Association of mecA gene and pvl gene in Methicillin Resistant *Staphylococcus aureus* Isolated from Clinical Specimens

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ABSTRACT

Objectives: The aim of this study was to explore the association of mecA and pvl genes in clinical isolates of MRSA.

Materials and Methods: A total of 748 clinical samples were processed by standard microbiological techniques. Colony morphology, Gram's staining and biochemical tests including catalase, oxidase and biochemical tests were used for the identification. Antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion technique and their resistance to Methicillin was evaluated using the Cefoxitin disc diffusion method. DNA was extracted by using a modified phenol-based DNA extraction method and finally, the presence of pvl and mecA genes were investigated by the polymerase chain reaction method.

Results: Out of 748 clinical samples, (29.4%; 220/748) samples had bacterial growth. Among 220 culture positive isolates, 19.1% (42/220) were positive for S. aureus. Among 42 S. aureus (66.7%; 28/42) were multiple drug resistant (MDR) and 38.1% (16/42) were MRSA. Among 16 MRSA; mecA and pvl genes were positive for 16 (100%) and 7 (43.7%) of the isolates respectively. Seven of mecA positive isolates was positive for pvl gene.

Conclusions: It can be concluded from these results that the prevalence of pvl gene is high in MRSA isolates in this study and there is association between the presence of pvl and mecA genes in these isolates.

Keywords: MRSA, panton valentine leukocidin (pvl), mecA, MDR, PCR.

INTRODUCTION

Staphylococcus aureus is a Gram-positive, facultative anaerobic bacterium, and is the most potent and significant species of staphylococci that causes infections from simple furuncles (boils) and carbuncles to deadly necrotizing pneumonia and toxic shock syndrome (Jr *et al.*, 2016). The emergence of multidrug resistant *S. aureus* strains, including methicillin resistant, is a global concern. Methicillin-resistant *S. aureus* (MRSA) was first described in 1961 in England and has become endemic and epidemic in hospitals worldwide (Özekinci *et al.*, 2014). Methicillin resistance results from the introduction of mobile genetic element called staphylococcal cassette chromosome mec (SCCmec) that contains the antibiotic resistance gene mecA (Chambers and Deleo, 2010). The mecA gene encodes for a variant type of penicillin-binding protein (PBP2a), which has a lower affinity for the β-lactam antibiotics (Motamedi *et al.*, 2015a). The ability of MRSA to cause various infections are mainly due to the presence of different extracellular and surface virulence factors with

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adhesive properties example is Panton Valentine Leukocidin (PVL) (Girgis and Ahmed, 2017). PVL is a bicomponent cytolytic toxin encoded by two genetic elements called PVL (LukF-PV [34kDa] and LukS-PV[33kDa] (Taromian *et al.,* 2016) shows cytolytic activity with a high cell specificity to leukocytes, which are carried by a group of specific bacteriophage(Bhatta *et al.*, 2016). After secretion of the two components, they form a pore-forming heptameric on neutrophil membranes, leading to either neutrophil lyses or apoptosis and contribute to tissue necrosis. PVL has been linked to skin, soft tissue infections and necrotizing pneumonia (Eed *et al.*, 2016).

This study explores the prevalence of MRSA infections, antimicrobial susceptibility patterns and detection of mecA gene and pvl gene by using molecular methods, PCR with objective to determine the association of pvl and mecA in MRSA strains isolated from clinical samples.

METHODS

Study design, samples and identification of S. aureus

The study was hospital based cross-sectional study conducted in Annapurna Neurological Institute and Allied Sciences (ANIAS) and Annapurna Research Center, Maitighar, Kathmandu from January 2019 to June 2019. The ethical approval was obtained from institutional review board of Nepal health research council (Reg. 506/2019). A total of 748 clinical samples were taken for the study. The samples were collected in clean, sterile and leak proof containers using aseptic technique by experienced medical officers and taken immediately into the microbiology laboratory for further processing. All the samples were inoculated into Blood Agar (BA), Mac Conkey agar (MA) and Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hours. The isolates were identified as S. aureus using standard microbiological techniques including biochemical and coagulase tests.

Antibiotic Susceptibility pattern

Antibiotic susceptibility test of the different clinical isolates towards the various classes of antibiotics was performed by modified Kirby-Bauer disk diffusion method on Muller Hinton Agar following CLSI guidelines (CLSI 2017). Antibiotics used were Ampicillin (10mcg), Ceftriaxone (30mcg), Cotrimoxazole (25mcg), Gentamycin (10mcg), Cefoxitin (30mcg), Ciprofloxacin (5mcg), Erythromycin (15mcg), Clindamycin (2mcg), Linezolid (30mcg) and Vancomycin30mcg). Results were reported as susceptible, intermediate or resistant based on the diameter of the inhibition zone. Cefoxitin (30mcg) was used for the detection of MRSA isolates for which size of zone of inhibition is ≥22mm for sensitive and ≤21mm for resistant. Also, isolates which were resistant to 3 or more classes of antibiotics were detected as Multi-Drug Resistant (MDR).

DNA Extraction

The isolates were cultured in Nutrient Broth (NB, HiMedia, India) for 18h at 37°C. Then, 3000µl (3ml) of bacteria culture was centrifuged (10000 rpm, 5minutes) and pellets was suspended in 567 µl TE buffer and mixed well by vortexing. The lyses of cellwall and protein was done by adding 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase K followed by mixing and incubation at 37°C for 45 min. After incubation, 100 µl 5M sodium chloride solution followed by 80 µl of CTAB/NaCl (10%CTAB/0.7M NaCl) were added and incubated at 65°C for 10 min. Then after equal volume of chloroform isoamyl alcohol (C: I) in the ratio (24:1) was added to the suspension and centrifuged (10000 rpm, 5 min). Upper aqueous phase was transferred to new tube and equal volume of (P: C: I) was added and centrifuged (14000 rpm, 5 min). After that supernatant was transferred to a new tube and solution was treated with iso- propanol and centrifuged (12000 rpm, 5 min), iso- propanol was removed carefully. The DNA was then precipitated and washed subsequently with absolute 70% ethanol. Finally, DNA was air dried for 10-15 min and resuspend in 50µl of TE buffer and store at -20°C till experiment (Nakaminami *et al.*, 2014).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed in a final 25µl volume reaction mixture containing 12.5µl master mixture, 0.5µl each of the forward and reverse primers, 2µl of the extracted crude DNA and 9.5 µl of distilled water was added in individual amplification tubes. Amplification reaction was carried out under the following thermal cycling conditions:

Thermal Cycling process for mecA consisted an initial denaturation step at 94°C for 2 min, followed by 33 cycles of denaturation 94°C for 1 min, annealing 55°C for 45 s, and 72°C for 45 s ending with Final extension step at 72°C for 4 min and followed by a hold at 4°C (Karmakar *et al.*, 2018). While, thermal cycling process for pvl consisted initial denaturation step at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s; annealing step at 56°C for 45 s and extension step at 72°C for 30 s. A final extension step was done at 72°C for 2 min (Bhatta *et al.*, 2016).

Gel Electrophoresis for characterization of mecA and pvl gene

For the separation of PCR products, the amplified PCR product along with positive control, negative control and ladder was run through 1.5% agarose gel stained with Ethidium bromide (EtBr). The gel was visualized under gel

documentation system (UVTEC Cambridge, UK) and image was captured.

Statistical Analysis

Standard statistical formulae were used to calculate frequencies and percentages of data that were compared using Pearson's Chi-square test. A P-value of <0.05 was considered as statistically significant.

RESULTS

A total of 748 samples (urine 301, blood 128, sputum 149, pus 24, CSF 91, throat swab 4, body fluids 13, wound swab 22, tissue 7, and stool 9) from patients attending the hospital for treatment were collected and analyzed. From the total of 748 samples, 42 (5.61%) were confirmed as *S. aureus* and were further tested for MRSA.

The drug profile of isolates was tested for 11 different antibiotics. The Cefoxitin disk detected 16 (38.1%) MRSA isolates. The maximum numbers of MRSA were found in age group>40 years (68.75%) and higher incidence of MRSA infections were found in males (75%). However, both failed to elicit a statistically significant difference. High proportion of blood samples contained MRSA, 31.2% (5/16) and a higher proportion 62.5% (10/16) of MRSA was derived from ward.

Among the antibiotics profile, vancomycin was the most effective drug to which all the isolates of MRSA were sensitive followed by Linezolid (81.3%) and Gentamycin (62.5%). While 66.7% (28 /42) isolates among S. aureus were multidrug resistant (MDR). Similarly, 16 (100%) out of 16 MRSA isolates were positive for MDR and statistically, there were significant association (P=0.0048) (Table 1).

Table 1; Pattern of MDR in MRSA

MRSA	MDR		Total	*P-value*
	Positive	Negative		
Positive	16(38.1%)	0	16	0.0048*
Negative	12(28.5)	14(33.3%)	26	

Among 16 MRSA isolates, mecA were positive for all isolates where as seven MRSA isolates were positive for pvl gene as shown in table 2. Statistically, there was significant association between mecA and pvl gene in MRSA (P= 0.005). Table 3 shows the PCR results of each sample.

Higher prevalence of pvl among >40 years of age was observed. pvl gene was highly prevalent in blood (18.7%). Statistically, there was no association (P=0.993).

Table 2: Detection of pvl gene in MRSA isolates

Specimen type	Total no. of MRSA	pvl positive
Urine	3	2
Sputum	3	2
Blood	5	3
CSF	1	0
Pus	2	0
Wound swab	2	0
Total	**16**	**7**

Table3: PCR results of mecA and pvl genes

Isolates	mecA	pvl
1	+	-
2	+	-
3	+	-
4	+	-
5	+	-
6	+	-
7	+	+
8	+	+
9	+	-
10	+	-
11	+	-
12	+	+
13	+	+
14	+	+
15	+	+
16	+	+

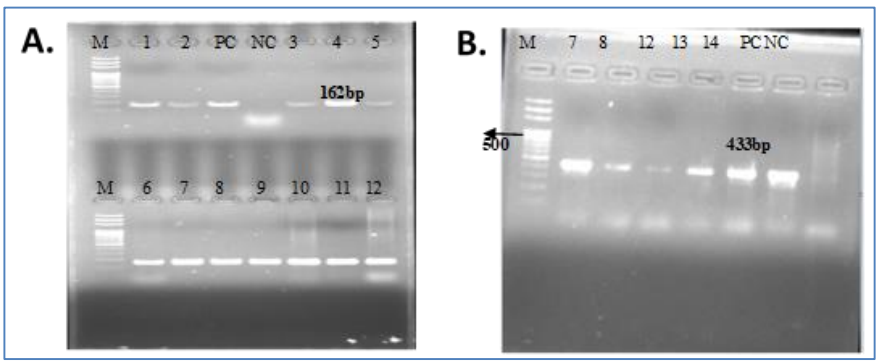

Figure 1: Gel electrophoresis results; A. mecA gene (162 bp) B. pvl gene (433 bp)

DISCUSSION

MRSA infections are prevalent in healthcare and community settings and more prevention efforts are needed to overcome newly emerging multidrug resistance (Mazi *et al.*, 2020). Having knowledge about the prevalence of MRSA and their virulence factors is useful for treatment and control. Following the emergence of MRSA strains, molecular studies showed that some of these isolates carry pvl gene (Shore *et al.*, 2011). The pvl positive strains lead to infections with different clinical appearance even in immunocompromised patients lead to necrotic pneumonia, which its mortality can be as much as 75% (Qiao *et al.*, 2014). Therefore, frequent monitoring of this pathogen, its antibiotic susceptibility and determining their virulence factors is of great importance in control and treatment of infections**.**

Of the 42 strains of S. aureus studied, 38.1% (16/42) strains were Methicillin resistant. This finding is similar as compared to the percentage of MRSA reported in the previous studies carried out in Nepal by Lamichhane et al (1999), Rajbhandari et al. (2002), Thapa et al (2004), Sapkota et al (2006) and Tiwari et al. (2007) have reported the percentage of MRSA out of the total S. aureus isolates to be as high as 25%, 54.9%, 29.23% 31.1%,, and 69.1% respectively.. This finding is also similar with previous studies that reported 40.7% MRSA in Nairobi by(Iliya *et al.* 2020) Eschetie et al (2016) and Wang et al (2017) have reported 32.5% MRSA in Ethiopia and 49.3% in china respectively. In contrast, higher rate 80.4% MRSA in Cameroon by (Kengne *et al*., 2019), 72% by (Jarraud *et al.*, 2018)and 65.7% by (Asiimwe *et al.*, 2017).

A higher proportion (62.5%) of MRSA was derived from Ward and higher incidence of MRSA infections were found in males (75%). Similarly, the isolation of MRSA was not related to gender as well ($\chi 2$ =0.006, P>0.05). Our report indicates high prevalence of MRSA among in-patients (ward) compared to OPD patients There was no significant difference between the isolation of MRSA from Hospital units ($\chi 2$ = 11.50, P>0.05) which is similar to previous research findings conducted in Nepal by (Shrestha *et al.*, 2014) and contrary to the finding of (Khanal, Adhikari and Guragain, 2018) where higher prevalence of MRSA among OPD patients Out of the 16 MRSA isolates, 5 (31.2%) from blood, 3 (18.7%) each from urine and sputum, 2(12.5%) each from pus and wound swab and 1 (6.2%) from CSF.

High proportion of blood samples contained MRSA (31.2%) which is similar to the study carried out in Nepal by(Raut *et al.*, 2017) has reported the percentage of MRSA out of the total *S. aureus* isolates to be as high as 49%.

All 16 MRSA isolates included in this study were positive for mecA gene Whereas pvl genes were detected in 43.7%. Higher prevalence of PVL among (>40 years of age) was observed as compared to children which is in contrary to another study from India by (Bhutia *et al*., 2010). Similarly, pvl gene is found more in male (85.7%; 6/7) than female. According to the study carried out in United States by(Darboe and Secka, 2019) reveals that PVL prevalence was high across all age groups.

Our results revealed that PVL-positive MRSA were associated with increased disease severity as blood stream 18.7% (3/16), Similar findings were also reported in previous studies(McClure *et al.*, 2006; Eed *et al.*, 2016; Gonzalez *et al.*, 2018). Epidemiological data suggest that high virulence of community acquired MRSA associated with pvl gene but direct evidence of association of pvl to pathogeneses remains limited (Hsu *et al.*, 2005), while a previous study in Greece revealed that a unique clone of PVL-positive MRSA had spread in both the community and hospital settings. In Central Gabon, Africa, PVL encoding genes were detected in 55.9% of study isolates, with authors concluding that the pygmies in that study faced a risk of developing necrotizing infections, due to the virulence characteristic of the PVL(Asiimwe *et al.*, 2017). Mortality rates also significantly differ between the two types: PVL-positive infection has a higher mortality rate than PVL-negative infection(Fujisaki *et al.*, 2014)

The obtained results in the present study are in contrast with the finding of (Hiramatsu *et al.*, 1992) and (Motamedi *et al.*, 2015b) that reported no pvl positive strain is there among tested MRSA strains and have suggested it is maybe that resistance determinants be carried on other mobile elements, such as plasmids, transposons, and phages; so, their elimination from bacterial cell would result in the absence of mecA gene and consequently no association with pvl gene.

Conclusion

Methicillin resistant S. aureus is one of the major infectious agents of infection in hospital. Only vancomycin was the

effective antibiotics towards the isolates of S. aureus followed by Linezolid while resistant to the rest of the antibiotics used in the study. The study also showed that there was a significant association between MDR and MRSA. Further, the study showed a significant association in between mecA and pvl gene in MRSA isolates.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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