Iranian J Publ Health, Vol. 39, No.1, 2010, pp.8-14

Original Article

Prevalence of Genes Encoding Bi-Component Leukocidins among Clinical Isolates of Methicillin–Resistant *Staphylococcus aureus*

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(Received 3 Sep 2009; accepted 17 Feb 2010)

Abstract

Background: *Staphylococcus aureus* has been recognized as a major human pathogen and is the major cause of nosocomial infections. Gamma-toxin, leukocidin and other bi-component toxins are a family of proteins encoded by the *hlg* and *luk-PV,* respectively. Panton-Valentine leukocidin (PVL) is an example of these toxins and causes leukocyte destruction and tissue necrosis. The aim of this study was to determine the prevalence of bi-component leukocidin in Methicillin – Resistant *Staphylococcus aureus* (MRSA) isolates in staphylococcal infections.

Methods: Collectively, 143 isolates of *S. aureus* were obtained from Tehran University of Medical Sciences hospitals and confirmed with biochemical tests. Then polymerase chain reaction was used to detect *luk-PV* loci and *luk-E/D*. Coagulase gene was used as internal control. The antibiotic susceptibility patterns of isolates were determined using disk diffusion method.

Results: Out of 149 *S. aureus* isolates 24.2% were *luk-PV* positive and 73.8% were *luk-E/D* positive.

Conclusion: There was PVL–positive MRSA isolates with high prevalence in evaluated hospitals. The diseases from these bacteria are with extensive necrosis, leucopenia and even death. We desire that, prevent from progress and death by diagnosis and right treatment.

Keywords: *Bi-component leukocidin, Methicillin-resistant Staphylococcus aureus, Staphylococcal infections, Iran*

Introduction

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is the most disease generating strain of staphylococci and is the most prevalent pathogen isolated from hospitalized patients (1); it is also resistant to a number of antibiotics classes because *mecA* induces methicilin resistance in the bacteria (2, 3). It causes a wide range of diseases from mild infections of the skin and soft tissue to life threatening pneumonia and poisonings like toxic shock syndrome (4).

S. aureus has several virulent factors and produces many toxins that are associated with pathogenicity, as well. Some of the exotoxins have the capacity to phagocyte destruction, particularly, polymorphonuclear (PMN) and monocytes. These exotoxins belong to the family of bi-component leukotoxins including S and F proteins (5).These proteins are transcribed by two adjacent and contra scribed genes, encoded and carried on a temperate bacteriophage (6). The mentioned proteins function synergically to create pores on the membrane of the phagocytes (5). These dimeric molecules which are called synergohymenotropic toxins assemble on the neutrophil membrane to create an octameric structure and open calcium channels by providing pores. The secretion of cytolytic enzymes and the production of super oxidize ions can also lead to tissue necrosis (7). The family of the staphylococcus leukotoxins includes Panton-Valentine leukocidin (PVL), γ Hemolysin

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(HlgA+HlgB+HlgC), LUKM (LUKM-PV+LUKF-PV) and LUKE/D (LUKE+LUKD) (8-11).
Currently, *S. aureus* carrying Panton-Valentine leukocidin has turned into a serious global problem. Although cutaneous infections and relapsing abscess are usually originated by the *S. aureus*, it is now ten years that PVL positive strains has caused the increase in pneumonia prevalence among the previously healthy youth; this is accompanied by a high mortality and morbidity rate (12). The prevalence of acute cutaneous infections has been observed among the American school children due to these isolates (13). Similarly, PVL-related cutaneous infections have also been reported among Dutch homosexuals (14), Switzerland schoolchildren (15) and the Scottish hospital staff (16). Today, the increasing number of reports on the PVL positive strains related to necrotizing pneumonia acquired from the community has caused a lot of concerns (5).
Lina et al. (1999) described the relationship between the presence of PVL- positive strains, pneumonia and cutaneous infections (17). Moreover, it was proved in other studies that PVL can be found in both MRSA and MSSA strains and that the possibility of PVL production by CA-MRSA is higher than HA-MRSA (18). Studies on the methicilin-resistant *S. aureus* (MRSA) isolates carrying PVL genes have also been carried out in hospitals of Florida (19), Germany (20), France (21), Minnesota (22), Latvia (23), Austria (24), Belgium (25), the Netherlands (26) and Middle Tennessee (27). In the present study, using PCR assay, the presence of Panton-Valentine leukocidin (PVL) and leukocidin (LUKE/D) genes in various infections caused by *S. aureus* is evaluated and the MRSA or MSSA nature of the isolates was determined.
The aim of this study was to determine the frequency of bi-component leukocidins in MRSA and MSSA isolates for epidemiological purposes and determine the relation of these genes with *S. aureus* infections.

Materials and Methods

Bacterial isolates

Collectively 143 isolates of *S. aureus* were obtained from Tehran University of Medical Sciences hospitals (Children's Medical Center, Shariati, Sina, Loghman, Imam Khomeini). These isolates were transferred to microbiology laboratory of School of Public Health and subcultured on blood agar. Then all of these isolates were reconfirmed with biochemical tests (Coagulase, Manitol fermentation and DNase tests).

Genomic DNA extraction

DNA was isolated by using genomic DNA extraction kit (Bioneer Inc, Korea) as recommended by the manufacturer, with the modification that 1.5 λ lysostaphin was added to bacterial suspension .Finally the genomic DNA extraction was used as the template for PCR.

PCR analysis

A collection of 149 isolates were screened for the presence of *pvl* and *luke/d* genes and *coa* gene as an amplification internal control by PCR, using previously described primers (17). Primers used in this study were as follow **5'CGAGACCAAGATTCAATAAC 3'** as forward and **5' AAAGAAAACCACTCACA TC ACA 3'** as reverse. These primers sequences correspond to 900bp of *coa* gene. Amplification of the *pvl* gene was performed as a single PCR with a forward **5'ATCATTAGGTAAAA TGT CTGCACATGATCCA3',** and reverse **5'GCA TCAASTGTATTGGATAGCCAAAAGC3'** and of *luke/d* gene with a forward **5' ATT CCATAGCATAAGCACTGC 3',** and reverse **5 'TGAAAAACCTTCAAAGTTGAT ACC AG 3'** primers as described before(17). These primers sequences correspond to 433bp of *pvl* gene and 269bp of *luke/d* gene respectively. In this study *Staphylococcus aureus* strain, NCTC 13300, was used as positive control and distilled water as a negative control for PCR. DNA amplification was performed on an Ependorf

cycler in a final volume of 50 µl containing 10 µl of 10X PCR Buffer, 3.5 µl of Mgcl2(10mM), 0.2 mM dNTPmix, 20 ρM of each primer,1U of Taq polymerase and 4 µl of template DNA . Amplification was carried out with first denaturation at 97 °C for 6min (First denaturation) followed by 35 cycles according to the following program: denaturation at 92 °C for 30 S, annealing at 55 °C for 30S,and extension at 72 °C for 45S, plus a final extension at 72 °C for 10 min to complete partial polymerization.

Detection of PCR products

The PCR products were resolved by electrophoresis through a 1/5% agarose gel containing Ethidium bromide.

Antimicrobial susceptibility testing

Susceptibility to oxacillin was determined by agar disc diffusion method using, Muller Hinton agar medium containing 2% NaCl and the plates were incubated at 35°C overnight.

Results

Coa gene was screened and detected in all 149 isolates of *S. aureus*. The standard *S. aureus* NCTC13300 produced amplified PCR products of 269bp and 433bp for (PVL) LUKS/FPR and LUKE/D, respectively; in this test, specialized primers were used for each gene (Fig 1 & 2). Among the 149 *S. aureus isolates*, there were 36(24/16%) PVL positive and 110 (73/82%) LUKE/D positive isolates (Table 1 & 2). Among the PVL positive *S. aureus* isolates, there were 61.8% MRSA, HA MRSA type.

Table 1: Presence of *pvl* gene in *S.aureus* isolates of different origin

Origin	Total number of isolates	Number (%) of positive isolates	*P* value
Cutaneous	52	21(40.38)	**0.002**
Tracheal	34	9(26.47)	0.65
Blood	28	4(14.28)	0.2
Urine	25	1(4)	**0.01**
Other	11	1(9.09)	0.40

The *P* values were assessed by x^2 test. Boldface value indicates statistical significance ($P \leq 0.05$).

Table 2: LUK E/D- positive isolates of *Staphylococcus aureus* in different staphylococcal infections

Origin	Total number of isolates	Number (%) of positive isolates	*P* value
Cutaneous	52	42(86.53)	**0.01**
Tracheal	34	29(85.29)	0.08
Blood	28	18(64.28)	0.34
Urine	25	14(56)	**0.02**
Other	11	5(45.45)	**0.009**

The *P* values were assessed by x^2 test. Boldface value indicates statistical significance ($P \leq 0.05$).

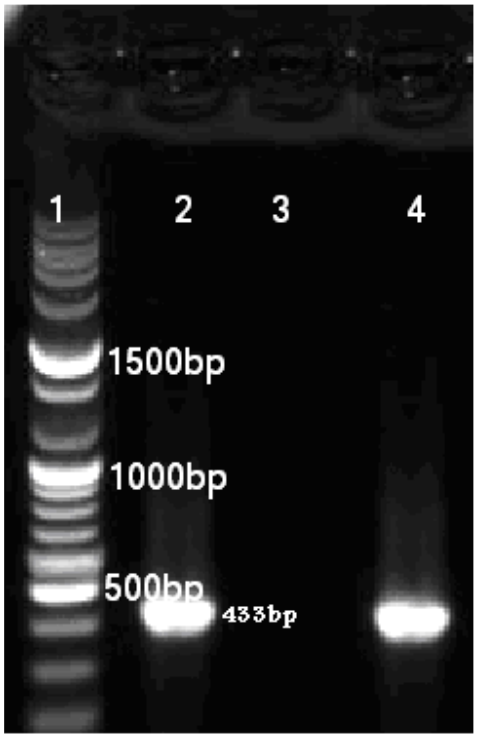

Fig. 1: Six µl PCR- product of *luk s/f-pv* (agarose 1%); Lane 1- molecular size marker (fermentase), Lane 2- Positive control for *luk s/f-pv* gene (433bp),Lane 3 – Negative control, Lane 4-Positive isolate from patient for *luk s/f-pv* gene

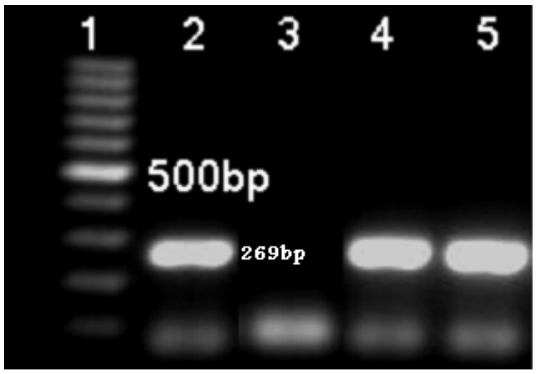

Fig. 2: Six µl PCR- product of *luk s/f-pv* (agarose 1%).Lane 1: molecular size marker (fermentase), Lane 2: Positive control for *luk e/d* gene (269bp), Lane 3: Negative control, Lane 4& 5: Positive isolates from patients for *luk e/d* gene

Discussion

We collected 149 isolates of *S. aureus* from five hospitals across Tehran and PCR assays showed that 24.16% of the isolates were LUKS/ F-PV (PVL)-positive and 73.82% were LUK E/D positive. The prevalence for *pvl* genes is higher than the European countries. For instance, in England and Wales in 2005, 1.6% of the isolates were PVL positive (5). In fact, in all studies, the prevalence of PVL positive *S. aureus* isolates was reported at 2-35% (3, 28). However, it is noteworthy in Argentina, the prevalence of the isolates was 56% (2). These differences may be due to various geographical areas or the type of assay used for detecting the gene. In the present study, 40.38%, 26.47%, 14.28%, 4% and 9.09% of the isolates were related to cutaneous, tracheal, blood, urine and other isolates, respectively. In fact, 94.4% of the cases were PVL positive isolates related to cutaneous, tracheal and blood while the rest was related to the other samples. In other studies also, PVL-positive *S. aureus* isolates were more prevalent in cutaneous and pulmonary isolates (5, 18) and our findings was the same. An interesting finding of our study was the presence of PVL genes in *S. aureus* isolates separated from urine isolates, because in the previous studies, such isolates were not found (18). Moreover, the prevalence of PVL positive *S. aureus* was high in blood isolates (18). 64.3% of the studied isolates belonged to men and 35.66% belonged to women; among these, 41.67% and 58.33% of the PVL positive *S. aureus* strains go to women and men, correspondingly. There was no significant difference and the rates were compatible with the previous findings (27). Lina et al. showed an association between *pvl* genes and cutaneous infections (17), confirming earlier findings by other workers (5). In our study, also there was a significant relation between cutaneous infections and the presence of *pvl* genes (Table 2). Studies have shown that *pvl* genes were first represented among the strains of CA-MRSA (29-33); *pvl* genes are rarely reported in HA-MRSA (12). However, the analysis of MRSA isolates has shown that PVL-containing MRSA did not merely exist in the community and they may be found in hospitals, as well (27). In the present study, 61.8% of the PVL

positive isolates were MRSA, type HA-MRSA. Conspicuously, methicillin-sensitive (MSSA) PVL containing S. aureus isolates were found in our study; however, such isolates were previously reported, as well (20). In an assay with microarray, it was demonstrated that MRSA isolates harboring genes for several bi-component toxins such as LUKE/D (23). We detected LUKE/D genes in nearly 73.8% of the *S. aureus* isolates separated from all staphylococcal infection types; harboring the genes was not associated with any blood and tracheal isolates and was compatible with the previous findings (34). But interestingly there was a significant relation between presence of the gene and coetaneous and urine isolates (Table 2)

In succinct, PVL-positive *S. aureus* exist in several infections, especially cutaneous ones. These strains are not only prevalent among CA-MRSA isolates but are abundantly found in HA-MRSA; carriers of the PVL positive isolates are also found (35) and this highlights the importance of these isolates in hospitals. Since PVL is a very remarkable virulence factor, it is recommended that early treatment is applied following an on-time diagnosis and performing an antibiogram. The reason is that such isolates are very dangerous and life threatening. We can prevent person-to-person transfer and fatal prevalence by making early diagnosis of the diseases in patients and the carriers with a simple PCR assay.

Acknowledgements

This research has been supported by Tehran University of Medical Sciences grant no: 86-03-27-5912. We are grateful to Dr Mohammad Iman Eini for the gift of control positive isolate. The authors declare that they have no conflicts of interest.

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