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Original Article

Molecular analysis of Panton-Valentine Leucocidin (*pvl*) gene among MRSA and MSSA isolates

Análise molecular do gene Panton-Valentine Leucocidin (pvl) entre isolados de MRSA e MSSA

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Abstract

The present study was conducted in order to determine the frequency of *pvl* gene among the pathogenic and healthy population isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA). For this purpose, nasal swab samples were collected from the healthy individuals (to be used as controls, all the samples were collected irrespective of the sex and age factors), the pathogenic samples were collected from different patients suffering from skin &soft tissue infections caused by *S. aureus* (to be used as test samples).Both of these population samples were analyzed for the presence of *pvl* gene. *S.aureus* were identified through conventional microbiological identification procedures. In the case of normal samples, 70 nasal swabs were collected and only 33 (47%) proved to be *S. aureus* while 20 pathogenic samples were collected and all (100%) were cleared as *S. aureus*. For further distribution of samples into MRSA and MSSA, antibiotic susceptibility pattern was checked by using the standard protocols of Kirby-Bauer disc diffusion method. Two antibiotic discs Oxacillin (OX: 1ug) and cefoxitin (FOX: 30ug) were used. Among healthy population, MRSA was found to be 79% (n=26) and MSSA were present as 21% (n= 7). Among pathogenic strains 100% MRSA was detected where n= 20. Detection of *pvl* gene among the MRSA and MSSA isolates was done by using the uniplex PCR followed by gel electrophoresis. MRSA and MSSA of normal healthy population carried 49% and 7% *pvl* gene respectively. While, pathogenic MRSA samples carried 46% *pvl* gene among them. Potentially alarming percentage of *pvl* gene is present among the normal healthy individuals which indicates a future threat and a major health concern.

Keywords: *pvl* gene, Methicillin Resistant *Staphylococcus aureus* (MRSA), MSSA, pathogenic strain, antibiotic susceptibility pattern.

Resumo

O presente estudo almejou determinar a frequência do gene PVL entre os isolados patogênicos e saudáveis da população de *Staphylococcus aureus* resistente à meticilina (MRSA) e Staphylococcus aureus sensível à meticilina (MSSA). Para este propósito, amostras de swab nasal foram coletadas de indivíduos saudáveis (utilizadas como controle, todas as amostras foram coletadas independentemente do sexo e idade), e de diferentes pacientes com infecções de pele e tecidos moles causadas por *S. aureus* (utilizadas como amostras de teste). Ambas as amostras populacionais foram analisadas quanto à presença do gene PVL. *S.aureus* foram identificados através de procedimentos convencionais de identificação microbiológica. No caso de amostras normais, 70 swabs nasais foram coletados e apenas 33 (47%) provaram ser *S. aureus*, enquanto 20 amostras patogênicas foram coletadas e todas (100%) foram eliminadas como *S. aureus*. Para distribuição posterior de amostras em MRSA e MSSA, o padrão de suscetibilidade a antibióticos foi verificado a partir dos protocolos padrão do método de difusão de disco de Kirby-Bauer. Foram utilizados dois discos de antibióticos Oxacilina (OX: 1ug) e cefoxitina (FOX: 30ug). Entre a população saudável, MRSA foi encontrado em 79% (n = 26) e MSSA estava presente em 21% (n = 7). Entre as cepas patogênicas, 100% de MRSA foi detectado onde n = 20. A detecção do gene PVL entre os isolados de MRSA e MSSA foi feita usando a PCR uniplex seguida de eletroforese em gel. MRSA e MSSA da população saudável normal carregavam 49% e 7% do gene PVL, respectivamente. Enquanto as amostras patogênicas de MRSA carregavam 46% do gene PVL entre elas. Uma porcentagem potencialmente alarmante do gene PVL foi detectada entre os indivíduos saudáveis normais, o que indica uma ameaça futura e um grande problema de saúde.

Palavras-chave: gene PVL, *Staphylococcus aureus* resistente à meticilina (MRSA), MSSA, cepa patogênica, padrão de suscetibilidade a antibióticos.

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1. Introduction

Humans have *S. aureus* as their normal flora upon their epithelial surfaces. Colonization of the nasopharynx is also commonly seen. Approximately 30% to 50% of the individuals carry *S. aureus* in their nasal cavity (Yamamoto et al., 2010). It only causes damage to the internal tissues if the cutaneous barrier gets damaged (Tong et al., 2015). *S. aureus* possesses a wide range of the virulence factors that helps it to breach immune system. These factors include various surface proteins as the protein A and different secretory proteins as Panton Valentine Leukotoxin (PVL) (Gordon and Lowy, 2008). *S. aureus* notoriously plays with the human immune system, more precisely the innate immunity, as it possesses the immune evasion molecules that helps it to trespass the defensive ability of the body and cause damage to the immune cells. for example, it can halt all the pathways of complement system by neutralizing the complement molecules using its own staphylococcal complement inhibitors (SCIN) and chemotaxis inhibitory proteins (CHIPS) evasion molecules (Rooijakkers et al., 2005; de Haas et al., 2004).

Methicillin resistant *S. aureus* (MRSA) has become a major challenge of this era in both the hospitals and the community settings. MRSA is further sub categorized in two divisions as hospital acquired (HA) MRSA and community acquired (CA) MRSA. Both of these are developed by the successful insertions of genomic island *staphylocccal cassette chromosome mec (SCCmec)* into the MSSA (Asghar, 2014). Methicillin resistance is encoded by *mecA* gene which translates for low-affinity penicillin-binding protein (PBP2A) (Javed et al., 2009). This gene is located in the *SCCmec* which has the ability to be inserted anywhere in the staphylococcal chromosome (Ahmad et al., 2009). Being a mobile genetic element and due to its ability of variable insertion in the staphylococcal genome, it can be exchanged in different species of staphylococci. This phenomena gave rise to seven distinct types of (Deurenberg and Stobberingh, 2008). CA MRSA and the HA MRSA varies in many aspects; CA-MRSA carries smaller *SCCmec* type iv or type v, a bacteriophage integrated gene (*phiSLT)* called *pvl* (Vandenesch et al., 2003),While HA-MRSA carries large *SCCmec* types i, ii or iii Its greater size indicates that they carry non b-lactam antibiotic resistance genes (Diep et al., 2006; Ahmad et al., 2009)

Panton valentine leukocidin (PVL) is a powerful yet lethal pore forming cytotoxin that is present in the MRSA and some of the MSSA. It is usually associated with the CA MRSA (Bukharie, 2010; Yoong and Torres, 2013). PVL is gamma hemolysin toxin and targets the cellular membranes of the polymorphonuclear leukocytes (PMN) of the humans and rabbit species. Its cytolytic effect results in the destruction of neutrophils preventing the phagocytosis (Arslan et al., 2016). PVL is comprised of two synergistically assembled protein possessing components; LukS-PV and LukF-PV. Both these components are co-transcribed and crucial for one another to function properly (Hu et al., 2015; Tong et al., 2015). Studies had reported that PVL is associated with various lethal diseases including primary skin and soft tissue infections, furuncles, cutaneous abscess, necrotizing pneumonia and osteomyelitis (Gillet et al., 2002; Francis et al., 2005; Haque et al., 2017).

As the PVL has become a threat to the health globally and the prevalence of PVL positive MRSA has been increased to a greater altitude in Pakistan. So, in these regards it is crucial to study the frequency of *pvl* gene amongst the pathogenic and normal population isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA).

2. Materials and methods

2.1. Sampling

The study was conducted over a period of 9 months (August 2018 April 2019) at the Department of Microbiology and Molecular Genetics (MMG), University of the Punjab, Lahore, Pakistan. A total of 70 nasal swab samples were collected from the healthy population of Lahore and the 20 nasal swab samples were collected from the General Hospital Multan, Pakistan to be considered as pathogenic samples from the diseased population. (Data of diseases not mentioned). The sample collection was irrespective of the age and gender of the donor. These samples were then tested for the screening of *Staphylococcus aureus* in the sterile conditions at the laboratory of department. Of 70 healthy population nasal swab samples only 33(47%) and of pathogenic samples all 20(100%) appeared to be *S. aureus*.

2.2. Characterization of bacterial strains

All the bacterial strains were tested for *S. aureus*, cultured on the blood agar for 24- 48 hours aerobically to check for the beta hemolysis. Identification was done by observing the colony morphology, Gram's staining. Further biochemical tests were done to confirm *S. aureus* by growing them on the mannitol salt agar medium (MSA OXOID, UK). Then catalase, DNase and Coagulase test were performed as described by (Perveen et al., 2013) Further screening for the MRSA and MSSA was done by incubating the strains for 24 hour at 37 °C on the Muller Hinton agar using Oxacillin (OX= 1ug) and Cefoxitin (FOX= 30ug) as per guidelines of the clinical laboratory standard institute CLSI (2020) using the Kirby-Boyer disc diffusion method according to the CLSI guidelines Broekema et al. (2009). MRSA screening was done. All the results were interpreted by measuring the zone of inhibition around the discs.

2.3. DNA extraction and detection of PVL gene by PCR

DNA of all the samples were extracted by sung the CTAB NaCl method as described by (Ahmed et al., 2014). For molecular analysis, amplification of *pvl* gene was conducted in the thermal cycler. For this purpose, specific forward and reverse primers were designed to attain a 433bp product in the form of *pvl*. Primers were synthesized by OLIGO-USA and were reconstituted as per advised by manufactures (Moussa and Shibl, 2009). Each 20ul PCR mixture was comprised of 4ul template DNA, 10ul PCR mix (Thermo Fisher Scientific), 0.5ul F and R primers and

Table 1. PCR primers and cycling parameters for gene presented in this study.

Target gene	Sequence of Primer (5'-3')	PCR conditions	PCR product size (bp)	Reference
Pvl	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R-GCATCAAGTGTATTGGATAGCAAAAGC	1 min 94 °C 1 min 50 °C 2 min 72 °C	433	(Moussa and Shibl, 2009)

5ul DNase free ampule water. PCR details are given in the Table 1. After the PCR amplification of gene, the products were observed upon gel electrophoresis considering 1% agarose gel in 0.5x TBE buffer. For comparison, ready to use, Fermentas Gene Ruler 1kb DNA ladder, was used.

3. Results

3.1. Division of strains

A total of 90 nasal swab samples were collected from healthy population and diseased population. The isolates corresponded to be 70 from healthy population (to run as control samples) and 20 from diseased population (to run as test samples). Of the 70 isolates obtained from the healthy individuals only 33 (47%) were classified as *S. aureus* while on the other hand all the samples collected from diseased population were categorized as *S. aureus* (100%).

3.2. Detection of MRSA and MSSA by Kirby Bauer disc diffusion method

A total of 33 sample isolates from normal healthy population were subjected to the antimicrobial susceptibility testing. Among these 33 isolates, 79% were found to be MRSA where n=26 and 21% were MSSA where was n=7. The antibiogram assay results are shown in the Figure 1.while, the pathogenic samples collected from hospital were all (100%) found to be MRSA where n=20. No MSSA was found in the pathogenic isolates. All the sample isolates in this study were considered as CA MRSA.

3.3. Evaluation of frequency of pvl gene among the MRSA and MSSA isolates by PCR

PCR was carried out with the DNA extracted from MRSA and MSSA isolates of normal samples as well as diseased samples. The expected band of *pvl* gene of 433 bp was amplified in all phenotypically confirmed MRSA and MSSA strains using PCR machine thermal cycler as shown in the Figure 2, Figure 3 and Figure 4. Out of 26 MRSA of normal healthy population 20 (49%) carried the *pvl* gene while out of 7 strains of MSSA only 2 (7%) were positive for *pvl* gene. While the (48%) pathogenic samples were positive for desired gene where n=20. As shown in Figure 5.

4. Discussion

Emergence of antibiotic resistance among bacterial strains is one the biggest challenge in developing countries like Pakistan. Moreover, various arsenals of virulent factors of those bacterial strains also augment their pathogenicity.

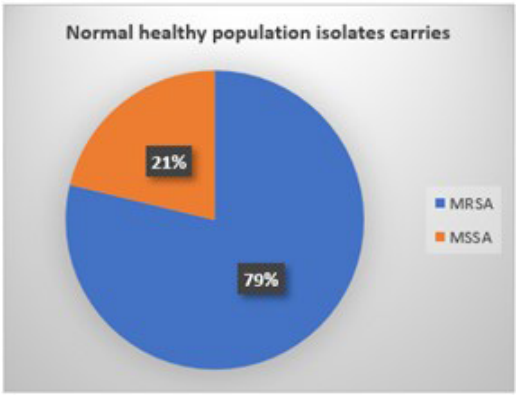

Figure 1. Distribution of MRSA and MSSA among the healthy individual's isolates

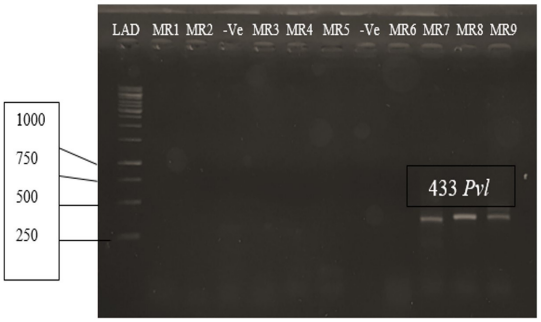

Figure 2. Normal healthy individual's MRSA carrying *pvl* gene. Agarose gel electrophoresis demonstrating the PCR amplification of *pvl* gene among the healthy individual's MRSA. From left to right lane1 well 1= fermentas gene ruler 1 kb ladder, well 2= sample MR1, well3= sample MR2, well 4= -Ve control, well 5= sample MR3, Well6= sample MR4, well 7= sample MR5, Well 8= -Ve control, well 9= sample MR6, Well 10= sample MR7 positive for gene *pvl*, Well 11= Sample MR8 positive for gene *pvl*, well12= sample MR9 positive for gene *pvl*

Resultantly, a number of resistant pathogen has emerged which includes CA MRSA and HA MRSA (Hannan et al., 2015). Amongst the many features enlisted for virulence of CA MRSA, the outstanding one is PVL. A cytotoxin that targets the leukocytes and responsible for tissue necrosis, skin and soft tissue infections and necrotizing pneumonia. Moreover, *pvl* gene is a very important marker for the identification of CA MRSA (Asghar, 2014). Not only

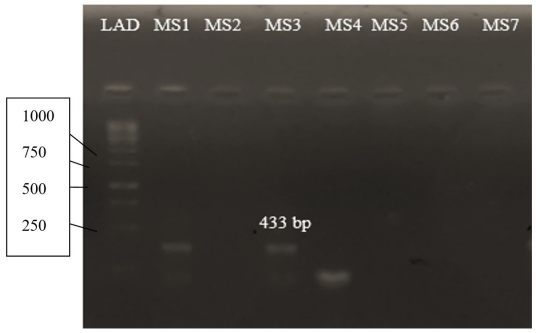

Figure 3. Normal healthy individual's MSSA carrying *pvl* gene. Agarose gel electrophoresis demonstrating the PCR amplification of pvl gene among the normal healthy individual's MSSA. From left to right, lane1 well 1= fermentas gene ruler 1 kb ladder, well2= sample MS1, well3= sample MS2, well 4= sample MS3, well 5= sample MS4, Well6= sample MS5, well 7=sample MS6, Well 8= sample MS7

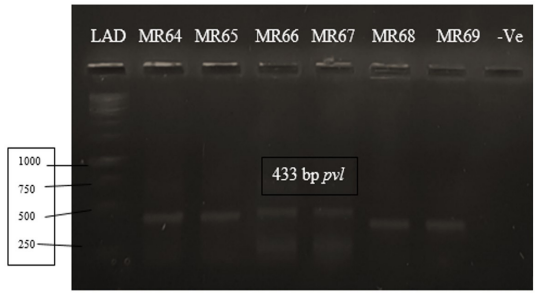

Figure 4. Diseased individual's MRSA carrying *pvl* gene. Agarose gel electrophoresis demonstrating the PCR amplification of *pvl* gene among the diseased individual's MRSA. From left to right lane1 well 1= fermentas gene ruler 1 kb ladder, well2= sample MR64, well3= sample MR65, well 4= sample MR66, well 5= sample MR67, Well6= sample MR68, well 7=sample MR69, Well 8= negative control.

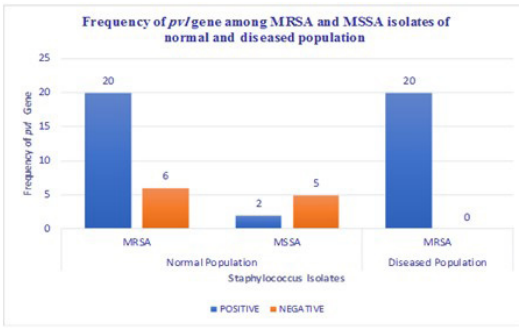

Figure 5. Frequency of *pvl* gene in *Staphylococcus aureus* isolates (MRSA vs MSSA) of healthy and diseased population samples.

MRSA but the MSSA is also responsible for HA MSSA and CA MSSA infections in China. And *pvl* gene also present among various sub classes of these china borne MSSA (Yan et al., 2016). There is no doubt that the CA MRSA is the leading cause of major health concerns but MSSA in hospitals has associated itself more commonly to the endocarditis, sepsis and bacteremia than did the MRSA (Piper Jenks et al., 2016)

In this study the frequency of *pvl* gene among the MRSA and MSSA isolates of healthy and unhealthy individuals from Lahore and Multan, Pakistan has been documented. The emergence of *pvl* positive CA MRSA is diversified from continent to continent which is >90% in the USA, lesser in European countries 1-3% and moderate valued in India as >65% (Sunagar et al., 2016). Western Africa reported a higher prevalence of 57% indicating that the third world and developing countries posse. Other countries around the globe are also threatened by the increasing prevalence rates of *pvl* positive MRSA as, Korea 0.9%, Germany 30%, Singapore 11.6%, turkey 4% and china 12.8% (Shrestha et al., 2014)

A china-based study is contradictory to this study which showed the 59% *pvl* negative CA MRSA from the normal population as our results concluded 47% *pvl* positive MRSA strains (Chen et al., 2017). This notion tells that the Chinese MRSA of general population infrequently carry the *pvl* gene. In their study and the study conducted in Italy by (Sanchini et al., 2011) *pvl* positive strains were found in a very few numbers as Sanchini only found 1 *pvl* positive strain of 18 CA MRSA. 15% prevalence of *pvl* positive CA MRSA has been recorded in the Netherlands which is quite a low percentage as compared to our study which shows a way higher percentage of 47% so, our study does not coincide with the study of (Wannet et al., 2005). Our study is also in contradiction with a study done in Indonesia by (Severin et al., 2008) which suggested the presence of 10% *pvl* positive MSSA in the general population which 1s 2x higher than our found value of 5%. All the pathogenic strains in our study came into being as MRSA and carried the *pvl* gene (48%) nevertheless, the study results opposed the shanghaiing study which showed the MRSA 1(8.4%) and MSSA (17.3%) (Han et al., 2010).

A study designed in Lahore city stated nearly matched 40.9% results regarding the prevalence of *pvl* gene among pathogenic samples as our results 48% shown in the diseased population samples (Iqbal et al., 2018). This number tracked along the highest percentage of *pvl* gene among the pathogenic samples which is a matter of grave concerns for the health management organization in Pakistan. Another study of Nepal showed different results 51.9% *pvl* positive mass and 26.1% *pvl* positive MRSA regarding pathogenic samples (Shrestha et al., 2014). This study is quite in paradox with ours as their percentage of *pvl* positive MSSA is quite higher on the other side we could not find a single MSSA among pathogenic strains during our study. A study in India mentioned that they had documented the higher percentages of *pvl* gene among MRSA and MSSA of healthy individuals, but they did not mention the data (Nadig et al., 2010). A study result of Madzgalla et al. (2016) does relate to our study as their percentage of *pvl* positive MRSA among the pathogenic strains were 49%. Another study also mentioned the higher frequency of *pvl* positive MRSA 35% among the pathogenic strains which has a little bit comparable to our results (Jamil et al., 2018). A comparative study conducted in KSA to

compare the Egyptian and Saudi Arabian isolates indicated that the both strains isolated from outpatients infrequently carry the *pvl* gene. Their results for PVL positive MRSA are quite low in percentages 15% for Egyptian originated strains EGOs and 11.5% for Saudi ArabiaSAOs originated strains. While, their *pvl* positive MSSA frequency 9% and 8% for EGOs and SAOs respectively, is in accordance to ours MSSA results.

5. Conclusion

The present study describes a high prevalence of *pvl* positive MRSA and MSSA in healthy as well as diseased population needs to be emphasized. As, in Pakistan there is lesser amount of data targeting the frequency of *pvl* gene among healthy normal populations MRSA and MSSA based upon molecular typing that's why we were able to refer towards a few studies available. Our study was limited by time constrains and data resources. However, the present data illustrates the occurrence of gene among MRSA and MSSA from normal flora as well as pathogenic forms.

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