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Full Length Research Paper

Isolation and identification of methicillin-resistant *Staphylococcus aureus* from students' coins

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial pathogen of increasing risk on community. This study aims at determining the risk of MRSA transfer from coins as silent and underestimated reservoir in community. One hundred swabs from coins were collected from college students in Malaysia. A series of identification and differentiating tests were conducted for precise identification of MRSA bacteria. Moreover, this study compared the efficacy of the different identification tests with gold standard, polymerase chain reaction (PCR) assay. The tests used were tube coagulase, DNase agar test, antibiogram, several routine biochemical identification tests and PCR assays. PCR assay used specific primers for resistance or ID -related genes: *mecA, ermA, ermB, ermC, msrA, linA, femA,* and *nuc* genes. A total of 37 bacterial isolates were isolated from college students' coins; non-PCR assays of identification and resistance detection revealed the presence and spread of MRSA in coins of 2 college students. PCR-amplification of the *nuc* gene was used as a baseline test to detect *S. aureus*. PCR showed only one isolate as true MRSA. False positive MRSA by disc diffusion assay might be attributed to low pH and high thymidine content of Muller Hinton agar medium. Collectively, coins proved to be possible source for the transfer of MRSA in community of college students in South East Asia. Moreover, PCR assay for identification of *S. aureus* resistance proved to be superior on other methods.

Key words: *Staphylococcus aureus*, MRSA, nail, PCR, resistance.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become more prevalent as nosocomial pathogens causing severe infections (Michael et al., 1996). MRSA is caused by a strain of Staphylococcus bacteria that became resistant to beta-lactams antibiotics especially methicillin, cefoxitin and gentamicin. The mean incidence of MRSA has drastically increased and become a worldwide problem (Liu et al., 2009). The emergence of MRSA infection can be acquired outside healthcare settings in the community (Kerttula et al., 2004).

The resistance to methicillin is due to a penicillin-binding protein encoded by a mobile genetic element

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called the methicillin-resistant gene (mecA) and erythromycin-resistance methylase (*erm*C). The *mec*A gene contained in the *mec* region primarily mediates MRSA's resistance towards methicillin (Tattevin et al., 2008). Recently, MRSA strains became resistant to several different antibiotics such as penicillin, oxacillin, and erythromycin (Kerttula et al., 2004).

Although, the respiratory tract, opened wounds, nails, coins, keys and hand phones are also potential sites for infection, MRSA usually colonizes the anterior nares (the nostrils). Hence, there is high opportunity of bacteria such as MRSA to get into fingers, nails and coins. Studies of bacteria on coins of money are scarce. A study in the Journal of the American Medical Association studied bacteria from 200 coins and found harmful germs, such as fecal bacteria and *S. aureus*, on 13 percent of coins in 1972 (Tolba and Loughrey 2007).

Healthy individuals may carry MRSA asymptomatically through coins for periods of times from a few weeks to many years. Compromised patients with weak immune systems have higher risks for symptomatic secondary infections. Most MRSA isolates are acquired from patients with prolonged hospital stay, patients from burn units and surgical wards, and from patients who have undergone invasive procedures (Valles et al., 1997). The important method for the control of MRSA infection is the prevention of transmission among hospitalized patients. Unrecognized MRSA carriers appear to be continuous sources of infection as they are not isolated (Jann et al., 2009). Therefore, we studied and investigated the presence of MRSA in coins among the college students. Therefore, the objectives of this study are isolation and identification of MRSA from college students' coins, identification and confirmation of MRSA by biochemical tests, identification of MRSA using antibiogram test, identification of MRSA using primer genes via molecular method (polymerase chain reaction), and determination of the circulation of MRSA through coins and spot out the potential site of colonization.

MATERIALS AND METHODS

One hundred sampling collection were consecutively carried out in this study. Among the samples coins from 100 college students (27 males and 73 females), the range of age was 19 to 25 years old. 71 Malays, 26 Chineses and 3 Indians were involved. A criterion for inclusion was the history of flu within one month. The study was performed from September 2010 to February 2011.

Phenotypic identification of *S. aureus*

After swabbing, cotton swabs were immediately put in the sterile screw cap test tube with 5 ml of nutrient broth at 37°C for 2 to 6 h. Samples were placed on blood agar and incubated at 37°C for 24 h. Positive control ATCC MRSA and negative control *Escherichia coli* were included. Bacterial growth was observed. Growth of large, golden or opaque colonies stained Gram positive under microscope was suspected to be *S. aureus*. The suspected *S. aureus* isolates were further confirmed by growing yellow colonies on mannitol salt agar (MSA), tube coagulase test (TCT), DNase test and catalase test (Rushdy et al., 2007)

Tube coagulase test is a definitive test for *S. aureus.* The capacity of plasma to clot depends on the presence of strain of *S. aureus*. Growth of yellow colonies on MSA surrounded by yellow zones after 24 h of incubation at 37°C indicated a positive result (David and Daum, 2010). Mannitol salt agar positive was considered as *Staphylococci.*

DNase test is an alternative test to differentiate *S. aureus* from the coagulase-negative staphylococci. *S. aureus* produces a DNase that can be diffused from a colony and hydrolyze DNA within a plate. A few drops of hydrochloric acid (HCl) were dropped on the DNase agar and covered all the colonies. Excess HCl were removed by Pasteur pipette and clear zones around the bacterial colonies indicated DNase positive colonies.

Antibiotic susceptibility tests (Disc diffusion)

The Clinical and Laboratory Standards Institute recommended that antimicrobial susceptibility tests were performed by disc diffusion methods. Eleven antimicrobial agents were tested, which were methicillin (10 µg), oxacillin (1 µg), penicillin G (1 µg), erythromycin (30 µg), ampicillin (2 µg), vancomycin (30 µg), gentamicin (10 µg), tetracycline (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg) and trimethoprim (5 ug). Positive control strain *S. aureus* ATCC was included in this study.

Direct colony suspension method in which a large single colony from fresh nutrient agar plates was inoculated into 5 ml of PBS and checked the turbidity of 0.5 McFarland. A sterile cotton swab was dipped into the culture and plated on Mueller- Hinton (MH) agar plate. The plate was then rotated to a 60° angle and swabbed in the same manner. The plate was rotated two times to ensure an even distribution of the whole agar. The rim of agar was also swabbed.
Eleven types of antibiotic discs were placed aseptically using a sterile forceps on the surface of MH agar plate. The disc must be pressed and diffused immediately upon contact with the agar. Therefore, extra care must be taken to avoid misplacement of antibiotic discs. MH agar plates were incubated inversely at 37°C for 24 h.

After 24 h, the MH agar plates were observed and the diameter of the zone of inhibition was measured in millimeter. The resistance or susceptibility of the isolates to the antibiotic discs was determined according to the CLSI standard and the results were recorded. The standard of each antibiotic are listed in Table 1.

Preparation of genomic DNA and PCR procedures (Genotypic identification)

Total bacterial DNA was extracted using GeneJET Genomic DNA Purification Kit. Two milliliter (2 ml) of bacterial suspension in microcentrifuge tube were centrifuged at 10000 rpm for 10 min and the supernatant was discarded. The pellet was resuspend in 200 µl of Gram-positive bacteria lysis buffer and then incubated for 30 min at 37°C. 200 µl of Lysis Solution and 20 µl of Proteinase K were added. Then, it was mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. The sample was incubated at 56°C while vortexing occasionally for around 30 min. Twenty milliliter (20 µl) of RNase A solution were added and mixed by vortexing and incubated for 10 min at room temperature. Afterwards, 400 µl of 50% ethanol were added and mixed by pipetting or vortexing. The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube and then the column was centrifuged for 1 min at 6000 rpm. The collection tube containing the flow-through solution was discarded and placed the column into a new 2 ml collection tube. Then, 500 µl of Wash Buffer I was added and centrifuged for 1 min at 8000 rpm. Then, the flow-

Table 1. CLSI standards of antibiotics inhibition zone diameter measurement.

Antimicrobial agent	Disk content (µg)	Diameter zone (mm)		
		R (resistant)	I (intermediate)	S (sensitive)
Methicillin (MET)	10	≤ 9	10-13	≥ 14
Penicillin G (P)	1	≤ 28	-	≥ 29
Oxacillin (OX)	1	≤ 10	11 – 12	≥13
Erythromycin (E)	30	≤13	14 - 22	≥23
Vancomycin (VAN)	30	-	-	≥15
Gentamicin (CN)	10	≤12	13 - 14	≥15
Tetracycline (TE)	30	≤14	15-18	≥19
Trimethoprim (W)	5	≤10	11-15	≥16
Ampicillin (AMP)	2	≤26	27-35	≥36
Chloramphenicol (C)	30	≤18	19-26	≥27
Cefoxitin (FOX)	30	≤18	19-26	≥27

Table 2. Primers used in this study.

Target genes	Primer sequence (5'-3')	bp
mecA (f)	TCCAGATTACAACTTCACCAGG	162
mecA (r)	CCACTTCATATCTTGTAACG	162
ermA (f)	GTTCAAGAACAATCAATACA	421
ermA (r)	GGATCAGGAAGGACATTT	421
ermB (f)	CCGTTTACGAAATTGGAACAGGTAAAGGGC	359
ermB (r)	GAATCGAGACTTGAGTGTGC	359
ermC (f)	GCTAATATTGTTTAAATCGTCAATTCC	572
ermC (r)	GGATCAGGAAAAGGACATTTTAC	572
msrA (f) `	GGCACAATAAGAGTGTTTAAAGG	940
msrA (r)	AAGTTATATCATGAATAGATTGTCCTGTT	940
linA (f)	GGTGGCTGGGGGGTAGATGTATTAACTGG	323
linA (r)	GCTTCTTTTGAAATACATGGTATTTTCGATC	323
femA (f)	CTTACTTACTGCTGTACCTG	684
femA (r)	ATCTCGCTTGTTATGTGC	684
nuc (f)	GCGATTGATGGTGATACGGTT	270
nuc (r)	AGCCAAGCCTTGACGAACTAAAGC	270

through was discarded and the purification column was placed back into the collection tube. The suspension was added with 500 µl of Wash Buffer II and centrifuged for 3 min at maximum speed 140,000 rpm. The collection tube containing the flow-through solution was discarded and the column was transferred to a sterile 1.5 ml microcentrifuge tube. Then, 200 µl of Elution Buffer were added to the column membrane to elute genomic DNA. Then, it was incubated for 2 min at room temperature and centrifuged for 1 min at 8000 rpm. The purification column was discarded. Extracted DNA was stored at -20°C.

PCR procedures and agarose gel electrophoresis

The crude cell lysate was used directly for PCR. PCR was performed using the primers that would detect the *nuc* gene which specifically in *S. aureus* and *mec A* gene is responsible for the resistance of methicillin in MRSA. The primers used for detection of *ermA, ermB, ermC, msrA, linA and fem* in this study are listed in the Table 2.

Four hundred microliter (400 µl) of PCR product and 100 µl of 6X loading dye were loaded into well comb of 1% agarose gel. Also 600 µl of 100 bp DNA ladder (5X dilution) were loaded into the first well to serve as a marker. The electrophoretic condition were set at 80 V and let to run for 45 min. The gel is stained with ethidium bromide for 10 min and shaken at 45 rpm. The gel was viewed under ultraviolet (UV) and the photo was taken using high performance UV Transilluminator with exposure time of 6 s.

RESULTS

Thirty-six out of 100 isolates (36%) collected from college students' coins were Gram-positive cocci in cluster form and catalase positive and eighteen out of thirty-six isolates fermented mannitol. Six out of eighteen isolates were tube coagulase positive. In addition, four out of eighteen isolates were DNase positive. Yellow colonies growth on mannitol salt agar, DNase test positive and

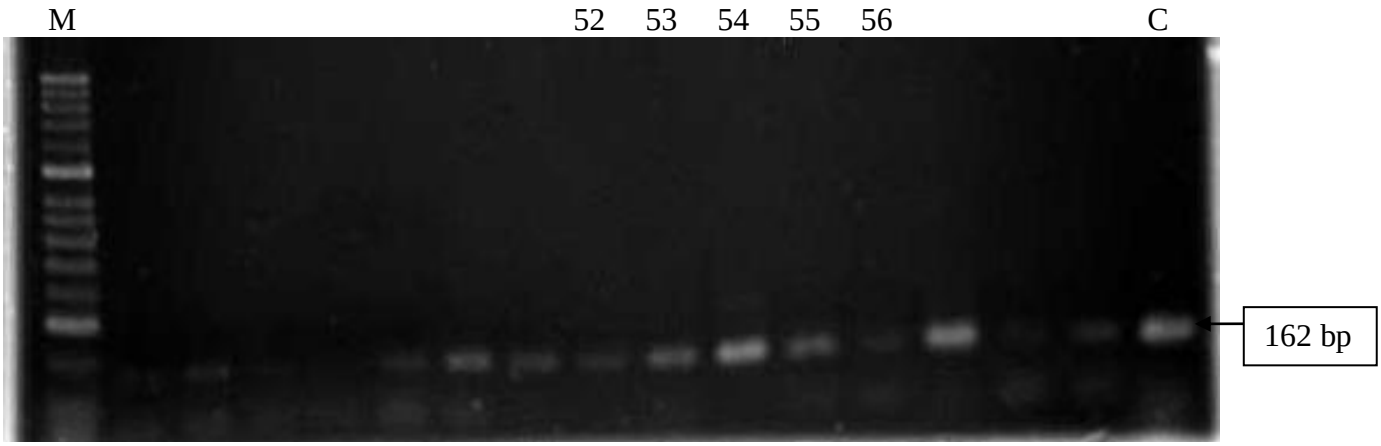

Figure 1. Agarose gel (1%) electrophoresis of amplified 162 bp DNA fragment *mec A* gene of *S.aureus* ; lane M is molecular marker; lane C is positive control ATCC MRSA and lanes 52-56 is bacterial isolates. Samples for 52-56 were positive for the presence of *mec A* gene.

tube coagulase test positive were presumptive as MRSA (David and Daum, 2010).

Tube coagulase test is the most common used method to identify *S. aureus*. Both human and rabbit plasma were used to perform tube coagulase test in this study. Human plasma is more widely used in most hospital settlings because the rabbit plasmas are costly and low quality. The results of TCT using rabbit plasma were varied. Four isolates were human plasma tube coagulase positive whereas two isolates were rabbit plasma tube coagulase positive yet not in human plasma. However, human plasma is sometimes obtained from blood banks as expired materials, therefore it is not recommended for tube coagulase test as it contains paramount of co-agulase reacting factor (Kateete et al., 2010).

In addition, human plasma produced the most satis-factory coagulase clotting while the rabbit plasma was less sensitive. In this study, human plasma tube co-agulase gave a positive reaction within one to two hour, whereas rabbit tube coagulase test required twice of the time (Ajuwape et al., 2001). Positive coagulase tubes were incubated for 24 h to detect the presence of fibrinolysin enzyme which is extracted from bacterial cultures. Fribrinolysin is commonly produced by most strains of *S. aureus*. Fibrinolysin can attack and inactivate the fibrin and discourage the clotting of the wounds.

DNase test was performed to differentiate *S. aureus* from the coagulase-negative staphylococci. *S. aureus* produces DNase that can be diffused from a colony and hydrolyze DNA within a plate. Five isolates samples have positive results out of eighteen isolates. Growth of yellow colonies on MSA (18%) was the most sensitive test in detecting *S. aureus* compared to TCT (6%) and DNase (5%).

Through the antibiotic disk diffusion methods, two out of 18 isolates was investigated for their susceptibility to methicillin; both of them were resistant to erythromycin, oxacillin and methicillin while other two isolates showed resistance to either erythromycin or oxacillin. Other isolates gave intermediate result to methicillin.

Five isolates were further analysed through PCR in this study. These five isolates were positive for the presence of *mec A* gene and only one of the samples was positive to *nuc* gene. *mec A* is the gene responsible for resistance towards methicillin while *nuc* gene is responsible in detecting the presence of *S. aureus.* Thus, this sample can be considered as MRSA along with the presence of *erm A, erm C, lin A and msr A* genes in this particular sample. It was found that this sample from college students' coin with the presence of *mec A* gene and *nuc* gene is UPM student aged 22, Chinese female who had history of flu within one month.

Those samples which lack *nuc* gene were not *S. aureus* and may belong to *S. epidermidis, S. saprophyticus* or other *Staphylococcus* spp. Primer gene such as *erm A, erm C* were erythromycin resistance methylase which are inducible antibiotic genes from *S. aureus*, which can detect all erythromycin-resistant MRSA isolates. And thus the MRSA sample was positive for the presence of *mec A, nuc*, *erm A and erm C* fulfilling the criteria of MRSA (Jonas et al., 2002) (Figures 1 to 4).

DISCUSSION

Illnesses and deaths caused by MRSA infection mainly cause death at a level higher than that of deaths caused by HIV infection (Lawrence et al. 1996). In comparison to molecular methods in identification of methicillin resistant *S. aureus*, antibiotic disk susceptibility test was less accu-rate and sensitive due to pH, moisture and excessive amount of thymidine. The pH of Müeller-Hinton agar should be between pH 7.2 to 7.4 at room temperature after solidification.

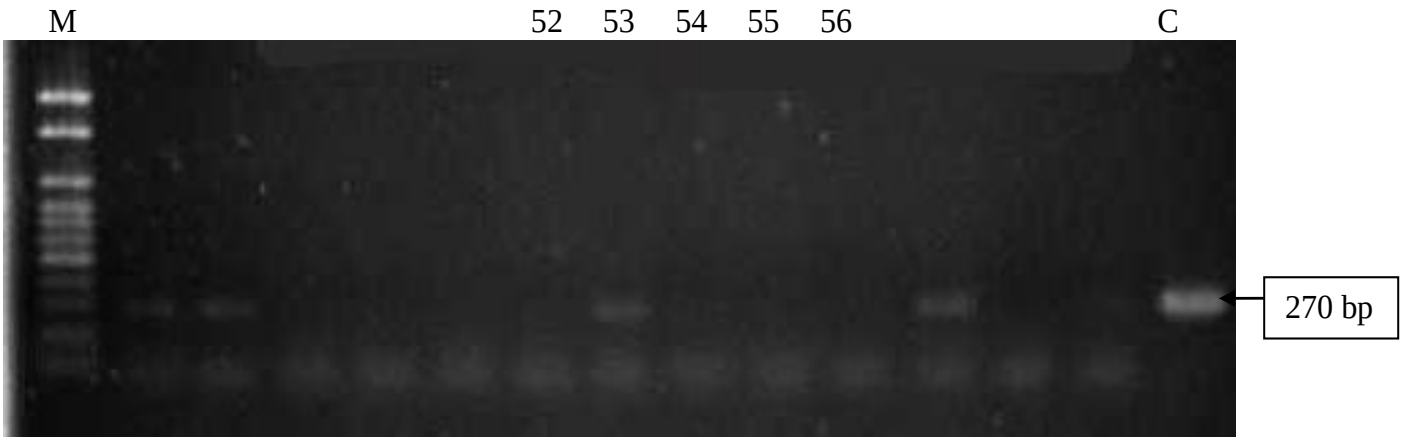

Figure 2. Agarose gel (1%) electrophoresis of amplified 270 bp DNA fragment *nuc* gene of *S.aureus*; lane M is molecular marker; lane C is positive control ATCC MRSA and lane 52-56 is bacterial isolates. Sample 53 was positive for the presence of *nuc* gene.

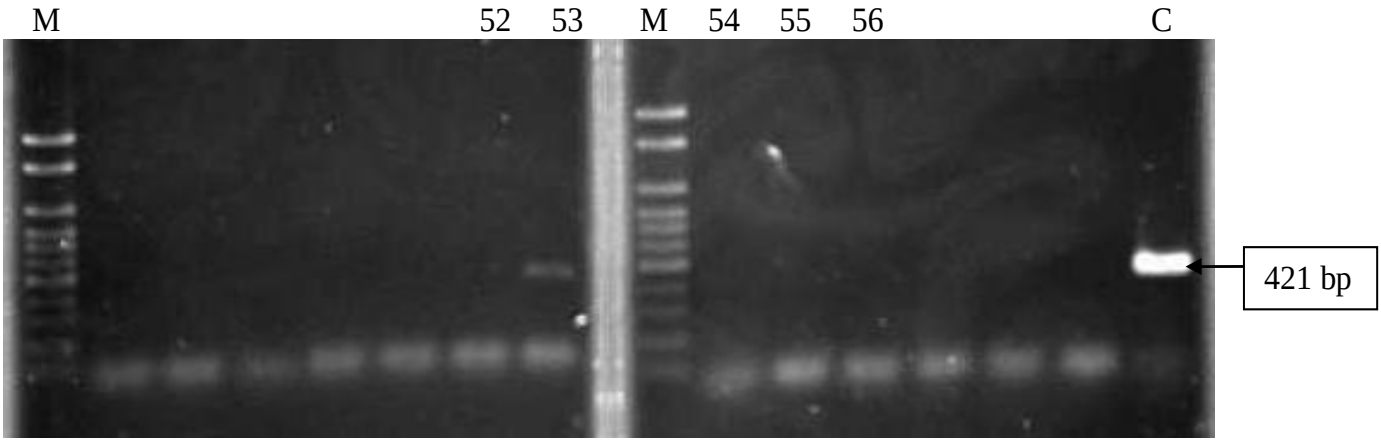

Figure 3. Agarose gel (1%) electrophoresis of amplified 421 bp DNA fragment *erm A* gene of *S. aureus* ; lane M is molecular marker; lane C is positive control ATCC MRSA and lanes 52-56 is bacterial isolates. Sample 53 was positive for the presence of *erm A* gene.

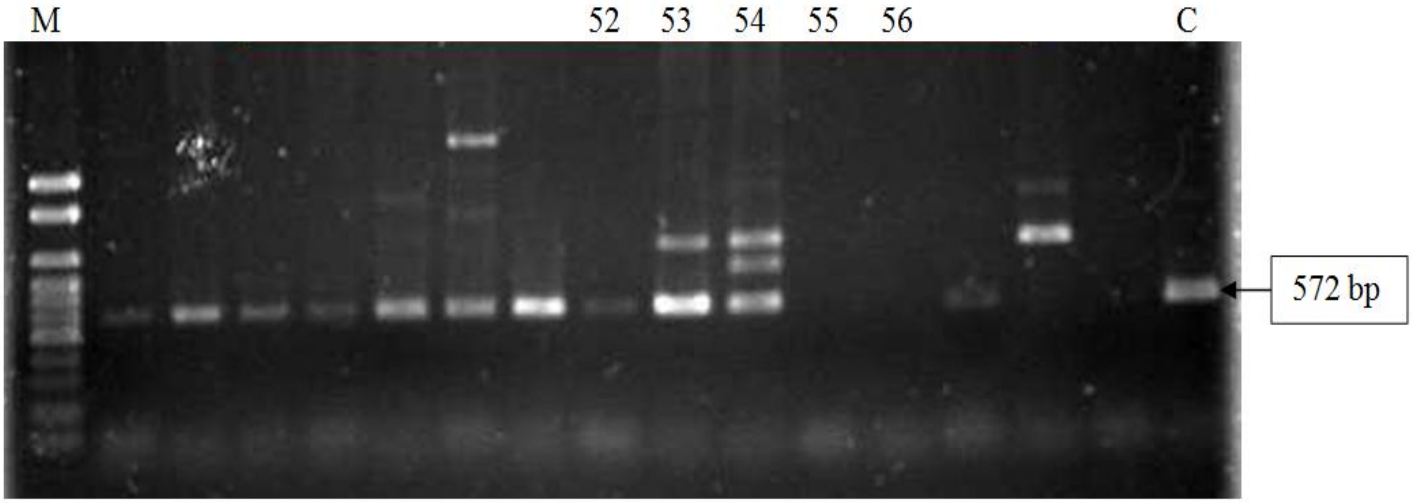

Figure 4. Agarose gel (1%) electrophoresis of amplified 572 bp DNA fragment *erm C* gene of *S.aureus* ; lane M is molecular marker; lane C is positive control ATCC MRSA and lanes 52-56 is bacterial isolates. Samples 52, 53 and 54 were positive for the presence of *erm C* gene.

In this study, the results of antibiotic susceptibility test were compared to amplification of DNA fragment (PCR). Some antibiotics may have excessive or less activity if the pH of Müeller-Hinton agar is too low. On the other hand, the opposite effects can happen due to high pH of agar medium. The defined MRSA sample was investigated in PCR with the presence of *mec A, nuc*, *erm A and erm C* genes which all indicate that this sample is MRSA. Whilst in antibiogram test, this sample was not resistant to methicillin but only had intermediate zone of inhibition. So, pH of agar medium may be a factor that causes this variation results (Coombs et al., 2004).

In addition, cross contamination might happen in this study during the experiment and hence affected the results. Agar medium may also contain excessive amounts of thymidine that can reverse the inhibitory effect of certain drugs, therefore had smaller or even no zones. Thymidine therefore might affect the role of drugs to become less sensitive and specific, which may result in false-resistance reports. Accordingly, low thymidine content of Müeller-Hinton agar is preferred to be used in such studies. Quality good media provide essentially satisfactory clear, distinct zones of inhibition 20 mm or greater in diameter. On the other hand, high content thymidine media yield un-satisfactory results such as no zone of inhibition, growth within the zone, or a zone of less than 20 mm (Coombs et al., 2004; Jones et al., 2002).

Money such as coins and paper money is another surface upon which bacteria can colonizes for a period of time. This phenomenon appears due to the occurrance of cross contamination. Most coins are touched by hundreds or thousands of people which come in contact with a variety of surfaces. Coins are porous which allowing bacteria to move deep inside the fibers to avoid environmental hazards (Tolba, and Loughrey, 2007; Edgeworth et al., 2007).

However, in comparing PCR results of coins with other types of samples collected from nasals, keys, handphone and nail (unpublished data), coins acted as source of transmission of MRSA but at level less remarkable. MRSA usually colonize in the nasal; so, coins have the possibility to be one of the potential sites of MRSA colonization too. In addition, MRSA has the opportunity to transfer from nasal to fingers and nails, followed by the transmission of MRSA through the hand contact with things such as coins and others (Scribel et al., 2009).

After handling coins using hands, transmission of MRSA is possible from any place. Colonization of few species of bacteria such as MRSA depends on the type of materials which are used to make the coins. Coins pass from one person to another continuously which increases the percentage of transmission of MRSA to the public and causes the outbreak of MRSA. Surface of coins provide a dry environment for the bacteria such as MRSA to colonize. However, dry surface of coins lacks nutrients and thus bacteria can only colonize on coins for few hours to one day. According to Hitachi, the ATM maker, there are a "clean ATM" which people can get their money by pressing down between rollers for one-tenth of a second at 392 degrees in order to kill many bacteria in Japan (Christoph, 1997).

Conclusions

Biochemical tests, antibiotic sensitivity tests, and PCR all succeeded in detecting MRSA. However, the detection of resistance genes such as *mec A*, *nuc* and others in PCR was more reliable, sensitive and specific than non-PCR means of detection. Coins proved to be possible source of MRSA circulation in the community. And it was concluded that hygiene among college students is not sufficiently adequate. To eliminate MRSA, complete surface sanitation and hand hygiene are necessary and important.

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