Detection of ST772 Panton-Valentine leukocidin-positive methicillin-resistant Staphylococcus aureus (Bengal Bay clone) and ST22 S. aureus isolates with a genetic variant of elastin binding protein in Nepal

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Abstract

Genetic characteristics were analysed for recent clinical isolates of methicillin-resistant and -susceptible Staphylococcus aureus (MRSA and MSSA respectively) in Kathmandu, Nepal. MRSA isolates harbouring Panton-Valentine leukocidin (PVL) genes were classified into ST1, ST22 and ST88 with SCCmec-IV and ST772 with SCCmec-V (Bengal Bay clone), while PVL-positive MSSA into ST22, ST30 and ST772. ST22 isolates (PVL-positive MRSA and MSSA, PVL-negative MRSA) possessed a variant of elastin binding protein gene (ebpS) with an internal deletion of 180 bp, which was similar to that reported for ST121 S. aureus previously outside Nepal. Phylogenetic analysis indicated that the ebpS variant in ST22 might have occurred independently of ST121 strains. This is the first report of ST772 PVL-positive MRSA in Nepal and detection of the deletion variant of ebpS in ST22 S. aureus.

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Keywords: Bengal Bay clone, elastin binding protein, MRSA, Nepal, Panton-Valentine leukocidin (PVL), ST22, ST772

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Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is recognized as one of the most common pathogens of both nosocomial and community-acquired infections worldwide. As a feature distinct from methicillin-susceptible S. aureus (MSSA), MRSA has a transmissible genome element, staphylococcal cassette chromosome mec (SCCmec), inserted in a specific site of the chromosome. The SCCmec in MRSA has been differentiated into at least 12 genetic types (I–XII) [1,2], among which types I–III have been traditionally associated with hospital-acquired MRSA (HA-MRSA), while type IV and V have been commonly found in community-acquired MRSA (CA-MRSA) [3]. However, in recent years, CA-MRSA with the dominant SCCmec types (IV and V) has been brought to healthcare settings causing nosocomial infections [4–6], which makes distinction between HA- and CA-MRSA more difficult in terms of SCCmec type. The pathogenesis of many CA-MRSA strains have been attributed to the production of Panton-Valentine leukocidin (PVL), a two-component toxin encoded by two genes, lukF-PV and luk-S-PV, which are carried on lysogenic bacteriophages [7,8]. The PVL causes leukocyte lysis or apoptosis via pore formation [9]. Accordingly, PVL-positive S. aureus is associated with severe symptoms in a wide spectrum of infections including skin and soft tissue infections and necrotizing pneumonia [10,11]. Prevalence of CA-MRSA harbouring PVL genes has been increasing recently in hospitalized patients as well as healthy individuals in the community [12,13].

Distribution and spread of MRSA clones on a global scale have been revealed by genetic classifications with multilocus sequence typing and SCCmec typing [14,15]. Several HA-MRSA clones including ST5-MRSA-SCCmec II (ST5-II, NY/Japan clone) and ST22-IV (EMRSA-15) are known as pandemic clones...
predominating in East Asia/North America and Europe, respectively. In contrast, various clones have been documented for CA-MRSA which are distributed locally or predominate in a region, often associated with international spread. Globally predominant CA-MRSA includes five clones, i.e. ST1 (USA400 clone), ST8 (USA300 clone), ST30 (South West Pacific clone), ST39 (Taiwan clone) and ST80 (European clone), among which ST8 and ST30 are considered pandemic as a result of its distribution to every continent [15]. In Asia, two pandemic HA-MRSA clones with ST5 and ST239 are disseminating, whereas various CA-MRSA clones including those with ST8, ST30, ST59, ST72 and ST772 have been reported [16].

In Nepal, the prevalence of MRSA from clinical specimens in hospitals has been described to be 26–69% in several studies via antimicrobial susceptibility testing [17–21], although the rate varies depending on the types of infections or specimens examined. A recent study revealed a high prevalence of PVL genes in nosocomial isolates of MRSA and MSSA (26% and 52% respectively) [22]. However, in Nepal, there have been no studies conducted on genotypes (ST and SCCmec types) of clinical MRSA isolates, particularly PVL-positive isolates.

We analysed recent clinical isolates of MRSA and MSSA in hospitals in Nepal. We found high prevalence of PVL in MRSA and MSSA, as well as the presence of PVL-positive ST772 MRSA-V (Bengal Bay clone). A deletion variant of elastin binding protein gene was first identified in ST22 S. aureus isolates and its origin was analysed.

Materials and Methods

Bacterial isolates and initial genetic analysis
From August 2012 to October 2012, about 200 S. aureus isolates were collected from two general hospitals (approximately 100 isolates each) with more than 500 beds in Kathmandu, Nepal. These isolates were transported to Genesis Laboratory and Research and processed. Of these, only 100 isolates recovered were included in this study. The main specimen of the isolates was pus (n = 84), followed by urine (n = 12), sputum and blood (n = 2 each). A single isolate from each individual patient was subjected to study. Bacterial isolation and species identification were performed by conventional microbiological methods, and the presence of nuclease gene was confirmed by multiplex PCR. Individual bacterial strains were stored in Microbank (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at ~80°C and recovered when they were analysed.

Staphylococcal 16s rRNA, nuc, mecA, PVL gene ( lukS-PV/lukF-PV) and ACME-arcA (arginine deaminase gene) were detected for all isolates by multiplex PCR assay as described by Zhang et al. [23]. SCCmec type was determined by multiplex PCR using previously published primers and conditions [24].

Antimicrobial susceptibility testing and detection of drug resistance genes
Minimum inhibitory concentrations against 18 antimicrobial agents based on the broth microdilution test were measured by using Dry Plate ‘Eiken’ DP32 (Eiken Chemical, Tokyo, Japan). Breakpoints defined in the Clinical Laboratory Standards Institute guidelines were used to distinguish between resistant and susceptible strains for most of drugs examined [25]. Antimicrobial resistance genes were detected by multiplex or uniplex PCR using primers described previously [26].

Genetic typing, detection and analysis of virulence factors
Staphylocoagulase genotype (coa type) of S. aureus isolates was determined by multiplex PCR using previously published primers and conditions [27]. For the strains for which the coa types were not determined for I–X by the multiplex PCR, partial coa sequences (D1, D2 and the central regions) were determined as described previously [28,29] to assign the coa genotype by sequence identity via BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For selected isolates, sequence type (ST) was determined according to the scheme of multilocus sequence typing [30], and agr group was classified as described previously [31].

Presence of genes encoding enterotoxins and other toxins, adhesins, other proteins related to virulence and antimicrobial resistance genes was analysed by multiplex or uniplex PCR.

| Genotype | MRSA PVL(+) | MRSA PVL(−) | MSSA PVL(+) | MSSA PVL(−) |
|----------|--------------|--------------|--------------|--------------|
| coa      |              |              |              |              |
| Ia       | 1            | 1            | 2            | 12           |
| Ila      | 2            | 0            | 0            | 0            |
| Iva      | 1            | 0            | 26           | 0            |
| Va       | 0            | 1            | 5            | 3            |
| Vla      | 16           | 3            | 6            | 0            |
| Vic      | 0            | 0            | 0            | 0            |
| Vila     | 2            | 0            | 2            | 2            |
| Vila     | 0            | 0            | 0            | 1            |
| Va       | 0            | 1            | 0            | 0            |
| SClcme   | 3            | 2            | 6            | 0            |
| IV       | 1            | 1            |              |              |
| V        | 17           | 3            |              |              |
| NI       | 7            | 3            |              |              |

| MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-susceptible S. aureus; PVL, Panton-Valentine leukocidin.
| 4 Not identified. Ten SCCmec-NI strains: two isolates, mec class untypeable (mec-UT)/ccr-UT; three isolates, mec-UT/ccrC1; three isolates, mec C2/ccr-UT; two isolates, mec-UT/ccr-UT. |
(three isolates) and a PVL-negative MSSA isolate (Table 2). The isolates), PVL-positive MSSA (six isolates), PVL-negative MRSA

17 isolates as representatives of PVL-positive MRSA (seven isolates), PVL-positive MSSA (six isolates), PVL-negative MRSA (three isolates) and a PVL-negative MSSA isolate (Table 2). The PVL-positive MSSA isolates belonged to ST1, ST22, ST88 or ST772. The ST772 was identified into MRSA with SCCmec V, coa-Vla and agr-II (one PVL-negative and four PVL-positive isolates), as well as two PVL-positive MSSA. ST22 was also identified in mecA-positive and/or PVL-positive isolates. MRSA isolates with or without PVL were mostly resistant to ampicillin, gentamicin and levofloxacin, and had generally more drug resistance genes than PVL-positive MSSA, although some mecA-positive isolates (MRSA) were susceptible to oxacillin (Supplementary Table S1). Although lukE-lukD and haemolysin genes were detected in most isolates examined, ST772 isolates (MRSA and MSSA) and ST22 MRSA had more enterotoxin genes than ST1 and ST88 MRSA and ST30 MSSA isolates (Table 2).

In the present study, we first demonstrated the presence of ST772-MRSA-V and ST22-MRSA-IV in Nepal. ST772 and ST22 have been reported as epidemic clones associated with infections in both community and healthcare settings in India [16,33–35]. ST772 MSSA was originally reported in Bangladesh [36]; thereafter, ST772-MRSA-V was identified in India, followed by transmission to East/Southeast Asia, Australia, New Zealand, the Middle East and Europe [15]. This clone is colloquially referred to as the Bengal Bay clone, and it is mostly PVL positive and relatively multiresistant compared to other CA-MRSA [37]. In addition to the increasing prevalence of ST772-MRSA-V in India, detection of this clone outside India has been related to travel history to or from India [14,37,38]. Because of its adjacent location, it is conceivable that ST772-MRSA-V in India might have been readily transmitted to Nepal, or it may have been originally endemic in Nepal as well as India.

Although the elastin binding protein (EbpS) gene (ebpS) was detected in all the isolates examined by PCR with primers

using primers described previously [26,32]. The sequence of the gene encoding elastin binding protein (ebpS) was determined by PCR and direct sequencing, as described previously [26]. Multiple alignment of ebpS sequences determined in the present study and those retrieved from the GenBank database was performed by the MultAlin interface (http://multalin.toulouse.inra.fr/multalin/). The LALIGN program (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/psa/lnalign/nucleotide.html) was used for pairwise alignment and calculation of identity between two ebpS sequences. A phylogenetic tree of ebpS was constructed by the neighbour-joining method by MEGA 5.01 software, statistically supported by bootstrapping with 1000 replicates.

Full-length ebpS sequences of strains NP173, NP177 and NP199 determined in the present study were deposited in the GenBank database under accession numbers KT951674–KT951676 respectively.

**Results and Discussion**

Among the 100 S. aureus clinical isolates examined, 32 isolates were MRSA which had SCCmec type V (n = 20, 62.5%) or type IV (n = 2, 6.3%) (Table 1), while SCCmec type was not identified for ten isolates. PVL genes were detected in 78% (25/32) of MRSA and 71% (48/68) of MSSA isolates. The most common coagulase genotypes of PVL-positive MSSA and MRSA were IaV and Vla, respectively. Genetic characteristics were analysed for 17 isolates as representatives of PVL-positive MRSA (seven isolates), PVL-positive MSSA (six isolates), PVL-negative MRSA (three isolates) and a PVL-negative MSSA isolate (Table 2). The PVL-positive MRSA isolates belonged to ST1, ST22, ST88 or ST772.
| LBR | 90 |
|-----|----|
| 71A-511 | NSRNFDDKTE KRWGEHEK VDSLLHEDT IETFDDQFP RNDPRKRRR DLATHHKKOV NNESQSSDQ VONEASTID |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | — |
| Y12 | — |
| USA300-FPR | — |
| COL | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | — |
| CA-347 | — |

| S | 100 |
|----|-----|
| 71A-511 | RFEVSE-HST ESQPPSHONS PGHHEFYYYY KNAFAMDKH RPEFLNDOHK DTKKNAKHNH EHSYVSHKSE VEGS0QPWPY FTISAGKSET |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | A S VN |
| Y12 | A S VN |
| USA300-FPR | — |
| COL | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | A S VN |
| CA-347 | A S VN |

| S | 270 |
|----|-----|
| 71A-511 | GSHHDNSV KQDQFPEK — |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | — |
| Y12 | — |
| USA300-FPR | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | — |
| CA-347 | — |

| S | 360 |
|----|-----|
| 71A-511 | AGTARRGA ASKSASAASK PHASNMAG HEHODHERD KERRKQOMAK VPLRIAAVL TIGALAFGB MABNHINGT KENKJANMK |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | — |
| Y12 | — |
| USA300-FPR | — |
| COL | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | — |
| CA-347 | — |

| S | 459 |
|----|-----|
| 71A-511 | MNSDESKOD TKDSKSSKS KSTDSSKSDK DDDATKSET DDDONANAA NDONANAMD ONDONQDOO QORGGGQRH TVNQENLYR |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | — |
| Y12 | — |
| USA300-FPR | — |
| COL | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | — |
| CA-347 | — |

| S | 487 |
|----|-----|
| 71A-511 | IAIOYSSGS PENVEKIRR NLSGNNITRN GOQIVIP |
| NP199 | — |
| NP173 | — |
| NP177 | — |
| 6850 | — |
| 93B-59 | — |
| Y12 | — |
| USA300-FPR | — |
| COL | — |
| H-EMRSA-15 | — |
| HD-5096-04 | — |
| LGA251 | — |
| CA-347 | — |
described previously [32], PCR products that were shorter than the expected size (652 bp) were found in four ST22 isolates (data not shown), among which three isolates (NP173, NP177 and NP199; PVL-positive MRSA, PVL-negative MRSA and PVL-positive MSSA respectively) were further analysed for their ebpS gene sequences. These ebpS genes were revealed to be a variant (ebpS-v) with an internal deletion of 180 bp encoding a 60 aa sequence. By BLAST search, sequences similar to ebpS-v were identified in strain 71A_S11 (ST22), 93b_S9 and Y12 (ST121) and 6850 (ST50). Alignment of the deduced amino

FIG. 2. Phylogenetic dendrogram based on nucleotide sequences of elastin binding protein genes (ebpS). Bootstrap values less than 80% are not shown. Three lineages (I, II, III) and ST22 cluster are indicated at right. Closed circle indicates strains with ebpS-v (ebpS with internal 180 bp deletion). Scale bar = 0.01 substitutions per nucleotide.

FIG. 1. Alignment of elastin binding protein (EbpS) amino acid sequences from 13 Staphylococcus aureus isolates including three ST22 isolates in Nepal. Amino acid numbers based on strain LG251 and CA-347 are shown above sequence. Dot indicates identical amino acid to that of strain 71A_S11 on top; dash denotes gap. LBR near N terminus represents ligand-binding region of EbpS, and H1, H2 and H3 denote three hydrophobic domains. Asterisk indicates position of amino acid deletion detected in lineage II ebpS (see Fig. 2).
acid sequences of the ebpS-v identified in the present study with those of other representative *S. aureus* strains is shown in Fig. 1. The deleted portion (60 aa) of ebpS-v in ST22 Nepalese strains, corresponding to aa 199–258 of Ebps from strain COL, was identical to those found in strains 71A_S11, 93b_S9, Y12 and 6850 (GenBank accession nos. CP010940, CP010952, JF706229 and CP006706, respectively). Phylogenetic analysis of the ebpS from various *S. aureus* strains, including ebpS-v, revealed the presence of three major lineages (I, II and III) (Fig. 2). Lineage II contained the ST22 cluster, which consisted of ebpS-v from Nepalese strains and intact ebpS in ST22 strains as EMRSA-15. In contrast, ebpS-v in ST121 strains clustered in lineage III. Nucleotide sequence identity of intact ebpS within the same lineage was more than 98.8%, while it was 95.2–98% between different lineages (Supplementary Table S2). ebpS-v of ST22 Nepalese strains showed >99% identity with each other, but slightly lower identity was found in ST121 strains (95.6–95.7%). The variant of ebpS with a 180 bp deletion was first reported for isolates from orthopaedic infections in Italy, although their ST was not identified [39]. Thereafter we identified a similar ebpS variant (ebpS-v) in ST121 MSSA isolates in Myanmar [26] as well as in ST121 isolates in Bangladesh [40] and Japan [41]. The present study elucidated that ebpS-v of ST22 and ST121 belong to different lineages, suggesting that the 180 bp deletion event in ebpS might have occurred in ST22 *S. aureus* and ST121 *S. aureus* independently.

EbpS, one of the adhesins that binds to host cellular matrix factors involved in biofilm formation, is produced by most MRSA examined so far [41–43]. EbpS is a cell-surface molecule and mediates binding of bacterial cell to soluble elastin peptides and tropoelastin [44,45], with its N-terminal region (aa 14–34) a ligand-binding domain exposed on the surface of the cell and two (H1 and H3) among the three putative hydrophobic domains spanning the cell membrane [45]. Although the functional and structural changes caused by the deletion in ST22 isolates are unknown, the N-terminal region must be exposed on the surface of cell for the normal function of EbpS instead of the deleted H1 region [45]. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

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| Conflict of Interest |
|----------------------|

None declared.

| Appendix A. Supplementary data |
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Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.nmni.2016.02.001.

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