Malaysian Journal of Microbiology, Vol 12(2) 2016, pp. 171-176
http://dx.doi.org/10.21161/mjm.69915

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Detection of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from Bagan Lalang recreational beach, Malaysia

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Received 9 January 2015; Received in revised form 3 November 2015; Accepted 13 November 2015

ABSTRACT

Aims: The aim of the study was to assess the presence of methicillin resistant *Staphylococcus aureus* (MRSA) at Bagan Lalang recreational beach, Selangor using the PCR method.
Methodology and results: Water samples collected from Bagan Lalang recreational beach were analyzed by culturing on selective solid media and by PCR using *mecA* gene. Water samples were collected in triplicate from three different locations at the sampling site including main recreational area that is accessible to the public. The result shows that out of 286 presumptive *S. aureus* enumerated by the culturing method, only 12 (4.2%) were confirmed as MRSA based on PCR detection of *mecA* gene. Interestingly, all of MRSA detections were found at the main area of recreational activity.
Conclusion, significance and impact of the study: Our results suggest that public marine beaches may be reservoirs for the transmission of MRSA to beach visitors as well as an ecosystem for exchange of antibiotic resistance genes among staphylococci and related genera. PCR is an effective method to detect MRSA from recreational beaches. This is the first report on the detection of MRSA in recreational beaches in Malaysia.

Keywords: Methicillin resistant *Staphylococcus aureus* (MRSA), recreational beach, Bagan Lalang, PCR

INTRODUCTION

Beaches have been suggested as a potential source of community acquired pathogens. Microbial contamination of marine waters worldwide is known to cause several infections in human communities annually. *Staphylococcus aureus* is an opportunistic pathogen to the humans causing skin and soft tissue infections (Eady and Cove, 2003). Infections from methicillin resistant *Staphylococcus aureus* (MRSA) have risen substantially and drug resistant infection places an additional $5 billion burden on the United States health care system annually (McDougal *et al*., 2003). *Staphylococcus aureus* and MRSA are shed by swimmers and both can be found within the sand and beach seawater. *Staphylococcus aureus* concentration has been correlated to bather density and is attributable to human activity (Elmir *et al*., 2007).

Staphylococcus aureus is a common cause of serious and life threatening infections. The first MRSA isolates were reported in England in 1961, one year after the introduction of methicillin (Jevons, 1961). The prevalence of MRSA has increased rapidly over the last decade due in large part to the emergence of community-acquired MRSA (CA-MRSA) infections. Most (90%) CA-MRSA infections are skin and soft tissue infections. However, more serious and deadly infections do occur (Eady and Cove, 2003; Miller and Diep, 2008). Repeated MRSA infections are common and a recent study found that 27% of the MRSA positive hospital patients were still colonized with MRSA a year after they had been discharged from the hospital (Datta and Huang, 2008) 2008. *Staphylococcus aureus* and MRSA are spread from fomite to person and from person to person (Khivsara *et al*., 2006; Zhang *et al*., 2009). Yet few environmental reservoirs outside the healthcare setting and closed communities such as schools, prisons and sports teams have been characterized (Eady and Cove, 2003). A 1987 report described the isolation of *Staphylococcus* spp. from Israeli coastal marine waters, while another report described the isolation of *S. aureus, S. epidermidis* and *S. hominis* from North Eastern Atlantic ocean and estuarine waters (Yoshpe-Purer and Golderman, 1987). More recently both *S. aureus* and MRSA isolates have been shown to survive river, sea and swimming pool conditions in the laboratory (Tolba *et al*., 2008).

Accurate and rapid identification of MRSA in recreational beaches is essential for identification and

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

decision on timely isolation procedure. Numerous approaches that improve turnaround time for the identification of MRSA have been described. Fluorescence test (Qadri *et al.*, 1994) or penicillin binding protein 2a (PBP2a) antibody agglutination tests have been described elsewhere (Nakatomi and Sugiyama, 1998). Yet, these require subculture and solid media and are unable to determine species and methicillin susceptibility at the same time. A simultaneous test of methicillin resistance by *mecA* has been proposed (Goodwin and Pobuda, 2009). Presence of the *mecA* gene in staphylococci has been considered as a molecular basis for identification of MRSA (Mason *et al.*, 2001; Goodwin and Pobuda, 2009). The *mecA* gene encodes the extracellular PBP2a which is unique to methicillin resistant staphylococci (Kim *et al.*, 2012). Therefore the current study screened for the presence of MRSA from Bagan Lalang recreational beach water samples using PCR amplification of the *mecA* gene.

MATERIALS AND METHODS

Study site

Seawater samples were collected from recreational beaches in Bagan Lalang (Figure 1). This coastal area has experienced rapid housing and tourism development in the past decade due to its proximity near to the Kuala Lumpur International Airport and the Sepang International Circuit.

Figure 1: A map shows the exact places with three different sampling area at Bagan Lalang recreational beach. Station 2 is the main recreational area with high number of recreational activities and visitors.

Sample collection

Samples collected for this study were part of a long-term surveillance program on the microbiology quality of water available for recreational purpose in Malaysia. Bathing water samples were taken from areas close to the swash zone of the bathing beach (i.e the zone of interface that is continually washed over by the waves where people are found of bathing). Sterile glass bottles (1000 mL) were used to collect water samples in triplicate.

Detection of MRSA by culturing methods

The water samples were filtered using membrane filtration as describe in APHA (1999). Volumes of 10 mL and 50 mL were filtered using 0.45 µm pore size membranes. The membranes were then placed on plates containing Mannitol Salt Agar (MSA) and incubated at 37 °C for 24 to 48 h. After this incubation period, characteristic yellow colonies were taken as presumptive *S. aureus*. A total of 286 colonies were randomly chosen and purified by subculturing on MSA. Purified colonies were then streaked on CHROMagar^{TM} MRSA (Focus Biotech Inc.) to observe their morphological characteristics. Oxicillin antibiotic screening agar was used to sub-culture the colonies from CHROMagar^{TM} MRSA. Bacteria growth on this agar indicated a positive result for MRSA. These isolates were subsequently used in the PCR screening.

Detection of MRSA by antibiotic susceptibility testing

A total of 65 (22.7%) isolates of *S. aureus* were selected randomly for antibiotic susceptibility testing from a total of 286 isolated from Bagan Lalang recreational beach. Seven different antibiotics were tested namely 10 µg ampicillin, 5 µg novobiocin, 30 µg chloroamphenicol, 10 µg streptomycin, 10 µg gentamycin, 30 µg tetracycline and 30 µg kanamycin.

Preparation of bacterial DNA samples

Methods described by Kobayashi *et al.* (1994) was used for DNA extraction. A bacteria colony was suspended in TAE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.5). After centrifugation, the pellet was resuspended in 10 µL of achromopeptidase (10,000 U/mL: Wako Pure

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Chemical Industries) and incubated at 37 °C for 10 min. Then 50 µL of 0.5 M KOH was added to lyse the bacteria cells and incubated for 5 min, followed by neutralization with 50 µL of 1 M Tris HCl (pH 6.75). The supernatant obtained after centrifugation was used as the DNA sample for PCR.

Detection of MRSA by PCR identification method

PCR confirmation MRSA utilized primers to amplify *mecA*1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and *mecA*2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) yielding an expected 310 bp amplicon. The primer was synthesized by First Base Sdn. Bhd., Malaysia and used at a final concentration of 0.1 µM. The PCR cycling condition were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 45 sec at 94 °C, 45 sec at 53 °C and 60 sec at 72 °C, with final extension step at 72 °C for 2 min. Each 50 µL PCR mixture used contained 1X PCR buffer, 2 mM $MgCl_2$, 200 µM each DNTP and 0.25 U of taq polymerase. PCR product was examined by agarose gel electrophoresis (1% agarose) using 1X Tris buffered EDTA at 80V for 45 min. The gel was then stained with 10 µg/mL ethidium bromide. Also included as a molecular size marker in the gel was a 100 bp DNA ladder (Fermentas). In each run of the PCR, a *mecA* positive MRSA from a clinical source was included to confirm successful amplification of the *mecA* gene. The PCR product obtained was purified and sequenced to confirm the identity of the gene.

RESULTS

This study focused on the detection of MRSA in recreational beaches. Three sites were identified for sampling location because of the varying density of bathers at the selected points. Interestingly, only samples collected from the main bathing area were found positive for MRSA. MRSA colonies detected in this study were typically yellow colonies on Mannitol Salt Agar (MSA) with diameter between 0.5 to 1.5 mm. The work flow for the identification of MRSA from a recreational beach is shown in Figure 2.

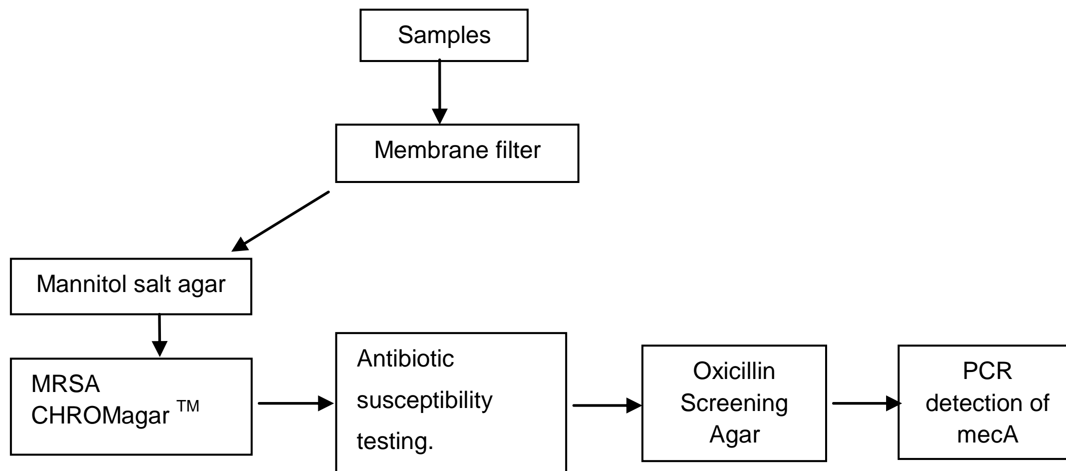

Figure 2: A combination of cultural and molecular approaches in the detection of MRSA from recreational beach samples (Goodwin and Pobuda, 2009; Schwartz *et al*., 2012).

Table 1: Detection of MRSA from recreational beach Bagan Lalang.

Method of isolation	No. of isolates	Characteristics
Mannitol Salt agar (MSA)	286	yellow colonies
CHROMagar™ MRSA and characterization	65	mauve with matte halo
Oxicillin antibiotic screening agar	12	white colonies
PCR for *mecA* gene	12	310 bp PCR product

While in the first instance up to 286 yellow colonies were obtained on MSA as presumptive *S. aureus* isolates, further screening by culturing on MRSA CHROMagar™ yielded a total of 65 isolates that showed characteristic mauve with matte halo colonies having diameters ranging from 0.5 to 0.8 mm. This was equivalent to 22.7% detection rate of presumptive MRSA among the *S. aureus* isolates. To reconfirm the validity of results on CHROMagar™ MRSA, antibiotic susceptibility testing and oxicillin antibiotic screening agar was necessary.

Based on antibiotic susceptibility test, 12.3% of the isolates were found to be resistant to 10 µg ampicillin, 30 µg chloroamphenicol, 10 µg kanamycin and 30 µg streptomycin while 10.8%, 6.2% and 4.6% were resistant

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

to 10 µg gentamicin, novobiocin and tetracycline respectively. Twelve out of the presumptive 65 isolates on MRSA CHROMagar^{TM} were found to be resistant to oxicillin based on culture characters on oxacillin antibiotic screening agar. This was equivalent to 18.5% detection rate. These 12 isolates were resistant to all antibiotics tested. These isolates were subsequently screened for the presence of *mecA* gene. All twelve colonies carried the *mecA* gene of 310 bp in size (Figure 3). BlastX result from Genebank showed the sequences were 99% similar to MRSA SCCmec typing type V.

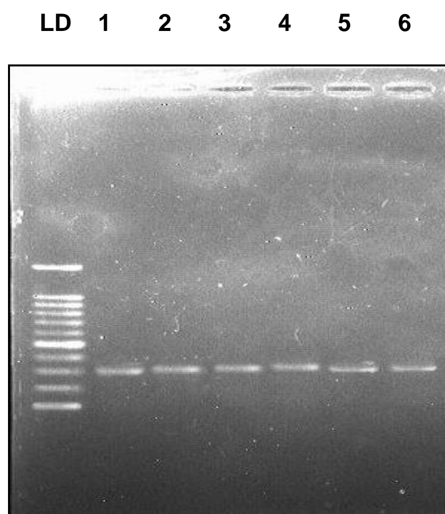

Figure 3: Presence of *mecA* gene PCR products sized 310 bp for some isolates recovered from Bagan Lalang recreational beach. In lane 1 is the 100 bp ladder and lane 2 is the positive control, the rest of the lanes are the isolates recovered in this study.

DISCUSSION

This study area was selected due to popularity of the recreational beach and increasing development of recreational facilities and resort, for example, the Sepang Gold Coast resort. Three sites were selected for sampling. Station 2 is the main recreational area with highest number of visitors and recreational activities compared to stations 1 and 3. A correlation between seawater exposure and MRSA infection rates suggests that the oceans may be one pathway by which community acquired infections are transmitted (Lee *et al.*, 2006; Bonilla *et al.*, 2007; Yamahara *et al.*, 2007, 2009; Goodwin and Pobuda, 2009; Halliday and Gast, 2011; Sabinro *et al.*, 2011; Zhu *et al.*, 2011; Shah *et al.*, 2011). High count of MRSA in recreational water is often considered a risk factor that contributes to many diseases that affect areas such as skin, eyes and ears. Recent studies suggest that adaptations to the external environment and lateral gene transfer among strains may play important roles in the evolution of pathogens (Agerso *et al.*, 2002).

Based on the results, all 12 MRSA isolates came from water samples collected from the main bathing area (Station 2). Water samples collected from the other stations where relatively few people go for recreational activity were negative for MRSA. Even though the total numbers of MRSA detected in this study is low, preventive infection control is needed to avoid the beach from being polluted by dangerous pathogens. There were also a lot of other activities happening around the beach including development of resorts and shops close to the shoreline. High exposure of human activity might contribute to the presence of this bacterium. The ability of MRSA to spread from person to person and survive in high pressure environment can pose threats to the public (Heaney *et al.*, 2009).

The usefulness of the PCR assay for the detection of bacterial and viral pathogens has been established (Cox *et al.*, 1995). Genotypic detection of drug resistance will undoubtedly become an important component of the diagnostic clinical and environmental laboratory. Early detection of MRSA in the environment is very important for preventive infection control strategies (Cheung *et al.*, 1990; Cimolai, 2008). Guidelines for interpretation of *mecA* gene result will need to be formally addressed as more laboratories begin to use this method. Besides being a marker gene for MRSA, *mecA* (Goodwin *et al.*, 2012), the gene is also known as a virulence gene that is believed to be a major contributor to methicillin resistance (Olivia *et al.*, 2005). This was supported in our study by the growth of bacteria on oxacillin antibiotic screening agar which all showed the presence of the *mecA* gene. This method is effective, saves time and accurately detects the presence of MRSA from different sources.

Methicillin resistance is the single most important clinical resistance trait acquired by *S. aureus*. It is able to confer cross-resistance to virtually all beta lactam antibiotics, which represent the single most commonly prescribed class of antibacterial agents. Since their first appearance in 1961, epidemic strains of MRSA have spread worldwide in hospitals and in the community. MRSA infections continue to present one of the major challenges to control of infectious diseases nowadays (Moran *et al.*, 2005).

Methicillin resistance in *S. aureus* is mediated by an acquired penicillin-binding protein (PBP) named PBP2A, which has an extremely low reactivity with beta lactam antibiotics because of two factors which is a high association constant for the antibiotic in the non-covalent complex and a poor first-order rate constant for the acylation of the protein by the antibiotic. PBP2A is a peptidoglycan transpeptidase that in cooperation with the transglycosylase domain of PBP2 of *S. aureus*, can catalyze cell wall biosynthesis in the presence of beta lactam antibiotics, thus enabling survival and growth of the bacteria (Hartman *et al.*, 1985; Pinho *et al.*, 2001). PBP2A is encoded by the imported *mecA* gene which is incorporated into the S. *aureus* chromosome as part of a large heterologous mobile genetic element, the *staphylococcal* cassette chromosome *mec*, or SCC*mec* (Ito *et al.*, 1999).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

The expenses culturing method demands for testing one specimen for the presence of MRSA is high. PCR methods offer a more efficient alternative. Moreover the sensitivity of this technique, which neither starts with nor requires single, visually questionable colonies, can be superior to that of routine diagnostic procedures. Recent studies proposed testing for presumptive MRSA identification by using Mannitol Salt Agar (MSA) for detection of *S. aureus* (Mir *et al*., 1998; Schwartz *et al*., 2012). This approach was also adopted in our study. The limitation with this approach however is that MRSA can sometimes be overlooked in culture because some stains grow as non-pigmented colonies or are clumping factor negative if tested on a slide with rabbit plasma (Wichelhaus *et al*., 1999). The heterogenous expression of methicillin resistance can make it difficult to determine the resistance phenotype definitively. Therefore the detection of the *mecA* gene using PCR remains the gold standard to identify MRSA (Schwartz *et al*., 2012).

CONCLUSION

The presence of MRSA at recreational beach Bagan Lalang recreational beach might pose health threats to the community despite low detection rate reported in this study. Bearing in mind the ability of this bacterium to survive in different environments and to spread virulence traits to other bacteria, control strategies are therefore needed to protect the public from associated infectious diseases. This study strengthens the assertion that the *mecA* gene using PCR offers a fast and accurate means of MRSA identification after selective enrichment, obviating the need for prior isolation of bacterial colonies on solid media. The benefit of accuracy and speed in simultaneous identification of species and methicillin susceptibility could make this method a valuable diagnostic tool, especially in hospital and environment areas where MRSA is endemic.

ACKNOWLEDGEMENT

We are grateful for the financial support provided by Universiti Kebangsaan Malaysia Research Grant (UKM-OUP-KPB-33-169).

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538