Prevalence of community-acquired methicillin-resistant *Staphylococcus aureus* from inmates of the Manila City Jail, characterization for SCC*mec* type and occurrence of Panton-Valentine leukocidin gene

Esperanza C. Cabrera[1*], Dahlia Teresa Ramirez-Argamosa[2] and Roslyn D.M. Rodriguez[3]

[1] Biology Department and the Center for Natural Science and Ecological Research, De La Salle University, 2401 Taft Ave., Manila

[2] Department of Pathology, College of Medicine, University of the Philippines, Manila

[3] Microbiology Laboratory Section, Philippine General Hospital, Taft Ave., Manila

Methicillin-resistant *Staphylococcus aureus* (MRSA) has established itself as a significant cause of hospital and community-acquired infections worldwide. Resistance to methicillin compromises clinical treatment options, as it results in cross resistances to all other β-lactam antibiotics, which are the most commonly prescribed antibacterial agents. The study determined the prevalence of MRSA among inmates of the Manila City Jail with and without pyoderma, their antibiograms (susceptibility to other antimicrobial agents), SCC*mec* type and occurrence of the *luks-lukf* Panton-Valentine leukocidin (PVL) virulence gene among the isolates. Methicillin resistance was determined using oxacillin and cefoxitin discs and the detection of *mecA* gene and its product, PBP2a. The MRSA isolates were studied for their SCC*mec* types and for the presence of the PVL gene using multiplex polymerized chain reaction and nucleotide sequencing. *Staphylococcus aureus* was isolated from 78% of 105 inmates with pyoderma, 41% of which were resistant to methicillin, giving an overall prevalence rate of 32% among the infected inmates. Nasal carriage rates of *S. aureus* and MRSA among 104 inmates without pyoderma were 12% and 5%, respectively. All MRSA were susceptible to vancomycin; 35% and 44% were susceptible to erythromycin and clindamycin, respectively. Intermediate susceptibility to erythromycin and clindamycin was seen in 59% and 50%, respectively, while 6% were resistant to each of erythromycin and clindamycin. PBP2a and *mec*A gene were present in 94% of the 35 MRSA isolates tested. SCC*mec* type IV was demonstrated in 30 of the 33 isolates with *mecA* gene, while the remaining three were of SCC*mec* type I. PVL gene was detected in 83% of MRSA isolates. Nucleotide sequencing of the PCR amplicons from representative isolates studied all showed a G to C transversion at position 1004 of the *luks-lukf* gene, resulting in the substitution of valine (GTT) with leucine (CTT). This is the first report on the prevalence of MRSA carrying SCC*mec* type IV and the PVL genes in the Philippines and in a jail setting. Type IV *mec*A is the most easily transferred SCC*mec*, and its acquisition converts methicillin susceptible strains to resistance, while *luks-lukf* PVL is a composite of virulence genes that results in more serious infections. The study shows that community-acquired infection with this strain exists, and can be a growing problem that has to be fully addressed immediately to prevent further spread not only within the crowded penitentiary, but in the community as well.

* Corresponding Author
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INTRODUCTION

Staphylococcus aureus is a facultatively anaerobic, Gram (+) bacterium that causes diseases ranging from common skin infections to life threatening septicemia. It is the most prevalent pathogen causing hospital infection throughout the world, and the incidence is still increasing (Ma et al. 2002; Klein et al. 2007). The drugs of choice for treatment of staphylococcal infections are the β-lactam antibiotics, such as penicillins, cephalosporins or cephems, monobactam and carbapenems. However, through the years, the bacterium has evolved several mechanisms that render it to be resistant to the antimicrobials. The most common mechanism is the production of β-lactamase that inactivates many of the β-lactam antibiotics (Haeggman et al. 2004; Garcia-Cobos et al. 2008).

Methicillin, a β-lactamase-resistant β-lactam antibiotic, was introduced in the late 1950s. However, soon after its introduction, methicillin-resistant *S. aureus* (MRSA) emerged (Jevons et al. 1963). Methicillin resistance in *S. aureus* is mediated by the production of an altered penicillin binding protein called PBP2a (or PBP2') that has remarkably reduced affinity for β-lactam antibiotics (Ma et al. 2002). PBP2a is encoded by the *mecA* gene, which is carried by a large mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* is integrated into the chromosome of MRSA. Aside from the *mecA* gene, SCC*mec* is also comprised of two site-specific recombinase genes designated *ccrA* and *ccrB* that catalyze precise excision of SCC*mec* from MRSA and its integration into the chromosome of another microorganism, resulting in the transmission of the resistance (Katayama et al. 2000). The *mec* gene complex also has insertion sites for plasmids and transposons that allow the acquisition of resistance to non-beta-lactam antibiotics, e.g. erythromycin, gentamicin, tetracycline and ciprofloxacin.

Methicillin- resistant *S. aureus* or MRSA cannot be eradicated with any of the wide array of β-lactams, presenting a serious treatment problem. Compounding this difficulty is the occurrence of the *luks-lukf* Panton-Valentine leukocidin (PVL) gene in certain strains of MRSA, notably those that are community-acquired (CA) (Vandenesch et al. 2003). CA-MRSA with the PVL gene has predominantly caused skin and soft-tissue infections. However, invasive life-threatening infections are now being reported, including necrotizing pneumonia even in previously healthy individuals (Gillet et al. 2002), septic shock and death (Miller et al. 2005; Kravitz et al. 2005). PVL stimulates the production of inflammatory mediators such as histamine from basophils, β-glucuronidases and lysozyme, which leads to tissue necrosis. It also inhibits phagocyte functions, and forms transmembrane pores in polymorphonuclear leukocytes, monocytes and macrophages that result in osmotic lysis. (Genestier et al. 2005). The combination of this enhanced virulence with resistance to the most commonly prescribed anti-staphylococcal antibiotics thus makes this organism an important public health problem.

Recently, numerous reports have focused on the outbreak of infectious skin diseases such as furuncles and carbuncles among inmates of several correctional institutions in Metro Manila, Philippines, where overcrowding and poor personal hygiene contribute to the rapid spread of the etiologic agents (Bureau of Jail Management and Penology Report 2007; GMANews.TV 2007; Buensuceso et al. 2005; Pimentel 2005). This study was conducted to determine the prevalence of CA-MRSA isolates among infected and noninfected inmates of the Manila City Jail, their susceptibility to other antimicrobial agents, the SCC*mec* type of the *mecA* gene, and the presence of the *luks-lukf* Panton-Valentine leukocidin gene.

MATERIALS AND METHODS

Selection of Study Participants

Manila City Jail is a minimum security state detention center which has approximately 4000 male and 800 female inmates. It is composed of 14 male wards and 5 female wards, with health care needs provided by two medical doctors and six nurses. All inmates with pyoderma (inflamed lesions with purulent material) at the time of sampling (designated as Group A, n=105), and randomly chosen inmates without any skin lesions for testing nasal carriage state (designated as Group B, n=104) were included in the study. Each participant was asked for his/her proper consent. All patients were physically examined and assessed by a dermatologist. Sampling was conducted twice, once in July and another time in August 2007. The research protocol was approved by the review committee of the Manila City Jail.

The following set of exclusion criteria, based on the CDC definition of CA-MRSA was followed in the choice of the subjects (CDC 2005): "Patients should have no medical history in the past year of (1) hospitalization, (2) admission to a nursing home, skilled nursing facility, or hospice (3) dialysis and (4) surgery. There should be no permanent indwelling catheters or medical devices that pass through the skin into the body".

Specimen Collection and Bacterial Identification

The infected skin area of the inmate was thoroughly cleaned with 70% ethyl alcohol and povidone-iodine. Exudates or aspirated pus from lesions were asceptically collected using sterile swabs. Samples were also taken from both the right and left anterior nares of non-infected inmates using sterile cotton swabs. The specimens were cultured on sheep blood agar and chocolate agar under 5% CO_2 and on mannitol salt agar or MSA (Difco, USA) for 18-24 hrs at 35^0C. *Staphylococcus aureus* was identified based on its Gram stain morphology, colonial morphology, growth and production of acid on MSA, catalase test and coagulase test. The StaphID™ latex agglutination test (Biomeriuex, France) was used to determine the presence of

Protein A and bound coagulase that are specific for *S. aureus*.

Test for Susceptibility to Methicillin and Other Antimicrobials

Methicillin susceptibility was phenotypically determined using the following methods: the standard disc diffusion method using cefoxitin (30 µg) and oxacillin (I µg) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2007); the Staph MRSA™ Latex Agglutination protocol (Biomeriuex, France) for the detection of PBP2a, and the oxacillin E-test MIC Macro Method (AB Biodisk, Solna, Sweden), both according to the instructions of the manufacturer. Oxacillin resistant *S. aureus* 3269, a kind gift from Dr. I. Spiliopoulou of the University of Patras, Greece was used as the positive control in all assays, while *S. aureus* ATCC 29213 was included as the negative control.

Susceptibility to other antimicrobials, namely: penicillin G (10 units), erythromycin (15 ug), clindamycin (2 ug), and vancomycin (30 ug) was determined using the standard disc diffusion based on the recommended procedure of CLSI (2007). *Staphylococcus aureus* ATCC 25923 was used as control.

Extraction of chromosomal DNA.

DNA was extracted following the boiling method described by Zhang et al. (2004). One to five colonies from an 18-24 hour MRSA culture grown in blood agar plate were suspended in 50 uls distilled water, and boiled for 10 mins. The supernatant with DNA was harvested after centrifugation at 20,000 x g at 4°C.

Multiplex PCR for the detection of *mecA* gene and and *luks-lukf* PVL genes

The primer sequences for detection of the different target genes and the expected sizes of the PCR products are found in Table 1. Primer pair Staph 756F and Staph 750R targets the *Staphylococcus* genus-specific 16s rRNA gene, and was included as an internal control to rule out the presence of amplification inhibitors. Primers MecA1 and MecA2 amplify a fragment of the *mecA* gene, while Luk-PV-1 and Luk-PV-2 target the PVL gene. PCR was carried out in a 25-ul final reaction volume following the method of McClure et al. (2006). The mixture consisted of 5-10 ng DNA template, 1X PCR buffer, 0.2mM of each dNTP, 2.5 mM $MgCl_2$, 0.04 U/ul Taq DNA polymerase (Intronbio™), 0.3 uM of each primer. The thermocycling conditions were as follows: initial denaturation at 94°C for 10 min, 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 a final extension at 72°C for 10 min.

Table 1. Primer sequences for detection of *mecA*, *luks-lukf* genes and 16srDNA for *Staphylococcus* and the expected amplicon sizes.

Primer	Nucleotide sequence (5' to 3')	Amplicon size (bp)	References
Staph 756F	AACTCTGTTATTAGGGAAGAACA	756	McClure et al. 2006; Zhang et al. 2004
Staph 750R	CCACCTTCCTCCGGTTTGTCACC		
MecA1	GTAGAAATGACTGAACGTCCGATAA	310	McClure et al. 2006; Zhang et al. 2004
MecA2	CCAATTCCACATTGTTTCGGTCTAA		
Luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	McClure et al. 2006; McDonald et al. 2005
Luk-PV-2	GCATCAAGTGTATTGGATAGCAAAAGC		

Multiplex PCR for the Determination of the SCC*mec* Type

Table 2 shows the primer sequences used in the determination of the SCC*mec* type, and the type to which the different amplicons are expected to be found (Oliveira and de Lencastre 2002). This typing system identifies types I to IV based on loci located upstream and downstream of the *mecA* gene, and does not take into account the specific *ccr* gene complex (Zetola et al. 2005). The amplification of the *mecA* gene was included to serve as an internal control. PCR was carried out in a 25-ul final reaction volume that consisted of the following: 5-10ng DNA template, 1X PCR buffer, 0.2mM of each dNTP, 2.5 mM $MgCl_2$, 0.05 U/ul Taq DNA polymerase (Intronbio ™), 0.4 uM of each primer. The thermocycling conditions were as follows: initial denaturation at 94°C for 4 min, and 30 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 75 sec, and a final extension at 72°C for 4 min. The PCR amplicons were visualized using a UV transilluminator after electrophoresis in 1.7% agarose at 50 volts for 1-1.5 hours, and staining with ethidium bromide.

Sequencing of PCR Products

The sequences of the primers for the amplification of the *mecA* gene and its sequencing are as follows: *mecA* forward 5' TGGCTATCGTGTCACAATCG3'; *mecA* reverse 5' CTGGAACTTGTTGAGCAGAG 3'. Those for the PVL gene, 16srDNA for the genus *Staphylococcus*, and for SCC*mec* typing are as shown in Tables 1 and 2. The amplicons were sent to Macrogen, Korea for purification and sequencing, and were submitted for nucleotide alignment and identification to the National Center for Biotechnology Information (http:ncbi.nlm.nih.gov). Representative sequences were deposited with Genbank under accession numbers GU360975, GU360976 (*mecA*), and GU360977, GU360978 (*luks-lukf* PVL).

Table 2. Primer sequences for *SCCmec* typing of methicillin-resistant *Staphylococcus aureus* (Oliveira and de Lancastre 2002).

Primer	Nucleotide sequence (5' to 3")	Amplicon size (bp)	SCC*mec* type
CIF2 F2 CIF2 R2	TTCGAGTTGCTGATGAAGAAGG ATTTACCACAATTACTACCAGC	495	I, IA
KDP F1 KDP R1	AATCATCTGCCATTGGTGATGC CGAATGAAGTGAAAGAAAGTGG	284	II
DCS F2 DCS R1	CATCCTATGATAGCTTGGTC CTAAATCATAGCCATGACCG	342	I, IA, II, IV, IVA
RIF4 F3 RIF4 R9	GTGATTGTTCGAGATATGTGG CGCTTTATCTGTATCTATCGC	243	III
IS431 P4 PUB110 R1	CAGGTCTCTTCAGATCTACG GAGCCATAAACACCAATAGCC	381	IA, II, IVA
MECA P4 MECAP7	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162	*mecA*

RESULTS

Prevalence of MRSA and Susceptibility to Other Antimicrobials

A total of 105 inmates with skin lesions were examined, 87 (83 %) were males and 18 (17 %) were females. *Staphylococcus aureus* was isolated from 82 (78%) of these infected inmates, and 34 (41%) out of the 82 were identified as MRSA using the cefoxitin and oxacillin disc diffusion method. This gives an overall prevalence of 32% MRSA among inmates with pyoderma. To assess if MRSA carriage existed among inmates without skin lesions, swab specimens of anterior nares were collected from randomly selected 104 subjects, 85 (82%) were males and 19 (18%) were females. Twelve (12%) were found to have *S. aureus* in their nasopharyngeal flora, and an overall carriage rate of MRSA of 5%.

As was expected, all MRSA isolates from both infected and non-infected inmates were resistant to the β-lactam penicillin G. All isolates were found to be susceptible to vancomycin. While a low 6% were resistant to erythromycin, 59% showed only intermediate susceptibility to the antibiotic, and 35% were completely susceptible to it. The results with clindamycin paralleled those with erythromycin, where 6% were resistant, 50% showed intermediate susceptibility and 44% were susceptible to it.

Detection of *mecA* and *luks-lukf* PVL gene, and SCC*mec* typing

Of the 39 MRSA isolates, 35 (31 from pyoderma, four from nasal flora) were available for testing for the *mecA* gene. Amplification of the gene was observed in all isolates except for A102 and B67 (Fig. 1). This confirms the isolates to be MRSA strains, since detection of the *mecA* gene is considered to be the gold standard in the determination of methicillin resistance (Felten et al. 2002; Velasco et al. 2005). Aside from being negative for *mecA*, isolates A102 and B67 were also found to be negative for PBP2a. However, on further testing, the minimum inhibitory concentration (MIC) of oxacillin for the two isolates was determined to be 8 ug ml^{-1}, which confirmed their resistance to methicillin (CLSI, 2007). The resistance may be due to the overproduction of β-lactamase (Hamdad et al. 2006), or due to an unidentified chromosomal mutation that does not involve *mec*A (Ryffel et al. 1994). In addition, among the 33 isolates found to be *mecA* positive, 30 were of *SCC mec* type IV, while the remaining three were of type I (Fig. 2).

The 35 MRSA isolates were also tested for the presence of the *luks-lukf* PVL gene, where 29 (or 83%) were found to be positive (Fig. 1). Two of these were from inmates with no pyoderma. A102 and B67 that were negative for the *mecA* gene and PBP2A were also negative for the PVL gene. Nucleotide sequencing of the PCR amplicons from the 10 representative isolates studied all showed a G to C transversion at position 1004 of the *luks-lukf* gene, resulting in the conservative substitution of valine (GTT) with leucine (CTT) (Genbank

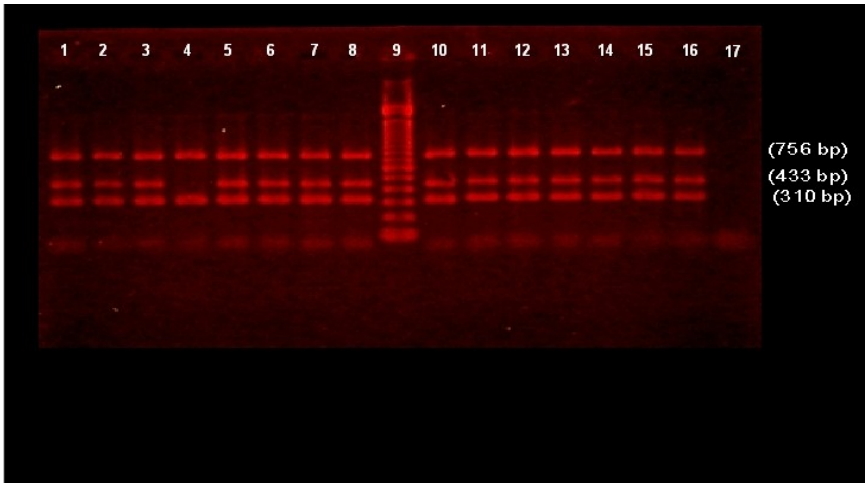

Figure 1. Multiplex PCR for the detection of *mecA* (310 bp), *lukF-luk-S* PVL (433 bp) and *Staphylococcus*-specific 16s rRNA genes (756 bp, internal control). Lanes 1-3, 5-8, 10-16: Isolates positive for *mecA* (MRSA), *lukF-luk-S* PVL and *Staphylococcus*-specific 16srRNA genes (Isolates A26, A29, A30, A34, A37, A42, A45, A47, A49, A51, A53, A54, A56, A57, respectively). Lane 4: MRSA negative for the PVL gene (A24). Lane 9: reference 100 bp ladder. Lane 17: negative control *Escherichia coli.*

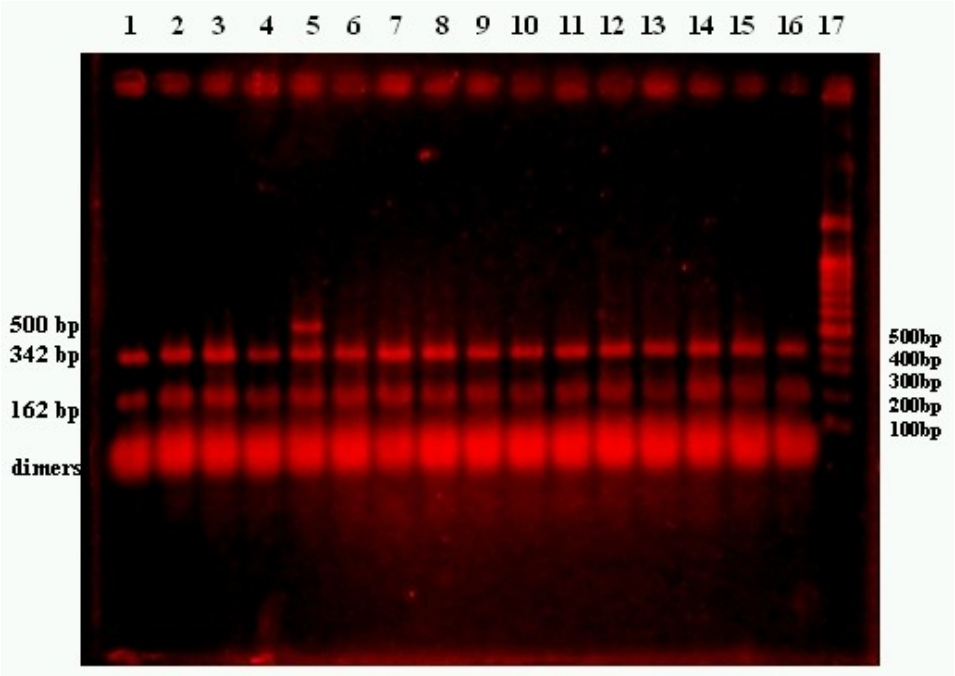

Figure 2. Multiplex PCR for SCC*mec* typing of methicillin-resistant *Staphylococcus aureus. mecA* internal control (162 bpP); SCC*mec* type IV (342 bp); SCC*mec* type I (342 bp and 500bp). Lanes 1-4, 6-16: SCC*mec* type IV (Isolates A24, A26, A29, A30, A37, A43, A45, A47, A49, A51, A53, A54, A56, A57, A61, respectively). Lane 5: SCC*mec* type I (Isolate A34). Lane 17: 100bp ladder.

accession numbers GU360977, GU360978). However, the effect of this mutation on the PVL protein was not determined further in the study.

DISCUSSION

The present study showed a high overall prevalence of 32% MRSA among inmates with pyoderma, or 41% MRSA among the *S. aureus* isolates. This suggests the ease of transmission of the pathogen among the inmates who are housed in very crowded, poorly-ventilated and dirty cells. Acquisition of the organism is further facilitated by the lack of facilities for personal hygiene and medical care (Buensuceso et al. 2005; Pimentel 2005). In addition, although 17% of the infected inmates were given antimicrobials, all admitted to not complying with the proper dosage and duration of drug intake as instructed by the medical personnel. Nonetheless, the most common antibiotics prescribed were the β-lactams amoxicillin (61%) and cloxacillin (17%), which are not effective for treatment of methicillin-resistant strains (CLSI 2007).

It is interesting to note that this prevalence is much lower when compared to results of some studies conducted among infected inmates in other correctional institutions, which reported prevalence rates of 63.5% in Chicago's Cook County Jail among isolates cultured in years 2004 to 2005 (David et al. 2008), and 29% in 1997 that rose to 74% in 2002 in the San Francisco County Jail system (Pan et al. 2003). MRSA was detected in 77.7% of *S. aureus* cultured between 2000 and 2007, also from inmates of the San Francisco County Jail system (Tattevin et al. 2008). Lowy et al. (2007) showed that 48.3 % of clinical *S. aureus* isolates from all New York state prisons collected in a 6-month period were MRSA. The proportion of *S. aureus* infections that were methicillin-resistant increased from 24% in 1998 to 66% in 2002 among inmates in correctional facilities operated by the Texas Department of Criminal Justice (MMWR 2003), despite the implementation in 1996 of a comprehensive set of treatment and prevention guidelines for MRSA skin infections. The lower prevalence of MRSA in the present study compared to those reported in the other studies can not be easily explained, considering that the other correctional institutions have better facilities and provisions for medical care for their inmates. One reason may be the heavier usage of β-lactams for the empirical treatment of the pathogen in some of these institutions (David et al. 2008).

The overall carriage rate of MRSA in the nasopharynx of non-infected inmates was 5%. While this result seems to be comparable to that reported in the study of Felkner et al. (2007) among newly-admitted non-infected Texas County Jail inmates where the prevalence rate of MRSA was 4.5%, this carriage rate is lower than expected if we consider that these sampled inmates in the present study were crammed under unhygienic conditions in the same cells with the MRSA-infected inmates,. The same observation can be said when the present result is compared to reports of studies with non-infected subjects in non-jail settings. Lu et al. (2005) found that 19.1% of subjects associated with health care-related facilities in Taiwan had nasal colonization with *S. aureus*, with 7.63% of the subjects carrying MRSA, while Huang et al. (2007) showed that between 2005 and 2006, 7.3% of healthy Taiwanese children between the ages of 2 months and five years were colonized by MRSA in their nares. The overall MRSA nasal colonization rate among injection drug users in Vancouver, Canada in 2000 was found to be 7.4%, which rose to 18.6% in 2006 (Al-Rawahi et al. 2008).

The PVL gene was detected in 83% of the MRSA tested. The occurrence of the PVL genes in MRSA is reported to enhance its virulence. It has been implicated in severe, necrotizing, soft tissue infections and systemic diseases such as septicemia, necrotizing pneumonia, hemorrhaging pneumonia and endocarditis, even in previously healthy individuals (Robinson et al. 2005; Lopez-Aguilar et al. 2007; Bocchini et al. 2006, Vandenesch et al. 2003), and often poor prognosis and high mortality rates are associated with it. MRSA with the PVL gene is reported worldwide, and is a serious threat to public health.

The SCC*mec* type IV of the *mecA* gene found in 91% of the MRSA isolates tested, and the presence of the *luks-lukf* PVL gene in 83% of these *mecA* gene positive isolates, are consistent with the characteristics of CA-MRSA reported in other studies (Dumitrescu et al. 2007; Bhattacharya et al. 2007; Huang et al. 2006). These two characteristics of CA-MRSA render it a cause for public health concern**.** Data of other studies support the hypothesis that *mecA* gene is transferred from cell to cell as a part of the SCC*mec* across staphylococcal species (Katayama et al. 2000; Wisplinghoff et al. 2003; Hanssen et al. 2004), although the mechanism of transfer of *mec* DNA from a donor to a recipient is not completely understood (Salmenlinna et al. 2002). In addition, compared to the other types, SCC*mec* IV has a relatively high rate of excision and integration (Huang et al. 2006; Corkill et al. 2004). Its small size likewise may favor its acquisition and retention due to the lower cost on fitness when acquired by *S. aureus* (Okuma et al. 2002). On the other hand, the presence of two isolates with the SCC*mec* type I suggests the transmission of hospital-acquired MRSA (HA-MRSA) in the community, since this type is characteristic of hospital strains (Huang et al. 2006; Hiramatsu et al. 2001; Ito et al. 2004).

Results of the present study firmly established the high prevalence of MRSA with the very mobile SCC*mec* type IV and the PVL virulence genes among inmates in this institution. The transfer of the strains among inmates that are housed together in substandard, humid and congested cells that have poor access to running water, detergents and other personal health care materials can not be overestimated. The ease of transmission of MRSA is shown by the results of the study by Al-Rawahi et al. (2008) where its nasal carriage among injection drug users rose from 7.4% in 2000 to 18.6% in 2006, and that of Pan et al. (2003) where MRSA prevalence among inmates rose from 29% in 1997 to 74% in 2002 in the San Francisco County jail system. Moreover, because inmates are released and incarcerated from time to time, it is very possible that those who harbor the organism could serve as foci for its dissemination into the urban communities. It is thus imperative that this problem be addressed with serious political will. The living conditions and health care

programs in correctional institutions should be thoroughly evaluated and improved. Education of inmates on personal hygiene and monitoring of the changing trends in bacterial infections and their antimicrobial susceptibility patterns should be instituted regularly. Implementation of the correct use of antimicrobials is mandatory to reduce the spread of MRSA (and other multiply resistant antibiotic strains) in the community.

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NO CONFLICT OF INTEREST STATEMENT

We certify that there is no conflict of interest or financial conflict in the conduct of the present study, in the preparation or submission of this manuscript.

Esperanza C. Cabrera
Dahlia Teresa Ramirez-Argamosa
Roslyn D.M. Rodriguez
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CONTRIBUTION OF AUTHORS

Esperanza C. Cabrera: collection of specimens, molecular analysis for *mecA*, SCC*mec* type, PVL gene, preparation of the manuscript, corresponding author, responded to comments; Dahlia Teresa Ramirez-Argamosa and Roslyn D.M. Rodriguez: arrangement with Manila City Jail for consent and approval, collection of specimens, isolation and identification of the bacterial isolates, determination of antimicrobial susceptibility patterns

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