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Short Communication

Occurrence of methicillin-resistant *Staphylococcus aureus* in milk samples from Serbian cows with subclinical mastitis

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The objective of this study was to analyze the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) from cattle with subclinical mastitis in two dairy herds in the northwest of Serbia. All quarters reacting positive in the California Mastitis Test were sampled for bacteriological analysis. Nasal and vaginal swabs from MRSA positive cattle, and nasal swabs from humans working on farms were also collected. From 1026 cows, 212 (20.7%) suffered from subclinical mastitis. *S. aureus* was detected in milk samples from 84 (39.6%) cows with subclinical mastitis. Among them, MRSA were isolated from 5 (5.9%) cows. Three out of five positive cows harboured MRSA in their nose and 1 cow harboured MRSA in the vagina. No MRSA was found in human nasal swabs. Seven out of 10 MRSA isolates in farm A, and two MRSA isolates in farm B, were resistant to gentamicin and tobramycin and exhibited SCC*mec* type IV. The three other isolates in farm A were resistant to tetracycline and carried SCC*mec* elements of type V. To our knowledge, this is the first description of MRSA from bovine subclinical mastitis in Serbia. The results of our study herald the emergence of MRSA in dairy farms in Serbia, so continued surveillance is recommended.

Key words: Methicillin-resistant *Staphylococcus aureus*, staphylococcal cassette chromosome *mec* typing, dairy cattle.

INTRODUCTION

Staphylococcus aureus is one of the most important pathogen in dairy cattle mastitis (Tenhagen et al., 2006; Piepers et al., 2007). Resistance of *S. aureus* to antimicrobial agents can complicate treatment of its infections (Lowy, 2003). Resistance to methicillin and other beta-lactams is due to presence of a modified penicillin binding protein (PBP 2a), which has a reduced affinity for beta-lactams. This protein is encoded by the *mec*A gene, located on a mobile genetic element called the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). MRSA isolated from bovine mastitis was first reported in 1972 (Devriese et al., 1972). After this first report, MRSA has been described in mastitis only occasionally (Kwon et al., 2005; Lee, 2006; Juhász-Kaszanyitzky et al., 2007; Moon et al., 2007; Fessler et al., 2010; Vanderhaeghen et al., 2010). From such studies, it seems that the prevalence of MRSA in mastitis is generally low.

Mastitis as a disease, especially the subclinical form, has received little attention in Serbia. Some studies on the prevalence and the major cause of bovine mastitis in the country have been conducted so far (Radanovic et al., 2011). The current study is the first report of MRSA isolation in milk samples from cows with subclinical mastitis in Serbia.

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MATERIALS AND METHODS

In 2009, a total of 1026 dairy cattle of two conventionally producing dairy farms (A, n = 674 cows, B, n = 352 cows) in the northwest of Serbia were examined. All tested dairy cattle were of Holstein Friesian breed and were apparently healthy with clinically sound udder secreting normal milk.

After udder sanitation, appraisal and discarding of foremilk, the California Mastitis Test (CMT) was performed for each quarter, as previously described (Leslie et al., 2002). Each positive CMT milk sample was collected under aseptic conditions in a sterile screw caped bottle numbered to identify the particular quarter. All milk samples were transported to the laboratory in ice cooled containers with a minimum of delay for routine culture techniques.

Milk samples were streaked onto Columbia agar plates (Torlak, Serbia) containing 5% sheep blood, MacConkey agar plates (HiMedia, India) and Baird-Parker (BP) agar plates (HiMedia, India). After incubation at 37°C for 24 h, the colonies were presumptively identified according to morphologic features, pigment production, Gram stain results, catalase test results, type of haemolysis and characteristic growth on BP agar. The isolates initially characterized as staphylococci were tested by coagulase tube test, BBL Crystal Gram-Positive ID Kit (Becton Dickinson, USA) and PCR for *nuc* (Brakstad et al., 1992) to confirm their identification as *S. aureus*. All *S. aureus* isolates were tested for presence of *mec*A gene by PCR (Bignardi et al., 1996). Antimicrobial drug susceptibility of MRSA isolates was determined by VITEK2 System (bioMérieux, France) using AST-P580 cards: benzylpenicillin, cefoxitin screen, clindamycin, erythromycin, fosfomycin, fusidic acid, gentamicin, levofloxacin, linezolid, moxifloxacin, mupirocin, nitrofurantoin, oxacillin, rifampicin, teicoplanin, tetracycline, tigecycline, tobramycin, trimethoprim/sulfamethoxazole and vancomycin. All cards were processed according to the manufacturer's directions. For interpretation of results, Clinical and Laboratory Standard Institute (CLSI, 2009) breakpoints were used. Determination of the SCC*mec* types was performed by a multiplex PCR (Boye et al., 2007). *S. aureus* strains HT20020290, HT20020285, HT20030826, HT20040068, HT20060580, and HT20020274 served as control strains.

In order to investigate the possible source of MRSA, we collected nasal and vaginal swabs from MRSA positive cattle, and nasal swabs from humans working on farms using sterile cotton swabs (Copan, Italy). (The study was approved by Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade, Serbia). All swabs were cultured in Mueller-Hinton broth (bioMérieux, France) containing 6.5% NaCl at 37°C for 24 h. 50 µl of broth was then streaked out on chromogenic media plate (MRSA-ID, bioMérieux, France). After incubation of 24 and 48 h at 37°C, green colonies were tested for the presence of *nuc* and *mec*A genes using PCR and further investigated as previously described.

RESULTS

From 1026 cows tested in this investigation, 212 (20.7%) suffered from subclinical mastitis. *S. aureus* was detected in milk samples from 84/212 (39.6%) cows. Among them, MRSA strains were isolated from 5 (5.9%) cows. In farm A, 4 (2.6%) cows were MRSA positive: three had MRSA in two quarters (both hind quarters) and one in one quarter (right hind quarter). In farm B, only one (1.8%) cow had MRSA in one quarter (right front quarter).

Three out of 5 positive cows harboured MRSA in their nose: two cows from farm A and the cow from farm B. One cow from farm A harboured MRSA in vagina. No MRSA was found in nasal swabs from humans.

All MRSA isolates detected in this study (n = 12) were confirmed by PCR for *nuc* and *mec*A genes. In farm A, seven out of 10 MRSA isolates were resistant to gentamicin and tobramycin and carried SCC*mec* type IV. The three other isolates discovered from a single cow (two from hind quarters and one from nose) were resistant to tetracycline and carried SCC*mec* type V. In farm B, two MRSA isolates were resistant to gentamicin and tobramycin and carried SCC*mec* type IV.

DISCUSSION

Given the importance of *S. aureus* as a cause of mastitis in cattle and the widespread usage of intramammary antibiotics in this species, it is perhaps not astounding that the first isolations of MRSA from animals were in milk from mastitic cows (Devriese et al., 1972). Following the initial report of MRSA in dairy cows, sporadic cases of MRSA mastitis in dairy cattle were described, typically at a low prevalence, among *S. aureus* isolates from clinical or subclinical mastitis. The long-term low prevalence of MRSA mastitis is quite surprising given the number of years since the first identification of MRSA in cattle and the close contact of humans with the udders of dairy cattle. One of the limiting factors contributing to the low prevalence of MRSA mastitis may be omitting routine susceptibility testing of *S. aureus* isolates to methicillin, or an appropriate analogue such as cefoxitin, in many laboratories worldwide.

The prevalence of methicillin resistance in *S. aureus* isolated from cows with subclinical mastitis within two Serbian dairy farms was 5.9%. This is a higher resistance rate than in the case of bacteria isolated from bovine mastitis in Korea, which showed 2.8% methicillin resistance (Moon et al., 2007). Nevertheless, reports can be found in which a higher prevalence of MRSA among *S. aureus* isolated from mastitis cases is described. In Turkey, the prevalence of methicillin resistance in *S. aureus* isolated from mastitis milk samples was 17.5% (Turutoglu et al., 2006). However, the detection in the Turkish study was based on phenotypic disk diffusion testing. Performing only phenotypic tests has previously been shown to lead to false positive or false negative results (Murakami et al., 1991; De Oliveira et al., 2000). Generally, it is now accepted that the detection of the *mec*A gene is the most reliable method to prove methicillin resistance, although new diagnostic guidelines for the detection of MRSA should consider the inclusion of tests for a novel *mec* gene (*mec*C or *mec*A_{LGA251} gene) (García-Álvarez et al., 2011). Still, even than, it remains difficult to make viable comparisons, due to differences in sampling methodology or a lack of information on the source of the strains (Vanderhaeghen et al., 2010).

As it was shown that within-cow transmission between quarters likely occurs in *S. aureus* mastitis (Barkema et al., 1997), the fact that one cow carried MRSA in only

one quarter could mean, that this isolate might be only a contaminant. However, *S. aureus* infection of only one quarter also certainly exists. Moreover, *S. aureus* was shown to more frequently infect the right and hind quarters. Seven out of eight MRSA isolates from milk samples in this study were originated from hind quarters while the eighth MRSA isolate was from a right front quarter.

This study was mainly undertaken to estimate the prevalence of MRSA in the udder of cows. However, nasal and vaginal colonization was also observed in lactating cows. Further studies are required to determine the importance of other body sites and the environment as a potential reservoir for MRSA in dairy herds.

In conclusion, concerns about MRSA in animals are reasonable and require careful study of various aspects to better understand the emergence and dissemination of MRSA in different species, including cows. Considering the fact that this is the first report of MRSA from bovine mastitis in Serbia, the actual presence of this pathogen in bovine mastitis in Serbia should urgently be studied in more depth, in order to profoundly assess its possible burden.

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