

Prevalence of mastitis pathogens in milk from clinically healthy cows

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ABSTRACT: A total of 669 individual cow milk samples originating from asymptomatic cows from 16 dairy farms were examined for the presence of microorganisms with the potential to cause mastitis. Coagulase-negative staphylococci clearly predominated (53.5% positive samples) followed by streptococci and enterococci (both occurring in 16.1% samples). Among streptococci, so-called mastitis streptococci (*S. uberis*, *S. dysgalactiae* and *S. agalactiae*) prevailed (11.7% positive samples). *Enterobacteriaceae* were found in 10.0% samples, most of which (6.6% samples) were positive for *Escherichia coli*. Yeasts (mainly *Candida* spp.) were found in 8.2% samples. One of the major mastitis pathogens, *Staphylococcus aureus* subsp. *aureus*, was isolated from 9.0% of samples. *S. aureus* isolates were further characterised in terms of their capability to form biofilm, antimicrobial susceptibility and clonality (PFGE). All *S. aureus* isolates were capable of biofilm formation and were generally susceptible to the majority of tested antibiotics. The exception was ampicillin, resistance to which was observed in 27.7% isolates. Therefore, the relatively frequent occurrence of *S. aureus* could be attributed to persistent intramammary infections due to biofilm formation rather than low efficacy of particular antibiotics. PFGE analysis revealed clonal spread of certain *S. aureus* isolates within and between farms indicating that certain lineages of *S. aureus* mastitis strains are particularly successful.

Keywords: mastitis; bovine; intramammary; IMI; etiology; epidemiology; macrorestriction

Bovine mastitis is a large-scale infectious disease with significant impact on the economy of milk production (Halasa et al. 2007; Awale et al. 2012). Besides health disorders of the mammary gland, mastitis can also cause significant losses in milk yield, alterations in its quality (impaired nutritive and technological properties of milk), fertility disorders and even systemic diseases (Gruet et al. 2001; Bradley 2002; Malinowski and Gajewski 2010; Le Marechal et al. 2011; Awale et al. 2012). Moreover, causative agents of mastitis with zoonotic potential may represent a health risk for human populations via the food chain (Bradley 2002).

Numerous microorganisms have been described as causative agents of bovine mastitis (Watts 1988;

Bradley 2002). According to their epidemiology, mastitis pathogens can be divided into contagious and environmental. The primary reservoir of contagious pathogens is an infected udder whereas a contaminated environment is the primary reservoir of pathogens causing environmental mastitis. *Streptococcus agalactiae*, *Staphylococcus aureus* subsp. *aureus* and *Mycoplasma* spp. are considered as typical contagious pathogens. Typical environmental pathogens are so-called environmental streptococci (streptococci other than *S. agalactiae* such as *Streptococcus uberis*; enterococci), *Enterobacteriaceae* and coagulase-negative staphylococci (CNS). *Streptococcus dysgalactiae* has been most commonly described as a contagious patho-

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gen but it can also behave as an environmental pathogen (Gruet et al. 2001; Bradley 2002; Barkema et al. 2009). Similarly, contagious transmission has also been reported in some CNS (Gillespie et al. 2009). Pathogens such as *Pseudomonas* spp., *Pasteurellaceae*, some pyogenic and anaerobic bacteria, yeasts and algae number among those which occur infrequently. In recent times, there is clear evidence for an increasing incidence of environmental mastitis while the incidence of contagious mastitis has decreased (Bradley 2002; Rysanek et al. 2007).

Mastitis can appear in a clinical and subclinical form, the latter being commonly found in most herds (Gruet et al. 2001; Awale et al. 2012). There is a known relationship between particular pathogens and the form of the disease. For example, *S. uberis*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa* and pyogenic bacteria are mainly considered as causative agents of clinical mastitis. On the other hand, *S. agalactiae*, CNS and *Enterococcus* spp. are associated with subclinical mastitis (Bradley 2002; Barkema et al. 2009; Awale et al. 2012). However, *S. aureus* has been designated as a causative agent of both clinical (Gruet et al. 2001) and subclinical (Awale et al. 2012) mastitis. Unlike the clinical form of the disease, subclinical mastitis is hard to recognise, and for this reason it may cause significant losses in milk production. Moreover, subclinically infected cows may repre-

sent a source of particular pathogens that can be spread via automatic milking systems (Barkema et al. 2009; Hovinen and Pyorala 2011). Therefore, to evaluate the prevalence of subclinically infected cows and their significance as a potential source of mastitis pathogens, we analysed in this study individual milk samples from animals expressing no clinical signs of the disease. Since *S. aureus* is the principal mastitis pathogen in the Czech Republic (Rysanek et al. 2007), isolates of this species were further analysed.

MATERIAL AND METHODS

Sampling and sample preparation. A total of 669 individual cow milk samples originating from 16 dairy farms were collected during 2012. The number of samples collected from the different farms is listed in Table 1. The samples were taken shortly prior to milking and only cows expressing no clinical signs of mastitis were sampled. Sampling of milk and subsequent processing of the samples were done according to the standards EN ISO 6887-2 and EN ISO 6888-3. The samples were then selectively cultivated as described below.

Isolation and confirmation of selected mastitis pathogens. The prevalence of *Staphylococcus* spp., *Enterobacteriaceae*, *Pseudomonas* spp., *Streptococcus* spp., *Enterococcus* spp., yeasts and moulds

Table 1. Numbers of samples positive for the monitored microorganisms

	Dairy farm																Total	
	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12	F13	F14	F15	F16	n	%
<i>Staphylococcus aureus</i>	23	7	0	1	2	1	7	0	1	1	0	14	0	0	3	0	60	9.0
Other CPS	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	4	0.6
CNS	16	25	11	15	39	79	43	18	11	24	1	23	17	7	21	8	358	53.5
Mastitis streptococci ¹	10	2	0	10	13	8	4	4	0	12	0	6	0	0	4	5	78	11.7
Other streptococci	2	2	2	4	3	9	1	0	0	3	0	0	3	0	4	1	34	5.1
<i>E. faecalis</i> , <i>E. faecium</i>	9	3	2	4	12	3	3	1	2	1	0	4	2	0	3	1	50	7.5
Other enterococci	2	15	3	3	2	13	3	3	4	7	0	0	3	0	4	0	62	9.3
coliform <i>Enterobacteriaceae</i>	1	16	18	2	8	3	1	0	0	3	0	2	0	0	2	0	56	8.4
non-coliform <i>Enterobacteriaceae</i>	0	0	9	0	2	0	0	2	0	1	0	0	0	0	2	2	18	2.7
<i>Pseudomonas aeruginosa</i>	0	0	0	0	0	0	4	6	0	1	0	1	0	0	0	0	12	1.8
Yeasts	6	17	0	1	4	12	0	0	0	1	0	2	2	0	5	5	55	8.2
Number of samples	34	38	30	33	80	110	59	25	18	25	5	40	30	30	56	56	669	100

CPS = coagulase-positive staphylococci (all *Staphylococcus hyicus*); CNS = coagulase-negative staphylococci

¹*Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*

was monitored in this study. One hundred µl of the analytical sample were cultivated on Baird-Parker (Merck, Darmstadt, Germany) and Kranep (HiMedia, Mumbai, India) agars for isolation of *S. aureus* and CNS, respectively, MacConkey agar (Bio-Rad, Marnes-la-Coquette, France) for isolation of *Enterobacteriaceae* and *Pseudomonas* spp., and Edwards Medium Modified (EMM) agar (Oxoid, Basingstoke, UK) for isolation of *Streptococcus* spp. and *Enterococcus* spp. Dichloran Rose Bengal Chloramphenicol (DRBC) and Sabouraud agars (both LabMediaServis, Jaromer, Czech Republic) were used for isolation of yeasts and moulds. The agars were cultivated aerobically at 37 °C for 20 h (MacConkey), 36 h (EMM) and 44 h (Baird-Parker, Kranep). DRBC (incubated at 25 °C) and Sabouraud (incubated at both 25 and 37 °C) agars were cultivated for one week with the first evaluation after 48 h of growth. Morphologically distinct colonies suggestive of the monitored species were further examined. For identification of *Staphylococcus*, *Enterococcus* and *Streptococcus* species STAPHYtest 24, EN-COCCUStest and STREPTOtest 24 were used, respectively (all Pliva-Lachema, Brno, Czech Republic). *Enterobacteriaceae* and *Pseudomonas* spp. were identified using ENTEROtest (Pliva-Lachema). In samples containing 10³ or more CFU/ml of yeasts, these were identified at a genus level using the AUXACOLOR™ 2 system (Bio-Rad). Isolates of *S. aureus* were additionally confirmed using PCR (Martineau et al. 1998) and further analysed as described below.

PCR based analysis of samples. A total of 53 randomly selected samples were subjected to qPCR analysis using a commercially available PathoProof Mastitis PCR Assay (Thermo Fisher Scientific, Vantaa, Finland). DNA from samples was isolated using the PathoProof DNA Extraction kit according to the manufacturer's instructions. Using the PathoProof Mastitis Complete-12 kit the presence of the following microorganisms was monitored: *S. aureus*, CNS group, *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *Enterococcus* spp., *E. coli* and *Klebsiella oxytoca*/*Klebsiella pneumoniae*. The PCR conditions were according to the manufacturer's instructions. The PCR was carried out on a Bio-Rad Chromo4 real-time PCR detection system using the Opticon Monitor 3.1. (Biorad, Benicia, USA). The data were analysed using the software Norden Lab Mastitis Studio General Edition 1.5.1. (Thermo Fisher Scientific).

Pulsed-field gel electrophoresis (PFGE). *S. aureus* isolates were subjected to PFGE analysis as previ-

ously described (Jaglic et al. 2010). The isolates were limited to one per sample except for five pairs of morphologically distinct isolates originating from five different samples. Briefly, DNA was digested with 8 IU of *Sma*I (New England BioLabs, Hitchin, UK) at 25 °C for 18 h. Electrophoresis was carried out on the CHEF-DR III System (Bio-Rad) with a voltage of 5.5 V/cm for 21 h with an initial switch time of 0.5 s, increasing to 50 s. Restriction endonuclease patterns (PFGE-types) were analysed with Gel Compare software (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient and the UPGMA algorithm with 1% tolerance and 0.5% optimisation settings.

Biofilm. The ability to form biofilm was tested in *S. aureus* isolates using polystyrene microtitration plates for tissue culture (Becton Dickinson Labware, Pont-de-Claix, France) according to Cucarella et al. (2001). The isolates were also examined for the presence of biofilm genes using PCR. To amplify a part of the *ica* operon, one of the essential factors involved in biofilm formation, *icaAB-F* and *icaAB-R* primers were used (Frebouret et al. 2000). The biofilm-associated protein gene (the *bap* gene) was amplified with the forward primer 5'-AATTTTCAGCGAATCCTTCTGG-3' and reverse primer 5'-TGCACCTCCAAATAAACCATC-3'. Each PCR was performed in a total volume of 40 µl containing 1 × DyNAzyme II PCR buffer and one unit of DyNAzyme II DNA polymerase (both from Finnzymes, Vantaa, Finland), 94 µM of each dNTP (Jena Bioscience, Jena, Germany) and 0.5 µM of each primer. The PCR amplification was performed in a PTC-0220 DNA Engine Dyad Thermal Cycler (Bio-Rad) under the following conditions: initial denaturation at 98 °C for 2 min, followed by 45 cycles of denaturation at 96 °C for 10 s, annealing at 59 °C for 10 s, and extension at 72 °C for 40 s with final extension at 72 °C for 2 min. *Staphylococcus epidermidis* CCM7221 and *S. aureus* V329 were used as positive PCR controls for *ica* and *bap*, respectively.

Antimicrobial susceptibility. Minimum inhibitory concentrations of ampicillin, ampicillin/sulbactam, cloxacillin, gentamicin, cotrimoxazol, tetracycline, clindamycin, neomycin, tylosin, cephalothin, cefotaxime, norfloxacin and vancomycin (all Sigma, St Louis, MO, USA) were determined for *S. aureus* isolates using the broth microdilution method according to the approved standard of the Clinical and Laboratory Standards Institute (CLSI document M07-A8). Susceptibility interpretation criteria were based on the CLSI guidelines

(documents M31-A3 and M100-S20). *S. aureus* isolates were further tested for inducible resistance to clindamycin by the D-test using erythromycin (15 µg) and clindamycin (2 µg) discs (both Oxoid) according to the CLSI document M100-S20. The isolates were also screened for potential methicillin resistance by testing their susceptibility to cefoxitin (30 µg disc, Oxoid) as recommended in the CLSI document M02-A10. Finally, using a nitrocefin-based test (Pliva-Lachema), *S. aureus* isolates were tested for the production of β-lactamases according to CLSI documents M07-A8 and M100-S20. When appropriate, *S. aureus* strains ATCC 25923, ATCC 43300 and ATCC 29213 served as reference strains for quality control purposes.

RESULTS

Prevalence of monitored microorganisms in individual cow milk samples

Table 1 summarises the prevalence of individual cow milk samples that were positive for the monitored microorganisms. The highest prevalence of positive samples was observed for CNS (358 samples; 53.5%). A total of 19 CNS species were identified, among which *Staphylococcus sciuri* prevailed (95 samples; 14.2%), followed by *Staphylococcus xylosum* (73 samples; 10.9%), *Staphylococcus arlettae* (56 samples; 8.4%), *Staphylococcus warneri* (47 samples; 7.0%), *Staphylococcus chromogenes* (39 samples; 5.8%) and *Staphylococcus epidermidis* (35 samples; 5.2%). A total of 55 samples (8.2%) were positive for non-typeable CNS. The same prevalence (108 samples; 16.1%) of positive samples was observed for

both streptococci and enterococci. Among streptococci, so called mastitis streptococci (*S. agalactiae*, *S. dysgalactiae* and *S. uberis*) clearly predominated, occurring in 78 (11.7%) samples. The most prevalent mastitis streptococcus was *S. uberis* (53 samples; 7.9%), followed by *S. dysgalactiae* (21 samples; 3.1%) and *S. agalactiae* (5 samples; 0.7%). Among other streptococci, *Streptococcus bovis* was most frequent (11 samples; 1.6%). *Enterococcus faecalis* was the most prevalent enterococcus species (44 samples; 6.6%), followed by *Enterococcus caecorum* (26 samples; 3.9%) and *Enterococcus solitarius* (10 samples; 1.5%). *Enterobacteriaceae* were found in 67 (10.0%) samples, most of which were positive for coliform enterobacteria (56 samples, 8.4%). Among coliform enterobacteria, *E. coli* prevailed (44 samples; 6.6%) while among non-coliform enterobacteria *Proteus* spp. was most frequent (8 samples; 1.2%). *S. aureus* occurred in 60 (9.0%) samples. Besides *S. aureus*, *Staphylococcus hyicus* was the only coagulase-positive staphylococcus found; it was detected in 4 (0.6%) samples. *P. aeruginosa* was isolated from 12 (1.8%) samples. A total of 55 (8.2%) samples were positive for yeasts (no moulds were isolated). Among samples ($n = 11$) containing 10^3 or more CFU/ml of yeasts, *Candida* spp. predominated (54.5% positive samples). In total, 159 (23.8%) samples were negative for the monitored microorganisms.

Comparison of cultivation and PCR-based analysis of samples

An overall correlation rate of 82% was observed between cultivation and PCR-based analysis of samples. The highest correlation rates were observed

Table 2. Comparison of cultivation and PCR-based analysis of 53 selected samples

	Culture+	Culture–	Total	Culture+	Culture–	Total	Culture+	Culture–	Total	Culture+	Culture–	Total
	<i>Staphylococcus aureus</i>			CNS			<i>Streptococcus agalactiae</i>			<i>Streptococcus dysgalactiae</i>		
qPCR+	16	5	21	21	15	36	3	4	7	3	5	8
qPCR–	4	28	32	5	12	17	1	45	46	1	44	45
Total	20	33	53	26	27	53	4	49	53	4	49	53
	<i>Streptococcus uberis</i>			Enterococcus spp.			<i>Escherichia coli</i>			<i>Klebsiella oxytoca/pneumoniae</i>		
qPCR+	22	5	27	5	5	10	3	7	10	3	2	5
qPCR–	3	23	26	12	31	43	2	41	43	1	47	48
Total	25	28	53	17	36	53	5	48	53	4	49	53

Culture +/- = positive/negative result of cultivation; qPCR +/- = positive/negative result of PCR-based analysis; CNS = coagulase negative staphylococci

with specific pathogens such as *K. oxytoca*/*K. pneumoniae* (94%), *S. agalactiae* (91%), *S. dysgalactiae* (89%), *S. uberis* (85%), *S. aureus* (83%) and *E. coli* (83%). Lower correlation rates were observed with *Enterococcus* spp. (68%) and the CNS group (62%) which could be explained by the wide range of detected species. Table 2 summarises the results of cultivation and qPCR. Except for CNS and *Enterococcus* spp., both methods gave comparable results, i.e., an agreement between cultivation and qPCR was observed in the majority of samples. On the other hand, cultivation and qPCR seemed to be more reliable for the detection of *Enterococcus* spp. and CNS, respectively.

Pulsed-field gel electrophoresis (PFGE)

Among the 65 *S. aureus* isolates, a total of 33 PFGE-types were found whereas four isolates were non-typeable. Discrimination of the isolates using a cut-off value of 79%, as proposed by Miragaia et al. (2008), revealed nine (I to IX) different clusters comprising 81.5% ($n = 53$) of the isolates (only those groups of isolates in which more than one PFGE-type were found were considered as clusters; Figure 1). This indicates generally close epidemiological relatedness among the *S. aureus* isolates originating from different dairy farms. Several different clusters were observed on one particular farm (i.e., farms F01, F07 and F12)

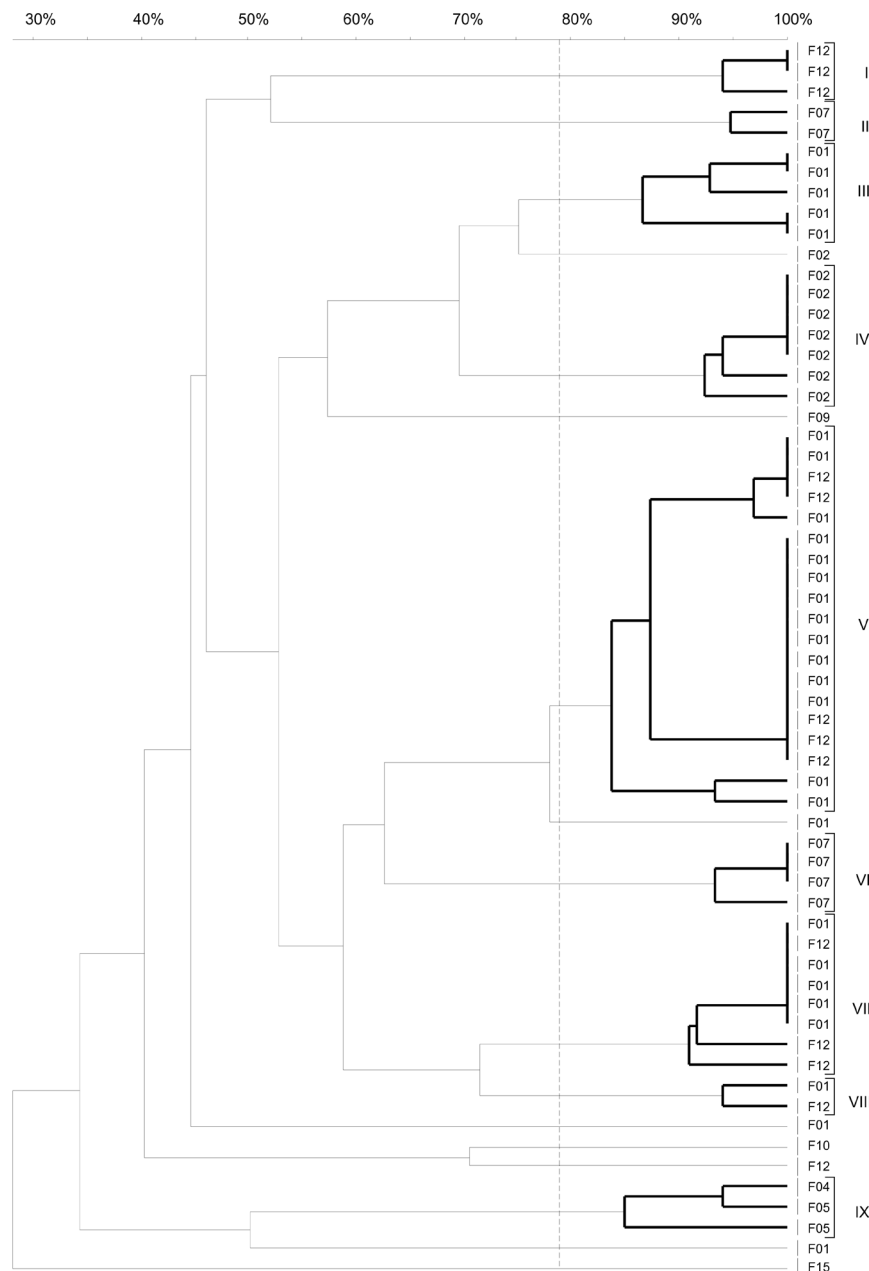


Figure 1. Dendrogram showing the levels of similarity among *SmaI* macrorestriction (PFGE) patterns of 61 *Staphylococcus aureus* isolates. The vertical dashed line indicates the cut-off value (set to 79%) discriminating particular clusters. The nine clusters are indicated with bold lines as well as Roman numerals I to IX. The origin of isolates is indicated by the farm code (e.g. F01) on the right side of the dendrogram

and some specific clusters occurred on more than one farm (i.e., clusters V, VII, VIII and IX). However, some clusters were specifically found only in one farm (i.e., clusters I, II, III, IV and VI were found in farms F12, F07, F01, F02 and F07, respectively). In addition, clonal spread of particular *S. aureus* isolates was typically observed in farm F01 and, to a certain extent, in farms F02 and F07. Moreover, three distinct PFGE clones were shared between farms F01 and F12.

Biofilm

The capability of biofilm formation at the phenotypic level was confirmed in all *S. aureus* isolates. In addition, all except for two isolates were positive for the *ica* operon by PCR. None of the isolates was positive for the *bap* gene.

Antimicrobial susceptibility

In general, *S. aureus* isolates were susceptible to the majority of tested antibiotics (Table 3). Resistance to ampicillin was most frequently observed (18 isolates; 27.7%) whereas resistance to other antibiotics was sporadic and observed only with tetracycline (seven isolates) and norfloxacin (three isolates). Nine isolates were intermediately

resistant to norfloxacin. Resistance to antibiotics belonging to two and three different classes was found in four and one isolate, respectively. Neither resistance to erythromycin nor clindamycin (including inducible resistance) was observed among the isolates. All isolates were susceptible to ceftiofur. It could be therefore concluded that the isolates harboured neither macrolide-lincosamide-streptogramin B (MLS_B) nor methicillin resistance. On the other hand, production of β -lactamase was frequently detected, i.e., in 40 (61.5%) isolates. Using the disc diffusion method, resistance to penicillin was confirmed in all β -lactamase-producing isolates (data not shown).

DISCUSSION

Although clinical mastitis may cause serious damage to the udder and even systemic disorders leading to the culling of affected animals, subclinical mastitis is in general a more insidious form of the disease because it is invisible to the farmer. This results in reduced milk quality and yield, which in turn leads to a reduction in the farmer's income as well as that of the dairy industry. This is mainly due to the prolonged diagnostics of the disease and long-term decrease in the milk yield and technological value (Mungube et al. 2005; Halasa et al.

Table 3. Minimum inhibitory concentrations (MICs; $\mu\text{g/ml}$) of 13 antimicrobial agents for *Staphylococcus aureus* isolates ($n = 65$)

	MIC range	MIC mod	MIC 50	MIC 90	S	I	R
Ampicillin	0.0625–2	0.25	≤ 0.25	≤ 1	47	0	18
Ampicillin/sulbactam	0.5–2	0.5	≤ 0.5	≤ 0.5	65	0	0
Cloxacillin	0.0625–0.25	0.125	≤ 0.125	≤ 0.25	65	0	0
Gentamicin	0.5–2	0.5	≤ 0.5	≤ 1	65	0	0
Cotrimoxazol	0.5–2	0.5	≤ 0.5	≤ 1	65	0	0
Tetracycline	0.5–64	0.5	≤ 0.5	≤ 16	58	0	7
Clindamycin	0.125–0.5	0.125	≤ 0.125	≤ 0.125	65	0	0
Neomycin	0.5–2	0.5	≤ 0.5	≤ 1	65	0	0
Tylosin	1–2	1	≤ 1	≤ 2	65	0	0
Cephalothin	0.25–0.5	0.5	≤ 0.5	≤ 0.5	65	0	0
Cefotaxime	1–2	1	≤ 1	≤ 1	65	0	0
Norfloxacin	0.5–64	4	≤ 4	≤ 8	56	6	3
Vancomycin	0.25–2	1	≤ 1	≤ 1	65	0	0

MIC mod = the MIC value most frequently occurred; MIC 50 = the MIC value for 50% isolates; MIC 90 = the MIC value for 90% isolates; S = susceptible isolates; I = intermediately resistant isolates; R = resistant isolates

2009). Moreover, unlike the clinical form of the disease, subclinical mastitis is widely spread among dairy herds (Gruet et al. 2001).

According to Awale et al. (2012), CNS are the most prevalent cause of subclinical mastitis. This is in accordance with the results of the current study, in which CNS also prevailed in cows showing no clinical signs of the disease. In recent times, the significance of CNS as causative agents of bovine subclinical mastitis has been well recognised (Taponen and Pyorala 2009). Either coagulase-positive staphylococci (CPS) or environmental pathogens have been described as the second most prevalent mastitis pathogens (Awale et al. 2012). In our study, environmental pathogens (such as *S. uberis*, *Enterococcus* spp., *Enterobacteriaceae*) prevailed compared to contagious pathogens (*S. aureus*, *S. agalactiae*), indicating that non-contagious transmission may play a dominant role in the occurrence of infection. This correlates with recent observations that the prevalence of environmental pathogens increases at the expense of contagious pathogens (Bradley 2002). This may further impede the early detection of infection since somatic cell count, a widely accepted criterion for measuring udder health, is a better tool for monitoring mastitis caused by contagious than that caused by environmental pathogens (Rysanek et al. 2007). It is also noteworthy that a range of environmental pathogens associated mainly with clinical mastitis were found to greater (*enterobacteria*, *S. uberis*) or lesser (*P. aeruginosa*) extents in asymptomatic cows. This further underlines the necessity of implementing proper hygiene management to prevent infection from the environment (McDougall et al. 2009).

In the current study, a relatively high occurrence of *S. aureus* was also observed. In fact, *S. aureus* was one of the most frequently isolated staphylococci (60 positive samples; 9.0%), supporting the assertion that this microorganism numbers among the main mastitis pathogens in the Czech Republic (Rysanek et al. 2007). It should, however, be mentioned that such levels of *S. aureus* are comparable with those described in some other European countries such as Denmark and Germany (Schwarz et al. 2010; Mahmmud et al. 2013). Biofilm formation in *S. aureus* is considered an important virulence factor in bovine mastitis. The ability of *S. aureus* to adhere to the mammary gland epithelium and form biofilms facilitates its persistence in the host due to the evasion of the immune response and increased tolerance to antimicrobials (Melchior et al. 2006;

Oliveira et al. 2006). In the current study, all *S. aureus* isolates were capable of biofilm formation and all except for two isolates were positive for the *ica* operon. However, unlike some CNS, the presence of *ica* in *S. aureus* does not seem to play a crucial role in biofilm formation (Cucarella et al. 2001).

Antimicrobial resistance represents a serious problem in the treatment of infectious diseases including mastitis. In recent times, an increasing antimicrobial resistance rate has been recognised in *S. aureus* from bovine mastitis (Saini et al. 2012; Wang et al. 2013). On the other hand and similarly to our findings, Oliveira et al. (2012) reported a relatively low occurrence of antimicrobial resistance in *S. aureus* isolates from clinical and subclinical mastitis. This may be surprising considering that antibiotics and chemotherapeutics have been commonly administered via the intramammary route for decades. We speculate that not only antimicrobial pressure but also other factors (such as colonisation of the mammary gland by resistant strains) may be involved in the epidemiology of antimicrobial resistance. In addition, methicillin resistance does not seem to be widely spread among *S. aureus* isolates from bovine milk, which is in accordance with our results as well (Lim et al. 2013; Unnerstad et al. 2013). This is in contrast to *S. epidermidis*, in which methicillin resistance was commonly found (Jaglic et al. 2010). However, similarly to the study of Overesch et al. (2013), we observed a high prevalence (61.5%) of β -lactamase-producing (penicillin-resistant) isolates. In any case, despite susceptibility to most of the antimicrobials tested, *S. aureus* was detected relatively frequently in our milk samples, which could be explained by its capability to form biofilms. As already reported, persistent or recurrent intramammary infections with *S. aureus* could be attributed to biofilm growth rather than the efficacy of particular antibiotics (Melchior et al. 2006).

We observed that the prevalence of *S. aureus* varied substantially between different farms. The majority of isolates (61.7%) originated from two farms (farms F01 and F12; Table 1). We observed that three distinct PFGE clones were shared between these two farms. In addition, three different PFGE clusters (V, VII and VIII) were common to both farms. These farms were approximately 3.5 km apart from each other and belonged to the same corporation. This strongly suggests that transmission of *S. aureus* occurs between these farms. On the other hand, other PFGE clusters (except for

cluster IX) were typically found only on one of the farms. Clonal spread of *S. aureus* mastitis isolates has been previously described at both the regional and farm level (Capurro et al. 2010; Fessler et al. 2010; Castelani et al. 2013). Such clonal spread indicates that certain lineages of *S. aureus* mastitis strains have proven to be particularly successful.

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