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Original article

Clustering of *Staphylococcus aureus* bovine mastitis strains from regions of Central-Eastern Poland based on their biochemical and genetic characteristics

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Abstract

Staphylococcus aureus strains were isolated from mastitic milk of cows with infected mammary glands. The animals were living in 12 different farms near Lublin, in Central-Eastern Poland. A biochemical identification method based on enzymatic assay was performed, followed by haemolytic and proteolytic tests. PCR-RFLP targeted on the *gap* gene allowed the genetic identification of strains at the species level and verified phenotypic identification results. A molecular typing method using triplex PCR was performed to recognize the genetic similarity of the analyzed strains. DNA microarray hybridization (StaphyType, Alere Technologies) was used for detection of antibiotic resistance and virulence associated markers. The results obtained indicate high genetic similarity in strains isolated from the same sites. High genetic similarities were also detected between strains isolated from cows from different farms of the same region. A slightly lower similarity was noted however, in strains from various regions indicating that the strains are herd specific and that the cow's infections caused by *S. aureus* were of a clonal character. In 21 representative isolates selected for DNA-microarray testing, only fosfomycin (*fosB*) and penicillin resistance markers (*blaZ, blaI, blaR*) were detected. The presence of genes coding for haemolysins (*lukF, lukS, hlgA, hla, hld, hlb)*, proteases (*aur, sspA, sspB, sspP*), enterotoxins (*entA, entD, entG, entI, entJ, entM, entN, entO, entR, entU, egc-cluster*), adhesins (*icaA, icaC, icaD, bbp, clfA, clfB, fib, fnbA, map, vwb*) or immune evasion proteins (*scn, chp, sak*) was common and, with exceptions, matched triplex PCR-defined clusters.

Key words: infection, mammary gland inflammation, mastitis, molecular grouping, *Staphylococcus aureus*

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Introduction

Staphylococci are part of the physiological biocenosis of both humans and animals; however, under specific conditions, these microorganisms can cause severe infections. For this reason they are often classified as opportunistic or commensal microflora (Międzobrodzki et al. 2008, Fluit 2012, Peton and Le Loir 2013). Mastitis, inflammation of the mammary gland, is one of the most common infections caused by staphylococci among livestock, and is associated with significant economic losses in farming and the dairy industry. Among the many species of bacteria that cause mastitis in cattle, *Staphylococcus aureus* is mentioned as one of the most contagious and common etiologic factors of this disease (Zadoks and Fitzpatrick 2009, Le Marechal et al. 2011, Peton and Le Loir 2013). Staphylococci are isolated from the mastitic milk of animals suffering from an acute inflammation of the udder, but more frequently from cows that exhibit or do not exhibit any clinical manifestation of the disease (subclinical mastitis). Treatment of infected individuals is difficult due to frequent recurrences, as staphylococci can be found commonly in the natural environment and also on the skin of people tending the animals and in animals from the same herd (Malinowski and Gajewski 2010, Peton and Le Loir 2013). The infection occurs after the mammary gland tissues are invaded by bacteria, triggering the action of various factors, especially proteases, enterotoxins and pyrogenic superantigens, such as the toxic shock syndrome toxin 1 (TSST-1), (Bukowski et al. 2010, Zdzalik et al. 2012). A set of identified virulence factors, and additionally slime production, in staphylococcal mastitis are reported (Pejsak and Tarasiuk 1989, Krukowski et al. 2008). Subclinical bovine mastitis is a particularly important stage of the disease. The infected, but not treated or isolated, animals act as a pathogen reservoir in the herd (Oliveira et al. 2011). Moreover, mastitic milk from an infected udder is a low quality product, with decreased levels of protein, fat and lactose. Untreated, the subclinical form of staphylococcal infection may evolve into a long-lasting and expensive clinical mastitis characterized by latent, acute or chronic symptoms (Saei et al. 2009). Due to the clonal nature of *S. aureus* infections it is important to determine the degree of relationship between the isolates analyzed because correctly--chosen molecular typing methods can allow the route of transmission and, most importantly, the probable origin of the infection to be determined. In response to the interest of veterinary epidemiologists and practitioners, one of aims of the present study was to determine whether the *S. aureus* strains associated with mastitis in Eastern Poland were of a clonal, subclonal or ecological character. The second aim was assessment of antibiotic resistance and virulence potential of representative isolates based on diagnostic DNA-microarray hybridization. The results of a biochemical test based on enzymatic assay (Lachema, Brno), a test widely used in veterinary medicine, were compared with the results of a PCR-Restriction Fragment Length Polymorphism analysis (PCR RFLP) in order to determine the accuracy of phenotypic identification of livestock-associated *S. aureus* isolates. An assessment of the degree of relationships dependent on the epidemiological factors was then performed using a PCR-based approach, by amplifying a part of the hypervariable region (*hvr*) adjacent to the *mecA* gene, a part of the *spa* gene coding for protein A, and a part of the *coa* gene coding for coagulase. Based on geographic origin and triplex PCR clustering representative isolates were tested using DNA-microarrays for the presence of resistance and virulence markers. The final aim was to recognize the genetic features of the analyzed strains, observe the clustering of strains into particular populations based on their DNA similarity, and link them to appropriate herds and regions.

Materials and Methods

Animals and bacterial isolates

The studies were conducted on 86 *S. aureus* strains obtained from cows affected by clinical mastitis, from the inflammatory secretion of the mammary glands of 79 cows from 12 dairy herds (consisting of 8-127 cows each) from 8 districts in Eastern Poland. Farms were located in Biłgoraj (1 isolate, Farm: B), Zamość (5 isolates, Farm: Z1, Z2), Lubartów (3 isolates, Farm: Lub), Łęczna (6 isolates, Farm: Le), Łuków (29 isolates, Farm: Luk), Garwolin (3 isolates, Farm: G), Świdnik (13 isolates, Farm: S), Tomaszów Lubelski (26 isolates, Farm: Tl1, Tl2, Tl3, Tl4). The collection was supplemented with one reference strain, *S. aureus* ATCC 25923, resulting in a total number of 87 analyzed strains. Firstly, the somatic cell count, California mastitis test (CMT) was performed in determination of milk samples from infected mammary glands of every individual quarter. Only in the case of three animals was more than one sample taken, due to existing infection in more than one mammary gland. From these animals, isolates were sampled and cultured from separate infected quarters. Microorganisms from cow A included isolates designated 108, 109, 110, from cow B 105, 106, 107, and isolates sampled from cow C were 118 and 120. The results obtained were predictors for bacteriological status and the basis for further discrimination of

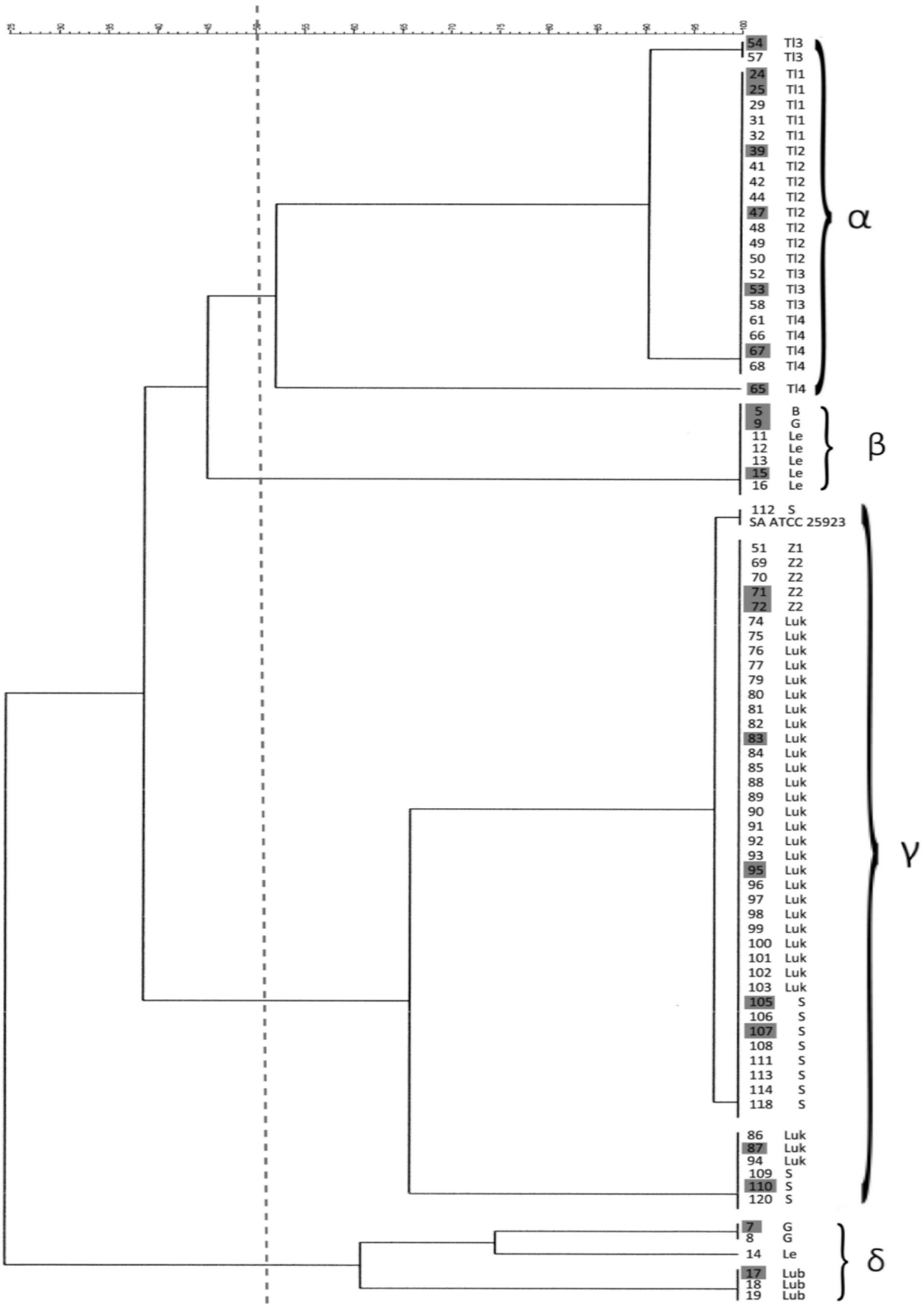

Fig. 1. Dendrogram illustrating phylogenetic similarity and clustering of bovine mastitis *S. aureus* isolates, determined by triplex PCR. Isolates selected for DNA microarray analysis are highlighted in grey.
Legend marks: B – Biłgoraj; G – Garwolin, Le – Łęczna; Lub – Lubartów; Luk – Łuków; S – Świdnik; TL1-4 – Tomaszów Lubelski; Z1-2 – Zamość.

samples to culture on a sheep blood agar medium to check the characteristics of colonies, their macro- and microscopic morphology (catalase activity and Gram-staining) and their haemolytic activity.

Phenotypic methods

The results of phenotypic identification were obtained from commercially available biochemical tests produced by Lachema Brno with semi-automatic measurement. Proteolytic and haemolytic activity assays were performed by streaking bacteria on tryptic soy agar (TSA) medium enriched with 10% skimmed milk or with 5% sheep blood, respectively.

Molecular methods

PCR-RFLP of *gap* gene: The genetic identification of the strains was achieved by carrying out a PCR-RFLP analysis by amplifying the *gap* gene, encoding glyceraldehyde-3-phosphate dehydrogenase, as previously described by Yugueros et al. 2001 (Yugueros et al. 2001). The obtained PCR product (933 bp) was digested with the restriction enzyme *AluI* (Thermo Fisher Scientific, Waltham, USA) and separated by electrophoresis gel. Species identification was based on the electrophoresis patterns of *S. aureus* reference strains (Yugueros et al. 2001, Oliveira and de Lencastre 2002).

Triplex PCR: Molecular typing was performed via the triplex PCR method by amplifying three genomic sequences encoding coagulase (*coa*), protein A (*spa*), and the hypervariable region (*hvr*) adjacent to the *mecA* gene (Sabat et al. 2006). The phylogenetic tree, showing the relationships between strains, was constructed using GelCompar II 5.1 software (Applied Maths, Kortrijk).

DNA microarray hybridization: Diagnostic DNA microarrays (StaphyType, Alere Technologies, Jena, Germany) and a StaphyType DNA microarray kit were used for detection of resistance and virulence associated genes according to protocols and procedures described previously (Monecke et al. 2008). The 21 microarray tested isolates (highlighted in dark grey in Fig. 1) were selected based on geographic source data and triplex PCR clustering.

Results

Species identification results

The phenotypic identification of strains was conducted by using a standard biochemical enzymatic test (Lachema, Brno), verified by carrying out a genetic restriction fragment length analysis of the *gap* gene using the PCR-RFLP method. From 86 analyzed isolates, 82 were identified as *S. aureus* by both methods. In four cases there was a discrepancy: in phenotypic assay one strain isolated in Lubartów was diagnosed as *Dermatococcus*, two strains from Tomaszów Lubelski were identified as *Staphylococcus lentus* and *Staphylococcus sciuri*, and one of the strains isolated in Zamość was diagnosed as *Staphylococcus gallinarum*. Genotypic assay determined that all tested strains belonged to *S. aureus* species.

Proteolytic and haemolytic activity

The entire collection of strains was then tested for proteolytic and haemolytic activity in order to assess the virulence determinants of mastitis isolates. Proteolytic activity was observed in 71 strains (83%), of which 58 (68%) of the strains displayed intensive (+) activity and 13 (15%) displayed very intensive activity (++). A group of 16 strains (17%) did not present proteolytic activity (-). In regard to haemolytic activity, a group of 75 (88%) *S. aureus* strains were β-haemolytic and the remaining 9 (11%) strains were not haemolytic (γ-haemolysin), and there was no data concerning the haemolytic capabilities of two strains (1%).

Molecular typing

The dendrogram illustrating genomic relatedness of *S. aureus* strains was generated automatically using GelCompar II 5.1 software (Applied Maths, Kortrijk) with the following parameters: optimization 0.5%, tolerance 0.8%. During triplex PCR typing, the PCR products of three isolates could not be obtained and thus were not included in the final comparison. The general overview of the clusters of strains exhibited in the dendrogram shows that at the level of 50% DNA similarity, four groups (named α, β, γ, δ) were discriminated from the 83 isolates subjected to triplex PCR analysis. Cluster α included two groups of isolates displaying 100% similarity: a pair of isolates, numbered 54 and 57, from farm Tl3, and a much bigger subgroup of 20 isolates collected in all Tl farms (Tl1-Tl4, Tomaszów Lubelski). The other population of seven identical strains received from Le farms (5 strains), and two additional strains from farms B and G comprise a separate cluster β showing a 45% similarity with the previous cluster α. The next cluster of 47 strains reported as γ comprised three subgroups of triplex PCR indistinguishable isolates.

The most numerous subgroup included 39 isolates: 26 isolates from farm Luk, 8 isolates from farm S, 4 isolates from farm Z2, and 1 isolate from farm Z1. A similarity of 97% to that population is exhibited by two other identical strains: 112 from farm S, and the reference strain *S. aureus* ATCC 25923. The third subgroup included 6 isolates: 3 from farm Luk, and 3 from farm S, all at a 60% similarity level to the biggest subgroup of the same cluster γ, and 38% similar to the clusters α and β. A 25% similarity to all the previously presented clusters was demonstrated by cluster δ, which comprised two subgroups of a 60% similarity including 3 strains each. One group comprised 3 identical strains from farm Lub, the next identical group was formed by 2 strains that originated from farm G, and 1 strain from farm Le. Isolates which were collected from multiple sampling of three cows with infection in more than one mammary gland (quarter) were assigned to cluster γ.

Three isolates that originated from cow A, designated as 105, 106 and 107 exhibited 100% similarity and were grouped together in the most numerous subgroup of the γ cluster. Isolates 108 and 118, collected from cows B and C, respectively, were indistinguishable from the three above – mentioned isolates, and were also assigned to the same subgroup. The remaining isolates, 109 and 110 collected from cow B, and 120 isolated from the infected quarter of cow C, were identical and exhibited 60% similarity to isolates 108 and 118. Overall, at 100% similarity level 10 types were defined by triplex PCR, indicating the existence of 10 *S. aureus* strains and the highly clonal character of mastits infection among the sampled farm animals.

DNA microarray-based detection of resistance and virulence genes

From the collection, 21 isolates representing triplex PCR clusters and geographic origin were selected. Antibiotic resistance and virulence associated genes, as determined by microarray hybridization, are presented in Table 1. The majority of analyzed isolates (19/21) carried *fosB* which confers resistance to fosfomycin. In 11 isolates, genes involved in β-lactamase production and penicillin resistance (*blaI, blaR, blaZ*) were detected.

Regarding the presence of genes coding for known virulence factors all 21 isolates harbored α, β, δ and γ-haemolysins or their components (*lukF, lukS, hlgA, hla, hld, hlb*), aureolysin (*aur*), V8-protease (*sspA*), staphopain A and B (*sspP, sspB*), intercellular adhesion proteins A, C and biofilm PIA synthesis protein D (*icaA, icaC, icaD*), fibrinogen binding protein (*fib*), fibronectin-binding protein A (*fnbA*), clumping factors A and B (*clfA, clfB*), major histocompatibility complex class II analog protein (*map*) and Willebrand factor – binding protein (*vwb*). The only difference between isolates from cluster δ, was the absence of the *fnbB* gene in one of them (marked, as 7). It was also the only such case observed during microarray analysis as all the other tested isolates carried the gene of fibronectin-binding protein. In the vast majority of isolates, the genes encoding enterotoxins G, I, M, N, O and U (*entG, entI, entM, entN, entO, entU, egc-cluster: seg/sei/sem/sen/seo/seu*) and serine proteases A and B (*splA, splB*) were detected. Bone sialoprotein-binding protein (*bbp*) was absent in only 5 tested isolates: two from cluster α (39, 47), one from β (15) and two from δ (7, 17). Carriage of chemotaxis inhibitory protein (*chp*), staphylococcal complement inhibitor (*scn*), staphylokinase (*sak*) and enterotoxins A, D, J, R (*entA, entD, entR, entJ*) was predominant in cluster γ, but also detected in strain 9 from cluster β. Isolates which were derived from multiple quarter infection in cow A (105, 107) and one (110) cow B exhibited an identical DNA microarray profile with regard to reported resistance and virulence markers. With the exception of isolate 15 (cluster β) all tested strains had capsule type 5. Isolates 15 (cluster β), 7, 17 (cluster δ) were the only ones positive for serine protease E (*splE*), but overall were characterized by the lowest number of virulence associated genes carried.

Discussion

In most countries *Staphylococcus aureus* is one of the most predominant mastitis pathogens causing significant impact on cows' health, milk quality and productivity as well as an increase in the use of medicine and veterinary services (Saei et al. 2009, Castelani et al. 2013). Subclinical mastitis is a major cause of economic loss in the dairy industry which, combined with the increasing phenomenon of medicine resistance, requires the monitoring of staphylococcal strains and their spread, and the implementation of prevention programmes intended for reducing such infections (Zadoks and Fitzpatrick 2009, Zadoks et al. 2011). For this purpose, the present study was aimed at demonstrating the nature of the mastitis infection in cows from 12 different herds originating from Central-Eastern Poland. Moreover, in view of the reports on emerging atypical *S. aureus* strains, characterized by altered biochemical properties, on which phenotypic identification is based, the molecular, PCR-based method was carried out in order to confirm the species of the mastitis isolates (Cuteri et al. 2003, Peton and Le Loir 2013). Among the 86 strains identified genetically as *S. aureus*, the biochemical

Table 1. Resistance and virulence associated genes from selected isolates of Central-Eastern Poland.

Cluster	Geographical code	Isolate designation	Resistance and virulence genes
α	TL1 TL3 TL4	24, 25 53, 54 65, 67	**Resistance**: *fosB* **Enterotoxins**: *entG, entI, entM, entN, entO, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP,* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb, bbp* **Capsule**: *capsule 5*
	TL2	39, 47	**Resistance**: *fosB* **Enterotoxins**: *entG, entI, entM, entN, entO, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb* **Capsule**: *capsule 5*
β	B	5	**Resistance**: *fosB* **Enterotoxins**: *entG, entI, entM, entN, entO, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb;* **Proteases**: *aur, sspA, sspB, sspP, splA, splB* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb, bbp,* **Capsule***: capsule 5*
	G	9	**Resistance**: *fosB, blaZ, blaI, blaR* **Enterotoxins**: *entG, entI, entM, entN, entO, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP, splA, splB* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb, bbp* **Immune evasion proteins**: *sak, scn, chp* **Capsule***: capsule 5*
	Le	15	**Resistance**: *fosB* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP, splA, splB, splE* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb* **Capsule***: capsule 8*
γ	Z2 Luk	71, 72 83, 87, 95	**Resistance**: *fosB, blaZ, blaI, blaR* **Enterotoxins**: *entA, entD, entG, entI, entJ, entM, entN, entO, entR, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP, splA, splB* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb, bbp* **Immune evasion proteins**: *sak, scn, chp* **Capsule***: capsule 5*
	S	105, 107, 110	**Resistance**: *fosB, blaZ, blaI, blaR* **Enterotoxins**: *entG, entI, entJ, entM, entN, entO, entU, egc-cluster* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb;* **Proteases**: *aur, sspA, sspB, sspP, splA, splB* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb, bbp* **Immune evasion proteins**: *chp* **Capsule**: *capsule 5*

cont. Table 1

Cluster	Geographical code	Isolate designation	Resistance and virulence genes
δ	G	7	**Resistance**: *blaZ, blaI, blaR* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb* **Proteases**: *aur, sspA, sspB, sspP, splA, splB, splE* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, map, vwb* **Capsule***: capsule 5*
	Lub	17	**Resistance**: *blaZ, blaI, blaR* **Haemolysins**: *lukF, lukS, hlgA, hla, hld, hlb;* **Proteases**: *aur, sspA, sspB, sspP, splA, splB, splE* **Adhesins and biofilm formation proteins**: *icaA, icaC, icaD, clfA, clfB, fib, fnbA, fnbB, map, vwb* **Capsule**: *capsule 5*

Legend: Resistance: *fosB* – putative marker for fosfomycin, fleomycin; *blaZ* - Beta-Laktamase; *blaI* – Beta Lactamase repressor (Regulatory Protein); *blaR* – Beta Lactamase regulatory protein.
Virulence: *entA* – enterotoxin A; *entD* – enterotoxin D; *entG* – enterotoxin G; *entI* – enterotoxin I; *entJ* – enterotoxin J; *entM* – enterotoxin M; *entN* – enterotoxin N, *entO* – enterotoxin O; *entR* – enterotoxin R; *entU* – enterotoxin U; *egc*-cluster – enterotoxins *seg*/*sei*/*sem*/*sen*/*seo*/*seu*; *LukF* – haemolysin gamma, component B; *lukS* - haemolysin gamma, component C; *hlgA* – haemolysin gamma, component A; *hla* – haemolysin alpha; *hld* – haemolysin delta; *hlb* – haemolysin beta; *aur* – aureolysin; *sspA* – V8-Protease; *sspB* – staphopain B*; sspP* – staphopain A; *icaA* – intercellular adhesion protein A; *icaC* – intercellular adhesion protein C; *icaD* – biofilm PIA synthesis protein D; *bbp* – bone sialoprotein-binding protein; *clfA* – clumping factor A; *clfB* – clumping factor B; *fib* – fibrinogen binding protein; *fnbA* – fibronectin-binding protein A; *map* – major histocompatibility complex class II analog protein; *vwb* – Willebrand Factor – binding protein; *sak* – staphylokinase; *chp* – chemotaxis inhibitory protein (CHIPS); *scn* – staphylococcal complement inhibitor (SCIN); *splA* – serine protease A; *splB* – serine protease B; *splE* – serine protease E.
Geographic origin: B – Biłgoraj; G – Garwolin, Le – Łęczna; Lub – Lubartów; Luk – Łuków; S – Świdnik; TL1-4 – Tomaszów Lubelski; Z1-2 – Zamość.

identification results were 95% consistent. The PCR-RFLP analysis should be treated as a reference method because the phenotypic method in a few cases did not allow for an unmistakable, clear identification at a species or even at a genus level. The necessity of introducing genetic methods in routine veterinary diagnosis has therefore been confirmed.

A mammary gland inflammation induced by *S. aureus* is characterized by very high somatic cell count (SCC), decreased milk production and a low therapy success rate. There are estimates that show that 19.4% of all cows are infected by this pathogen. Initially, *S. aureus* damages the tissues lining the teat and gland cisterns to move up into the duct system and establishes deep-seated pockets of infection in the alveolar tissue (Nickerson 1993). The diversification of symptoms relies on bacterial expression systems responsible for up- or down-regulation of potential virulence factors, biofilm formation and adhesion abilities enhanced by the presence of milk in the environment (Naidu et al. 1991, Międzobrodzki et al. 2008, Szweda et al. 2012). In this study an assessment of virulence was conducted through haemolytic and proteolytic assays because during invasion into animal or human tissue these enzymes function as pathogenicity determinants and factors facilitating spread within the host organism (Feng et al. 2008, Peton and Le Loir 2013). Haemolysins produced by staphylococci are responsible for destroying haemoglobin and contribute to reducing the number of erythrocytes in the blood, with all its consequences (Le Maréchal et al. 2011). The secretion of proteases however, provides nutrients for staphylococci. These enzymes are also responsible for tissue destruction during the course of infection (Zdzalik et al. 2012, Camussone and Calvinho 2013). Therefore, the amount of produced proteases and haemolysins is considered a prerequisite for the pathogenicity of staphylococci. In the haemolytic assay, 88% of the isolates exhibited β-haemolysis and 11% did not induce the dissolution of red blood cells, displaying what is known as γ-haemolysis. Proteolysis was observed in 83% of the strains and 15% exhibited intensive proteolytic activity.

In many countries, during the mastitis prevention programme, *S. aureus* bacteria have been reported as a difficult to control, persistent and contagious pathogen, prone to recurrence (Chrobak et al. 2011, Le Maréchal et al. 2011, Kot et al. 2012). This is mainly due to the difficulties in routine diagnostics, persistence of staphylococcal infections and abundance of *S. aureus* in the environment. The bacteria can be found on the skin of bovine and non-bovine animals, on the

skin of staff, on milking equipment, flies, and throughout the barn or farm area (Zadoks and Fitzpatrick 2009). It is therefore important to determine the source and the nature of the mastitis infection, which may demonstrate a clonal, subclonal or ecological character.

In the current study, the genotyping of *S. aureus* strains was conducted using triplex PCR (*coa, spa, hvr*) with the exception of four isolates for which the PCR products could not be obtained. For 7 isolates (8.3%) the *spa* fragment amplification was not detected. This lack of PCR product might have resulted from mutation in the protein A gene which occurs naturally in *S. aureus* (Baum et al. 2009). Among the 87 fully analyzed strains (86 isolates and 1 reference strain) all had the *coa* fragment and thus were coagulase-positive and no isolate was identified as MRSA.

As presented in the dendrogram (Fig. 1) the majority of the analyzed strains showed a clonal nature of infection. Seven strains originating from farms Lub, Tl1, Tl2 and Z2 were distributed to other different clusters. A slightly lower, genetic similarity was observed in the strains from farms Tl3, Tl4, Luk and S, which clustered on adjacent branches of the phylogenetic tree. Only in two cases – farms G and Le – did the isolated strains show no clonality. A high correlation between the strains in particular groups and their farm of origin, indicates the clonal nature of the *S. aureus* mastitis infection in cows. Among the collection, 3 sets of isolates in a total number of 8, were derived from three animals (cows A, B, C) in which more than one mammary gland was infected. Five of these isolates, recovered from cow A (105, 106, 107), B (108) and C (118) were assigned by triplex PCR in the most numerous subgroup of cluster γ at 100% similarity, indicating that they were representatives of one *S. aureus* strain. The remaining isolates collected from cow B (marked as 109, 110) and one from cow C (120) were identical, placed in the same cluster γ and related to previous samples at 60% similarity. The results obtained indicate that only in case of cow A was the same strain a cause of infection in all tested mammary glands. Further genetic relatedness (60%) observed among isolates collected from separate mammary glands of cows B and C does not preclude the existence of the clonal nature of the infection but suggests the occurrence of microevolution within the strain. The presented results are in accordance with global data as the occurrence of clonal or closely related mastitis *S. aureus* strains have often been reported and molecular typing methods confirm that *S. aureus* strain distribution is mainly herd-specific (Zadoks et al. 2011, Castelani et al. 2013).

The presence of genes coding for antibiotic resistance was very poor in the tested isolates. Only fosfomycin (*fosB*) and penicillin resistance markers (*blaZ, blaI, blaR*) were detected. In contrast, carriage of virulence-associated genes was common in the tested isolates although major markers such as PVL (*luKS-PV/lukF-PV*), TSST-1, (*tst*), exfoliative toxins (*etA, etB, etD*) or biofilm-associated protein (*bap*) were not detected. Genes and their products involved in haemolysis (*hla, hld, hlb*), proteolytic degradation of host tissues (*aur, sspA, sspB, sspP*), adhesion and biofilm formation (*icaA, icaC, icaD, clfA, clfB, fib, fnbA, map, vwb*) were present in all tested isolates. Serine proteases A and B (*splA, splB*) were absent only in cluster α, yet serine protease E (*splE*) was detected only in 3 tested isolates from clustes β and δ. The vast majority of isolates were positive for enterotoxins G, I, M, N, O, U, bone sialoprotein-binding protein (*bbp*) and had capsule type 5. Genes involved in immune evasion such as staphylococcal complement inhibitor (*scn*), staphylokinase (*sak*) or chemotaxis inhibitory protein (*chp*) were detected in clusters γ and β but not in all tested isolates (Zecconi and Scali 2013, Foster et al. 2014). Additional four enterotoxins, A, D, R and J (*entA, entD, entR, entJ*), were present only in cluster γ, and predominantly originated on farms Lk (Luków) and Z2 (Zamość). Isolates designated as 15 (cluster β, Łęczna), 7 and 17 (cluster δ, Garwolin, Lubartów) had the lowest number of harbored virulence markers. Isolates 105, 107 and 110, derived from cows in which more than one mammary gland was infected were assigned to cluster a by triplex PCR. Although the genetic relatedness of isolate 110 to the identical pair of 105 and 107 was determined at 60%, the DNA hybridization profiles of the three samples were identical in regard to analyzed resistance and virulence markers. Overall, the representative isolates tested by DNA microarray showed genetic potential for virulence, in accordance with their mastitis association. The observed patterns of virulence markers and under-representation of antibiotic resistance genes might be the result of isolate selection, as no entire collection could have been microarray tested.

Although triplex PCR allowed for clustering of isolates reflecting their geographical origin, the microarray-based gene patterns indicate that more complex molecular methods could be used for further differentiation of the collection. The most acknowledged typing methods such as PFGE (Pulsed-field Gel Electrophoresis) or *spa* typing are however time-consuming and still too expensive for use in routine veterinary practice (Cuteri et al. 2003, Sabat et al. 2006, Karyński et al. 2008).

The application of PCR-based molecular methods in mastitis diagnostics and research will contribute to increase the understanding of the epidemiological characteristics of mastitis, which in turn will allow the

recognition of transmission routes to be improved and the treatment of staphylococcal infections to be accelerated (Truszczyński 1998). A high level of genetic diversity of *S. aureus* strains indicates the necessity of monitoring clusters of these strains and discriminating specific populations in order to identify the nature of the infection so as to reduce the costs of complex diagnostics and treatment as well as to limit economic loss.

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