

## Occurrence of *Staphylococcus aureus* in cattle, sheep, goat, and pig rearing in the Czech Republic

Marcela Klimešová<sup>1</sup>, Ivan Manga<sup>2</sup>, Ludmila Nejeschlebová<sup>1</sup>, Jiří Horáček<sup>2</sup>, Antonín Ponižil<sup>2</sup>, Eva Vondrušková<sup>1</sup>

<sup>1</sup>Dairy Research Institute, Prague, Czech Republic

<sup>2</sup>Agritec Plant Research, Šumperk, Czech Republic

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### Abstract

The study focused on the prevalence of *Staphylococcus aureus* and resistant strains in livestock. In this study, 114 different samples from three cattle farms (84 from two farms of dairy cows and 30 from one farm of suckler cows), 132 samples from one sheep farm, 120 samples from one goat farm, and 82 samples from three pig farms were examined. Strains identified as *Staphylococcus aureus* were further analysed by the polymerase chain reaction method for detection of the *mecA* gene and for confirmation of the sequence type 398. Positive incidence of *Staphylococcus aureus* was confirmed in farms of suckler cows, sheep, goats and pigs. The incidence of methicillin-resistant *Staphylococcus aureus* was confirmed at a goat farm, with all strains belonging to the sequence type 398. Repetitive element palindromic-polymerase chain reaction analysis was performed to compare the relatedness of selected human and animal *S. aureus* strains at the goat and sheep farms. The obtained data from repetitive element-polymerase chain reaction analysis showed significant clonal similarity among the tested isolates and indicated the possibility of mutual transmission between animals or animal and human and possible transfer in the food chain.

*Breeding environment, food animals, methicillin resistant S. aureus*

*Staphylococcus aureus* is a gram-positive bacterium commonly found on the skin or mucous membranes of both humans and animals. It is associated with many diseases, from less serious skin problems to very serious infections such as bacteraemia or pneumonia.

Problems arise during the treatment, in case methicillin-resistant *S. aureus* (MRSA) strains occur (Naimi et al. 2003). Despite all the measures taken in livestock breeding, the incidence of *S. aureus* persists in the environment, in milk, on the animal's body, and also in humans (Golding et al. 2010; Harrison et al. 2014). Methicillin-resistant *S. aureus* strains were detected in humans and in animals, including those that are intended for the production of food, especially in pigs (Bardoň et al. 2012; Zutic et al. 2012; Kolář et al. 2008). It was shown that infected animals can spread resistant strains not only in humans but also in raw food materials intended for further processing and for consumption (Janštová et al. 2014; Trnčíková et al. 2010; Normanno et al. 2007; van Loo et al. 2007; Lončarić et al. 2013; Jackson et al. 2013; Paterson et al. 2013; Harrison et al. 2013), or as well as in the case of other raw materials, e.g. spices (Klimešová et al. 2015). The incidence of MRSA has been reported in cattle, horses, small ruminants, camels, poultry but especially pigs (Stastkova et al. 2009; Köck et al. 2009; Alzohairy 2011; Harrison et al. 2014). Based on the EC Commission Decision 2008/55, the European Union countries conducted the first study on the prevalence of MRSA in pigs in 2008. Samples were taken from the stable environment of various pig farms. The results of monitoring in the Czech Republic showed the incidence of MRSA in 1.8% of samples (5/283) and all isolates of MRSA were assigned in a single sequence type ST 398. Regarding legislative measures

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#### Address for correspondence:

Marcela Klimešová  
Dairy Research Institute, Ltd.  
Ke Dvoru 12a, 160 00 Prague, Czech Republic

Phone: +420 608 260 395  
E-mail: [marcela.vyletelova@seznam.cz](mailto:marcela.vyletelova@seznam.cz)  
<http://actavet.vfu.cz/>

for the occurrence of *Staphylococcus aureus* and their enterotoxins in raw materials or foodstuffs, the criteria for coagulase-positive staphylococci and/or enterotoxins apply for cheese, milk powder, whey and products from crustaceans and molluscs in the Czech Republic (according to Commission Regulation (EC) no. 2073/2005; Annex 2.2 and 2.4). Microbiological criteria and limit values for microorganisms identified as agents of foodborne diseases (such as *Staphylococcus aureus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Salmonella* spp., *Escherichia coli*, and others) are defined in the Czech standard CSN 56 9609 (2008, Annex B). For coagulase-positive staphylococci, tolerance levels are given for meat and meat products, fish, milk and dairy products, egg products, ice cream, pasta, bakery and confectionery products, starch and potato products, food supplements, dehydrated products for the preparation of subsequent meals, spices, delicatessen products and semi-finished products. Newly in 2015, the control of *S. aureus* was introduced in raw milk, and the limit of 500 cfu in 1 ml was determined (Regulation no. 11/2015, Annex no. 6).

The work is focused on the prevalence of *S. aureus* and resistant strains (methicillin-resistant *S. aureus*, MRSA) in livestock breeding (cattle, sheep, goats and pigs).

### Materials and Methods

#### Origin and sampling

Samples were obtained from animals (milk, nostril swabs, rectum surroundings), from the stable staff (hands, nasopharynx, nasal swabs), from the excrements and from different locations of the rearing. The farms breeding cattle (Holstein, Blonde d'Aquitane), sheep (Tsigaiia), goats (short white and brown) and pigs were located in Moravia in the Czech Republic. The monitored animals were not treated with antibiotics and were not in the protective period as a result of antibiotic treatments. The examined persons were in contact with animals for about 8 h a day (on cattle, sheep, and pig farms) and for 12 h in the case of goat farm. A total of 114 samples were collected from the cattle rearing environment (60 animal samples, 10 environmental, 2 excremental and 12 samples from 4 persons from two different dairy farms; 20 animal samples, 3 environmental, 1 excremental and 6 samples from 2 persons from one farm with suckler cows); 132 samples from one sheep farm (90 animal samples, 15 environmental, 3 excremental and 24 samples from 4 persons); 120 samples from one goat farm (90 animal samples, 15 environmental, 3 excremental and 12 samples from 2 persons); and 82 samples from three pig farms (66 animal samples, 9 environmental, 1 excremental and 6 samples from 2 persons). The origin of the samples is presented in detail in Table 1.

Table 1. List of collected samples.

Animal	Farm/Animals (n)	Milk	Nostrils	Rectum	Environment	Persons/Samples	Excrements
Dairy cows	2/20	20	20	20	10	4/12	2
Suckler cows	1/10	xxx	10	10	3	2/6	1
Sheep	1/30	30	30	30	15	4/24	3
Goats	1/30	30	30	30	15	2/12	3
Pigs	3/66	xxx	66	xxx	9	2/6	1

xxx = samples were not obtained

Milk samples of 10 ml were collected into sterile sample containers from the whole udder. Samples from nasal mucosa were taken using swabs with Amies transport medium. For sampling from the rectum and the environment sponges were used (3M Sponge-Stick 1.5 × 3 Sponge/10 ml buffered peptone water). Faecal samples from the cattle, sheep and goats from different places (stable, space in front of the parlour, in parlour, feeding, milking equipment, barriers) were collected into sterile bags. The samples were stored in a thermobox with a temperature of 6 ± 2 °C and processed immediately after transport to the laboratory.

#### Microbiological analyses

Samples (sponges, swabs, and faeces) were thoroughly homogenised in buffered peptone water BPW (swabs in 13 ml and the others in 30 ml of BPW; HiMedia, India) and then 5 ml of suspension was pipetted into 5 ml

of salty broth (SB, Mueller Hinton Broth + 6.5% NaCl, Merck, Germany). The prepared samples were incubated at  $36 \pm 1$  °C for 24 h and then were subcultured by 1 ml into 9 ml of tryptic soy broth (TS broth) with antibiotics aztreonam AZT and cefoxitin CXT (LabMediaServis TS broth + AZT + CXT, Czech Republic) and again cultivated at  $36 \pm 1$  °C for 24 h. Thereafter, they were plated onto surface “Brilliance” MRSA2 Agar (MRSA2, Oxoid, UK) and again cultivated at  $36 \pm 1$  °C / 24 h.

In parallel with this procedure, the samples suspended only in BPW were cultured at  $36 \pm 1$  °C for 24 h and (without subsequent enrichment in the SB and TS broth) they were plated also onto the surface of baird parker agar (BP agar; HiMedia, India) and incubated at  $36 \pm 1$  °C / 24 h. Suspected colonies from MRSA2 and BP agar were subcultured on blood agar (Oxoid, UK), incubated at  $36 \pm 1$  °C for 24 h, and then identified using biochemical tests STAPHYtest and identification application software (TNW pro7.0, Erba Lachema, Germany).

Strains identified as *S. aureus* were confirmed by polymerase chain reaction (PCR) method for detection of specific deoxyribonucleic acid (DNA) *S. aureus* fragment SA442 (Martineau et al. 1998), and they were subjected to testing for resistance to antimicrobial agents using disc diffusion method (Clinical and Laboratory Standards Institute - CLSI 2012, Table 2). The resistance/sensitivity of isolated *S. aureus* was observed for selected antibiotics: OX (oxacillin), T tetracycline, E (erythromycin), DA (clindamycin), SXT (trimethoprim-sulfamethoxazole), AMC (amoxicillin-clavulanic acid), C (chloramphenicol), CN (gentamicin), CIP (ciprofloxacin), VAN (vancomycin), TEC (teicoplanin), RD (rifampicin), FOX (cefoxitin), NV (novobiocin used for *S. aureus* identification).

The presence of the *mecA* gene was confirmed in all strains identified as *S. aureus* by PCR method (regardless of the result of resistance to oxacillin; Boşgelmez-Tinaz et al. 2006). In case of positive MRSA strains, it was detected whether they belong to the sequence type 398 (van Wamel et al. 2010).

Milk samples were inoculated directly (without the enrichment process) on the surface blood and BP agar. After incubation at  $36 \pm 1$  °C for 24 h, the procedure for species identification, confirmation of MRSA and determination of susceptibility to oxacillin was identical with the above described procedure.

### Molecular typing

Two persons (one at goat and one at sheep farm) were identified to be positive for *S. aureus* in their nasal mucosa or throat. We subsequently selected five representative *S. aureus* strains isolated from animals and milk, featured with a similar pattern of antibiotic susceptibility to those two strains isolated from humans. The analysed samples and their origins are shown in Tables 2 and 3.

Table 2. Origin of chosen *S. aureus* for repetitive element palindromic-polymerase chain reaction (rep-PCR) analysis.

Strain no.	Origin	Farm
584	Nostrils	goat
922	Milk	
956	Personnel nose	
625	Milk	sheep
881	Nostrils	
882	Nostrils	
910	Personnel nasopharynx	

Repetitive element palindromic-polymerase chain reaction (rep-PCR) analysis of selected *S. aureus* was performed with regard to comparing the clonal relationship of these strains and thus investigating the possibility of transmission between humans and animals on the farms. The strains were typed with the rep-PCR method using (GTG)<sub>5</sub> primer as described before (Manga et al. 2015). The obtained image data with fingerprints were processed using the BioNumerics v. 6.6 software (Applied Maths NV, Belgium). The unweighted pair-group method using arithmetic averages (UPGMA) clustering method with the Dice correlation coefficient and 2% of Tolerance coefficient was utilized for interpretation of the results (BioNumerics, Applied Maths NV, Belgium). The fingerprints with similarity  $\geq 90\%$  were considered to represent clusters with significant clonal similarity.

Table 3. Prevalence of *S. aureus* and methicillin resistant *S. aureus* on farms.

	Goats		Sheep		Pigs		Suckler cows		Dairy cows
	SA	MRSA	SA	MRSA	SA	MRSA	SA	MRSA	SA
Milk	1	-	2	-	xxx	xxx	0	-	0
Nostrils	12	2	7	-	10	-	0	-	0
Rectum	2	1	1	-	xxx	xxx	0	-	0
Environment	4	3	1	-	0	-	0	-	0
Excrements	0	-	0	-	0	-	0	-	0
Personnel	7	3	6	-	0	-	1	-	0

SA = *Staphylococcus aureus*, MRSA = methicillin resistant *S. aureus*

Note: all MRSA strains belong to ST398

## Results

### Occurrence of *S. aureus* and MRSA

The results of *S. aureus* and resistant strains of MRSA prevalence on the monitored farms are summarized in Table 3. Positive incidence of *S. aureus* was confirmed in sheep, goat, and pig farms both in animal and human samples. In case of cattle rearing (suckler cows), *S. aureus* was confirmed only in the nasopharyngeal swab from one person. Negative results in cows may be explained by the fact that only a part of all animals was investigated, and also that high standards of hygiene during milking and a mastitis control program are applied there. According to documentation, higher incidence of environmental pathogens, mainly *Streptococcus uberis*, was observed there (personal communication). The negative incidence in suckler cows can also be explained by good keeping conditions. The highest incidence of *S. aureus* and MRSA was confirmed repeatedly in goat farming. There, 26 strains were isolated, mostly from the nostrils of animals ( $n = 12$ ). Also, the incidence of MRSA was confirmed only in this farm, and all the strains belong to the sequence type 398 (2 MRSA from the nostrils, 1 from the rectum, 3 from the environment, and 3 from the same person). Due to the recurrence of MRSA in both animals and personnel, we can assume that a spread of one strain occurs here.

The prevalence of *S. aureus* in sheep was lower ( $n = 17$ ), with the highest incidence detected in the nostrils ( $n = 7$ ), as in goats. None of the *S. aureus* strains were confirmed as MRSA.

Similar results were obtained in pig farming. The occurrence of *S. aureus* was detected only in the nostrils ( $n = 10$ ) and none of the *S. aureus* strains were confirmed as MRSA.

### Sensitivity to antibiotics

The results of sensitivity to antibiotics are summarized in Table 4. Higher incidence of resistant strains was found in goat breeding, in which resistance to 7 tested antibiotics (OX, T, SXT, AMC, CN, CIP, FOX) was detected. The highest resistance was confirmed in AMC (46.2%) and in OX, T, and FOX (all 34.6%). In sheep, we identified resistance to two antibiotics (T, AMC) and the higher rate was for AMC (47.1%), as in goats.

Comparison of the resistance/sensitivity in animals (milk, nostrils, rectum) and in human samples showed comparable results regarding the type of antibiotics. However, different (higher) resistance was evident for AMC, CN, and FOX in human isolates and conversely, higher resistance in case of T and CIP in strains isolated from animals. In our study, the results acquired using both methods (disc diffusion and PCR) were identical.

### Rep-PCR cluster analysis

Data obtained from rep-PCR cluster analysis showed substantial similarity of strains isolated from both the goat and sheep farm. Strain no. 922 isolated from milk at the goat farm showed significant similarity to human strain no. 956. Furthermore, strains no. 881 (animal nostrils) and 910 (human) from the sheep farm showed significant clonal similarity. This result may indicate a possible transfer of *S. aureus* between animals and humans. Moreover, the macrorestriction profiles of strains no. 625 (sheep milk) and 882 (sheep nostrils) from two different animals were identical and therefore refer to an identical clone. Our observation indicates that *S. aureus* of animal origin was capable of further spreading at the farm and contaminating the food chain (milk, meat).

## Discussion

The incidence of *S. aureus* and MRSA strains on dairy farms (cow, goat, and sheep) and pig farms is still a widespread problem throughout the world. Vyletelova et al. (2011)

Table 4. Resistance of *S. aureus* to antimicrobial agents (summary; in %).

	OX	T	E	DA	SXT	AMC	C	CN	CIP	TEC	RD	FOX	NV
Goat farm	n	9	9	0	0	1	12	0	5	1	0	9	0
(n = 26)	%	34.6	34.6	0	0	3.8	46.2	0	19.2	3.8	0	34.6	0
Sheep farm	n	0	3	0	0	0	8	0	0	0	0	0	1
(n = 17)	%	0	17.6	0	0	0	47.1	0	0	0	0	0	5.9
Pig farm	n	0	7	0	9	0	0	3	0	7	0	0	0
(n = 10)	%	0	70	0	90	0	0	30	0	70	0	0	0
Cow farm	n	0	0	0	1	0	0	1	0	0	0	0	0
(n = 1)	%	0	0	0	100	0	0	100	0	0	0	0	0
Animals	n	6	14	0	0	0	10	3	1	7	0	3	1
(n = 35)	%	17.1	40	0	0	0	28.6	8.6	2.9	20	0	8.6	2.9
Humans	n	3	3	0	1	0	7	1	3	1	0	3	0
(n = 14)	%	21.4	21.4	0	7.1	0	50	7.1	21.4	7.1	0	21.4	0
Environment	n	0	2	0	0	1	3	0	1	0	0	3	0
(n = 5)	%	0	40	0	0	20	60	0	20	0	0	60	0
Total	n	9	19	0	10	1	20	4	5	8	0	9	1
(n = 54)	%	16.7	35.2	0	16.0	1.6	37.0	7.4	9.3	14.8	0	16.7	1.6

OX1 (oxacillin), T30 tetracycline, E15 (erythromycin), DA2 (clindamycin), SXT25 (trimethoprim-sulfamethoxazole), AMC20 (amoxicillin-clavulanic acid), C30 (chloramphenicol), CN10 (gentamicin), CIP5 (ciprofloxacin), VAN30 (vancomycin), TEC30 (teicoplanin), RD5 (rifampicin), FOX30 (cefoxitin), NV5 (novobiocin for identification)

monitored 703 bulk milk samples from dairy cows in the Czech Republic, and isolated 326 *S. aureus* strains, of which 20 (6.1%) were confirmed as MRSA. Bogdanovičová et al. (2015) investigated 212 samples of bulk tank milk from 25 dairy farms also in the Czech Republic and detected *S. aureus* in 68 cases (32.1%). Overall, only one MRSA strain was found in this study. Ben Slama et al. (2011) investigated the incidence of MRSA in 423 people in their work. A positive result was found also in one man, namely a veterinarian. From 3,687 human samples Golding et al. (2010) isolated 5 strains LA-MRSA (livestock-associated methicillin resistant *S. aureus*) that belonged to ST 398-associated *spa* types. The prevalence of *S. aureus* in sheep was studied also in our previous work Vyletětlová et al. (2011a). They confirmed the presence of MRSA also in cow's and goat's milk. No MRSA was found in sheep's milk. The occurrence of MRSA was not confirmed on pig farms in our study, either. Bardoň et al. (2012) performed the first nationwide survey on the prevalence of MRSA

in herds of breeding pigs in the Czech Republic. The MRSA isolates were detected with a prevalence of 1.8%. These results confirm that MRSA incidence does not constitute a serious risk for the human population in case of pig farms.

We have confirmed a higher incidence of strains resistant to OX, T, SXT, AMC, CN, CIP, and FOX in goats, and to AMC, OX, T, and FOX in sheep. In our previous work, we found strains with low resistance to E, DA, CN, CIP, and FOX but no resistance to OX, T, and AMC in goat's and sheep's milk (Vyletětlová et al. 2011b). This study confirms the

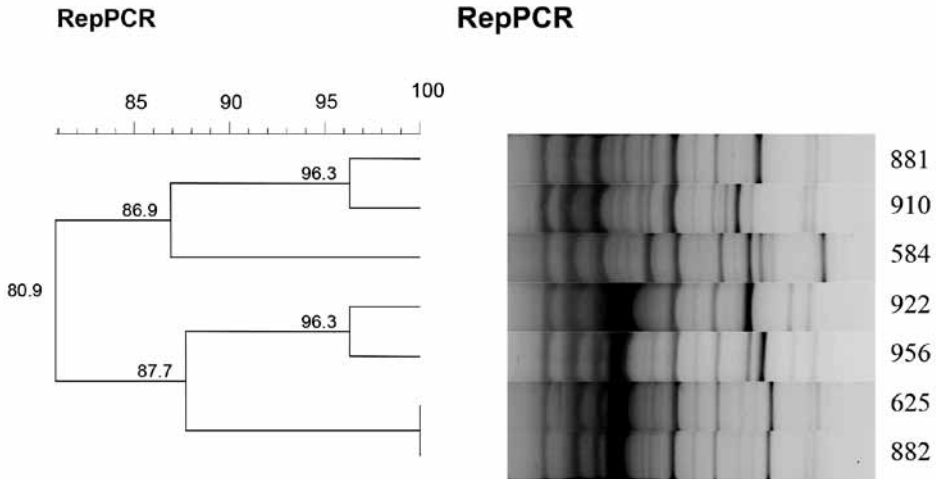


Fig. 1. Dendrogram of repetitive element palindromic-polymerase chain reaction (rep-PCR) with (GTG)<sub>5</sub> primer

increasing number of resistant strains in animal production. In pigs, the highest resistance was confirmed in case of DA, T, and CIP. Ben Slama et al. (2011) detected the percentages of antimicrobial resistance (resistance genes) for T (22%), E (5%), CIP (14.5%) and SXT (2%), similarly to our study of humans isolates.

The oxacillin-susceptible methicillin-resistant *Staphylococcus aureus* (OS-MRSA) and the difference in results between methods (PCR method and disc diffusion method) have been already reported. Vyletelova et al. (2011a) confirmed 23 isolated strains as MRSA by the PCR method (*mecA* gene), but 25 strains were resistant to oxacillin by the disk diffusion method. Saeed et al. (2014) also found six (1.2%) OS-MRSA isolates phenotypically identified and reported as MRSA by conventional methods, originated from human samples. The occurrence of OS-MRSA means a hidden resistant mechanism that can have the impact on the correct treatment of *S. aureus* infections. For this reason, the PCR method resistance identification appears to be more reliable.

Reinoso et al. (2007) used also rep-PCR for determination of the similarity in *S. aureus* strains isolated from bovine mastitis. They found most of the strains (75%) to be grouped in one cluster. Their results confirmed that the genotypes were similar in different herds. Manga et al. (2015) analysed *S. aureus* strains from different types of spice. The rep-PCR analysis with the (GTG)<sub>5</sub> primer showed that the tested strains belonged to only two strain clusters, moreover exhibiting similarity just below the significance level at a given setting. This indicated one source of *S. aureus* contamination. Manga and Vyletlová (2012) used rep-PCR also for characterization of MRSA originating from farms and humans. Analysis of fingerprints with the unweighted pair-group method using arithmetic averages (UPGMA) clustering method revealed the presence of six strain clusters with a similarity below 85%. The fingerprint of MRSA strain infecting the human differed significantly from the remaining fingerprints. This indicated the strains of the farm origin were not the source of the infection in the human. Nováková (2007) described the utilization of rep-PCR for the identification of clinically important species of the genus *Staphylococcus*. The cluster analysis showed that all included species formed the species-specific and clearly distinguishable profiles. Švec et al. (2010) confirmed also the suitability of rep-(GTG)<sub>5</sub>-PCR assay for rapid and reliable identification of *Staphylococcus* spp.

The results indicate that the prevalence of *S. aureus* was higher in breedings of small ruminants. Resistant strains of *S. aureus* were confirmed on the goat farm both in animal and in human samples, and belonged to the sequence type 398. The molecular methods used confirmed the possibility of a mutual transmission between animals, and between animals and humans.

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