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Original Article

Detection of A2058G and A2059G on the 23S rRNA Gene by Multiplex Nested PCR to Identify *Treponema pallidum* Resistance to Azithromycin in Indonesia

Yeva Rosana[1]*, Andi Yasmon[1], Wresti Indriatmi[2], Ida Effendi[3,4], Raden Lia Kusumawati[5], Rasmia Rowawi[6], Sunarjati Sudigdoadi[7], Gita Widya Pradini[7], Anak Agung Gde Putra Wiraguna[8], Ni Made Dwi Puspawati[8], Maryam Kusumawaty[9], and Muhammad Nasrum Massi[10]

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ABSTRACT: Azithromycin is an antibiotic used to treat syphilis, especially in the context of penicillin allergy. Although resistance to azithromycin has been widely reported to be associated with one- and/or two-point mutations on the 23S rRNA gene, it has yet to be described in Indonesia. Specimens were collected from 220 patients diagnosed with secondary syphilis. A multiplex nested polymerase chain reaction (PCR) testing system using the 23S rRNA target gene of *Treponema pallidum* was designed using three primer pairs. The first step involved the use of PCR primer pairs to detect *T. pallidum*. In the second step, two PCR primer pairs were constructed to identify azithromycin-resistant *T. pallidum* based on A2058G and A2059G point mutations. *T. pallidum* detected in samples from Jakarta or Bandung were not resistant to azithromycin. However, azithromycin-resistant *T. pallidum* were found in samples from Makassar, Medan, and Bali. Specimens from heterosexual males and patients with HIV accounted for the majority of azithromycin resistance noted in the study. This study demonstrated that the azithromycin-resistant *T. pallidum* detected in Indonesia appear to be a novel variant of resistance, containing both the A2058G and A2059G mutations found in Medan and Makassar.

INTRODUCTION

Syphilis is a complex, sexually transmitted disease with diverse clinical manifestations. According to World Health Organization data in 2005 and 2008, there was an increase in the prevalence of syphilis in Southeast Asian countries, from 1.37% to 6.1% in women and 1.25% to 6.3% in men (1). The prevalence of syphilis among direct and indirect female sex workers (FSWs) in Indonesia was reported as 7.5% and 3.1%, respectively. Moreover, an increasing prevalence was found among brothel-based FSWs from 7.8% to 14.5% in 2005–2007 (2). A problem in diagnosing syphilis is the incapability of *Treponema pallidum* to be cultured outside the human body; this impairs the ability to detect and identify *T. pallidum* and makes it difficult to determine the antibiotic sensitivity pattern (1,3–5).

The most commonly used diagnostic test for syphilis is a nontreponemal serological examination followed by a treponemal confirmation test (5–7). To minimize false positive and negative results in serological examinations, such as rapid plasma reagin (RPR) and *T. pallidum* hemagglutination (TPHA) vs. clinical symptoms, molecular methods such as polymerase chain reaction (PCR) having suitable sensitivity

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and specificity to detect *T. pallidum* in primary and secondary syphilis as well as the potential to simultaneously identify antibiotic-resistance *T. pallidum* have been used (7–9).

Syphilis treatment has evolved since the discovery of penicillin. Penicillin-based regimens remain the standard treatment for syphilis at all stages (5,10). Recommended penicillin regimens include deep intramuscular preparations, which patients may refuse and could yield a Jarisch-Herxheimer reaction. The risk of needlestick injuries also exists for medical personnel. Moreover, allergic reactions could cause anaphylactic shock, which may lead to death. Finally, penicillin desensitization may not be available and could cause delays in treatment.

In Indonesia, macrolide antibiotics are used as an alternative to penicillin for syphilis treatment, according to the sexually transmitted infections (STIs) treatment manual by the Ministry of Health of 2012 (11). Oral administration of azithromycin is an alternative treatment that has been shown to have similar efficacy to penicillin (12,13). The benefits and ease of oral administration, suitable tissue penetration, minimal side effects, and long half-life (68 hours) make azithromycin one of the most widely used macrolide antibiotics in the treatment of syphilis. Azithromycin treatment failure is reported predominantly in patients with HIV, first described in San Francisco in 2002. Since then, azithromycin resistance has been reported in multiple settings, including the Americas, Europe, and Asia (14–16). To our knowledge, there are no reports of azithromycin resistance in Indonesia. To determine the prevalence of *T. pallidum* resistance to azithromycin in Indonesia, we used a multiplex nested PCR molecular method to detect A2058G and A2059G mutations of *T. pallidum* on the 23S rRNA gene, which have been widely reported to be responsible for the resistance of *T. pallidum* to azithromycin worldwide.

MATERIALS AND METHODS

Study population: This study was carried out over two years. Blood samples were collected from 220 patients over the study period. In the first year, samples were taken from 62 patients from 4 STIs Clinic in Jakarta (Ciptomangunkusumo Hospital, Jatinegara Clinic, community health centers in Pasar Rebo and Tambora). Optimization of all PCR parameters to obtain optimal reaction conditions was carried out in the first year of study and has been tested using all clinical samples. In the second year, this PCR system method was tested on 158 clinical samples, including 33 clinical samples from Medan and 32 clinical samples from Bandung representing Western Indonesia, 33 clinical samples from Bali representing the middle part of Indonesia, and 60 clinical samples from Makassar representing Eastern Indonesia. This study was approved by the Ethical Committee of the Hospital and Faculty of Medicine University of Indonesia (no: 158/UN2.F1/ETIK/2015), and all patients gave their written consent before enrollment in the study.

Clinical specimens: A pair of venous blood samples were collected as clinical specimens without and with EDTA anticoagulant. After arriving at the laboratory, blood samples from the tube with EDTA anticoagulant were transferred to a 1.5-mL microtube PCR and stored at −80 °C. Blood samples from tubes without anticoagulants were centrifuged at 2,500 rpm for 10 min to obtain serum. The serum was transferred to a 1.5-mL microtube PCR and stored at −80 °C.

Serology test: The RPR test was used as a nontreponemal test, and the TPHA test was used as a treponemal test. The qualitative and quantitative RPR test used a commercial kit (RPR AIM Cat. No E RPR 2; Germaine Laboratories, San Antonio, TX, USA) according to the kit protocol. The positive results of the RPR test showed smooth black to coarse blobs. If the qualitative RPR shows a positive result, the quantitative RPR test can proceed. All samples tested by RPR were confirmed by TPHA tests using a commercial kit (TPHA AIM Cat. No. TPHA E 100; Germaine Laboratories) in accordance with existing protocols. TPHA qualitative and quantitative tests were carried out simultaneously. The positive results of the TPHA test showed agglutination between the patient's serum containing antibodies and the antigenic component of *T. pallidum* (Nichol's strain).

DNA extraction and multiplex nested PCR: DNA extraction was performed using a High Pure PCR DNA Template Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions with a final elute of 60 µL. DNA extraction results were stored at −35°C until used. Multiplex nested PCR test was performed in accordance with our previous report (17,18). The first step of PCR used a pair of primers to detect *T. pallidum*. In the second step, the PCR used two pairs of primers to identify azithromycin-resistant *T. pallidum* based on mutations of A2058G and A2059G. Oligonucleotide primer sequences used in PCR-1 and PCR-2 can be seen in Table 1. The total volume of PCR-1 was 20 µL, while that of PCR-2 was 20 µL. When amplification was complete, 10 µL of the amplification products were separated using 3% agarose gel electrophoresis with a 50-bp molecular mass standard. Electrophoresis was performed at 100 volts for 30–35 minutes, then stained with red staining gel and visualized using UV light with GelDoc BioRad®. The positive control used synthetic DNA with a length of 194 bp, while the negative control was DNase free water.

RESULTS

Specimens were collected from 220 patients diagnosed with secondary syphilis in Indonesia based on clinical symptoms. Redness and maculopapular erythema were found in almost all parts of the body, particularly on the palms and soles. Some patients also had genital ulcers in the area of the shaft and dorsum penis, which were isolated and painless to mildly painful. Hair loss instances with moth-eaten alopecia (alopecia syphilitica) and thinning eyebrows were observed. Some patients also complained of mild visual impairment, blurred vision, and condyloma lata.

In the first year of this study, multiplex PCR and multiplex nested PCR tests were performed on specimens collected in Jakarta, with tests completed on all the remaining blood specimens in the second year

Table 1. Oligonucleotide primer sequences used in nested multiplex PCR

Primer	Sequence	Product
PCR-1		
Primer PCR 1F	5'- CGA ATG GTG TAA CGA CTC TG -3'	20 mer
Primer PCR 1R	5'- TTC ACT GTT GAC TCC GCC TA -3'	20 mer
PCR-2		
Primer 2058F	5'- CGG TTA CCC ATA GTT AGA CAG G -3'	22 mer
Primer 2058R	5'- CTG TTG ACT CCG CCT AAC CT -3'	20 mer
Primer 2059F	5'- ACT CTG GAC ACT GTC TCG ACG -3'	21 mer
Primer 2059R	5'- GAT GAA GGT TCA CGG GGT ATC -3'	21 mer

of this study. Multiplex nested PCR test for *T. pallidum* using blood specimens showed positive results with a 187-bp band on electrophoretic agarose gels. Of the 62 specimens from Jakarta, 22.6% carried *T. pallidum*. Azithromycin-resistant *T. pallidum* were not detected in any of the patients with HIV (53% of the total samples).

In the second year of this study, 158 specimens were collected from patients diagnosed with secondary syphilis in Indonesia. Specimens from western Indonesia were collected from Medan and Bandung. In the 33 specimens from Medan, azithromycin-resistant *T. pallidum* were detected, with 130-bp and 100-bp bands on electrophoretic agarose gels (Fig. 1). There was no history of azithromycin use and no recorded HIV status among these patients.

Of the 32 specimens collected from Bandung, the presence of *T. pallidum* was detected in one specimen by multiplex nested PCR; the strain was not identified as being resistant to azithromycin. Of the total number of patients included in the study in this region, 65.6% of patients had HIV.

Of the 33 specimens from Bali, 4 specimens indicated the presence of *T. pallidum* by multiplex nested PCR, and 1 indicated the presence of azithromycin-resistant *T. pallidum*. Visualization on electrophoretic agarose gels showed that, among 39.4% of patients with HIV, resistance to azithromycin was represented by a 130-bp band, corresponding to the A2058G mutation.

In Makassar, specimens were collected from 60 patients. In 2 specimens, the presence of *T. pallidum* was detected by multiplex nested PCR, and resistance to azithromycin was demonstrated for both specimens. Visualization on electrophoretic agarose gels showed 130-bp and 100-bp bands, representing A2058G and A2059G mutations, respectively; 40% of the specimens were obtained from patients with HIV.

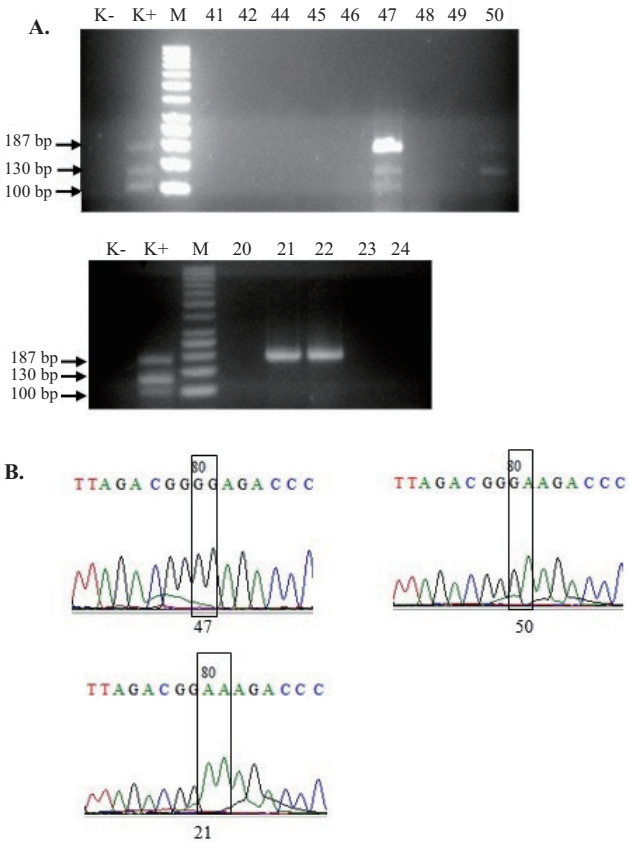

Fig. 1. (Color online) Mutation analysis using multiplex nested PCR. **A.** Results of agarose gel electrophoresis. The band at 187 bp indicates *Treponema pallidum* 23S rRNA gene (bacterial detection), the band at 130 bp indicates A2058G mutation, and the band at 100 bp indicates A2059G mutation. Sample 47 indicates mutation at both nucleotide positions 2058 and 2059, sample 50 indicates mutation at single nucleotide position 2058, whereas samples 21 and 22 indicate no mutation at both nucleotide positions 2058 and 2059. Samples 20, 23, 24, 41–46, 48, and 49 show negative PCR results. M represents 50bp DNA Ladder (from bottom to top: 100, 150, 200, 250–1000 bp). bp means base pair. K+ and K- represent positive and negative controls, respectively. **B.** Results of DNA sequencing for samples 47, 50, and 21.

DISCUSSION

Of the 220 patients from Indonesia diagnosed with secondary syphilis, the majority of male patients were MSM. This study is similar to another study conducted on patients from Makassar, Indonesia, which also reported a higher number of syphilis cases among MSM (19). A previous study in six Indonesian cities reported data indicating that MSM are at an elevated risk for HIV infection in many low and middle-income countries (20,21). There is a significant risk for an increasing number of transmissions between syphilis and HIV among MSM groups (19,22,23). Therefore, testing for HIV should be prioritized in patients diagnosed with syphilis in Indonesia, particularly among MSM groups. This is a significant issue in Indonesia given that HIV comprises a large, undiagnosed proportion nationally, very low rates of people with HIV are on antiretroviral

Table 2. Serology test for *Treponema pallidum*

Region in Indonesia	Sexual status (%)			RPR[1] (%)			TPHA[2] (%)			PCR (+) *n*
	Hetero	Bisex	H/L[3]	(-)	≤ 1:4	≥ 1:8	(-)	≤ 1:80	≥ 1:160	
Western										
Jakarta (62 patients)[4]										
Male (59)	2	10	88	5	10	85	16	20	64	14
Female (3)	100	0	0	0	0	100	0	0	100	0
Medan (33 patients)[5]										
Male (21)	24	14	62	33	0	67	33	0	67	0
Female (12)	100	0	0	50	0	50	50	0	50	1
Bandung (32 patients)[6]										
Male (29)	7	10	83	21	48	31	41	38	21	1
Female (3)	100	0	0	0	67	33	33	33	33	0
Middle part										
Bali (33 patients)[7]										
Male (28)	43	0	57	7	4	89	7	4	89	3
Female (5)	80	0	20	0	0	100	20	0	80	1
Eastern										
Makassar (60 patients)[8]										
Male (25)	48	0	52	24	24	52	24	40	36	2
Female (35)	100	0	0	66	28	6	66	31	3	0

[1]: RPR, the rapid plasma reagin test.
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[3]: H/L, Homosexual/Lesbian.
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therapy (ART), and data on those virally suppressed through ART are limited.

Only a few laboratory tests can support definite syphilis diagnoses. Serological examination is a commonly used diagnostic test. Serological tests can detect antibodies at all stages of syphilis and are therefore used for a variety of purposes, such as screening in low-risk populations (e.g., pregnant women, blood donors), screening in high-risk populations (e.g., HIV-AIDS and MSM population), and diagnostic testing in patients with syphilis symptoms and monitoring therapeutic responses. Nontreponemal and treponemal tests for secondary syphilis using titers in RPR and TPHA tests were equal or more than 1:8 and 1:160, respectively (Table 2). Based on serology tests, the highest positive rates were found in Bali, followed by Medan, Jakarta, Makassar, and Bandung.

In this study, the bacterial loads of *T. pallidum* detected by multiplex nested PCR were low compared to those observed in the serological examination, possibly because *T. pallidum* counts in the patient's blood were below the threshold of PCR detection. Based on the results of published meta-analysis test, the best PCR sensitivity value (78.4%) was obtained from the examination of lesions in primary stage syphilis (24). The best type of specimen is taken from the infection site, where the highest number of bacteria can be sampled. In secondary stage syphilis, *T. pallidum* bacteria spreads from the primary infection site through the blood to manifest on the skin. Due to the difficulty of obtaining samples from skin lesions of secondary syphilis patients, on account of the collection techniques being invasive and causing discomfort to patients, blood samples are the primary choice for PCR examinations.

The PCR sensitivity for secondary stage syphilis using blood samples shows moderate values (24,25). The low positive value of the *T. pallidum* multiplex nested PCR compared to serology tests suggests that this method should not be used to rule out syphilis diagnoses in patients presenting a clinical picture of secondary syphilis. This method is more useful as a confirmatory test, especially if paired with serological tests that show uncertain results, as well as for characterizing resistance.

Antibiotic resistance is accelerated by the misuse and overuse of antibiotics, as well as poor infection prevention and control. Since syphilis can cause symptoms similar to many other diseases, an undiagnosed patient can unconsciously transmit the disease to their partners as well as to society (26,27). Several patients possibly carrying syphilis (early–primary/secondary syphilis in particular) might not

have access to STI services and are likely to self-treat by attending local pharmacies. Since often pharmacists recommend oral antibiotic treatment such as azithromycin (not IM penicillin), this could be a potential factor causing azithromycin resistance. In this study, although the majority of syphilis cases was found in MSM, azithromycin resistance was found among heterosexual males with HIV. Therefore, surveillance, diagnosis, treatment, and contact tracing are necessary for high-risk populations, such as MSM and their partners.

In this study, azithromycin-resistant *T. pallidum* were detected and identified by multiplex nested PCR, using 23S rRNA with 3 pairs of primers. The A2058G and A2059G mutations appear to be responsible for causing resistance in all cases in Makassar and Medan. In Bali, one in four specimens positive for *T. pallidum* also showed azithromycin resistance and presented with the 130 bp band on gel electrophoresis, representative of the A2058G mutation. Azithromycin-resistant *T. pallidum* were not found in Jakarta and Bandung.

The discovery of *T. pallidum* resistance to azithromycin in Makassar, representative of Eastern Indonesia, and Medan, located in the westernmost part of Indonesia, with the two-point mutation A2058G and A2059G is very interesting. To my knowledge, this form (both alleles in the same sample) has not been found in any previous study. To date, resistant genomes always contain either A2058G or A2059G (28). This is a novel variant containing both the A2058G and A2059G mutations found only in Indonesian samples. On the other hand, azithromycin resistance genomes in Bali as the representative of central Indonesia were the same as those described in previous studies (i.e., one single mutation in A2058G). Detection of resistant genomes should be considered to evaluate the government policy on using azithromycin as an alternative treatment for syphilis cases in Indonesia.

In conclusion, multiplex nested PCR can increase laboratory capabilities to support the clinical diagnosis of syphilis as "the great imitator" as it may cause symptoms similar to many other diseases. Azithromycin-resistant *T. pallidum* have been found in Indonesia, as also reported in America, Europe, and Asia. The azithromycin-resistant *T. pallidum* in Indonesia appear to be a novel variant of resistance, containing both the A2058G and A2059G mutations, as found in samples from Medan and Makassar. Therefore, special consideration should be given to using azithromycin to treat syphilis in Indonesia and worldwide.

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Conflict of interest None to declare.

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Original Article

Detection of A2058G and A2059G on the 23S rRNA Gene by Multiplex Nested PCR to Identify *Treponema pallidum* Resistance to Azithromycin in Indonesia

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ABSTRACT: Azithromycin is an antibiotic used to treat syphilis, especially in the context of penicillin allergy. Although resistance to azithromycin has been widely reported to be associated with one- and/or two-point mutations on the 23S rRNA gene, it has yet to be described in Indonesia. Specimens were collected from 220 patients diagnosed with secondary syphilis. A multiplex nested polymerase chain reaction (PCR) testing system using the 23S rRNA target gene of *Treponema pallidum* was designed using three primer pairs. The first step involved the use of PCR primer pairs to detect *T. pallidum*. In the second step, two PCR primer pairs were constructed to identify azithromycin-resistant *T. pallidum* based on A2058G and A2059G point mutations. *T. pallidum* detected in samples from Jakarta or Bandung were not resistant to azithromycin. However, azithromycin-resistant *T. pallidum* were found in samples from Makassar, Medan, and Bali. Specimens from heterosexual males and patients with HIV accounted for the majority of azithromycin resistance noted in the study. This study demonstrated that the azithromycin-resistant *T. pallidum* detected in Indonesia appear to be a novel variant of resistance, containing both the A2058G and A2059G mutations found in Medan and Makassar.

INTRODUCTION

Syphilis is a complex, sexually transmitted disease with diverse clinical manifestations. According to World Health Organization data in 2005 and 2008, there was an increase in the prevalence of syphilis in Southeast Asian countries, from 1.37% to 6.1% in women and 1.25% to 6.3% in men (1). The prevalence of syphilis among direct and indirect female sex workers (FSWs) in Indonesia was reported as 7.5% and 3.1%, respectively. Moreover, an increasing prevalence was found among brothel-based FSWs from 7.8% to 14.5% in 2005–2007 (2). A problem in diagnosing syphilis is the incapability of *Treponema pallidum* to be cultured outside the human body; this impairs the ability to detect and identify *T. pallidum* and makes it difficult to determine the antibiotic sensitivity pattern (1,3–5).

The most commonly used diagnostic test for syphilis is a nontreponemal serological examination followed by a treponemal confirmation test (5–7). To minimize false positive and negative results in serological examinations, such as rapid plasma reagin (RPR) and *T. pallidum* hemagglutination (TPHA) vs. clinical symptoms, molecular methods such as polymerase chain reaction (PCR) having suitable sensitivity

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and specificity to detect *T. pallidum* in primary and secondary syphilis as well as the potential to simultaneously identify antibiotic-resistance *T. pallidum* have been used (7–9).

Syphilis treatment has evolved since the discovery of penicillin. Penicillin-based regimens remain the standard treatment for syphilis at all stages (5,10). Recommended penicillin regimens include deep intramuscular preparations, which patients may refuse and could yield a Jarisch-Herxheimer reaction. The risk of needlestick injuries also exists for medical personnel. Moreover, allergic reactions could cause anaphylactic shock, which may lead to death. Finally, penicillin desensitization may not be available and could cause delays in treatment.

In Indonesia, macrolide antibiotics are used as an alternative to penicillin for syphilis treatment, according to the sexually transmitted infections (STIs) treatment manual by the Ministry of Health of 2012 (11). Oral administration of azithromycin is an alternative treatment that has been shown to have similar efficacy to penicillin (12,13). The benefits and ease of oral administration, suitable tissue penetration, minimal side effects, and long half-life (68 hours) make azithromycin one of the most widely used macrolide antibiotics in the treatment of syphilis. Azithromycin treatment failure is reported predominantly in patients with HIV, first described in San Francisco in 2002. Since then, azithromycin resistance has been reported in multiple settings, including the Americas, Europe, and Asia (14–16). To our knowledge, there are no reports of azithromycin resistance in Indonesia. To determine the prevalence of *T. pallidum* resistance to azithromycin in Indonesia, we used a multiplex nested PCR molecular method to detect A2058G and A2059G mutations of *T. pallidum* on the 23S rRNA gene, which have been widely reported to be responsible for the resistance of *T. pallidum* to azithromycin worldwide.

MATERIALS AND METHODS

Study population: This study was carried out over two years. Blood samples were collected from 220 patients over the study period. In the first year, samples were taken from 62 patients from 4 STIs Clinic in Jakarta (Ciptomangunkusumo Hospital, Jatinegara Clinic, community health centers in Pasar Rebo and Tambora). Optimization of all PCR parameters to obtain optimal reaction conditions was carried out in the first year of study and has been tested using all clinical samples. In the second year, this PCR system method was tested on 158 clinical samples, including 33 clinical samples from Medan and 32 clinical samples from Bandung representing Western Indonesia, 33 clinical samples from Bali representing the middle part of Indonesia, and 60 clinical samples from Makassar representing Eastern Indonesia. This study was approved by the Ethical Committee of the Hospital and Faculty of Medicine University of Indonesia (no: 158/UN2.F1/ETIK/2015), and all patients gave their written consent before enrollment in the study.

Clinical specimens: A pair of venous blood samples were collected as clinical specimens without and with EDTA anticoagulant. After arriving at the laboratory, blood samples from the tube with EDTA anticoagulant were transferred to a 1.5-mL microtube PCR and stored at −80 °C. Blood samples from tubes without anticoagulants were centrifuged at 3,500 rpm for 10 min to obtain serum. The serum was transferred to a 1.5-mL microtube PCR and stored at −80 °C.

Serology test: The RPR test was used as a nontreponemal test, and the TPHA test was used as a treponemal test. The qualitative and quantitative RPR test used a commercial kit (RPR AIM Cat. No E RPR 2; Germaine Laboratories, San Antonio, TX, USA) according to the kit protocol. The positive results of the RPR test showed smooth black to coarse blobs. If the qualitative RPR shows a positive result, the quantitative RPR test can proceed. All samples tested by RPR were confirmed by TPHA tests using a commercial kit (TPHA AIM Cat. No. TPHA E 100; Germaine Laboratories) in accordance with existing protocols. TPHA qualitative and quantitative tests were carried out simultaneously. The positive results of the TPHA test showed agglutination between the patient's serum containing antibodies and the antigenic component of *T. pallidum* (Nichol's strain).

DNA extraction and multiplex nested PCR: DNA extraction was performed using a High Pure PCR DNA Template Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions with a final elute of 60 µL. DNA extraction results were stored at −35°C until used. Multiplex nested PCR test was performed in accordance with our previous report (17,18). The first step of PCR used a pair of primers to detect *T. pallidum*. In the second step, the PCR used two pairs of primers to identify azithromycin-resistant *T. pallidum* based on mutations of A2058G and A2059G. Oligonucleotide primer sequences used in PCR-1 and PCR-2 can be seen in Table 1. The total volume of PCR-1 was 20 µL, while that of PCR-2 was 20 µL. When amplification was complete, 10 µL of the amplification products were separated using 3% agarose gel electrophoresis with a 50-bp molecular mass standard. Electrophoresis was performed at 100 volts for 30–35 minutes, then stained with red staining gel and visualized using UV light with GelDoc BioRad®. The positive control used synthetic DNA with a length of 194 bp, while the negative control was DNase free water.

RESULTS

Specimens were collected from 220 patients diagnosed with secondary syphilis in Indonesia based on clinical symptoms. Redness and maculopapular erythema were found in almost all parts of the body, particularly on the palms and soles. Some patients also had genital ulcers in the area of the shaft and dorsum penis, which were isolated and painless to mildly painful. Hair loss instances with moth-eaten alopecia (alopecia syphilitica) and thinning eyebrows were observed. Some patients also complained of mild visual impairment, blurred vision, and condyloma lata.

In the first year of this study, multiplex PCR and multiplex nested PCR tests were performed on specimens collected in Jakarta, with tests completed on all the remaining blood specimens in the second year

Table 1. Oligonucleotide primer sequences used in nested multiplex PCR

Primer	Sequence	Product
PCR-1		
Primer PCR 1F	5'- CGA ATG GTG TAA CGA CTC TG -3'	20 mer
Primer PCR 1R	5'- TTC ACT GTT GAC TCC GCC TA -3'	20 mer
PCR-2		
Primer 2058F	5'- CGG TTA CCC ATA GTT AGA CAG G -3'	22 mer
Primer 2058R	5'- CTG TTG ACT CCG CCT AAC CT -3'	20 mer
Primer 2059F	5'- ACT CTG GAC ACT GTC TCG ACG -3'	21 mer
Primer 2059R	5'- GAT GAA GGT TCA CGG GGT ATC -3'	21 mer

of this study. Multiplex nested PCR test for *T. pallidum* using blood specimens showed positive results with a 187-bp band on electrophoretic agarose gels. Of the 62 specimens from Jakarta, 22.6% carried *T. pallidum*. Azithromycin-resistant *T. pallidum* were not detected in any of the patients with HIV (53% of the total samples).

In the second year of this study, 158 specimens were collected from patients diagnosed with secondary syphilis in Indonesia. Specimens from western Indonesia were collected from Medan and Bandung. In the 33 specimens from Medan, azithromycin-resistant *T. pallidum* were detected, with 130-bp and 100-bp bands on electrophoretic agarose gels (Fig. 1). There was no history of azithromycin use and no recorded HIV status among these patients.

Of the 32 specimens collected from Bandung, the presence of *T. pallidum* was detected in one specimen by multiplex nested PCR; the strain was not identified as being resistant to azithromycin. Of the total number of patients included in the study in this region, 65.6% of patients had HIV.

Of the 33 specimens from Bali, 4 specimens indicated the presence of *T. pallidum* by multiplex nested PCR, and 1 indicated the presence of azithromycin-resistant *T. pallidum*. Visualization on electrophoretic agarose gels showed that, among 39.4% of patients with HIV, resistance to azithromycin was represented by a 130-bp band, corresponding to the A2058G mutation.

In Makassar, specimens were collected from 60 patients. In 2 specimens, the presence of *T. pallidum* was detected by multiplex nested PCR, and resistance to azithromycin was demonstrated for both specimens. Visualization on electrophoretic agarose gels showed 130-bp and 100-bp bands, representing A2058G and A2059G mutations, respectively; 40% of the specimens were obtained from patients with HIV.

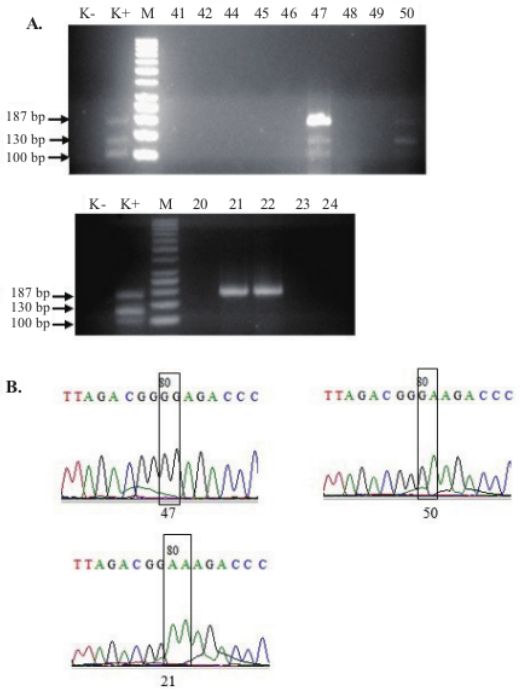

Fig. 1. (Color online) Mutation analysis using multiplex nested PCR. **A.** Results of agarose gel electrophoresis. The band at 187 bp indicates *Treponema pallidum* 23S rRNA gene (bacterial detection), the band at 130 bp indicates A2058G mutation, and the band at 100 bp indicates A2059G mutation. Sample 47 indicates mutation at both nucleotide positions 2058 and 2059, sample 50 indicates mutation at single nucleotide position 2058, whereas samples 21 and 22 indicate no mutation at both nucleotide positions 2058 and 2059. Samples 20, 23, 24, 41–46, 48, and 49 show negative PCR results. M represents 50bp DNA Ladder (from bottom to top: 100, 150, 200, 250–1000 bp). bp means base pair. K+ and K- represent positive and negative controls, respectively. **B.** Results of DNA sequencing for samples 47, 50, and 21.

DISCUSSION

Of the 220 patients from Indonesia diagnosed with secondary syphilis, the majority of male patients were MSM. This study is similar to another study conducted on patients from Makassar, Indonesia, which also reported a higher number of syphilis cases among MSM (19). A previous study in six Indonesian cities reported data indicating that MSM are at an elevated risk for HIV infection in many low and middle-income countries (20,21). There is a significant risk for an increasing number of transmissions between syphilis and HIV among MSM groups (19,22,23). Therefore, testing for HIV should be prioritized in patients diagnosed with syphilis in Indonesia, particularly among MSM groups. This is a significant issue in Indonesia given that HIV comprises a large, undiagnosed proportion nationally, very low rates of people with HIV are on antiretroviral

Table 2. Serology test for *Treponema pallidum*

Region in Indonesia	Sexual status (%)			RPR[1] (%)			TPHA[2] (%)			PCR (+) *n*
	Hetero	Bisex	H/L[3]	(-)	≤ 1:4	≥ 1:8	(-)	≤ 1:80	≥ 1:160	
Western										
Jakarta (62 patients)[4]										
Male (59)	2	10	88	5	10	85	16	20	64	14
Female (3)	100	0	0	0	0	100	0	0	100	0
Medan (33 patients)[5]										
Male (21)	24	14	62	33	0	67	33	0	67	0
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Middle part										
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Male (28)	43	0	57	7	4	89	7	4	89	3
Female (5)	80	0	20	0	0	100	20	0	80	1
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Male (25)	48	0	52	24	24	52	24	40	36	2
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[5]: 18.2% of them were HIV patients.
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[8]: 40% of them were HIV patients.

therapy (ART), and data on those virally suppressed through ART are limited.

Only a few laboratory tests can support definite syphilis diagnoses. Serological examination is a commonly used diagnostic test. Serological tests can detect antibodies at all stages of syphilis and are therefore used for a variety of purposes, such as screening in low-risk populations (e.g., pregnant women, blood donors), screening in high-risk populations (e.g., HIV-AIDS and MSM population), and diagnostic testing in patients with syphilis symptoms and monitoring therapeutic responses. Nontreponemal and treponemal tests for secondary syphilis using titers in RPR and TPHA tests were equal or more than 1:8 and 1:160, respectively (Table 2). Based on serology tests, the highest positive rates were found in Bali, followed by Medan, Jakarta, Makassar, and Bandung.

In this study, the bacterial loads of *T. pallidum* detected by multiplex nested PCR were low compared to those observed in the serological examination, possibly because *T. pallidum* counts in the patient's blood were below the threshold of PCR detection. Based on the results of published meta-analysis test, the best PCR sensitivity value (78.4%) was obtained from the examination of lesions in primary stage syphilis (24). The best type of specimen is taken from the infection site, where the highest number of bacteria can be sampled. In secondary stage syphilis, *T. pallidum* bacteria spreads from the primary infection site through the blood to manifest on the skin. Due to the difficulty of obtaining samples from skin lesions of secondary syphilis patients, on account of the collection techniques being invasive and causing discomfort to patients, blood samples are the primary choice for PCR examinations.

The PCR sensitivity for secondary stage syphilis using blood samples shows moderate values (24,25). The low positive value of the *T. pallidum* multiplex nested PCR compared to serology tests suggests that this method should not be used to rule out syphilis diagnoses in patients presenting a clinical picture of secondary syphilis. This method is more useful as a confirmatory test, especially if paired with serological tests that show uncertain results, as well as for characterizing resistance.

Antibiotic resistance is accelerated by the misuse and overuse of antibiotics, as well as poor infection prevention and control. Since syphilis can cause symptoms similar to many other diseases, an undiagnosed patient can unconsciously transmit the disease to their partners as well as to society (26,27). Several patients possibly carrying syphilis (early–primary/secondary syphilis in particular) might not

have access to STI services and are likely to self-treat by attending local pharmacies. Since often pharmacists recommend oral antibiotic treatment such as azithromycin (not IM penicillin), this could be a potential factor causing azithromycin resistance. In this study, although the majority of syphilis cases was found in MSM, azithromycin resistance was found among heterosexual males with HIV. Therefore, surveillance, diagnosis, treatment, and contact tracing are necessary for high-risk populations, such as MSM and their partners.

In this study, azithromycin-resistant *T. pallidum* were detected and identified by multiplex nested PCR, using 23S rRNA with 3 pairs of primers. The A2058G and A2059G mutations appear to be responsible for causing resistance in all cases in Makassar and Medan. In Bali, one in four specimens positive for *T. pallidum* also showed azithromycin resistance and presented with the 130 bp band on gel electrophoresis, representative of the A2058G mutation. Azithromycin-resistant *T. pallidum* were not found in Jakarta and Bandung.

The discovery of *T. pallidum* resistance to azithromycin in Makassar, representative of Eastern Indonesia, and Medan, located in the westernmost part of Indonesia, with the two-point mutation A2058G and A2059G is very interesting. To my knowledge, this form (both alleles in the same sample) has not been found in any previous study. To date, resistant genomes always contain either A2058G or A2059G (28). This is a novel variant containing both the A2058G and A2059G mutations found only in Indonesian samples. On the other hand, azithromycin resistance genomes in Bali as the representative of central Indonesia were the same as those described in previous studies (i.e., one single mutation in A2058G). Detection of resistant genomes should be considered to evaluate the government policy on using azithromycin as an alternative treatment for syphilis cases in Indonesia.

In conclusion, multiplex nested PCR can increase laboratory capabilities to support the clinical diagnosis of syphilis as "the great imitator" as it may cause symptoms similar to many other diseases. Azithromycin-resistant *T. pallidum* have been found in Indonesia, as also reported in America, Europe, and Asia. The azithromycin-resistant *T. pallidum* in Indonesia appear to be a novel variant of resistance, containing both the A2058G and A2059G mutations, as found in samples from Medan and Makassar. Therefore, special consideration should be given to using azithromycin to treat syphilis in Indonesia and worldwide.

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Conflict of interest None to declare.

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