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The prevalence of methicillin resistance and Panton-Valentine leukocidin synthesis genes in *Staphylococcus aureus* isolates of bovine and human origin

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ABSTRACT

Using the California Mastitis Test (CMT), on 46 highly productive dairy farms in Serbia, cows with milk secretion disorder were identified. Milk samples were taken from cows with positive CMT and from cows with clinical mastitis. Standard microbiological analysis of milk samples and API Staph confirmed the presence of 75 isolates of *Staphylococcus aureus*. Those 75 isolates, as well as 11 isolates of *Staphylococcus aureus* originating from humans were analyzed for the presence of genes encoding Panton-Valentine leukocidin (*PVL*) and PBP2A protein, responsible for methicillin resistance. The presence of gene encoding *PVL* was determined by PCR in 5 out of 75 (6.67%) and in 7 out of 11 (63.63%) bovine and human isolates of *Staphylococcus aureus,* respectively. The presence of the *mecA* gene was determined by PCR in 1 of 75 (1.33%) and in 2 of 11 (18.18%) bovine and human isolates of *Staphylococcus aureus,* respectively. The presence of the *mecC* gene was not determined in analyzed isolates. Further research is needed to investigate the genetic relationship between

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bovine and human *Staphylococcus aureus* isolates, to determine the exact impact of bovine *Staphylococcus aureus* strains from the cow udders on animal and public health.

Key words: cows, udder, milk, Panton-Valentine leukocidin, methicillin resistance

Introduction

Staphylococcus aureus is a major cause of food poisoning (ANONYM., 1996; ANONYM., 2013) may also cause pneumonia, toxic shock syndrome and wound infections (VAN DEN BROEK, 2003), and it is one of the most frequently isolated pathogens from both subclinical, clinical and chronic bovine mastitis (WATTS, 1988; FOURICHON et al., 2001; ESMAT and BADER, 1996; EL-SEEDY et al., 2010).

Exotoxins constitute essential components of the virulence mechanisms of *Staphylococcus aureus*. Some strains produce additional exoproteins that may be responsible for a particular clinical manifestation, including staphylococcal enterotoxins, the Toxic-Shock Syndrome Toxin-1, exofoliative toxins, and the Panton-Valentine leukocidin (*PVL*) (DINGES et al., 2000; RAHIMI and ALIAN, 2013; BENIĆ et al., 2012).

PVL is a *Staphylococcus aureus* - specific exotoxin, which belongs to the family of bicomponent synergohymenotropic toxins (SUPERSAC et al., 1993; GRAVET et al., 1998). It is a leukocytolytic toxin, which disrupts the membranes of polymorphonuclear neutrophils, creating pores in the cell membrane. (KANEKO and KAMIO, 2004). It is encoded by two contiguous and cotranscribed genes carried on bacteriophage, causing leukocyte destruction and tissue necrosis (SZMIGIELSKI et al., 1999; MELLES et al., 2006; HOLMES et al., 2005). This cytotoxin has been associated with necrotic lesions involving severe cutaneous infections with severe necrotizing pneumonia (CRIBIER et al., 1992; LINA et al., 1999; GILLET et al., 2002).

PVL may be found in both methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains. It is encoded by two contiguous and cotranscribed genes, designated as lukF-PV and lukS-PV (PREVOST et al., 1995).

During nationwide surveillance conducted in Serbia in 2008, 162 MRSA strains were collected in 26 Serbian hospitals (mostly emergency hospitals). The presence of *PVL* was demonstrated in four (2.5%) of these isolates. All *PVL* positive MRSA strains were isolated from younger patients with skin infections, and were from different hospitals (CIRKOVIC et al., 2012).

TAVAKOL et al. (2012) identified *PVL* negative MRSA strains resistant to two or more classes of antibiotics, in 14 different dairy herds located in the provinces Overijssel and Gelderland in The Netherlands. They concluded that MRSA is transmitted between various animal species and may be considered as an etiological agent of mastitis in dairy

cows. Many authors have examined the presence of the gene for *PVL* synthesis in MRSA in humans, in food as well as in hospitals, which is often associated with methicillin resistance genes (CIRKOVIC et al., 2012). On the other hand, the presence of *PVL* genes in *Staphylococcus aureus* isolates from cow udders is still insufficiently explored.

The findings of BENHAMED and KIHAL (2013), showed that only one of 141 *Staphylococcus aureus* strains, isolated from dairy cows in West Algeria, had a *PVL* gene.

The aim of our research was to investigate the prevalence of genes encoding *PVL* synthesis as well as PBP2A in *Staphylococcus aureus* isolates from cow' udders with secretion disorders, on farms in Vojvodina and central Serbia, as well as in isolates of human origin.

Materials and methods

Our research involved highly productive cows that produce over 5,000 L of milk per year, from 46 dairy farms in Vojvodina and central Serbia in 2012. The farms had populations ranging from fewer than 5 cows to over 100 dairy cows. The occurrence of clinical mastitis on these farms was not frequent, however, an increased somatic cell count (over 400,000 SCC/mL) in bulk tank milk samples was recorded.

All the cows were tested using the California Mastitis Test, according to the manufacturer's instructions.

Isolates of *Staphylococcus aureus* were biochemically confirmed using API Staph (BioMérieux, France) and by a PCR targeting nuclease gene -characteristic for *Staphylococcus aureus*.

Bacterial DNA was extracted from a single *Staphylococcus aureus* colony using 25 µL of nuclease-free water and 25 µL of PrepMan Ultra reagent (Applied Biosystems, Foster City CA, USA) placed in a 1.5 mL micro centrifuge tube. The samples were heated in boiling water for 10 minutes, allowed to cool to room temperature and centrifuged at 16000×g for 2 min. The supernatant (containing the DNA) was transferred to a clean 1.5 mL microcentrifuge tube.

A 255-bp fragment of the nuclease gene was amplified using the following primer pair:

nuc-F (TCAGCAAATGCATCACAAACAG) and

nuc-R (CGTAAATGCACTTGCTTCAGG).

PCR amplification was performed using a PCR kit (Invitrogen, Carlsbad, CA, USA) in a total volume of 50 µL containing 5 µL of 10× reaction buffer, 1 µL of dNTPs, 5 µL of each primers, 1 µL of template DNA, 0.25 µL of Taq DNA (5 U/µL) and 32.75 µL of PCR water to make up the final volume. Amplification was performed using an AB 2720 thermocycler (Applied Biosystems, CA, USA). Thermal cycling conditions was as

follows: Initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s with a final extension at 72 °C for 5 min. Ten microliters of PCR product were used for electrophoresis in a 1.5% agarose gel at 120 V for 30 min.

PCR amplification of an 85-bp fragment of the *PVL* gene, a 162-bp fragment of *mecA* gene and a 138-bp fragment of *mecC* gene used primer pairs as follows:

PVL-F (GCTGGACAAAACTTCTTGGAATAT);

PVL-R (GATAGGACACCAATAAATTCTGGATTG);

mecA-f (TCCAGATTACAACTTCACCAGG);

mecA-r (CCACTTCATATCTTGTAACG);

mecC-f (GAAAAAAAGGCTTAGAACGCCTC);

mecC-r (GAAGATCTTTTCCGTTTTCAGC);

PCR amplification was performed using a PCR kit (Invitrogen, Carlsbad, CA, USA) in a total volume of 50 μL containing 5 μL of 10× reaction buffer, 1 μL of dNTPs, 2 μL of each primers, 1 μL of template DNA, 0.25 μL of Taq DNA (5 U/μL) and 30.75 μL of PCR water to make up the final volume. Amplification was performed using an AB 2720 thermocycler (Applied Biosystems, CA, USA). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 60 s with a final extension at 72 °C for 5 min. Ten microliters of PCR product were used for electrophoresis in a 1.5% agarose gel at 120 V for 30 min.

Nucleotide sequences encoding Staphylococcal protein A of isolates of *Staphylococcus aureus* originating from cows in our research, are deposited in GenBank under accession numbers from KJ023978 to KJ024046 at the US National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

Results

In our study *Staphylococcus aureus* was isolated, using standard microbiological analysis, API Staph and nuc gene detection, from 62 milk samples taken from CMT positive cows from Central Serbia, 13 isolates originated from cows with clinical mastitis from farms in Vojvodina and 11 isolates originated from humans from the Novi Sad City Hospital.

The presence of the *PVL* gene was determined in 5 out of 75 (6.67%) bovine and 7 out of 11 (63.63%) human isolates of *Staphylococcus aureus* (Fig. 1 and 2). 5 *PVL*-positive bovine strains, labeled as samples 58, 59, 60, 61 and 62, were also *mecA* and *mecC* negative, and they originated from imported cows, housed on 2 out of the 46 farms. The presence of the gene for the *PVL* synthesis was not determined in milk samples from cows with clinical mastitis.

The presence of the *mecA* gene was determined by PCR in 1 (1.33%) bovine isolate of *Staphylococcus aureus*. It originated from a cow with clinical mastitis and it was *PVL* negative.

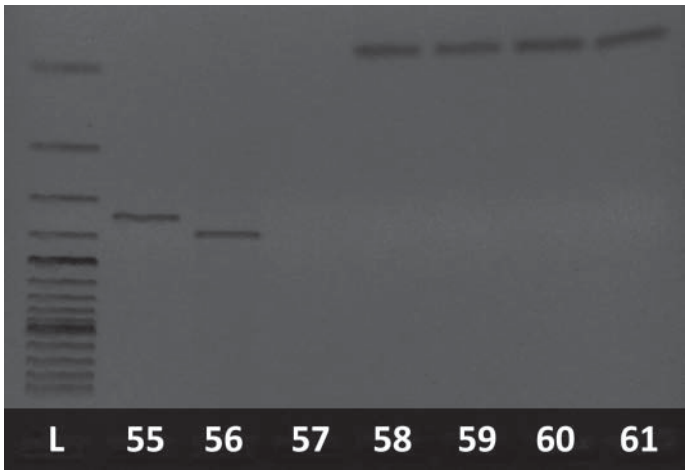

Fig. 1. Agarose gel electrophoresis - determination of *PVL*-positive *Staphylococcus aureus* strains (samples from 58 to 61), L-100 bp ladder

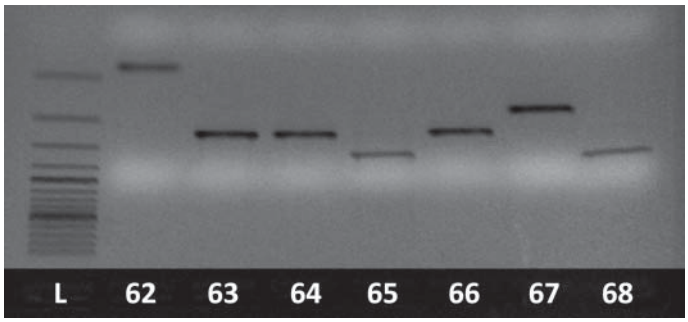

Fig. 2. Agarose gel electrophoresis -determination of *PVL*-positive *Staphylococcus aureus* strains (sample 62), L-100 bp ladder

The *mecA* gene was found in 2 (18.18%) human isolates of *Staphylococcus aureus.* Both samples were also *PVL* negative. The presence of the *mecC* gene was not determined in the analyzed isolates (Table 1).

Table 1. Findings of *PVL*, *mecA* and *mecC* genes in CMT positive and cows with clinical mastitis and in humans

Presence of gene for synthesis		Number of *Staphylococcus aureus* isolates taken from		
		CMT positive cows (total of 62)	Cows with clinical mastitis (total of 13)	Humans from city hospital (total of 11)
PVL	+	5 (8.1%)	/	7 (63.6%)
	-	57 (91.9%)	13 (100%)	4 (36.4)
mecA	+	/	1 (7.7%)	2 (18.8%)
	-	62 (100%)	12 (92.3%)	9 (81.2%)
mecC	+	/	/	/
	-	62 (100%)	13 (100%)	11 (100%)

Discussion

Staphylococcus aureus was isolated from 56% milk samples taken from CMT positive cows with no clinical symptoms of mastitis. These results are somewhat higher than earlier findings by BOBOŠ (1985) in Vojvodina and RADINOVIĆ et al. (2009), in Vojvodina, (52% and 43%, respectively). Our results were also higher those by VIEIRA-DA-MOTTA (2001), who isolated 35% strains of *S. aureus* from milk samples of CMT-positive cows, and JÁNOSI and BALTAY (2004) who isolated 32.5%. On the other hand, our results correspond to the conclusions of FOX and GAY (1993), who reported *Staphylococcus aureus* infection in 7% to 40% of all cows (not only CMT positive).

The presence of the *PVL* synthesis gene was not determined in milk samples from cows with clinical mastitis in our research. In samples originating from CMT positive cows, with no clinical symptoms of mastitis, the *PVL* synthesis gene was found in 8.1%. In research in West Algeria, the presence was found of the *PVL* gene in *Staphylococcus aureus,* from bovine mastitis cases isolates in one strain of 11 (9%) (BENHAMED and KIHAL, 2013).

The presence of the gene for *PVL* synthesis is often associated with genes for methicillin resistance (CIRKOVIC et al., 2012). In England, in 2005, among *S. aureus strains* sent to the Staphylococcus Reference Unit for epidemiological purposes, 1.6% (8 of 515) were positive for the PV locus (HOLMES et al., 2005).

During nationwide surveillance conducted in Serbia in 2008, 162 MRSA strains were collected in 26 Serbian hospitals (mostly emergency hospitals). The presence of *PVL* was demonstrated in four (2.5%) of these isolates. All *PVL* positive MRSA strains were isolated from younger patients with skin infections, and were from different hospitals (CIRKOVIC et al., 2012). In our research *PVL* positive strains of *Staphylococcus aureus* were *mecA* and *mecC* negative. There was only one *mecA* positive strain found which had

been isolated from cow udders and to the best of our knowledge this is the first report of MRSA isolated from cow udders in Serbia.

Conclusion

The presence of gene encoding *PVL* was determined in 5 out of 75 (6.67%) and in 7 out of 11 (63.63%) bovine and human isolates of *Staphylococcus aureus,* respectively. The presence of the *mecA* gene was determined in 1 of 75 (1.33%) and in 2 of 11 (18.18%) bovine and human isolates, respectively. Both bovine and human isolates of *Staphylococcus aureus* which were *mecA* positive, were found to be *PVL* negative. The presence of the *mecC* gene was not determined in either bovine or in human isolates.

Further research is needed to investigate the relationship between bovine and human *Staphylococcus aureus* isolates, to determine the exact impact of bovine *Staphylococcus aureus* strains from cow udders on animal and public health.

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SAŽETAK

Uporabom kalifornijskog testa za upalu vimena na 46 visoko produktivnih mliječnih farmi u Srbiji identificirane su krave s poremećajem sekrecije mlijeka. Uzeti su uzorci mlijeka krava pozitivnih kalifornijskim testom i krava s kliničkim mastitisom. Standardnom mikrobiološkom pretragom uzoraka mlijeka i identifikacijom kompletom API Staph potvrđena je prisutnost 75 izolata bakterije *Staphylococcus aureus*. Tih 75 izolata, kao i 11 izolata te bakterije podrijetlom iz ljudi bili su analizirani lančanom reakcijom polimerazom na prisutnost gena koji kodiraju za Panton-Valentine leukocidin (*PVL*) i PBP2A protein odgovoran za meticilinsku rezistenciju. Prisutnost gena koji kodira za *PVL* dokazana je u pet od 75 (6,67%) izolata podrijetlom iz goveda i

u sedam od 11 (63,63%) izolata podrijetlom iz ljudi. Prisutnost *mecA* gena dokazana je u jednog od 75 (1,33%) izolata podrijetlom iz goveda i u dva od 11 (18,18%) podrijetlom iz ljudi. Prisutnost *mecC* gena nije utvrđena u analiziranim izolatima. Potrebna su daljnja istraživanja kako bi se istražio odnos između izolata *Staphylococcus aureus*-a podrijetlom iz ljudi i iz goveda i odredio točan utjecaj sojeva vrste *Staphylococcus aureus* iz vimena krava na zdravlje životinja i zdravlje ljudi.

Ključne riječi: krave, vime, mlijeko, Panton-Valentine leukocidin, rezistencija na meticilin