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Short Communication

Genetic Characterization of *Staphylococcus aureus* Isolates Carrying Panton-Valentine Leukocidin Genes in Bangladesh

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SUMMARY: To assess the spread and genetic characteristics of Panton-Valentine leukocidin (PVL) gene-carrying *Staphylococcus aureus* in Bangladesh, we investigated 59 strains (49 isolates from clinical specimens and 10 isolates colonized in the nasal cavities of medical staff), including 26 methicillin-resistant *S. aureus* (MRSA) strains. The PVL gene was detected only in methicillin-susceptible *S. aureus* (MSSA) strains (7 clinical strains and 2 colonizing strains). PVL gene-positive MSSA strains were found to belong to coagulase serotypes III or VI and were classified into sequence types ST88 (CC88), ST772, and ST573 (CC1) by multilocus sequence typing, and *agr* types 2 or 3. These types were different from those determined for MRSA (coagulase serotypes I and IV, ST240 and ST361, and *agr* type 1). PVL gene-positive MSSA possessed a larger number of virulence factor genes than MRSA, although they were susceptible to more antimicrobials. These findings suggest that the PVL gene is distributed to limited populations of *S. aureus* clones with specific genetic traits that are distinct from MRSA in Bangladesh, but genetically close to CA-MRSA clones in the CC1 lineage reported in the United States and European countries.

Staphylococcus aureus is an important human pathogen causing community- and hospital-acquired infections. Methicillin-resistant *S. aureus* (MRSA) has been recognized as the most important nosocomial pathogen worldwide because of the increased incidence of multidrug-resistant strains among hospital-acquired (health care-associated) MRSA (HA-MRSA) (1). Furthermore, community-acquired MRSA (CA-MRSA), which is derived from individuals without risk factors for hospital acquisition of MRSA, has also been recognized as an important infectious agent, and its prevalence has been increasing globally during the past decade (1,2). CA-MRSA strains are isolated originally from skin and soft tissue infections, and occasionally cause deadly systemic infections.

MRSA is defined by the presence of the *mecA* gene, which encodes an altered penicillin-binding protein (PBP-2´). The *mecA* gene is located in staphylococcal cassette chromosome *mec* (SCC*mec*), which is a genetic element inserted at a specific site in the *S. aureus* chromosome (3). Sequence analyses of *S. aureus* genome defined the presence of three major SCC*mec* types (I, II, and III) among HA-MRSA and two major types (IV and V) in CA-MRSA (1,3). These two groups of SCC*mec* types in HA- and CA-MRSA are distinguishable with regard to their sizes, the presence of functional recombinase, and antibiotic resistance markers. Furthermore, the majority of CA-MRSA is distinguishable from HA-MRSA based on the production of Panton-Valentine leukocidin (PVL), which is encoded by *lukS-PV-lukF-PV*, a component of the phage genome inserted in bacterial chromosome. The PVL is a bicomponent cytotoxin that is preferentially linked to furuncles, cutaneous abscess, and severe necrotic skin infections (4,5).

In Bangladesh, although only limited information is available regarding the prevalence of MRSA, the detection rates of MRSA in hospitals at different cities were recently reported as 32 - 63%, which is a high incidence comparable to the United States and European countries (6). It was also described that the prevalence of MRSA has increased substantially over the past 4 - 5 years in hospital patients. However, neither phenotypic and genetic characteristics of MRSA nor the prevalence of PVL among *S. aureus* have been studied in Bangladesh. Since skin infections, including furuncle and carbuncle, are still prevalent due to the low hygienic status in this country, the distribution of PVL among *S. aureus* has also been receiving increasing attention.

In this study, we investigated the prevalence and genetic characteristics of MRSA and PVL gene-carrying *S. aureus* in Bangladesh. A total of 59 *S. aureus* strains comprising 49 clinical isolates from pus or wound swabs of skin infections and suppurative diseases, and 10 colonizing strains from 10 medical staff members were analyzed. The clinical strains were collected from patients admitted to the Dhaka Medical College (DMC) hospital in Dhaka and Mymensingh Medical College hospital in Mymensingh, Bangladesh, during a period from September to December 2004. Mymensingh is located approximately 200 km to the north of Dhaka. The isolation rate of *S. aureus* was 28% among the clinical specimens examined. Colonizing strains were isolated from the anterior nasal cavities of healthy doctors and nurses working in the

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wards of surgery, gynecology, and orthopedics in the DMC hospital in September 2004. Of the 47 strains of *S. aureus* isolated from 102 medical staff members (detection rate, 46%), 10 strains were selected for this study.

MRSA was identified by the detection of *mecA* gene, and the SCC*mec* types were determined based on the results of polymerase chain reaction (PCR) assay, as described previously (7-9). The PVL gene (*lukS-PV-lukF-PV*) was detected by PCR as described previously (5), and the nucleotide sequences of PVL-gene of some representative strains were determined by PCR and direct sequencing. The coagulase serotype was determined by coagulation-inhibition test with coagulase serotype I - VIII-specific antisera (staphylococcal coagulase antiserum kit; Denka Seiken, Inc., Tokyo, Japan) (10) in accordance with the manufacturer's instructions. For genetic typing, a repeat number of polymorphisms of the protein A gene (*spa*) and coagulase gene (*coa*) were analyzed for all the strains by PCR. The 3′-end side of *spa* and *coa* genes contain repeat regions with 24-bp and 81-bp units, respectively. The repeat number in *spa* was estimated as described previously (11). The repeat region of *coa* was amplified by primers coa-PR1 (TAGAGAT GCTGGTACA GGTA) and coa-PR2 (GAGTTATAAACTTATTTGTTACT CTAGG), and repeat numbers were estimated based on the size of the PCR product (product size = 81×repeat no. + 84 nucleotides).

Among the 49 strains from clinical specimens, 26 strains (53.1%) were identified as MRSA (53.3% in Dhaka; 52.6% in Mymensingh), while all the colonizing strains were MSSA. SCC*mec* from most of the MRSA strains (24 strains, 92.3%) was grouped into type III (Table 1). The other two strains were found to have type IV SCC*mec*, which was further classified into subtype IVa for one strain, but the subtype of the other strain was not determined. All the MRSA having type III SCC*mec* were classified into coagulase serotype IV, *coa*-repeat type C6 (*coa*-C6), and *spa*-repeat type S7 or S9 (*spa*-S7, S9) (Table 1). Two MRSA stains with type IV SCC*mec* belonged to coagulase serotype I, *coa*-C5, and *spa*-S6. In contrast, MSSA strains were grouped into various coagulase serotypes (III-VII), *coa*-repeat types (C5-C8), and *spa*-repeat types (S4, S6-S11), although coagulase serotype VII, *coa*-C6 type, and *spa*-S4 type were predominant.

PVL genes were detected in nine MSSA strains (seven clinical strains and two colonizing strains with detection rates of 30.4 and 20%, respectively), while no MRSA harbored the PVL gene (Table 1). PVL gene-positive strains were isolated from patients with wound infections at burn sites or diabetic ulcer, and those with gunshot injury (5 cases), carbuncle (1 case), or suppurative otitis media (1 case). Patients with PVL gene-positive *S. aureus* had fever at a significantly higher rate (6 in 7 patients, 86%) than those with PVL gene-negative bacteria (3 in 42 patients, 7%) ($P < 0.001$), although there was no apparent difference in other symptoms between these groups of patients. All the PVL gene-positive clinical strains were grouped into coagulase serotype VI, *coa*-C6, and *spa*-S8 or S10. However, two colonizing strains with the PVL gene belonged to different types (coagulase serotype III, *coa*-C7, and *spa*-S9). PVL-gene sequences from the two clinical strains (D64, M25) were identical to those of ϕSLT (GenBank accession no. AB045978), while the PVL gene of a colonizing strain DC14 (accession no. EU368821) was different from that of ϕSLT by only one nucleotide.

Five representative MSSA strains with PVL gene (four clinical strains and one colonizing strain) and three MRSA strains were further genetically characterized by multilocus sequence typing (MLST) using sequence data from seven housekeeping genes that were determined by PCR and direct sequencing, as previously described (12) (Table 2). The sequence types (STs) of the four PVL gene-positive clinical strains were determined as ST573 or ST772, and a colonizing strain was assigned to ST88 and then further grouped as clonal complex (CC) CC1 or CC88, respectively. In contrast, the STs of three MRSA strains with the SCC*mec* types III and IV were assigned to ST240 (CC8) and ST361 (CC not classified), respectively.

The *agr* (accessory gene regulator) specificity group (I - IV) was classified by PCR with specific primer pairs, as described previously (13). PVL gene-positive strains were grouped into *agr* types 2 and 3, while PVL gene-negative strains were assigned to *agr* type 1 (Table 2). The presence of the 28 staphylococcal virulence genes was examined by PCR assay using previously reported primers (14-16). Clinical MSSA strains with PVL gene were found to harbor more genes encoding toxins and virulence factors than the colonizing strain or PVL gene-negative MRSA strains. The enterotoxin gene cluster (*egc*) including *seg*, *sei*, *sem*, *sen*, and *seo* was found in all the PVL gene-positive MSSA clinical strains and one PVL gene-negative MRSA strain, and enterotoxin genes *sel*, *sem*, and *seo* were detected only in PVL gene-positive clinical strains. SCC*mec* type III MRSA strains were resistant to more antibiotics than PVL gene-positive strains (Table 2).

While PVL is rarely produced by *S. aureus* (less than 5% of strains), the PVL gene is detected at high rates (more than 50%) in isolates from community-acquired skin infections

Table 1. Coagulase serotype, *coa*-repeat type, and *spa*-repeat type of *S. aureus* isolates

Typing and type	No. of isolates			
	MRSA		MSSA	
	Total	(SCC*mec* type)	Total	PVL-positive
Coagulase serotype				
I	2	(type IV: 2)	0	0
III	0		5 (3)[2)]	2 (2)
IV	24	(type III: 24)	3	0
V	0		2	0
VI	0		8	7
VII	0		11 (7)	0
ND	0		4	0
coa-repeat type[1)]				
C5	2	(type IV: 2)	4	0
C6	24	(type III: 24)	24 (7)	7
C7	0		3 (3)	2 (2)
C8	0		2	0
spa-repeat type[1)]				
S4	0		14 (7)	0
S6	2	(type IV: 2)	4	0
S7	22	(type III: 22)	2	0
S8	0		3	3
S9	2	(type III : 2)	3 (3)	2 (2)
S10	0		5	4
S11	0		2	0
Total	26		33 (10)	9 (2)

1): *coa*-repeat type and *spa*-repeat type represent repeat numbers of 24-bp or 81-bp units at the 3′-end side of *coa* or *spa*, and expressed as the nos. with C or S, respectively.
2): nasal colonizing strain.
ND, not detected.

Table 2. Characteristics of the representative MRSA and MSSA strains in Bangladesh

Type, gene or resiatance	MRSA strains			MSSA strains				
	D28	M38	D118	D64	D122	M25	M183	DC14
SCC*mec* type	III	III	IV					
lukS-PV-lukF-PV	–	–	–	+	+	+	+	+
Coagulase serotype	IV	IV	I	VI	VI	VI	VI	III
CC	8	8	N[1)]	1	1	1	1	88
ST	240	240	361	772	772	573	772	88
Allele								
arcC	2	2	4	1	1	1	1	22
aroE	3	3	3	1	1	1	1	1
glpF	1	1	1	1	1	1	1	14
gmk	1	1	1	1	1	1	1	23
pta	21	21	11	22	22	12	22	12
tpi	4	4	72	1	1	1	1	4
yqil	3	3	64	1	1	1	1	31
agr type	1	1	1	2	2	2	2	3
Genes for virulence factors[2)]	*sea* *sek* *seq* *cna* *lukE-lukD* *hla* *hld* *hlg-2*	*sea* *sek* *seq* *cna* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*	*seb* *seg* *sei* *sen* *seo* *sem* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*	*sec* *seg* *sei* *sel* *sem* *sen* *seo* *cna* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*	*sea* *sec* *seg* *sei* *sel* *sem* *sen* *seo* *cna* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*	*sea* *sec* *seg* *sei* *sel* *sem* *sen* *seo* *cna* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*	*sea* *sec* *seg* *sei* *sel* *sem* *sen* *seo* *cna* *lukE-lukD* *hla* *hld* *hlg-2*	*sea* *sep* *lukE-lukD* *hla* *hld* *hlg* *hlg-2*
Resistance[3)]	OXA GM KM ERY TC CIP	OXA GM KM ERY TC CIP	OXA KM TC	KM CLDM			KM	ABPC

[1)]: Not classified.
[2)]: Virulence factors (genes) : staphylococcal enterotoxins (enterotoxin-like toxins); SEA (*sea*), SEB (*seb*), SEC (*sec*), SEG (*seg*), SEK (*sek*), SEL (*sel*), SEM (*sem*), SEN (*sen*), SEO (*seo*), SEP (*sep*), SEQ (*seq*), collagen adhesion protein (*cna*), Leukocidine ED (*lukE-lukD*), Alpha-hemolysin (*hla*), Delta-hemolysin (*hld*), Gamma-hemolysin components A, B, and C (*hlg*), Gamma-hemolysin variant (*hlg-2*). Genes encoding SED, SEE, SEH, SEJ, exfoliative toxin A and B, TSST-1, Luk-M, Beta-hemolysin, and EDIN-A were not detected.
[3)]: None of the strain was resistant to vancomycin and chloramphenicol.
OXA, oxacillin; GM, gentamicin; KM, kanamycin; ERY, erythromycin; TC, tetracycline; CIP, ciprofloxacin; CLDM, clindamycin; ABPC, ampicillin.

and necrotic hemorrhagic pneumonia (5,17). Although less numbers of isolates were analyzed in this study, the detection rates of PVL gene (14.3% in all the clinical isolates from skin infections and suppurative diseases; 30.4% in MSSA) may be similar to those reported in a recent hospital-based study in Singapore in which PVL gene was positive in only MSSA, 11.6% of all the *S. aureus* strains (MRSA rate, 43%), and 27.2% in MSSA from skin and soft tissue infections (4). In the present study, PVL gene-positive MSSA strains were distinct from PVL gene-negative MRSA and MSSA strains based on the coagulase serotype, various genetic types, and the presence of virulence factor genes, indicating that PVL gene is distributed to certain specific populations of *S. aureus* clones. The four MSSA clinical strains carrying PVL gene were assigned to ST772 or ST573, which are single-locus variants of ST1 and are classified into CC1. The ST1 was assigned for the MW2 strain, which is known as a prototype of CA-MRSA, with PVL genes causing fatal infection in the United States, and has also been reported for other PVL-positive MRSA in the United States and European countries (2,18). Another sequence type that was identified in a PVL gene-positive colonizing strain in this study was ST88, which has also been reported for PVL-positive MRSA in Belgium and Singapore (19,20). The detection of PVL gene-positive MSSA of CC1 and CC88 in Bangladesh may suggest that these genetic clones harboring PVL gene might have originally been distributed worldwide. Accordingly, it may be hypothesized that the PVL-positive MRSA of CC1 and CC88 might have emerged via dissemination of SCC*mec* elements among preexisting PVL-positive MSSA. A similar view has been presented based on a study of PVL-positive ST80 and ST8 strains in Germany (21).

In the present study, PVL gene was detected only in MSSA strains in the CC1 lineage, while the predominant MRSA clones in Bangladesh seemed to belong to the CC8 lineage. The ST8 (CC8) is known as one of the predominating lineages in CA-MRSA, and acquisition of PVL phage by ST8-MRSA is thought to be as an evolutionary event that makes possible the occurrence of ST8 CA-MRSA (epidemic CA-MRSA USA300) (22). Attention should therefore be paid to

the emergence of PVL-positive CA-MRSA in Bangladesh, as well as the spread of PVL gene-positive MSSA.

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