Occurrence and Characteristics of Methicillin Resistant *Staphylococcus aureus* and Methicillin Resistant Coagulase-negative Staphylococci in Raw Milk Manufacturing

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Abstract

Vyletělová M., Vlková H., Manga I. (2011): **Occurrence and characteristics of methicillin resistant *Staphylococcus aureus* and methicillin resistant coagulase-negative staphylococci in raw milk manufacturing**. Czech J. Food Sci., **29** (Special Issue): S11–S16.

For monitoring the occurrence of MRSA (methicillin resistant *Staphylococcus aureus*) and MR-CNS (methicillin resistant coagulase-negative staphylococci), cow's, goat's, and sheep's milks (bulk milks and individual samples) were investigated. Human nasal and throat swabs of the farm staff and nasal swabs of animals were also investigated as well. In total 1729 samples were examined and 634 strains were isolated by means of the cultivation method and used in this study. Generic identification of the staphylococci isolates was done performed by biochemical tests and all *S. aureus* and CNS isolates were checked by the PCR method for the presence of *mecA* gene which is responsible for methicillin resistance. The presence of the staphylococcal cassette chromosome *mec* (SCCmec), Panton-Valentine leukocidin (*pvl*) and genes encoding toxic shock syndrome toxin (*tst*) was detected in all strains confirmed as MRSA. The species were also examined for antimicrobial susceptibility by using disk diffusion method with antibiotic disks. *S. aureus* was the most frequently identified species from the samples tested (n = 557; 32.2%), followed by *S. haemolyticus* (n = 32; 1.9%), *S. chromogenes* (n = 24; 1.4%), *S. epidermidis* (n = 20; 1.2%), and *S. caprae* (n = 1; 0.16%). Among the resistant staphylococci (n = 49), *S. aureus* (n = 25; 51%) was found the most frequently, followed by *S. epidermidis* (n = 17; 34.7%), *S. chromogenes* (n = 6; 12.2%), and *S. haemolyticus* (n = 1; 2%). The resistant *Staphyloccocus* sp. occurred mainly in cow's milk (MRSA, *S. epidermidis*, *S. chromogenes*, *S. haemolyticus*) and in animal's swabs (*S. epidermidis*). One MRSA was also found in goat's milk and one was isolated from human swab. No resistant strains were found in sheep's milk. The negative results of the analysed genes presence (*pvl, tst*) were identical with all MRSA tested. The staphylococcal cassette chromosome *mec* (SCC*mec*) was classified as type IV or V.

Keywords: *Staphylococcus*; bulk milk; cow; sheep; goat

Staphylococcus aureus is one of the most important mammary gland pathogens responsible for bovine mastitis that can cause enormous economic losses to dairy farmers (Hata *et al.* 2008). *S. aureus* can gain access to milk either by direct excretion from udders or by secondary contamination (Scherrer *et al.* 2004). Moreover, *S. aureus* is an important causal bacterium of various human diseases such as impetigo, abscesses, endocarditis, toxic shock syndrome, foodborne intoxication, and staphylococcal scalded skin syndrome (Ladhani *et al.* 1999; Dinges *et al.* 2000). Several studies

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QH81111, by the Ministry of Education, Youth and Sports of the Czech Republic, Projects No. MSM 2678846201 and No. MSM LA10030.

suggest that the transfer of *S. aureus* between humans and cows is also possible (Matos *et al*. 1991; Roberson *et al*. 1994; Zadoks *et al*. 2002).

Methicillin-resistant *S. aureus* (MRSA) were found primarily in humans, later they were detected also in animals (Lee *et al*. 2004). In recent years, the increase of staphylococci strains that show resistance to methicillin/oxacillin has become a serious clinical and epidemiological problem. MRSA strains harbour the *mecA* gene, which encodes a modified PBP2 protein with a low affinity for methicillin and all β-lactam antibiotics (Velasco *et al*. 2005). It is assumed that methicillin-resistance encoding gene has evolved in coagulase-negative staphylococci (CNS) (Archer *et al*. 1994; Barbier *et al*. 2010). Furthermore, *S. aureus* has developed multidrug resistance with a wide variation from herd to herd (Waage *et al*. 2002).

Methicillin-resistant *S. aureus* are divided into three groups according to their epidemiological and genetic characteristics: hospital acquired (HA-MRSA), community-associated (CA-MRSA), and livestock-associated (LA-MRSA). The groups differ in sensitivity to antibiotics, in the location and size of the chromosomal cassette (SCC*mec*), and in the presence of Panton-Valentine leukocidin gene (PVL) (Karpíšková *et al*. 2009; Huber *et al*. 2010).

The objective of this study was to characterise the phenotypic and genotypic traits and antibiotic resistance of staphylococci isolates obtained from milk and human and animal swabs.

MATERIAL AND METHODS

Samples. Milk samples (Table 1) obtained from farms (n = 139) in the Czech and Slovak Republic and used in this study were collected from the herds of Holstein cows (bulk milk samples n = 703; individual composite samples n = 724), goat herds – White short-haired breed (bulk milk samples n = 1; individual samples n = 75), and from sheep herds – Tsigai breed (bulk milk samples n = 8; individual samples n = 89). The sampling from small ruminants was carried out from the whole dairy herd. The collection of the individual milk composite samples from dairy cows was focused on the animals with suspected disease according to NK-test results (Ryšánek & Renda 1971).

Human nasal and throat swabs of the farm staff (n = 18) and nasal swabs of animals (cow's = 59; sheep's = 19; goat's = 3) were taken and transported in the Amies medium (Med-Lab trade, s.r.o., Brno, Czech Republic). The swabs were transported to the laboratory for further procedure in a cooling box.

Detection and identification of staphylococci. Milk samples (1 ml) were enriched in 9 ml and swabs in 5 ml of Mueller-Hinton broth (BioRad, Hercules, USA) supplemented with 6.5% NaCl. After incubation at 37°C for 20 h, 1 ml was enriched in 9 ml of tryptone soya broth with antibiotics (TSB + 3.5 mg/l cefoxitin + 75 mg/l aztreonam) (LabMediaServis, Jaroměř, Czech Republic) and incubated at 37°C for 20 h and then inoculated in parallel onto Baird-Parker medium (Oxoid, Basingstoke, UK) and selective chromogenic medium MRSAselect™ (BioRad, Redmond, USA) and ORSAB (Oxoid, Basingstoke, UK). The suspected colonies from all types of media were then inoculated onto Blood agar (Oxoid, Basingstoke, UK), cultivated at 37°C for 24 h and subsequently identified biochemically using the STAPHYtest with the identification programs TNW Pro 7.5 (Erba Lachema, s.r.o., Brno, Czech Republic) and BIOLOG III (Biolog Ltd., Hayward, USA). The tentatively identified *S. aureus* isolates were confirmed by the multiplex PCR method for the detection of the species specific fragment SA442 (Martineau *et al*. 1998). PCR for coagulase negative staphylococci was performed using the PPP polymerase (Top-Bio, Prague, Czech Republic) and primers synthetised by the Generi Biotech Ltd. (Hradec Králové, Czech Republic). MRSA and MR-CNS were screened for the presence of *mecA* gene which encodes the resistance to methicillin (Boşgelmez-Tinaz *et al*. 2006).

For all strains confirmed as MRSA, the analysis followed of the staphylococcal cassette chromosome *mec* (SCCmec) type according to Milheirico *et al*. (2007), then Panton-Valentine leukocidin (*pvl*) by Lina *et al*. (1999), and the presence of genes encoding toxic shock syndrome toxin (*tst*) by Mehrotra *et al*. (2000).

Antibiotic susceptibility of staphylococci isolates. All identified staphylococci species were examined for antimicrobial susceptibility by disk diffusion method with antibiotic disks (Oxoid, Basingstoke, UK) as follows: vancomycin (30 μg), amoxicillin/clavulanic acid (20/10 μg), rifampin (5 μg), oxacillin (1 μg), tetracycline (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), clindamycin (2 μg), gentamicin (10 μg), ciprofloxacin

Table 1. Sample type, indentification of S*taphylococcus* sp. and their resistance to methicillin

Sample	Origin	Number of samples	Identification	Number of isolates	MRS (%)	MRS from isolated MRS (%)
Cow milk	bulk milk	703	*S. aureus*	326	20 (6.1)	40
			S. epidermidis	7	7 (100)	14
			S. haemolyticus	1	0 (0)	0
	individual	724	*S. aureus*	180	3 (1.7)	6
			S. epidermidis	4	1 (0.3)	2
			S. haemolyticus	29	1 (3.5)	2
			S. chromogenes	24	6 (25)	12
Goat milk	bulk milk	1	*S. aureus*	1	0 (0)	0
	individual	75	*S. aureus*	20	1 (5)	2
			S. epidermidis	3	3 (100)	6
Sheep milk	bulk milk	38	*S. aureus*	11	0 (0)	0
	individual	89	*S. aureus*	1	0 (0)	0
			S. haemolyticus	2	0 (0)	0
			S. caprae	1	0 (0)	0
Nasal swabs	animals	81	*S. aureus*	15	0 (0)	0
			S. epidermidis	6	6 (100)	12
Nasal and throat swabs	humans	18	*S. aureus*	3	1 (33.3)	2
Total		1729		634	49 (7.7)	100

(15 μg), teicoplanin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), and co-trimoxazole (25 μg). Mueller Hinton Agar (HiMedia, India) was used to perform the assay. The results were interpreted according to CLSI standards (Clinical and Laboratory Standards Institute, USA, 2006).

RESULTS AND DISCUSSION

Totally 634 staphylococci were isolated out of 1729 different samples (Table 1). *Staphylococcus aureus* (557 isolates) was the most frequent species in all the samples. The methicillin-resistant *S. aureus* (n = 23) were mostly isolated from cow's milk samples and there was only one isolated strain of MRSA from goat's milk samples. One MRSA was isolated from human swabs as well (a person of the goat farm). *S. epidermidis* (n = 17) was the most common species identified among coagulase-negative staphylococci (70.8% of identified MR-CNS). No resistant strain was found in sheep's milk but 6 methicillin-resistant *S. epidermidis* were isolated from sheep's nasal swabs (Table 1).

Similar results of *S. aureus* occurrence were obtained in another work (Vyletělová 2009; Vyletělová *et al.* 2010). *S. aureus* (together with *Streptococcus uberis*) was the main pathogen caus-

Table 2. Genotypic characteristics of MRSA isolates

Origin of isolates	*mecA*[3]	*tst*[4]	*pvl*[5]	*SCCmec* IV[6]	*SCCmec* V
BM[1] – cows	+	–	–	13[7]	7
IM[2] – cows	+	–	–	1	2
BM – goat	+	–	–	1	–
Human sample	+	–	–	1	–

[1]bulk milk samples; [2]individual milk samples; [3]presence of *mecA* gene; [4]presence of gene encoding toxic shock syndrome toxin 1; [5]presence of gene encoding Panton-Valentine leukocidin; [6]type of staphylococcal cassette chromosome *mec;* [7]number of strains

Table 3. Resistance to antimicrobials in methicillin-resistant staphylococci (No. (%) of methicillin/oxacillin-resistant species)

Antimicrobial agents	*S. aureus*	*S. epidermidis*	*S. haemolyticus*	*S. chromogenes*
Oxacillin (100 %)	25	17	1	6
Tetracycline	25 (100)	7 (41)	1 (100)	0
Erythromycin	2 (8)	3 (18)	1 (100)	0
Chloramphenicol	0	1 (6)	0	0
Co-trimoxazole	6 (24)	5 (29)	1 (100)	0
Amoxicillin/clavulanic acid	25 (100)	7 (41)	1 (100)	0
Clindamycin	1 (4)	4 (24)	0	0
Gentamicin	14 (56)	3 (18)	1 (100)	0
Ciprofloxacin	0	0	1 (100)	0
Vancomycin	0	0	0	0
Teicoplanin	0	0	0	0
Rifampin	2 (8)	3 (18)	0	0
Cefoxitin	21 (84)	8 (47)	0	0
Cefotaxime	22 (88)	3 (18)	0	0

ing mastitis in cows and small ruminants in the Czech Republic. In addition to the findings of *S. aureus*, other isolates of coagulase-negative staphylococci (CNS) were recorded, especially *S. haemolyticus*. Contrary to our results, in the study of Moroni *et al*. (2005) coagulase-negative staphylococci were reported to be the most common pathogens causing mastitis (in 96% of infections). In the study carried out by Pitkälä *et al*. (2004), 12 661 milk samples were collected from 3282 dairy cows at 216 farms in Finland. Coagulase-negative staphylococci remained the most common bacterial group (16.6%), whereas the relative number of the *Staphylococcus aureus* isolates decreased since the time of the previous study. MRSA has become a widespread problem in Korea. The rate of methicillin resistance among human *S. aureus* isolates in Korea is over 50% (Lee *et al*. 2001). In the case of the nasal swabs, MRSA alone was carried by 56 (8.2%) of 680 patients in UK (Dall'Antonia *et al*. 2005). Interesting facts appeared in the study by Hata *et al*. (2008) who did not isolate MRSA and MR-CNS from Japanese dairy farms or dairy cows.

The results of genotyping characteristics of MRSA confirmed are summarised in Table 2. The positive results of *mec*A gene presence and the negative results of the presence of any other genes analysed (*pvl, tst*) were identical with all strains tested. The staphylococcal cassette chromosome *mec* (*SCCmec*) was classified as type IV or V. Similar results for LA-MRSA are described in the works by Karpíšková *et al*. (2009), Šťástková *et al*. (2009), and Huber *et al*. (2010).

The results concerning antimicrobial susceptibility are shown in Table 3. The *mecA*-positive methicillin-resistant strains isolated in this study showed the following frequencies of resistance to another 13 antibiotics. All isolated MRSA were resistant to tetracycline and amoxicillin, 84% of them were resistant to cefoxitin and 88% to cefotaxime. Methicillin-resistant *S. epidermidis* strains showed frequent resistance to cefoxitin (47%), amoxicillin (41%) and tetracycline (41%). All MRSA and *S. epidermidis* isolates were susceptible to ciprofloxacin, vancomycin and teicoplanin, and furthermore MRSA isolates were susceptible to chloramphenicol. The resistance of *S. aureus* can be also caused by transfer of resistance from *S. epidermidis* (Forbes & Schaberg 1983). These authors described the transfer of resistance plasmids (gentamicin, erythromycin, tetracycline). Bloendaal *et al.* (2010) confirmed the hypothesis of *SCCmec* horizontal transfer from *S. epidermidis* to methicillin-susceptible *S. aureus* (MSSA), the latter becoming MRSA.

S. aureus are frequently resistant to other antibiotic agents in clinical use, including β-lactams, fluorquinolones, aminoglycosides, rifampin, and mupirocin (Carbon 2000). *S. epidermidis* strains

have acquired resistance to several antibiotics as well, including gentamicin, tetracycline, chloramphenicol, erythromycin, clindamycin, and sulphonamides (Rogers *et al*. 2009). Recent findings of the resistance patterns among methicillin-resistant coagulase-negative staphylococci are frequent. In the Netherlands, 71% of the coagulase-negative staphylococci strains resistant to methicillin were also resistant to gentamicin, 30% to clindamycin, and 37% to ciprofloxacin (De Neeling *et al.* 1998). In Korean dairy farms with livestock with mastitis problems, antibiotics (penicillin, ampicillin) are largely used for the treatment, although oxacillin and methicillin are rarely used because of the increasing incidence of MRSA strains (Lee 2003).

CONCLUSION

The determination and elimination of mastitis pathogens have been considered the most important steps for the prevention of the pathogens expansion to other animal species, humans involved in animal care and in food processing. The increasing occurrence of MRSA and MR-CNS should be under consideration from the point of view of antibiotic selection for mastitis treatment, especially if the possibility exists of the resistance transfer in or between microbial species.

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Received for publication July 25, 2011
Accepted after corrections November 23, 2011

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