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Panton Valentine Leukocidin (PVL) and thermonuclease (NUC) profile in clinical isolates of *S. aureus* and an assessment of impact of variations in nucleotide sequences of *fem* A, B and X genes

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Abstract

Introduction: *S. aureus* produces more than 32 extracelleular molecules which include a number of virulence factors, toxins, enzymes and haemolysins, which allow it to adhere to surface, invade or avoid the immune system and in addition causes toxicity to the host. Virulence factors like coagulase, thermonuclease and Panton Valentine Leukocidin (PVL) are all secreted. We investigated the distribution and expression of PVL and thermonuclease in clinical isolates of *S. aureus.* Spontaneous sequence variations were observed in the *fem* genes of clinical isolates of *S. aureus* (data under communication) and we investigated if this would impact the secretion of thermonuclease and PVL.

Materials and Methods: Genomic DNA was isolated from157 *S. aureus* clinical isolates and screened for *fem*, *nuc* and *pvl* through PCR. To evaluate whether the sequence variations in *fem* genes had any effect on the expression of *pvl*, we randomly selected 43 *fem* (*fem* A, B and X) variants from the 109 *pvl* positive isolates and measured *pvl* transcript by qPCR. The isolates were genotyped for *nuc* by PCR and the transcript was measured by qPCR while DNAase secretion was screened by methyl green DNA agar plate method.

Results: 109 (77%) out of 157 isolates were *pvl* PCR positive. PVL expression was marginally down regulated when compared with the reference gene in these variants. Almost all isolates were *nuc*-PCR positive. No remarkable change in the expression of thermo nuclease was observed among the *fem* sequence variants.

Conclusion: Variations in the nucelotide sequences of *fem* A, B and X genes were observed in several clinical isolates of *S. aureus*. These variations did not affect the secretion of coagulase, thermonuclease and transcription of *pvl*. PVL has long been known as virulence factor associated with and a molecular marker for CA-MRSA. However, our study has shown a relatively high prevalence of *pvl* in *S. aureus* isolated from hospital acquired infections.

Keywords: *pvl, fem* (A, B and X), *nuc,* MRSA, MSSA

Introduction

Staphylococcus aureus (*S. aureus*) causes a range of infections (1-4) among patients in hospitals (hospital acquired infections) and people in the community. Blood stream infection and sepsis due to MRSA is life threatening unless diagnosed early and treated with appropriate antibiotics. Reliable and rapid identification of *S. aureus* is necessary for successful management of patients with sepsis (5, 6) and PCR based methods are increasingly being used for this (7-9). Since *mec*A could be carried by non-*S. aureus* bacteria also, it is important for any molecular method to be able to differentiate and identify *S. aureus* in addition to detecting *mec*A for the diagnosis of MRSA. Our laboratory evaluated *fem* and *nuc* for species identification and reported direct detection of MRSA in uncultured clinical samples using a simple sample processing protocol and multiplex PCR (under communication). The severity of *S. aureus* infection is largely attributed to the large number of virulence factors (more than 32 extracelleular products) which include toxins, enzymes, adhesins and haemolysins (10-13). It is useful to determine the virulence profile of the *S. aureus* simultaneously while screening for MRSA. We investigated the profile of coagulase, thermonucelase and PVL in these isolates. Coagulase, though plays an important role in the pathogenesis of *S. aureus* disease, is invariabley used for identification and classification of *S. aureus* (14, 15) as it is constitutively expressed by *S. aureus*. Thermonuclease gene has been widely used as a specific marker to detect *S. aureus* contamination, in PCR-based methods. It can resist heat upto 100^0C for 1 h and has the ability to degrade DNA, RNA (16, 17) and it apparently plays important role in biofilms (18, 19). Besides these, one of the important exo-toxins secreted by *S. aureus* is Panton Valentine Leukocidin (PVL) which is encoded by two contiguous and co-transcribed genes LukS-PV and LukF-PV. PVL is known to induce cell death in soft tissue infections, pneumonia and sepsis (11, 13, 20, 21) by necrosis or apoptosis. PVL is reportedly present more often in community acquired (CA-MRSA) infections than in hospital acquired (HA-MRSA) infections and therefore it is used as molecular marker for identification of CA-MRSA (22). Prevalence of

PVL among *S. aureus* isolates is being increasingly reported in hospital acquired infections also in recent years which probably indicates a shift in the profile of the *S. aureus* reservoir in the hospitals as more and more patients from the community get admitted. Data from across the globe in recent years seem to indicate that PVL is important even in HA-MRSA infections. Reports indicate that in hospital acquired *S. aureus* infections a PVL prevalence of 30% in Germany, 57% in Central and Western Africa and 97% in United States (23-25). Since *fem* genes are involved in synthesis and assembly of the cell wall, we studied if sequence variations in *fem* interfered with the expression and secretion of PVL, coagulase and thermonuclease.

Materials & Methods

Bacterial strains and phenotypic tests

S. aureus isolates (n=157) isolated from different clinical specimens of hospital acquired infections over a period of one year. Isolates were identified as *S. aureus* on the basis of, isolation on Mannitol Salt Agar, coagulase test and Methyl Green DNAse Agar (Himedia Pvt Ltd) test. Thermonuclease activity was screened by streaking all isolates on Methyl Green DNAse Agar and incubating for 24 hrs at 37^0C and the clear zone was scored as (+), (++) and (+++) depending on the area of the zone. All *S. aureus* isolates were screened with Cefoxitin (30µg) and Oxacillin (1µg) disc on Mueller Hinton Agar to identify MRSA and MSSA.

DNA Isolation and *fem*-PCR for Species Identification

Initial optimisation of PCR was done with genomic DNA isolated by Sambrook method from a few isolates of *S. aureus* (26). For subsequent screening of all the isolates for genotyping of *fem*, *nuc* and *pvl* by PCR, cell free DNA lysate was prepared by TEX (Tris buffer-EDTA-Triton X-100) method (27) and later used as template for PCR amplification in a reaction volume of 20µl. The primers and thermal cycling conditions are listed in Table 1.

Table 1: PCR Conditions Used In Study

Gene	Sequence 5'-3'	PCR conditions	Product size	Reference
*mec*A-F	ACG AGT AGA TGC TCA ATA TAA	94ºCx5 min, 94ºCx30s, 55ºCx30s, 72ºCx50s } x35 and 72ºC x 10 min	293bp	(28)
*mec*A-R	CTT AGT TCT TTA GAG ATT GA			
femA-F	AGA CAA ATA GGA GTA ATG AT	94ºCx 5min 94ºCx30s, 50ºCx1min 72ºCx1min } x35 and 72ºCx10min,	509bp	(29)
fem A-R	AAA TCT AAC ACT GAG TAA TGA T			
fem B-F	TTA CAG AGT TAA CTG TTA CC	94ºCx5min, 94ºCx30s, 50ºCx1min, 72ºCx1.5min } x40 and 72ºCx10min.	651bp	(29)
fem B-R	ATA CAA ATC CAG CAC GCT CT			
fem X-F	ATT GTT AAA TAG AAG GAG ATA TC	94ºCx5min 94ºCx30s, 53ºCx30s, 72ºCx30s } x35 and 72ºCx10 min,	621bp	(30)
fem X-R	CCC CAG TGA TTT TCA TTA ATT C			
pvl-F	CAGGAGGTAATGGTTCATTT	94ºCx5min, 94ºCx30s, 56ºCx30s, 72ºCx30s } x35 and 72ºC for 10 min,	131bp	(28)
pvl-R	ATGTCCAGACATTTTACCTAA			
nuc-F	GCGATTGATGGTGATACGGTT	94ºC for 5 min 94 ºCx30s, 55ºCx30s, 72ºCx50s } x35 and 72ºCx10 min.	270bp	(16)
nuc-R	AGCCAAGCCTTGACGAACTAAAGC			

RNA isolation

We randomly selected 43 *fem* (*fem* A, B and X) variants from the 109 *pvl* positive isolates. One ml of broth culture were centrifuged at 10,000 rpm. Total RNA was isolated using RNAeasy mini kit following manufacturer's instructions (Qiagen, Cat. No. 74104). Total RNA concentrations and quality were determined using Nanodrop (Model No. ND200) and 50 ng of total RNA was used for qPCR.

cDNA preparation and Real Time PCR assay

cDNA conversion was performed in Analytika Jena qTower 2.2 using Reverse Transcriptase Core kit (Eurogentec) at 25^0C for 10 mins, 48^0C for 30 mins and finally followed by 95^0C for 5mins. The cDNA was stored at -20^0C if not subjected to RTPCR immediately. Real-time PCR was performed using the Takyon™ No Rox SYBR® MasterMix dTTP Blue (Eurogentec) PCR master mix. Each reaction tube contained in a final volme of 20 µl, 1X (7.5 µl) SYBR Green master mix, 10 pmol each of *pvl* forward and reverse primers (28) and 1 µl of cDNA. Amplification was performed under following conditions: 95°C for 10 min followed by 35 cycles at 95°C for 15s and 56°C for 30s and finally 60°C for 1 min. Each qPCR run included a *pvl* positive control cDNA (ATCC 25923) and *pvl* negative control (Reference strain of *S. aureus* 43300 which is negative for *pvl* gene). Thermonuclease (*nuc*) gene (16) was used as house keeping gene to normalize the transcript quantitation. Each reaction was analyzed in duplicate including the negative control and normalised to the value of the house keeping gene. Expression of *pvl* was determined by comparison to identical quantities of total RNA from all *S. aureus* isolates and relative to the expression of *nuc,* the housekeeping gene.

Results

Thermonuclease Profile

We screened 157coagulase positive isolates for methicillin resistance by Cefoxitin and Oxacillin Disc diffusion test, out of which 105 were MRSA and 52 were MSSA. 142 isolates showed nuclease activity on Methyl Green DNAse agar and the enzyme activity was graded as 1+, 2+ and 3+ based on the size of the clear zone produced by the colony on the agar plate (Table 2). 15 isolates including a few wild *fem* type *S. aureus* isolates did not show any nuclease activity though they were *nuc* genotype positive. One of the reasons for this lack of correlation between the genotype and Methyl Green DNAse agar method could probably be the poor sensitivity of the methyl green agar test for nucelase activity. The sequence variations in *fem* genes apparently did not impact the expression/secretion of nuclease in these clinical isolates.

Table 2: Thermonuclease Phenotyping and genotyping in *fem* variants

***fem* variants**	**DNAse Activity (n=157)**				***nuc*-PCR**
	-ve	**1+**	**2+**	**3+**	**(+)**
fem A (n=3)	0	0	1	2	3
fem B (n=48)	5	7	17	19	43
fem X (n=6)	0	0	1	5	6
fem A + B (n=8)	2	2	0	4	6
fem B + X (n=12)	3	3	3	3	11
Wild(n=80)	5	10	10	55	80

Correlation of *fem*-PCR and *nuc*-PCR

We bench-marked all our PCR results (*fem*-PCR and *nuc*-PCR) against the coagulase slide agglutination test as the gold standard for species identification. The prevalence of *fem* variants based on the PCR results was as follows: 11/157 (7%) were *fem*A, 68/157 (43%) were *fem*B and 18/157 (11.5%) were *fem*X. On the contrary, 8 isolates (8/157) were not detected by *nuc*-PCR. *mec*A PCR confirmed 106 as MRSA and the remaining 51 isolates as MSSA demonstrating absolute correlation with Cefoxitin disc diffussion method. In another study we observed that *nuc* is a better genetic marker for *S. aureus* species identification (under communication) than the *fem* genes. (Table.2).

3. 3 *pvl* and *nuc* genotyping of clinical isolates of *S. aureus* from different specimen

pvl and *nuc* have different biological characteristics and functions though both are virulent factors. We assessed the relative distribution of these two virulence factors among the clinical isolates from different clinical specimen, 77% (109/157) isolates were *pvl*-PCR positive (Figure 1). In addition to being species specific and constitutively expressed in *S. aureus*, the thermonucelase gene was detectable in almost all the isolates. It was remarkable that *pvl* was fairly widespread among these isolates and it seems that *pvl* is no longer an exclusive virulence marker of CA-MRSA. Several isolates of *S. aureus* from blood (33/49, 67%), pus (28/39, 71%), and wound swab specimen (15/21, 71%) were *pvl* positive where a definitive role for pvl may be ascribed. Distribution of the virulence factors, *pvl* and *nuc* (Figure 2), in different clinical specimens is presented in Table 3.

Table 3: PVL Detection Through PCR (n=157)

Specimen Type	**No. of Isolates**	***pvl*-PCR (+)**	***nuc*-PCR (+)**
Blood	49	33 (67%)	46 (94%)
Pus	39	28 (71%)	38(97.4%)
Wound Swab	21	15 (71%)	19(90.4%)
Tissue	7	4 (57%)	7(100%)
ET Secretion	5	2 (40%)	5(100%)
Sputum	2	2	2
Reteropharyngealabscess	1	1	1
Urine	1	1	1
MRSA SCREEN	2	2	2
Central line tip	2	0	2
Vaginal swab	2	2	2
Fluid	4	1(25%)	2(50%)
Foot Lar	1	0	1
Skin Peel	1	0	1
Endothelia	1	1	1
Unknown	19	17(89%)	19(100%)
Total	157	109	149

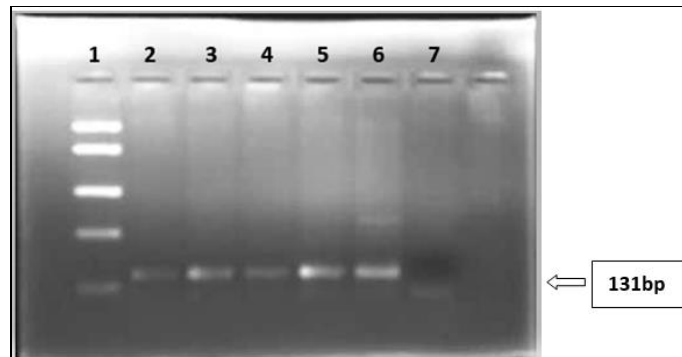

Fig 1: PVL genotyping: Cell free DNA lysates prepared from clinical isolates were screened for *pvl* by PCR and analysed on 2% agarose gel. From Left to Right: Lane 1-100bp DNA Ladder; Lane 2-PCR Positive Control; Lane 3-6 pvl positive (131bp) *S. aureus* isolates, Lane 7-PCR Negative control

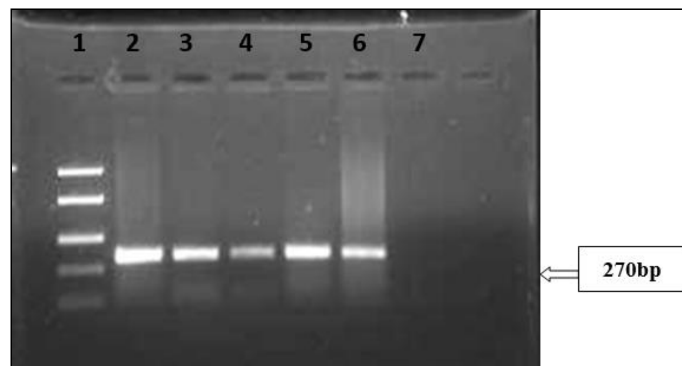

Fig 2: *nuc* genotyping: Cell free DNA lysates prepared from clinical isolates were screened for *nuc* and analysed on 2% agarose gel. From Left to Right: Lane 1-100bp DNA Ladder; Lane 2-PCR Positive Control; Lane 3-6-*nuc* positive (270bp) *S. aureus* isolates, Lane 7-PCR Negative control

Reverse Transcriptase Real Time PCR (qPCR)

Species specific marker thermonuclease (*nuc)* gene was used for normalization. Relative transcription levels of *pvl* in several *fem* variants were analyzed by $2^{\Delta\Delta Ct}$ method after normalization to *nuc* gene (reference gene) using the Livak formula. (Fig. 3). *pvl* transcripts were quantitated in 43 *fem* variant *pvl* positive isolates, a *pvl* negative control (ATCC 43300) and a *pvl* positive control (ATCC 25923) were included. The *pvl* transcripts were measurable in all 43 isolates and were marginally reduced (10-20%) in *fem* A, B and X variants, compared to the reference gene (*nuc*) and no difference was noticeable between MRSA and MSSA phenotypes. Mean and SD were calculated for the respective *fem* (A, B and X) variant group and represented in fig 4.

Fig 3: *pvl* qPCR Results

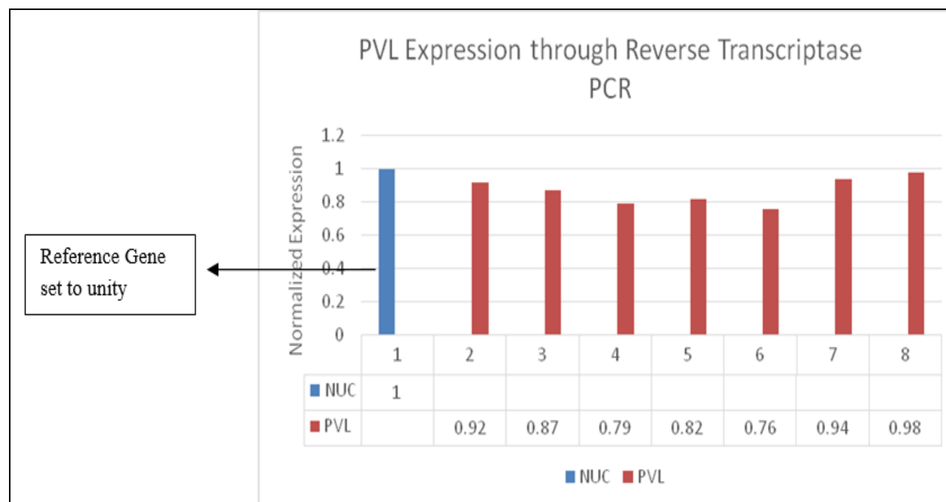

Fig 3: 1- *nuc* positive (house keeping gene), 2-*fem* A variant MRSA, 3- *fem* X variant MRSA, 4 &5 –*fem* B variant MRSA, 6- *fem* B variant MSSA, 7 – *fem* X variant MSSA, 8 –ATCC25923 *pvl* positive isolate

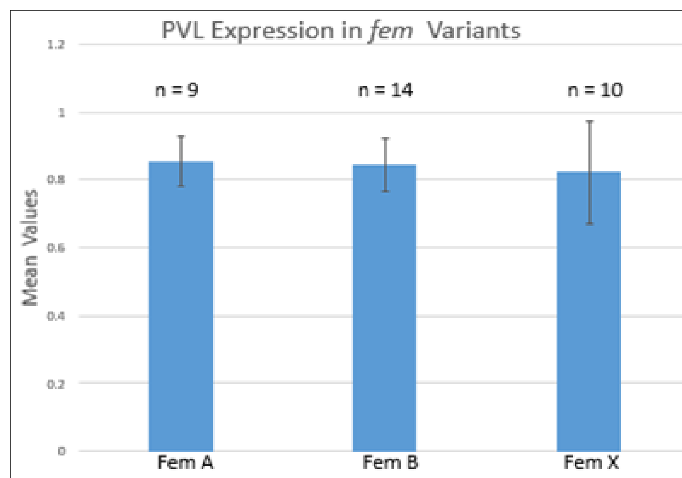

Fig 4: PVL expression in *fem* variants

Discussion

For decades, it has been known that *S. aureus* secretes *pvl* leukotoxin and thermostable nuclease enzyme. Nuclease activity is highly conserved among *S. aureus* and has been used as a marker for direct detection of *S. aureus* (16) in clinical microbiology. To establish infections, *S. aureus* expresses a multitude of virulence factors, including secreted toxins, enzymes and peptides (*PVL*) and surface proteins (protein A) which contribute to disease development (31, 32).

In this study we screened the clinical isolates for three important virulence factors: coagulase test to confirm if the isolate is coagulase positive or negative; thermonucelase (DNAse agar test) test to assay the nuclease activity and PVL as a virulent marker to check if it is carried by clinical isolates from hospital infections. Morphological changes and changes in cell lysis have been reported in *fem* mutants (36, 37). Defective cross linking in the peptidoglycan layers of cell wall due to inactive *fem* activity also have been reported affecting the cell wall function like secretion (38). Since these virulence markers are secreted, we attempted to correlate their profile with the *fem* sequence variations. All 157 isolates were coagulase positive and *nuc* seemed to be a reliable genetic marker as reported by others (Costa *et al*, 33). Though *fem* variants showed variable expression of nuclease on DNAse agar (zone of clearance of methyl green), there was no direct correlation of nuclease clearance zone with the *fem* sequence variation. We also noticed that several isolates did not show any enzyme actvity on DNAase agar though they contained the *nuc* gene, which we believe, was due to low sensitivity of the agar plate assay compared to *nuc*-PCR (34,35). PVL, once believed to be a genetic indentity to CA-MRSA seems to be quite prevalent even among clinical isolates of *S. aureus* perhaps helping in the pathogenesis of the disease.

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