Short communication: Antimicrobial susceptibility profiling and genotyping of *Staphylococcus aureus* isolates from bovine mastitis in Poland

T. Jagielski,*¹ E. Puacz,† A. Lisowski,‡ P. Siedlecki,§ W. Dudziak,# J. Międzobrodzki,# and H. Krukowski‡

*Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, 02-096 Warsaw, Poland

†National Chamber of Medical Laboratory Specialists, 03-428 Warsaw, Poland

‡Department of Animal and Environmental Hygiene, University of Life Sciences, 20-950 Lublin, Poland

§Department of Plant Molecular Biology, Institute of Plant Experimental Biology and Biotechnology, Faculty of Biology, University of Warsaw, 02-096 Warsaw, Poland

#Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 30-387 Cracow, Poland

ABSTRACT

Staphylococcus aureus is the predominant causative agent of bovine mastitis, a disease that remains a major economic burden for the dairy industry worldwide. In this study, the antimicrobial resistance patterns and the genetic composition of 80 *S. aureus* mastitis isolates collected from 14 dairy farms in Eastern Poland were determined. Of the 10 antimicrobial agents evaluated, only testing for penicillin G produced drug resistance. As 41% of the *S. aureus* isolates were penicillin resistant, this drug along with other β -lactamase-sensitive β -lactams, should rather not be considered for the treatment of bovine mastitis caused by *S. aureus*. Upon genotyping, with a triplex PCR method, a total of 11 distinct PCR types were produced. The population structure of *S. aureus* isolates was highly clonal, with 1 predominant genotype circulating on each farm. The observed similarities in the genotype composition of *S. aureus* populations from geographically distant farms underscore the significance of interfarm transmission of *S. aureus* in Poland. This, in turn, argues for the establishment of a nationwide surveillance program for bovine mastitis due to this pathogen.

Key words: *Staphylococcus aureus*, bovine mastitis, drug resistance, genotyping

Short Communication

Bovine mastitis persists as the most prevalent disease of dairy herds worldwide and represents one of the most significant economic challenges for the global dairy industry. The total economic burden from mastitis, both clinical and subclinical, has been calculated at up to

€200 per cow yearly (Huijps et al., 2008). The losses are mainly due to a drastically decreased milk production and also due to the costs of veterinary treatment, early culling, and replacements. A wide spectrum of microorganisms has been implicated in the causation of bovine mastitis, including bacteria, viruses, fungi, and algae (Krukowski et al., 2006; Malinowski et al., 2006; Miranda-Morales et al., 2008; Tenhagen et al., 2009; Botrel et al., 2010; Persson et al., 2011). Yet, the vast majority of bovine mastitis cases are of bacterial etiology, with only a few species predominating. Among these, *Staphylococcus aureus* ranks first, producing prevalence rates of 10 to 30% in mastitis-affected cattle (Malinowski et al., 2006; Miranda-Morales et al., 2008; Tenhagen et al., 2009; Botrel et al., 2010; Persson et al., 2011). *Staphylococcus aureus* mastitis presents with varying degrees of severity, ranging from mild and self-limiting to rapidly progressing and fatal. However, the disease most often presents in a subclinical form, which usually evolves into chronic status, characterized by extensive fibrosis, microabscess formation, and occlusion of the ducts and alveoli of the secretory tissue (Zadoks et al., 2011). Of particular concern is that *S. aureus* mastitis infections are often refractory to standard intramammary treatment regimens; one reason for this is increased resistance of the staphylococci to different antimicrobial agents. Indeed, several studies have reported on *S. aureus* mastitis isolates resistant, at different levels, to a wide array of commonly used antibiotics (Malinowski et al., 2002; Moroni et al., 2006; Sakwinska et al., 2011b; Gao et al., 2012). Monitoring of drug resistance in *S. aureus* from dairy herds has been considered as an essential part of mastitis control strategies, as it guides the selection of the most appropriate and efficient therapy for staphylococcal mammary infections.

The advent of molecular typing methods has considerably contributed to the understanding of the epidemiology of many human and animal pathogens. Genotyping has also become an important clinical tool

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¹Corresponding author: t.jagielski@biol.uw.edu.pl

to investigate outbreaks, infection sources, and transmission routes of bovine mastitis caused by *S. aureus*. Recently, an increasing amount of research has been conducted into the molecular epidemiology of *S. aureus* mammary infections (Jørgensen et al., 2005; Peles et al., 2007; Ikawaty et al., 2009; Sakwinska et al., 2011b; Gao et al., 2012). However, molecular data on isolates from Poland are still relatively scarce. The purpose of this study was to determine the antimicrobial resistance patterns and the genetic composition of *S. aureus* mastitis isolates collected from dairy herds in Poland.

Study Design and Sampling

The study was conducted on 14 dairy farms (I–XIV) located in Lublin Province, Eastern Poland, over a 3-yr period (2007–2009). The herds comprised between 12 and 168 cows (median herd size of 33 cows) of the Polish Holstein-Friesian breed and raised under similar conditions of maintenance, feeding, and veterinary care. Throughout the study period, a total of 815 milk samples from 419 cows (out of 673 examined) with clinical and subclinical mastitis were tested. The diagnosis of mastitis was made on the basis of the California mastitis test, clinical examination of the udder, and microbiological examination of milk samples. The milk samples were collected aseptically into sterile glass vials from all quarters that were California mastitis test positive or upon clinical examination, or both. All milk samples for microbiological culturing were collected before milking. The surface of the teat ends was disinfected by wiping it with clean cotton dipped in 95% alcohol. The samples were cooled and immediately transported to the laboratory.

Isolation and Identification of *S. aureus*

Equal volumes (0.1 mL) of milk samples were inoculated onto 5% Columbia sheep blood agar (Merck KGaA, Darmstadt, Germany) plates and incubated at 37°C for 24 to 48 h. Colonies presumptive of staphylococci based on morphology, hemolysis, and Gram staining, were further tested for catalase, coagulase, and clumping factor production (Slidex Staph Plus; bioMérieux SA, Marcy-l'Étoile, France). Final species identification was achieved by using 2 commercially available biochemical identification systems: STAPHYtest 24 (Pliva-Lachema AS, Brno, Czech Republic) and Phoenix (Becton, Dickinson and Co., Franklin Lakes, NJ). Species assignment was valid if the identification results were congruent and the confidence levels of identification equaled or exceeded 98% in both systems.

Antimicrobial Susceptibility Profiling

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK) by the disc diffusion method, following the recommendations of the Clinical Laboratory Standards Institute (CLSI, 2012). The antimicrobial agents tested included penicillin (10 U), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), doxycycline (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), and ceftiofur (30 µg) as a surrogate marker for methicillin resistance. In addition, susceptibility to vancomycin was determined with E-test stripes (AB Biodisk, Solna, Sweden) in accordance with the manufacturer's instructions. Isolates were categorized as susceptible, intermediate, or resistant, under the Clinical Laboratory Standards Institute breakpoints (CLSI, 2012). The reference strain *S. aureus* ATCC 25923 served as quality control for the disc diffusion method, whereas vancomycin-susceptible *Enterococcus faecalis* ATCC 29212 and vancomycin-resistant *E. faecalis* ATCC 51299 strains were used to validate the performance of the Epsilometer test (E-test) assay.

Preparation of DNA

The bacterial genomic DNA was extracted using the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland), according to the protocol provided by the manufacturer. The purified DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and stored at –20°C until used.

Genotyping

Genotyping was carried out with a triplex PCR coamplifying 3 loci within the *S. aureus* genome; that is, part of the *coa* gene coding for coagulase, part of the *spa* gene coding for protein A, and part of the hypervariable region adjacent to the *mecA* gene, essentially as described elsewhere (Strandén et al., 2003). The DNA fingerprints were analyzed with the PyElph software (version 1.4; source code available from <http://sourceforge.net/projects/pyelph/>; Pavel and Vasile, 2012). A dendrogram was constructed by the unweighted pair group method with arithmetic averages (UPGMA) algorithm, with a band position tolerance of 5%. A cluster was defined as 2 or more isolates exhibiting 100% identity of their triplex PCR patterns, whereas those nonclustered were referred to as unique. Clusters were

designated after the respective PCR patterns, with Latin uppercase letters.

One hundred two (12.5%) of the quarter milk samples yielded *S. aureus* cultures, as evidenced on initial screening (positivity in both slide and tube coagulase tests). Eighty (78.4%) of these cultures were confirmed as *S. aureus* using the STAPHYtest 24 and Phoenix identification systems combined. Thus, the overall positive culture rate of *S. aureus* was 9.8% (80 positive samples out of 815 samples tested). The *S. aureus* isolates were recovered from all but 3 (IV, VI, and IX) dairy farms under the study, with 1 farm (XII) accounting for over one-third of the isolates (27/80).

All 80 *S. aureus* isolates were subjected to antimicrobial susceptibility testing, the results of which are presented in Figure 1. All of the isolates were fully susceptible to cefoxitin, chloramphenicol, gentamicin, doxycycline, ciprofloxacin, and sulfamethoxazole/trimethoprim. Likewise, all isolates were susceptible to clindamycin and erythromycin, although the susceptibility to these drugs was intermediate in 4 and 1 isolate, respectively. None of the isolates were resistant or intermediate resistant to vancomycin, as determined by the E-test method. The MIC values for this antibiotic were within the range of 0.38 to 2 µg/mL, with a mean value of 0.94 µg/mL. Penicillin was the only drug whose antistaphylococcal activity varied widely, with the inhibition zone diameters spanning from 6 to 52 mm. Forty-seven (59%) isolates were susceptible to penicillin, whereas the remaining 33 (41%) were penicillin resistant.

Genotyping was performed on 80 confirmed isolates of *S. aureus*. Three isolates did not yield any PCR amplification product and were, thus, excluded from the analysis. Among the remaining 77 *S. aureus* isolates, a total of 11 distinct PCR patterns (A–K) were produced (Figure 2). Of these, 7 were defined as many clusters containing 73 (94.8%) isolates, whereas 4 patterns were unique and corresponded to single isolates. The 2 largest clusters representing the 2 most prevalent PCR types (D and I) contained, each, 22 isolates. Clusters designated as A, J, and F contained 9, 8, and 7 isolates, respectively. Cluster B comprised 3 isolates, and cluster G comprised 2 isolates. Regarding the distribution of specific PCR types on different dairy farms, 7 types were observed on single farms only, 2 (A and D) on 4 farms, 1 (I) on 3 farms, and another (F) on 2 farms (Table 1). Of the 11 dairy farms positive for *S. aureus*, 5 (III, V, VII, XI, and XIV) were homogeneous in terms of their bacterial population (i.e., all *S. aureus* isolates within a farm had the same PCR type). On 3 farms, isolates of 3 PCR types were observed. On another 3 farms, 2 subpopulations of *S. aureus* isolates were found, each represented by a distinct PCR type.

The antimicrobial susceptibility testing demonstrated high activity of 9 out of 10 agents evaluated against *S. aureus* mastitis isolates. The only exception was penicillin G to which 41% of the staphylococci were resistant. Resistance to penicillin among *S. aureus* from bovine mastitis has been encountered with increasing frequency throughout the world. However, the reported prevalence rates have varied extensively in different geographical regions. The percentage of penicillin-resistant *S. aureus* isolates was found to be as high as 87% in China (Shi et al., 2010), 62% in Turkey (Turutoglu et al., 2006), and 61% in Estonia (Kalmus et al., 2011). A lower but still high percentage of penicillin resistance, similar to that found in the current study, was detected among *S. aureus* isolated from Argentina (40%; Gentilini et al., 2000), France (30%; Sakwinska et al., 2011b), and Hungary (30%; Peles et al., 2007). Contrastingly, in Germany, the United States, and Norway, the prevalence of penicillin-resistant *S. aureus* was low, with isolation rates of 17, 10, and 6%, respectively (Jørgensen et al., 2005; Anderson et al., 2006; Tenhagen et al., 2006). It is noteworthy that drug susceptibility patterns of bovine mastitis *S. aureus* determined in the current study somewhat mirrored those previously reported in Poland. According to several studies performed over the last decade, resistance to penicillin was consistently the most frequently observed phenotype, whereas resistance to other antimicrobial agents was much less common (Malinowski et al., 2002, 2008). However, when comparing more recent reports, including the present one, with those previously published from Poland, a downward shift in the frequency of drug resistance can be observed. For instance, penicillin resistance among *S. aureus* isolates has long remained at a level of around 70%, which is much higher than the 41% reported herein or 24% reported in a recent study of Szveda et al. (2014). Nevertheless, β -lactams are a group of antibiotics to which mastitic staphylococci are resistant most frequently and this is linked to the fact that β -lactams rank first regarding the number of compounds commercially available and in terms of their use in the treatment of bovine mammary infections.

Several molecular methods have been used over the last years for estimating the genetic diversity and population structure of *S. aureus* isolates from bovine mastitis cases. The methods most frequently applied include pulsed-field gel electrophoresis (PFGE; Jørgensen et al., 2005; Anderson et al., 2006; Peles et al., 2007; Rabello et al., 2007; Ikawaty et al., 2009; Kozytska et al., 2010), multilocus sequence typing (Jørgensen et al., 2005; Smith et al., 2005; Rabello et al., 2007; Ikawaty et al., 2009; Kozytska et al., 2010), coagulase gene (*coa*) typing (Schlegelová et al., 2003; Karahan and Cetinkaya, 2007; Dastmalchi Saei et al., 2009), and

IZD (mm)	Antimicrobial agent										MIC ($\mu\text{g/mL}$)
	PEN	FOX	CHL	ERY	CLI	GEN	DOX	CIP	SXT	VAN	
6	2									1	0.38
10											
12	2									9	0.5
13	5									24	0.75
14	3									35	1.0
15	1									9	1.5
16	6									2	2.0
17	2										4.0
18						1					8.0
19	1				2						16.0
20	1				1						
21					1	2					
22				1	3	3					
23	2		6	1	3	15					
24	1		11	1	13	18	1	1			
25	1	1	11	7	4	16	1				
26	2	1	8	10	15	15	3	3			
27	3	7	7	5	1	5	1		1		
28	1	10	25	29	15	2	8	6	5		
29	2	5	3	6	1		2	3	3		
30		23	7	10	20	1	16	8	2		
31	1	3		1			2	6	9		
32		9	1	6		1	19	12	16		
33	1	9	1	2			19	16	27		
34	1	5		1			2	10	10		
35		1				1	1	6	5		
36		2				1	2	3	1		
37								4			
38	1	3					3	1	1		
39											
40	6	1						1			
>40	35										
Mean	32.5	30.8	26.7	28.1	26.4	24.9	31.3	32.2	32.3	0.94	
IZD/MIC											
	(mm)										($\mu\text{g/mL}$)

Figure 1. Distribution of inhibition zone diameter values (or MIC values) of different antimicrobial agents for the 80 *Staphylococcus aureus* strains evaluated in the study. The MIC values refer only to vancomycin (VAN). The black squares represent resistant zone diameter break-points. The squares shaded in dark and light gray represent zone diameters in the resistant and intermediate range, respectively. IZD = inhibition zone diameter; PEN = penicillin; FOX = ceftiofur; CHL = chloramphenicol; ERY = erythromycin; CLI = clindamycin; GEN = gentamicin; DOX = doxycycline; CIP = ciprofloxacin; SXT = trimethoprim/sulfamethoxazole.

spa gene typing (Ikawaty et al., 2009; Sakwinska et al., 2011b). In the current study, a triplex PCR method, targeting 3 heterogeneous loci, namely the *spa* gene, the *coa* gene, and the hypervariable region adjacent to the *mecA* gene, was used. This method, designed as an alternative to the PFGE gold standard, provides an excellent tool for rapid and cost-effective typing of *S. aureus* in outbreak settings (Strandén et al., 2003; Sabat et al., 2006). The genotyping results showed a low level of genetic differentiation among the *S. aureus* isolates studied. With only 11 PCR types obtained, the overall genetic diversity rate, calculated as the number of different patterns divided by the number of isolates, was 14.3%. This was very similar to what was observed

in other studies that exploited the polymorphism of the *coa* gene. The genetic diversity rates in those studies ranged from 12.5 to 15.5% (Schlegelová et al., 2003; Karahan and Cetinkaya, 2007; Dastmalchi Saei et al., 2009). Much higher genotype diversity (26.4–47%) was demonstrated only when PFGE profiling was used (Peles et al., 2007; Rabello et al., 2007; Ikawaty et al., 2009; Kozytska et al., 2010). However, regardless of the genetic variation for the entire population, the genotype diversity for the individual farms was usually very low. In other words, a very limited number of genotypes existed among *S. aureus* isolates within farms. Moreover, on farms where 2 or more *S. aureus* genotypes were circulating, usually one clearly predominated

Figure 2. A dendrogram of triplex PCR patterns of the 77 *Staphylococcus aureus* isolates recovered from bovine mastitis cases. The boxed letters (A–K) designate clusters represented by different triplex PCR types.

over the others (Schlegelová et al., 2003; Rabello et al., 2007; Gao et al., 2012). In line with these observations are the findings in the present study. Here, up to 3 *S. aureus* genotypes were found on each of the farms evaluated. The majority of the genotypes (7/11; 63.6%) were unique to a particular dairy farm. Four genotypes were distributed among 2 to 4 different farms, some of which were located within an over-100-km radius of each other. The finding of the same genotype in such distant locations can be explained in various ways. First, the interregional herd movement might be the

cause. Second, the pathogen can be spread with humans as carriers. This hypothesis is likely, as cases of both human-to-cow and cow-to-human transmission have recently been evidenced (Sakwinska et al., 2011a). Finally, the retrieval of identical genotypes from widely separated locations can be explained by a phenomenon of homoplasy; that is, independent acquisition of similar structures without common ancestors. Another possible reason may relate to the insufficient discriminatory power of the genotyping method applied. Of particular notice are 2 *S. aureus* genotypes, designated as D

Table 1. Distribution of *Staphylococcus aureus* triplex PCR types among dairy farms evaluated in this study¹

Item	No. of <i>S. aureus</i> isolates with a PCR type:											No. of isolates	
	A	B	C	D	E	F	G	H	I	J	K	<i>S. aureus</i>	Non- <i>S. aureus</i>
Dairy farm													
I	1						2					3	0
II	5							1				6	1
III		3										3	0
IV												0	3
V				5								5 + <u>1</u>	3
VI												0	1
VII				8								8	2
VIII			1	5								6	1
IX												0	2
X				4	1						1	6	2
XI									3			3	1
XII						4			15	8		27	1
XIII	2					3			4			9 + <u>2</u>	4
XIV	1											1	1
No. of isolates	9	3	1	22	1	7	2	1	22	8	1	77 + <u>3</u>	22

¹Underlined numbers indicate isolates for which no PCR product was obtained.

and I, both accounting for 57% of the isolates tested. Genotype D was found on 4 dairy farms located in the same municipality, suggesting transmission of this type between the farms. Here, the direction of transmission of the staphylococcal infection might have followed the chronology of strain isolation (Table 2).

Genotype I was demonstrated on 3 farms, far away from each other, making the transmission less plausible. On those farms, where the genotypes D and I co-occurred with 1 or 2 additional genotypes, the former were the most abundant. The predominance of these 2 genotypes can be associated with their increased virulence, resistance to the host immune response, and enhanced potential for persistence and replication in the host mammary tissue. Interestingly, half (12/22) of the genotype I isolates were resistant to penicillin.

These isolates amounted to one-third (12/33) of all penicillin-resistant *S. aureus* isolates from this study.

In conclusion, the present work showed the population structure of *S. aureus* isolates from mastitis-affected dairy cows to be highly clonal, with either exclusively one or one predominant genotype circulating within a farm. The observed similarities in the genotype composition of *S. aureus* populations from geographically distant farms underscore the importance of interfarm transmission of *S. aureus* in Poland. This, in turn, argues for the establishment of a nationwide surveillance program for bovine mastitis due to *S. aureus*. Of the antimicrobial agents, whose in vitro activity against *S. aureus* was evaluated, only testing for penicillin G produced drug resistance. More than 40% of the bacterial isolates were penicillin resistant, indicating a limited role of that drug and other β -lactamase-sensitive β -lactams in the treatment of bovine mastitis caused by *S. aureus*.

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Table 2. Distribution of 4 triplex PCR types that occurred on more than 1 farm and the dates of isolation of *Staphylococcus aureus* strains harboring those types

PCR type	No. of isolates	Dairy farm	Date of isolation
A	1	I	May 2007
	5	II	April 2008
	2	XIII	May 2009
	1	XIV	May 2007
D	5	V	August 2008
	8	VII	October 2008
	5	VIII	October 2008
	4	X	March 2009
F	4	XII	April 2009
	3	XIII	May 2009
I	3	XI	March 2009
	15	XII	April 2009
	4	XIII	May 2009

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