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Microscopic examination using negative staining for rapid diagnosis of syphilis

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ABSTRACT

BACKGROUND

Syphilis is a global health problem, especially in developing countries including Indonesia. *Treponema pallidum*, the etiologic agent of syphilis, cannot be cultured in vitro. Syphilis has several clinical manifestations, making laboratory testing a very important aspect of diagnosis. Microscopic examination may support the diagnosis but is rarely used in Indonesia. The aim of this study was to evaluate negative staining using the light microscope to detect *T. pallidum* in syphilitic lesions.

METHODS

A cross-sectional study was conducted involving 27 subjects who came to several dermato-venereology clinics in Jakarta. Exudates were collected from genital ulcers, condylomata lata, and dry mucocutaneous rash on palms and soles of syphilis patients. Negative staining using one drop of Indian ink was used to examine for treponemas under the light microscope at 10x100 magnification.

RESULTS

Microscopic examination using negative staining showed a few clusters of small and spiral shaped bacteria. Of the 39 specimens from 27 subjects, microscopic examinations were successfully done on 10 specimens. Observations could only be conducted on 5 specimens, 3 (60.0%) of which showed the morphology of spirochetes. This examination is the easiest method for detecting the bacteria. Moreover, the bacteria that were isolated from painless genital ulcers could be observed more clearly than those from erythematous maculopapular lesions.

CONCLUSION

Treponema pallidum was successfully detected by microscopic examination in all moist lesions, but was difficult to detect in dry lesions. Negative staining under the light microscope appears to be simple, affordable, and available in most microbiology laboratories in Indonesia.

Keywords: Microscopic examination, light microscope, negative staining, *Treponema pallidum*

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INTRODUCTION

Syphilis, known in Indonesian as the "lion king", is a disease that is transmitted primarily through sexual contact. The disease shows multiple clinical manifestations, a long course of disease and serious complications. The course of syphilis in patients who do not receive treatment or receive inadequate treatment will develop from a local infection (primary stage), after which the infection enters the circulation and spreads throughout the body, causing a severe inflammatory reaction (secondary stage), leading to complications in the central nervous system and the vital organs, including the heart and eyes (tertiary stage). This disease may be without any symptoms at the latent stage, so that the patient can go undiagnosed. Syphilis can also be transmitted through the placenta from an infected mother to her fetus, who is at risk of developing fetal defects and intrauterine fetal death.[1,2]

Syphilis is a global health problem, especially in developing countries including Indonesia.[3,4] The diagnosis of syphilis is difficult to establish. Limitations in laboratory facilities become an obstacle in detecting *T. pallidum*. Moreover, this bacteria cannot survive outside the human body, has a long generation time (30-33 hours), minimal metabolic ability, and is highly dependent on the host. *T. pallidum* cannot be cultured on bacteriological media outside the human body.[2,5] Serology can only be used for the detection of antibodies with the risk of getting false negative and false positive results.[6,7]

The simplest and most rapid diagnostic test for syphilis is microscopic examination that directly detects *T. pallidum* bacteria from the lesions. This examination requires a special type of microscope, such as the darkfield microscope, fluorescence microscope, or electron microscope, or needs special stains such as the silver stain.[8-10] Currently, the availability of microscopic examination facilities for syphilis in the laboratory is very limited. There has been no recent study about negative staining for syphilis because most patients come with no specific symptoms, therefore it is not easy to collect specimens. Also, due to the limited number of facilities and competent technicians, microscopic examination for the detection of *T. pallidum* in Indonesia is rarely performed. Since most microbiology laboratories in Indonesia use the light microscope for direct specimen examination, negative staining with light microscopy was applied in this study. This method is simple, affordable, and possible to use in most microbiology laboratories in Indonesia. The aim of this study was to evaluate negative staining using the light microscope to detect T. pallidum in syphilitic lesions.

METHODS

Research design

A cross-sectional laboratory experimental study was conducted in 2015 at the Clinical Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia- Cipto Mangunkusumo Hospital, Jakarta. This study was part of a larger study to evaluate *Treponema pallidum* drug resistance which was finished in 2019. We published this study because as far as we know there has been no recent study about this topic using a cheap and simple method that is important to be applied in Indonesia.

Study subjects

The study subjects were collected using consecutive sampling from patients who came to dermato-venereology clinics in Jakarta, including the Dermato-venereology Clinic of Cipto Mangunkusumo Hospital (Central Jakarta), the Sexually Transmitted Disease (STD) clinic in Pasar Rebo District, East Jakarta, the STD clinic in Tambora District, West Jakarta, and the Indonesian Family Planning Association ProCare clinic in Jatinegara District, East Jakarta. Syphilis patients with signs and symptoms of primary or secondary syphilis, and willing to become study subjects by filling out an informed-consent form were included in this

study. Specimens were taken from adult patients, more than 18 years of age. Patients with latent and tertiary stage syphilis, neurosyphilis, or congenital syphilis were excluded from the study. Patients who received antibiotics in the three days before specimen collection were also excluded.

Sample size determination

The sample size was determined by considering the sensitivity of darkfield microscopic examinations to be 80% [11] and using the following formula [12]:

$$n = \frac{(Z\alpha)^2 S(1-S)}{d^2}$$

$$n = \frac{(1.96)^2 0.80(1-0.80)}{0.15^2}$$

where n= sample size
Zα= 1.96
S = sensitivity of dark-field microscopy= 80%
d = desired level of absolute accuracy = 15%
so that the minimal sample size was 27.

Intervention

Sterile normal saline was used to clean the clinical lesion of syphilis. The exudate was collected using a cotton swab by gently pressing and rotating it on the moist lesion. The exudates from dry lesions were collected by gentle abrasion using a scalpel and pressing until the exudate was seen. Serous fluid was put directly on a glass slide, or taken using a swab or a bacteriologic loop. If necessary, a drop of physiologic saline was placed on the slide to prevent drying of the specimen. One drop of Indian ink was mixed with the exudate and spread out on the slide using another glass slide. The glass slide was covered using a cover glass and examined under the microscope at 10x100 magnification using immersion oil.

Measurements

Microscopic examinations were performed qualitatively to observe *T. pallidum*. Due to the difficulty of visualizing its typical motility, the negative staining was focused more on its morphology. The observation was started by rotating the objective lens to the lowest power, and placing the slide on the stage, with the coverslip centered. The objective lens was focused using the coarse focus knob and continued using fine focus. When nothing was seen, the slide was slightly moved while viewing and focusing. A negative result was reported if no spiral shape was found after observing all fields of the slide.

Laboratory analysis

Proper specimen collection and handling was critical for optimizing the sensitivity of negative staining. After obtaining the serous exudate, minimizing contamination with blood or pus would increase the sensitivity of this method. Microscopy visualization at 10x100 magnification and immersion oil showed that *T. pallidum* was a delicate, tightly spiraled, corkscrew-shaped organism, which was occasionally bent in the middle when obstructed by cellular elements or debris in the field. On negative staining, against the black background on the glass slide, the treponemes appeared colorless or pale.

Statistical analysis

The results of microscopic examination using negative staining were recorded and photographed. Descriptive methods were used to analyze the data.

Ethical clearance

This study was approved by the Ethics Committee, Faculty of Medicine, University of Indonesia - Cipto Mangunkusumo Hospital, under no. 158/UN2.F1/ETIK/2015, and all patients gave their written consent before enrollment into the study.

RESULTS

The study subjects were dominated by men with a median age of 26 years and an overall

age range of 18 to 40 years, among whom 88.5% were men who have sex with men (MSM), and most of them (54.10%) were HIV patients.

In this study, many clinical manifestations were found. The most frequent was redness of the skin in the form of maculopapular erythema (100%) that was found in almost all parts of the body, particularly on the palms and soles. Other clinical manifestations found were genital ulcers in the area of the shaft and the dorsum of the penis (18.03%). Most of the genital ulcers found were solitary, but some were multiple with an ulcer base of granulomatous tissue while the ulcer margins were not raised. The ulcers ranged from painless to mildly painful. Complaints of hair loss were found in 6.56% of patients with features of moth-eaten alopecia (alopecia syphilitica) and thinning eyebrows. Among the patients 4.92% complained of mild visual disturbances in the form of blurred vision. Condylomata lata were found in 3.28% of female subjects.

Microscopic examination was conducted in this study using negative staining and observed under the light microscope. Microscopy visualization at 10x100 magnification using immersion oil showed a few clusters of small and spiral-shaped bacteria (Figure 1). Of the 39 specimens**,** microscopic examinations were done on 10 specimens, but reliable observations could only be made on 5 specimens, with 3 (60.0%) of them showing spirochete morphology.

DISCUSSION

Syphilis is a disease that is transmitted mainly through sexual contact with infected mucosal lesions. Other body fluids are also infectious when patients have bacteremia. Syphilis can cause early complications such as irreversible loss of vision, so rapid diagnosis of this infection is important for primary care clinicians.[13]

Primary syphilis classically presents as a single, painless, indurated genital ulcer (chancre), but this presentation is only 31% sensitive; lesions can be painful, multiple, and extra-genital. Since the symptoms appear 10-90 days (mean = 21 days) after exposure, [14] the patients often do not consult a doctor. Mostly patients come to a clinic at the secondary stage of syphilis. The clinical manifestations of secondary stage of syphilis are very diverse and confusing. Therefore, syphilis has been known as "the great imitator" as it may cause symptoms similar to many other diseases.[15-17] Bacterial dissemination in secondary syphilis classically presents as a diffuse, symmetric, copper-colored, maculopapular, possibly pruritic rash of any morphology except vesicular. Mucous

Figure 1. *Treponema pallidum* microscopic picture using negative staining of exudate specimens

lesions, patchy alopecia, fever, headaches, and generalized painless adenopathy may also occur.[18,19] Similar to previous reports, many clinical manifestation were found in this study, including maculopapular erythema in nearly all parts of the body, particularly on the palms and soles, painless to mildly painful genital ulcers, alopecia, thinning eyebrows, blurred vision and condylomata lata. Therefore, laboratory examination is needed to support the diagnosis of syphilis.

There has been very limited use of laboratory examinations supporting a definite diagnosis of syphilis. The incapability of culturing *Treponema pallidum* outside the human body becomes an obstacle in detecting this spirochete.[5,20] The routine method is serology, but this gives many false negative and false positive results.[6,7] Moreover, the diagnosis of syphilis is more difficult in HIV-positive patients, because HIV can alter the clinical picture of syphilis and can cause more serious complications. Unusual serological responses such as high titers as well as false negative reactions have been reported for VDRL tests in HIV-reactive patients.[6,21] Therefore specific tests will be needed particularly in all HIV-reactive patients.

Microscopic examination may support the diagnosis of syphilis by directly detecting *T. pallidum* bacteria in the lesions. This method is simple and rapid but rarely used in Indonesia. It requires a special type of microscope such as the darkfield microscope, fluorescence microscope, or electron microscope, or needs special stains such as the silver stain. Darkfield microscopy requires a special microscope and a trained microscopist in close proximity to where patients are examined. The fluorescence microscope or the electron microscope are also of limited availability in most Indonesian laboratories. Special stains are expensive and need more skill in interpreting the results.

This study investigated the use of a simple microscopic examination to support the diagnosis of syphilis using the light microscope. Negative staining with the light microscopy is simple, affordable, and available in most laboratories in Indonesia. In our study, negative staining successfully detected *Treponema pallidum* in all moist lesions, but had difficulty in detecting the bacterium in dry lesions such as mucocutaneus rash on the palms and soles (Figure 2). This method is simple and reliable for detecting *T. pallidum* directly in exudates and fluids from lesions, and needs only one drop of Indian ink

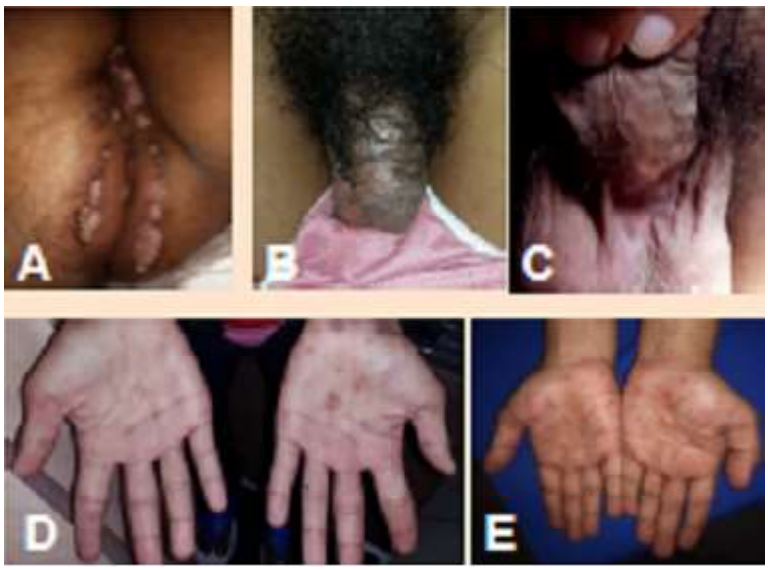

Figure 2. Clinical picture of secondary syphilis.
A,B,C Genital Ulcers; D,E maculopapular rash

and the light microscope. It can overcome the limitations of darkfield microscopy as the gold standard for syphilis microscopic examination which is not available in most laboratories in Indonesia.

There are no recent studies about negative staining for syphilis using the light microscope. Compared to darkfield microscopy which is the gold standard for microscopic examination of syphilis, negative staining is simpler and presumably more reliable for detecting *T. pallidum* directly in exudates and fluids from lesions, when darkfield microscopy and expertise is not available. While the wet mount method using the darkfield microscope can examine the morphology and motility of the spirochete,[8,11] microscopic examinations using negative staining has difficulty in visualizing its typical motility and is more focused on its morphology. However, negative staining is simpler, cheaper, and more applicable, because the examination uses the light microscope which is available in most laboratories in Indonesia.

The present study was able to show the possibility of using negative staining to assist clinicians in the rapid diagnosis of syphilis. One of the limitations of this study is the fact that a limited number of microscopic slides were examined for the diagnosis of syphilis due to the difficulties in collecting exudates from the lesions of syphilis in all patients. Further studies are required to examine more specimens and explain the benefit of negative staining for routine laboratory examination to support the diagnosis of syphilis.

CONCLUSION

Microscopic examination using negative staining can be used to support the early diagnosis of syphilis in moist lesions, but the results should be carefully interpreted in the case of dry lesions. Negative staining using the light microscopy appears to be a simple, affordable, and highly available method in most microbiology laboratories in Indonesia.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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CONTRIBUTORS

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

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