

Detection and identification of azithromycin resistance mutations on *Treponema pallidum* 23S rRNA gene by nested multiplex polymerase chain reaction

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ABSTRAK

Latar belakang: Saat ini telah terjadi peningkatan kasus *T. pallidum* resisten azitromisin akibat mutasi gen 23S ribosome-ribonucleic acid (rRNA). Hingga kini belum ada kit pengujian laboratorium untuk mengidentifikasi mutasi yang terkait resistensi *T. pallidum*. Oleh karena itu, pada penelitian ini dikembangkan sistem uji nested multiplex polymerase chain reaction (PCR) untuk mendeteksi dan mengidentifikasi mutasi titik A2058G dan A2059G gen 23S rRNA.

Metode: Tiga pasang primer digunakan pada reaksi nested PCR. Untuk mendapatkan kondisi uji yang optimal, dilakukan optimasi parameter yang penting pada proses PCR. Uji spesifisitas dilakukan terhadap beberapa mikroorganisme yang dapat menyebabkan reaksi silang. Selain itu, dilakukan uji sensitivitas untuk mendapatkan deteksi deoxyribonucleic acid (DNA) minimal. Metode PCR yang sudah dioptimasi diujikan pada 45 sampel klinis darah yang hasilnya dikonfirmasi dengan DNA sekuensing.

Hasil: Uji nested multiplex PCR dapat mendeteksi 4.400 jumlah copy DNA dan tidak bereaksi silang terhadap mikroorganisme yang potensial menyebabkan hasil positif palsu. Melalui uji pada 45 sampel klinis darah ditemukan 13 sampel positif *T. pallidum* dan tidak ditemukan mutasi baik A2058G maupun A2059G. Dua sampel positif dikonfirmasi dengan DNA sekuensing dan menunjukkan tidak ada mutasi.

Kesimpulan: Uji nested multiplex PCR yang dikembangkan pada penelitian ini menunjukkan uji yang spesifik dan sensitif untuk mendeteksi *T. pallidum* dan mengidentifikasi mutasi A2058G dan A2059G gen 23S rRNA.

ABSTRACT

Background: Azithromycin-resistant strains of *Treponema pallidum* is associated with the mutation of 23S rRNA gene of *T. pallidum*. Although these strains are now prevalent in many countries, there is no laboratory test kit to detect and identify these mutations. Thus, in this study we developed a nested multiplex polymerase chain reaction (PCR) to detect and identify A2058G and A2059G mutations in 23S rRNA gene.

Methods: Three primer sets were designed for nested PCR reactions. To obtain maximum PCR reaction, all parameters were optimized. The specificity of the primer sets was evaluated towards some microorganisms. A sensitivity test was conducted to get detection limit of deoxyribonucleic acid (DNA). Forty-five whole blood specimens were tested by PCR, and positive results were confirmed by the DNA sequencing.

Results: The assay could detect at least 4,400 DNA copy number and showed no cross reaction with other microorganisms used in the specificity test. A total 13 of 45 whole blood specimens were PCR positive for *T. pallidum*, and no single mutations (either A2058G or A2059G) were detected. Two positive specimens were confirmed by the DNA sequencing and showed no mutation.

Conclusion: Nested multiplex PCR developed in this study showed a specific and sensitive test for the detection and identification of A2058G and/or A2059G mutations of 23S rRNA *T. pallidum* gene.

Keywords: PCR, syphilis, *Treponema pallidum* resistance

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Following decades of low prevalence, syphilis is now resurgent in many developed countries.¹ Although penicillin remains the preferred treatment for syphilis, the convenience of single-dose oral administration of azithromycin, its long half-life in tissue, and its efficacy, which is equivalent to penicillin, have led to the frequent use of this antibiotic for the treatment of syphilis.¹⁻⁴ Widespread use of azithromycin for the treatment of syphilis has resulted in the emergence of resistant strains to the antibiotic. Some research also showed that recent macrolide used for unrelated infections (e.g., oral, skin, respiratory, and genital infections) contributed to the increased prevalence of macrolide-resistant *Treponema pallidum* by providing a selective pressure.^{1,5,6}

Azithromycin resistance is often associated with alteration of the target site (i.e., the peptidyl transferase region in domain V of 23S ribosome-ribonucleic acid (rRNA)) via mutation an adenine (A)-to-guanine (G) transition at position A2058G or A2059G in the 23S rRNA gene, that alters the active site of antibiotic on ribosomes.^{1-3,6-8} Studies have shown the increased prevalence of azithromycin-resistant *T. pallidum* in many developed countries over the years.⁹ The use of macrolide especially azithromycin is increasing in developing countries, and therefore it is likely that azithromycin-resistant *T. pallidum* will eventually emerge or will be introduced via travel and/or tourism.¹

Antibiotic resistance indicates the need to determine the sensitivity of *T. pallidum* using molecular method because this bacterium cannot be cultured on artificial medium.^{10,11} Although resistant strains are now prevalent in many countries, no laboratory test kit is available to detect and identify these mutations. Thus, in this study we developed a nested multiplex PCR to detect and identify A2058G and A2059G mutations of *T. pallidum* 23S rRNA gene. Several methods could be used to detect mutation such as the polymerase chain reaction–restriction fragment length polymorphism (PCR RFLP), real time polymerase chain reaction (PCR), and deoxyribonucleic acid (DNA) sequencing.¹²⁻¹⁴ However, real time PCR and DNA sequencing are highly expensive (reagents and instruments) and time consuming, as well as needs special skills especially for the DNA sequencing.^{15,16} PCR-RFLP and nested PCR are not expensive methods, in which PCR-RFLP is less sensitive than nested

PCR.¹⁷ Therefore, nested multiplex PCR was developed as an alternative assay for the detection of A2058G and A2059G mutations of *T. pallidum*.

METHODS

Clinical specimen

Forty-five whole blood specimens were obtained from patients with secondary syphilis from the sexually transmitted diseases (STD) clinic of Cipto Mangunkusumo Hospital, the STD clinic of Puskesmas Pasar Rebo, the STD clinic of Tambora, and Perkumpulan Keluarga Berencana Indonesia (PKBI) Jatinegara. All patients agreed to enroll in this study by signing informed consent forms. This study was approved by Fakultas Kedokteran Universitas Indonesia (FKUI) research ethical committee no. 158/UN2.F1/ETIK/2015.

Primer design

Three sets of primers were designed using primer designer (please kindly contact corresponding author to get the primer sequences). One set of the primer was used to detect *T. pallidum* 23S rRNA gene while the other two primer sets were used to detect the mutations (A2058G and A2059G) of *T. pallidum* 23S rRNA gene; thus, the nested multiplex PCR produced three DNA segments (187 bp, 130 bp and 100 bp).

Positive control

A synthetic positive control was purchased from gBlocks® gene fragments (194 bp length) having the sequences of *T. pallidum* 23S rRNA gene with mutations A2058G and A2059G.

DNA extraction

The DNA of *T. pallidum* from 200 µl whole blood specimens were extracted using high pure PCR template preparation kit (Roche) according to the manufacturer's instructions with final elution of 70 µL.

Nested multiplex PCR

The reaction mixture of PCR 1 (20 µL): 1x PCR buffer, 1.75 mM MgCl₂, 0.2 µM dNTP mix, 1x Q-solution, 0.2 µM of each primer (PCR1-F and PCR1-R), 0.5 U enzyme *Taq* DNA polymerase (HotStarTaq® Qiagen), and 5 µl of DNA template. The thermal cycles of PCR I: 95°C for 15 min (initial activation); 25 cycles of 95°C for 30 sec 58°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min (final

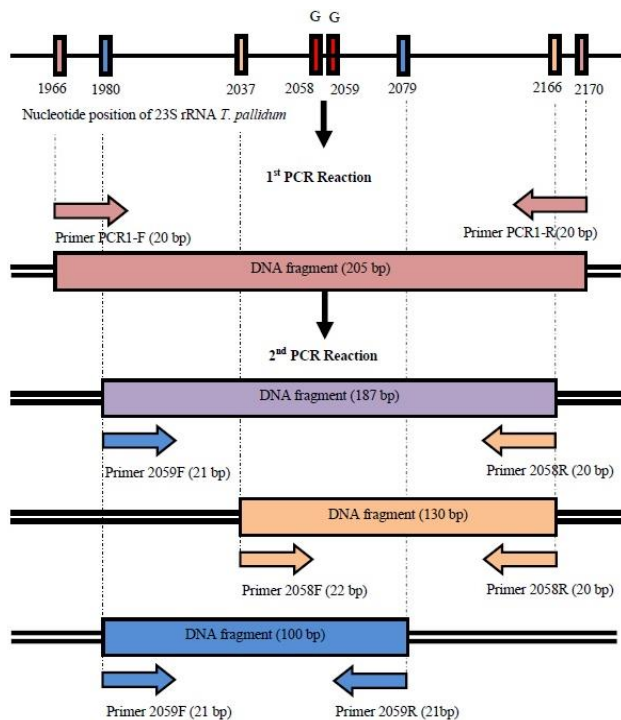


Figure 1. Scheme illustrating primer positions and products (DNA segments) produced by first and second round of nested multiplex PCR. 130-bp and 100-bp DNA segments will be amplified if there is mutation A2058G and/or A2059G respectively. The 3' end of primer forward (2058 F) and reverse (2059 R) have been designed that they can only hybrid with A2058G and A2059G mutations of 23S rRNA respectively. A 187-bp DNA segment will be amplified only from *T. pallidum* 23S rRNA gene (internal control for bacterial detection)

extension). The reaction mixture of PCR 2 (20 μ L): 1x PCR buffer, 1.13 mM $MgCl_2$, 0.35 μ M dNTPmix, 1x Q-solution, 0.45, 0.25, 0.15, 0.4 μ M of 2058F, 2058R, 2059F, and 2059R primers, respectively; 1.5 U enzyme *Taq* DNA polymerase, and 1 μ L of PCR1 product. The thermal cycles of PCR II: 95°C for 15 min (initial activation); 35 cycles of 95°C for 30 sec 61°C for 30 sec, and 72°C for 1 min; and 72°C for 10 min (final extension). DNA amplification was done using the PCR machine (BioRad) according to the manufacturer's instructions. All parameters in the PCR reactions, including annealing temperature, primer concentration, enzyme concentration, annealing time, magnesium concentration, extension time, and cycle number of PCR, have been optimized before applying for clinical samples. Nested multiplex PCR products were analyzed by electrophoresis using gel agarose 2% and colorized by GelRed™. Nucleotide bands visualized by the UV trans-illumination of Gel Doc machine BioRad®. GeneRuler 50 bp DNA ladder (Thermo Scientific) used as marker size molecules.

Specificity and sensitivity of PCR

Nested multiplex PCR specificity was tested against DNA of some various pathogens potential causing false positive results including: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Candida albicans*, Epstein Barr virus, cytomegalovirus, herpes simplex virus, and varicella zoster virus. The sensitivity of nested multiplex PCR was performed using distilled water solutions containing serial dilution ($1/10^4$ - $1/10^{12}$) of purified *T. pallidum* DNA. This test was used to find the detection limit of DNA in the PCR-reactions.

DNA sequencing

In order to verify nested multiplex PCR assays, the A2058G and A2059G mutations were confirmed by DNA sequencing.

RESULTS

Primer design

In this study, we designed three sets of primers (one set of primer for PCR 1 and two sets of primers for PCR 2). The second PCR will produce three DNA fragments with different length (Figure 1). One DNA segment (187 bp) indicates *T. pallidum* 23S rRNA gene. The other two of DNA segments (130 bp and 100 bp) indicated A2058G and A2059G mutations respectively.

Optimization of nested multiplex PCR

Optimization of nested multiplex PCR reaction was conducted in order to obtain optimum PCR result. From optimization of the annealing temperature, the results showed that the optimum annealing temperature for the amplification (both PCR 1 and PCR 2) was 60.9°C (61°C). Optimization of primer concentration showed that concentration of 0.13 μ M, 0.3 μ M, 0.4 μ M, and 0.47 μ M for each primer, gave the optimum result (at total volume reaction of 20 μ L). Optimization of enzyme concentration showed that 1.5 units of *Taq* DNA polymerase at a total reaction volume of 20 μ l gave the optimum result. From optimization of the annealing time, the results showed that the optimum annealing time for the amplification was 30 seconds. Optimization of magnesium ion concentration showed that concentration 0.33 mM magnesium

ion at a total reaction volume of 20 µL gave the optimum result. From the optimization of the number of cycles, the results showed that the optimum one was 35 cycles.

Specificity and sensitivity of nested multiplex PCR

To determine the specificity of nested multiplex PCR, we tested a panel of microorganisms including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Candida albicans*, Epstein Barr virus, cytomegalovirus, herpes simplex virus, and varicella zoster virus. The results showed no cross reacting with those microorganisms. Specificity

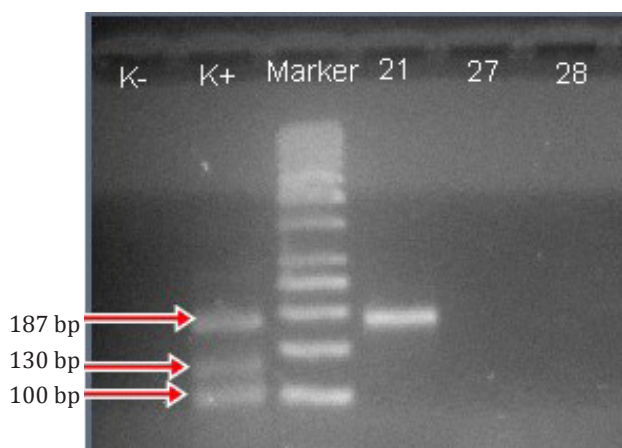


Figure 2. Examples of polymerase chain reaction (PCR) positive and negative results from whole blood samples. K- and K+: negative and positive controls. Positive control consisted of three DNA segments (187 bp, 130 bp and 100 bp). 187 bp: a DNA band indicating *T. pallidum* 23S rRNA gene (bacterial detection). 130 bp and 100 bp: DNA bands indicating A2058G and A2059G mutations respectively

of the primers was also analyzed by primer basic local alignment search tool (BLAST) program and showed no cross reacting with the DNA fragment from other microorganisms except *T. pallidum*. The detection limit of DNA of nested multiplex PCR was 4,400 DNA copy number.

Detection of *T. pallidum* in clinical specimens

PCR results showed that 13 of 45 whole blood specimens were positive *T. pallidum* 23S rRNA gene with no mutations (A2058G and/or A2059G)

Confirmation of PCR results by DNA sequencing

Two positive samples with no mutations were confirmed by DNA sequencing. The DNA sequencing results clarified that there were no mutations in 2058 and 2059 position of 23S rRNA gene and in accordance with PCR results.

DISCUSSION

The inability to cultivate *T. pallidum* on artificial medium and the limitations of conventional methods for direct detection of pathogenic treponemes in clinical specimens, have made clinical decision-making of syphilis is often based upon serological tests despite the fact that their results may not correlate with disease activity.¹⁸ The increased prevalence of azithromycin-resistant *T. pallidum* indicates the importance of determining the sensitivity of *T. pallidum* to antibiotic. The sensitivity test using conventional method cannot be conducted because the inability to cultivate *T. pallidum* on artificial medium. As a result, molecular method would enable rapid detection to detect and identify mutations of *T. pallidum*.

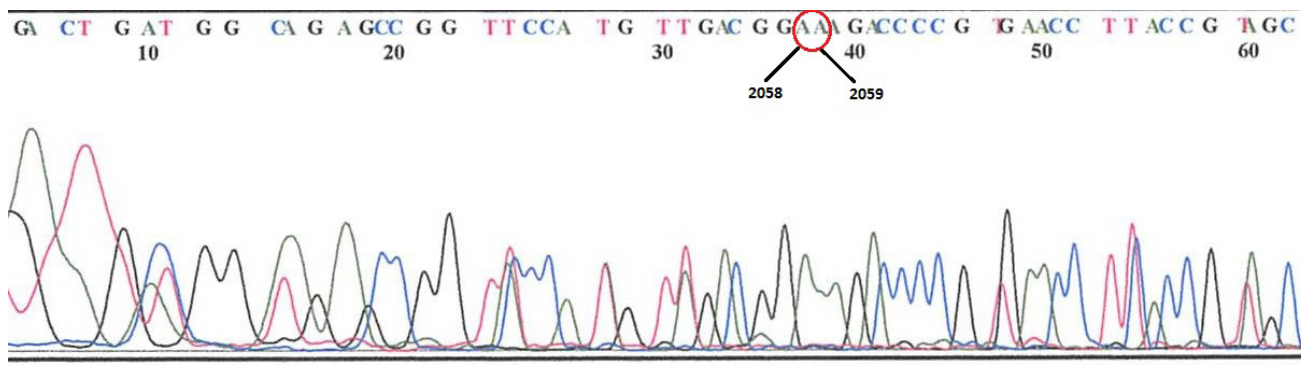


Figure 3. An example of deoxyribonucleic acid (DNA) sequencing result from positive sample. Red circle shows adenine (A) in position 2058 and 2059 of *T. pallidum* 23S rRNA gene, indicating azithromycin-sensitive *T. pallidum*

In this research, we developed a nested multiplex PCR using three primer sets to detect *T. pallidum* and to identify mutations (A2058G and A2059G) of *T. pallidum* 23S rRNA gene that caused azithromycin and macrolide resistant *T. pallidum*. The 3' end of primers (2058F and 2059R) were designed to hybrid only at A2058G and 2059 positions of 23S rRNA gene resistant *T. pallidum*. Besides the mutation identifications, the assay also could detect the *T. pallidum* that was indicated by a 187-bp DNA segment (Figure 1). The PCR method to detect the mutations of 23S rRNA has also been developed by other researchers with different approaches. Lukehart et al.⁷ developed a conventional method for the detection of A2058G mutation used a nested PCR followed by MboII restriction enzyme digestion and agarose gel electrophoresis. An additional restriction enzyme (BsaI) is needed to detect the A2059G mutation as developed by Matejkova et al.⁶ and Chen et al.¹⁹ developed a real-time multiplex PCR to simultaneously detect both mutations without two separate enzymatic digestions.

All parameters in the PCR reactions including annealing temperature, primer concentration, enzyme concentration, annealing time, magnesium ion concentration, extension time, and cycle number of PCR were optimized to obtain ideal results. There is no single protocol or single set of conditions that is optimal for all PCR.^{20,21} Therefore, each new PCR application is likely to require specific optimization to overcome problems, such as no detectable or low yield of PCR product, the presence of nonspecific bands or smeary background; the formation of "primer-dimers" that compete with the chosen template/primer set for amplification; or mutations caused by errors in nucleotide incorporation.^{20,21} Furthermore, syphilis specimens, such as blood, may contain extremely low concentrations of treponemes.¹⁸ It was, therefore, essential to improve an assay's sensitivity with optimization of some parameters such as annealing temperature, primer concentration, enzyme concentration, annealing time, magnesium ion concentration, extension time, and cycle number of PCR.²⁰⁻²²

To determine the specificity of nested multiplex PCR, we tested a panel of microorganisms including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Candida albicans*, Epstein

Barr virus, cytomegalovirus, herpes simplex virus, and varicella zoster virus. The results showed no cross reacting with those microorganisms. Chen et al.¹⁹ determined the specificity of the triplex PCR assay with DNA extracted from an in-house specificity panel comprising closely related *T. pallidum* subsp. *endemicum* and *T. pallidum* subsp. *pertenue*, nonpathogenic treponemes (*T. denticola*, *T. refringens*, and *T. phagedenis*), and pathogenic and commensal genital tract microorganisms, as well as normal skin flora. The number microorganisms for specificity study were limited. However, the specificity of the primers were also analyzed by primer-BLAST program and showed no cross reacting with DNA fragment from other microorganisms beside *T. pallidum*. We recommend that it is important to perform the specificity test against larger representative microorganisms in the future.

From the sensitivity test, we found that the detection limit of DNA of the assay was 4,400 DNA copy numbers. This result was different from Liu et al.²³ and Orle et al.²⁴ which had a sensitivity of 10 organisms. These differences might occur as a result of using different gene target, set primers or specimen in each study. Liu et al.²³ and Orle et al.²⁴ using *polA* as gene target and swab from genital ulcer as sample. Many studies showed that swab from genital ulcer specimens or from skin active lesions have better sensitivity than blood specimens.^{10,25,26} However, in this study we could not obtain the skin specimens because not all of patients showed wet skin lesion; thus, we only obtained the whole blood samples. Although the sensitivity of *T. pallidum* bacteremia detection is lower, it varies according to stage.¹⁰ Moreover, we also used more than one primer pair (three sets of primers) that might affect the sensitivity assay.²⁷

A total 13 of 45 whole blood specimens were PCR positive for *T. Pallidum*, and no single point mutation (either A2058G or A2059G) were detected. Nested multiplex PCR could only detect mutations from positive control that always ran parallel with PCR reactions from samples. This result was different from other studies of mutations A2058G and A2059G of 23S rRNA gene that caused azithromycin and macrolide resistant *T. pallidum*. Studies from many countries showed the increase of resistant *T. pallidum* strain.^{2,3,9,10} It is not enough evidence to conclude that resistance strains of *T. pallidum* do not emerge in Indonesia

because of the samples we tested only taken from one city that is Jakarta. We suggest to conduct further studies by using clinical specimens from other provinces in Indonesia to obtain national data of azithromycin-resistant *T. pallidum*.

In conclusion, we have developed nested multiplex PCR for simultaneous detection and identification of A2058G and A2059G mutations 23S rRNA *T. pallidum* gene by using three sets of primers. This assay has been optimized to obtain the optimal condition of PCR with high specificity and sensitivity. Optimized PCR have been tested for clinical specimens and showed the same results as confirmatory DNA sequencing. Based on this study, the PCR developed is potential to apply for clinical specimen.

Conflicts of Interest

The authors declare that they have no conflict of interests.

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