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RESEARCH ARTICLE

Detection and Purification of Panton – Valentine Leukocidin toxin Produced by Methicillin resistant *Staphylococcus aureus*

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Abstract

Panton - Valentine leukocidin (PVL) produced by community acquired methicillin *Staphyloccous aureus* involved in skin and soft tissue infection comprised of two fraction namely PVLS and PVLF . In the present study , one of aims was to identify resistance profile for MRSA, and other aims are detection and purification of PVL toxin. A total of (100) MRSA isolates were recovered from hospitalized patients in Baghdad in 2013 . The percentage of PVL – positive was represented by 27% of isolates and 55.6% of them isolated from wound and 40.7% of them from abscess . All isolates were resistant to cloxacillin , followed by cefoxitin and cephalexin and lincomycin (86, 51 and 23)% respectively. Result of PVL toxin purified by sequatinal ammonium sulphate precipitation ion exchange and gel filtration with sepharos 6B then hydroxyl aptite chromatography revealed the protein concentration was 22.4 µg/ml with molecular weight 35.4kDa.

Introduction

Staphylococcus aureus is an opportunistic pathogen often carried on the human body. Methicillin-resistant *S. aureus* (MRSA) includes those isolates that have a gene giving them resistance to methicillin and other beta-lactam antibiotics **(Dufour et al.,2002).** This bacteria has since emerged as a problem in human medicine. MRSA was first recorded as a nosocomial pathogen in human hospitals ,and as other *S. aureus* hospital associated isolates have become resistant to most common antimicrobials, and treatment can be difficult **(Boyle-Vavra and Daum,2007**). Among important virulence factors *S.aureus* expresses avariety of toxins including panton-valentine leukocidine (PVL) . PVL was first described by panton and valentine in 1932 (**Kaneko and Kamio, 2004**). The toxin comprised of Two subunits namely luk F – PV and luk S –PV that assemble in to an octameric pore on the myeloid cell surface, including polymorphonuclear neutrophils (PMN) **(Tacconelli et al.,2008**). The diagnosis of PVL producing strain is mainly based on polymerase chain reaction (PCR) , nucleic acid hybridization kits for the detection of luk S – PV and luk F – PV genes **(Tang et al.,2007**). Sequence of peptide data indicate that the lukFis a 36.8KDa and luk S is a 35.7 KDa protein **(Yamamoto et al.,2010**). this work was carried out to determine the antibiotic resistance of the MRSA isolates and purification of Panton – Valentine Leukocidin toxin .

Materials and Methods

Bacterial isolates

A total of one hundred *S aureus* isolates were obtained from clinical specimens (abscess and wound) from patients who were admitted to Baghdad hospital in 2013 . the isolates were identified by convential biochemical reaction according to the criteria established by **Forbes et al.(2007)** .The isolates were inoculated on a CHROM agar MRSA plate ,the result were read after 24-48 hrs of incubation at 35c the growth of colonies showing mauve coloration was considered to be positive **(Diederen et al.,2005)**

Antimicrobial susceptibility test :

Antimicrobial susceptibility of the isolates were tested by using Kirby – Bauer disk diffusion method following CLSI guideline (CLSI,2009) using commercially available 6 mm discs (Bio analyse / Turkey). The susceptibility of the isolates was determined against 12 antibacterial agents on Muller – Hinton plate (Lab M limited Topley house ,UK), using over night culture at a 0.5 Mcfarland .

PCR assay

All isolates were tested for the presence of *pvl* genes using a PCR assay with Specific primers were performed according to **Jarraud et al.(2002)** to identify these genes for each MRSA isolates (*pvl*-Forward 5-ATCATTAGGTAAAATGTCTGCACATGATCCA -3 and *pvl*-Reverse 5-GCATCAASTGTATTGGATAGCCAAAAGC -3). DNA template was prepared as described by **Olsvik et al.(2008)** using boiling method. DNA template (25ml) of PCR amplification mixture contained deionized sterile water (12.5ml) green go taq master mix pH (8)(promega,USA).The thermocyling were as follows: lnitial denaturation at 94c for 10 min and 35 cycles of denaturation at 94 c for 1min, annealing at 51c for 1min and extension at 72 c for 1 min and afinal extension was performed at 72c for10 min . All PCR products were analyzed by electrophoresis through 1% agarose gels.

Purification of PVL toxin .

Preparation of crude toxin:

It was prepared according to **(Diep et al.,2008)** with some modification as follows:

The isolates of *S.aureus* were incubated in trypticase soy broth for 24 hr at 37c with shaking at 225 rpm for 24 hr .cells were separated by centrifugation at 6000 rpm for 10 min at 4c . the resulting supernatants were filtered through 0.22 µm membrane filter .

Purification of toxin:

The toxin present in the supernatant fraction was concentrated by ammonium sulfate precipitation (80% saturation) at 4c for 16 hr .and precipitate peleted by centrifugation (15000xg for 20 min at 4c) was dissolved in phosophate buffer(30mm sodium phosphate buffer,pH 6.5). Proteins were dialyzed against the same butter for 5hr.the dialysate was used for purification by cation exchange column (DEAE cellulose column) and elution was performed by using a linear gradient from zero to 0.5 M NaCl in phosphate buffer . fraction containing LUK S –PV was purified by a sepharose 6B column .LUK S-PV was eluted with a linear gradient of zero to 0.25M NaCl in phosphate buffer .And ammonium sulfate was added to LUK F-PV and LUK S- PV fraction to 1.5 ml . pH =6.8 for 40 min . and proteins were eluted with the same buffer at flow rate of 2ml /min. protein concentration in each eluted fraction was determined at 280 nm .concentrated protein at each step was determined by Bradford method **(Bradford ,1976**).

Results and Discussion

All (100) clinical bacterial isolates were characterized according to Bergeys Manual of systematic bacteriology **(William et al.,2009)** as well as , other characters reported by **(Brooks et al.,2007)** . Cultural ,Morphological , Biochemical characteristics and using CHROMO agar MRSA, revealed that these isolates being Methicillin resistant *S.aureus* (MRSA).

One of aims of this study was to identify resistance profile for MRSA isolates. The antimicobial susceptibility of these isolates showed that all isolates were resistant to cloxacillin (100)% , followed by cefoxitin and cephalexin and lincomycin in (86 , 51 , 26) % respectively (table – 1), result mentioned that MRSA is the most important common nosocomial bacterial pathogen isolates in different part of the world , this agreement with this finding **Shittu and Lin(2006) and Grundmann et al.(2006)** . The result revealed that 18% of these isolates were resistant to gentamicin and 17% were resistant to vancomycin (table 1) , where **(Suod ,2005)**mentioned that his isolates were sensitive to vancomycin 100% . the first clinical isolates of vancomycin resistant *S.aureus* was reported by researcher from Brazil and Jordan in 2002 **(Ng et al.,2011) .**

Table : 1 . Susceptibity of 100 isolates of Methicillin resistant *S.aureus* to the Antimicrobials.

NO	Antimicrobials		Rate of resistant
1-	rifampicin	**RA**	**22%**
2-	Clindamycin	**DA**	**13%**
3-	Lincomycin	**L**	**26%**

4-	Levofloxacine	**LEV**	**13%**
5-	Trimethoprime	**TEM**	**23%**
6-	Cloxacillin	**CX1**	**100%**
7-	Azethromycine	**AZM**	**16%**
8-	Cefoxitine	**CX30**	**86%**
9-	Gentamicin	**CN**	**18%**
10-	Cephalexine	**CL**	**51%**
11-	Tecoplanine	**TEC**	**4%**
12-	Vancomycin	**VA**	**17%**

All of isolates of MRSA(100 isolates) were tested for the presence of *pvl* genes using Polymerase Chain Reaction assay . The results showed that (27) of isolates were positive,40.7% from abscess and (55.6% and 3.7%) from wound and Burnes respectively (table-2) (figure 1).

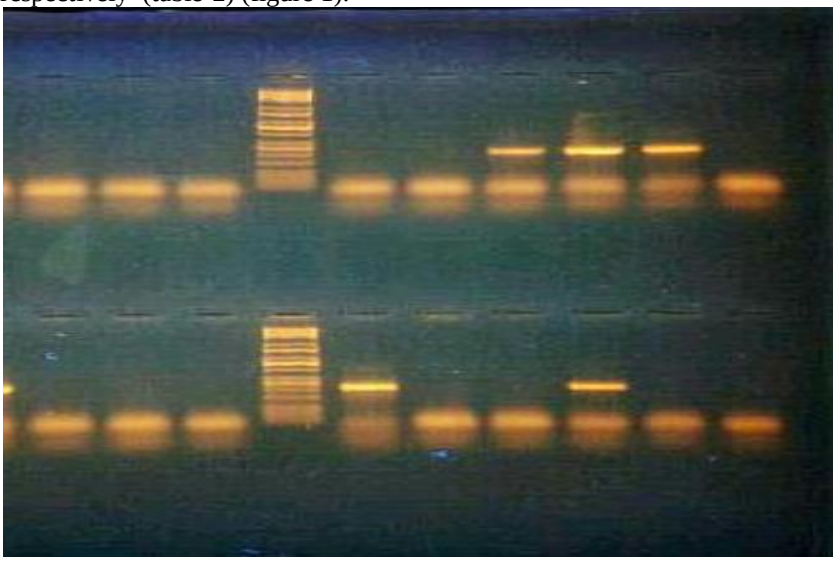


Figure (1) :Gel electrophoresis (1.2% agarose, 50V, for 1.30 hour) of *pvl* gene (433bp), using 1000bp DNA ladder.

Table 2 :Percentage of *pvl* genes in MRSA isolates .

Infection	**Pvl(27%)**	
	No. of isolates from source	**%**
Wound	**15**	**55.6**
Abscess	**11**	**40.7**
Burnes	**1**	**3.7**
Total	**27**	**100**

Narita et al.(2001) showed that the temperate phage θSLT infected 3% of clinical *pvl* negative *S.aureus* strains leading to PVL production . **Cabrera et al .(2010)** mentioned that 83% of *S. aureus* isolates carrying pvl genes when isolates were categorized according to type of staphylococcal infection. The *pvl* genes were common associated with abscesses .the results are in agreement with the results abtained by other study , they reported that 70% of *S.aureus* isolates from abscess a carrying pvl gene.

The partial purified PVL toxin was applied on DEAE cellulose column , this column had been equilibrated with sodium phosphate buffer (pH:6.5) and which later served as the elute , each eluted (2ml) fraction read at 280 nm . And the curved was plotted between the absorbance and fraction number (figure-2). Sepharose 6Bis stable at pH values(4-9)and it is suitable to separate proteins with range of molecular weight between 10^4 to 10^6 Dalton **(Janson et al .,1998)** , and the gel filtration is performed using porous beads as the chromatographic support and it is unique in that fractionation is beaded on the relative size of proteins molecules a good resolution of different size of proteins could be obtained by using this technique ,if some criteria follow such as volume of matrix to volume of samples ,low flow rate ,appropriat diameter of column with high length , sample quality application and a absence of any denaturizing a gen in elution buffer **(Stellwagen ,1990)**.The elution profile in sepharose 6B was carried out at aflow rate of 2ml/min .fraction 13-25 through sepharose 6B were selected sapartially purified , the eluted fraction of this step apptied on the adsorption chromatography using hydroxyl apatite . The protein concentration at each purification steps were revealed in (Table 3).

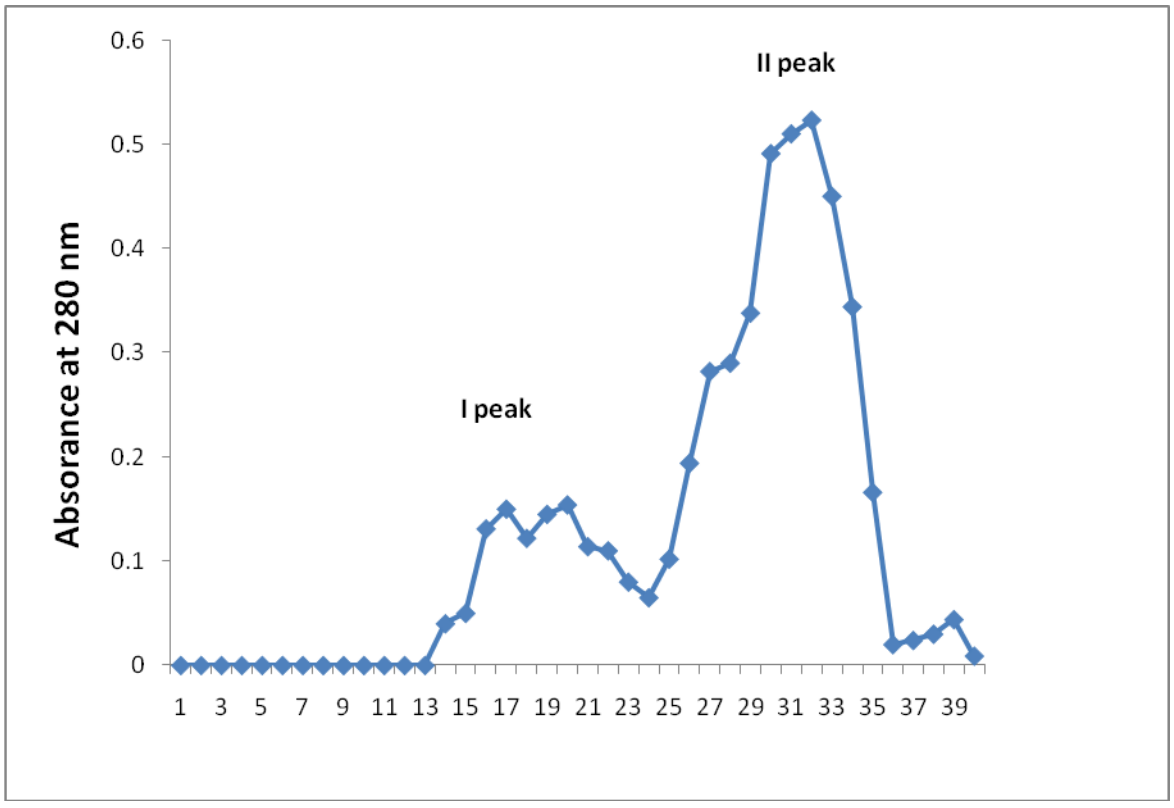


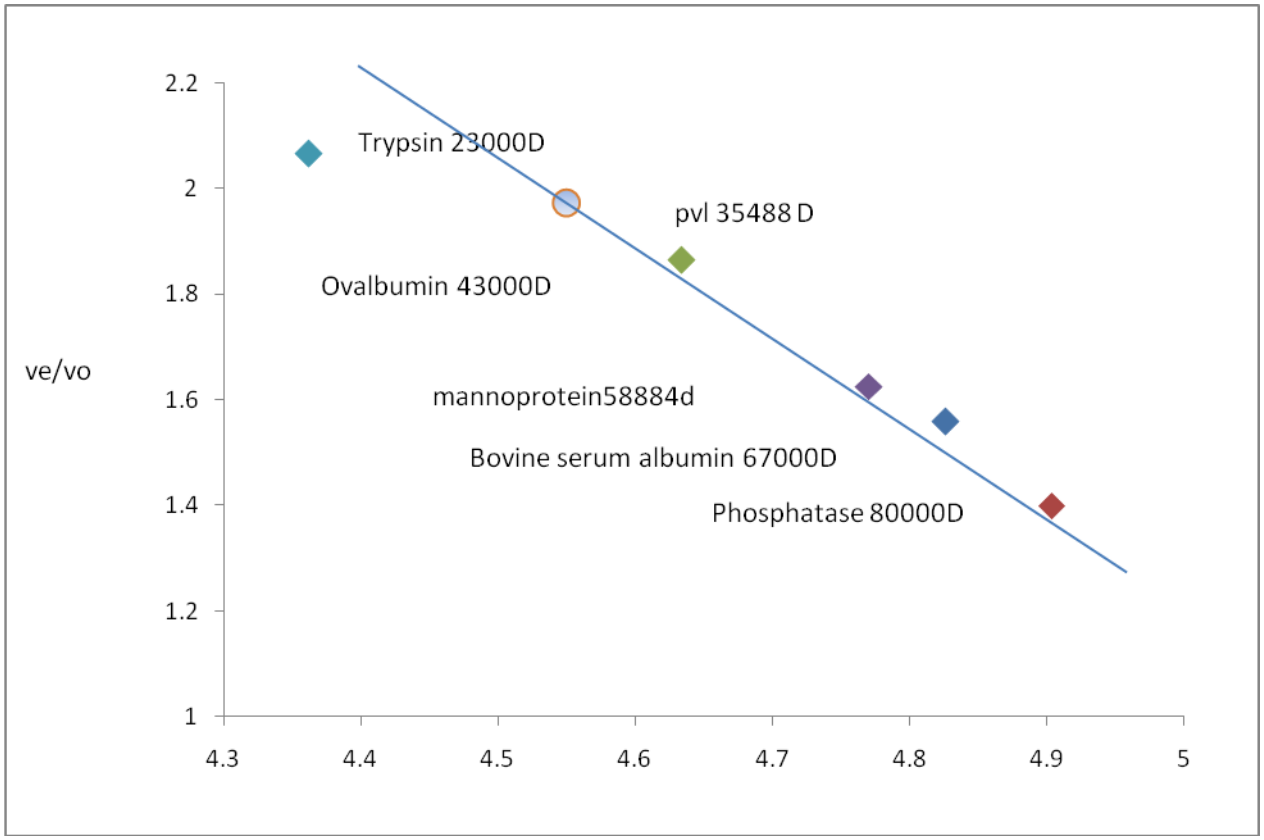
Figure 2: The peak II for PVL activity by Sepharose 6B

Table (3) :Estimation of protein concentration (Toxin) at each purification step.

Steps	Protein concentration(µg/ml)
Ion exchange chromatographyUsing DEAE-Cellulose	21.92
Gel-filtration using sepharose 6B	17.005
Adsorption chromatographyUsingHydroxy apatite	22.435

The molecular weight of PVL toxin was determine. The standard curve that represent the relationship between log of molecular weight and relative mobility (Rm) for standard proteins was use to estimate the molecular weight of PVL by SDS – PAGE . As shown in figure 3 . The molecular weight of PVL toxin was determined as 35.44KDa , which is in agreement with **Yamamoto et al.(2010**) who mentioned that lukS was a 35.7 KDa and LUK F was a 36.8 KDa protein.

Figure 3 : PVL toxin molecular weight



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