Polymorphism Analysis of the Coagulase Gene in Isolates of Methicillin-Resistant *Staphylococcus aureus* with *AluI* Restriction Sites

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Abstrak

Latar belakang: *Analisis polimorfisme suatu gen penting dilakukan untuk memperoleh informasi lebih awal dalam identifikasi penanda genetik yang berhubungan dengan sifat yang ingin dilihat. Metode RFLP menjadi salah satu metode yang dipilih karena dapat melihat polimorfisme urutan DNA yang dapat dideteksi melalui adanya perbedaan fragmen DNA setelah dipotong dengan menggunakan enzim endonuclease tertentu sehingga mampu menggambarkan polimorfisme dari suatu gen. Tujuan penelitian adalah untuk mengetahui adanya polimorfisme gen coagulase S. aureus resisten methicillin.*

Metode: *Penelitian yang dilakukan secara deskriptif pada 25 sampel. Isolatbakteri MRSA diidentifikasi menggunakan pemeriksaan bakteriologis dan PCR gen mecA dan coagulase dengan menggunakan primer spesifik. Analisis polimorfisme gen coagulase dengan situs restriksi AluI isolat S. aureus resisten methicillin dilakukan dengan menggunakan metoda PCR-RFLP.*

Hasil: *Amplifikasi menunjukkan produk PCR (amplicon) gen mecA dan coagulase dengan primer spesifik ke 25 isolate bakteri MRSA mempunyai positivitas sebesar 100%. Hasil dari PCR-RFLP menunjukkan empat pola RFLP dengan situs restriksi AluI pada 25 isolat. Proposi terbesar (64%) pada pola RFLP I (pola yang tidak terdigesti enzim restriksi AluI).*

Kesimpulan: *Terdapat polimorfisme isolate S. aureus resisten methicillin berdasarkan analisis gen coagulase.* ***(Health Science Journal of Indonesia 2016;8(1):19-24)***

Kata kunci: *Staphylococcus aureus,gen coagulase, PCR-RFLP, AluI*

Abstract

Background: *Analysis of the polymorphism of a gene is important to obtain early information in identifying genetic markers related to the characteristics to be seen. The RFLP method becomes one of the chosen methods because it can see polymorphism that can be detected by using the different fragments of DNA that have been cut by using certain endonuclease enzyme so that it is possible to describe the polymorphism of a gene.The aim of the study is to discover the gene polymorphism of methicillin-resistant Staphylococcus aureus.*

Methods: *This was a descriptive study using 25 isolates. Isolates of MRSA tested bybacteriological examination and PCR of mecA and coagulase gene using specific primers. Polymorphism analysis of the coagulase gene in isolates of methicillin-resistant Staphylococcusaureus with AluI Restriction Sites tested by PCR-RFLP.*

Results: *The Amplification showed that PCR product (amplicon) of mecA and coagulase gene from specific primers of all 25 isolate samples, had a positivity of 100%.The PCR-RFLP of coagulase gene showed that all 25 samples underwent polymorphism into four RFLP patterns with AluI restriction sites. The largest proportion* (64%) *was found polymorphism in clinical samples MRSA with RFLP I pattern (un-digested pattern of AluI restriction enzyme*).

Conclusion: *There is polymorphism in the samples MRSA from the analysis of the coagulase gene.* ***(Health Science Journal of Indonesia 2016;8(1):19-24)***

Keywords: *Staphylococcus aureus, coagulase gene, PCR-RFLP, AluI*

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a *Staphylococcus aureus* that become insusceptible or resistant by methicillin antibiotic types.[1] MRSA become resistant because of genetic changes that caused by exposure of irrational antibiotic therapy.[1] Patients with *S. aureus* colonization are the major transmission sources of *S. aureus* at hospitals and are also responsible for the clinical infections of other patients.[2]

The prevalence of MRSA in various hospitals in the world ranges from 2-70% with an average of 20%.[3]In recent decades there is an increased prevalence of *S. aureus* and MRSA in the world. A study based on the population of North America and Europe indicated that the prevalence of *S. aureus* was between 18-30%.[4] In overall Asia, MRSA prevalence has reached 70%[5], while MRSA publication and prevalence in Indonesia are still very limited and difficult to obtain. MRSA prevalence in 2003 at Atmajaya Hospital of Jakarta has reached 47% and MRSA at Dr. Moh. Hoesin Provincial Public Hospital of Palembang in 2010 has reached 46%.[6,7]

S. aureus is able to produce *coagulase*, an enzyme protein which is capable of coagulating plasma oxalate or plasma citrate. Bacteria producing *coagulase*s are considered having the potential to become invasive pathogens. A *coagulase* enables *S. aureus* to avoid phagocytosis through the formation of protective wall layers in the forms of fibrin. *S. aureus* pathogenicity is due to the toxin production working after bacteria successfully enter and survive in the hosts' body. In this initial phase, a *coagulase* serves as a virulence factor which is capable of coagulating proteins by protecting bacteria from phagocytosis and inhibiting the antibiotic penetration. As the treatment is difficult to perform, those bacteria may cause infection and antibiotic resistance.[8]

A gene polymorphism analysis is necessary to conduct to obtain earlier information in identifying genetic markers related to the desirable characteristics to see. Restriction fragment length polymorphism (RFLP) is a method selected to see the DNA sequence homologues which may be detected using DNA fragment differences cut using a certain endonuclease enzyme which is capable of describing a gene polymorphism. The analytical studies on methicillin-resistant *S. aureus coagulase* gene polymorphism using *AluI* restriction enzyme have been conducted on methicillin-resistant *S. aureus* isolate population in Iran, India, and Europe, which result in different RFLP patterns.[8-11] In Indonesia, studies on clinicalisolates of methicillin-resistant *S. aureus coagulase* gene polymorphism with *AluI* restriction enzyme have never been conducted before that the researcher is interested in conducting polymorphism analysis of the *coagulase* gene in clinical isolates of methicillin-resistant *S. aureus* with *AluI* restriction sites by RFLP. The application of RFLP in this research is especially used to see the presence of methicillin-resistant *S. aureus* polymorphism *coagulase*s. Based on above descriptions, the researcher is interested in conducting analysis on *coagulase* gene with *AluI* restrictive site of methicillin- resistant *S. aureus*isolates.

METHODS

Bacterial Isolates

The samples (25) were collected from patients suspected for MRSA infections in RSUD DR. Soetomo Surabaya. In addition one strains of *Staphylococcus aureus* subsp 6850 were maintained as positive control for identification of *coagulase* gene polymorphism. The collected samples were streaked on blood agar medium and incubated at 37^{o}C for about 24 hours.

Identification of *S.aureus*

Several tests were carried out for the identification of *S. aureus*. Initially Gram's staining was performed to identify the morphology of the organism, and various biochemical tests such as catalase test, oxidase test, mannitol salt agar test, slide *coagulase*, motility, citrate, and fermentation of carbohydrates (fructose, galactose, lactose, maltose, mannose, sucrose) test were carried out.

DNA extraction

Genomic DNA from the bacteria was extracted from the samples with chloroform-phenol method. Initially 1.5 ml bacterial culture was taken and subjected to centrifugation. The resulting pellet was add with 1000 µl of DNAzol and itwas incubated at 25°C for 20 minutes, after that add 200 µl of chloroform, vortex. Then the suspension was centrifuged. Later 600 µl of isopropanol was added and centrifuged. The pellet was washed with 75% ethanol and dried. Finally, the DNA pellet was stored in nuclease free water. Then the DNA fragments were measured by Nanodrop ™ 2000 Thermo Scientific spectrophotometer.

Identification of *mecA* gene methicillin-resistant in *S. aureus* by PCR

The *mecA*gene was amplified by using two sequence of the primers by using forward primer: 5′ TGGCTATCGTGTCACAATCG 3′ and reverse primer: 5′ CTGGAACTTGTTGAGCAGAG 3′.[3]*S. aureus* isolates were tested for the presence of the 304-bp PCR product of the *mecA* gene. The initial denaturation was at 94°C for 30 second. Denaturation of 94°C for 1 minute, annealing at 52°C for 30 second and elongation temperature of 72°C for 1 minute was maintained for 30 cycles. The final elongation was at 72°C for 5 minutes and the reaction was held at 4°C. The amplified products were subjected to 2% agarose gel electrophoresis.

Identification of *coagulase* gene methicillin-resistant in *S. aureus* by PCR

The *coagulase* gene was amplified by using two sequence of the primersby using forward primer: 5′ ATAGAGATGCTGGTACAGG 3′ and reverse primer: 5′ C GCTTCCGATTGTTCGATGC 3′.[11]*S. aureus* isolates were tested for the presence of the 756-bp PCR product of the *coagulase* gene. The initial denaturation was at 94°C for 45 second. Denaturation of 94°C for 20 minute, annealing at 57°C for 30 second and elongation temperature of 72°C for 30 minute was maintained for 30 cycles. The final elongation was at 72°C for 3 minutes and the reaction was held at 4°C. The amplified products were subjected to 2% agarose gel electrophoresis.

PCR- RFLP of *coagulase* gene *S. aureus*

The RFLP of *coagulase* gene was carried out by using *AluI*, 10µl of *coagulase* gene PCR products were digested with 2U *AluI* restriction enzyme according to the manufacturer's instructions (*AluI* Thermo Scientific) and incubated at 37ºC for 3 hour. The resulting restricted fragments were separated in polyacrylamide gel.

RESULTS

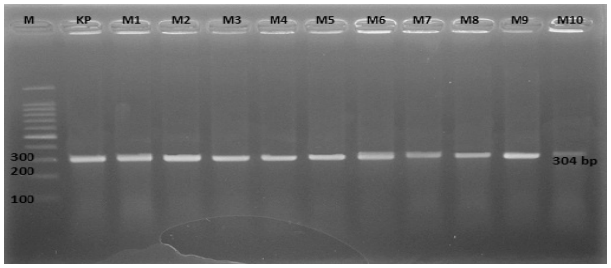
Isolation and identification of *S. aureus*

Twenty five different samples were collected from clinical patients. Among these samples, 25 samples resulted in individual spherical colonies of greyish white color. The isolates with individual spherical, greyish white colonies were again sub cultured in a new blood agar medium and were incubatedat 37°C for 24 hours. The results of bacteriological examination are conducted using a conventional method.[12] The following results were obtained (Figure 1), it can be concluded that all of the samples are *S. aureus*.

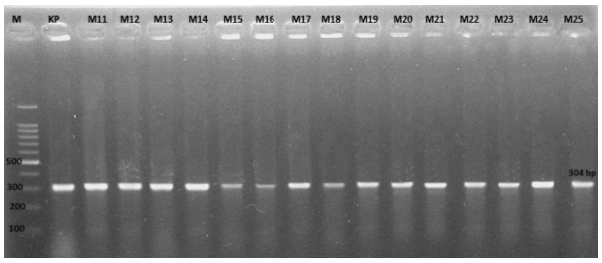
Figure 1. S.aureus in a new blood agar medium. The resulted of S. aureus in individual spherical colonies of greyish white colour

Identification of *mecA*gene methicillin-resistant in *S. aureus* by PCR

Identification of *mecA* gene methicillin-resistant in *S. aureus* was performed by PCR. The results were observed under 2% agarose gel electrophoresis with 100-1500bp DNA marker. The following results were obtained (Figure 2), it can be concluded that 100% samples are positive*mecA* gene.



(a)



(b)

Figure 2. Agarose gel electrophoresis of PCR-amplified meca genes from of S. aureus.M=Marker (DNA Ladder 100 bp), KP = Positive Control (304 pb). (a) M1-M10 = Isolate samples of MRSA (304 bp). (b) M11-M25 = Isolate samples of MRSA (304 bp)

Identification of *coagulase* gene methicillin-resistant in *S. aureus* by PCR

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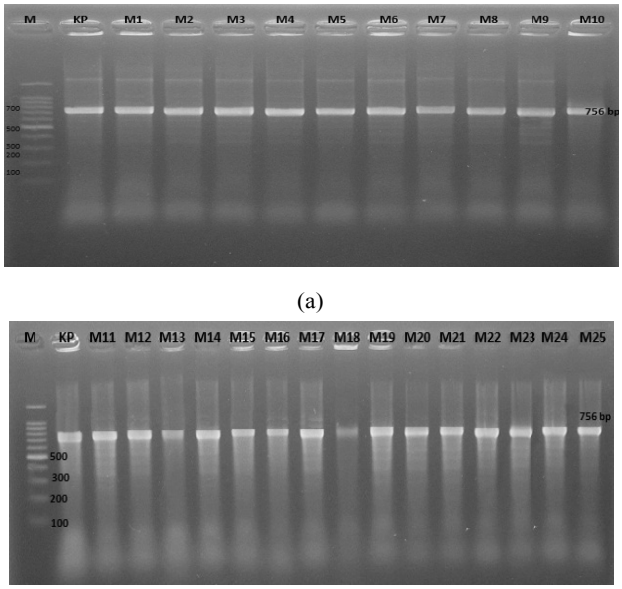

Figure 3. Agarose gel electrophoresis of PCR-amplified coa genes from of S. aureus. M=Marker (DNA Ladder 100 bp), KP= Positive Control (756 bp). (a) M1-M10 = Isolate samples of MRSA (756 bp), (b) M11-M25 = Isolate samples of MRSA (756 bp)

PCR- RFLP of *coagulase* gene *S. aureus methicillin resistant* (MRSA)

The PCR amplicons were digested with *AluI* restriction enzyme and five different restriction patterns were obtained (Figure 4) (Table 1). This difference in restriction pattern may be due to the polymorphism existing in the *coagulase* gene of different MRSA isolates.

DISCUSSIONS

Table shows 1 that *S. aureus coagulase* gene in patients at Dr. Soetomo hospital of Surabaya is polymorphic as the most dominant allele proportion are only 64%. A gene is considered polymorphic when the most dominant allele proportion is less than 95%[13], and based on Table 1, it is obtained that from RFLP patterns of *coagulase* gene, the most dominant pattern is RFLP I by 64%. It is then respectively followed by RFLP IV by 24% and RFLP II by 8%. Meanwhile, RFLP III pattern has the lowest proportion by only 4%.

The study the *coagulase* gene polymorphism on methicillin-resistant *S. aureus* population in Europe shows that RFLP I pattern which is undigested by *AluI* restriction enzyme may become RFLP pattern with the lowest proportion of only about 2%.[11]A similar study is also stating similar finding that *coagulase* gene polymorphism in methicillin-resistant *S. aureus* population in Iran suggests that RFLP I pattern which is undigested by *AluI* restriction enzyme may become RFLP pattern with the lowest proportion of only about 2%.[7] In India, the results of RFLP *coagulase* gene patterns are divided into31. Those thirty-one pattern variations show that all samples may be well digested by *AluI*.[10] A similar latest study that in India, the study of *S. aureus coagulase* gene polymorphism on the population in India shows that RFLP *S. aureus* patterns are divided into 5. Those five pattern variations also show that all samples may be well digested by *AluI*.[9]

There are several variation differences of RFLP MRSA patterns in *coagulase* gene found in this study when compared with those found by the other researchers which shows that the strains of methicillin-resistant is highly *S. aureus* polymorphic shown by the sequential changes of *AluI* enzyme introduction in *coagulase* gene since there is a point mutation. These sequential changes may cause genetic diversity of *S. aureus coagulase* genes. The presence of genetic diversity in *coagulase* gene is in accordance with the results of research that the *coagulase* gene polymorphic is also due to the differences in sequence 3' of *coagulase* gene regional variable on *S. aureus* strains which may result in various amino acid sequential encoding in *coagulase* gene.[8]

It is predicted that this *coagulase* gene polymorphism has an important role in antibiotic resistance occurring in *S. aureus* bacteria. *Coagulase* enzyme has the function to coagulate plasma oxalate or plasma citrate as there is a reactive *coagulase* factor in the serum which reacts with the enzyme. The esterase resulted may increase the coagulation activity that fibrin deposit is formed on the surface of bacterial cells. Fibrin deposit on the surface of bacterial cells may cover pores of the bacterial cell walls which inhibit the antibiotic penetration as to many antibiotics enter the bacterial cells through the pores of the cell walls and membranes. The antibiotic concentration in cells is equal with the number of pores in the cells that bacteria resistant to antibiotics have the mechanism to reduce or close the pores on cell membranes by maximizing the activities of *coagulase* enzymes to inhibit the antibiotic absorptions and enable the number of antibiotics entering the cell is inadequate to damage or kill the bacteria.[14]

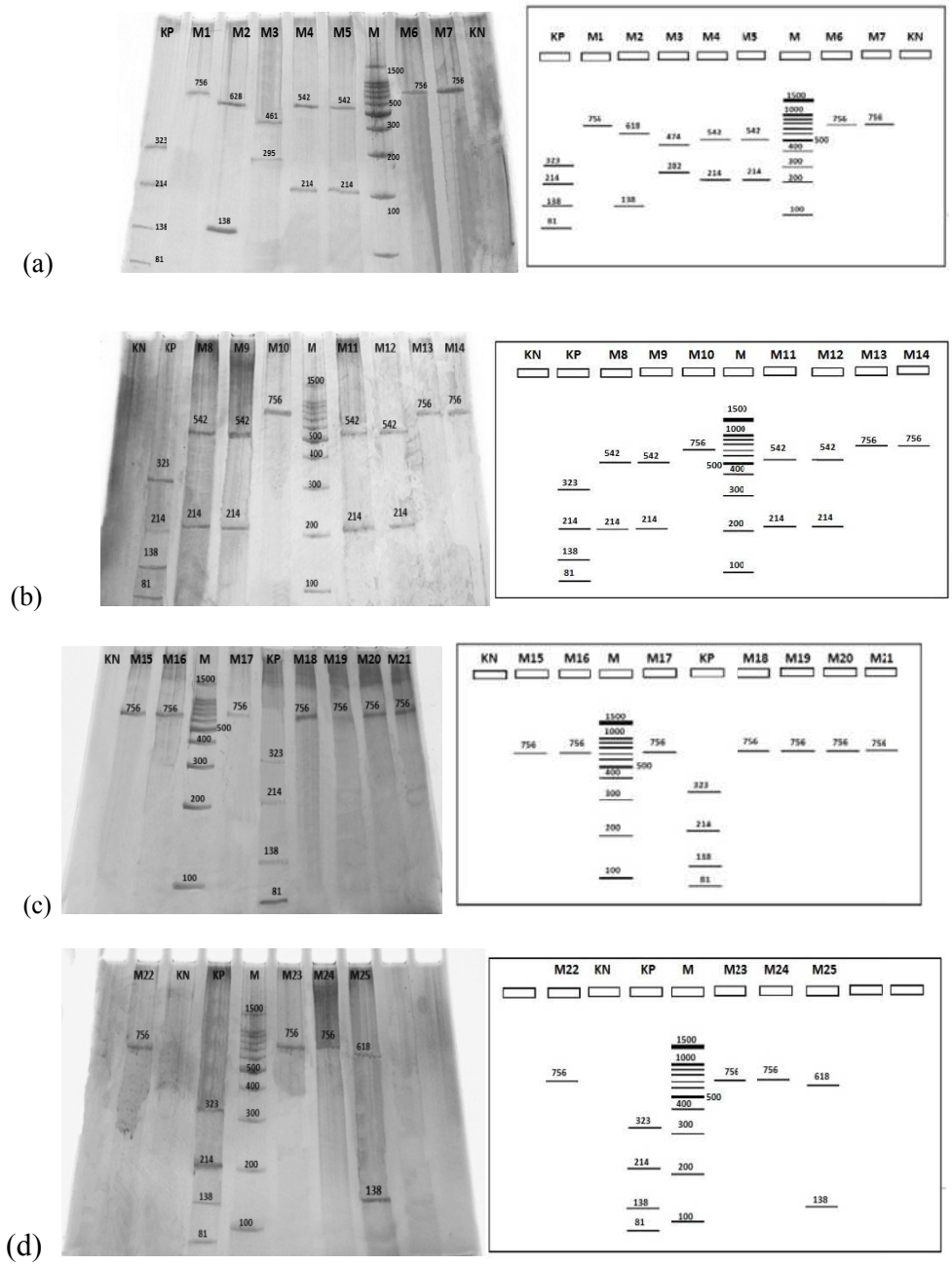

Figure 4. Acrylamide gel electrophoresis of PCR-RFLP coa genes from of MRSA. M=Marker (DNA Ladder 100 bp), KP = Positive Control (756 bp), KN = Negative Control. (a) M1-M7 = Isolate samples of MRSA, (b) M8-M14 = Isolate samples of MRSA, (c) M15-M21 = Isolate samples of MRSA, (d) M22-M25 = Isolate samples of MRSA

Table 1. Patterns of restriction *AluI S. aureus coagulase* gene

Sample Number	Amplicon *coagulase* gene (bp)	Restriction Fragment (bp)	Pattern	(%)
M1, M6, M7, M10, M13, M14, M15, M16, M17, M18, M19, M20, M21, M22, M23, M24	756	756	I	64
M2, M25	756	138, 618	II	8
M3	756	282, 474	III	4
M4, M5, M8, M9, M11, M12	756	214, 542	IV	24

Antibiotic resistance is also caused by the horizontal resistance transfer through methicillin-resistant antibiotic sensitivity test on *Staphylococcus*. This antibiotic resistance is caused by PBP2a protein expressions which are synthesized by *mecA* gene. Similarly, methicillin-resistant on staphylococcus strains may be transferred through staphylococcal cassette chromosome (SCC) elements which have *mecA* gene (SCCmec). The occurring resistance transfer possibility is only seen in methicillin antibiotics as SCCmec is an element which is easy to transfer. The reported *mecA* gene transfer occurs from Staphylococcus positive *coagulase*, for example *Staphylococcus intermedius* to *Staphylococcus aureus*. In addition, *S. aureus* antibiotic resistance is also caused by the inappropriate use of antibiotics. Thus, antibiotic abuses are the common causes of antibiotic resistance, including the first antibiotic uses for viral infections and less optimized antibiotic dosage uses may result in bacterial antibiotic resistance.[15,16]

Analysis on various virulence and resistance factors which are responsible for *S. aureus* pathogenicity is essential to conduct. This research emphasizes on the importance of methicillin-resistant *S. aureus* positive *coagulase* molecular diagnosis. Polymorphism analysis in *coagulase* gene is extremely helpful in treating various infections caused by methicillin-resistant *S. aureus* based on its genotype.

In conclusion, it was found polymorphism in the samples MRSA from the analysis of polymorphism of the *coagulase* gene.

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