ORIGINAL ARTICLE

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 SCC*mec*-IV and ST772 with SCC*mec*-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.*

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* (SCC*mec*), inserted in a specific site of the chromosome. The SCC*mec* 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 SCC*mec* 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 SCC*mec* 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 SCC*mec* typing [14,15]. Several HA-MRSA clones including ST5-MRSA-SCC*mec* II (ST5-II, NY/Japan clone) and ST22-IV (EMRSA-15) are known as pandemic clones

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TABLE 1. Frequency of staphylocoagulase (*coa*) genotypes and SCC*mec* types in *Staphylococcus aureus* isolates

	MRSA		MSSA	
	PVL(+)	PVL(−)	PVL(+)	PVL(−)
Genotype	(n = 25)	(n = 7)	(n = 48)	(n = 20)
coa				
IIa	1	1	2	12
IIIa	2	0	0	1
IVa	1	0	26	0
Va	0	1	5	3
VIa	16	3	6	0
VIc	0	0	0	1
VIIa	2	0	2	2
VIIb	0	0	0	1
Xa	0	0	1	0
XIa	3	2	6	0
SCC*mec*				
IV	1	1		
V	17	3		
NI[a]	7	3		

MRSA, methicillin-resistant *Staphylococcus aureus;* MSSA, methicillin-susceptible *S. aureus;* PVL, Panton-Valentine leukocidin.
[a]Not identified. Ten SCC*mec*-NI strains: two isolates, mec class untypeable (mec-UT)/ccrA2B2; three isolates, mec-UT/ccrC1; three isolates, mec C2/ccr-UT; two isolates, mec-UT/ccr-UT.

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), ST59 (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 SCC*mec* 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

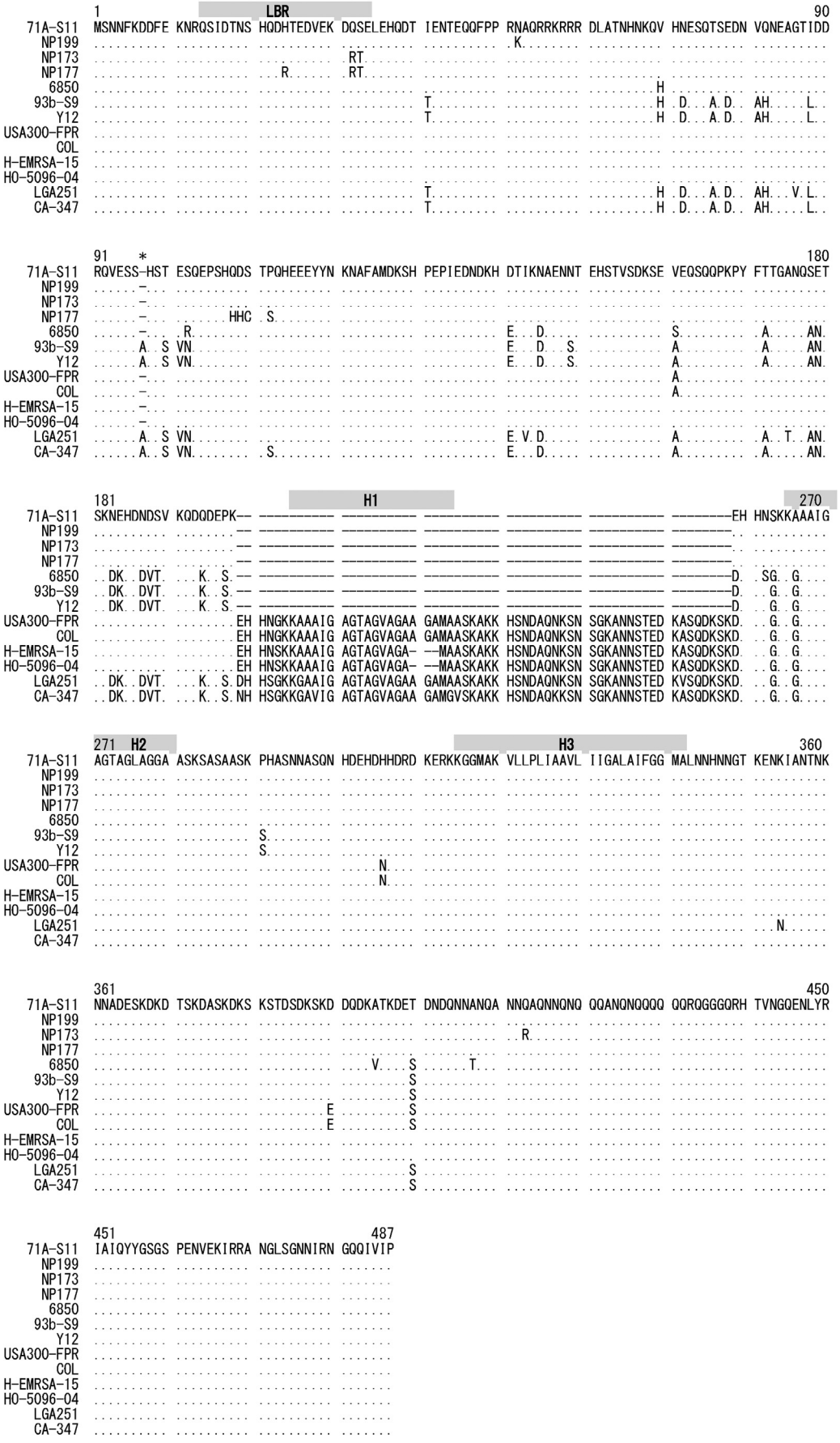
Bacterial isolates and initial genetic analysis

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

TABLE 2. Genetic characteristics and virulence factors in 17 *Staphylococcus aureus* isolates in Nepal

			Genotype							
*mec*A/PVL	Isolate no.	Isolate source	SCC*mec*	*coa*	*agr*	ST	Leukocidins, haemolysins[a]	Enterotoxins[b]	Adhesins[a,b,c]	Other[b]
mecA	NP154	Urine	V	IIa	III	ST1	*lukE-lukD, hla, hld, hlg2*	*sea, sec, seh, sei, sek, sel, seq*	*cna, ebpS, fnbA, fib, sdrD, sdrE*	*tst-1*
mecA	NP177	Pus	IV	XIa	I	ST22	*hla, hld*	*sec, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS-v, fnbA, sdrD, sdrE*	*chp, tst-1*
mecA	NP18	Urine	V	VIa	II	ST772	*hla, hld, hlg2*	*sea, sec, seg, sei, sel, sem, sen, seo*	*cna, ebpS, fnbA, fib, sdrD, sdrE*	
mecA/PVL	NP160	Pus	V	VIa	II	ST772	*lukE-lukD, hla, hlg2*	*sea, sec, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, fib, sdrD, sdrE*	
mecA/PVL	NP171	Blood	V	VIa	II	ST772	*lukE-lukD, hla, hlg2*	*sea, sec, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, fib, sdrD, sdrE*	
mecA/PVL	NP173	Pus	IV	XIa	I	ST22	*lukE-lukD, hla*	*sec, seg, seh, sei, sel, sem, sen, seo*	*cna, ebpS-v, fnbA, sdrD, sdrE*	*tst-1*
mecA/PVL	NP185	Pus	V	VIa	II	ST772	*lukE-lukD, hla, hlg2*	*sea, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, sdrD, sdrE*	
mecA/PVL	NP189	Urine	V	VIa	II	ST772	*lukE-lukD, hla, hlg2*	*sea, sec, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, sdrD, sdrE*	
mecA/PVL	NP190	Pus	V	VIIa	III	ST1	*lukE-lukD, hla, hld, hlg2*	*sea, seh, sek, sel, seq*	*cna, ebpS, fnbA, fib, sdrD, sdrE*	
mecA/PVL	NP27	Pus	V	IIIa	III	ST88	*lukE-lukD, hla, hld, hlg2*	*sed, sek, seq*	*ebpS, fnbA, fib, sdrD, sdrE*	
PVL	NP163	Pus		IVa	III	ST30	*lukE-lukD, hla*	*seg, seh, sem, sen, seo, sep*	*cna, ebpS, fnbA, bbp*	
PVL	NP169	Pus		IVa	III	ST30	*hla, hld*	*seg, sem, sen, seo*	*cna, ebpS, fnbA, fib, bbp*	
PVL	NP172	Pus		XIa	I	ST22	*lukE-lukD*	*seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS-v, fnbA, sdrD, sdrE*	
PVL	NP166	Pus		VIa	II	ST772	*lukE-lukD, hlg2*	*sea, sec, seg, seh, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, sdrD, sdrE*	
PVL	NP193	Pus		VIa	II	ST772	*lukE-lukD, hla, hlg2*	*sea, seg, sei, sel, sem, sen, seo, sep*	*cna, ebpS, fnbA, sdrD, sdrE*	
PVL	NP199	Urine		XIa	I	ST22	*lukE-lukD, hla*	*seg, sei, sem, sen, seo*	*cna, ebpS-v, sdrD, sdrE*	
—	NP195	Pus		IIa	I	ST672	*lukE-lukD, hla, hld, hlg2*	*seg, sei, sem, seo*	*ebpS, fnbA, fib, sdrD, sdrE*	

[a]The following genes were detected in all strains: *hlg, icaA, icaD, eno, fnbB, clfA, clfB, sdrC.*
[b]The following genes were not detected in any strain: *seb, see, sej, ser, ses, set, eta, etb, etd, edin-A, edin-B, lukM, bnp, sak, scn.*
[c]Elastin binding protein gene (*ebpS*) with internal deletion (180 bp).



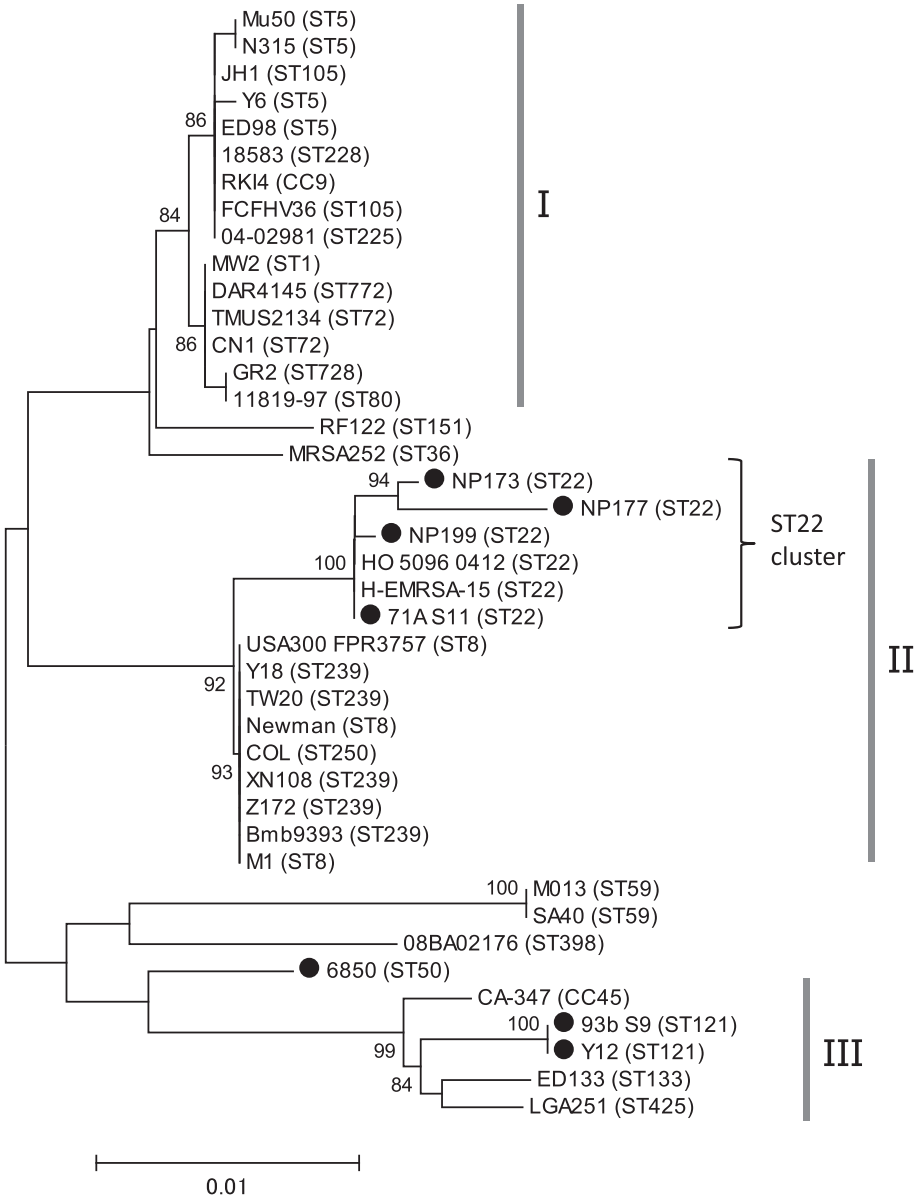


FIG. 2. Phylogenetic dendrogram based on nucleotide sequences of elastin binding protein genes (*ebpS*). Bootstrap values less than 80% are not shown. Three lineages (I, II, III) and ST22 cluster are indicated at right. Closed circle indicates strains with *ebpS-v* (*ebpS* with internal 180 bp deletion).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

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 deiminase gene) were detected for all isolates by multiplex PCR assay as described by Zhang *et al.* [23]. SCC*mec* type was determined by multiplex PCR using previously published primers and conditions [24].

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).

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 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/lalign/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 SCC*mec* type V (n = 20, 62.5%) or type IV (n = 2, 6.3%) (Table 1), while SCC*mec* 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 IVa and VIa, 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. The ST772 was identified into MRSA with SCC*mec* V, *coa*-VIa 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 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

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-v;* accordingly, the H2 region is necessary to span the membrane instead of the deleted H1 region (Fig. 1, Supplementary Fig. S1). In that case, only the form of the cytoplasmic portion of *ebpS-v* may be different from that of EbpS, which may be possible to cause any functional difference.

It was notable in the present study that all the ST22 *S. aureus,* including MRSA and MSSA, harboured *ebpS-v,* while intact *ebpS* was also found in other previously reported ST22 strains including EMRSA-15. In contrast, all the ST121 *S. aureus* analysed to date harboured *ebpS-v* [26,40,41]. The ST121 clone is known to be a common cause of skin and soft tissue infection disseminating globally, mostly *mecA* negative but PVL positive, exhibiting higher virulence [46]. Although the association of *ebpS-v* with the enhanced virulence of the ST121 clone is not known, if the presence of *ebpS-v* affects phenotypic trait of ST22 *S. aureus* (e.g. virulence or fitness advantage to environment), it is possible that the proportions of ST22 isolates with *ebpS* and *ebpS-v* may change over time epidemiologically. Further studies thus may be necessary to monitor the prevalence of ST22 *S. aureus* with *ebpS-v.*

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Conflict of Interest

None declared.

Appendix A. Supplementary data

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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