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Carriage Patterns and Susceptibility Testing of *Staphylococcus aureus* in Healthy Nasal Carriers in UiTM

Staphylococcus is a group of gram-positive bacteria that can cause illness ranging from mild to a number of severe infectious diseases. These bacterial species of the genus *Staphyloccus* are normal inhabitants of the nostrils in the healthy population with carrier rates between 20% to 55%. This study aims to classify these adults into persistent, intermittent or noncarriers, to differentiate *Staphyloccoccus aureus* (*S. aureus*) from other Gram positive cocci and also to obtain the antibiotic sensitivity patterns in this population. From 422 nasal swabs collected, 66 (16%) were *S. aureus* carriers. Thirty (7%) were persistent carriers (index >0.8-1.0), 36 (9%) were intermittent carriers (index between 0.1-0.7) and 356 (84%) were non-carries (index 0). Only 202 samples were further examined for species differentiation. A total of 37 (18.3%) were identified as *S. aureus*, 156 (77.2%) *S. epidermidis*, 8(4%) *S. saprophyticus* and only 1(0.5%) methicillin resistant *S. aureus*. From these 202 samples, 13/38 (34.2%) were susceptible to all the 12 antibiotics tested, and 20/38 (52.6%) were resistant to penicillin alone. Tetracycline resistance was 13.2% and low percentages of this *S. aureus* were resistant to erythromycin (2.6%) and gentamycin (2.6%). Apart from the phenotypic methods, further reconfirmation was performed by the amplification of *nuc* gene. It is recommended that both molecular and phenotypic analysis for *S. aureus* can be used to achieve a rapid and accurate identification. In conclusion, this study found that some of the students are intermittent carriers or persistent carriers while majority are noncarriers with no serious threat to methicillin or vancomycin resistance.

Keywords: *Staphylococcus aureus*, nasal carriers, phenotypic analysis, molecular diagnosis

INTRODUCTION

The anterior nostril is the primary resident of *Staphylococcus aureus* (*S. aureus*). Various cross-sectional studies of healthy adults have shown that the prevalence of *S. aureus* range from 19% to 64% {1}. *S. aureus* nasal carriage has been extensively studied in patients and healthy individuals {1,2,3,4}. About 30% of human population carry *S. aureus* in the nose, which is an important risk for nosocomial infection {4}. However, by longitudinal study, carrier patterns with different percentages were reported to differ between individuals. Three types of nasal carriers have been described: 20% persistent carriers, 60% intermittent carriers and 20% non-carriers {1}. Although the criteria for these classification varies in terms of number of specimens collected and the follow-up period, many studies have indicated the importance of distinguishing persistent and intermittent carriers. The persistent nasal carriers have been associated with higher numbers of *S. aureus* in the nostrils and more extensive dispersion in the environment and nosocomial infection in patients undergoing surgery {3}. *S. aureus* nasal carrier have a three-fold higher risk for bacteremia compared to non-carrier {5}.

Traditionally, bacterial infection by *S. aureus* is confirm by culture and followed by phenotypic analysis based on the characteristic bacteria morphology on blood agar, mannitol salt agar and nutritional requirements. However, these techniques can lead to misidentification and time consuming. Rapid and accurate identification of clinically important disease-causing agent including antibiotic testing is now a prerequisite for disease control and epidemiological surveillance {6}. With the advent of PCR method, the potentials for the rapid diagnosis of *S. aureus* have been applied which targets the Sa442 fragments {7), *femA/femB* gene {8} and *mecA* gene {9}.Antibiotic choices are classified into preferred and alternative recommendations based on clinical evidence of effectiveness {10}. Although all Malaysian hospitals have microbiological culture and antibiotic sensitivity testing facilities, only 20% of therapeutic prescriptions were based on microbiological test results {11}. The remaining 80% prescriptions were not based on laboratory testing on bacteria cultures. Antibiotic resistance is belief to be caused by use or misuse of antibiotics in the hospitals. This study therefore investigate the identification of the bacteria present in the nostrils with specific focus on *S. aureus, S. aureus* carrier types, and *S. aureus* antibiotic sensitivity patterns among the healthy individuals.

MATERIALS AND METHODS

Specimen collection and culture isolation

Single right nostril samples were taken from 422 students from the Nursing Program and Medical Laboratory Technology Program (MLT), Faculty of Health Sciences, UiTM. The sex, age, ethnic group and record of health exposure were recorded using a Survey Form which has been approved by the Research Ethics Committee [REC/UiTM/2007(17)] UiTM. To determine the *S. aureus* carrier index, swabs were taken from the same students on the 0^{th} week, 5^{th} week and 9^{th} week.

The nasal swab specimens were taken using sterile dry cotton-wool swabs. The anterior nasal vestibule of the right nostril were swabbed with cotton-wool swabs which were moistened with sterile phosphate-buffered saline prior to inserting into the nostrils. The swab specimens were inoculated immediately onto mannitol salt agar (MSA) (BML.01225) and incubated at 37ºC for 24 to 48 hours in an aerobic atmosphere. *S. aureus* (ATCC® 25923) was included as positive control.

Culture identification

All isolates were identified by conventional methods: gram-positive cocci by Gram-stain, catalase and mannitol positive. On MSA, *S. aureus* colonies were recognised as yellow colonies and *S. epidermidis* were red colonies, a distinguishing test between the two strains {12}.

S. aureus were confirmed by the coagulase plasma test and production of clumping factor using a commercial test, Avipath STAPH OD044 (Omega Diagnostics). To confirm for presence of any methicillin resistant *S. aureus* (MRSA), a qualitative slide latex agglutination test was used. All the *S. aureus* positive colonies were then subcultured onto blood agar and fresh cultures (19-24 h) were extracted for penicillin-binding protein 2'(PBP2') and finally the supernatant was used to test for MRSA by latex agglutination (MRSA-Screen, Denka Seiken).

Antibiotic susceptibility testing

For antibiotic susceptibility tests (AST) by disc diffusion method, three to five similar colonies (both control strain and tests) were picked up using sterile disposable wire loop. The colonies were suspended in 5 ml of Mueller-Hinton (MH) broth which were incubated at 37^0C. The 0.5 McFarland standard containing approximately 1 x 10^8 CFU/ml {12,13} was used as the turbidity standards for AST. This was done by optical density measurement at 625 nm using a spectrophotometer. The optical density should be around 0.08 to 0.10.

The inhibition zone diameters around each disc on MH agar were measured (in mm) and interpreted based on National Committee for Clinical Laboratory Standards (NCCLS) zone diameter interpretative standard.

Extraction of Bacteria Genomic DNA

For extraction of genomic DNA, 5 ml of overnight *S. aureus* culture in brain heart infusion suspension was centrifuged at 4,000 x g for 5 min. The pellet was resuspended in 2 ml ice-cold 70% ethanol for 30 min and centrifuged at 12,470 x g for 5 min in refrigerated microcentrifuge. The resulting precipitate was resuspended in 150 μl Tris-EDTA buffer pH 8.0 and the cell was lysed by adding 10 μl of 10 mg/ml lysozyme. This suspension was vortexed and incubated for 30 min at 37°C in waterbath. The lysate was boiled at 100°C in boiling water for 10 min and centrifuged at 12,470 x g for 5 min. The clear supernatant was removed into sterile 1.5 ml microcentrifuge tube and 10 μl of 20 mg/ml Proteinase K was added, mixed for 30 min and incubated for 30 min at 37°C in waterbath and finally boiled for 15 min to stop enzyme activity. The extracted DNA was kept in freezer at -20°C until further use {14}.

Polymerase Chain Reaction Amplification of Bacterial DNA

The 267-bp sequence of *nuc* gene was amplified from previous extracted bacterial DNA by polymerase chain reaction (PCR). The primer sequence were as follows: forward primer (*nuc* 1: 5'-GCG ATT GAT GGT GAT ACG GTT-3') and reverse primer (*nuc* 2: 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3') {15}. This PCR amplification was performed in a Px2 Thermal Cycler (Thermo Electron, USA) using a 5 μl DNA extracted from the bacterial culture described above. The extracted DNA was added to 20 μl amplification mixture containing 2.5 μl of 10X amplification buffer (100 mM Tris-Cl pH 8.3, 500 mM KCl and 15 mM $MgCl_2$), 1.5 μl of each 10 μM *nuc* 1 and *nuc* 2 primers, 0.5 μl of 10 mM dNTP mixture (Vivantis), 0.4 μl of 5u/μl *Taq* DNA polymerase (Vivantis) and 13.6 μl of sterile distilled water. The reaction mixture was briefly centrifuged for 30 sec (Sorvall Legend Micro 17R centrifuge) to bring down all reagents and extracted DNA to the bottom of the thin wall PCR tubes.

The target gene was amplified using the following profile: one cycle of initial denaturation at 94°C for 2 min; thirty five cycles of PCR consisting of denaturation, annealing, and elongation at 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min, respectively; and one cycle of final elongation at 72°C for 1 min and final hold time at 4°C.

Agarose Gel Electrophoresis.

The PCR-amplified samples were analyzed by using a 1.5% agarose gel using the Sub-cell® GT Agarose Gel Electrophoresis System (Bio-Rad). Once solidified, the gel was placed into the buffer

chamber containing 1X TBE buffer (45mM Tris-borate and 1 mM EDTA, pH 8.3). A 3μl DNA ladder (VC100bp Plus, Vivantis) was mixed with 2 μl 6X loading dye (Vivantis) and loaded in the first well of the gel as a DNA marker. For negative control, positive control and each amplicon, 5 μl were mixed with 2 μl 6X loading dye (Vivantis) and loaded into respective wells. Next, the electrophoresis was carried out by applying constant voltage at 90V for approximately 1 h. At the end of the electrophoresis, 0.003% (w/v) ethidium bromide solution was used as the stain and the PCR products were visualized and photographed on a transilluminator (Bio-Rad). Amplified products were sent to an independent laboratory and sequencing results obtained were then analysed phylogenetically.

RESULTS

Culture Isolation and Antibiotic Testing

The carrier index were defined as the number of nasal swab specimen cultures that grew *S. aureus* divided by the total number of nasal swab specimen cultures performed for that person. Persistent nasal carriers comprised those persons with carrier indices of 0.80 or higher, intermittent carriers were those with carrier indices between 0.1 and 0.70, and non-carriers were those with indices of zero {1}.

Sixty-six out of the 422 (16%) samples were Gram-positive cocci, non-motile, non-spore formers, catalase-positive, coagulase positive and mannitol fermenters. Amongst the 66 *S. aureus* carriers, 30(7%) were persistent carriers (index >0.8-1.0), 36(9%) were intermittent carriers (index between 0.1-0.7) and 356(84%) were non-carriers (index 0) (Table 1). Our results showed the distribution of *S. aureus* among the students by gender, age, programme and carrier status. Only 202 samples were further examined for other than *S. aureus* out of which 156 (77.2%) were *S. epidermidis*, 8(4%) *S. saprophyticus* and 1(0.5%) MRSA.

For antibiotic sentitivity testing, the average zone size was interpreted according to NCCLS standard. The percentage of sample resistant, moderate resistant and susceptible to antibiotics is shown in Figure 1. A total of 34.2% (13/38) of the strains were fully susceptible to all the 12 antibiotics tested; 52.6% (20/38) resistant to penicillin alone; 5.3% (2/38) were resistant to two antibiotic (penicillin and tetracycline); 2.6% (1/38) was resistant to penicillin and tetracycline but moderately resistant (intermediate) to fusidic acid; 2.6% (1/38) was moderately resistant (intermediate) to three antibiotics (erythromycin, fusidic acid and tetracycline); and 2.6% (1/38) were resistant to more than three antibiotics (oxacillin, penicillin, tetracycline and gentamicin). Tetracycline resistance was 13.2% and low percentages of this *S. aureus* were resistant to erythromycin (2.6%) and gentamycin (2.6%). One strain was MRSA. There was no zone of inhibition around the oxacillin disc and for tetracycline, penicillin and gentamicin discs, the zone size were very small {16}.

Confirmation of S. aureus by Genomic Amplification and Sequencing

PCR analysis showed that 16 of the isolates that were further reconfirmed by amplification of *nuc* gene were in concordance with catalase, coagulase and DNAse test. The PCR product appeared as a sharp band with 279-bp thus confirming the *nuc* gene in the amplified products (Figure 2). Further analysis of the amplicons by sequencing and phylogenetic tree shows that the local strain labelled as UiTM 115 was the closest strain to the ATCC strains (Figure 3), and clustered into the ATCC 19258. The rest belong to other clusters which probably are novel species and have yet to be studied further by other method like DNA-DNA hybridization method.

DISCUSSION

S. aureus is one of the most common cause of community and health-care associated infection, however there is a need to gather more information on the carrier types and its effects in the population as a whole. In 1986, Soon *et al.* reported 55% *S. aureus* within the outpatient community and 76% within the hospital staff {17}. Other studies showed that the percentage varies from 37% in USA, 19% in Scotland, and 22% in Japan {1}. In 2007, screening for resistant strains of Staphylococci and MRSA has been carried out in a medical college to help monitor the student population who might pose a risk to patients and hospital personnel and the community at large {18}. They found that the carrier rate was lower in the Indian medical students (16.6%), while the Chinese medical students were higher *S. aureus* carrier rate. In another report, the *S. aureus* colonization rate in adults was reported to be 40% at any given time {19}. Our study showed that 16% of the students were *S. aureus* carriers. This carrier rate was lower than and the variation compared to those reported rates may be due to sampling, culture techniques and study population. Therefore, for follow-up, it is recommended that the same protocol should be strictly carried out. The low *S. aureus* positive rate among the students is expected since most of them have no record of hospital stay, frequent hospitalization, surgery, dialysis, living in a long-term care facility during the previous 12 months, or prior MRSA carriage.

Since nasal carriage is a recognized source of *S. aureus* with severe consequences, persistent and intermittent carriers should be follow-up {20, 21}. A very recent report have showed that staphylococcal infections were reported to have no racial predilection {19}. Since majority of the students in

UiTM are Malays therefore our study cannot validate this result. However, this can be expanded later to the general population.

Age was identified in earlier studies as a factor influencing nasal colonization with *S. aureus* {20}. Newborns have high rates of *S. aureus* colonization ranging from 59% to 100% within a few days of birth, and these rates were reported to decrease to "adult" carrier rates (10% to 35%) by 5 to 6 years of age {20}. From many longitudinal studies, persistent carriage was reported to be more common in children than in adults, and the carrier state changed in many people between the ages of 10 and 20 years and over time for some individuals {22}.

This trend toward a decline in *S. aureus* carriage of advancing age cannot be concluded in our present study since no *S. aureus* carrier index was performed for those in the 5-6 years old. In our study the mean age of students was 20 years. Male gender has also been reported in several studies as a risk factor for nasal carriage of *S. aureus*, unfortunately the reason for this association remains unclear. In this present study, the male population was only 68 and results showed that only 2(0.5%) males were persistent carriers and 4(1%) males were intermittent carriers. In the females, 28(8%) were persistent carriers and 32(9%) were intermittent carriers. The presence of *S. aureus* among the nursing students and medical laboratory technology (MLT) students were higher (76%) in the former compared to 24% in the latter.

Most healthy carriers have *S. aureus* on their skin without showing any active infection or disease or colonization. Proportions of nasal carriage patterns differ, depending on the study design with respect to the number of nasal specimen cultures that are performed, the population studied, the sampling and culture techniques applied, the follow-up period, and the definitions for persistent, intermittent and non-carrier in a longitudinal study {21}. Despite this lack of consistency, several studies have shown the importance of distinguishing persistent from intermittent nasal carriage. Other risk factors for infection include age, dialysis, repeated rupture of the skin, and underlying conditions such as renal or liver disease, and diabetes.

In an attempt to define the prevalence of *S. aureus* according to types of carrier status, 24% were persistent carriers and 57% intermittent carriers in Scotland, and 12% persistent carrier and 19% intermittent carrier in Japan {1}. Our present study indicated a lower rate, 30 (7%) persistent carriers and 36 (9%) intermittent carriers. Previous study by Choi {21} showed the prevalence of *S. aureus* nasal carriage was 23.4% among healthy adults but no assessment on carrier types were performed. In general, nasal carrier rates among hospital personnel and patients were 60%-70% as compared to community carriers (30-50%) {4}. In several studies, the elimination of nasal carriage reduced the incidence of *S. aureus* infections. Klutymans et al {2} observed a significant reduction in the rate of surgical-would infection after intervention with mupirocin nasal ointment and reduction by a factor of four in the incidence of *S. aureus* bacteremia per patient year in carriers receiving hemodylisis. A substantial proportion of cases of *S. aureus* bacteremia appear to be endogeneous origin originating from colonies in the nasal mucosa {22, 21}. Therefore, measurement of colonization prevalence provides a useful estimate of the potential for development of staphylococcal disease in the population {23}.

A comparison of the antibiotic sensitivity patterns of *S. aureus* in the community showed that penicllin resistant was 82.7% but 100% sensitive for vancomycin {22}, which is in concordance to our findings. Methicillin sensitive *S. aureus* showed multiple-resistance trait against amplicillin, norfloxacin and fusidic acid {24} which was similar to our report on fusidic acid. Resistance towards fusidic acid may develop if the drug is used alone {25}. In Malaysia, a fusidic acid-rifampicin combination is used as an alternative oral regimen for the treatment of bacteraemia, musculokeletal and cardiovascular infections caused by MRSA {26}. Rifampicin, vancomycin, chloramphenical, ciprofloxacin, clindamycin, and trimethoprim/sulfamethoxazole remain susceptible in our study.

As seen in Figure 1 clindamycin was fully sensitive compared to erythromycin. Both belonged to the macrolide groups and this was expected since this antibiotic was not available in the hospital {27}. There is no report of increased resistance levels in the hospital strains {28, 29}, thus the precise relationship between antimicrobial use and antimicrobial resistance in both the hospital and community settings is not absolute {30}.

As a comparison to a local study done by a private practitioner in the Klang Valley, 18.4% *S. aureus* were mainly isolated from wound, pus and ear swabs. Not one of the 218 strains were resistant to methicillin however 91% were resistant to penicillin while 23% were resistant to tetracycline and 13% to erythromycin {31}. The AST pattern from the healthy carriers (students) revealed less resistance compared to clinical isolates (65.8% resistant to penicillin, 13.2% to tetracycline and 2.6% to erythromycin), respectively.

Community-associated methicillin-resistant *S. aureus* infections are an emerging problem in the US. If adequate measures are not taken to thoroughly understand and control its changing epidemiology and clinical presentation, it may

become a significant public health problem in the near future {32}.

DNA-based typing methods are universally applicable to all bacteria with differences only in the use of particular restriction enzymes, hybridization probes and PCR primers. The most often used method for typing is PFGE for surveillance typing system. More sophisticated ones include the QPCR, DNA-microarrays and PNA probes. As they are technically demanding and expensive, these methods are unlikely to be routine used in the foreseeable future {33}. Thus, a simple and precise method for the specific identification of *S. aureus* using *nuc* gene will contribute to future clinical findings for *S. aureus* infections.

PCR can discriminate between species correctly, furthermore conventional processing of samples takes 2 to 3 days before a definitive identification can be achieved. Genotyping sequence obtained from a strain can be compared with that of other strain and thus help in identifying the genetic differences between the clones {32}. Sequence typing shows relatedness of the local strains to other strains available in the GenBank database. This study therefore warrants further testing with *nuc* gene but using other samples like pus, blood or body fluids. To increase the validity and reliability of the results, larger number of samples should be tested. The PCR for amplification of the *nuc* gene has potential for the rapid diagnosis of *S. aureus* infections by direct clinical testing of clinical specimens, including speciments from patients with ongoing antimicrobial therapy {15}. Active surveillance of *S. aureus* infections and molecular analysis of virulence genes will help in understanding the trends, meanwhile, treatment of all *S. aureus*-related infection originating in the community should be guided by susceptibility tesing of the community strains.

In conclusion, this study confirms the students are health carriers of *S. aureus*. Using the carrier index calculation, majority are non-carriers and among the positive cases, most of them are intermittent carriers and the remaining are persistent carriers. The carrier rate is higher in the female (17%) and only 8% in the male students. It also shows that the rate of MRSA carriage remains very low in the community. The antibiotic resistance is lower compared to clinical cases. The present findings indicate the potential danger of dissemination of *S. aureus* among the nursing students when they are going to perform their duties among the patients. Since this is only a preliminary work among the students, it is best if clinicians and hospital infection control personnel be aware of *S. aureus* prevalence among these potential health workers. There is little prospect of eradicating *S. aureus* disease in the short term, so what will be important is collaborative research with free dissemination of information like this. Continued surveillance of *S. aureus* carriage will help to determine future trends in the characteristics of carriage and the potential effectiveness of targeted population-based intervention.

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Table I. Distribution of Gender, Age and Program by SA Nasal Carriage State in Healthy Adults.

Characteristics	*S. aureus* nasal carrier state			
	Noncarriage (n=356)	Intermittent (n=36)	Persistent (n= 30)	Overall (n=422)
Male	62	4	2	68
Female	294	32	28	354
Male/female ratio	0.2 (84%)	0.1 (8.5%)	0.1 (7.1%)	0.2 (100%)
Age (yr) (mean)	20	21	20	20
Programme (no. [%] of subjects)				
Nursing	269[76]	29[81]	23[77]	321[76]
MLT	87 [24]	7[19]	7[23]	101[24]
Overall	84.3%	8.5%	7.1%	100%

n=422, p=0.55, CI=95% , d=0.05

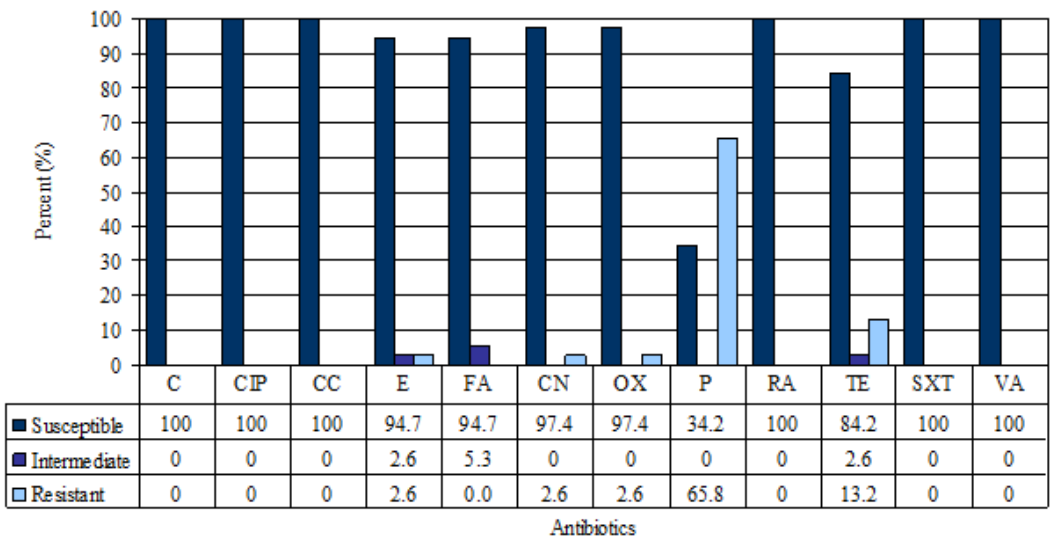

Figure I. Percentage of antibiotic susceptibility of Staphylococcus aureus.

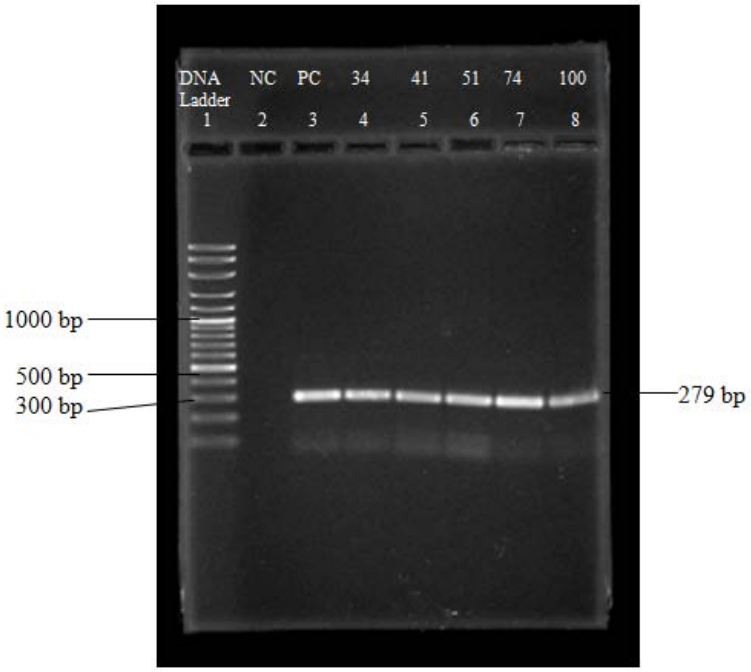

Figure II. Agarose gel electrophoresis of amplified DNA fragment with 279 bp size corresponding to *nuc* gene of *Staphylococcus aureus.*

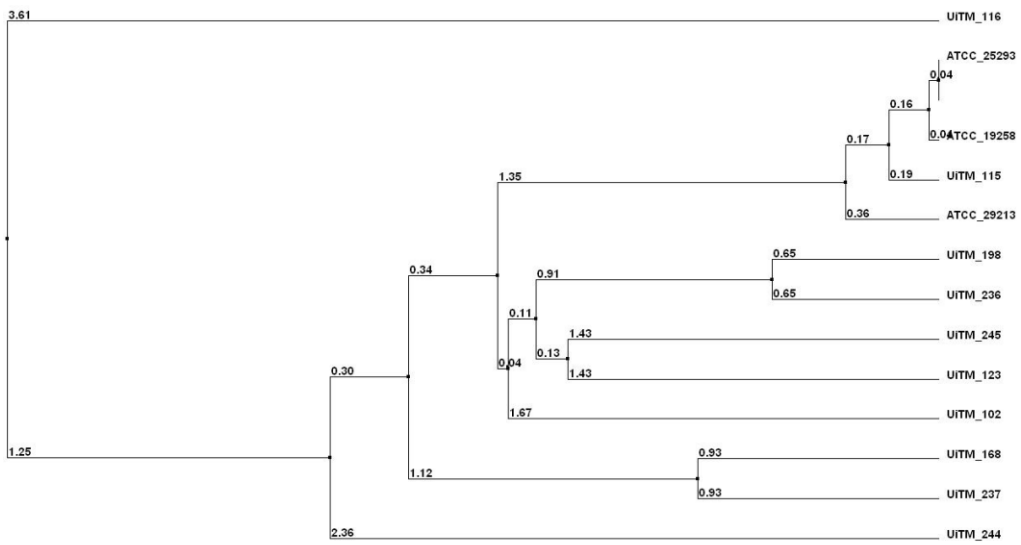

Figure III. Phylogenetic tree of local strains labeled as UiTM 116, 115, 168, 237, 198, 236, 245, 123, 102 and 244 which were amplified in this study. The tree was built with three reference strains *Staph.aureus* ATCC 25293, ATCC 19258 and ATCC 29213 for comparison.