

Research design

A cross-sectional study was performed from April 2015 to April 2016.

Study subjects

The study subjects were screened with consecutive methods, i.e. patients aged >18 years with the clinical picture of secondary syphilis who attended the Dermatoveneorology polyclinic in Cipto Mangunkusumo Hospital, the Sexually Transmitted Disease (STD) clinic of the Pasar Rebo subdistrict Puskesmas, the STD clinic of the Tambora subdistrict Puskesmas and the Indonesian Familty Planning Association (*Perkumpulan Keluarga Berencana Indonesia*, PKBI) ProCare clinic. Patients who had received treatment were excluded from the study.

Sample size determination

The sample size was obtained by considering the proportion of positives of the PCR test with blood samples of secondary syphilis to be 38%.[10] The sample size determination used the following formula:

$$n1 = n2 = \left[\frac{\left(Z\alpha\sqrt{2PQ}\right) + \left(Z\beta\sqrt{P1Q1 + P2Q2}\right)}{(P1 - P2)^2} \right]^2$$

where n1=n2= sample size
Zα= 1.96
Zβ= 0.842
P1 = proportion of standard effect 1
P2 = proportion of investigated effect (clinical judgment)
[proportion of positives]
P = ½ (P1+P2)
P2 = 38% =0.38
Q2 = 1-P2 = 0.62
P1-P2 = 25% = 0.25
P1 = 0.63
Q1 = 0.37
P = 0.50
Q = 0.5
so that the minimal sample size was 61 per group.

Intervention

From all patients, blood was drawn from the vein in the cubital fossa into two vacutainer tubes using sterile 23G ¼ inch needles. The blood was drawn to a volume of 3-4 ml into the red-capped tube without anticoagulant and to a volume of 1-2 ml into the violet-capped tube containing EDTA anticoagulant. The blood in the tube without anticoagulant was processed into serum, which was used for the RPR and TPHA serological tests. Whole blood and serum were used for *Treponema pallidum* multiplex nested PCR testing.

Measurements

The RPR and TPHA tests were performed quantitatively to determine the titration concentrations. The serological test results in patients with the picture of clinical secondary syphilis were considered to be positive if an RPR titer of >1:8 and a TPHA titer of >1:80 were obtained. A positive *Treponema pallidum* multiplex nested PCR test was shown by a 187-bp band on visualization in 3% agarose gel after electrophoresis at 100 volts for 30 minutes, as internal control indicating the presence of *Treponema pallidum.* If bands of 130 bp and 100 bp were also found, these show the presence of mutations at 2 points in the DNA sequence of azithromycin-resistant *Treponema pallidum.*

Laboratory analysis

Qualitative RPR testing used a commercial kit (RPR AIM Cat. No. E RPR 2) on the sera of all patients according to the protocol of the kit. The results that were reactive on qualitative RPR testing were to be followed with the quantitative RPR test. All samples subjected to the RPR test were confirmed with the quantitative TPHA (TPHA AIM Cat. No.TPHA E 100) according to the protocol of the kit.

The PCR test used the DNA resulting from the extraction of blood and serum that were stored at a temperature of -35°C. Blood was extracted according to the protocol of the Roche kit (High Pure PCR DNA Template Preparation Kit). Serum was extracted according to the protocol of the Qiagen kit (Qiagen QIAmp DNA mini kit). The multiplex nested PCR test used *Treponema pallidum* 23S rRNA target genes and was already optimized using 3 (three) pairs of primers.[16] The PCR 1 reaction used one pair of primers (forward-reverse) for detecting the 23S rRNA component of *Treponema pallidum.* The reaction mixture with total volume of 20 µl consisted of distilled water (DNase-free water) 7.5 µl, 10x HotStar PCR buffer 2.0 µl, 25 µM $MgCl_2$ 0.2 µl, 10 µM dNTP mix 0.4 µl, 5x Q solution 4.0 µl, 10 µM PCR1 F primer 0.4 µl, 10 µM PCR1 R primer 0.4 µl, and HotStar DNA polymerase enzyme 0.1 µl. A volume of 5 µl DNA was introduced to the reaction mixture as template. The PCR 1 reaction consisted of a total of 25 cycles at a predenaturation temperature of 95ºC for 15', denaturation at 95ºC for 30", annealing at 58ºC for 30", elongation at 72ºC for 30", and final extension at 72ºC for 10'.

The PCR 2 reaction used two pairs of primer (forward-reverse) with the purpose of amplifying the DNA sequence of *Treponema pallidum* where there are mutation points to identify azithromycin-resistant *Treponema pallidum* based on the mutation patterns of adenine bases into guanine in the DNA sequence positions 2058 and 2059 of the 23S rRNA gene. The reaction mixture at a total volume of 20 µl consisted of distilled water (DNase-free water) 9.2 µl, 10x HotStar PCR buffer 3.0 µl, 25 µM $MgCl_2$ 0.2 µl, 10 µM dNTP mix 0.4 µl, 5x Q solution 4.0 µl, 10 µM PCR 2058F primer 0.9 µl, 10 µM PCR 2058R primer 0.5 µl, 10 µM PCR 2059F primer 0.3 µl, 10 µM PCR 2059R primer 0.8 µl, and HotStar DNA polymerase 0.3 µl. A volume of 1 µl PCR 1 products were introduced into the mixture as a template. The PCR 2 reaction comprised 35 cycles at predenaturation temperature of 95ºC for 15', denaturation at 95ºC for 30", annealing at 61ºC for 30", elongation at 72ºC for 1' and final extension at 72ºC for 5'. The positive controls used were 194 bp-long positive synthetic DNA controls, which were used in the PCR 2 reaction. The negative controls in the PCR 1 reaction used DNase free water.

The PCR 2 products were subjected to electrophoresis using 3% agarose. The marker used was the 50 bp marker. The agarose gel was subjected to electrophoresis at 100 volts for 30 minutes, then stained with Gel Red Staining and visualized using UV radiation with the BioRad® GelDoc™ EZ Imager. The PCR 2 products were shown as bands of 187 bp, 130 bp and 100 bp, respectively.

Statistical analysis

The collected data were processed with the SPSS software version 20 and Microsoft Excel

2007. To obtain the significance of the *Treponema pallidum* multiplex nested PCR test results, sensitivity and specificity were used to evaluate the diagnostic test.

Ethical clearance

This study obtained ethical clearance from the Health Research Ethics Committee, Faculty of Medicine,Universitas Indonesia, under no. 158/ UN2.F1/ETIK/2015. This study also obtained approval from Cipto Mangunkusumo Hospital (RSCM), the Jakarta One-Door Integrated Service Board (*Badan Pelayanan Terpadu Satu Pintu Jakarta)*, recommendation and research permit from the Jakarta Health Service head office *(Dinas Kesehatan Jakarta)*, and recommendation and research permit from the West Jakarta Health Service sub-office (*Suku Dinas Kesehatan Jakarta Barat)*.

RESULTS

The collected study sample numbered 122 clinical specimens, consisting of 61 blood specimens and 61 sera from 61 patients with the clinical picture of secondary syphilis. The study subjects were predominantly males with median age of 26 years and overall age range of 18-40 years. A total of 88.5% (54 study subjects) had a homosexual orientation, i.e. males who had sexual intercourse with males. The majority of study subjects, i.e. 54.10% (33 of 61) had a reactive HIV status (Table 1).

Table l. Demographic and clinical characteristics of the study subjects (n=61)

Characteristic	n (%)
Sex	
Male	58 (95.08)
Female	3 (4.92)
Sexual Orientation	
Homosexual (MSM)	54 (88.52)
Heterosexual	6 (9.84)
Transvestite	1 (1.64)
Status HIV	
Reactive HIV	33 (54.10)
Nonreactive HIV	24 (39.34)
No data	4 (6.56)
Clinical Manifestation	
Maculopapular erythema	61 (100)
Genital ulcer	11 (18.03)
Moth-eaten Alopecia	4 (6.56)
Uveitis	3 (4.92)
Condyloma lata	2 (3.28)

All sera (61 specimens) were serologically tested for syphilis. A total of 42 of the 61 showed positive results for the RPR and TPHA tests, signifying that the proportion of positives of the serological tests for secondary syphilis in the present study was 68.85%. The *Treponema pallidum* multiplex nested PCR test was performed for all blood and serum specimens. In blood, the test showed positive results in 14 of 61 specimens (22.95%), which were marked by the presence of a 187 bp band on visualization with agarose gel. On the other hand, only 4 of the 61 serum specimens (6.56%) were positive.

Figure 1 shows visualization of the PCR test results on agarose as seen under ultraviolet light.

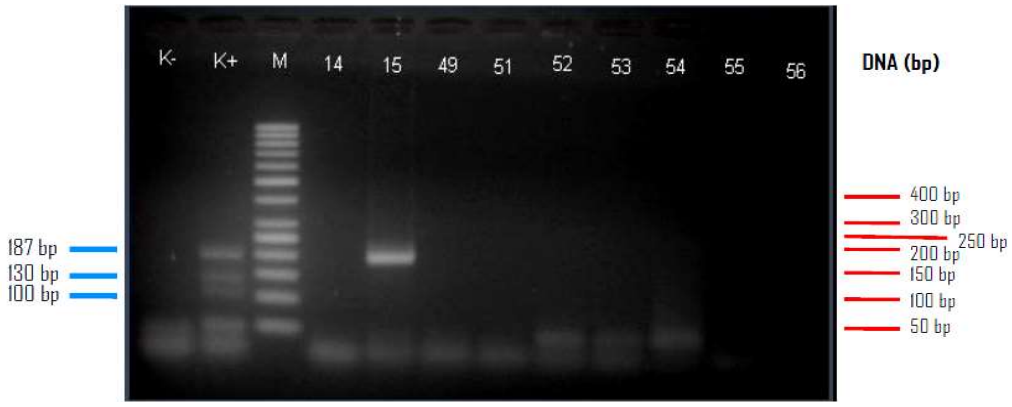

Figure 1. Visualization of positive electrophoresis results band 187 bp specimen no. 15
Treponema pallidum multiplex nested PCR test in blood; K- = negative control, K+ = positive control, M =50 bp marker; Numbers indicate the numbers of specimen teste

Table 2. *Treponema pallidum* multiplex nested PCR test in blood and serological tests of the patients (n=61)

		Serological test		Total
		Positive	Negative	
T.pallidum multiplex nested PCR blood	Positive	13	1	14
	Negative	29	18	47
Total		42	19	61

The results of the *Treponema pallidum* multiplex nested PCR test using blood and serum were compared with the results of the serological tests. The proportion of positives of the *Treponema pallidum* multiplex nested PCR test using blood was 22.95%, compared with the proportion of positives of the serological tests of 68.85%. Nearly all patients with positive blood results on the *Treponema pallidum* multiplex nested PCR test had positive serological test results. There was one patient (5.26%) whose blood test result on multiplex nested PCR was positive, but whose serological test results did not support secondary syphilis (RPR titer >1:8 and TPHA titer ≤1:80). The proportion of positives in the *Treponema pallidum* multiplex nested PCR test for serum was 6.56% compared with the positive serological test results of 68.85%. All patients' sera with positive *Treponema pallidum* multiplex nested PCR test results also had serological test results that supported secondary syphilis.

Treponema pallidum multiplex nested PCR for blood showed a sensitivity of 30.95% and a specificity of 94.7% (Table 2), while for serum the test showed a sensitivity of 9.52% and a specificity of 100% (Table 3).

Under the conditions of the *Treponema pallidum* multiplex nested PCR test in this study, it was found that the test for blood was able to detect *Treponema pallidum* 3.25 times higher positive serological test results, compared with serum, and statistically showed a significant difference.

DISCUSSION

The study subjects were predominantly males, particularly the homosexuals, i.e. male who had sex with males (MSM). According to the results of a survey by the Integrated Surveillance of Behavioral Biology program (*Surveilans Terpadu Biologi Perilaku*, STBP) in 2011 in 23 large Indonesian cities, the highest prevalence of syphilis was in the transvestite group, but the MSM were the most at risk population group for infection with syphilis.[3] If compared with the STBP surveys of 2007 and 2011, the prevalence of syphilis increased 3-fold in the MSM population.[3] The increased prevalence of syphilis in the MSM group had also been reported by several studies in developed countries.[17]

The *Treponema pallidum* multiplex nested PCR test using blood was able to detect *Treponema pallidum* at 22.95%. The proportion of positives of the test using blood was lower than in other studies. The *Treponema pallidum* PCR test using blood has been evaluated in several studies.[8,18] The proportion of positives of the PCR tests vary among a number of

Table 3. *Treponema pallidum* multiplex nested PCR test in serum and serological tests of the patients (n=61)

		Serological test		Total
		Positive	Negative	
T.pallidum multiplex nested PCR blood	Positive	4	0	4
	Negative	38	19	57
Total		42	19	61

studies on patients with secondary syphilis, which comprise the studies conducted by Martin et al.,[18] Tipple et al.,[8] and Grange et al.[10] with a proportion of positives of 44%, 58% and 38%, respectively. In these studies the PCR test with the target gene *PolA* resulted in the detection of *Treponema pallidum* using blood in patients with reinfection of syphilis, primary syphilis, secondary syphilis, and latent syphilis. In meta-analytic studies, the sensitivity of PCR tests using blood in patients with secondary syphilis reached 52.2%.[19,20]

The *Treponema pallidum* multiplex nested PCR test performed on serum could detect *Treponema pallidum* at 6.56%. The proportion of positives of the *Treponema pallidum* multiplex nested PCR test using serum was lower than in other published studies. The study by Gayet–Ageron et al.[15] and Grange et al.[10] found that the sensitivity of serum examination in secondary syphilis was 47% and 15%, respectively. Both studies used the same target gene tpp47.

The difference in the proportion of positives in the *Treponema pallidum* multiplex nested PCR test using blood and serum with the other studies may presumably have been caused by the different target genes used, the PCR method, the sample preparation, the active phase of the disease, and the presence of immunodeficiency. The use of the *Treponema pallidum* target gene and the differing clinical specimens affected the results of the multiplex nested PCR.[13,14,21] The target gene and PCR method used in this study refers to the target gene and PCR method used by Gultom et al.[16] The study by Gayet–Ageron et al.[20] used the 47kDa *Treponema pallidum* target gene with the real-time PCR method. The study by Grange et al.[10] used the tpp47 *Treponema pallidum* target gene with the nested PCR method. In the meta-analytic study by Gayet-Ageron et al.[9] that compared the use of the target genes tpp47 and *polA,* the investigators stated that the most used target gene in molecular studies of *Treponema pallidum* is the tpp47 gene (78%). From the results of the comparison it was apparent that both target genes, the tpp47 as well as the *polA* gene showed the same accuracy. There have been no previous publications on the use of the 23S rRNA target gene to detect *Treponema pallidum* in molecular PCR tests.

Spirochetemia may be detected in the blood particularly in the active phase. In the course of syphilis the highest concentration of bacteria are found in the secondary stage.[22] In the present study, the low number of *Treponema pallidum* detected with multiplex nested PCR, was presumably because the concentration of *Treponema pallidum* in the blood of the patients were below the detection threshold of the PCR test or there was DNA damage in the specimens that were not immediately processed.

The presence of immunodeficiency in the patients will affect the proportion of positives of the *Treponema pallidum* multiplex nested PCR test.[23] In this study the majority of the patients showed reactive HIV results (54.09%). The PCR test successfully detected *Treponema pallidum* in one patient with reactive HIV, the RPR test results showed a high titer (1:16 to 1:64), but the TPHA test yielded a low titer (1:20 to 1:80). This patient was diagnosed as having syphilis with the serological tests, which needs to be confirmed. In patients with reactive HIV, the immune response may be suppressed so that the formation of antitreponemal antibodies is inhibited. As a result, the TPHA test shows a low or negative titer.[23,24] In contrast, the condition of immunodeficiency causes a reduction in the numbers and ability of the macrophages to eliminate pathogenic bacteria, so that the number of *Treponema pallidum* may be higher in the blood circulation, particularly in the active phase of syphilis.[22] High spirochetemia will increase the sensitivity of the PCR. In the present study, the majority of patients who showed positive results for the *Treponema pallidum* multiplex nested PCR test both in blood and serum had a reactive HIV status.

The purpose of the *Treponema pallidum* PCR test is to detect *Treponema pallidum* DNA.

Various specimens may be used for the PCR test, but not all specimens have the same diagnostic value. The differing processes in the blood and serum may presumably cause differences in the numbers of bacteria that are present. In the blood, all blood components are preserved and *Treponema pallidum* exists in greatest numbers. In serum, there is separation of the cellular components from the liquid as a result of the centrifugation process. *Treponema pallidum* may most probably be precipitated together with the cellular components, so that the number of *Treponema pallidum* in serum is not as great as in the blood. This is thought to be the cause of the higher proportion of positives of the *Treponema pallidum* multiplex nested PCR test in the blood as compared with serum.

Based on the published results of a meta-analysis, the highest sensitivity of the *Treponema pallidum* PCR of 78.40% was obtained from the examination of smears of lesions in primary syphilis.[23] In examinations using blood samples the sensitivity of PCR in primary and secondary syphilis was moderate.[15,20] The low proportion of positives in the *Treponema pallidum* PCR test shows that the PCR test should not be used to rule out the diagnosis of syphilis in patients with the clinical picture of secondary syphilis. The PCR test is more useful as a confirmatory test, particularly in serological testing with dubious results.[20]

In specimens with positive serological test results, the sensitivity of the *Treponema pallidum* multiplex nested PCR test using blood was 30.95%, whereas when using serum, the sensitivity of *Treponema pallidum* multiplex nested PCR test was only 9.52%. The results of this study are supported by the study conducted by Grange et al.[10] who found proportions of positives of serum and blood of 15% and 38%, respectively, in the PCR test for *Treponema pallidum* in patients with secondary syphilis.

One of the limitations in this study was the fact that no microscopic examination was performed as gold standard for the diagnosis of syphilis for comparison with the multiplex nested PCR test, due to the difficulties in collecting exudates from lesions of syphilis in all patients.

The study was able to show the best specimens between blood and serum of patients with the clinical picture of secondary syphilis for the *Treponema pallidum* multiplex nested PCR test with the 23S rRNA target gene to assist clinicians in determining the diagnosis and instituting rapid and appropriate management.

Further studies are required using molecular tests with different target genes and PCR methods for blood with positive serological test results, to explain the low proportion of positives in the *Treponema pallidum* multiplex nested PCR test.

CONCLUSIONS

The sensitivity of the *Treponema pallidum* multiplex nested PCR test using blood specimens is 3.25 times higher than using serum, i.e. 30.95% compared with 9.52%. In patients with the clinical picture of secondary syphilis, using blood is better than using serum for the *Treponema pallidum* multiplex nested PCR test.

CONFLICT OF INTEREST

Competing interests: no relevant disclosures.

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CONTRIBUTORS

IE, YR, and AY designed the study. AY and WI supervised fieldwork and gathered data and samples. IE and WI did statistical analyses. IE, YR and AY wrote the first draft of the report, with revisions and input from WI. All authors contributed to revisions and approved the final version.

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