Prevalence of the Panton-Valentine Leukocidin in Staphylococcus aureus associated with Upper Respiratory Tract Infections

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Abstract

*S. aureus* with lukS-PV and lukF-PV genes, responsible for encoding the cytotoxin panton valentine leukocidin (PVL) becomes a growing problem throughout the world. Initially, this pore-forming cytotoxin in human leukocytes was associated with skin and soft tissue infections, especially in recurrent abscesses. However, in the last decade a growing association of these PVL positive strains has been verified, with devastating pneumonia with a high mortality rate affecting mainly young adults, apparently healthy. In this study we were to determine the prevalence of PVL in *S. aureus* associated with upper respiratory tract infections. We were isolated 60 strains of *S. aureus* from respiratory tract infections in humans; these were tested for different antibiotics. Then, from DNA we analyzed the presence or absence of the lukS-PV and lukF-PV using the PCR technique. The phenotypic profile for different antibiotics tested revealed only one strain sensitive for all, 59 strains showed resistance: 2 (34%) to gentamicin and trimethoprim/sulfamethoxazole, 46 (78%) to levofloxacin, 51 (86.4%) to oxacillin and all (100%) to Penicillin G. The lukS-PV gene was detected only in 2 strains (3.3%), the lukF-PV gene was detected in 20 strains (33.3%) and both genes were detected simultaneously in only two strains, which were classified as PVL positive (3.3%). The average age of the infected individuals with PVL positive strains is 11 years, which may indicate that PVL positive strains infect younger individuals. The 18 positive strains for the lukF-PV gene suggest the use of other techniques to confirm the non - gene amplification for lukS-PV gene. The 60 strains showed a high resistance pattern to β-lactam antibiotics including penicillin and oxacillin, however, it was not established a link between PVL positive strains and resistance to a specific antibiotic or MRSA phenotype.

Keywords: *S. aureus*, LukS-PV, LukF-PV, PVL.

INTRODUCTION

*Staphylococcus* spp. is a Gram positive bacteria, pathogenic or commensal for humans and animals. These organisms can resist to adverse conditions and can recover from non-physiological environments several months after inoculation. They grow easily in different environments and are classified based on response to coagulase. Coagulase positive strains are classified as *Staphylococcus aureus* (*S. aureus*), which is the most importantly pathogen within the *Staphylococcus genera*. (Lisa and Plano, 2004). The emergence of multidrug-resistant strains is a major problem associated with this bacteria. The resistance to methicillin and other β-lactam antibiotics is conferred by the presence of the mecA gene, located on the mobile genetic element *SSCmec* (staphylococcal cassette chromosome mec), which encodes altered proteins capable of

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binding to penicillin (PBP-2). These strains are designated as methicillin resistant S. aureus (MRSA). (Afroz et al., 2008).

MRSA strains can be classified as HA-MRSA – hospital acquired strains – or CA-MRSA – community acquired strains. However, due to the constant bacterial evolution became very unclear the limit for the distinction between these two types. The fact that CA-MRSA strains become endemic in several European countries, USA, Australia and New Zealand led Friedman et al. in 2002, to suggest the creation of a broader concept that includes infections associated with health care (IACS). (Afroz et al., 2008; Johannes, 2008; Monecke et al., 2002; Monecke et al., 2007; Boyle et al., 2009; Holmes et al., 2005).

Infections caused by S. aureus can result from the direct invasion of tissues or from the action of many exotoxins produced as virulence factors, including the cytolytic toxins α-hemolysin (HLA), β-hemolysin, Y-hemolysin (Hlg), δ-hemolysin, leukocidin (Luk) and Panton-Valentine leukocidin (PVL). (Monecke et al., 2007; Said-Salim et al., 2005)

PVL is a cytotoxin from the family of sinergohimenotropic toxins, composed by two classes of proteins secreted in association, protein S and F, which are encoded by the lukS-PV and lukF-PV genes, respectively. The protein complexes form non-specific heptameric pores in the host’s defense cells, in particular, polymorph nuclear leukocytes, monocytes and macrophages thereby increasing the virulence of these bacteria. (Monecke et al., 2007; Lina et al., 1999; Boyle-Vavra and Daum, 2007; Ramos et al., 2009; Holmes et al., 2005).

The literature describes a greater association between clones of MRSA acquired in community and the production of PVL in recurrent or chronic skin infections, tissue infections and necrotizing pneumonia, hemorrhagic and fatal prevalent in children and young adults. Although PVL is not the only virulent toxin in MRSA clones, it is the most frequently used to explain the severity of community acquired infections. (Lisa and Plano, 2004; Monecke et al., 2007; Boyle-Vavra and Daum, 2007; Gillet et al., 2007; Tristan et al., 2007; Labandeira-Rey et al., 2007; Masiuk et al., 2010; Thomas et al., 2009; Kaneko and Kamio, 20; Blaine et al., 2010)

The relationship between necrotizing pneumonia and S. aureus has been reported several years ago, however, the association of this type of pneumonia with the specific cytotoxic action of PVL is relatively new, and was first described by Gillett et al. in 2002. Since this date, several cases were referred throughout the world, which contributes to the growing interest in the study of this cytotoxin. (Gillet et al., 2007)

Pneumonia positive for the lukSF-PV genes present a similar pathogenesis since the action target of the cytotoxin is constant. The severity of that cytotoxic effect varies depending on the host. In humans, PVL is correlated with long periods of infection, which may extend to invasion and mortality in pediatric patients with osteomyelitis and septic arthritis; thereby human polymorph nuclear leukocytes are susceptible to the cytotoxic effects of PVL. Other investigators didn’t report differences between PVL positive strains and PVL negative strains regarding the size of the abscess, bacteria density, lyses of neutrophils, bacteraemia and sepsis in mice. Based on these results, mice polymorphonuclear leukocytes appear to be relatively resistant in vitro to PVL. (Blaine et al., 2010; Diep et al., 2010; Löffler et al., 2010)

A pathological response to PVL is only seen in transfected strains with multiple copies of the PVL operon. Significant differences can’t be detected in that same response in strains expressing a single operon. (Blaine et al., 2010)

The detection of lukSF-PV genes may be performed using PCR, multiplex or real-time techniques. (Said-Salim et al., 2005; Schlebusch et al., 2009; Woltering et al., 2008; Karahan et al., 2009). Given the different studies of lukSF-PV genes prevalence in S. aureus, observed variations have been reported depending on the country and the moment at which the studies are carried out, also differences in the virulence of the toxin, in the presence of demographic variants and detection of new MRSA PVL positive clones have reported. (Said-Salim et al., 2005; Schlebusch et al., 2009; Woltering et al., 2008; Karahan et al., 2009)

The aim of this study was to determine the prevalence of the lukS-PV and lukF-PV genes in S. aureus isolated from samples of the upper respiratory tract of hospitalized patients in central Portugal.

MATERIAL AND METHODS

Study population

We isolated 60 S. aureus from infections of the upper respiratory tract in humans, in the Microbiology unit of the Center and University Hospital of Coimbra, during July until September (2010). The strains were selected randomly without taking into account the age and sex of the infected individuals.

Identification and antibiotic susceptibility test of S. aureus

The strains of S. aureus were isolated from samples of bronchial aspirate and expectoration. To perform the bacterial identification, the samples were inoculated on blood agar with 5% sheep blood and incubated at 37°C
in capnophily for 18-24 hours. After the growth, the *S. aureus* strains were subcultured to new blood agar plates and incubated under the same conditions for 18 hours. The strain identification was made automatically, using the equipment Vitek®2 - bioMérieux™. The antibiogram was carried out on a letter of susceptibility to Gram positive microorganisms (AST-P580) in this same equipment. The susceptibility test was made by disc diffusion method, for following antibiotics: levofloxacin, gentamicin, trimethoprim/sulfamethoxazole, vancomycin, oxacillin and penicillin G.

**DNA extraction from *S. aureus***

We made a suspension with one pure colony into a Luria Bertani medium (LB), which was incubated overnight at 37°C, with agitation. Then, the inoculum was centrifuged 10 min. at 13000g, the supernatant was discarded and the pellet was resuspended in 200µL of Tris-EDTA (TE). The DNA extraction was made using the Genomic DNA purification Kit #K0512 (Fermentas) following the manufacturer instructions. The DNA solution obtained was stored at -20°C for the next steps.

**Detection of lukS-PV and lukF-PV genes in *S. aureus***

The DNA extracted from the isolates was subjected to amplification of the *lukS-PV* and *lukF-PV* genes; we used the primers *PVS-F1* 5'- GCA AGG TTT TAT CAA TTC AAA GAC TAC TT -3' and *PVS-R1* 5'- GGG TCA TTT GTG AGA CCA ATA T -3' to amplify 111pb in lukS-PV gene, *PVF-F1* 5'- TAC ACA GTT AAA TAT GAA GTG AAC TGG A -3' and *PVF-R1* 5'- AGC AAA AGC AAT GCA ATT GAT G -3' to amplify 154pb in lukF-PV gene. (Nakagawa et al., 2005).

For each PCR reaction (25µL) contained 1x buffer, 2.5mM MgCl₂, 0.2mM dNTP’s, 2.5pmol of each primer, 2.5U of Taq Polymerase and ~50ng of DNA solution. The amplification program consisted of an initial denaturation step at 94°C for 5min, 30 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1 min, extension at 72°C for 1min and a final extension step at 72°C for 7min. The PCR reactions were performed in a mini-BioRad MJ thermocycler.

The PCR products were observed into an agarose gel electrophoreses (2%) stained with ethidium bromide (50mg/mL). The image acquisition was obtained through of Gel Doc™ XR BioRad.

**RESULTS**

**Sample characterization**

The 60 strains were isolated from biological products of individuals with an average age of 71 years; 44 were collected from male subjects with an average age of 70 years and 16 from female subjects with an average age of 77 years. The *S. aureus* were isolated from 27 expectoration samples and 33 bronchial aspirates.

The individuals infected with *S. aureus* were hospitalized into different departments of hospital. The 60 strains, 13 were isolated from individuals in the Care Medicine, 10 of the Surgery, 10 of the Internal Medicine, 6 of the General Emergency, 3 of the Nephrology, 1 of the Neurology, 5 of the Neurotraumatology, 2 of the Pulmonology, 2 of the Functional Respiratory Physiopathology Unit (FRPU), 1 of the Cerebral Vascular Disease Unit (CVDU), 3 of the Burn Unit, 1 of the Infectious Diseases, 1 of the Psychiatry and 1 of the Cardiology.

**Antibiotic Susceptibility Test**

All strains showed sensitivity to vancomycin, only one was sensitive to all antibiotics tested and 59 strains were resistant to Penicillin G. Only 2 (3.4%) of these 59 strains were resistant to the gentamicin and trimethoprim/sulfamethoxazole, 46 (78%) were resistant to levofloxacin and 51 (86.4%) were resistant to oxacillin.

**Prevalence of the lukS-PV and lukF-PV genes**

The LukS-PV and PV-lukF genes were simultaneously present in two strains, indicating a prevalence of 3.3% for PVL. The LukS-PV gene was detected only in two strains, prevalence of 3.3%. The LukF-PV gene was detected in 20 strains, prevalence of 33.3% (Figure 1).

**Relationship between the resistant profile, origin of the isolates and presence of lukS and lukF-PV genes in *S. aureus***

The two positive strains for PVL, both were resistant to penicillin G, one was resistant to levofloxacin and oxacillin and don’t showed resistance to trimethoprim/sulfamethoxazole and vancomycin. The 20 positive strains for LukF-PV gene, 13 were resistant to levofloxacin, 1 was resistant to gentamicin, 15 were resistant to oxacillin, 20 were resistant to penicillin G, 1 was resistant to trimethoprim/sulfamethoxazole and don’t showed resistance to vancomycin.

Both PVL positive strains belong to samples from patients hospitalized in the surgical unit. The 20 positive strains for the LukF-PV gene were isolated from samples of patients hospitalized in different services: 1 in UFFR, 1 in Neurology, 1 in Nephrology, 3 in General Emergency, 3 in Internal Medicine, 6 in Surgery and 5 in Intensive Care Medicine.
DISCUSSION

The prevalence of the coding genes for the cytotoxin varies among different countries worldwide, in a range of 1 to 30% (Monecke et al., 2007; Lina et al., 1999; Holmes et al., 2005). In the present study we obtained a 3.3% percentage of prevalence to PVL. However, the prevalence of the lukF-PV gene is slightly higher than the range mentioned for the presence of PVL worldwide. According to Wolter et al. in 2007, different alleles can occur for the PVL genes that are associated with specific strains of S. aureus. This author identified seven polymorphisms, one of which corresponds to the substitution of the arginine amino acid for histidine at position 176, which leads to an increase in molecular weight of the LukS protein. This fact supports the hypothesis that some strains that have integrated the study may contain changes in the nucleotide sequence of the genes coding for PVL, particularly in the annealing zone of the specific primers for the lukS-PV gene, which restrains the proper pairing of these primers and therefore the detection of this gene. This could be a possible explanation for the difference between the prevalence of the lukS-PV and lukF-PV genes. Wolter et al. 2007 also reported that the genes encoding PVL in S. aureus have been transmitted by bacteriophages and that different sequence for the lukS-PV and lukF-PV genes have been identified in those phages. (Wolter et al., 2007)

Several studies refer that PVL can only exert its cytotoxic effect in the presence of two protein subunits, the S and F proteins, respectively encoded by the lukS-PV and PV-lukF genes. Thus, the presence of lukF-PV gene by itself is not enough to occur a pathological effect of the PVL cytotoxin. (Monecke et al., 2007; Wolter et al., 2007; Boyle-Vavra and Daum, 2007; Diep et al., 2010; Otter et al., 2009).

According to Holmes et al. in 2005, one of the interests in studying the PVL was the fact that is involved in severe diseases among children and young adults exposed to health care institutions. (Holmes et al., 2005). This fact could not be verified in this study population because it showed a high mean age (71 years). However, it was found that the average age of
the individuals infected with *S. aureus* PVL positive was inferior in 11 years than the average age of the individuals infected with *S. aureus* PVL negative. This reduction in almost a decade in the average age of PVL positive individuals indicates that there may actually be a relationship between age and presence of PVL.

The strains subjected to the antibiogram showed a pattern of β-lactam resistance, namely to oxacillin and penicillin G, which in accordance with various studies, is conferred by the presence of the mecA gene located on the mobile genetic element SCCmec. (Valsesia et al., 2010; Holmes et al., 2005; Tristan et al., 2007; Conceicao et al., 2010).

This study could not find any association between PVL positive strains and resistance to oxacillin (ORSA/MRSA strains). According Monecke et al. in 2007, MRSA and MSSA strains are PVL positive, and there isn't necessarily a pattern of association. However, most recent studies have verified an increase in MRSA - PVL positive strains, since the mobile genetic element SCCmec may include genes coding for PVL. (Monecke et al., 2007; Wolter et al., 2007; Boyle-Vavra and Daum, 2007; Diep et al., 2010; Tristan et al., 2007).

This study couldn't also establish a relationship between the PVL positive strains and the service in which the infected individual were hospitalized. Most authors refer that there is an association between the presence of PVL and CA-MRSA strains (community acquired), which can't be verified in this study due to lack of information regarding the origin of the strain: if in hospital environment or in community. Furthermore, according to Friedman et al. in 2002, the distinction between CA-MRSA and HA-MRSA strains is no longer clear, and that created a new concept called infections associated with healthcare. (Johannes, 2008; Friedman et al., 2002)

The strains, in which the lukF-PV gene was detected, came in greater numbers from the individuals in Surgery, Internal Medicine and Critical Care Medicine Services. Blaine et al. in 2010, said in their study that about 31% of their PVL positive strains were found in patients subjected to health care for more than 48 hours, which can justify the higher number of strains positive for the lukF-PV gene in services that require a greater number of interment days as in the case of Internal Medicine and Critical Care Medicine. (Johannes, 2008; Friedman et al., 2002; Blaine et al., 2010)

CONCLUSION

The prevalence of PVL in the studied strains was 3.3%. However, it would be important to confirm with other methods if the lukS-PV gene is actually absent in the strains which it was only detected the presence of LukF-PV gene and that they are not falsely negative strains for this gene.

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