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Inhibition of Panton-Valentine Leukocidin Toxin Induced Neutrophil Cell Lysis by Vancoplus in Methicillin-Resistant *Staphylococcus aureus* Infections

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Abstract: Pantone Valentine Leukocidin (PVL) is a cytotoxin associated with virulence of Methicillin-Resistant *Staphylococcus aureus* (MRSA). This study was undertaken to analyze the prevalence of PVL among MRSA strains and to study the effect of adjuvants (L-lysine, L-arginine, Ethylenediamine Tetraacetic Acid (EDTA) as well as drugs on PVL induced neutrophil cell lysis. Further, *in vitro* activity of Vancoplus, vancomycin, linezolid, teicoplanin and daptomycin against PVL positive MRSA strains was analyzed. A total of 67 *Staphylococcus aureus* strains collected were screened for the prevalence of methicillin resistance and PVL by Polymerase Chain Reaction (PCR). Effects of various adjuvants and drugs on PVL induced neutrophil cell lysis were studied by Lactate Dehydrogenase (LDH) assay. Antibiotic susceptibility was conducted using Clinical and Laboratory Standards Institute (CLSI) guidelines. Our results showed that of 67 clinical isolates, 71.6% (48/67) isolates were confirmed to be MRSA. Of these MRSA strains, 75% (36/48) isolates were observed to be PVL positive. Pus swab (86.3%; 19/22) had more proportion of PVL than urine (75%; 6/8); sputum (70%; 7/10) and blood (50%; 4/4). When diverse adjuvants alone (L-lysine, L-argine and EDTA) or in combinations were tested on PVL induced neutrophil cell lysis, 10 mM L-lysine alone and combinations of 10 mM L-lysine +10 mM EDTA and 100 mM L-arginine +10 mM EDTA produced the highest 78.7±7.1, 92.6±8.8 and 93.4±6.3%, respectively, inhibition of LDH activity (known marker of cell lysis). Further, when Vancoplus reconstituted with solvent produced 93.1±6.4% inhibition of LDH activity whereas other comparator drugs produced only 12.7±2.4 to 30.5±3.1% inhibition of LDH activity. Vancoplus appeared to be the most efficacious against 94.4% of the MRSA strains with MIC values 0.25 to 4 μg mL^{-1}. The susceptibility of other drugs vancomycin, linezolid, teicoplanin and daptomycin against MRSA varied from 22.3 to 77.8% of isolates with MICs 0.5 to 32 μg mL^{-1}. This study provides new insight into the prevalence of PVL toxins among MRSA. Results of this study showed that Vancoplus is the most active against MRSA and it additionally protects the neutrophil cells from PVL toxins and prevents from sequels of MRSA infections caused by these cytotoxins.

Keywords: Adjuvants, Clinical Isolates, Methicillin-Resistant *Staphylococcus aureus*, Panton-Valentine Leukocidin

Introduction

Staphylococcus aureus is an opportunistic Gram-positive pathogen that is frequently associated with various types of life-threatening hospital and community acquired infections (Nizet, 2007; Nubel *et al*., 2008). It commonly causes necrotic lesions in skin, necrotic hemorrhagic pneumonia as well as endocarditis (Holmes *et al*., 2005; Corey, 2009). Approximately 80% of the human population has this bacteria in skin (Kluytmans *et al*., 1997).

The presence of antibiotic resistant genes as well as production of virulence factors makes *S. aureus* highly pathogenic organism. Methicillin-Resistant *S. aureus* (MRSA) is one of the most prevalent forms of antimicrobial-resistant bacteria (Baba-Moussa *et al*., 2010). The virulence factors which are produced by this organism includes exotoxins, such as Exfoliative Toxins

(ETs), along with Toxic Shock Syndrome Toxin-1 (TSST-1), Staphylococcal Enterotoxins (SEs), leukocidins (Panton-Valentine Leukocidin; PVL, LukE/D) and hemolysins (α, β, γ, δ). These toxins create a survival advantage for the bacteria by forming pores into the membrane of target cells, inducing cell death and weakening the host during infections (Dinges *et al*., 2000; Sina *et al*., 2013). Among these virulence factors, PVL is reported to be an important virulence factor for *S. aureus* (Clinic *et al*., 1992). It is made up of two components called LukS-PV and LukF-PV which work synergistically to damage the membranes of macrophages and polymorphonuclear leukocytes, important components of the immune response (Prevost *et al*., 1993).

According to a study, approximately 93% of the community-associated *S. aureus* causing skin infections such as furnucles, possessed the PVL genes (Lina *et al*., 1999). Another study reported that around 96% cases of furuncles are associated with PVL positive strains of *S. aureus* (Durupt *at al*., 2007). PVL has also been shown to be involved in necrotizing pneumonia (Labandeira-Rey *et al*., 2007) and osteomyelitis (Gillet *et al*., 2007).

It has been proposed that MRSA strains that have the ability to cause severe skin and soft tissue infections, do so in a PVL-dependent manner (Lina *et al*., 1999). Upon infection with PVL-expressing *S. aureus*, the recruited neutrophils are rapidly killed by PVL, resulting in uncontrolled release of neutrophil proteases that damage the airway epithelium and thus damage immune system causing necrotizing pneumonia (Niemann *et al*., 2012). It indicates that proteases released by PVL-damaged neutrophils are mainly responsible for lung injury and neutrophil killing during infection with PVL-producing strains. The host counteracts this pathogen strategy by using PVL-neutralizing beta-sheet breakers/terminal blocking groups or by neutralizing the released proteases via protease inhibitors (Korkmaz *et al*., 2008). Lysine and arginine are essential amino acids known to be protease inhibitors (Korkmaz *et al*., 2008). A variety of antibiotics including vancomycin have routinely been used for the treatment of *S. aureus* infections specially MRSA strains. However, persistence use of vancomycin caused development of Vancomycin-Resistant *S. aureus* (VRSA) and Vancomycin-Intermediate *S. aureus* (VISA). The resistant genes may also affect toxin production (Sina *et al*., 2013).

A study in last deacde showed a 76% treatment failure rate with vancomycin (Howden *et al*., 2004). Several reports from India recorded the emergence of various degree of vancomycin resistance (Veer *et al*., 2010; Thati *et al*., 2011). Other parts of the world also reported the prevalence of vancomycin resistance (Perichon and Courvalin, 2009; Reynolds *et al*., 2012; Jang *et al*., 2012). Similarly, the rate of non-susceptibility of penicillin-resistant *Streptococcus pneumoniae* to cephalosporins particularly ceftriaxone is increasing significantly (Karunakaran *et al*., 2012). In such scenerio, where cephalosporins and vancomycin are getting resistant individually, an unmet medical need to cater growing vancomycin resistance and to counter the toxins produced by MRSA is required.

This study was aimed to study the prevalence of PVL in MRSA and to study the effect of adjuvants (L-lysine, L-arginine, EDTA) and drugs on PVL induced neutrophil cell lysis. In addition, *in vitro* activity of different drugs used to treat MRSA infections was studied to check their susceptibility behaviour.

Materials and Methods

Chemicals

Primary monoclonal antibody (Mouse anti-Luks-PV mAB), secondary antibody (anti Horseradish Peroxidase (HRP) conjugate) and detection antibody (polyclonal anti PVL LukS) were obtained from Institute of Biosciences and Technology (IBT), USA. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was obtained from Cell Signaliing Technologies (CST), USA. Taq polymerase was purchased from Merck Milipore Genei (Banglore). Ficoll-paque PLUS and Latacte Dehydrogenase (LDH) assay kit were obtained from Sigma Aldrich, USA. RPMI-1640 medium, Bovine Serum Albumin (BSA), sodium chloride (NaCl), sodium Hydroxide (NaOH), monosodium phosphate (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), agarose, ammonium sulfate, tween-20, proteinase K, Soyabean Casein Digest Medium (SCDM), lysine, arginine, Ethylenediamine Tetraacetic acid Disodium (EDTA) and mannitol salt agar were purchased from Hi-Media (Mumbai, India). HEPES buffer was purchased from Spectrochem Limited (Mumbai, India).

Antibacterial Agents

The following antibiotics were used in this study: A novel antibiotic adjuvant entity of ceftriaxone sodium and vancomycin hydrocloride with VRP1020 (Vancoplus; Venus Remedies Limited, Chandigarh, India), teicoplanin (T-planin, Glenmark, India), linezolid (Linospan injection, Cipla Limited, Mumbai, India), daptomycin (cubicin; Novartis Pharmaceuticals, United Kingdom) and vancomycin (vancocin-CP, Astra Zeneca Pharma India Limited, Banglore, India). All the drugs were reconstituted according to the instructions of the manufacturer.

Bacterial Isolates Collection and their Identification

A total of 67 clinical isolates of *S. aureus* were collected from Department of Microbiology of Vijayanagara Institute of Medical Sciences, Bareilly, Utter Pradesh, India, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow, India and Baba Farid University of Health Sciences, Faridkot, Punjab, India. These isolates were collected from blood, urine, pus swab and sputum of patients with their informed consent. All specimens were inoculated onto mannitol salt agar and incubated at

37°C and observed for 3 days. *S. aureus* isolates were confirmed by Gram staining, catalase test, oxidase test, coagulase test and growth characteristics on mannitol-salt agar (Akbar *et al*., 2013).

DNA Isolation

DNA from all clinical isolates was isolated according to the methods described earlier (Chaudhary and Payasi, 2013).

Identification of MRSA

MRSA isolates were identified by the presence of *mecA* gene as reported earlier (Chaudhary and Payasi, 2013).

Identification of PVL in MRSA Strains

All the isolates confirmed to be MRSA were analyzed for the presence of PVL gene using the following primers: Luk-PV-F-5-ATCATTAGGTAAA-ATGTCTGGACATGATCCA-3 and Luk-PV-R-5 GCATCAASTGTATTGGATAGCAAAAGC-3. These primers were obtained from Sigma Aldrich Chemicals Private Limited, Bangalore, India. For PCR amplification, about 200 pg of DNA was added to 20 µL of reaction system containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Banglore Genei) in 1x PCR buffer. Amplification was performed in a Eppendorf thermocycler (Germany) with cycling parameters: Initial denauration at 94°C for 5 min followed by 30 cycles (30 sec of denaturation at 94°C, 30 s of annealing at 55°C, 1 min of extension at 72°C) and final extension at 72°C for 1 min. PCR products were analyzed by 1.0% agarose gel electrophoresis. The expected amplicon size was 433 base pairs.

Minimun Inhibitory Concentration (MIC) Testing

MICs of all drugs were determined by the agar dilution method following the Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines. MIC was defined as the lowest concentration of drugs that inhibit the visible growth of a microorganism when incubated at 37°C for 18 h. Two fold serial dilutions of drugs were used for the MIC study. Strains were classified as resistance or susceptible according to the criteria recommended by CLSI (2013).

Isolation of PVL Toxin from S. aureus

Ten selected PVL positive MRSA strains were used for the study. Twenty mililitre of each overnight grown MRSA strain was pelleted at 5000 rpm for 5 min, washed twice with phosphate buffer saline (PBS, 0.5 M, pH 7.4) and was suspended in 10 mL of the PBS. Following addition of 500 µL of SDS (10% w/v) into each tube, suspension was incubated for 3 h at 37°C, centrifuged at 12000 rpm for 15 min and resulting supernatant was used as a crude PVL for further study. *S. aureus* ATCC 49775 served as the reference strain for PVL production. Isolated PVL from clinical isolates was pooled and used for study.

PVL Quantification

PVL quantification was performed by an antibody-sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with primary monoclonal antibody (Mouse anti-Luks-PV mAB), secondary antibody (anti Horseradish Peroxidase (HRP) conjugate) and detection antibody (polyclonal anti PVL LukS) following the manufacturer instructions. Briefly, 50 µL of anti-LukS-PV monoclonal antibody (20 µg mL^{-1} in phosphate-buffered saline (PBS, pH 7.4, 0.05 M) was added into each well of microtiter plate (Greiner Bio-One, USA) and incubated overnight at 4°C for binding. After incubation, plate was washed twice with PBS to remove unbound monoclonal antibody and nonspecific binding sites were blocked with a PBS-BSA solution (1%) for 2 h at 37°C and again plate was washed twice with PBS and then 100 µL of standard recombinant LukS-PV dilutions (from 0.005 to 1.0 µg mL^{-1} in PBS-BSA (1%) or samples (diluted in PBS-BSA (1%) was added to duplicate wells and incubated for 2 h at 37°C. After incubation plate was washed twice with PBS and 100 µL of polyclonal anti PVL-LukS antibody (20 µg mL^{-1} in PBS) was added to each well, incubated for 2 h and washed twice with PBS. Subsequently, 100 µL of secondary antibody HRP conjugated rabbit polyclonal anti-LukS-PV, diluted in the ratio of 1:1000 in blocking buffer, was added into each well and incubated for 30 min at 37°C and washed the plates with PBS for three times. Finally, 100 µL of TMB solution was added into each well and incubated for another 20 min. After 20 min incubation, the reaction was stopped by adding stop solution (1 N HCl). The plate was read at 450 nm with ELISA reader (Bio-Rad). Values are shown as mean ± SD of three experiments.

Neutrophil Cell Isolation

Human neutrophils were isolated according to the method described elsewhere (Kobayashi *et al*., 2003).

Cell Culture

The neutrophils were cultured in RPMI 1640 culture medium (Hi-Media, Mumbai, India) supplemented with 10% FBS and 1% antibiotic/antimyotic solution at 37°C in an atmosphere of 5% CO_2 in a humidified incubator.

Effect of Different Concentrations of PVL on Neutrophils

To study the effect of various concentrations of PVL on neutrophils, 100 µL of neutrophils (1×10^7 cells mL^{-1}) were seeded into 96-well plate and incubated for 2 h at 37°C with 5% CO_2 in the absence and presence of different concentrations of PVL (0.025, 0.05, 0.1 and 0.15 µg mL^{-1}). After incubation, plate was centrifuged at 1000 rpm for 7 min at 4°C. Aliquots (100 µL) from each well were transferred to a another 96-well plate and percent LDH release was determined as described below.

Lactate Dehydrogenase Activity (LDH) Assay

Lysis of neutrophils by PVL was assessed by the release of LDH. LDH was assayed using a kit (Sigma Aldrich, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm. Values are shown as mean ± SD of three experiments. Percentage of LDH release was calculated using followings formula:

$$LDH\ release(\%)\frac{Control_{OD}-Test_{OD}}{Control_{OD}}\times 100$$

Effect of Adjuvants (L-lysine, L-arginine, EDTA) on PVL Induced Lysis of Neutrophils

To study the ability of amino acids, L-lysine, L-arginine and EDTA to inhibit PVL induced lysis of neutrophils, isolated neutrophils were seeded into 96 well plate and divided into 17 groups designated as group A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P and Q. Cells in group A were treated with PBS served as a negative control. Cells in group B to Q were stimulated with PVL (0.1 μg mL^{-1}) and cells in group B served as positive control. Cells in C to Q groups were simultaneously treated with 5, 10, 50, 100 and 150 mM concentrations of each of EDTA, L-lysine and L-arginine. Only cell culture media in another group R was served as a blank. The plate was then incubated for 2 h in CO_2 incubator (37°C, 5% CO_2). Following 2 h of incubation, plate was centrifuged at 1000 rpm for 7 min at 4°C. Aliquots (100 μL) from each well were transferred to another 96-well plate and LDH was measured and calculated as described above.

Further, different combinations of adjuvants, L-lysine + EDTA, L-Lysine + L-arginine and L-arginine + EDTA were made and evaluated on PVL induced neutrophils lysis. For this, neutrophils were divided into 5 groups named as group A, B, C, D and E. Cells in group a treated with PBS and served as negative control. Cells in group B to E were stimulated with PVL (0.1 μg mL^{-1}) and cells in froup B served as positive control. Cell in group C to E simultaneously treated with 10 mM L-lysine +10 mM EDTA, 10 mM L-Lysine +100 mM L-arginine and 100 mM L-arginine +10 M EDTA, incubated for 2 h. Only cell culture media in another group F served as blank. After incubation and centrifugation, 100 μL of supernatant was used to measure LDH as described above.

Effect of Drugs on PVL Induced Neutrophil Lysis

To evaluate the effect of drugs, Vancoplus, vancomycin, linezolid, daptomycin and teicoplanin, on PVL induced neutrophils lysis, neutrophils were seeded into 96 well plate and grouped as follows; group A, B, C, D, E, F and G. Cells in group A were treated with PBS which served as a negative control. Cells in group B to G were stimulated with PVL (0.1 μg mL^{-1}) and cells in group B served as positive control. Cells in C, D, E, F and G groups were simultaneously treated with half of MIC of each of drugs Vancoplus, vancomycin, linezolid, daptomycin and teicoplanin. Only cell culture media in another group H served as a blank. Here, Vancoplus was reconstituted with solvent (provided with pack by manufacturer as per manufacturer's instructions) whereas remaining drugs were reconstituted in water. The procedure for LDH measurement was the same as described above.

Statistical analysis

All data were represented the mean ± SD of three independent experiments. The data were further analyzed using Graph pad prism 5.01 in different groups compared with control by one way ANOVA and Tukey's test. Values of $p>0.05$ were considered not to be significant.

Results

Identification of clinical isolates

All the 67 isolates were confirmed to be *S. aureus* according to their biochemical test results. Out of 67 clinical isolates, 71.6% (48/67) isolates were found to be MRSA as confirmed by the presence of *mecA* gene through PCR amplification. Amplification of *mecA* in some isolates is shown in Fig. 1.

Prevalence of PVL in Clinical Specimens

All 48 MRSA were analysed for the presence of PVL gene. Of these, 75% (36/48) isolates were noted to be PVL positive as evident by PCR producting 433 base pair amplicon (Fig. 2). The proportions of the PVL in the strains varied depending upon the clinical specimens. The frequency of PVL in the different specimens is shown in Table 1. Pus swab (86.3%; 19/22) had more proportion of PVL than Urine (75%; 6/8); sputum (70%; 7/10) and blood (50%; 4/4).

MIC

MIC testing was carried out in only PVL positive MRSA strains and results are depicted in Table 2. Of the tested drugs, vancoplus was found to be the most efficacious drugs with MIC values 0.25 to 4 μg mL^{-1}.

Table 1. Frequency of PVL gene in MRSA isolates obtained from different specimens

	PVL gene frequency		
Specimens	Present	Absent	Total
Urine	6	2	8
Pus swab	19	3	22
Blood	4	4	8
Sputum	7	3	10
Total	36	12	48

Table 2. MIC of drugs against PVL positive isolates

Drugs	MIC (μg/mL)	½ MIC (μg/mL)
Vancoplus	0.25 to 4	0.125 to 2
Linezolid	1 to 32	0.5 to 16
Teicoplanin	2 to 32	1 to 16
Vancomycin	1 to 64	0.5 to 32
Daptomycin	0.5 to 32	0.25 to 32

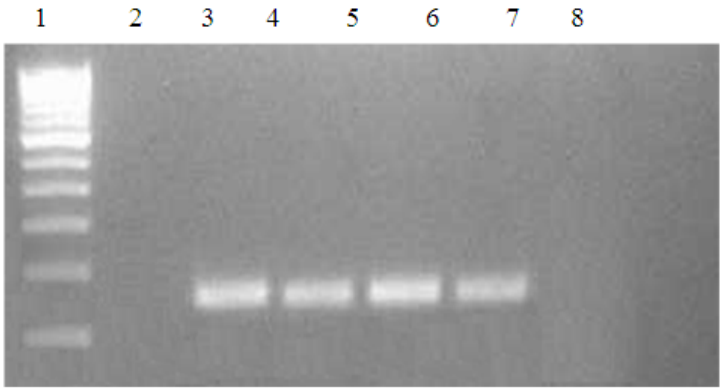

Fig. 1. Amplification of mec A gene in clinical isolates of *S. aureus* by polymerase chain reactionas

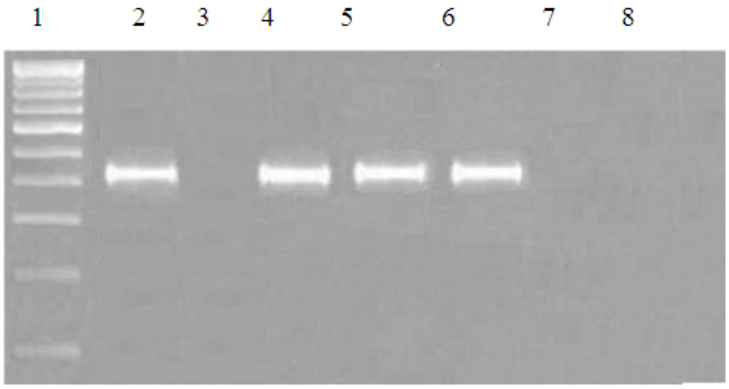

Fig. 2. Amplification of PVL gene in clinical islates of MRSA

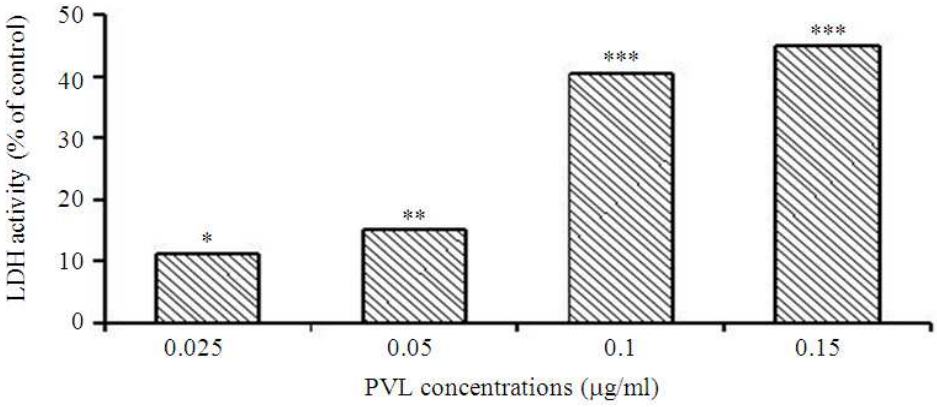

Fig. 3. Percentage increase in LDH activity

The MICs of linezolid and teicoplanin were ranged 1-32 and 2-32 µg mL^{-1}, respectively. The MICs of vancomycin and daptomycin varied 1-64 and 0.5 to 32 µg mL^{-1}. For further study, half of MIC of drugs were used. All the drugs displayed variability in MIC values indicating strains have different susceptibility behaviour towards drugs due to presence of various types of resistant determinants. The types of resitant determinants were not studied here as it was not part of this study.

Effect of Different Concentration of PVL on Neutrophils

To determine whether PVL induces neutrophil lysis, we measured LDH release into the media following 2 h PVL exposure. Treatment with different concentrations of PVL (0.025 to 0.15µg mL^{-1}) resulted in a concentration dependent increase in LDH activity which were statistically significant ($p<0.05$ to $p<0.001$) in

comparision to control (Table 3). Treatment of neutrophil cells with 0.025, 0.05, 0.1 and 0.15 μg mL^{-1} resulted in 11.1, 15.2, 40.6 and 45.1% increase in LDH activity, respectively (Fig. 3).

Effect of Adjuvants (L-lysine, L-arginine, EDTA) on PVL Induced Lysis of Neutrophils by LDH Assay

According to the results presented in Table 4, all the adjuvents (L-lysine, L-arginine, EDTA) inhibited the PVL induced neutrophil cell lysis in a concentration dependent manner. The inhibition of LDH increased with increasing the concentrations of these adjuvants. L-lysine caused 33.8±2.8 and 78.7±7.1% LDH inhibition at 5 and 10 mM in comparision to control, respectively and further increasing its concentrations had no significant increase in LDH inhibition. On the other hand, 18.8±2.3, 32.3±3.6, 67.5±7.1, 79.5±8.2 and 83.5±8.1% inhibition of LDH activities were observed with 5, 10, 50, 100 and 150 mM of L-arginine in comparison to control. These results suggest that the 10 mM of L-lysine produced significant (78.7±7.1%) LDH inhibition at 15 times lower concentration which is comparable to 100 mM of L-arginine (79.5±8.2% LDH inhibition).

EDTA was the least effective in the inhibition of PVL induced neutrophil cell lysis. It displayed only 12.5±1.7, 20.2±2.4, 24.2±2.6, 27.4±3.2 and 29.7±3.3% LDH inhibition at 5, 10, 50, 100 and 150 mM when compared with control. Further, our results showed that EDTA up to 10 mM produced sharp increase in LDH inhibition and thereafter remained constant. Overall, results suggest that 10 mM L-lysine and 150 mM L-arginine more efficiently inhibit the PVL induced neutrophil cell lysis.

When the best performing combinations of L-lysine + EDTA, L-Lysine + L-arginine and L-arginine + EDTA were tested on PVL induced neutrophil cell lysis, an additive effect was observed and 100 mM L-arginine +10 mM EDTA could inhibit up to 93.4±6.3% LDH activity (Table 5).

Effect of Drugs on PVL Induced Neutrophil Lysis

When various drugs were tested to evaluate their ability to inhibit PVL induced neutrophils lysis, we noted that Vancoplus efficiently inhibit PVL induced neutrophil lysis as it produced 93.1±6.4% inhibition of LDH activity. While vancomycin, linezolid, daptomycin and teicoplanin showed only 12.7±2.4, 26.7±3.4, 16.5±2.3 and 30.5±3.1% inhibition of LDH activity (Table 6).

Table 3. Effect of different concentrations of PVL on neutrophils

PVL concentration (μg/ml)	LDH activity (nmol/min/ml)	P-value
Control	0.3±0.03	
0.025	3.5±0.3	p<0.05 (*)
0.05	4.7±0.4	p<0.01(**)
0.1	12.6±1.4	p<0.001 (***)
0.15	14.0±1.4	p<0.001 (***)

Table 4. Effect of adjuvants on PVL induced lysis of neutrophils

Name of adjuvants	Concentration (mM)	LDH inhibition (%)
L-lysine	5	33.8±2.8
	10	78.7±7.1
	50	81.3±7.2
	100	83.1±8.4
	150	84.5±8.1
L-arginine	5	18.8±2.3
	10	32.3±3.6
	50	67.5±7.1
	100	79.5±8.2
	150	83.5±8.1
EDTA	5	12.5±1.7
	10	20.2±2.4
	50	24.2±2.6
	100	27.4±3.2
	150	29.7±3.3

Table 5. Effect of combinations of adjuvents on PVL induced lysis of neutrophils

Combinations of adjuvants	LDH inhibition (%)
10 mM L-lysine +10 mM EDTA	92.6±8.8
10 mM L-lysine +100 mM L-arginine	90.6±8.4
100 mM L-arginine +10 m M EDTA	93.4±6.3

Table 6. Effect of drugs on PVL induced lysis of neutrophils

Drugs	LDH inhibition (%)
Vancoplus	93.1±6.4
Vancomycin	12.7±2.4
Linezolid	26.7±3.4
Daptomycin	16.5±2.3
Teicoplanin	30.5±3.1

The gel lanes are: 1 = molecular weight ladder (100 bp), 2 = *S. aureus* ATCC 25923 (negative control), 3 = *S. aureus* ATCC 43300 (positive control), 4-6 = *S. aureus* (mecA positive isolates), 7-8 = *S. aureus* (mecA negative isolates).

The gel lanes are: 1 = molecular weight ladder (100 bp), 2 = *S. aureus* ATCC 49775 (positive control), 3 *S. aureus* MTCC 737 (negative control) = 4-6 = *S. aureus* (PVL positive isolates), 7-8 = *S. aureus* (PVL negative isolates).

Discussion

Over the past several decades, the incidence of resistant gram-positive organisms has risen throughout the world. Among these, MRSA are predominant pathogens associated with serious infections. Both human and rabbit neutrophils are highly sensitive to the pore-forming properties of PVL and rapidly undergo cell death (Ma *et al*., 2012). Plenty of studies have reported that PVL is one of the major virulence factors contributing to the morbidity and mortality attributed to *S. aureus* (Omuse *et al*., 2013; Gillet *et al*., 2008; Gillet *et al*., 2002). Current

investigation demonstrated the prevalence of MRSA to be 71.6%. Previous studies demonstrated the incidence of MRSA in India to be 30-70% (Singh *et al*., 2012; Muralidharan, 2009; Chaudhary and Payasi, 2013). Further analysis of these MRSA for prevalence of PVL, overall prevalence of the PVL gene in MRSA isolates was around 75%. The prevalence of PVL in MRSA obtained in our study is little higher than that of previous studies performed in Africa and Kenya where PVL prevalence was reported to be 57 and 58%, respectively (Breurec *et al*., 2010; Omuse *et al*., 2013). However, our result is close to the 70 and 72% obtained in Benin and Algeria, respectively (Sina *et al*., 2013; Campbell *et al*., 2008). In the current study PVL was the most prevalent in pus swab followed by urine, sputum and blood. Our observations clearly indicates that prevalence of PVL varies with geographical locations and clinical specimens. Previous study also noted that PVL prevalence vary with clinical specimens (Goering *et al*., 2008). It has been reported that compared with PVL-negative *S. aureus* isolates, PVL-positive isolates are more pathogenic because the lytic activity of PVL directly affects monocytes, macrophages, polynuclear neutrophils and metamyelocytes, although erythrocytes are not lysed by PVL (Prévost *et al*., 2001; Barr *et al*., 2006; Kazakova *et al*., 2005). Moreover, PVL toxin is reported to have a cytolytic effect and as such polynuclear neutrophils were identified as important indicators of staphylococcal virulence (Cribier *et al*., 1992).

In the last decade dramatic changes have occurred in the epidemiology of MRSA infections. The antibiotic sensitivity pattern of MRSA isolated from clinical specimens were found to be highly variable. Our susceptibility data demonstrated that vancomycin appeared to be resistant to 77.7% (28/36) strains of PVL positive followed by daptomycin 55.5% (20/36), teicoplanin 33.3% (12/36), linezolid 22.2% (8/36) and Vancoplus 5.5% (2/36). No previous study of this nature has been done.

In our study, 22.2% isolates were resistant to linezolid which is very close to the study performed by Thool *et al*. (2012) in which 23.5% resistant to linezolid was observed. Resistance to linezolid may be explained by the fact that mutations in the 23 s rRNA of the 50 s ribosomal subunit resulting in alteration of linezolid-binding sites of the bacteria (Long *et al*., 2010; Nannini *et al*., 2010). Our data showed that there is a drastic rise of resistance of glycopeptide (vancomycin, teicoplanin) and lipopeptide (daptomycin). The emergence of the glycopeptide resistance is of serious concern for the treatment of infections caused by Gram positive organisms particluarly MRSA. A number of other studies have also pointed out the reduced susceptibility of glycopeptide and lipopeptide to MRSA (Estes and Derendorf, 2010; Judge *et al*., 2012). The reduced susceptibility of glycopeptide is explained by the fact that trapping of glycopeptide in the peptidoglycan of the bacterial cell causing decreased reaching of glycopeptide to the cytoplasmic membrane where the targets of glycopeptides are located (Westerlind, 2012). Resistance of daptomycin has also been associated with alteration in the structure and function of the cell envelope and surface charge.

Our results showed that 94.5% of PVL positive MRSA were sensitive to Vancoplus, the novel antibiotic adjuvant entity. The enhanced susceptibility of Vancoplus is because of synergistic activity. When ceftriaxone and vancomycin are used in combination there are less chances of trapping of these molecules in the peptidoglycan as a result enhanced susceptibility. Additionally, Vancoplus contains animo acids (VRP1020) for chemical compatibility and is reconstituted with solvent, it neutralizes more than 95% of PVL toxins. By neutralizing PVL toxins it can avoid the side effects caused by PVL when treated during MRSA infections, especially in immuno-compromised patients.

Conclusion

This study provides new insight into the prevalence of PVL toxins among MRSA. Results of this study showed that of the tested drugs, Vancoplus is the most active against MRSA positive with PVL toxins and is highly effective in PVL neutralization thereby causing lesser neutrophil lysis in comparison to comparator drugs and can be a safer and more effective therapy for MRSA management.

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Author's Contributions

MC and AP designed the experimental work and analyzed the results. SKP carried out all experiments.

Ethics

We declare that we have no ethical issue.

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