The first outbreak of methicillin-resistant *Staphylococcus aureus* in dairy cattle in Poland with evidence of on-farm and intrahousehold transmission

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ABSTRACT

Staphylococcus aureus is a widely recognized pathogen responsible for many serious diseases in both humans and animals. It is also one of the major causative agents of bovine mastitis. Methicillin-resistant *S. aureus* (MRSA), although relatively rare in this pathology, has been increasingly reported in livestock animals, mainly in pigs, but also cattle, sheep, and poultry. The recent emergence of livestock-associated (LA-)MRSA is cause for an immediate public health concern due to the risk of zoonotic transmission to humans, and is of particular concern for people who work in animal husbandry or have prolonged contact with livestock animals. This study reports on the first LA-MRSA outbreak in dairy cattle and the first probable case of MRSA transmission between humans and cows in Poland. A single dairy farm located in Eastern Poland was monitored on a regular basis for the occurrence of mastitis. Over a 1-yr study period, 717 quarter-milk samples from 583 cows were collected and examined microbiologically. A total of 5 MRSA isolates from as many cows with subclinical mastitis were cultured. They all belonged to the same outbreak, given a 2-mo time window in which they were identified. During the outbreak, 24 oral and nasal swabs were voluntarily taken from 6 people: a milker, a veterinarian, and 4 members of the veterinarian's family. Eight swabs from a milker, veterinarian, and 2 family members yielded positive MRSA cultures. All MRSA isolates were genotyped with a combination of multiple-locus variable number tandem repeat analysis, multilocus sequence typing, and staphylococcal protein A gene (*spa*) typing. Eleven bovine ($n = 5$; 5 cases) and human ($n = 6$; 4 cases) isolates showed an

identical drug-susceptibility profile and were indistinguishable upon multiple-locus variable number tandem repeat analysis (pattern A), multilocus sequence typing (ST398) and *spa* (t034) typing. The results of this study provide the evidence of transmission of MRSA between humans and cows, and between humans in the family setting. This work, despite being a preliminary investigation, underscores the risk of intra- and interspecies transmission of LA-MRSA and urges enhancement of the existing biosecurity measures aimed at preventing MRSA (and other milk pathogens) spread at both the farm- and household levels.

Key words: bovine mastitis, outbreak, transmission, methicillin-resistant *Staphylococcus aureus*, humans

INTRODUCTION

Staphylococcus aureus is one of the major human pathogens that cause a wide variety of clinical manifestations ranging from localized, superficial skin lesions to severe, life-threatening invasive diseases (Turner et al., 2019). The emergence of methicillin-resistant *S. aureus* (MRSA) strains since the early 1960s has gravely compounded the management of *S. aureus* infections, posing a significant therapeutic challenge (Boswihi and Udo, 2018; Turner et al., 2019).

Before the mid-1990s, MRSA strains were mostly associated with hospitals and other healthcare facilities. However, in the early 2000s, MRSA infections escalated among patients without prior exposure to healthcare environments. These cases, referred to as community-acquired, are caused by strains that are genetically distinct from nosocomial MRSA and, unlike nosocomial strains, they exhibit enhanced virulence and fitness, which contribute to their rapid dissemination in the community (Udo, 2013; Boswihi and Udo, 2018). Finally, there is a growing evidence that livestock constitutes an important reservoir of MRSA strains with the potential of zoonotic transmission (Pantosti, 2012). These livestock-associated (LA-)MRSA strains

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have been isolated from pigs, cattle, horses, and poultry (Nemati et al., 2008; van Duijkeren et al., 2010; Tavakol et al., 2012; Locatelli et al., 2017). Intra- and interspecies transmission of LA-MRSA, including zoonotic transmission of LA-MRSA strains from livestock to humans, has been amply described in the literature, with people living and working in close proximity to farm animals consistently identified as being at higher risk of MRSA acquisition (Cuny et al., 2009; van Duijkeren et al., 2010; van Duijkeren et al., 2016; Schnitt and Tenhagen, 2019).

In this study, we describe the first LA-MRSA outbreak in dairy cattle in Poland and document the first putative case of direct transmission of MRSA between cows and humans in our country.

MATERIALS AND METHODS

Study Sample

The study was conducted on a dairy farm (herd size: 806 cows) located in Lublin Province, Eastern Poland, over a 1-yr period (from January to December 2018). Clinical examination of the animals and milk-quality assessment were carried out as described previously (Jagielski et al., 2019a). A total of 717 quarter-milk samples (positive on California mastitis test) were collected from 583 cows with clinical (28) and subclinical (555) mastitis, following aseptic procedures as described by the National Mastitis Council (2017).

Twenty-four oral and nasal swabs were collected voluntarily from 6 people: a milker, a veterinarian, and 4 members of the veterinarian's family (his wife, 2 sons, and daughter). The swabs were taken from both nostrils and the throat (3 sites) from each person. All swabbing was performed once, except for the veterinarian and the milker, who were sampled twice. The samples were taken upon specific request of the veterinarian. All samples were transported under refrigeration (4°C) to the laboratory for microbiological evaluation.

Ethical Considerations

Informed consent was obtained from all human subjects and confidentiality was ensured by anonymization of the data. Thus, ethical approval was not required with local legislation.

Primary Isolation, Culture, and Species Identification

Quarter-milk samples (10 µL) were inoculated onto Columbia agar with 5% sheep blood (Oxoid, Basingstoke, UK). Cultures were incubated aerobically at 37°C for up to 96 h.

Human oral and nasal swabs were first transferred into the brain heart infusion broth with 2% NaCl (Oxoid) and, after overnight incubation at 37°C, 100-µL culture samples were plated onto mannitol-salt-agar (Oxoid) supplemented with 75 mg/L aztreonam and 6 mg/L oxacilline (Sigma-Aldrich, St. Louis, MO). Cultures were incubated aerobically at 37°C for up to 96 h.

Colonies suspected to be *S. aureus* were subjected to species identification, which included Gram staining, oxidase, catalase, and coagulase-activity testing, performed according to standard laboratory procedures (Lancette and Tatini, 1992).

Drug-Susceptibility Testing

Antimicrobial susceptibility was performed on Mueller-Hinton agar (Oxoid) using the disk diffusion method according to EUCAST recommendations and clinical zone diameter breakpoints provided therein (EUCAST, 2018). Briefly, 24-h cultures of *S. aureus* isolates were used to prepare suspensions in sterile 0.85% NaCl equivalent to 0.5 McFarland turbidity standard. The suspensions were subsequently used to inoculate the Mueller-Hinton agar plates. Disks impregnated with antimicrobial agents (Oxoid) were placed on the surface of each of the inoculated agar medium. Incubation was conducted aerobically at $35 \pm 1^\circ\text{C}$ for 18 ± 2 h. After incubation, the development of the zones of inhibition of bacterial growth around the disks was observed and their diameters measured (mm). The *S. aureus* reference strain ATCC 29213 was used as the control.

The cefoxitin (30 µg) disk diffusion method was used to detect methicillin resistance. An inhibition zone diameter of <21 mm was indicative of a methicillin-resistant phenotype.

All MRSA isolates were further subjected to supplementary antimicrobial susceptibility testing by disk diffusion method, with amikacin (30 µg; sensitive (SV) ≥ 18 ; resistant (R) < 16), chloramphenicol (30 µg; SV ≥ 18 ; R < 18), ciprofloxacin (5 µg; SV ≥ 21 ; R < 21), clindamycin (2 µg; SV ≥ 22 ; R < 19), co-trimoxazole (23.75/1.25 µg; SV ≥ 17 ; R < 14), fusidic acid (10 µg; SV ≥ 24 ; R < 24), gentamicin (10 µg; SV ≥ 18 ; R < 18), mupirocin (20 µg; SV ≥ 30 ; R < 18), norfloxacin (10 µg; SV ≥ 17), and tetracycline (30 µg; SV ≥ 22 ; R < 19).

DNA Extraction

Genomic DNA was extracted from *S. aureus* cultures grown on brain heart infusion agar with a GeneMATRIX Environmental DNA and RNA purification kit (EURx, Gdańsk, Poland) according to the manufacturer's pro-

tolol. The DNA concentration was quantified with the NanoDropOne spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -20°C until used.

Genotyping

All strains were genotyped with 3 molecular methods: multiple-locus variable number tandem repeat analysis (MLVA; Sabat et al., 2003), multilocus sequence typing (MLST; Enright et al., 2000), and staphylococcal protein A gene (*spa*) typing (Harmsen et al., 2003).

MLVA Typing. A set of PCR primers to simultaneously amplify the hypervariable regions of the *spa*, *sspA*, *clfA*, *clfB*, *sdrC*, *sdrD*, and *sdrE* genes was used, as described by Sabat et al. (2003). The DNA fragments were separated electrophoretically in 2% agarose gels and visualized by staining with ethidium bromide (0.5 $\mu\text{g/mL}$) and exposure to UV light ($\lambda = 320\text{ nm}$) with BioDoc-It UVP Transilluminator (Analytik Jena, Jena, Germany).

MLST Typing. Seven housekeeping genes (i.e., *arc*, *aroE*, *glp*, *gmk*, *pta*, *tpi*, and *yqiL*) were interrogated in the MLST scheme and their respective fragments were amplified by using primers and conditions described elsewhere (Enright et al., 2000). Amplicons were purified with the Clean-Up kit (A&A Biotechnology, Gdynia, Poland) and sequenced in both directions at the Genomed DNA sequencing laboratory (Warsaw, Poland). Sequence data were assembled and analyzed with the Clone Manager version 8 software (Sci-Ed Software, <https://www.scied.com>), and the resulting consensus sequences were aligned against the *S. aureus* MLST database (<http://saureus.beta.mlst.net/>).

Spa Typing. The variable repeat region X of the protein A-encoding *spa* gene was amplified by PCR with primers and conditions provided elsewhere (Ridom Bioinformatics, <https://www.ridom.de/>). Sequence data were assembled and analyzed with Clone Manager, and the resulting consensus sequences were aligned against the spaTyper 1.0 database (Bartels et al., 2014).

RESULTS

During the 12-mo study period, 12 *S. aureus* isolates were recovered from milk samples (out of 717 tested; 1.7%) collected from 12 cows (out of 583 tested; 2.1%), all with subclinical mastitis. Five (41.7%) *S. aureus* isolates were MRSA. The first isolations of methicillin-susceptible and methicillin-resistant *S. aureus* were achieved in April and June, respectively. The last MRSA isolate from milk was cultured in July (Table 1).

Eight body swabs (out of 24 tested; 33.3%), including 5 nasal and 3 oral samples, yielded growth of *S. aureus*. All of these isolates exhibited MRSA phenotype, as

Table 1. Distribution of MLVA,¹ MLST,² *spa* types,³ and drug-susceptibility profiles among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates included in this study

Item	MRSA1	MRSA2	MRSA3	MRSA4	MRSA5	MRSA6	MRSA7	MRSA8	MRSA9	MRSA10	MRSA11	MRSA12	MRSA13
Isolation source	Milk	Milk	Milk	Milk	Milk	Nasal swab	Nasal swab	Nasal swab	Oral swab	Nasal swab	Oral swab	Oral swab	Nasal swab
Date of isolation (year 2018)	10.06	17.06	02.07	06.07	06.07	27.06	27.06	19.06	19.06	27.06	27.06	27.06	27.06
Host ⁴	Cow	Cow	Cow	Cow	Cow	Milker	Milker	Veterinarian	Veterinarian	Veterinarian	Veterinarian	Veterinarian's son	Veterinarian's wife
MLVA type	A	A	A	A	A	A	A	A	A	B	A	A	A
MLST type	ST398	ST398	ST398	ST398	ST398	ST398	ST398	ST398	ST398	ST45	ST398	ST398	ST398
<i>Spa</i> type	t034	t034	t034	t034	t034	t2633	t2633	t034	t034	t2633	t034	t034	t034
Tested drug ⁵	S	S	S	S	S	S	S	S	S	S	S	S	S
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R
Clindamycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Cotrimoxazole	S	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S
Mupirocin	S	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefoxitin	R	R	R	R	R	R	R	R	R	R	R	R	R

¹MLVA = multiple-locus variable number tandem repeat analysis.

²MLST = multilocus sequence typing, according to MLST database (<http://saureus.beta.mlst.net/>); ST398 refers to the following number of repeats: *arc* 3, *aroE* 35, *glpF* 19, *gmk* 2, *pta* 20, *tpi* 26, *yqiL* 39; ST45 refers to the following number of repeats: *arc* 10, *aroE* 14, *glpF* 8, *gmk* 6, *pta* 10, *tpi* 3, *yqiL* 2.

³Number of repeats and *spa* type according to spaTyper 1.0 database (Bartels et al., 2014); t034 refers to the 08-16-02-25-34-24-25 *spa* type, whereas t2633 to the 08-16-02-16-34-13-17-34-16-34 *spa* type.

⁴The isolates were recovered from 5 bovine and 4 human hosts (i.e., milker, veterinarian, veterinarian's wife, and one of their sons).

⁵Isolates were categorized as either resistant (R) or susceptible (S).

they were resistant to cefoxitin. They were all collected in June and originated from a veterinarian ($n = 4$), his wife ($n = 1$), his son ($n = 1$), and a milker ($n = 2$).

All milk isolates ($n = 5$) were fully susceptible to amikacin, chloramphenicol, cotrimoxazole, fusidic acid, gentamicin, and mupirocin, yet showed resistance to ciprofloxacin, clindamycin, norfloxacin, and tetracycline. All human isolates had exactly the same drug-susceptibility profile, except 1 isolate (MRSA6) that was resistant to cefoxitin only (Table 1).

Genotyping was performed on all ($n = 13$) MRSA isolates (Table 1). Five bovine (5 cases) and 6 human (4 cases) isolates were indistinguishable upon MLVA typing (pattern A), and harbored identical MLST (ST398) and *spa* (t034) types (Figure 1; Table 1). The other 2 human isolates (2 cases) shared identical MLVA (pattern B), MLST type (ST45), and *spa* type (t2633) (Figure 1; Table 1).

DISCUSSION

In the etiology of bovine mastitis, *S. aureus* plays a leading role, accounting for 6 to 74% of infections (Persson et al., 2011; Hoque et al., 2018; Poutrel et al., 2018; Pumipuntu et al., 2019). Since the first description of MRSA in dairy cattle in the 1970s, isolations of MRSA from mastitic cows have been reported from across the world, representing up to 29% of all *S. aureus* strains cultured (Prashanth et al., 2011). In Poland, the preva-

lence of staphylococcal mastitis among dairy herds has been calculated to be 17 to 44% (Malinowski et al., 2006; Sztachńska et al., 2016; Jagielski et al., 2019b). Until this study, however, no MRSA isolates have been reported from bovine samples in Poland. In contrast, studies performed in Poland's neighbors have shown the prevalence of MRSA in bulk-tank milk samples from dairy herds in Germany, Ukraine, and Czech Republic at levels of 5.7, 6.5, and 38%, respectively (Tenhagen et al., 2014; Berhilevych et al., 2017; Tegegne et al., 2019).

Several molecular typing methods have been used for epidemiological investigations of *S. aureus* infections (Tenover et al., 2009; Jagielski et al., 2014; Locatelli et al., 2017). Currently, MLST typing with 7 housekeeping genes (Enright et al., 2000), and *spa* typing based on the protein A-coding gene (Bartels et al., 2014) are considered frontline tools in the molecular epidemiology of MRSA worldwide. By using these techniques, it has been demonstrated that many *S. aureus* lineages display strong host specificity and others may colonize or infect a variety of animal species (Pantosti, 2012; Aires-de-Sousa, 2017). Furthermore, different lineages show a considerable variation in their geographical distributions (Monistero et al., 2018).

Although several studies have addressed the issue of transmission of MRSA among humans and animals (van Duijkeren et al., 2004; Angen et al., 2017; Zhou et al., 2018; Pirolo et al., 2019), few have demonstrated, directly or indirectly, that such transmission occurs

Figure 1. Multiple-locus variable number tandem repeat analysis patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates under the study. Lane designations (1–13) correspond to isolate numbers (MRSA 1–13; see Table 1); M = size marker (Gene Ruler 1 kB, Thermo Fisher Scientific, Waltham, MA).

among humans and dairy cows (Juhász-Kaszanyitzky et al., 2007; Sakwinska et al., 2011; Locatelli et al., 2017; Sato et al., 2017; Schmidt et al., 2017).

In this study, a triple-modality typing approach showed that all bovine MRSA isolates, and all but 2 human MRSA isolates, were identical, representing the MSLT sequence type (ST)398 and *spa* type t034 genotype.

The ST398 represents the most prevalent LA-MRSA lineage in Europe and North America, and is quite common in Asia. It was first identified among pigs (Armand-Lefevre et al., 2005), but subsequent studies have reported its recovery from other animal species including dogs, horses, poultry, and dairy cattle (Nemati et al., 2008; Nienhoff et al., 2009; Fessler et al., 2010; Vanderhaeghen et al., 2010; van Duijkeren et al., 2010; Sakwinska et al., 2011; Tavakol et al., 2012; Sharma et al., 2016; Kadlec et al., 2019). With the escalating dissemination of the ST398 among food-producing and companion animals, there has been an increased prevalence of human colonization with that genotype, especially among people occupationally exposed to animals and their products (e.g., farmers, veterinarians, abattoir workers). The MRSA ST398 was shown to colonize up to 86% of pig farmers, 37% of cattle- or poultry-farmers, and 45% of pig-care veterinarians (Goerge et al., 2017). Furthermore, several studies have pointed to the accumulation of MRSA ST398 in households of colonized livestock workers, suggesting human-to-human transmission (Cuny et al., 2009; Verkade et al., 2014; Bosch et al., 2015). Finally, although generally associated with colonization, MRSA ST398 has been implicated in human infections of variable clinical spectrum and relevance, including fatal events (Becker et al., 2017).

In Poland, MRSA strains of ST398 were first isolated in 2008 on pig farms during the first MRSA baseline survey on dust samples from pig holdings in the European Union (EFSA, 2009). Subsequently, the ST398 MRSA strains were recovered from nasal swabs from pig veterinarians (Marszałek et al., 2009). More recently, ST398 has been described as a predominant MRSA clade on Polish swine farms (Mroczkowska et al., 2017). However, never before has this genotype been cultured from bovine milk in Poland. Noteworthy, the finding of MRSA ST398 in dairy milk cows was described in 2 countries neighboring Poland, namely Germany (Fessler et al., 2010; Kadlec et al., 2019) and Czech Republic (Teagene et al., 2019). In this study, all MRSA-infected cows presented with subclinical mastitis. The overall number of MRSA-positive cows was 0.9% (5/583), which was similar to that in Germany (0.3%), but drastically lower compared with what was observed in a single herd in Italy (60%; Locatelli et al.,

2017; Kadlec et al., 2019). Interestingly, the proportion of MRSA among *S. aureus* isolates cultured from our herd (41.7%) was much higher than in other studies (1.2–9.3%) (Juhász-Kaszanyitzky et al., 2007; Vanderhaeghen et al., 2010; Sakwinska et al., 2011; Luini et al., 2015; Kadlec et al., 2019), except the Italian work, where almost all *S. aureus* isolates tested were methicillin-resistant (Locatelli et al., 2017). As in this study, cases of MRSA mastitis from other European countries were either exclusively or predominantly caused by ST398 (Vanderhaeghen et al., 2010; Sakwinska et al., 2011; Tavakol et al., 2012; Luini et al., 2015; Kadlec et al., 2019). All ST398 isolates in our study were also identified as belonging to the *spa* type t034, which has regularly been associated with the ST398 lineage and, together with *spa* type t011, represent the most common MRSA *spa* types circulating in both bovine and porcine populations (Fessler et al., 2010; Sharma et al., 2016; Teagene et al., 2019).

Despite its multihost specificity and clearly evidenced potential for interspecies transmission (including zoonotic route), the ST398 lineage contributes only marginally (<2%) to the overall MRSA burden in human population. However, marked differences exist between countries, depending on the intensiveness of the dairy agriculture and efficacy of infection surveillance and control programs for MRSA in the healthcare sector (Aires-de-Sousa, 2017).

Still, human-to-human transmission of MRSA ST398 occurred much less frequently than transmission of MRSA non-ST398 strains (Verkade et al., 2014). In this study, out of 6 human MRSA isolates sharing an identical phenotype (drug-susceptibility profile) and genotype determined by 3 typing methods with bovine MRSA isolates, 1 was from a milker, 3 from a veterinarian, 1 from the veterinarian's wife, and 1 from their son. This suggests transmission of 2 types: on-farm transmission between dairy cattle and livestock professionals and within-household transmission between the veterinarian and his family. The primary source of infection in the herd remains unknown. Consequently, it is unclear whether on-farm transmission was from the bovine-to-human or human-to-bovine route. Given that the clonal complex 398 is particularly associated with pig farming, with its *spa* t034 variant being a typical pig-borne genotype, it is possible that the MRSA strain was introduced into the dairy herd by the veterinarian, as he paid regular visits to pig facilities. The pathogen might then have spread within the herd either through a direct contact between the animals or during the milking process via the contaminated milker's hands, udder clothes, or teat cup liners. In the few studies which explored transmission of LA-MRSA strains between farm personnel and dairy cattle, the direction

of transmission could not be unequivocally established (Juhász-Kaszanyitzky et al., 2007; Fessler et al., 2010; Sakwinska et al., 2011; Locatelli et al., 2017; Sato et al., 2017; Schmidt et al., 2017). Seldom was it conjecturally inferred from comparison of specific genotype frequencies in human and bovine populations. Thus, finding a bovine isolate carrying a typically human genotype served as a proxy for human-to-bovine transmission, and vice versa (Sakwinska et al., 2011).

In accordance with previous studies, our present study demonstrated the potential of LA-MRSA to be transmitted between humans (Cuny et al., 2009; Bosch et al., 2015). Here, transmission between a veterinarian and his 2 family members was supported by the fact that those 2 had no direct contact with livestock animals or their environment.

Last, it is worth commenting briefly on culturing 2 different MRSA genotypes from the same anatomical site of 2 individuals. The nasal swabs from the veterinarian and the milker, which yielded 2 strains each (1 of ST398 and 1 of ST45) were concerning because ST45 represents one of the predominant, essentially hospital-acquired, human clones in Europe and beyond (Stefani et al., 2012). The clonal complex CC45-related genotypes are common in both asymptomatic carriage isolates and invasive isolates involved in severe nosocomial infections (Moore et al., 2010). The occurrence of the ST45 MRSA in a veterinarian and a milker may be because they have known each other and have worked together for many years during routine veterinary inspections of the herds.

The co-existence of multiple *S. aureus* genotypes (strains) in human nasal carriers has already been reported, albeit the clinical significance of such co-colonization remains obscure. Speculatively, it may influence the pathogenicity and virulence of individual strains, not excluding their transmission capacities (Votintseva et al., 2014).

CONCLUSIONS

This study documents, for the first time in Poland, an outbreak of subclinical mastitis in dairy cows due to MRSA. The strain involved in the outbreak belonged to the major LA-MRSA clonal lineage CC398. Our results also provide the evidence of transmission of that strain between humans and cows, and between humans in the family setting. This work was only preliminary, and further investigations need to be developed to better recognize the prevalence of MRSA among dairy cattle and the extent of MRSA carriage among farm professionals and their household members in Poland. Nevertheless, our findings underscore the risk of intra- and interspecies transmission of LA-MRSA and urge enhancement

of the existing biosecurity measures aimed at preventing MRSA (and other milk pathogens) spread at both farm- and household levels.

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The authors have not stated any conflicts of interest.

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