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Antibacterial Effect of *Gracilaria changii* and *Euchema denticulatum* on Molecular Properties of *Staphylococcus aureus* Genes *mecA*, *mecR1* and *mecI*

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Abstract: Gram-positive bacteria are more sensitive to antibiotics than gram-negative bacilli because of the lack of outer membrane which prevent easy access of the drug into the bacterial cells. However, there are many gram-positive organisms with natural, intrinsic resistance to antimicrobials. In addition, these bacteria are able to acquire resistance to frequently used drugs rapidly through selective pressure of the environment and also via the genetic evolution of bacteria. The resistance of those bacteria to antibacterial agents is mediated by antibiotic resistant genes. Therefore, the current study was designed to explore the effect of seaweed extracts on several antibiotic resistant genes in *Staphylococcus aureus mec* genes *mecA*, *mecR1* and *mecI* that regulate the expression of methicillin resistance was investigated by PCR.

Key words: Methicillin resistanc *Staphylococcus aureus* seaweed, polymerase chain reaction, gene, Malaysia

INTRODUCTION

Staphylococcus aureus which is namely by Sir Alexander Ogston, is an important opportunistic human pathogen and also a part of the normal microbial flora in the upper respiratory tract of human. It is a yellow pigmented species, non-motile, non-spore forming, catalase positive and gram positive cocci that is measure approximately 0.7-1.2 μm in diameter (David *et al*., 1999; Washington *et al*., 2006). It has low GC (Michael *et al*., 2005) content and is resistant to drying and readily dispersed in dust particles through the air. It is also a facultative aerobe bacterium which produces acid from glucose both aerobically and unaerobically. The expression of the *mecA* gene and the resulting production of PBP2a is regulated by proteins encoded by the penicillinase-associated *blaR1-blaI* inducer-repressor system and the corresponding genomic *mecR1-mecI* elements (Hackbarth and Chambers, 1993; Tesch *et al*., 1990; Sharma *et al*., 1998). Hiramatsu *et al*. (1992) identified in *Staphylococcus aureus* N315 the *mecR1-mecI* regulator element, which is located upstream of the *mecA* gene and is divergently transcribed from *mecA*. The *mecI* gene codes for a repressor protein and the *mecR1* gene for a ß-lactam-sensing transmembrane signalling protein. Methicillin and oxacillin are, however, not good inducers for this system, often resulting in slow induction of methicillin resistance. Phenotypically susceptible strains, known as pre-methicillin-resistant *S. aureus* (pre-MRSA) and pre-methicillin-resistant coagulase-negative staphylococci (pre-MRCNS), have been discovered, which do not express methicillin resistance, as *mecA* is fully repressed by *mecI* (Hiramatsu, 1995; Weller, 1999) The induction of *mecA* transcription is very slow and might be due to mutations of *mecI* (Weller, 1999) Distribution of *mec* regulator genes among methicillin-resistant *Staphylococcus* strains from various countries has already been studied by hybridization and sequencing, which showed that the loss or inactivation of the *mecI* gene leads to derepression of *mecA* gene transcription (Weller, 1999; Shimaoka *et al*., 1994; Suzuki *et al*., 1993). In this study, we have amplified the

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antibiotic resistance genes of *S. aureus mecA*, *mecR1* and *mecI* by Polymerase Chain Reaction after treated and untreated with marine seaweeds. Majority of the seaweeds grow by attaching to the hard surfaces like rocks and shells and can be found as far as 130 feet (40 m). The rich tropical waters surrounding the coast and islands, harbor a variety of seaweeds such as red algae (Rhodopyta), brown algae (Phaeopyta), green algae (Chlorophyta) and blue green algae (Cyanophyta), representing a potential source of useful products. Red algae which are found at where the water is much calmer can be utilized as a source of superfood for centuries. It comes in a variety of colors which gives rise to their variety of uses. In China, Japan and the Indo-Pacific region, several dozen species of red algae are used. This therapeutic superfood provides the body with a full array of nutrients including complete protein, complex carbohydrates, essential fatty acids, fiber, vitamins, minerals, trace elements, enzymes and sulfated polysaccharides. Red algae are capable of working on multiple levels to strengthen the body and solidify the body's primary defense system. Efficacy of red algae as antibacterial agent is few in mention especially the local red algae found around Malaysian coasters. Therefore, this study is in search for the antibacterial properties in red algae in order to find a new antibacterial agent that can inhibit or reduce the growth of bacteria in human body. Red algae that are used in this study are *Gracilaria changii*, which grow mild in Pantai Morib, Selangor and *Euchema denticulatum* which grow mild and cultivated in Pantai Sabah. Several approaches of molecular biology tools through genomic analysis were used to explore and to understand antibacterial mechanism of the red algae extract in this study. In the present study, the advantages of molecular tools through genomic analysis could be used to prove that both seaweed extracts will work very well as a new antibacterial agent in a new decade not only just by prescreening test but also through genomic analysis for understanding the mechanism of inhibition. The recent advances in gene amplification methods have been used in this study such as Polymerase Chain Reaction.

MATERIALS AND METHODS

Genomic study: Propagation of Bacterial Culture Treated with *Gracilaria changii* and *Euchema denticulatum* Extract: a single pure colony of *S. aureus* isolates was inoculated to a 250 mL capacity Enlermeyer flask containing an adequate volume of sterilized Luria Bertani broth (Invitrogen Inc.) containing either 50 mg mL^{-1} *G. changii* or 40 mg mL^{-1} *E. denticulatum* extract. The broth was then incubated 4-5 h at 37°C with constant shaking on the shaker. This was followed by the procedure of the DNA extraction using the GeniSpin™ Bacterial DNA Kit (BST^{Techlab}).

DNA extraction: Five isolates of *S. aureus* including MRSA and non-MRSA strains were extracted using the GeniSpin™ Bacterial DNA Kit (BSTTechlab). One *E. coli* strain, which serve as negative control, was extracted by using the same method. The culture was aliquot into 1.5 mL microcentrifuged tube and pelleted until an adequate quantity of bacteria was obtained. Cells then were resuspended in 100 μL TE buffer and the bacterial cell wall was removed by lysozyme (10 mg mL^{-1}) digestion and followed by buffer BTL and proteinase K (15 mg mL^{-1}) digestion. RNaseA (25 mg mL^{-1}) was added to remove the RNA, which normally co-purifies with genomic DNA. Following lysis, binding condition was adjusted and the sample was applied to an I-Spin™ column after adding the Buffer BDL and absolute ethanol. Two rapid wash steps using Wash Buffer will removed trace salt and protein contaminants and finally DNA was eluted in water or low ionic strength buffer. This DNA can be directly used in downstream applications without the need for further purification. The eluted DNA was then run onto 0.8% agarose gel electrophoresis to check the present of genomic DNA and stained with ethidium bromide to visualize under transilluminator. Lastly, the DNA was stored at-20°C in a refrigerator.

Polymerase Chain Reaction (PCR): All the primers that were utilized in this study were synthesized by Research Biolabs, Singapore. The sequences of primers for the amplification of *mecA*, *mecR1* and *mecI* genes were in accordance to previously published data (Murakami *et al*., 1991; Suzuki *et al*., 1993) (Table 1). Using the Thermal Block Cycler (T-Personal) provided by Biometra, amplification of *mecA, mecRI* and *mecI* genes were achieved according to data represented in Table 2 and 3.

Table 1: Sequences of primers used in detection and amplification of genes

Gene	Sequences	Size (bp)
mecA	5'-AAAATCGATGGTAAAGGTTGGC-3'	
	5'-AGTTCTGCAGTACCGGATTTGC-3'	533
mecR1	5'-GTCTCCACGTTAATTCCATT-3'	
	5'-GTCGTTCATTAAGATATGACG-3'	310
mecI	5'-AATGGCGAAAAAGCACAACA-3'	
	5'-GACTTGATTGTTTCCTCTGTT-3'	481

Table 2: The reaction mixture for each primer

Primer	*scv101*	*adaB*	*mecA*	*mecR1*	*mecI*	*ogt*
	(μL)					
MgCl2	0.3	0.3	0.4	0.4	0.4	0.4
dNTP	0.4	0.4	0.4	0.4	0.4	0.4
PCR buffer	2.5	2.5	2.5	2.5	2.5	2.5
Forward primer	0.1	0.1	0.1	0.1	0.1	0.1
Reverse primer	0.1	0.1	0.1	0.1	0.1	0.1
*Taq*DNA olymerase	0.2	0.2	0.2	0.2	0.2	0.2
Genomic	1.0	1.0	2.0	2.0	2.0	2.0

Table 3: Thermal cycling profile for each primer

Primer	Initial denaturation	Extension denaturation	Annealing temperature	Elongation	Final Extension	Cycle
mecA	96°C	95°C	59°C	55°C	55°C	
	(3 min)	(1 sec)	(30 sec)	(30 sec)	(30 sec)	25
mecRI	96°C	95°C	59°C	55°C	55°C	
	(3 min)	(1 sec)	(30 sec)	(30 sec)	(30 sec)	25
mecI	95°C	95°C	55°C	72°C	72°C	
	(1 min)	(1 min)	(1 min)	(2 min)	(10 min)	30

RESULTS AND DISCUSSION

The total genomic DNA of high molecular weight were successfully extracted from 5 isolates of untreated and treated *S. aureus* (Fig. 1), including MRSA and non-MRSA strains and one isolate of *Escherichia coli* (Fig. 2) using GeniSpin™ Bacterial DNA Kit (BST$^{\text{Tech Lab}}$). The ratio of absorbance at 260-280 nm (A260:A280) ranged from 1.238-2.000. The DNA concentrations were between 40-560 μg mL^{-1}. Amplification of *mecA*, *mecRI* and *mecI*, Genes by Polymerase Chain Reaction (PCR). Amplification of various genes through Polymerase Chain Reaction (PCR) with respective specific primers, were sensitive at the DNA template concentration of 100 ng μL^{-1} for *Staphylococcus aureus* genomic DNA. This study demonstrates that the *mecA* (Fig. 3), *mecRI* (Fig. 4) and *mecI* (Fig. 5) genes have been successfully amplified and isolated from 3 local isolates of MRSA at 533, 310 and 481 bp, respectively. All the isolates treated with either *Gracilaria changii* or *Euchema denticulatum* extracts also showed the amplification of these genes whereby a single band corresponding to the respective PCR products were observed.

An increasing number of investigators have employed the tools of molecular biology to facilitate the diagnostic process. In the current study, since the preliminary screening reveals the significant finding of antibacterial activity of *Gracilaria changii* and *Euchema denticulatum* extracts against *Staphylococcus aureus* isolates, the molecular biology tools through genomic analysis was used to explore and to understand this inhibitory activity to predict the antibacterial mechanisms of both extracts on several selected genes of *S. aureus* including the antibiotic resistance genes, DNA repair gene and cell wall synthesis gene. In this study, a high molecular weight and good quality of DNA without RNA contamination was successfully extracted from all the treated and untreated *S. aureus*. Good quality of DNA was obtained as evidenced from the agarose gel analysis which is a crucial technique in detecting the presence of genomic DNA. To amplify the gene of interest, the most important component is the primer, a short segment of nucleotides, which have complementary base pairs to the length of the DNA.

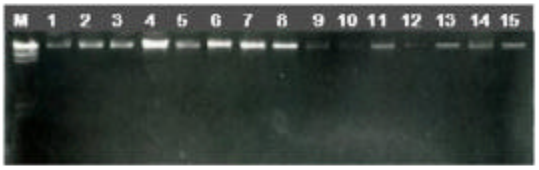

Fig. 1: The genomic DNA extracted from untreated and treated *Staphylococcus aureus*. Lanes 1-5 are the non-treated isolates, lanes 6-10 are the isolates treated with *Gracilaria changii* while lanes 11-15 are the isolates treated with *Euchema denticulatum* extract. Lane M in the Lambda

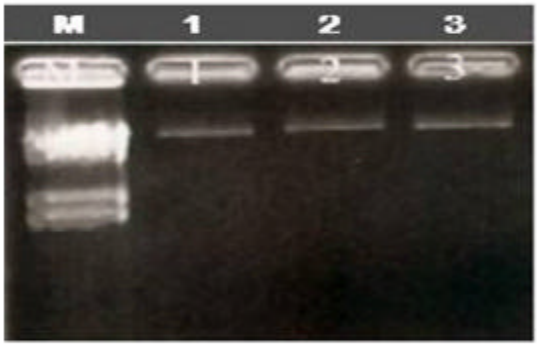

Fig. 2: The genomic DNA extracted from untreated and treated *Escherichia coli*. Lanes 1 is the untreated isolate, lane 2 is the isolate with *Gracilaria changii* and lane 3 is the isolates treated with *Euchema denticulatum* extract. Lane M in the Lambda Hind3 molecular weight marker

The correct identification of *S. aureus* and the detection of the methicillin resistant genes based on molecular methods have evolved as the method of choice for definitive identification of antibiotic resistance pattern. Antibiotic resistant genes that are chosen in this study were *mecA*, *mecRI* and *mecI* gene. According to Hiramatsu *et al*. (1992), *mecA* encoded a penicillin-binding protein, PBP2a, *mecI* codes for a repressor protein while *mecRI* codes for a β-lactam-sensing transmembrane-signalling protein. These antibiotic resistant genes are believed responsible for the expression of methicillin resistance (Ubukata *et al*., 1990) and make the treatment of infection by this strains become difficult. *S. aureus strains* which conferred by these genes known as Methicillin-Resistant *S. aureus* (MRSA). In this study, 3 isolates of *S. aureus* obtained from Universiti Malaya Medical Centre (UMMC) were screened for the presence of *mecA*, *mecRI* and *mecI* gene. The primers used for the detection of the *mecA* gene have already been published by Murakami *et al*. (1991) while the primers used for the

Fig. 3: Amplification of *mecA* gene of MRSA isolates by PCR. Lanes 1-3 are the band of gene of untreated genomic, lane 4 is the gene of genomic treated with *G. changii*, lane 5 is the gene of genomic treated with *E. denticulatum* while lane M is the 100 bp molecular weight marker. *mecA* gene positive isolates have a single band present in the region between the ladders of 500-600 bp. The actual band position is at 533 bp. Lane C is a negative control showing no band indicating that there was no contamination during the process

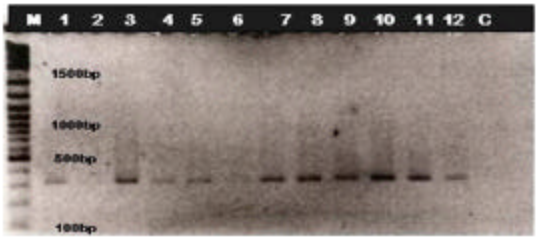

Fig. 4: Amplification of *mecRI* gene of MRSA isolates by PCR. Lanes 1-6 are the band of gene of untreated genomic, lanes 7-9 are the gene of genomic treated with *G. changii*, lanes 10-12 are the gene of genomic treated with *E. denticulatum* while lane M is the 100 bp molecular weight marker. *mecRI* gene positive isolates have a single band present in the region between the ladders of 300-400 bp. The actual band position is at 310 bp. Lane C is a negative control showing no band indicating that there was no contamination during the process

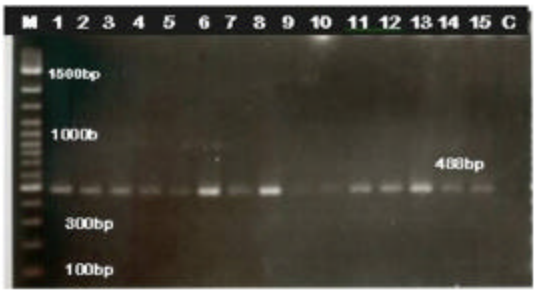

Fig. 5: Amplification of *mecI* gene of MRSA isolates by PCR. Lanes 1-6 indicate the band of gene of untreated genomic, lanes 7-10 are the gene of genomic treated with *G. changii*, lanes 11-15 are the gene of genomic treated with *E. denticulatum* while lane M is the 100 bp molecular weight marker. *mecI* gene positive isolates have a single band present in the region between the ladders of 300-400 bp. The actual band position is at 481 bp. Lane C is a negative control showing no band indicating that there was no contamination during the process

detection of the *mecRI* (membrane spanning part) and *mecI* gene were those published by Suzuki *et al*. (1993). The polymerase chain reaction assays then were performed using the template of untreated genomic and genomic treated with either *Gracilaria changii* or *Euchema denticulatum* extract.

For detection and amplification of those genes, the experimental conditions were re-optimized from the earliest time to obtain the best possible results whereas the template, primer, $MgCl_2$ and dNTP mix concentrations are optimized in the present study. For *mecA* and *mecRI* genes, the annealing temperature was increased to 59°C for 30 sec, the primer extension temperature was reduced to 55°C for 30 sec and the number of cycle was reduced to 25 cycles. Since the hot start technique was used, the denaturation time also were re-optimized for all genes.

All the isolates tested including the isolates treated with both seaweed extracts, harbored selected the antibiotic resistance genes as indicated by successful amplification shown by the strong positive signal of single DNA band for *mecA*, *mecRI* and *mecI* genes through PCR assay.

The successful amplification of the gene of interest is dependent upon the amount and quality of the template DNA. Nevertheless, in this study, the PCR was successfully performed even though the purity and the concentration of the genomic were low since DNA can be amplified even with 30-100 ng μL^{-1} of concentration.

CONCLUSION

As a conclusion from the results discussed above, the findings in this study assumed that the supplement of the methanol extract of *G. changii* and *E. denticulatum* into the MRSA isolates, will not deleting the antibiotic resistance genes in those isolates since the *mecA*, *mecR1* and *mecI* genes still were amplified in the treated samples.

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