ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Application of Recombinant LUK-SF Protein of Panton-Valentine Toxin of Community Acquired Methicillin Resistant *Staphylococcus aureus* for Toxin Detection and Neutralization Properties

Niveditha Sundar Poojary*, Shylaja Ramlal, Murali Harishchandra Sripathy, Harsh Vardhan Batra
Division of Microbiology, Defence Food Research Laboratory, Siddartha Nagar, Mysore-570011. Karnataka, India.

Manuscript Info

Manuscript History:

Received: 14 November 2013
Final Accepted: 25 November 2013
Published Online: December 2013

Key words:
Staphylococcus aureus, Panton-Valentine leukocidin (PVL), chimeric gene, Affinity chromatography, Western blot

Corresponding Author

Niveditha Sundar Poojary
Defence Food Research Laboratory-DRDO, Siddartha Nagar,
Mysore-570011
Karanataka
INDIA

Abstract

Panton-Valentine leukocidin (PVL) is a unique cytolytic toxin found associated with severe community-associated methicillin-resistant *Staphylococcus aureus*(CA-MRSA) infections that normally result in severe necrotizing infections mostly in the form of pneumonia. In the present study, cloning of *luk-S* and *luk-F* genes of PVL toxin and a chimeric gene comprised of both *luk-S* and *luk-F* was undertaken employing pRSETA vector. Isopropyl thiogalactoside (IPTG) induction with 1 mM concentration provided optimal expression of all the three recombinant proteins that were further purified by using Ni-NTA (nickel nitrilotriacetic acid) affinity chromatography. Murine hyperimmune sera raised against individual r-PVL-S and r-PVL-F specifically reacted to native 35.7 kDa and 36.8 kDa toxin protein components when culture supernatants of *S. aureus* strains positive for PVL-S and F were analyzed in Western blot. The hyper immune polyclonal antibodies raised against recombinant PVL S-F fusion protein could also specifically detect both the fractions of PVL toxin simultaneously in Western blot immunoassay. Detection of these two toxin components simultaneously by a single immunoassay definitely has the advantages in terms of rapidity, convenience and cost saving. The same polyclonal antibodies raised against r-PVL S and r-PVL F and also against r-PVL S-F fusion protein could neutralize the native PVL toxin activity in an *invitro* cell viability assay suggesting their potential as toxoid / candidate vaccine molecules. Hence, the recombinant PVL-S and PVL-F protein as well as PVL SF fusion protein reported here could serve as novel molecule both for diagnostic applications and for therapeutic intervention against CA-MRSA infections.

Introduction

Staphylococcus aureus is an important human pathogen that expresses a variety of exoproteins, including Panton–Valentine leukocidin (PVL). PVL genes are carried by community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clones that are spreading throughout the world (Vandenesch *et al.,* 2003; Tristan *et al.,* 2007). CA-MRSA infections are generally seen in healthy children or young athletes, with unexpected cases of diseases and also in elderly inpatients (Yamamoto *et al.,* 2010). The major cause of concern about CA-MRSA is that the strains carrying *luk-S* and *luk-F* (Panton-Valentine leukocidin) genes are increasingly found associated with severe necrotizing infections leading to pneumonia and proving fatal among children as well as with furuncles and primitive cutaneous abscesses (Wilks *et al.,* 2003; Niveditha *et al.,* 2012). PVL-positive CA-MRSA is easily transmissible within families but also, on a larger scale, in communities with increased promiscuity. From 1997 to 1999, deaths from childhood pneumonia/sepsis caused by CA-MRSA having the *lukS/F-PV* gene were reported in succession in Minnesota and North Dakota in the U.S (Takashi *et al.,* 1999; Boubaker *et al.,* 2004).

PVL is a cytolytic toxin comprised of LukF-PV and LukS-PV subunits that assemble into an octameric pore on the surface of myeloid cells, including polymorphonuclear neutrophils (PMNs). Strains of *S. aureus* that produce PVL have a particular affinity for basement membrane exposed by desquamated ciliated epithelium, and they rapidly establish themselves in the lung, producing the leukocidin. Membrane piercing PVL then destroys newly recruited polymorph cells, liberating inflammatory mediators (Francis *et al.,* 2005). Mortality due to such necrotising pneumonia is nearly 75% (Gillet *et al.,* 2002). PVL toxin acts on cell membranes by two separate and synergistic components (S and F). PVL exhibits cytolytic activity specific to leukocytic cells when the two components function in combination (Cribier *et al.,* 1992; Löffler *et al.,* 2010). Peptidic sequence data indicate that the LukSis a 35.7kDa and LukF is a 36.8kDa protein (Tatsuo *et al.,* 2010). The experimental evidence of PVL having leukotoxic activity that probably mediates the toxicity seen in CA-MRSA infections and also the fact that *lukS/F-PV* genes have broader role to play as a global regulator of virulence factors collectively create the problematic clinical phenotype which is associated with CA-MRSA strains (Rachel *et al.,* 2008).

With the emergence of the CA-MRSA strains throughout the world, the challenge lies with laboratories to efficiently identify these strains for surveillance, infection control, and treatment protocols to limit the dissemination of CA-MRSA (Fosheim *et al.,* 2011; Ryan *et al.,* 2005). To date, the diagnosis of PVL producing strains is mainly based on Polymerase Chain Reactions (PCRs), Real-Time PCR assays and nucleic acid-hybridization kits (AdvanDx® PVL Evigene™) for the detection of *lukF-PV* and *lukS-PV* genes (Yi-Wei Tang *et al.,* 2007; Carrol and Karen, 2008). Though the detection systems based on the PCRs are specific and accurate, the main disadvantage happens to be their inability to correlate to the expression of toxin components by the organism. Commercially available IVIg for PVL-specific antibodies can prevent the cytopathic effect of PVL on polymorphonuclear cells in vitro (Gauduchon *et al*., 2004). Though immunoassays are simple, robust and possess the ability to differentiate various toxins, commercially available antibodies and immunoassays are expensive.

Therefore in the present study, we have generated antibodies against recombinant proteins Luk-S and Luk-F and chimeric protein LukSF-PV. The antibodies were successfully evaluated for the detection of PVL in various strains of *S. aureus* isolated from different sources. Furthermore, polyclonal antibodies raised against these proteins were tested for their ability to neutralize the toxins.

MATERIAL AND METHODS

2.1 Bacterial cultures, chemicals and reagents:

Bacterial cultures used in the study are listed in Table 2. Dehydrated media and antibiotics were purchased from Himedia laboratories, India. DNA polymerases, dNTPs, restriction enzymes and T4 DNA ligase were purchased from MBI, Fermentas. The primers used in this study were synthesized at Eurofins Genomics Pvt. Ltd, India. *E. coli* host strains and vectors were purchased from Invitrogen and maintained in Luria Bertani broth/agar.

The murine macrophage cell line RAW 264.7 (ATCC® TIB-71™) was obtained from National Center for Cell Sciences (NCCS) Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Thermo scientific ltd, India), 5mM L-glutamine, 1mM sodium pyruvate, penicillin 100U mL^{-1}, streptomycin 100 µg mL^{-1} and 0.2% $NaHCO_3$ (Sigma Aldrich India) (Chiou WF 2000). Cultured cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

The *S. aureus* strains were grown in Brain Heart Infusion broth (BHI) and plated on Baird Parker agar (BPA) supplemented with Egg yolk tellurite emulsion. The plates were incubated at 37 ^{O}C for 24 hours. The isolates were identified morphologically and biochemically by standard laboratory procedures and maintained in 15% glycerol stocks in -80 °C as described by Murray and coworkers (2007).

For exotoxin extraction, the bacterial stocks were cultured in CCY medium containing 3% w/v yeast extract, 2% Bacto-Casamino acids, 2.3% sodium pyruvate, 0.63% Na_2HPO_4, and 0.041% KH_2PO_4 and the pH was adjusted to 6.7 (Diep *et al.,* 2008).

2.2 *Staphylococcus aureus*genomic DNA extraction:

DNA from *S. aureus* was extracted by phenol-chloroform method. A 3-5ml of overnight culture was centrifuged and incubated at 70 ^{O}C for 2 h in the presence of 400 mg of proteinase K ml-1, 1% sodium dodecyl sulfate, 2.5 mM disodium EDTA, and 25 mM sodium acetate. The suspension was sequentially extracted with phenol and then chloroform. The DNA was precipitated with 0.3 M sodium acetate and then with absolute ethanol, washed with 70% ethanol, dried and then dissolved in 50 µl of distilled water (Sambrook*et al.* 1989). The microfuge tube is than stored at -20 ^{O}C for further use.

2.3 Primer designing and Construction of *luk-S, luk-F* gene and chimeric fusion gene lukS/F:

The primers (Table 1) used in the construction of recombinant *luk-S* and *luk-F* genes and chimeric gene *luk-SF* were designed using the Generunner software. The gene sequences were retrieved from NCBI database. Each PCR reaction was performed with 1x *pfu* buffer with 2 mM $MgSO_4$, 200 µM of dNTPs, 10picomole each of forward and reverse primers, 50 ng of DNA template, one unit of *pfu* DNA polymerase and the final volume was adjusted to 20

µl with nuclease free water. The PCR was performed as follows: initial denaturation of 4 minutes at 94 ^{O}C followed by 30 cycles of denaturation for 30 seconds at 94 ^{O}C, annealing at 57 ^{O}C for 30 seconds, extension of one minute at 72 ^{O}C followed by a final extension of ten minutes at 72 ^{O}C. PCR products were analyzed in 2% agarose with ethidiumbromide.The PCR products (*luk-S* and*luk-F)* were digested with *Bam*HI and *Hind*III and inserted into pRSET A vector pre-digested with *Bam*HI and *Hind*III. The ligated product was transformed into BL21(DE3)-pLysS *E. coli* host strain. The resultant colonies were screened by colony PCR for the presence of insert by flanking T7 sequencing primers. The plasmid was extracted from transformed strain and sequenced with T7 primers. The strategy employed for the fusion of two genes is depicted in Figure 2.

2.4 Expression studies of the proteins:

The *E. coli* host cells harboring recombinant plasmids were grown overnight at 37 ^{O}C in LB broth with 100 µg/ml ampicillin. One ml of this culture was further inoculated in to flasks containing 25 ml LB broth containing 100 µg/ml ampicillin and grown at 37 ^{O}C with vigorous shaking until the OD_{600} reached 0.7. One mM IPTG was added to the cells and allowed to grow at 37 ^{O}C with vigorous shaking. One ml of cells were collected after 5 hrs of induction, lysed, subjected to 12 % SDS-PAGE and stained with Coomassie brilliant blue to determine the expression of the proteins. The proteins separated on SDS-PAGE were transferred on to a nitrocellulose membrane by Western blot procedure (Towbin *et al.,* 1979), blocked with phosphate buffered saline (PBS) containing 5% skim milk for 1 h followed by incubation with mouse monoclonal anti-histidine antibodies. After washing with PBST (PBS + 0.05% Tween 20), the membrane was incubated for 30 minutes with anti-mouse HRP labelled antibodies raised in goat. The membrane was thoroughly washed with PBST and developed with 3,3`, 5, 5` -d Diaminobenzidine tetra chloride and 0.004 % H_2O_2 in PBS. Later the reaction was stopped by washing the membrane with distilled water. For bulk production, the culture was inoculated in 100 ml LB broth with antibiotics. The recombinant proteins were purified under denaturation conditions using urea as denaturant by immobilized metal affinity chromatography (IMAC) using Ni-NTA agarose (Qiagen, Germany) as chelating resin and amino terminal hexa-histidine residues as affinity tag according to manufacturer's recommendations. The purity of the purified protein was analyzed by 12 % SDS-PAGE. The elutions were pooled and the protein concentration was quantified by Lowry's calorimetric assay using BSA as standard.

2.5 Immunization of the Mice:

4–6 weeks old female BALB/c mice were used for immunization. The mice were maintained and used in accordance with the recommendations of the Institutional Animals Ethics Committee. Mice were immunized i.m. with 50 ug of recombinantproteins. First injection was administered with Freund's complete adjuvant on the day 0, followed by booster immunization in emulsion with Freund's incomplete adjuvant on days 7, 14, 28. The mice with high anti-sera against r-luk-S, r-luk-F and r-luk-S/F were finally boosted by i.p. 3 days before hybridomafusion. The antibody titer of mice was measured by indirect ELISA with recombinant proteins. Hyperimmune sera obtained 14 days after the last boost was used for Western blots**.**

2.6 Exoprotein preparations:

Overnight cultures of *S. aureus* grown in BHI broth were centrifuged at 10,000g for 3 min and the proteins in supernatant were concentrated by Ammonium Sulphate precipitation (80% saturation) at 4 ^{O}C for 16 hours. Protein concentrations were determined by using the Lowry method further analyzed by Western blot analysis.

For PVL toxin production, overnight cultures from CCY medium were either used directly or diluted 1:200 into fresh CCY media and incubated for 8 h with shaking at 225 rpm at 37 ^{O}C till the OD_{600} reached 0.75. Bacteria were removed from the culture media by centrifugation. Culture supernatants were sterilized by passed through 0.22 µm filter and stored in aliquots at −80 ^{O}C for future use.

2.7 Toxin neutralization assay:

The toxin neutralization ability of antisera raised against recombinant proteins was assessed on RAW 264.7macrophage cell lines. The cell lines were grown upto approximately 4×10^4 cells/well in DMEM containing 10% FBS and appropriate antibiotics. The sera were diluted two folds in sterile PBS and preincubated in equal volumes with native 10µg/ml PVL toxin for 1 h at 37 ^{O}C under 5% CO_2. The medium present in the wells were replaced by 100 µl of the toxin-sera mixture or PBS and further incubated for 4 h at 37 ^{O}C under 5% CO_2. After 72h incubation, the media was replaced by 0.5 mg ml^{-1} MTT dissolved in DMEM and incubated for additional 1 h at 37 ^{O}C at 5% CO_2. The MTT reagent was replaced by 150 µl DMSO to solubilize the formazan crystals of MTT. The absorbance was read at 570 nm. Percent neutralization was calculated using the following formula. Percent neutralization = (sample OD value- standard OD value)/(cells only value-PVL toxin standard OD value) × 100 (Wang *et al.,* 2011).

RESULTS

3.1 Construction, Cloning and expression of *luk-S*, *luk-F* and *lukS/F* fusion genes:

The individual genes *luk-S* and *luk-F* were amplified from *S. aureus* genomic DNA (Fig1). The fusion gene was generated by restriction digestion method as depicted in figure 2. After cloning these genes into pRESTA vector, they were sequenced to determine if any mutations have appeared. Positive clones were induced with 1mM IPTG for 5 hrs at 37 ^{O}C and the expression of recombinant proteins were detected in 12% SDS-PAGE gel stained with Coomassie blue. The relative sizes of the proteins **r-luk-S, r-luk-F and r-lukS/F** were in agreement with the predicted size i.e. 35, 32 and 39 kDa respectively (Fig 3). Subsequently, the expression or the proteins were confirmed by Western blot analysis with anti-His antibodies.

3.2 Purification of the recombinant protein:

The recombinant proteins were purified from 100 ml induced culture by using immobilized metal affinity chromatography on Ni-NTA agarose column (Qiagen). The cells were induced for 5 h with 1 mM IPTG and they were collected and purified by urea denaturation according to manufacturer's protocol (Qiagen). The purified proteins were pooled and dialysed into PBS + 10 mM Urea for 2 h at 4 ^{O}C (fig 3). After buffer exchange, the protein concentration of luk-S, luk-F and luk-SF proteins were quantified to be 4.0 mg/ mL, 7.0 mg/mL, 3.0 mg/mL respectively by Lowry's calorimetric assay using bovine serum albumin as standard.

3.3 Antigenicity testing of recombinant protein:

Balb/C mice were immunized with recombinant proteins. When the end point titer reached 32,000, the blood was collected and sera were separated. The antisera rasied against these proteins were tested for their reactivity with luk-S, luk-F and luk-SF fusion proteins by indirect ELISA showed that mouse anti-r-luk-S, r-luk-F and r-luk-SF serum reacted strongly with native toxin was shown by Western Blot analysis (Fig 4). Hyperimmuneantiserum against r-luk-S, r-luk-F and r-luk-S/F did not exhibit any cross-reactions with other organisms tested (Table 2).

3.4 Assessment of toxin neutralization:

Polyclonal antibody raised against luk-S, luk-F and luk-SF can neutralize native PVL toxin on raw J7747 macrophage cell lines. This colorimetric assay is based upon the reduction of MTT by living cells. The percentage of inhibition was depicted in Figure 5.

Figure 1: Agarose gel showing amplification of individual genes and fusion gene .

Lane 1: PCR amplification of *luk-S* gene (798 bp); Lane 2: 1 kb DNA ladder; Lane 3: PCR amplification of *luk-F* (695 bp) gene; Lane 4: 1 kb DNA ladder; Lane5: fusion gene of *luk-S* and *luk-F* (948 bp); Lane 6: 1 kb DNA ladder

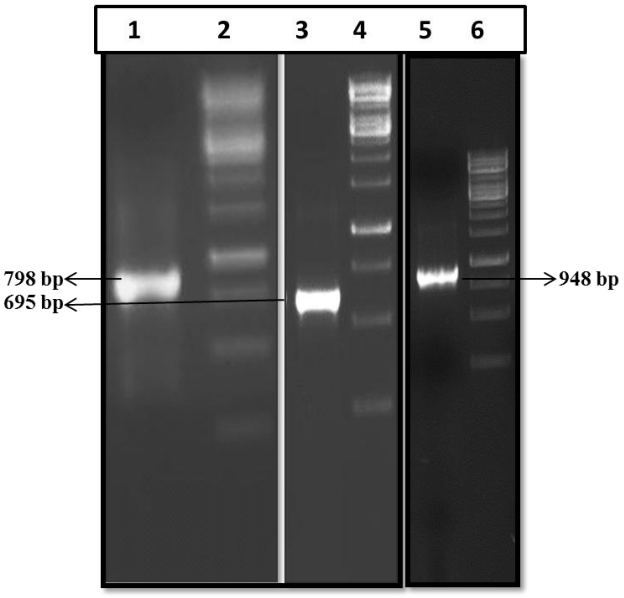

Figure 2: Strategy for fusion of genes

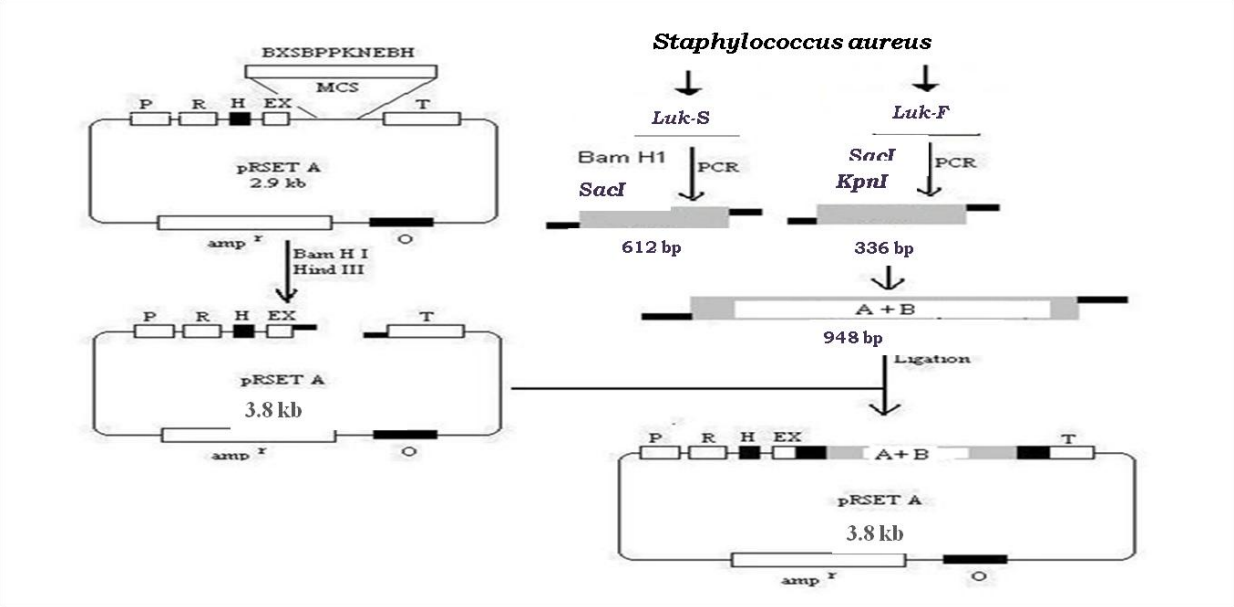

Figure 2: Strategy for fusion of genes

Figure 3: Coomassie blue stained SDS-PAGE gel showing expression and purification of r-luk-S, luk-F, chimericluk-SF proteins.

Polyacrylamide gel (12%) showing the expression and purification of the recombinant proteins from the IPTG induced clones.

Lane1: Prestained protein ladder; lane 2: Induced *E. coli* BL21 (DE3) carrying pRSET A-*luk-S* vector; lane 3: unstained protein ladder lane 4 & 5: Purified r-luk-S; lane 6: Induced *E. coli* BL21 (DE3) carrying pRSET A-*luk-F* vector; lane 7 & 8: Uninduced *E. coli* BL21 (DE3) host; lane 9 and 10: unstained protein ladder; lane 11 & 12: Purified r-luk-F; lane 13: unstained protein ladder; lane 14 & 15 : Induced *E. coli* BL21 (DE3) carrying pRSET A-chimeric fusion *luk-S &luk-F* vector; lane 16: unstained protein ladder; lane 17-23: purified r-luk-SFchimeric protein.

Figure 4:

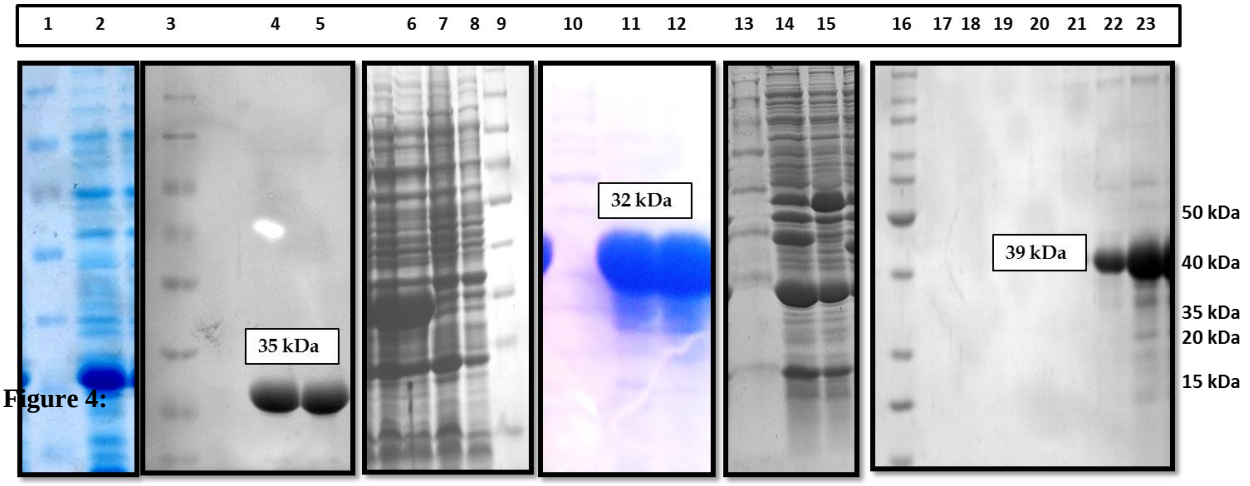

Figure 4: Western blot analysis with anti-chimeric luk-SF antibodies to check protein reactivity with crude protein.
Lane M: Prestained protein ladder; Lane 1 & 2 crude protein, Lane 3: r-chimeric luk-SF protein

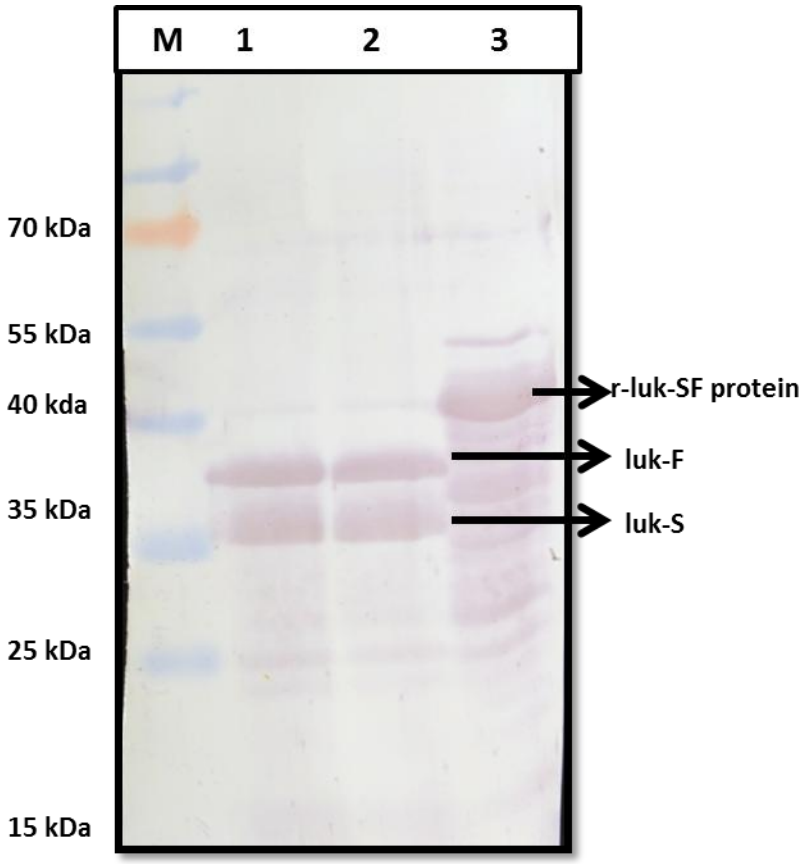

Figure 5: Percentage of neutralization of antibody raised against luk-S, luk-F, chimeric fusion luk-SF proteins

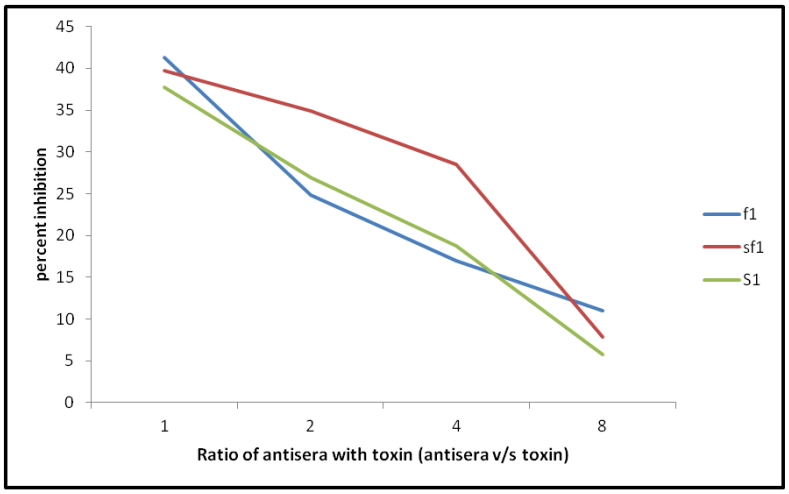

Figure 5: Percentage of neutralization of antibody raised against luk-S, luk-F, chimeric fusion luk-SF proteins

DISCUSSION

Panton Valentine Leukocidin (PVL) is a stable marker of CA-MRSA strains, which enhances the virulence potential of *Staphylococcus aureus*(Szmiegielski *et al.,*1999). It is important for clinicians to be familiar with the incidence of CA-MRSA in their communities, as prevalence can vary from region to region. Moreover, since CA-MRSA has a unique antibiotic resistance profile, knowledge of prevalence can inform empirical antibiotic therapy in more-severe cases that are handled as outpatients. This necessitates surveillance for PVL containing MRSA by the use of most efficient laboratory methods available. Currently, PCRs, nucleic acid based probes and triplex Real-Time PCRs are among the methods being employed for the detection of PVL genes (Ryan *et al.,* 2005; Fred *et al.,* 2006). With the surge observed in antibiotic resistance coupled to lack of rapid and low cost detection systems and the presence of multiple superfluous virulence factors among *S. aureus*strains, enables them to create havocs and this poses a challenge both to the researchers and the clinicians. Specific and highly sensitive detection of the toxin protein is difficult with the use of conventional techniques, and it is very time-consuming to obtain detection results. On the other side, targeting the expressed toxin employing appropriate immunoassays rather than the gene could provide more useful clinical relevance of the disease.

In the present study, an attempt was made to obtain individual recombinant proteinsluk-S &luk-F of staphylococcal Panton-Valentine leukocidin toxin as well as recombinant fusion protein encompassing both of these fractions (PVL–SF, GenBank accession no, JX951174.1). Murine hyperimmune sera raised against individual fractions of the PVL toxin recognized their corresponding native toxin components among the confirmed PVL toxin containing *S. aureus* strains whereas antisera against the fusion recombinant protein detected both the toxin fractions simultaneously. Therefore, all these antiseras could be successfully utilized in developing immunoassays for the specific detection of PVL toxin containing *S. aureus* that in turn confirmed the CA-MRSA strains. Antisera raised against single molecule of recombinant fusion protein of S and F components had merit over the antiseras raised against individual fractions of PVL toxin. Since PVL toxin is reported to exhibit cytolytic activity when the two components function in combination (Cribier *et al.,*1992; Löffler *et al.,* 2010), the antisera raised against recombinant fusion protein was much more relevant in detecting both the toxin components simultaneously among *S. aureus* strains. Moreover, generation, utilization and interpretation becomes relatively easier in a simple immunoassay like Western blot with antisera raised against recombinant fusion PVL-SF protein to identify the PVL toxin associated virulent MRSA *S. aureus* strains.

Toxin neutralization assay was carried out to study the interactions between these three types of polyclonal antibodies and the PVL toxin in RAW J7747 murine macrophage cell line. All the three types of polyclonal antibodies generated against recombinant proteins were successful enough to neutralize the toxic effect of PVL toxin as demonstrated onto murine macrophage cell line suggesting that all these antibodies have the ability to bind to the epitopes present in the native toxin to affect neutralizing capability. Therefore, it could be assumed that generated polyclonal antibodies had a significant binding capability even to the conformational epitopes present on the native toxin. More so, even antibodies generated against the individual sub component of the PVL toxin either against S or F had a highly effective neutralizing capability against the native toxin. Thus, it appears that for the toxic capability of the native PVL toxin, the two individual components of the PVL toxin are equally relevant to provide a synergistic effect for the activity.

In conclusion, this paper has described the successful construction and expression of r-luk-S, luk-F and chimeric r-PVL-S/F fusion protein with specificity tailored towards S and F fraction of PVL toxin coupled to *E. coli*-based expression with a simple one-step purification procedure for cost-effective production. Generation of fusion proteins by DNA manipulations is of high importance in applications like therapeutics and diagnostics. However, a method for directly detecting PVL toxin produced by *Staphylococcus aureus* has not been established. This protein may be useful in rapid and reliable immunoassays for proper surveillance and for initiating a timely response to clinical infections or foodborne outbreaks. The recombinant chimeric protein-based immune detection approach may eventually provide advantages over PCR formats. It is an objective of the present invention to provide an antibody specific to LukF-PV, LukS-PV and PVL-S/F and to provide a kit for specific and highly sensitive assay of the relevant substance with the use of such an antibody. Now it has become possible to detect PVL toxin directly from diverse sources. Antibody to PVL appeared to decrease the ability of PMNs to control the proliferation of PVL-producing *S. aureus* in the community. It proves to be a potential candidate vaccine molecule based on the cytotoxin may benefit in therapeutics. The assay has the potential to improve individual patient management and both community-acquired and nosocomial infection control procedures.

Acknowledgments:

Authors are thankful to the Director, DFRL for providing necessary facilities to conduct the study.

Table 1: Primers used in the study for individual gene cloning:

Primer	Sequence	Accession number	Amplicon size (bp)
luk-S For	5'CGCggatccATCACTCCTATTGCTACTTCG3'	AB678714.1	798
luk-S Rev	5'CCGggtaccGCCATAGTGTGTTGTTCTTCT3'		
luk-F For	5'CGCggatccTACAAAACAACTGCAACATCA3'	EF571802.1	695
luk-F Rev	5'CCGggtaccTTTTGCAGCGTTTTGTTTTCG3'		
Fusion gene	5'CGCggatccATCACTCCTATTGCTACTTCG3'	JX951174.1	948
luk-S For	5'CgagctcGCCATAGTGTGTTGTTCTTCT 3'		
luk-S Rev			
luk-F For	5'CgagctcAACGGCTTATCAGGTGGA3'		
luk-F Rev	5'CGGggtaccTTTTGCAGCGTTTTGTTTTCG 3'		

The sequence corresponding to restriction enzymes were given in lowercase.

Table 2: Evaluation of Polyclonal antibodies raised against r-luk-S, r-luk-F and chimeric r-luk-SF proteins on *Staphylococcus aureus* strains and other organisms:

Strains	Western Blot			Plate ELISA		
	PVL-S PoAb	PVL-F PoAb	PVL-SF PoAb	PVL-S PoAb	PVL-F PoAb	PVL-SF PoAb
S. aureus strains						
ATCC 700699	-	-	-	-	-	-
Japan TSST	+	+	+	+	+	+
FRI 722	+	+	+	+	+	+
ATCC 43300	-	-	-	-	-	-
ATCC 29213	-	-	-	-	-	-
ATCC 6538	-	-	-	-	-	-
NCIM 2079	-	-	-	-	-	-
NCIM 2901	-	-	-	-	-	-
NCIM 2654	-	-	-	-	-	-
NCIM 2672	-	-	-	-	-	-
NCIM 2792	+	+	+	+	+	+
NCIM 2127	+	+	+	+	+	+
NCIM 2124	-	-	-	-	-	-
NCIM 2121	-	-	-	-	-	-
NCIM 2122	+	+	+	+	+	+
RAB (DRDE)	-	-	-	-	-	-
IVRI	-	-	-	-	-	-
E 2147	+	+	+	+	+	+
E2533	-	-	-	-	-	-

E2279	-	-	-	-	-	-
E1345	-	-	-	-	-	-
E1357	-	-	-	-	-	-
E1988	+	+	+	+	+	+
E1975	+	+	+	+	+	+
E1977	+	+	+	+	+	+
E1978	+	+	+	+	+	+
FI 1	-	-	-	-	-	-
FI 2	-	-	-	-	-	-
FI 3	-	-	-	-	-	-
FI 4	-	-	-	-	-	-
FI 5	-	-	-	-	-	-
FI 6	-	-	-	-	-	-
FI 7	-	-	-	-	-	-
CI 1	+	+	+	+	+	+
CI 2	-	-	-	-	-	-
CI 3	+	+	+	+	+	+
CI 4	-	-	-	-	-	-
CI 5	-	-	-	-	-	-
CI 6	+	+	+	+	+	+
Other bacterial strains						
Escherichia coli	-	-	-	-	-	-
Vibrio vulnificus	-	-	-	-	-	-
Shigellaspp	-	-	-	-	-	-
Salmonella typhii	-	-	-	-	-	-
Proteus vulgaris	-	-	-	-	-	-
Aeromonasspp	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-
Listeria spp	-	-	-	-	-	-
Klebsiellaspp	-	-	-	-	-	-

ATCC: American Type Culture Collection, Manassas, Va; DRDE: Defence Research and Development Establishment, Gwalior, India; FRI: Food Research Institute, India; IVRI: Institute of Veterinary Research Institute, E: SDM College of Medical Sciences and Hospital isolates, Dharwad; Karnataka India; FI: Food isolate; CI: Clinical isolate.

REFERENCES:

1. BoubakerK, Patrick Diebold, Dominique S. Blanc, François Vandenesch, Gérard Praz, Georges Dupuis and Nicolas Troillet (2004); Panton-Valentine Leukocidin and Staphyloccoccal Skin Infections in Schoolchildren, Emerging Infectious Diseases, Vol(10)
2. Carrol, Karen C, Rapid Diagnostics for MRSA: current status, Molecular diagnosis and Therapy, Infectious diseases(2008); vol 12, 1, 15-24
3. Cribier, B., G. Prevost, P. Couppie, V. Finck-Barbanqon, E. Grosshans, and Y. Pitmont. (1992); Staphylococcus aureusleukocidin: a new virulence factor in cutaneous infections. Dermatology 185:175-180
4. DiepBinh An, Amy M. Palazzolo-Ballance, Pierre Tattevin, Li Basuino, Kevin R. Braughton, Adeline R. Whitney, Liang Chen, Barry N. Kreiswirth, Michael Otto, Frank R. DeLeo, Henry F. Chambers (2008); Contribution of Panton-Valentine Leukocidin in Community-Associated Methicillin-Resistant Staphylococcus aureus Pathogenesis, PLoSPathog 9(3)
5. Fosheim GE, AC Nicholson, VS Albrecht, BM Limbago, A Multiplex Real-time PCR Assay for Detection of Methicillin-1 Resistant *Staphylococcus aureus* and Associated Toxin Genes, J. Clin. Microbiol, JCM.00795-11, 2011
6. GravesShawna F., Scott D. Kobayashi, Kevin R. Braughton, Adeline R. Whitney, Daniel E. Sturdevant, Devon L. Rasmussen, Liliya N. Kirpotina§, Mark T. Quinn§ and Frank R. DeLeo. (2012); Sublytic concentrations of *Staphylococcus aureus* Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity. Journal of leukocyte biology

7. Graves Shawna F., Scott D. Kobayashia, Kevin R. Braughtona, Binh An Diepc, Henry F. Chambersc, Michael Ottod, and Frank R. DeLeoa (2010); Relative contribution of Panton-Valentine leukocidin to PMN plasma membrane permeability and lysis caused by USA300 and USA400 culture supernatants, *Microbes Infect*. June; 12(6): 446–456
8. Gillet Y, Issartel B, Vanhems P, Fournet JC, Lina G, Bes M, Vandenesch F, Piemont Y, Brousse N, Floret D, Etienne J. Association between *Staphylococcus aureus*strains carrying gene for Panton- Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet 2002;359:753–759
9. Löffler B, Hussain M, Grundmeier M, Brück M, Holzinger D (2010); *Staphylococcus aureus* Panton-Valentine Leukocidin Is a Very Potent Cytotoxic Factor for Human Neutrophils,*Staphylococcus aureus* Panton-Valentine Leukocidin Is a Very Potent Cytotoxic Factor for Human Neutrophils. PLoSPathog 6(1)
10. MurrayPatrick R., Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, Michael A. Pfaller (2007); Manual of Clinical Microbiology, 9th ed., American Society for Microbiology.
11. Niveditha. S, Shylaja. R, Murali. H. S, Batra. H. V (2012); A novel mPCR for the detection of prominent toxins in MRSA strains of *S. aureus* recovered from diverse sources. International Journal of Research in Biological Sciences; 2 (1): 26-32
12. Otokunefor K, SloanT, Angela M. Kearns and JamesR (2012); Molecular characterization and PVL typing of community-acquired methicillin-sensitive *Staphylococcus aureus* clinical isolates. J ClinMicrobiol 50(9):3069. doi:10.1128/JCM.00602-12
13. Rachel J, Gordon1 and Franklin D. Lowy (2008); Pathogenesis of Methicillin-Resistant *Staphylococcus aureus*Infection, Clinical Infectious Diseases; 46:S350–9
14. Ryan R. McDonald, Nick A. Antonishyn, Toni Hansen, Laelie A. Snook, Evelyn Nagle, Michael R. Mulvey, Paul N. Levett and Greg B. Horsman (2005); Development of a Triplex Real-Time PCR Assay for Detection of Panton-Valentine Leukocidin Toxin Genes in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*, JCM.43.12.6147–6149
15. Sambrook J, Fritsch EF,Maniatis T (1989); Molecular Cloning: A laboratory manual, 2nd ed. 3 volumes. Cold Springs Harbor Laboratory, Cold Springs Harbor, NY
16. Szmiegielski S, Prevost G, Monteil H (1999); Leukocidal toxins of staphylococci, ZentralblBakteriol **289:**185–201
17. Takashi Ito,MakikoIijima,Takayoshi Fukushima, Masato Nonoyama, Masahiro Ishii, Tatiana Baranovich, Taketo Otsuka,Tomomi Takano, and Tatsuo Yamamoto (1999); Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*— Minnesota and North Dakota, 1997–1999. JAMA; 282:1123–5
18. Takizawa Y, Taneike I, Nakagawa S, OishiT,Nitahara Y, IwakuraN,OzakiK,TakanoM,Nakayama T and Yamamoto T (2005); A Panton-Valentine Leucocidin (PVL)-Positive Community-Acquired Methicillin-Resistant *Staphylococcus aureus* (MRSA) Strain, Another Such Strain Carrying a Multiple-Drug Resistance Plasmid, and Other More-Typical PVL-Negative MRSA Strains Found in Japan. J ClinMicrobiol43(7): 3356–3363
19. Tatsuo Yamamoto, Akihito Nishiyama, Tomomi Takano, Shizuka Yabe, Wataru Higuchi, Olga Razvina, Da Shi(2010); Community-acquired methicillin-resistant Staphylococcus aureus: community transmission, pathogenesis, and drug resistance, J Infect Chemother16:225–254
20. Towbin H, Staehelin T, Gordon J, (1979); Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. ProcNatlAcadSci U S A.;76(9):4350-4
21. Wang, Y., Jiang, N., Zhan, D., Tao, H., Yuan, S., Wang, P., Wang, L., Zhang, Z. and Liu, C. (2011); Surface display of the 20-kDa N-terminal fragment of anthrax protective antigen based on attenuated recombinant Bacillus anthracis. World J MicrobiolBiotechnol. 27: 2575-2581
22. Wilks D, Mark Farrington, David Rubenstein (2003); The Infectious Diseases Manual, Wiley-Blackwell, London
23. Yamamoto T, Nishiyama A, Takano T, Yabe S, Higuchi W, Razvina O and Shi D (2010);Community-acquired methicillin-resistant *Staphylococcus aureus*: community transmission, pathogenesis, and drug resistance. Infect Chemother 16:225–254. Doi: 10.1007/s10156-010-0045-9
24. Yi-Wei Tang, Abdullah Kilic, Qunying Yang, Sigrid K. McAllister, Haijing Li, Rebecca S. Miller, Melinda McCormac, Karen D. Tracy, Charles W. Stratton, Jian Han, and Brandi Limbago, (2007); StaphPlex System for Rapid and Simultaneous Identification of Antibiotic Resistance Determinants and Panton-Valentine Leukocidin Detection of Staphylococci from Positive Blood Cultures, J ClinMicrobiol. June; 45(6): 1867–1873