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Isolation and Identification of Methicillin-resistance *Staphylococcus aureus* (MRSA) from Nasal of College Students

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In this study, classical and molecular methods were involved for isolation and identification of Staphylococcus aureus (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA). Identification of *S.aureus* involved tests such as Mannitol fermentation test, coagulase test and DNase test. Antibiotic susceptibility test which was carried out to explore MRSA antibiotic susceptibility patterns. Antibiotics involved were methicillin, oxacillin, vancomycin, tetracycline, cefoxitin, erythromycin, gentamycin, chloramphenicol, penicillin, ampicillin, and trimethoprim. Polymerase chain reaction (PCR) was performed to obtain more reliable result. Among 100 samples collected, totally 18 samples were confirmed as *S. aureus* based on molecular method. From these isolates, 16 of them showed presence of *mecA* gene and also *fem* gene(gene involved in the resistant of methicillin) thus making it positive as MRSA.

Key words: MRSA, nasal, *Staphylococcus*, antibiotics.

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium in a family of staphylococcae which is known as an important nosocomial pathogen. It is responsible in the infections associated with both hospital- and community- acquired diseases. Commonly, its colonization in human is found approximately 40% in the nasal passages. According to the Centers for Disease Control and Prevention (CDC), it is one of the common causes which bring about the human skin and soft tissue infections in the United States. The infections range from minor skin conditions such as pimples, boils and impetigo to more life threatening disease such as osteomyelitis, necrotizing pneumonia and infective endocarditis. Although the means of its treatment involve the use of antibiotics, however, it becomes more challenging especially when methicillin-resistant strains have emerged.

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In 1959, methicillin was introduced for the treatment of staphylococcal infection due to the development of resistance against penicillin. Though, an isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported later in 1961 thus becoming an endemic case worldwide in following years. Based on data from the National Nosocomial Infections Surveillance system, it was shown there is an increasing proportion of cases of MRSA, up to 59.5%-64.4%. Furthermore, it was reported that approximately 75% of hospital strains were resistant towards the effectiveness of methicillin[1]. In Malaysia, MRSA epidemiology studies conducted in certain hospitals in 1996 showed incidence rate as high as 40% [2].

S. aureus is resistant towards penicillin due to the production of B-lactamases which enables bacteria to hydrolyze the penicillins. Methicillin, an antibiotic that resists the action of this B-lactamase-producing strains, were then developed. However, strains resistant towards these agents soon emerged (3Barber, 1961). Three known mechanisms have been identified for *S. aureus* to become resistant to methicillin. It includes hyper production of B-lactamaes (4McDougal and Thornsberry, 1986), modification of normal penicillin-binding protein (PBPs)[5], and the presence of an acquired penicillin-binding protein, PBP2a (6Ubukata, 1985). MRSA strains possess the *mecA* gene, the structural determinant gene that encodes modified PBP2 protein which has low binding affinity for methicillin and all B-lactam antibiotics[7]). Thus, detection of this gene can be considered as marker for the detection of MRSA.

This study intended to isolate and identify *S. aureus* among college students in Malaysia as representative for the region of South East Asia by performing nasal swabs and detect the presence of MRSA among them with antibiotic susceptibility pattern and molecular method.

MATERIALS AND METHODS

Specimen Collection

This study involved 100 college students from University Putra Malaysia. Data collected in this study consists of age, gender, race, course of study, and history of flu or cold within one month which were collected for epidemiology purposes. Samples from nasal were collected from each of the participants by using sterile cotton swabs moistened with sterile phosphate buffer saline solution (PBS solution). The samples were then transported to the laboratory and incubated for 4-6 hours before cultured on blood agar. It is noteworthy to mention that this study was done according to the ethical committee regulations based on Helsinki agreement of humans and animals ethics of research.

Identification

Protocols on identification of *S. aureus* included gram staining and determination of its morphology, catalase test, mannitol salt fermentation, oxidation and fermentation test, tube coagulase test (using human plasma and rabbit plasma) and DNAse test.

Antibiotic susceptibility test

The antibiotic sensitivity test was done according to the Bauer-Kirby method[8]. Single, pure colony from the sample was inoculated in phosphate buffer saline solution (PBS solution).The density was adjusted to 0.5 MacFarland turbidity standard by using spectrophotometer. By using sterile cotton swab, the inoculum was inoculated on the entire surface of Muller-Hinton Agar (MHA). The step is repeated few times to get even distribution of inocula. The antimicrobial discs were placed onto the surface and the plate was incubated aerobically in inverted position for 24 hours in 37ºC. Antibiotic discs used were ampicillin, gentamycin, erythromycin, methicillin, vancomycin, tetracycline, oxacillin, cefoxitin, chloramphenicol, trimethoprim, and penicillin. The results obtained from this test were interpreted according to the recommendation of the National Committee for Clinical Laboratory Standards.

Preparation of genomic DNA

For DNA extraction, a commercial extraction kit was used (GeneJET™ Genomic DNA Purification Kit #K0721, #K0722) (Fermentas). Before that, single and pure colonies of bacterial cells were inoculated in LB broth and incubated overnight (~16-18 hours). $2x10^9$ bacterial cells were harvested in a 1.5 or 2.0 ml microcentrifuge tube by centrifugation for 10 mins at 5000 xg. The supernatant was discarded. The pellet formed was resuspend in 200µl of Gram-positive bacteria lysis buffer and then incubated for 30 min at 37ºC. 200µl

of Lysis solution and 20µl of Proteinase K were added and mixed thoroughly by vortexing or pipetting. The samples were incubated at 56ºC while vortex occasionally until the cells were completely lysed (~30 min).19µl of RNAse A solution were added, mixed by vortexed and incubated for 10 min at room temperature. 400µl of 50% ethanol is added and mixed by pipetting or vortexing. The prepared lysate was then transferred to a GeneJET™ Genomic DNA Purification collection tube containing the flow-through solution discarded. The GeneJET™ Genomic DNA Purification Column was placed into a new 2 ml collection tube. 500µl of Wash Buffer I (with ethanol added) were added and centrifuged for 1 min at 8000xg. The flow-through was discarded and the purification column was placed back into the collection tube.500µl of Wash Buffer II (with ethanol added) were added to the GeneJET™ Genomic DNA Purification Column and centrifuged for 3 min at maximum speed ($\geq$ 12000 xg). The collection tube containing the flow-through solution was discarded and the GeneJET™ Genomic DNA Purification Column was transferred to a sterile 1.5 ml microcentrifuge tube. 200µl of Elution Buffer were added to the center of the GeneJET™ Genomic DNA Purification Column membrane to elute genomic DNA. The sample was incubated for 2 min at room temperature before centrifuged for 1 min at 8000xg. The purification column was discarded. The purified DNA was ready to be used immediately in downstream applications or store at -20ºC.

Polymerase Chain Reaction (PCR) for Antibiotic Resistance Genes

PCR was performed to detect the presence of *ermA , ermB, ermC, msrA , linA* (9), *nuc ,fem* and *mecA* gene[10].

The master mix containing PCR buffer, dNTP mix, primer, Taq DNA polymerase and MgCl2 and template DNA was subjected for hot start PCR. The PCR conditions required for each gene are shown in Table 1. Following the amplification gel electrophoresis which was performed on 1% agarose gel, visualizing was carried out with ethidium bromide under UV transilluminator.

Statistical analysis

Data of the current study was processed using SPSS version 12.0.0.l. Data was checked for normal distribution curve and was found normally distributed. Therefore, parametric tests were used. P value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

From 100 samples collected, 26 % (26 out of 100 isolates) was suspected to be *S. aureus* since they showed positive results in mannitol salt fermentation test. From 26 samples, tube coagulase test (using human coagulase plasma) was performed. Based on the results obtained experimentally, 11 samples from them were identified as coagulase positive *S. aureus*. In contrast, when rabbit coagulase plasma was used in the tube coagulase test, an additional 2 more samples were observed for positive results in tube coagulase test. Thus, making it a total of 13 samples were identified as coagulase positive *S. aureus.* For an additional confirmation of identification of *S. aureus*, DNAse test was performed. Based on the results obtained, 16 isolates were shown positive by the formation of clear zone around the colony when a drop of hydrochloric acid (HCl) was dropped onto it.

The resistance of the identified *S. aureus* was checked by two methods conventional method which is represented by the antibiotic disk diffusion assay (Table 2) and molecular method targeting the resistance genes which is represented by PCR assay (Table 3). The results shown in tables 2 and 3 indicate clearly that the molecular method, namely PCR is more accurate and reliable.

Identification of *S. aureus* by tube coagulase test is commonly used by many developing countries either by using human or rabbit coagulase plasma. Although many practices prefer using human plasma in coagulase tests; however, it is reported that it tend to give discordant results[11]. Some of the reasons behind this is using outdated material from blood banks which contains variable amounts of Coagulase-Reacting Factor (CRF) and anti-staphylococcal antibodies[12]. Furthermore, there is a possibility that it contains high burden viral infections such as HIV/AIDS, Hepatitis B and C thus bring risks towards laboratory personnel. Besides, it brings about the issue of using human specimens[13]. Yet; research done has shown that the results obtained by the coagulase tests can be improved by testing the isolates with mannitol salt agar and DNAse test. Among these three phenotypic tests, a study

has shown that growth on MSA is the most sensitive then TCT (human and sheep plasma) and the least sensitive is DNase test[13]. The reason behind the effectiveness of MSA in detecting *S. aureus* may be due to its capability in isolating pathogenic *S. aureus*[14].

According to the data, 100% of this strain was resistant towards penicillin and ampicillin. Other than that, 61.5% showed resistance towards oxacillin, 57.7% resistance towards methicillin, 50% resistance towards erythromycin and cefoxitin, 42.3% towards vancomycin, 23.1% towards tetracycline and trimethoprim and 7.7% towards gentamycin and chloramphenicol. From the antibiogram results, all 26 isolates showed multi-drug resistance. Among the 11 types of antibiotics used, results were concerned towards 4 types of antibiotics which are: erythromycin, methicillin, oxacillin and cefoxitin.

For coagulase positive isolates, 7.7% (1 isolates) showed erythromycin resistance; 46.2% (6 isolates) were intermediate and 46.2% (6 isolates) were susceptible. In contrast, for coagulase negative, 12 isolates (92.3%) were resistant towards erythromycin, and 1 isolates (7.7%) was intermediate. For methicillin, coagulase positive isolates showed 30.8% (4 isolates) resistance, 38.5% (5 isolates) intermediate, and 30.8% (4 isolates) were susceptible. Vice versa, 84.6% (11 isolates) were resistant towards methicillin, and 2 isolates (15.4%) were susceptible for coagulase negative isolates. Among the coagulase positive isolates, 30.8% (4 isolates) were resistant for oxacillin and 69.2% (9 isolates) were susceptible. Whereas, 12 isolates (92.3%) from 13 isolates of coagulase negative were resistant towards oxacillin and only 1 isolate (7.7%) was susceptible by this antibiotic. For cefoxitin, 38.5% (5 isolates) from coagulase positive isolates were resistant and 8 isolates (61.5%) were susceptible. Among coagulase negative, 8 isolates (61.5%) were resistant towards cefoxitin, and 5 isolates (38.5%) were susceptible towards cefoxitin. In conclusion, among the 26 isolates, there were 9 MRSA, 4 methicillin-susceptible *S. aureus* (MSSA), 11 methicillin-resistant coagulase-negative staphylococci (MRCoNS) and 2 methicillin-susceptible coagulase-negative staphylococci (MSCoNS).

For PCR, the results are shown in Table 3.

Table 1. Lists of target genes

Target Gene	Primer sequences	PCR condition	Size (bp)	Ref.
ermA	5'-GTTCAAGAAC AATCAATACA GAG3' 5'-GGATCAGGAA AAGGACATTT TAC-3'	32 cycles of 94ºC for 30s, 52ºC for 30s and 72ºC for 60s	421	(13)
ermB	5'CCGTTTACGAAATTGGAACAGGTAAAGG 3' 5'-GAATCGAGAC TTGAGTGTGC-3'	32 cycles of 94ºC for 30s, 55ºC for 30s and 72ºC for 60s	359	(13)
ermC	5'-GCTAATATTG TTTAAATCGT CAATTCC-3' 5'-GGATCAGGAA AAGGACATTT TAC-3'	32 cycles of 94ºC for 30s, 52ºC for 30s and 72ºC for 60s	572	(13)
msrA	5'-GGCACAATAA GAGTGTTTAA AGG-3' 5'-AAGTTATATCATGAATAGATTGTCCTGTT3'	30 cycles of 94ºC for 60s. 50ºC for 60s, 72ºC for 90s	940	(13)
linA	5'GGTGGCTGGGGGGTAGATGTATTAACTGG3' 5'GCTTCTTTTGAAATACATGGTATTTTCGATC3'	32 cycles of 94ºC for 30s, 57ºC for 30s, 72ºC for 60s	323	(13)
mecA	Forward: TCCAGATTACAACTTCACCAGG3 Reverse: CCACTTCATATCTTGTAACG	4min at 94ºC: 30 cycles of 94ºC for 30s, 53ºC or 30s, and 50s; 4min at 72ºC	162	(14)
nuc	Forward : GCGATTGATGGTGATACGGTT Reverse:AGCCAAGCCTTGACGAACTAAAGC	94ºC for 4min; 32 cycles of 94ºC for 35s, 52ºC for 35s, 72ºC for 50s and 72ºC for 10 min	270	(14)
fem	Forward : CTTACTTACTGCTGTACCTG Reverse : ATCTCGCTTGTTATGTGC	94ºC for 4min, 32 cycles for 94ºC for 40s, 48ºC for 40s, 72ºC for 50s, and 72ºC for 10min.	684	(14)

PCR was used for the detection of antibiotic resistance genes in isolates. This technique has been proven to be an effective tool in the identification of *S. aureus* strains and in the detection of antibiotic resistance genes[15]. Amplification of 8 target genes was involved: *mecA, ermA, erm B, ermC, msrA, linA, nuc* and *fem* gene. The *nuc* gene encodes for a thermostable nuclease specific for *S.aureus* and the *mecA* gene encodes for PBP2a that confers resistance to B-lactam antibiotics[16]. Presence of erm genes (ermA, ermB, ermC) confers resistance to macrolide, lincosamide and streptogramin B (MLS_B) by target site alteration of the ribosome[9]. The *msrA* gene confers the so-called MS phenotype (inducible resistance to 14- and 15-membered ring macrolides and resistance to streptogramin type B after induction with erythromycin. The *linA* confers resistance to lincosamides only[17] while *fem* gene encodes a factor crucial for methicillin resistance and usually present in all *S. aureus* isolates[18]

According to the results of PCR, 16 out of 26 isolates (61.5%) yielded *mecA* gene , *nuc* gene and *fem* gene thus making it positive as MRSA. Among 18 isolates of *S. aureus*, only 2 isolates showed absence of *mecA* gene.

Two methods were involved for comparison of antibiotic susceptibility, namely between PCR and disc diffusion test. Firstly, we compared the results obtained for methicillin resistance by disc diffusion method and PCR. From the disc diffusion test, 15 isolates were found to be methicillin resistant. However, in PCR, 22 isolates showed the presence of *mecA* gene. Secondly, we compared the results obtained for the detection of erythromycin resistance between these 2 methods. Based on the disk diffusion test, it was found that 13 isolates were resistant towards antibiotic erythromycin. However in PCR, only 2 isolates were positive in showing the presence of *ermA* gene. Based on this study, it is clear that disc diffusion method gave different results from that of PCR;

Table 2. Antimicrobial susceptibility pattern by coagulase positive and coagulase negative isolates

Antibiotics	Coagulase positive			Coagulase negative			General for all isolates		
	S No. (%)	I No. (%)	R No. (%)	S No. (%)	I No. (%)	R No. (%)	S No. (%)	I No. (%)	R No. (%)
Erythromycin	6 (46.2%)	6 (46.2%)	1 (7.7%)	0 (0%)	1 (7.7%)	12 (92.3%)	6 (23.1%)	7 (26.9%)	13 (50%)
Penicillin	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	26 (100%)
Vancomycin	13 (100%)	0 (0%)	0 (0%)	2 (15.4%)	0 (0%)	11 (84.6%)	2 (7.7%)	0 (0%)	11 (42.3%)
Chloramphenicol	13 (100%)	0 (0%)	0 (0%)	10 (76.9%)	1 (7.7%)	2 (15.4%)	23 (88.5%)	1 (3.8%)	2 (7.7%)
Tetracycline	11 (84.6%)	0 (0%)	2 (15.4%)	2 (15.4%)	7 (53.8%)	4 (30.8%)	13 (50%)	7 (26.9%)	6 (23.1%)
Ampicillin	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	26 (100%)
Trimethoprim	5 (38.5%)	5 (38.5%)	3 (23.1%)	7 (53.8%)	3 (23.1%)	3 (23.1%)	12 (46.2%)	8 (30.8%)	6 (23.1%)
Methicillin	4 (30.8%)	5 (38.5%)	4 (30.8%)	2 (15.4%)	0 (0%)	11 (84.6%)	6 (23.1%)	5 (19.2%)	15 (57.7%)
Cefoxitin	8 (61.5%)	0 (0%)	5 (38.5%)	5 (38.5%)	0 (0%)	8 (61.5%)	13 (50%)	0 (0%)	13 (50%)
Oxacillin	9 (69.2%)	0 (0%)	4 (30.8%)	1 (7.7%)	0 (0%)	12 (92.3%)	10 (38.5%)	0 (0%)	16 (61.5%)
Gentamycin	13 (100%)	0 (0%)	0 (0%)	7 (53.8%)	4 (30.8%)	2 (15.4%)	20 (76.9%)	4 (15.4%)	2 (7.7%)

this indicates that the phenotypic methods are not 100% reliable as used to be reported by earlier research[19]. According to the current research done, classical susceptibility testing needs 2-3 days in obtaining the results, with several shortcomings. Different bacterial species have different susceptibility patterns to a given antibiotic; susceptibility tests give different readings when tested with the same antibiotic in triplicates under the same condition[19]. Hence, utility of PCR has been found as most reliable, fast and accurate in the detection of the mecA gene along with the possibility of simultaneous identification of *S. aureus* and detection of mecA gene[20].

Table 3.Isolate PCR results for all target genes

No. of	Presence of fragments							
Isolate	*mecA*	*nuc*	*ermA*	*ermB*	*ermC*	*msrA*	*linA*	*fem*
6	+	+	-	-	+	+	+	+
16	+	+	-	-	+	+	+	+
18	+	-	-	-	+	+	+	+
22	-	+	-	-	+	+	+	+
32	+	-	-	-	-	+	-	-
34	+	-	-	-	-	+	-	-
36	+	+	+	-	+	+	+	+
40	+	+	-	-	-	+	+	+
44	+	-	-	-	+	+	+	+
46	+	-	-	-	-	+	+	+
50	+	+	-	-	+	+	+	+
51	+	+	-	-	+	-	+	+
53a	+	+	-	-	-	+	-	+
53b	+	+	-	-	+	-	+	+
57	+	+	-	-	+	+	+	+
61	+	+	-	-	+	+	+	+
65	+	+	-	-	+	+	+	+
70	+	+	-	-	+	+	+	+
72	-	+	-	-	+	+	-	+
73	+	+	-	-	+	+	+	+
76	-	-	-	-	+	+	-	+
81	+	+	-	-	+	+	+	+
84	-	-	-	-	-	+	-	+
88	+	+	-	-	+	+	+	+
90	+	-	-	-	-	+	-	-
96	+	+	-	-	+	+	+	+

CONCLUSION

Taken together, nasals were shown to act as reservoir for MRSA in a high social class of community like university students. This indicated that no population sector is safe from getting MRSA. Moreover, this provided evidence that hygiene among university students must be improved in order to hinder MRSA spread and circulation. In addition, molecular methods of detection of MRSA were shown superior on other non-molecular methods.

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