Orginal Article

Accuracy of Serological, Chemiluminescene Immunoassays, and Polymerase Chain Reaction Examination in Identification of *Treponema Pallidum*: Diagnostic Tests in High-Risk of Sexually Transmitted Infections Individuals

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Abstract

Aim: The diagnosis of syphilis can involve serological tests, such as venereal disease research laboratory (VDRL) and chemiluminescence immunoassay (CLIA), or molecular tests, such as polymerase chain reaction (PCR). However, research on the diagnostic accuracy of these three methods in detecting syphilis is still limited. This study aimed to analyze the association between VDRL, CLIA, and PCR serological examinations in patients with syphilis.

Materials and Methods: A cross-sectional diagnostic study was conducted on eligible individuals with high-risk sexually transmitted infection at Wahidin Sudirohusodo Hospital and its network hospitals from January to November 2023. Qualitative VDRL and CLIA were used to determine whether a sample is positive for syphilis. Nested PCR was used to identify *Treponema pallidum* bacteria.

Results: Among the 49 samples, according to the VDRL examination, the highest examination result was positive (87.8%). Based on the CLIA examination, the highest examination result was positive (89.8%). Based on the nested PCR examination, the highest examination result was negative (55.1%). There was no association between the syphilis stage and the results of VDRL ($P = 0.805$), CLIA ($P = 0.678$), and nested PCR ($P = 0.678$). There was no agreement between the VDRL results and nested PCR ($P = 0.678$) and between the CLIA results and nested PCR ($P = 0.646$).

Conclusion: The VDRL and CLIA examination results had different diagnostic accuracy than the nested PCR examination in patients with a diagnosis of syphilis. Although these three examinations have no relationship to the degree of syphilis, this study strengthens the recommendation for the need for nested PCR examination in clinical conditions and other examinations that suggest syphilis.

Keywords: Immunoassay, polymerase chain reaction, serodiagnosis, syphillis, *Treponema Pallidum*

Introduction

Syphilis is an infection caused by *Treponema subspecies* pallidum. The manifestations of the disease are diverse, with different stages occurring over time in untreated infections. The stages of syphilis are divided into primary, secondary, latent, and tertiary stages.[1,2]

Data from the Centers for Disease Control and Prevention (CDC) in 2019 revealed that 129,813 cases of all stages of syphilis were reported, including 38,992 cases of primary and secondary syphilis, which are the most infectious stages

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of syphilis.[3] World Health Organization (WHO) estimates that around 7 million new syphilis cases in 2020.[4] The Indonesian Ministry of Health estimated 76.923 new cases in 2020.[5]

The diagnosis of syphilis should be made in patients with signs or clinical symptoms of syphilis infection. In addition, asymptomatic patients should be screened for syphilis if they are at high risk of the disease or for transmission of the disease to others. Serologic testing is commonly used for the diagnosis of syphilis, and the test was first described by Wasserman in 1906.[2,6]

Patients suspected of having syphilis are usually screened with the venereal disease research laboratory (VDRL) non-treponemal test and rapid plasma reagin (RPR) test, which will be confirmed with a treponemal serology test if positive.[7] Treponemal tests such as the immunofluorescence test (FTA-Abs) or *Treponema pallidum* (*T. pallidum*) hemagglutination agglutination or *T. pallidum* agglutination (TP-PA) are among the tests to confirm *T. pallidum* infection.[8,9] Non-treponemal tests become positive within 3 weeks of *T. pallidum* infection, allowing these tests to be negative early in the infection. On the one hand, VDRL is better than RPR in terms of sensitivity. However, the specificity shows that RPR is superior to VDRL. VDRL has a sensitivity of 44.4-100% specificity of 74.0-100%.[10]

Another treponemal diagnostic test for syphilis infection is the chemiluminescence immunoassay (CLIA) examination to detect serum *T. pallidum*, which is specific to antibodies with high sensitivity and specificity for detecting syphilis. Another study in Türkiye showed that CLIA examination in patients who donated blood was positive in 10 out of 5000 people who donated blood, and none of them were positive for VDRL examination results in these individuals.[11]

The polymerase chain reaction (PCR) molecular diagnostic test can detect syphilis infection early before it is detected by serologic examination.[2,6] PCR provides high-sensitivity results for detecting treponema DNA in ulcer lesion samples from patients with primary syphilis. *T. pallidum* DNA can be detected in blood samples from patients with latent syphilis. This method is particularly useful in situations where DarkField examination is not available and serology tests are non-reactive. The FDA has approved PCR for the diagnosis of syphilis.[9,12] Research regarding the diagnostic accuracy of the three previously mentioned methods is limited. This study aimed to analyze the concordance between the VDRL, CLIA, and PCR serology tests in patients with syphilis.

Materials and Methods

Study Design

This study used a cross-sectional diagnostic test. The diagnostic suitability of VDRL against *T. pallidum*-positive and -negative PCR and between CLIA against *T. pallidum*-positive and -negative PCR were analyzed.

Study Setting and Sampling Methods

In this study, the diagnosis of clinically active primary, secondary, and latent syphilis at an early stage was made according to the CDC criteria. In this study, all patients with syphilis were confirmed to have syphilis infection. The diagnosis of primary syphilis was made if the patient showed one or more painless chancres. Patients are confirmed to have skin and mucosal lesions, both localized and spread throughout the body, with or without regional lymphadenopathy, and can be diagnosed with secondary syphilis. Positive results in both non-treponemal and treponemal serologic tests in patients with these clinical criteria may be the basis for the diagnosis of primary and secondary syphilis. Asymptomatic patients who contracted an initial infection within the last year were diagnosed with early latent syphilis if they met one of the following criteria: 1) Documented seroconversion or a fourfold increase in non-treponemal test titers in the past 12 months; 2) Symptoms consistent with primary or secondary syphilis in the last year; 3) Sexual contact with a partner diagnosed with confirmed or probable primary or secondary syphilis or likely early latent syphilis (independently documented for less than one year); or 4) positive results on both non-treponemal and treponemal tests following likely exposure in the past 12 months. Individuals at high-risk of sexually transmitted infections (STI) were admitted to Wahidin Sudirohusodo Hospital and its network hospitals in Makassar from January to November 2023. The entire reach population willing to participate in the study and meet the inclusion criteria, including individuals at risk of STIs, will be recruited as samples. Patients who do not agree to participate in this study will be excluded.

Blood sampling, 3 mL of blood was drawn from the patient's fossa cubiti vein and stored in EDTA tubes at 2-8 °C for VDRL, CLIA, and PCR testing.

The VDRL examination was performed using a qualitative method according to standard procedures. Carbon antigen is dripped into the patient's serum with 0.9% NaCl on the VDRL card. The card is then placed on a rotator at 100 rpm for 8 min and interpreted as positive if fine to coarse blackish clots are present.

The CLIA inspection procedure using the COBAS E 601 device followed the manufacturer's guidelines. Sample examination can be performed with or without a barcode. After that, the nested PCR examination was carried out by extracting DNA and mixing it with PCR primers for *T. pallidum*. PCR analysis was then performed until the results were obtained as gel photos.

The approximate sample size was calculated using the single proportion formula. The proportion of positive test results was 60%, whereas the proportion of no difference between positive and negative results was 40%. This study's alpha and beta values are 5% and 20%, respectively. The study should include a minimum sample size of 45.

Ethical Consideration

Each respondent who met the inclusion criteria had their identity recorded and received information, as well as a detailed explanation of what would be done during the study. Furthermore, the participants were asked for their willingness to be involved in the study by signing a 10-informed consent letter. The Health Research Ethics Committee of the Hasanuddin University Faculty of Medicine (approval number: 579/UN4.6.4.5.31/PP36/2022, date: 11.10.2022) has evaluated and approved the entire research protocol. No personal data were obtained, and confidentiality was ensured.

Statistical analysis

This study presented the proportion of each category as percentages. The primary analysis involved a chi-square diagnostic test using a 2x2 contingency table, in which a *P* value of 0.05 or less indicated statistical significance. All statistical analyses were performed using SPSS version 17.0 (IBM Software, USA). This rigorous approach ensured the reliability and validity of the findings and provided a robust framework for interpreting the diagnostic accuracy of the various tests examined in this study.

Results

Characteristics of the Research Sample

The total number of subjects included in this study was 49. Table 1 lists the characteristics of the study participants. The majority of the samples were male (93.8%), with an age range of 20-44 years. In addition, based on the syphilis stage, the majority of samples were in the latent stage, followed by the secondary and primary stages. Based on the VDRL examination, the highest examination result was positive for 43 respondents (87.8). Based on the CLIA examination, the highest examination result was positive for as many as 44 respondents (89.8%). Based on the nested PCR examination, the 13 highest examination results were negative for as many as 27 respondents (55.1%).

Concordance Between the Syphilis Stage and VDRL, CLIA, and Nested PCR Results

Table 2 presents the suitability of diagnosis of syphilis stage with VDRL. It is known that patients in the primary stage with a positive category were 2 respondents (100%). In the secondary stage, with as many as 5 respondents (83.3%) and a negative category as many as 1 respondent (16.7%). In the latent stage, with a positive category, as many as 36 respondents (87.8%) and a negative category, as many as 5 respondents (12.2%). There was no concordance between the diagnosis of the syphilis stage and the VDRL results ($P = 0.805$).

Table 3 presents the suitability of syphilis stage diagnosis results with CLIA. It is known that patients in the primary stage with a positive category were 2 respondents (100%). In the secondary stage, with a positive category, as many as 6 respondents (100%). In the latent stage, there is a positive category of as many as 36 respondents (87.8%) and a negative category of as many as 5 respondents (12.2%). There was no concordance between the diagnosis of the syphilis stage and the CLIA results ($P = 0.678$).

Table 4 presents the suitability of the results for the diagnosis of syphilis stage using nested PCR. It is known that patients in

Table 1. Characteristics of the participants

Variable	Frequencies	
	n	%
Gender		
Male	46	93.8
Female	3	6.2
Syphillis stadium		
Primary	2	4.1
Secondary	6	12.2
Latent	41	83.7
VDRL results		
Positive	43	87.8
Negative	6	12.2
CLIA resuts		
Positive	44	89.8
Negative	5	10.2
PCR nested		
Positive	22	44.9
Negative	27	55.1

CLIA: Chemiluminescence immunoassay, PCR: Polymerase chain reaction, VDRL: Venereal disease research laboratory

the primary stage with a positive category were 2 respondents (100%). In the secondary stage, there is a positive category with as many as 3 respondents (50%) and a negative category with as many as 3 respondents (50%). In the latent stage, there is a positive category of as many as 17 respondents (41.5%) and a negative category of 24 respondents (58.5%). There was no concordance between the diagnosis of syphilis stage and the results of nested PCR ($P = 0.678$).

Agreement Between the VDRL and CLIA Results for Nested PCR

Table 5 presents the concordance of VDRL and CLIA results against nested PCR. This study found no concordance between VDRL results and nested PCR ($P = 0.678$) and between CLIA results and nested PCR ($P = 0.646$).

Discussion

In this study, most (93.8%) participants were male, with a male to female ratio of 15.3:1. This shows that men's prevalence is higher than that of women. The research subjects had a homosexual/MSM orientation and sexual relations with men (heterosexual). These results are from research 3 conducted in America in 2016, and the rate of primary and secondary syphilis is higher in men (15.6 cases per 100,000 men) than in women (1.9 cases per 100,000 women). One of the reasons for the high rate of case incidence is the large number of same-sex relationships. In 2020, the WHO estimated that 7.1 million adults aged 15-49 years contracted syphilis worldwide. Several countries that systematically monitor syphilis have shown a significant increase in syphilis cases among men who have sex with men, including congenital syphilis.[13] Previous research conducted in Makassar by Kusumawaty et al.[14] reported similar results, in which the majority of samples were

Table 2. Concordance between syphilis stage diagnosis and VDRL

Diagnosis of syphillis stages	VDRL				Total		*P* value
	Positive		Negative				
	n	%	n	%	n	%	
Primary	2	100	0	0	2	100	0.805
Secondary	5	83.3	1	16.7	6	100	
Latent	36	87.8	5	12.2	41	100	
Total	43	87.8	6	12.2	49	100	

VDRL: Veneral disease research laboratory

Table 3. Concordance between syphilis stage diagnosis and CLIA

Diagnosis of syphillis stages	CLIA				Total		*P* value
	Positive		Negative				
	n	%	n	%	n	%	
Primary	2	100	0	0	2	100	0.678
Secondary	6	100	0	0	6	100	
Latent	36	87.8	5	12.2	41	100	
Total	44	89.8	5	10.2	49	100	

CLIA: Chemiluminescence immunoassay

Table 4. Concordance between syphilis stage diagnosis and nested PCR

Diagnosis of syphillis stages	PCR nested				Total		*P* value
	Positive		Negative				
	n	%	n	%	n	%	
Primary	2	100	0	0	2	100	0.526
Secondary	3	50	3	50	6	100	
Latent	17	41.5	24	58.5	41	100	
Total	22	44.9	27	55.1	49	100	

PCR: Polymerase chain reaction

Table 5. Conformity between VDRL and CLIA results in nested PCR

	PCR nested				Total		*P* value
	Positive		Negative				
	n	%	n	%	n	%	
VDRL							
Positive	20	46.5	23	53.5	43	100	0.678
Negative	2	33.3	4	66.7	6	100	
CLIA							
Positive	19	43.2	25	56.8	44	100	0.646
Negative	3	60.0	2	40.0	5	100	

CLIA: Chemiluminescence immunoassay, PCR: Polymerase chain reaction, VDRL: Venereal disease research laboratory

of MSM sexual orientation and had human immunodeficiency virus (HIV) infection. This indicates a lack of improvement in the prevention of STIs, especially syphilis, among MSM in Makassar.[14]

Most of the participants in this study probably had syphilis infection based on the VDRL method. Serological tests for syphilis are categorized into non-treponemal and treponemal tests, both of which are essential for diagnosis. Non-treponemal tests can monitor treatment progress but have low specificity.[15] The VDRL test, a non-treponemal test for syphilis, utilizes cardiolipin as an antigen and is favored for screening because of its simplicity, sensitivity, and cost-effectiveness. *T. pallidum* infection leads to the rapid production of two antibody types: specific antibodies targeting bacterial polypeptide antigens and non-specific antibodies (reagin antibodies) that react with non-treponemal antigens known as cardiolipins.[16]

The prozone phenomenon and biological false-positive reactions are notable limitations of this test. Serological tests provide indirect evidence of syphilis and may yield reactive results even in the absence of clinical, historical, or epidemiological evidence of the disease.[17]

In individuals treated for primary syphilis, non-treponemal tests become non-reactive in 60% of cases by four months and in almost all patients by 12 months. For patients treated for secondary syphilis, tests generally become non-reactive 12-24 months after treatment. If treatment is administered during the early latent stage, non-treponemal tests might remain reactive at low titers for up to 5 years or longer. Patients with late latent syphilis may have non-reactive non-treponemal test results even without a history of treatment. In some cases, non-treponemal antibodies can persist at low titers for extended periods, sometimes lifelong, a condition known as seroxat reactions, which may be more common in HIV-infected individuals.[16] A prospective study of early syphilis therapy found that 14% of patients had less than a fourfold decrease in serology titers within 12 months post-treatment; patients with HIV infection who had primary or secondary syphilis were more likely to have an insufficient response compared to those without HIV infection.[18]

The performance of the CLIA test in detecting syphilis was similar to that of the VDRL test in this study. CLIA is also a serological examination method for syphilis detection. Unlike VDRL, CLIA is a category of treponema antibody detection. The treponemal test is highly specific, but it can remain positive for life and is not useful for patient follow-up. In contrast to enzyme immunoassay (EIA), CLIA is a more advanced and automated method that uses paramagnetic particles coated with recombinant antigens to capture immunoglobulin M (IgM) and IgG, followed by the addition of a chemiluminescence substrate to generate a relative signal proportional to the amount of bound antigen-antibody complex. This method has recently become available for EIA and CLIA, making it the preferred screening tool for syphilis in large diagnostic laboratories. This test may be suitable for large-scale screening as a replacement treponemal test for TP-PA.[19] CLIA appears to have higher sensitivity than TP-PA in primary syphilis. Compared with ELISA, CLIA is more reliable, sensitive, and accurate for detecting *T. pallidum*-specific antibodies in serum. In the future, this method may be used as an alternative test with higher sensitivity than ELISA.[20]

Automated EIA, CLIA, and multiplex flow immunoassay tests allow for the detection of disease at an early stage but have limitations, with an increased risk of false-positive results in low prevalence populations.[6] This makes CLIA usable as an automated method to detect treponemal antibodies in human serum with high sensitivity and can be used to screen large-scale samples after non-treponemal screening tests.[19]

In contrast, the nested PCR test significantly differed from the previous two tests. The positivity rate of this test was relatively low. In the previous two serological examinations, more positive results were found than negative results.

PCR is an essential technique for molecular diagnosis and is considered a valuable resource for diagnosing early-

stage syphilis, particularly in individuals with conspicuous erythema. According to some researchers, PCR may enhance the detection rate of syphilis in patients with symptoms that are typically hidden by other infectious diseases. such as HIV/AIDS.[21]

Although serological tests have high specificity and sensitivity, they also have certain limitations. including reduced sensitivity in the early and late stages of syphilis, the risk of false-positive reactions due to other acute or chronic infections, and the tendency of non-treponemal tests to produce false-negative results because of the prozone effect. Since 1990, direct detection of treponemal DNA using PCR has become common; however, this method is still not a standard practice. Previous research has also shown that the most reliable samples for detecting treponemal DNA are swabs taken from syphilitic ulcers rather than whole blood samples.[22] Nested PCR is more specific and sensitive than routine PCR or single PCR using probes, which can improve the accuracy of amplification products. The specificity of nested PCR was 95%, whereas its sensitivity was 70%. Recently, Wang et al.[23] demonstrated that nested PCR is more sensitive, particularly in the early stages or infectious stages of syphilis. They also reported that the DNA load of *T. pallidum* was correlated with the RPR titer. These findings suggest that nested PCR may be a useful tool for the early diagnosis and prognosis of syphilis; however, further investigation is needed to determine the applicability of PCR screening, as the sample size in their study was limited.[21] As shown in European research, nested PCR can enhance the diagnosis of syphilis, especially in seronegative patients and those with varying serologies.[6]

The suitability for detecting syphilis between VDRL and CLIA against nested PCR in this study was not statistically significant. Previous research conducted by Vrbová et al.[22] in 2020 investigated the connection between serology and nested PCR in diagnosing syphilis. The study examined 126 samples, all of which tested negative for both treponemal and non-treponemal serological tests. Of these samples, nearly 9% (n = 11) were positive by PCR, indicating that PCR can detect *T. pallidum* in early-stage infections when patients may be seronegative. All samples, except for one whole blood sample, were collected from the genitoanal swabs. In conclusion, swab samples were found to be significantly higher than whole blood samples, and PCR detection in whole blood samples from patients with primary and secondary syphilis exceeded 40%.[22]

Study Limitations

In this study, the positivity rate of nested PCR was relatively low. This differs from the results of previous studies that have been discussed, which found that nested PCR detects false positives and false negatives because of its relatively high specificity and sensitivity. However, these results can be achieved if the nested PCR examination is performed in the early phase, particularly in individuals with conspicuous erythema. The majority of samples in this study were in the latent stage and were infected with HIV/AIDS, which is one of the main limitations of this study. Sampling using a similar research method is recommended for primary syphilis or ulcer lesions to provide more accurate results on comparative sensitivity and specificity comparative analysis between serological examinations, CLIA, and nested PCR for *T. pallidum* infection. Moreover, the differences between VDRL, CLIA, and nested PCR examinations in patients infected with *T. pallidum* may be due to the working principle of each examination and the stage of infection at the time of the examination.

Conclusion

The results of the VDRL and CLIA examinations did not correlate with the results of the nested PCR examination in patients with syphilis. In addition, these three tests were not associated with the degree of syphilis suffered by the patient. Nevertheless, this study strengthens the recommendation that a nested PCR examination is necessary in clinical conditions and other examinations that lead to suspected syphilis.

Ethics

Ethics Committee Approval: The Health Research Ethics Committee of the Hasanuddin University Faculty of Medicine (approval number: 579/UN4.6.4.5.31/PP36/2022, date: 11.10.2022) has evaluated and approved the entire research protocol.

Informed Consent: It was obtained.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: F.S.K., Concept: F.S.K., Design: F.S.K., K.D., F.T., S.T., M., Data Collection or Processing: F.S.K., Analysis or Interpretation: F.S.K., K.D.,

F.T., S.T., M., F.I., M.N.M., M.I., Literature Search: F.S.K., F.I., M.N.M., M.I., Writing: F.S.K.

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