Comparison of the population structure of *Streptococcus uberis* mastitis isolates from Austrian small-scale dairy farms and a Slovakian large-scale farm

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ABSTRACT

Streptococcus uberis, a major mastitis pathogen associated with intramammary infections (IMI), can be found ubiquitously in the cow's environment. Although *Strep. uberis* is reported to be susceptible to most antimicrobials, in practice poor responses to treatment and recurrent mastitis are observed. This can be explained by reinfection or by persistence of strains. We hypothesized that among a heterogeneous group of *Strep. uberis* mastitis isolates, some predominant host-adapted clones might be recurrently isolated from IMI. Therefore, the aim of this pilot study was to determine the *Strep. uberis* genotype variety found among small-scale dairy herds (127 Austrian dairy farms) and compare this with a large-scale herd (a Slovakian dairy farm). We determined the occurrence and strain diversity of *Strep. uberis* (n = 309) isolates using molecular analysis. *Streptococcus uberis* isolates from aseptically collected quarter milk samples were genotypically characterized using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing. The *Strep. uberis* strain set covered isolates from 4 Austrian federal areas [Lower Austria (n = 67), Upper Austria (n = 8), Salzburg (n = 51), and Styria (n = 1)] and the Bratislava Region of Slovakia (n = 1). The PFGE analysis resulted in 187 *Sma*I profiles with 151 unique profiles. Simpson's index of diversity was 0.988. Individual cows (n = 17) harbored up to 3 different PFGE types in the udder. Dairy cows shared distinct PFGE types within a farm. Seven PFGE types were widely distributed among Austrian dairy farms. In the Slovakian farm, 10 predominant PFGE types were recurrently isolated from the same quarters; these genotypes were assigned as persisters. We identified novel sequence types (ST) using multilocus sequence

typing related to the global clonal complexes ST5 and ST143. We concluded that *Strep. uberis* IMI are caused by strains with a wide heterogeneity of PFGE types. This large number of unique subtypes indicates a high diversity of *Strep. uberis* in the environment. In the large herd, molecular epidemiological results revealed that specific strains might be involved in contagious transmission events and potentially lead to persistence. **Key words:** *Streptococcus uberis*, mastitis, persistence, pulsed-field gel electrophoresis

INTRODUCTION

Streptococcus uberis is considered a major bovine mastitis pathogen. It is associated with IMI and can be found ubiquitously throughout the cow's environment. Reservoirs of *Strep. uberis* include the cow's skin surface, genital and gastrointestinal tract, manure, and bedding material. Inadequate stall management, poor udder hygiene, and damage to teat ends are recognized to contribute to the risk of infection with *Strep. uberis* (Krömker et al., 2014).

Streptococcus uberis infections lead to milk production losses and increased SCC (Reyher et al., 2012; Heikkilä et al., 2018). *Streptococcus uberis* IMI are to a considerable extent clinically apparent, with visible changes to the milk secretion, palpable udder tissue alterations, and increased body temperature. Recently, *Strep. uberis* has become increasingly important as a cause of subclinical mastitis (Blowey and Edmondson, 2010; Krömker et al., 2014) and is mainly acquired during the drying-off period (De Vliegher et al., 2012; Krömker et al., 2014; Verbeke et al., 2014). Recent *Strep. uberis* incidence rates are fluctuating in a global context. Data from Belgium reported that *Strep. uberis* occurred in 18% of clinical cases (Verbeke et al., 2014), and the prevalence in France ranged between 18 and 37% for clinical mastitis and between 6 and 24% for subclinical mastitis (Poutrel et al., 2018).

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Strategies to decrease *Strep. uberis* prevalence in dairy herds include implementation of hygiene measures, enhancement of the immune status of cows, and pathogen-specific treatment concepts (Krömker et al., 2014; Ruegg, 2014; Mansion-de Vries et al., 2015). Risk assessment based on monitoring data (Samson et al., 2016; Soltau et al., 2017; Taponen et al., 2017), the implementation of novel diagnostic tools (Archer et al., 2017; Leimbach and Krömker, 2018), improved strain characterization (Hossain et al., 2015; Davies et al., 2016; Vélez et al., 2017; MLST, 2018c), and vaccination (Collado et al., 2018) are contributing to improved epidemiological understanding of *Strep. uberis* IMI and new approaches to control and prevent infections.

Consistent with its environmental origin, many authors have reported a high level of *Strep. uberis* diversity within investigated herds (Douglas et al., 2000; Khan et al., 2003; McDougall et al., 2004; Tomita et al., 2008; Haenni et al., 2010; Lasagno et al., 2011; Lundberg et al., 2014; Reinoso et al., 2015; Loures et al., 2017). Some *Strep. uberis* genotypes have even been suspected to be host adapted, capable of cow-to-cow transmission (Zadoks et al., 2003; Tassi et al., 2013; Klaas and Zadoks, 2018). Both increased cow susceptibility to infections and persistence in udder tissue are plausible causes of *Strep. uberis* IMI (Moyes et al., 2010; Abureema et al., 2014; Krömker et al., 2014). Recurrent *Strep. uberis* udder infections with novel genotypes have been observed, indicating a heterogeneous strain reservoir in the barn environment. In some cases, recurrences after therapy were linked to the same predominant *Strep. uberis* genotype [e.g., sequence types (ST) ST5 and ST143; Phuektes et al., 2001; Tomita et al., 2008; Abureema et al., 2014; Davies et al., 2016]. Current discussions concerning *Strep. uberis* mastitis pathogenesis relate to the effect of host immune status, pathogen interaction, and strain virulence potential (Tassi et al., 2013; Günther et al., 2016).

Our aim was to investigate the *Strep. uberis* population structure involved in bovine subclinical and clinical mastitis among Austrian small-scale dairy farms and compare results with those from a large dairy farm in Slovakia. We determined the *Strep. uberis* strain variety based on restriction enzyme-based genetic fingerprinting (pulsed-field gel electrophoresis; PFGE) and sequence-based analysis of housekeeping genes (multi-locus sequence typing; MLST), respectively (Coffey et al., 2006; MLST, 2018c). We hypothesized that among a heterogeneous group of *Strep. uberis* strains, some predominant host-adapted clones could be recurrently isolated from IMI and were geographically widespread. Implicitly, it may be that these strains are involved in contagious transmission and responsible for herd infections.

MATERIALS AND METHODS

Isolate Collection, Identification, and Farm Structure

Streptococcus uberis (n = 309) was isolated as pure cultures from quarter milk samples during 2014 and 2015 at the diagnostic laboratory of the University Clinic for Ruminants of the University of Veterinary Medicine, Vienna, Austria, and originated from cows with subclinical or clinical IMI. The samples were either sent to the laboratory for bacteriological examinations or obtained during herd visits. Biochemical characterizations and confirmation with 16S rRNA gene sequence analysis are described elsewhere (Wald et al., 2017). The partial rRNA gene sequence data have been deposited in the GenBank database under accession number KX389930 to KX390233 (<https://www.ncbi.nlm.nih.gov/genbank/>).

Streptococcus uberis isolates (n = 214) from 127 Austrian dairy farms in Lower Austria (LoA; n = 67), Upper Austria (UA; n = 8), Salzburg (SB; n = 51), and Styria (STM; n = 1) were included. Further, *Strep. uberis*-positive quarter milk samples (n = 95) were obtained from a large-scale dairy farm in Slovakia (SK) located close to the Austrian border in the Bratislava Region. *Streptococcus uberis*-positive cows were resampled 14 and 21 d later after antimicrobial treatment with β -lactams for a follow-up examination.

The isolate distribution according to geographical regions, individual cows, dairy farms, and information on resampling is provided in Table 1. Austria's special geographical feature as an alpine republic results in small-scale farms with a typically high proportion of grassland and mountain grazing. One-third of conventional farmers house their cattle in tiestalls, whereas the remaining farms have cubicle housing or straw or compost yards (Firth et al., 2017c). The most important breed is Simmental, which makes up to 81% of stock. An average holding has fewer than 30 dairy cows (Firth et al., 2017a; Federal Ministry for Sustainability and Tourism, 2018). Only about 3% of the farms keep more than 50 cows. Farm structure and management information of the large-scale dairy herd in SK are shown in Table 2.

If recorded, data on clinical history, DIM, and treatment regimen were collected. The form of mastitis was defined as subclinical if visual signs were absent and SCC was elevated ($\geq 200,000$ cells/mL) or as clinical if local or systemic symptoms were apparent (De Vliegher et al., 2012; Adkins and Middleton, 2018). Adapting the definition of Döpfer et al. (1999), recurrent cases of mastitis were defined by reisolation of *Strep. uberis* in the same quarter in subsequent sampling events at least 14 d after enrollment of the previous case of clinical

Table 1. Origin of *Streptococcus uberis* isolates characterized with pulsed-field gel electrophoresis (PFGE)

Country and region	Farms		Cows		Clinical mastitis		Subclinical mastitis		Clinical and subclinical mastitis		Cows repeatedly positive for <i>Strep. uberis</i> (no.)		Isolates		PFGE unique profiles (<i>SmaI</i>)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Austria																
Lower Austria	67	52.34	92	39.66	65	28.02	25	10.78	2	0.86	3		101	32.69	76	50.33
Salzburg	51	39.84	73	31.46	58	25.00	13	5.60	2	0.86	2		80	25.89	61	40.40
Upper Austria	8	6.25	13	5.6	9	3.88	4	1.72	0	0.00	0		26	8.41	4	2.65
Styria	1	0.78	5	2.16	0	0.00	5	2.16	0	0.00	0		7	2.27	1	0.66
Slovakia																
Bratislava Region	1	0.78	49	21.12	39	16.81	6	2.59	4	1.72	12		95	30.74	9	5.96
Total	128	100.00	232	100.00	171	73.71	53	22.84	8	3.45	17		309	100.00	151	100.00

mastitis. Temporal distances between recurrent infections varied from 2 to 3 wk (shortest time interval in SK, including all cows bacteriologically positive in the posttreatment samples) to several months (cows with recurrent clinical episodes within 1 lactation).

Molecular Epidemiology: *Streptococcus uberis* Subtyping

Streptococcus uberis isolates were stored as cryocultures (15% glycerol solution). In a literature review, protocols for PFGE typing were compared to develop an optimized protocol for *Strep. uberis* (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16930>). The PFGE analysis of *Strep. uberis* (n = 309) followed standards established by PulseNet International (2016). The isolates were cultivated on Columbia blood agar with 5% sheep blood (Oxoid Ltd., Basingstoke, UK) at 37°C for 18 h. Colonies were suspended in 2 mL of TE buffer (10 mM Tris:1 mM EDTA; pH 8.0). Subsequently, the optical density of the suspension was measured at 610 nm and finally adjusted to 1.2 optical density in a final volume of 400 µL. Lysozyme (20 mg/mL; Sigma-Aldrich, St. Louis, MO) was added to each tube and incubated in a thermomixer (Eppendorf, Hamburg, Germany) at 56°C for 30 min. During this time, 0.25 g of SeaKem Gold agarose (Lonza Group, Basel, Switzerland) was melted in 25 mL of TE buffer and tempered in a water bath (56°C). Proteinase K (20 mg/mL; Roche Diagnostics GmbH, Mannheim, Germany) was added to the cell suspension, mixed with 400 µL of 1% Seakem Gold agarose, and filled into plug molds. After 5 min of storage in the refrigerator at 4°C, the solidified plugs were transferred into falcon tubes, which contained a mixture of 5 mL of cell lysis buffer (1 M Tris, pH 8; 0.5 M EDTA, pH 8; sodium lauryl sulfate; aqua bidestillata) and 25 µL of proteinase K (20 mg/mL; Roche Diagnostics GmbH). The cell lysis and protein digest were performed overnight in a shaking water bath (54°C, 120 rpm). The next day the plugs were washed twice with sterile ultrapure water (54°C) and then 3 times with TE buffer (54°C). Plugs were stored refrigerated in 5 mL of TE buffer at 4°C. The plugs were incubated in 20 U of *SmaI* (Thermo Fisher Scientific Inc., Waltham, MA) at 25°C in the DNA macrorestriction digest for 4 h. The PFGE global standard *Salmonella* serotype Braenderup (H9812) was digested in 20 U of *XbaI* at 37°C for 4 h. Restricted samples were separated in a 1% (wt/vol) SeaKem Gold agarose gel in 0.5× Tris-borate-EDTA buffer at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories Inc., Hercules, CA). A linear ramping factor with pulse times from 5.0 to 50.0 s at 14°C and an inclined angle of 120° was applied for 22.5 h. The gels were stained with ethidium bromide

(Sigma-Aldrich) and digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories Inc.) in TIFF image format. The normalization of PFGE patterns and cluster analysis unweighted pair group method, including arithmetic averages and the Dice correlation coefficient with a position tolerance of 1.5%, was performed by applying BioNumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium). The PFGE types with no band difference were considered 100% similar and respectively genetically indistinguishable, according to Tenover et al. (1995). *Streptococcus uberis* PFGE types with identical profiles within the same quarter during 2 or more sampling events were classified as persistent genotypes, whereas recurrent infections with a different genotype were defined as reinfections (Abureema et al., 2014). The PFGE types with a unique *SmaI* profile pattern were defined as sporadic within this study. Simpson's index of diversity of PFGE profiles among *Strep. uberis* isolates (*SmaI* profiles) was calculated by applying the online tool of comparing partitions (<http://www.comparingpartitions.info/>).

The global distribution of predominant *Strep. uberis* PFGE types was assigned via MLST typing (MLST, 2018c). The DNA extraction followed the protocol for gram-positive bacteria (20 mg/mL lysozyme added to the cell lysis step) included with the Nucleospin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). *Streptococcus uberis* MLST typing based on the 7 housekeeping loci [glucose kinase (*gki*), transketolase

(*recP*), D-ala-D-ala ligase (*ddl*), thymidine kinase (*tdk*), carbamate kinase (*arcC*), triose phosphate isomerase (*tpi*), and acetyl CoA acetyl-transferase (*yqiL*)] was performed according to Coffey et al. (2006). An allele number was assigned to each housekeeping gene, and ST were determined and compared using the pubMLST sequence and isolate database (MLST, 2018b,d). Clonal complexes were defined as groups of ST sharing at least 4 housekeeping genes with another member of the group (Coffey et al., 2006).

Antimicrobial Susceptibility

In total, 36 *Strep. uberis* isolates were tested for antimicrobial susceptibility by applying the commercially available MIC microtiter plate assay Micronaut-S Mastitis 3 (Merlin Diagnostika GmbH, Bornheim, Germany). The assay covered a panel of the following antimicrobial classes: penicillins (penicillin G, 0.125–8 µg/mL; ampicillin, 4–16 µg/mL; oxacillin, 1–4 µg/mL; amoxicillin:clavulanic acid, 4:2–32:16 µg/mL), cephalosporins (first-generation cefazolin, 4–32 µg/mL; third-generation cefoperazone, 2–16 µg/mL; fourth-generation cefquinome, 1–8 µg/mL), macrolide (erythromycin, 0.25–4 µg/mL), aminoglycoside: first-generation cephalosporin (kanamycin:cephalexin, 4:0.4–32:3.2 µg/mL), third-generation fluoroquinolone (marbofloxacin, 0.25–2 µg/mL), and lincosamide (pirilmycin, 1–4 µg/mL).

Table 2. Data and herd parameters for the commercial dairy farm in Slovakia

Parameter	Data
Personnel	
No. of personnel	160
Training and education	2 farm veterinarians, animal technicians, and agricultural laborers
Production	
Age of facilities	~12 yr
Milking parlor	2 rotary (2 × 40 stalls)
Animals and milk production	
No. of cows on farm	2,500 dairy cows
Herd size	5,000
Breed	Holstein Friesian predominantly
Daily milk yield	28 kg
Bulk milk	Average 140,000–170,000 cells/mL
Calving	Year-round
Feeding	TMR, zero grazed
Mastitis	
Mastitis prevention actions	Milking order (fresh cows, then higher DIM, then mastitis group); cotton towels; cluster disinfection; postdipping with iodine-based agents
Mastitis treatment	Farm-specific regimen based on clinical symptoms and cow's history
Pathogens	History of <i>Staphylococcus aureus</i> , <i>Streptococcus uberis</i> , and <i>Escherichia coli</i> infections
Drying-off	
Time of drying-off	>220 d carried calf or milk yield <20 kg/d
Method	Antimicrobial drying agents and internal teat sealer
Housing	
Lactating cows	Freestall with concrete floor, concrete cubicles with rubber mats
Cows before calving	Straw
Fresh cows, dry cows, and mastitis	Recycled manure (separate)

Streptococcus uberis isolates were grown on Columbia agar containing 5% sheep blood (Oxoid Ltd.) for 24 h at 37°C incubation. The overnight cultures were suspended in sterile saline solution (0.85% NaCl) to achieve a turbidity of McFarland standard 0.5, and then were diluted 1:110 (Micronaut) and 1:367 (Sensititer), respectively, according to the manufacturer's instructions before use. The breakpoints for MIC were determined following the Clinical Laboratory Standards Institute (CLSI) standards for bacteria isolated from animals and recommendations for *Strep. uberis* provided in reference works (Pillar et al., 2009; Feßler et al., 2012; CLSI, 2018).

Statistical Analysis

Clinical data were analyzed using SPSS Statistics 23.0 (IBM, Armonk, NY) and Excel 2013 (Microsoft Corp., Redmond, WA). The chi-squared statistic was used to determine whether IMI were more frequent in front or hind quarters. Statistical significance was defined at $P \leq 0.05$.

RESULTS

Animals and Clinical Data

The majority of cows included in this study suffered from clinical signs of mastitis ($n = 171/232$; 73.71%), and 22.84% ($n = 53/232$) were affected by subclinical mastitis. The minority of cows indicated both clinical and subclinical mastitis during the whole study period (3.45%; $n = 8/232$). Details are provided in Table 1.

Most clinical IMI (83.14%) occurred during the first 200 DIM. Rear quarters ($n = 205$; 66.34%) were more affected by IMI (chi-squared statistic, $P < 0.001$) than front quarters ($n = 104$; 33.66%).

Some cows had multiple episodes of *Strep. uberis* mastitis in the same quarter (cows $n = 14/232$; $n = 11$ in Slovakian farm and $n = 3$ in Austrian farms) or in different quarters (cows $n = 3/232$ in Austrian farms) over time. In cows with repeated examinations, we could observe recurrent episodes of mastitis (cases $n = 35$) and cases without subsequent *Strep. uberis* detection ($n = 37$). In 21 cows (LoA, $n = 4$; UA, $n = 7$; SB, $n = 4$; STM, $n = 2$; SK, $n = 4$), more than 1 quarter was affected at the same sampling event.

Streptococcus uberis Subtyping

The *Strep. uberis* isolates ($n = 309$) clustered into 187 *SmaI* profiles represented by 301 isolates and 8 nontypable isolates. Simpson's index of diversity was 0.988. A total of 151 *SmaI* profiles were unique within this

data set and linked to sporadic mastitis cases (Table 1; Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16930>), and 36 *SmaI* profiles were repeatedly isolated and classified as sporadic when isolated during the same sampling event more than once or as persistent when isolated during 2 or more sampling events (Figure 1). The highest amount of heterogeneous profiles was obtained from *Strep. uberis* isolated from Austrian dairy farms located in LoA and SB (Table 1; Supplemental Table S2). Individual cows ($n = 17$) harbored up to 3 different PFGE types in the udder (SK, $n = 6$; LoA, $n = 5$; SB, $n = 5$; UA, $n = 1$; Supplemental Table S2). Dairy cows shared distinct PFGE types within a farm (SK, $n = 44$ cows and 11 PFGE profiles; LoA and SB each, $n = 6$ cows and 3 PFGE profiles; UA, $n = 5$ cows and 1 PFGE profile; STM, $n = 4$ cows and 1 PFGE profile; Supplemental Table S2).

Seven PFGE types were detected in Austria interfarms: LA14-1 = SB21, LA19 = LA31 = LA72-1, LA21 = LA46, LA70 = SB6, SB14 = SB15-2, SB25-2 = SB52-1, and LA79-5 = UA7-2 (Figure 1). Among the Austrian dairy farms, only 1 cow showed recurrent infection with an identical PFGE type (LA72-3). Other recurrent cases of mastitis (PFGE types SB31-3, SB31-4; SB11-1, SB11-2; LA24-1, LA24-2; and LA35-1, LA35-2) were clearly caused by new *S. uberis* strains evidenced by PFGE analysis. In the Slovakian farm, 10 predominant PFGE types circulated between cows ($n = 34$) and were recurrently isolated from the same quarters. Types SK2, SK11, and SK16 were even isolated from the same individual cow during several sampling events. The latter *Strep. uberis* genotypes were assigned as persisters (Figure 1). Intraherd present PFGE types were observed to be sporadic in individual cows and at the same time persistent in other cows (Figure 1; Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16930>).

The MLST analysis was performed for persistent and sporadic genotypes isolated from several cows. The *Strep. uberis* isolates could be assigned to novel ST ($n = 12$): ST876, ST879, ST881, ST886, and ST877 were isolated from Austrian dairy cattle, and ST878, ST880, ST882, ST883, ST884, ST885, and ST887 were isolated from Slovakian dairy cattle (Figure 1). The ST876 ($n = 6$, PFGE profile STM2-1) and ST886 ($n = 3$; PFGE profile S25-2 = S52-1) isolates from Austria could be assigned to ST-5 and ST-143 complexes, respectively. Generally, the investigated ST showed closest matches to the ST-5 and ST-143 complex isolates already present in the MLST database ($n = 1,179$ isolates; MLST, 2018a). The latter ST were different in 1 or 2 alleles.

Sequence type 879 ($n = 5$; PFGE type LA80) was isolated from Austrian dairy cattle and showed the closest match to ST880, which was the most abundant ST in

the Slovakian dairy farm (n = 25; PFGE profile SK7). Sequence type 881 (n = 10; PFGE type UA7-1) was related to ST143 complex isolates. In the PFGE cluster analysis, ST879 and ST880 showed different band patterns, underlining the highest discriminatory power of PFGE compared with MLST to identify genetic clones.

The PFGE profiles SK13 (n = 7), SK14 (n = 3), and SK14ST (n = 7), all closely related in the PFGE cluster analysis, were assigned to ST883 with the closest match to the ST-5 complex (Figure 1). The PFGE profile SK3 (n = 6) was identified as ST304, which had also been isolated in Italy from subclinical mastitis cases. ST884 isolates from SK showed the closest match to ST47 isolated from a subclinical mastitis event in the United Kingdom. ST877 (n = 6; PFGE type SK1) and ST880 (n = 25; PFGE type SK7) were closely related to ST863

and ST874, both of which had previously been isolated from Canadian mastitis cases. Sequence type 882 was the nearest neighbor to ST241 that had been isolated in Sweden from bovine mastitis cases. Sequence type 885 (n = 7; PFGE type SK4) and ST887 (n = 6; PFGE type SK8) had no relatives in the *Strep. uberis* MLST database.

Antimicrobial Susceptibility

Each of the persistent and recurrently isolated PFGE types (Figure 1) represented by distinct isolates (n = 36) had lowest MIC values for penicillin, ampicillin, amoxicillin/clavulanic acid, cefazolin, and cefquinome (Table 3) and was susceptible to cefoperazone (MIC breakpoint ≤ 2 $\mu\text{g/mL}$) and kanamycin/cephalexin

Figure 1. Pulsed-field gel electrophoresis (PFGE) cluster analysis (*SmaI*) of persistent and sporadic *Streptococcus uberis* genotypes (n = 36) isolated more than once. n = number; ST = sequence type; SK = Slovakia; UA = Upper Austria; LoA = Lower Austria; SB = Salzburg; STM = Styria; SC = subclinical mastitis; C = clinical mastitis; SPOR. = sporadic; PERS. = persistent; Intrafarm = intrafarm occurrence; Interfarm = interfarm occurrence.

(MIC breakpoint ≤ 8 $\mu\text{g/mL}$). Almost all isolates ($n = 34$) were considered to be susceptible (MIC breakpoint ≤ 2 $\mu\text{g/mL}$) to pirlimycin, but 2 strains, PFGE type SK8 (ST887) and PFGE type STM2-1 (ST876), were resistant (MIC breakpoint ≥ 4 $\mu\text{g/mL}$). The PFGE type SK8 showed growth at the highest concentrations in the dilution series of erythromycin.

DISCUSSION

The present work contributes to knowledge of the *Strep. uberis* population structure involved in bovine subclinical and clinical mastitis among Austrian small-scale dairy farms and a large dairy farm in SK. All field isolates were confirmed as *Strep. uberis* phenotypically and with molecular tools (Wald et al., 2017), and the isolates were further classified by genetic fingerprinting (PFGE) and sequence-based analysis of housekeeping genes (MLST). Antimicrobial susceptibilities were estimated for persistent and sporadic *Strep. uberis* genotypes isolated more than once ($n = 36$; Figure 1; Table 3).

Generally, the strain diversity was found to be high (Simpson's index of diversity = 0.988), which is in accordance with other studies (McDougall et al., 2004; Lundberg et al., 2014; Perrig et al., 2015). This could be explained by the strict host specificity of *Strep. uberis* leading to an increased nucleotide diversification compared with pathogens adapted to both humans and animals (Chen et al., 2018; Haenni et al., 2018). Most individual profiles were obtained from *Strep. uberis* isolated from Austrian dairy cattle, suggesting herd-specific environmental sources of *Strep. uberis* infection, confirming the findings of Wang et al. (2013). It is possible that distinct PFGE types spread within a dairy farm from environment- or milk equipment-associated reservoirs. Rato et al. (2013) suggested that inadequate implementation of management programs for the control of contagious pathogens (e.g., *Streptococcus agalactiae*) at farm level may even promote the transmission of environmental pathogens, such as *Strep. uberis*, among dairy cattle. Important hygiene control points include the type and frequency of changing bedding material and efficient sanitation of milking equipment (Krömker et al., 2014).

In our study, 2 important findings underline the high prevalence, strain diversity, and global spread of environment-associated *Strep. uberis* (Werner et al., 2018; Käppeli et al., 2019). First, in a small number of dairy cows, the udder was colonized by up to 3 unrelated genotypes during the same sampling event. Second, 7 genotypes were found among unrelated Austrian dairy farms (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16930>).

In the Slovakian farm, predominant PFGE types ($n = 10$; Figure 1) were recurrently isolated over several weeks from the same quarters. This was defined as persistence (McDougall et al., 2004; Pullinger et al., 2007; Abureema et al., 2014). Abureema et al. (2014) identified a small number of recurrences that were caused by the initial *Strep. uberis* strain, indicating persistence. The authors observed 2 recurrences in a new quarter with the initial *Strep. uberis* strain, indicating either spread between quarters or reactivation of a previous subclinical infection. This was also observed in our study for the PFGE type LoA 72-3 (LoA dairy farm), which was isolated twice from rear and front quarters within weeks. We defined this strain also as persistent due to PFGE fingerprinting. In contrast to Werner et al. (2018), the in-herd similarity of *Strep. uberis* strains was higher than the similarity from different herds (Figure 1). This can be explained by the relatively high band position tolerance of 5% and 80 to 95% similarity cut-off in the PFGE cluster analysis of Werner et al. (2018).

Bedding material such as straw and recycled manure, which was used in the large Slovakian herd, might have served as an important source of infection and reinfection with the environmental pathogens (Krömker et al., 2014; Verbeke et al., 2014). Lundberg et al. (2016) also suggested a certain influence of bedding material during the late housing season on the severity of persistent *Strep. uberis* infections and observed a lower prevalence during the pasture season. Additionally, herd size and milking hygiene are important factors determining the cow-to-cow spread of *Strep. uberis* (Lundberg et al., 2016). In contrast, Phuektes et al. (2001) and Khan et al. (2003) suggested the dominance of certain contagious clones of *Strep. uberis* in infected herds. We are in agreement with Wang et al. (2013) that predominant PFGE types in one herd indicate either infection with isolates from the same environmental reservoirs or cow-to-cow transmission, which is a well-described transmission route for cow-associated contagious pathogens.

The MLST analysis provided further insight into the global spread of the most abundant *Strep. uberis* strains included in the present study. Generally, all genotypes were assigned to novel ST (ST876, ST879, ST881, ST886, and ST877 from Austrian dairy cattle, and ST878, ST880, ST882, ST883, ST884, ST885, and ST887 from Slovakian dairy cattle), except for ST304, which is already present in the MLST database. The closest genetic traits could be identified with highly abundant ST-5 and ST-143 complex isolates included in the MLST database. Global clonal complexes ST-5 and ST-143, highly associated with bovine IMI, are genetic lineages of virulent isolates present in Australia, New Zealand, the United States, Canada, India, and Europe

Table 3. Minimum inhibitory concentrations ($\mu\text{g/mL}$) of *Streptococcus uberis* strains representative of predominant pulsotypes (isolates n = 36)

Pulsed-field gel electrophoresis type ¹ (<i>SmaI</i>)	Antimicrobial ³ (μg/mL)												
	Category ²	Amoxicillin: clavulanic acid 2:1 (4-32:2-16)					Kanamycin: cephalexin 10:1 (4-32:2-16)						
		Ampicillin (4-16)	Penicillin (0.125-8)	Oxacillin (1-4)	Cefazoline (4-32)	Cefoperazone (2-16)	Cefquinome (1-8)	Pirlimycin (1-4)	Marbofloxacin (0.25-2)	Erythromycin (0.25-4)			
Isolated in Austria													
LoA1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA14-1 = SB21	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA19 = LoA31 = LoA72-1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA21 = LoA46	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA70 = SB6	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA72-3	P	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA79-4	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
LoA79-5 = UA7-2	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
LoA80	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
LoA95-1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SB12	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
SB14 = SB15-2	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SB22	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	2	≤0.125		
SB23	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	2	≤0.125		
SB25-2 = SB52-1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SB51-1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
STM2-1	S	≤4	≤0.125	2	≤4	≤2	≤1	≤4	≤1	>4 ⁴	1	≤0.125	
UA1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
UA6	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
UA7-1	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
UA8	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
UA9	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
Isolated in Slovakia													
SK1	P/S	≤4	≤0.125	2	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK2	P	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK3	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK4	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	2	≤0.125		
SK7	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
SK8	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	>4 ⁴		
SK11	P	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK12	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		
SK13	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK14	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	2	≤0.125		
SK14-subtype	P/S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK16	P	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK20	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	1	≤0.125		
SK21	S	≤4	≤0.125	≤1	≤4	≤2	≤1	≤4	≤1	0.5	≤0.125		

¹LoA = Lower Austria; SB = Salzburg; UA = Upper Austria; STM = Styria; SK = Slovakia.²S = sporadic; P = persistent.³The resistance breakpoint for cefoperazone is ≥ 8 (Fefler et al., 2012; *Streptococcus uberis*, bovine mastitis), for kanamycin:cephalexin 10:1 is ≥ 32 (Pillar et al., 2009; *Streptococcus uberis*, bovine mastitis), and for pirlimycin is ≥ 4 (CLSI, 2018; *Streptococcus uberis*, bovine mastitis). No breakpoints for *Streptococcus uberis* were determined for the remaining antimicrobials.⁴Resistant isolate.

(Rato et al., 2008; Tomita et al., 2008; Shome et al., 2012). More recently, Käppeli et al. (2019) identified ST-5 complex to 30.7% ($n = 47/153$) present among bovine mastitis isolates collected in the Swiss Midlands. The geographic conditions, herd size, and breeds are most comparable with Austrian dairy cattle farming. A strong environmental link in *Strep. uberis* transmission, indicated by high ST variability, was evident and comparable with this study. Global clonal complex ST-5 and ST-143 isolates were significantly associated with clinical and subclinical mastitis and may represent a lineage of virulent isolates, whereas isolates belonging to global clonal complex ST86 were associated with cows with low cell counts (Tomita et al., 2008). A paper by Abureema et al. (2019) highlighted the difference in strain capacities of *Strep. uberis* to cause clinical mastitis: in a DNA hybridization array, genes were present among clinical strains related to carbohydrate and lipid metabolism (possible role in the growth or survival of *Strep. uberis* in milk) and enhanced virulence compared with *Strep. uberis* commensals.

It has been suggested that the virulence gene *hasA*, necessary for capsule formation in the udder tissue, was enriched in clonal complexes ST-5 and ST-143 complex isolates (Coffey et al., 2006; Pullinger et al., 2006; Tomita et al., 2008). For some *Strep. uberis* genotypes (ST879, ST880, ST883), the PFGE analysis was more discriminating in identifying genetically related isolates (Rato et al., 2008).

Streptococcus uberis isolates included in this study showed broad susceptibility to the majority of antimicrobial test agents, especially β -lactam antibiotics (penicillins and cephalosporins). This is in accordance with Käppeli et al. (2019), who determined a resistance rate of 11.8% for pirlimycin and elevated MIC 90 values for marbofloxacin and erythromycin in Switzerland. We found no evidence for resistance to the antimicrobial classes frequently used in IMI therapy in Austria: intramammary penicillin, cefquinome, cefoperazone, and kanamycin/cephalexin (Firth et al., 2017b). Furthermore, persistence could not be linked to AMR in certain genotypes. Studies revealed that extended intramammary treatment of *Strep. uberis* mastitis is necessary to increase bacterial cure rates in some herds (Krömker et al., 2014). Wente et al. (2019) assumed that transmission appears to be farm specific: inadequate treatment protocols together with high pressure for *Strep. uberis* reinfection from the environment and bovine reservoirs are possible reasons for persistence and recurrence of *Strep. uberis* mastitis within a herd. Therefore, we recommend a farm-specific mastitis control strategy, implementation of hygiene measures, culling of chronically infected cows, and extended therapy of acute mastitis cases. This includes regular monitoring of

mastitis pathogens in both large-scale and small-scale dairy herds.

CONCLUSIONS

Streptococcus uberis subclinical and clinical mastitis in dairy cattle are caused by multifaceted genotypes linked to the environment and to a lesser extent to global clonal complexes (e.g., ST-5, ST-143). The molecular epidemiological results in the large-scale herd examined indicate that specific strains of *Strep. uberis* might be involved in contagious transmission events and could lead to persistence.

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