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Prevalence of Panton-Valentine leukocidin genes in community-associated methicillin-resistant *Staphylococcus aureus* in the District of Pomoravlje

Prevalencija Panton-Valentin leukocidin gena u vanbolničkim meticilin-rezistentnim *Staphylococcus aureus* u Pomoravskom okrugu

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Abstract

Background/Aim. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains appear to have rapidly disseminated among population in the community without established risk factors for MRSA worldwide. Panton–Valentine leukocidin (PVL) is a cytolytic toxin, encoded by the *lukF-PV* and *lukF-PV* genes. PVL may be the key toxin responsible for enhanced virulence of CA-MRSA. The aim of this study was to detect the genes encoding PVL in CA-MRSA isolates from healthy people from the District of Pomoravlje. **Methods.** We took throat and nose swabs from healthy, employed persons with mandatory sanitary examinations and analyzed the presence of MRSA, between January 2011 and December 2012 in the District of Pomoravlje. Susceptibility of isolated strains to cefoxitin was investigated by using disc diffusion according to the recommendation of CLSI (Clinical Laboratory Standard Institute), and by E test. The presence of penicillin–binding protein 2a (PBP2a) in *Staphylococci* was detected using latex agglutination Slidex®MRSA Detection test. The gold standard, polymerase chain reaction (PCR) assay, was used for detection of *mecA* gene and PVL gene, and typing of SCC*mec* region. **Results.** Our investigation showed that staphylococcal carrier state was present in 2.58% of 52,910 throat and nasal swabs, and in 50 of them (3.67%) MRSA was isolated. Among these MRSA, 2 (4%) isolates were PVL-positive. **Conclusion.** The prevalence of CA-MRSA and the presence of PVL gene among healthy, employed population in the District of Pomoravlje were low. The values obtained in this study show that, our region is not significantly different from the other parts of our country, nor from the other European countries.

Key words:
methicillin resistance; staphylococcus aureus; community acquired infections; polymerase chain reaction; panton-valentine leukocidin; serbia.

Apstrakt

Uvod/Cilj. Vanbolnički (*community-associated* – CA) meticilin rezistentni *Staphylococcus aureus* (CA-MRSA) se brzo širi u opštoj populaciji, pa i među onima koji nisu bili izloženi riziku boravka u bolnici. Panton Valentin leukocidin (PVL), kodiran *lukF-PV* i *lukF-PV* genima, može biti ključni toksin odgovoran za virulenciju vanbolničkog MRSA. Cilj rada bio je detektovati gene koji kodiraju PVL u izolatima vanbolničkog MRSA u Pomoravskom okrugu. **Metode.** Prisustvo *S. aureus* rezistentnog na meticilin testirano je tokom dvogodišnjeg perioda, na 52 910 briseva grla i nosa poreklom od zdravih, radno sposobnih ljudi koji podležu sanitarnom nadzoru. Brisevi su zasejavani na krvni agar i inkubirani 24 h. Rezistencija na meticilin detektovana je disk difuzionim testom sa diskom cefoksitina i E-testom, a test aglutinacije primenjen je za dokazivanje penicillin-vezujućih proteina 2a (PVP2a). Za detekciju *mecA* gena PVL gena i tipizaciju SCCmec regiona primenjena je *polymerase chain reaction* (PCR) metoda. **Rezultati.** Stafilokokno kliconoštvo bilo je prisutno kod 1 363 (2,58%) ispitanih, a MRSA je potvrđen kod 50 izolata *S. aureus* (3,67%). PVL gen je otkriven u dva (4%) CA-MRSA izolata. Jedan od PVL-pozitivnih izolata sadržao je SCCmec region tipa IV, a drugi tipa V. **Zaključak.** Prevalenca MRSA kod zdravih kliconoša, kao i zastupljenost PVL gena bili su niski. Vrednosti dobijene u ovoj studiji, pokazuju da se naš region ne razlikuje značajno od drugih delova naše zemlje i drugih evropskih zemalja.

Ključne reči:
meticilin, rezistencija; staphylococcus aureus; infekcije, vanbolničke; polimeraza, reakcija stvaranja lanaca; panton-valentin leukocidin; srbija.

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Introduction

Until recently, methicillin-resistant *Staphylococcus aureus* (MRSA) was considered as the prototype of a nosocomial pathogen [1]. Since the mid-1990s [2], this pathogen has emerged as a cause of infection in young, previously healthy people in general community, and the term community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) was established. From their health care-associated MRSA (HA-MRSA) counterparts, these isolates differ clinically, in the virulence factors, epidemiology and frequency of occurrence [3, 4].

Methicillin resistance is conferred by the *mecA* gene, which is part of a mobile genetic element called the "staphylococcal cassette chromosome (SCC) *mec*". CA-MRSA and HA-MRSA can been distinguished by molecular methods, based on the differences of SCC*mec* region. HA-MRSA isolates carry a relatively large SCC*mec* belonging to type I, II, or III. Beside methicillin, they are often resistant to many classes of non-β-lactam antimicrobials. In contrast, CA-MRSA isolates harbor smaller SCC*mec* elements, type IV or V [5], having a size up to 30 kb, and are presumable more mobile. They are resistant to fewer non-β-lactam classes of antimicrobials.

MRSA, like other *S. aureus* strains, has numerous mechanisms to produce disease and to evade host defense. In establishing an infection, numerous surface proteins mediate adherence to host tissues or prosthetic materials. After adhesion, it is able to grow and persist in various ways: it can form biofilms, invade and survive inside epithelial cells, including endothelial cells; form small-colony variants which may contribute to persistent and recurrent infection, produce antiphagocytic microcapsule that help it evade the host immune system or produce leukocidins that cause leukocyte destruction [6].

Panton-Valentine leukocidin (PVL) is a two-component *S. aureus* protein encoded by the *lukF-PV* and *lukS-PV* genes. Its ability to lyse leukocytes was first described in 1894 by Van de Vald [7]. Panton and Valentine in 1932 linked the presence of leucotoxin with skin and soft tissue infections (SSTI) [8]. Some authors indicate that infections with PVL-positive strains are more severe: pneumonia caused by PVL-positive MRSA or methicillin-sensitive *Staphyloccocus aureus* (MSSA) strains is accompanied by high fever, sepsis, hemoptysis, pleural effusion, and even death [9]. PVL is commonly observed in CA-MRSA strains, and the frequency of PVL in the United States is increasing along with the spread of CA-MRSA clones [10, 11]. Subsequently, there have been reports of PVL-positive clones emerging in the hospital [12]. While some authors proposed PVL as a genetic marker of CA-MRSA [13], a group of authors from Australia did not find a significant association between CA-MRSA-SCC*mec* type IV and PVL gene [14].

The objective of this study was to establish the prevalence of PVL in MRSA isolates associated with community.

Methods

Bacterial isolates

During 2011 and 2012 we analysed 52,910 throat and nose swab samples taken from adult, healthy population from 16 to 60 years of age, from the District of Pomoravlje.

The swabs were cultured on blood agar (Bio-Merieux, France) and then incubated for 24 h aerobically at 37°C. All isolates were stored frozen in dextrose broth at -20ºC, and recultivated on blood agar prior to each experiment. The isolates of *S. aureus* were identifided by tube coagulase test with rabbit plasma (Torlak, Belgrade) after incubation for 4 h and 24 h. Test negative after 4 h had to be reexamined after 24 h.

Antibiotic susceptibility test

The sensitivity of S. *aureus* to methicillin and other groups of antibiotics was tested by the disk diffusion (DD) method according to the recommendation of Clinical Laboratory Standard Institute (CLSI) [15]. Mueller-Hinton agar (MHA) (Bio-Merieux, France) was inoculated with suspension of 24-hour culture of staphylococci, density of 0.5 McFarland. After 15 min antibiogram disks were placed: cefoxitin (30 μg), gentamicin (30 μg), amikacin (30 μg), tetracycline (30 μg), ofloxacin (5 μg), erythromycin (15 μg), clindamycin (2 μg), trimethoprim-sulfamethoxazole − SXT (1.25 + 23.75 μg), fusidic acid (30 μg), vancomycin (30 μg) (BD, England), and incubated for 18−24 h at 35−37°C.

Methicillin resistance was also determined by agglutination test "Slidex MRSA Detection" to prove PBP2a (Bio-Merieux, France). The "Slidex MRSA Detection" test is a rapid slide agglutination assay designed to detect the presence of PBP2a in *S. aureus*. Test was performed as recommended by the manufacturer.

MIC of cefoxitin was determined by E test (Bio-Merieux, France). The test conditions recommended by the manufacturer are based and providing results comparable with CLSI methods and include incubation of inoculum whose density is equivalent to 0.5 McFarland standards, on MH agar with 2% NaCl, for 24 h at 35−37 °C.

The isolates were considered CA-MRSA according to criteria established by Centers for Disease Control and Prevention (CDC) [16]: they were derived from healthy people that had not been hospitalized within the preceding year.

PCR detection of the mecA gene and PVL genes and typing of SCCmec region

For PCR amplification, bacterial DNA was prepared by the use of kit for DNA isolation (B-DNA Sorb, Sacace, Italy). The resistance to methicillin was confirmed by amplifying a 162 bp fragment of *mecA* gene by primers and conditions described previously Oliveira et al. [17]. The primers used to amplify a 433 bp region of *lukF-PV* genes and PCR conditions were previously described by Lina et al. [18]. Typing of SCC*mec* region was performed using the primers and protocols described by Milheiriço et al. [19].

Results

A total of 52,910 throat and nose swabs were analysed, and in 1,363 (2.58%) *S. aureus* was isolated. By the use of antibiogram disks with cefoxitin, E test for cefoxitin, and agglutination test for MRSA detection, and according to the

criteria established by CDC, among these *S. aureus* isolates 50 (3.67%) of them belonged to CA-MRSA. In PCR amplification with primers specific for *mecA* gene, all 50 isolates were positive and proven MRSA.

Beside cefoxitin, CA-MRSA isolates were tested for sensitivity to other groups of antibiotics: fusidic acid, SXT, quinolones, aminoglycosides, macrolides and tetracyclines. The least number of isolates was resistant to fusidic acid, only 4 (8%), to SXT 8 (16%) isolates, and to amikacin 9 (18%) isolates. Resistance to ciprofloxacin was detected in 15 (30%) of isolates, to gentamicin and clindamycin in 26 (52%) of isolates each, to erythromycin in 27 (54%), and to tetracyclin in 28 (56%) of isolates (Table 1). Our CA-MRSA isolates showed multiple drug resistance (MDR) patterns: 24 (48%) of the isolates were resistant to three or more antibiotics, 9 (18%) were resistant to two, 7 (14%) showed resistance to one antibiotic, but 10 isolates (20%) were susceptible to non-β-lactam antibiotics such as fusidic acid, SXT, quinolones, aminoglycosides, macrolides and tetracyclines.

Table 1

Resistance to non-β-lactam antibiotics of community-associated methicillin-resistant *Staphylococcas aureus* (CA-MRSA) isolates (n = 50)

Antibiotic	Resistant n (%)
Tetracyclin	28 (56)
Erythromycin	27 (54)
Clindamycin	26 (52)
Gentamicin	26 (52)
Ciprofloxacin	15 (30)
Amikacin	9 (18)
SXT	8 (16)
Fusidic acid	4 (8)

SXT − sulfamethoxazole/ trimethoprim.

PCR amplification with primers specific for genes encoding PVL detected these genes in only two CA-MRSA isolates (Figure 1), so the prevalence of the PVL-positive isolates was 4%. Molecular typing of two PVL-positive isolates reveal that one of them contained type IV SCC*mec* region, specific for CA-MRSA. Another PVL-positive isolate contained type V SCC*mec* region, that is also specific for CA-MRSA.

One of the PVL positive isolates was resistant to erythromycin, clindamycin gentamicin and tetracyclin, and other, except resistance to erythromycin and gentamicin, showed inducible clindamycin resistance.

Discussion

The anterior nares are the most frequent site of colonization for *S. aureus.* It is estimated that in some individuals (about 20%) this site is persistantly colonized with *S. aureus,* while in others (about 30%) colonization is only periodical [6]. Colonized individuals represent the main reservoir of *S. aureus,* and they contribute to the spreading of this bacteria in hospitals and community. Beside that, colonized strains are increasing the rate of infection especially in the case of host defence weakening, when they can easily be introduced.

The results of prevalence examination of *S. aureus* nasal carriage vary, depending on the studied population and study design. In this study *S. aureus* was isolated from 2.58% throat and nose swabs from healthy and employed population from the the District of Pomoravlje. In another study on healthy population in Belgrade, similar results were obtained [20].

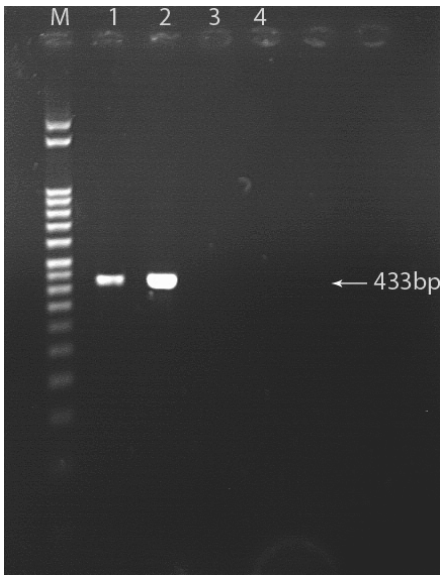

Fig. 1 – Polymerase chain reaction (PCR) detection of Panton-Valentine Leucocidin (PVL) genes. Line M: 50bp DNA ladder; lines 1, 2: PVL-positive community-associated methicillin resistant (CA-MRSA) strains; line 3: American Type Culture Collection (ATCC) 33591; line 4: ATCC 25932.

The prevalence of commensal *S. aureus* nasal colonization differed significantly in European countries, and the differences could not be explained by differences in age, gender or general practitioner (GP) practice, according to a recent research published in The Lancet Infectious Diseases [21]. A total of 32,206 nasal swabs from patients in nine countries were analysed in the study, and *S. aureus* was isolated from 6,956 (21.6%). The most extreme prevalence was in Hungary (12.1%) and in Sweden (29.4%).

In the study of von Eiff et al. [22], conducted at a single institution, 1,640 *S. aureus* strains were isolated from nasal swabs, and 5.8% of them were methicillin resistant. Among 1,363 *S. aureus* isolates in our study, 50 (3.67%) were methicillin resistant. The nasal carriage rate of MRSA in the population of medical students in Belgrade was low: 0.37% [23]. The discordant rates of colonization, probably, were driven by changes in the ecology and epidemiology of MRSA.

The strains of CA-MRSA carry SCC*mec* IV or SCC*mec* V, which are the smallest of the SCCs. In contrast to the multidrug-resistant nosocomial MRSA strains that carry larger SCC*mec* types, CA-MRSA strains are generally susceptible to several non-β-lactam antibiotics. But for some CA-MRSA strains, like epidemic clone USA300, it was noted that are becoming resistant to several non-β-lactam antibiotics [24].

The same situation is in the District of Pomoravlje. Compared to a similar research in 2009 [25], percentage of macrolide-resistant and aminoglycoside-resistant CA-MRSA isolates is higher in this study: 54% CA-MRSA isolates resistant to erythromycin *versus* 42.4% in 2009, and 52% isolates resistant to gentamicin *versus* 30.3% in 2009. "Older" antibiotics, such as fusidic acid and SXT, have retained their activity against CA-MRSA.

The basis for the apparent increased virulence of CA-MRSA strains is incompletely understood. Because these strains usually contain PVL, which is usually absent in HA-MRSA strains, this protein is postulated by some researchers to be responsible for that [6]. Highly virulent CA-MRSA strains USA400 and USA300 both harbor PVL operon and SCC*mec* IV [6]. In our CA-MRSA genes encoding PVL were present in only two (4%) isolates. Typing of SCC*mec* region conformed on molecular level that these isolats belonged to CA-MRSA. In Austria, the percentage ranges from 3% to 7% [26], and in Portugal, among healthy children colonized with MRSA, PVL gene was detected in only 1% of isolates [27]. In Canada, PVL positive CA-MRSA strains were detected in less than 5% of isolates [28]. In contrast, in the study of Vandenesch et al. [9], methicillin resistance was conferred in all CA-MRSA isolates by the truncated SCC*mec* type IV element, and all the isolates contained the PVL locus.

In our country, the first finding of PVL-positive MRSA was reported in 2013 [29]. The presence of PVL genes was demonstrated in 2.5% (4 of 162) MRSA isolates from 26 hospitals in Serbia. The three of these isolates carried SCC*mec* type V element, and one carried SCC*mec* IV element.

Conclusion

The prevalence of MRSA among carriers in the District of Pomoravlje is 3.67%. Also, only 4% of CA-MRSA isolates are PVL-positive. Because of a low percentage, the presence of PVL gene cannot be used as a marker of PVL-MRSA.

R E F E R E N C E S

1. *Archer GL.* Staphylococcus aureus: a well-armed pathogen. Clin Infect Dis 1998; 26(5): 1179−81.
2. *Jones ME, Mayfield DC, Thornsberry C, Karlowsky JA, Sahm DF, Peterson D.* Prevalence of oxacillin resistance in Staphylococcus aureus among inpatients and outpatients in the United States during 2000. Antimicrob Agents Chemother 2002; 46(9): 3104−5.
3. *Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB,* et al. Methicillin-resistant S. aureus Infections among Patients in the emergency department. N Eng J Med 2006; 355(7): 666−74.
4. *Paez A, Skiest D.* Methicillin-resistant Staphylococcus aureus: From the hospital to the community. Curr Infect Dis Rep 2008; 10(1): 14−21.
5. *Lazarevic V, Beaume M, Corvaglia A, Hernandez D, Schrenzel J, François P.* Epidemiology and virulence insights from MRSA and MSSA genome analysis. Futur Microbiol 2011; 6(5): 513−32.
6. *Gordon R, Lowy F.* Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis 2008; 46(Suppl 5): S350−9.
7. *Van de Velde H.* Etude sur le mécanisme de la virulence du staphylocoque pyogène. La Cellule 1894; 10: 401-10.
8. *Wright J.* Staphylococcal leucocidin (Neisser-Wechsberg type) and antileucocidin. Lancet 1936; 227(5879): 1002−5.
9. *Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H,* et al. Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 2003; 9(8): 978−84.
10. *Brown ML, O'Hara FP, Close NM, Mera RM, Miller LA, Suaya JA, Amrine-Madsen H.* Prevalence and Sequence Variation of Panton-Valentine Leukocidin in Methicillin-Resistant and Methicillin-Susceptible Staphylococcus aureus Strains in the United States. J Clin Microbiol 2011; 50(1): 86−90.
11. *Carleton HA, Diep BA, Charlebois ED, Sensabaugh GF, Perdreau-Remington F.*Community-adapted methicillin-resistant Staphylococcus aureus (MRSA): population dynamics of an expanding community reservoir of MRSA. J Infect Dis 2004; 190(10): 1730−8.
12. *Hultén KG, Kaplan SL, Lamberth LB, Slimp K, Hammerman WA, Carrillo-Marquez M,* et al. Hospital-acquired Staphylococcus aureus infections at Texas Children's Hospital, 2001-2007. Infect Control Hosp Epidemiol 2010; 31(2): 183−90.
13. *Shukla SK, Stemper ME, Ramaswamy SV, Conradt JM, Reich R, Graviss EA*, et al. Molecular characteristics of nosocomial and Native American community-associated methicillin-resistant Staphylococcus aureus clones from rural Wisconsin. J Clin Microbiol 2004; 42(8): 3752−7.
14. *O'Brien FG, Lim TT, Chong FN, Coombs GW, Enright MC, Robinson DA,* et al. Diversity among community isolates of methicillin-resistant Staphylococcus aureus in Australia. J Clin Microbiol 2004; 42(7): 3185−90.
15. *Clinical Laboratory Standards Institute.* Performance standards for antimicrobial susceptibility testing. Approved standard 11th ed.. CLSI document M02-A11. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
16. *Centers for Disease Control and Prevention.* Community associated MRSA information for clinicians. Infection control topics. [cited 2005 February 3]. Available from: http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca_clinicians.html#4.
17. *Oliveira DC, de Lencastre H.* Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 2002; 46(7): 2155−61.
18. *Lina G, Piémont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V,* et al. Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin Infect Dis 1999; 29(5): 1128−32.
19. *Milheiriço C, Oliveira DC, de Lencastre H.* Update to the multiplex PCR strategy for assignment of mec element types in Staphylococcus aureus. Antimicrob Agents Chemother 2007; 51(9): 3374−7.
20. *Obradović B, Kovačević L, Miloradović-Aćimović M, Purtić-Kljajić D, Relić T.* Prevalence of methicellin-resistant strains of *Staphylococcus aureus* in the healthy Belgrade population. Zdravstvena zaštita 2009; 38(2): 27−31. (Serbian)
21. *den Heijer CD, van Bijnen EM, Paget WJ, Pringle M, Goossens H, Bruggeman CA,* et al. Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study. Lancet Infect Dis 2013; 13(5): 409−15.

22. *von Eiff C, Becker K, Machka K, Stammer H, Peters G.* Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. N Engl J Med 2001; 344(1): 11−6.
23. *Ćirković I, Đukić S, Vuković D, Stevanović G, Švabić -Vlahović M, Stepanović S.* Nasal carriage of methicillin-resistant Staphylococcus aureus among medical students of Belgrade University**.** Srp Arh Celok Lek 2013; 141(5−6): 349−53. (Serbian)
24. *Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG,* et al. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant Staphylococcus aureus. Lancet 2006; 367(9512): 731−9.
25. *Petrović Jeremić LJ.* Sensitivity of methicillin-resistent *Strephylococcus anreus* in hospital and non-hospital settings to the ather groups of antibiotics. PONS 2009; 16: 18−25. (Serbian)
26. *Krziwanek K, Luger C, Sammer B, Stumvoll S, Stammler M, Metz-Gercek S,* Mittermayer H. PVL-positive MRSA in Austria. Eur J Clin Micribiol Infect Dis 2007; 26(12): 931−5.
27. *Gouveia C, Friães A, Neves CM, Melo J, Ramirez CM.* MRSA and PVL positive Staphylococcus aureus are rarely found in community-acquired osteoarticular infections in children in Portugal, a country with high MRSA Prevalence. Online Int J Micr Res 2013; 1(2): 20−4.
28. *Zhang K, McClure J, Elsayed S, Tan J, Conly JM.* Coexistence of Panton-Valentine leukocidin-positive and -negative community-associated methicillin-resistant Staphylococcus aureus USA400 sibling strains in a large Canadian health-care region. J Infect Dis 2008; 197(2): 195−204.
29. *Cirkovic I, Sorum M, Radenkovic D, Vlahovic MS, Larsen AR.* National surveillance reveals findings of Panton-Valentine leukocidin positive meticillin-resistant Staphylococcus aureus in Serbia. J Med Microbiol 2012; 62: 342−4.

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