Occurrence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) among the Health Workers of Rizal Provincial Hospital and Characterization for the Presence of *luks-lukf* PVL Gene

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major problem in the hospital as well as in the community setting. The resistance of MRSA to all β-lactam antibiotics, the most commonly prescribed group of antimicrobials for staphylococcal infections, poses a significant limitation on the treatment of diseases caused by this multiple drug resistant strain. The study determined the prevalence of MRSA among the hands and nasal cavities of hospital workers of the Rizal Provincial Hospital, their susceptibility to antimicrobial agents, and the occurrence of *mecA* gene and *luks-lukf* Panton-Valentine leukocidin virulence gene among the isolates. Methicillin resistance was determined using oxacillin and cefoxitin. *Staphylococcus aureus* was isolated from the nose of 26 (22.61%), from the hand of 1 (0.87%), and from both the hand and nose of 3 (2.61%) of the sampled 115 hospital health workers, giving an overall prevalence of *S. aureus* of 26.09%. Among the 30 health workers found to have *S. aureus*, 5 or 16.67% of them were found to have MRSA, with 4 health workers carrying the strain in the nasal cavity, and 1 health worker carrying the strain both on the hand and nasal cavity. The overall MRSA carriage rate is 4.35% among all the subjects sampled. All MRSA isolates were susceptible to doxycyclin, gentamicin, vancomycin, erythromycin, ciprofloxacin, and linezolid. *mecA* gene was found in all MRSA confirming their methicillin resistance, while the *luks-lukf* Panton-Valentine leukocidin virulence gene was present in 50% of the 6 MRSA isolated. The results of the study identified the presence of a significant yet easily overlooked source for transmission of MRSA, which may also carry additional virulence genes, in the local setting - the hospital workers themselves. This underscores the need for the consistent review and strict implementation of the hospital policy on infection control, such as mandatory requirement for thorough hand washing by hospital workers before and after handling each patient, which remains the cornerstone of prevention.

INTRODUCTION

Nosocomial infections or hospital-acquired infections, defined as infections that become evident 48 hours after admission, comprise one of the major problems in health care. These infections are caused by opportunistic microorganisms, most often those that survive antibiotic treatments, which manage to infect vulnerable hospitalized patients. These may be transmitted from person to person, from hospital equipment, materials, and hospital environment to the patient, or may be from the endogenous flora of the patient. One of the most common bacteria associated with nosocomial infections is *Staphylococcus*

Staphylococcus aureus (Enright et al. 2002), a spherical gram-positive bacterium known for causing a wide range of infections. The rapid increase in the spread of antibiotic resistant S. aureus worldwide through the years has become a major public health problem (Marquis 2008). A strain known for its multiple resistance to antibiotics is methicillin-resistant S. aureus or MRSA. MRSA carries the mecA gene and regulatory sequences that encode for production of a mutated penicillin-binding protein called PBP-2' or PBP2A. This protein has low affinity for β-lactam antibiotics, and is bifunctional, possessing both a transpeptidase (TPase) domain and a transglycosylase (TGase) domain that allows the synthesis of bacterial cell wall even in the presence of beta-lactam antibiotics. MRSA is clinically resistant to all beta lactams, the most commonly used group of antibiotics against staphylococcal infections (Micek et al. 2005). As a result, MRSA infection is becoming more common, and has been identified as one of the leading causes of hospital infections (McGrath et al. 2008; Lina et al. 1999).

In the Philippines, Atilano et al. (2001) reported a prevalence rate of 11.7% for hospital- or healthcare-acquired MRSA (HA-MRSA) isolated from August 2000 to May 2001 in a tertiary hospital in Metro Manila, while We et al. (1999) showed that the prevalence of nosocomial MRSA at the Philippine General Hospital was 53% among those isolated from December 1996 to April 1998. Carlos reported the prevalence of MRSA gathered by the Antimicrobial Resistance Surveillance of the Philippines to be 18% in 1999 (Carlos 2000), 24% in 2000 (Carlos 2001), 18% in 2001 (Carlos 2002), and 18% in 2002 (Carlos 2003). No data on MRSA prevalence among cases in the Rizal Provincial Hospital, the site of the present study are available, for the reason that culture and antimicrobial susceptibility testing are not done in the hospital.

Recently, MRSA cases have also been reported in healthy community-dwelling persons without having risk factors associated with MRSA infections acquired in the hospital environment, and are now termed as community-acquired or CA-MRSA infections. CA-MRSA was reported initially in the 1990’s and it was then thought to be from HA-MRSA strains. However, there are reports that the genetic background of CA-MRSA does not correspond to that of HA-MRSA, which suggests that CA-MRSA did not emerge from local HA-MRSA (Vandenesch et al. 2003). It often carries SCCmec types IV and V and the virulence factor Panton Valentine leukocidin (PVL) lukS-lukF gene locus. The PVL toxin is comprised of two subunits, LukSPV and LukF-PV, and belongs to the pore-forming toxin family. It causes lysis of human neutrophils, monocytes and macrophages (Prevost et al. 1995). Studying the frequency of MRSA that produces PVL has become important as another way of working on treatment and preventive measures against infection (Holmes et al. 2005). Outbreaks due to MRSA with PVL have steadily increased in the past few years among athletes, prisoners, and military personnel in the Philippines, Europe, North America, Oceania and the rest of the world (Cabrera et al. 2010; Vandenesch et al. 2003; Buensuceso et al. 2005; Zetola et al. 2005; Holmes et al. 2005).

Health care workers (HCW) may be carriers of MRSA acquired either from the community or from hospital patients, and may serve as sources of transmission of nosocomial infections (Creamer et al. 2010; Lessing et al. 1996; Kampf et al. 2003). Albrich and Harbarth (2008) reported that both HCWs that were transiently and persistently colonized with MRSA were responsible for several MRSA clusters. In another study, a distinct strain of MRSA was transmitted to patients by an HCW on three separate occasions over 27 months (Lessing et al. 1996). Over this same period, nine other small clusters were observed in the Oxford Hospital Group, comprised of 66 patients and 22 HCWs who were either colonized or infected with several strains of MRSA.

Considering the significant role of MRSA as a commonly occurring nosocomial pathogen, its transmission through person to person contact, its established association with community-acquired infections and its reported presence as a normal flora in some individuals (Williams 1961; Kluytmans et al. 1997), the present study aimed to determine the occurrence of MRSA among health workers of the Rizal Provincial Hospital. Phenotypic identification of methicillin resistance was confirmed genotypically with the detection of the mecA gene. The isolates were also studied for the presence of the lukf-luks PVL gene.

**MATERIALS AND METHODS**

**Specimen Collection and Isolation of Staphylococcus aureus**

The study subjects were hospital personnel of Rizal Provincial Hospital, an emergency hospital in Morong, Rizal, Philippines. It has more than 150 beds with an emergency room, pediatric ward, medical ward, pay ward, and licensed to do minor and major operations. Doctors, registered and student nurses, nursing aides in the pediatric ward, medical ward, pay ward and surgical ward were included in the study. The subjects were not informed beforehand about the time of collection. Each participant was requested to sign a consent form after the objectives of the study and the collection procedure were thoroughly explained. Nasal swabs and handprints were taken for the reason that results from other studies
showed that these are the areas where *S. aureus* is more commonly found as normal flora (Williams 1961; Kluytmans et al. 1997). After thorough explanation and demonstration of the collection procedure, the subjects were requested to swab both nasal cavities using separate cotton swabs. The swabs were inoculated into nutrient broth (NB) with 7.5% NaCl. Handprint from the predominant hand was done on mannitol salt agar plate (MSA). These were incubated at 37°C, after which growth from the NB was subcultured on MSA. Growth characteristics on MSA were used to identify the organisms.

**Identification of Bacterial Isolates**

Colonial morphology on MSA was described after culture for 18 hours at 37°C. Acid-producing, halotolerant bacteria that produced smooth, round, raised and glistening colonies on MSA were considered possible *S. aureus*. On further characterization, isolates showing Gram positive cocci in clusters that were also catalase and coagulase producers were identified as *S. aureus*.

**Antimicrobial Susceptibility Testing**

The disk diffusion method was used to determine the antimicrobial susceptibility of the isolates following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012). The antimicrobials tested were cefoxitin (30 μg), oxacillin (1 μg), penicillin G (10 units), erthyromycin (15 μg), clindamycin (2 μg), vancomycin (30 μg), gentamicin (10 μg), linezolid (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), chloramphenicol (30 μg) and cotrimoxazole (25 μg). The inoculum was prepared by suspending 18-hr bacterial colonies in sterile distilled water until turbidity was comparable to that of 0.5 McFarland standard. It was swabbed on Mueller Hinton agar plates, incubated at 37°C with ambient air, and read after 16 to 18 hours. Plates with oxacillin, cefoxitin, and vancomycin discs were read after 24 hours. The diameters of the inhibition zones were measured in millimeters and interpreted using the CLSI (2012) zone diameter interpretive standards for *Staphylococcus* spp.

**DNA Extraction**

Bacterial colonies from nutrient agar plates were suspended in 1mL of autoclaved water and centrifuged at 14,000xg for 1 minute. After decanting, 200μL of InstaGene™ were added to the pellet. The tube was left to incubate at 56°C for 10 minutes, then vortexed at high speed for 10 seconds, placed in a 100°C waterbath for 8 minutes, vortexed at high speed for 10 seconds and centrifuged for 2-3 minutes at 10,000 rpm. The supernatant was used as the source of DNA.

**Multiplex PCR for Amplification of meca gene, luk-s-lukf PVL Genes, and 16s rDNA for Staphylococcus spp.**

PCR was carried out in a 25uL reaction volume that consisted of the following: 5-10ng DNA template, 1X PCR buffer, 0.2mM of each dNTP, 3mM MgCl₂, 0.04 U/μL Taq polymerase, and 0.3μM of each primer. Sequences of primers used are found in Table 1. Primers for the 16s rDNA of *Staphylococcus* were included to serve as internal control for PCR.

The thermocycling conditions were as follows: initial denaturation at 94°C for 10 minutes, and 10 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 75 sec. This was followed by 25 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 75 sec, and final extension at 72°C for 10 minutes (McClure et al. 2006). PCR amplicons were visualized using UV transilluminator after electrophoresis in 1.7% agarose at 50 volts for 1- 1.5 hours and stained using ethidium bromide.

**Sequencing of PCR Products**

PCR products were sent to MACROGEN, Korea for DNA sequencing. ChromasLite was used to view the gene sequence. The nucleotide sequences of the PCR amplicons were identified using BLAST (Basic Local Allignment Search Tool) analysis accessed from the NCBI Database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
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<tbody>
<tr>
<td>Staph 756F</td>
<td>AACTCTGTATTAGGGAAGAAACA</td>
<td>756</td>
</tr>
<tr>
<td>Staph 750R</td>
<td>CCACTCTTCTCGGGTTGTCACC</td>
<td></td>
</tr>
<tr>
<td>MecA1</td>
<td>GTAGAAATGACTGAAACGTCGGATAA</td>
<td>310</td>
</tr>
<tr>
<td>MecA2</td>
<td>CCAATCTACATGTTTGGGTCTA</td>
<td></td>
</tr>
<tr>
<td>Luk-PV-1</td>
<td>ATCATTAGTAAATGTCGTCGATA</td>
<td>433</td>
</tr>
<tr>
<td>Luk-PV-2</td>
<td>GCAATCGTGATGGATGCAAAGGC</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer sequences for detection of meca, luk-s-lukf PVL genes, and 16s rDNA for Staphylococcus and their amplicon sizes (McClure et al. 2006).
RESULTS

Isolation and Phenotypic Identification of S. aureus
A total of 230 specimens comprised of nasal swabs and handprints from 115 hospital personnel were cultured for the presence of S. aureus. Of the 230 specimens, 33 or 14.35% were found to be positive for the bacterium. These consisted of 26 from the nasal swabs and 7 from the handprints, and belonged to 30 or 26.09% of the sampled hospital personnel. Of these 30 personnel, 3 or 10% had S. aureus in both the nose and hand, 26 or 86.67% had it in the nose only, and 1 or 3.33% had it on the hand only.

Antimicrobial Susceptibility Patterns of S. aureus Isolates
Of the 33 S. aureus isolates that were studied, 6 (18% of S. aureus) were found to be methicillin-resistant (MRSA), and were taken from five hospital employees: five were isolated from the nose and one was isolated from the hand. This gives an overall MRSA prevalence of 4.35% among the hospital employees. All MRSA isolates were resistant to cefoxitin but were found to be susceptible to vancomycin, gentamicin, erythromycin, ciprofloxacin, linezolid, chloramphenicol, cotrimoxazole, and clindamycin. Of the 27 methicillin susceptible S. aureus (MSSA), 18 were resistant to penicillin and 2 were resistant to both doxycyclin and erythromycin, while the remaining 7 were susceptible to all antimicrobials.

Detection and Identification of mecA and luksluk/PVL gene
The six (6) MRSA isolates were studied for the presence of mecA and lukslukf PVL genes. Amplification of mecA gene was observed in all isolates (Figure 1). This confirms that the isolates were MRSA strains, since detection of the mecA gene, which encodes for the production of PBP-2a or PBP-2’, is the standard indicator in the determination of methicillin resistance (Bignardi et al., 1996; Skov et al., 2006). The isolates were also tested for the presence of lukslukf PVL virulence gene, where 3 (50%) of MRSA were found to be positive (Figure 1). All of these isolates were from the nose. The identity of the amplicons was confirmed through DNA sequencing.

Figure 1. Agarose gel showing amplicons of multiplex PCR for the mecA gene (310 bp), PVL gene (433 bp), and internal control 16s rDNA for Staphylococcus spp. (756 bp) of MRSA isolates from hospital personnel. Lane 1: reference 100bp ladder. Lane 2: negative control. Lane 3: positive control MRSA with PVL gene. Lanes 4-9: Isolates are all positive for mecA (MRSA) and Staphylococcus-specific 16sRNA genes (isolates 3B, 19B, 26B, 39B, 41B, 26P respectively). Lanes 3, 5, 7, 8: MRSA isolates are positive for lukF-lukS PVL gene. Lane 4, 6 and 9: MRSA negative for lukF-lukS PVL gene.
DISCUSSION

The carriage rate of *S. aureus* was found to be 26.09% among the sampled 115 hospital personnel, with 79% of the bacterium being isolated from the nose, while the remaining 21% were from the hand. In addition, among the six isolates of *S. aureus* found to be methicillin resistant (MRSA), five were likewise from the nose, confirming the reported predilection of the organism to establish itself in the nasal area, allowing the site to be a reservoir for the organism, and hence serves as a good source for its transmission (Kluytmans et al. 1997).

The methicillin resistance was confirmed by the detection of the meca gene in all the isolates, phenotypically found to be resistant to cefoxitin using the disc diffusion method, confirming the specificity of its use for detection of MRSA. On the other hand, all MRSA isolates were susceptible to all the non-beta lactam antimicrobials tested against them, a characteristic that commonly distinguishes the more susceptible CA-MRSA from HA-MRSA (Milstone et al. 2010; Marx 2004; Zuger 2004). Moreover, the presence of the lukS-lukF PVL gene in three of the six MRSA isolates is also characteristic of CA-MRSA, although not all strains are reported to carry this. The results suggest that the MRSA from the health workers were acquired in the community and not from the hospital. Although it has been reported that infected and colonized patients are the major sources of MRSA (Marshall et al. 2004), the results of the present study imply the possibility of transmitting strains from the community by HCWs into the health-care setting should the carriers incur lapses in personal hygiene during patient care.

Results of the present study show the presence of MRSA in 4.26% of the total health workers sampled. This is higher than the 1% colonization reported in a previous study (CDC 2003), although it is comparable to the 4.6% global prevalence of MRSA carriage by HCWs as reported by Albrich and Harbarth (2008). This is a concern because transmission of nosocomial infections has been reported to be mainly through the hospital staff (McDonalds 1997). Furthermore, hospitalized patients are more susceptible to infections due to the lower status of their immune system, which is a factor that contributes to the frequency of MRSA infections (Prakash 2010). MRSA can also be positively selected for, especially in a hospital environment where heavy usage of antimicrobials is an established practice. The resistance of the MRSA to all β-lactams should also be considered, which further limits the treatment options for patients in this government-funded hospital, who are mostly from the lower-income group. No vancomycin resistant *S. aureus* (VRSA) strains were reported, which is important to note since vancomycin is considered to be one of the last options for patients that are infected with MRSA (Pitz et al. 2011).

The lukS-lukF PVL gene was detected in three (50%) of the MRSA isolated. These MRSA were isolated from three healthy HCW. MRSA strains with the PVL gene, typical of CA-MRSA, are known to cause diseases such as necrotizing pneumonia and necrotizing skin infections even in previously healthy individuals. It has relatively faster growth rate compared to HA-MRSA and is also highly transmissible (Milstone et al. 2010), and is becoming more prevalent in the hospital settings. These characteristics would further increase the risk of MRSA infections in the hospital, and would further compound the problem of having few options in treating MRSA infections.

In the United Kingdom, pre-employment screening and routine screening of staff for MRSA carriage is not recommended due to the transient nature of bacterial nasal carriage (Royal College of Nursing 2005). It is however, recommended that those who have been colonized by MRSA for long periods of time, be managed by the occupational health department and infection control staff. Those already showing symptoms of the infections are advised to be treated with the appropriate antimicrobials, and to return promptly for consultation should the symptoms worsen (Gorwitz et al. 2006).

Results of the study underscore the necessity for the continuous strict implementation of hospital control measures to prevent the occurrence and increase in the incidence of nosocomial infections. The different infection control programs for MRSA vary in scope and stringency in different hospitals and in different localities. It is recognized though, that the minimum effective measures required for MRSA containment have not been defined (Marshall et al. 2004). The 2009 Norwegian methicillin-resistant *Staphylococcus aureus* guidelines include screening of certain groups of patients and HCW for MRSA, case tracing, isolation and work restriction, decolonization therapy and follow-up of MRSA carriers (Elstrom et al. 2012). On the other hand, questions on their effectiveness to control MRSA have been raised (Barrett et al. 1998; Marshall et al. 2004).

RECOMMENDATION

Thorough hand washing by HCW before and after handling each patient remains the cornerstone of prevention, since the predominant mode of transmission is from patient to patient through the contaminated hands of the HCW (Marshall et al. 2004). In addition, proper management of patients through prudent use of antimicrobials that averts the positive selection for MRSA and other resistant strains to survive and be disseminated cannot be overemphasized.
ACKNOWLEDGEMENTS

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REFERENCES


