Associations between *Streptococcus uberis* strains from the animal environment and clinical bovine mastitis cases

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ABSTRACT

Bovine clinical mastitis quarter foremilk samples were collected from 15 German dairy farms for the isolation of *Streptococcus uberis* strains. Samples were also collected from the 8 spots where *Streptococcus uberis* was most expected in the dairy environment to investigate the transmission behavior of *Streptococcus uberis* within the farm. The selected environmental spots for sampling were the inner surface of the milking liner, drinking troughs (on pasture and in the barn), exit area of milking parlor, bedding material from the lying area in the barn, passageway to pasture, lying area of soil or vegetation on pasture, and the barn area in front of the milking parlor. We performed pulsed-field gel electrophoresis on 237 *Streptococcus uberis* isolates to identify environmental strains that matched those from mastitis milk. The same strains were detected on the passageway to the pasture, milking parlor waiting area, in one of the liners, and a drinking trough. *Streptococcus uberis* strains showed high variability within farms and because identical strains (in mastitis milk and environment) were found in different environmental localizations, its transmission appears to be farm specific. Thus, to establish a farm-specific mastitis control strategy, the main environmental sources of *Streptococcus uberis* must be analyzed for matching strains. A molecular method such as pulsed-field gel electrophoresis is an important tool that can be used to obtain the necessary information.

Key words: *Streptococcus uberis*, mastitis, environmental, strain typing, epidemiology

INTRODUCTION

Bovine mastitis causes huge financial losses and is spread by cow-associated and environmental pathogens. Studies conducted over several decades have shown the value of preventive methods, which have led to a reduction in the mastitis prevalence caused by cow-associated pathogens (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*) and that are transferred primarily during milking (Bramley and Dodd, 1984; Hillerton et al., 1995; Leigh, 1999; Hillerton and Booth, 2018). In contrast, the environmental pathogens (e.g., *Strep. uberis*, coliform bacteria) have come into focus as the most frequently found mastitis-causing pathogens (Smith and Hogan, 1993; Bey et al., 2004; Klaas and Zadoks, 2018). Mastitis caused by these pathogens is, unfortunately, less manageable despite the implementation of a 5-point plan developed in 1984 by Bramley and Dodd (Leigh, 1999) because infection of the mammary gland takes place mainly outside the milking procedure. Habitat is the source of infection in the dairy environment (e.g., bedding material, feces). However, some of these strains may behave like cow-associated pathogens and appear to be transmitted mainly during milking (Leigh, 1999; Zadoks et al., 2003; McDougall et al., 2004).

Streptococcus uberis, the most frequently found and difficult-to-handle environmental pathogen, causes acute clinical and recurrent mastitis (Bramley and Dodd, 1984; Todhunter et al., 1995) and colonizes within cows and their environment (Kruze and Bramley, 1982; Wendt et al., 1994; Krömker, 2007). This bacterium can lead to a spectrum of mastitis cases, ranging from mild to severe. Its infection rate is influenced not only by environmental exposure but also by the efficiency of the host response to the bacterial challenge. Moreover, it is misleading to classify *Strep. uberis* as an environmental pathogen because it also has cow-associated transmission characteristics. The latter

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makes it necessary to analyze the type of transmission (environmental/cow-associated) for an appropriate control strategy.

Streptococcus uberis has previously been isolated from cows' skin, lips, tonsils, respiratory tract, rumen, teat skin and canal, udder, rectum, and feces. Transmission via mucosae of the digestive tract and feces appears to be the most frequent route of distribution within the dairy environment. This bacterium has also been isolated from pasture and the indoor environment (Bramley, 1982; Hejlícek, 1994; Wendt et al., 1994; Krömker, 2007; Paduch et al., 2013). *Streptococcus uberis* is shown to produce biofilms (Crowley et al., 2011; Schönborn et al., 2017) and capsules (Matthews and Oliver, 1993; Coffey et al., 2006) and invade mammary gland cells (Matthews et al., 1994). Its persistence within the udder, which can lead to a chronic infection of the mammary gland, is due to its ability to resist phagocytosis (Thomas et al., 1994) and withstand intracellular destruction by leukocytes (Leigh et al., 1990). This special condition can transform environment-associated *Strep. uberis* into a cow-associated pathogen (Baseggio et al., 1997; Phuektes et al., 2001). All of these characteristics make *Strep. uberis* mastitis difficult to control, even when only a few strains are detected within a farm. Although the presence of a small variety of *Strep. uberis* strains in one farm raises suspicion of its cow-associated contagious nature, it could also result from some predominant strains being prevalent in environmental places (hotspots). Therefore, in this study, we sampled the most-frequented areas in the cows' environment within a farm, which potentially harbor a high number of *Strep. uberis* strains (Lopez-Benavides et al., 2005), to search for strains matching those previously found in mastitis milk samples. Pryor et al. (2009) infected cows with a mixture of environmental and mastitis strains to observe the predominant constellation of strains that remained in the mammary quarter. They found that selection may take place within the mammary gland, resulting in a single strain that is identified in the diagnosis of mastitis. To our knowledge, this study is the first to compare mastitis and environmental *Strep. uberis* isolates within a farm. An understanding of *Strep. uberis* sources in the cow's environment would help in discerning its mode of transmission and finding the appropriate tactics to control *Strep. uberis* mastitis.

MATERIALS AND METHODS

Study Design

Sixty-two farms in Lower Saxony (northern Germany) participated in the present study, which was conducted

between June 2014 and December 2015. The sample collection time for each farm was 12 mo, and farmers collected quarter foremilk samples for microbiological analysis from cases of clinical mastitis. All isolates of *Strep. uberis* obtained from these samples were collected and stored. Only one case of mastitis caused by *Strep. uberis* per cow, during the study, was taken into account so that no isolates of persistent mastitis were used repeatedly. All farms having at least 5 identified *Strep. uberis* clinical mastitis cases within a year were chosen and visited by trained researchers for collection of environmental samples at specified locations. We sampled the environment close to the time when we identified the fifth *Strep. uberis* mastitis case on a farm but continued collecting mastitis isolates until the end of the year. Then, *Strep. uberis* isolates from environmental samples taken at 8 locations were compared, using pulsed-field gel electrophoresis (PFGE), with those previously isolated from clinical mastitis cases. Additionally, all bacteriologically identified *Strep. uberis* strains were confirmed as *Strep. uberis* by using PCR.

Selection of Farms

The present study on the epidemiology of *Strep. uberis* was a part of a project that focused on the comparison between pasture and indoor farming. Farms with diverse access to pasture were chosen, with the categorization of the pasture during the main cycle (1), half-day pasture (2), year-round stable keeping and running or grazing (3), or all-year stable keeping (4). The farms were selected based on their willingness to participate in this project. The mean herd size was 143 lactating cows (range: 62–620 cows; median: 117). At the start of the study, the average milk yield at farm level ranged from 7,500 to 11,750 L/cow per year (arithmetic mean: 9,437.4; median: 9,500.0). There were no further specifications regarding udder health parameters such as the incidence of clinical mastitis or the spectrum of mastitis pathogens.

Collection of Milk Samples

In the case of clinical mastitis, quarter foremilk samples were taken by the farmers, who were trained by study staff veterinarian, according to the guidelines of GVA (2009). The udder quarter was dry-cleaned to remove particles and teat ends were disinfected for about 15 s with disposable wipes saturated with ethanol (70%). The first 3 milk streams were discarded followed by milking into a sterile 13-mL sampling tube containing a boric acid-based preserving agent (Ly-20; Heeschen et al., 1969). The quarter foremilk samples

Table 1. Sampling spots and techniques

Spot ¹ (described by)	Applied sampling technique
Liner (Zadoks et al., 2003)	Inner liner surface was sampled by swabbing the wall in 2 rounds in the upper barrel near the mouthpiece lip.
Drinking troughs (barn/pasture) (Döpfer et al., 2005; Zadoks et al., 2005)	One template area of 20 cm ² was treated by double-swab technique at the upper and outer edge of the drinking trough.
Exit area milking parlor (Lopez-Benavides et al., 2007) Lying area pasture (Cullen and Little, 1969; Döpfer et al., 2005; Zadoks et al., 2005) Passageway to pasture (Lopez-Benavides et al., 2007)	The surface layer of soil, ground vegetation, or mud was sampled from the most by cows frequented ground area.
Barn bedding material (Bramley, 1982; Döpfer et al., 2005)	One cubicle was sampled at a distance of one-third of cubicle length from the hind edge.

¹One sample at each spot was taken; one liner for each farm was swabbed.

were sent, within 2 d, to the microbiology laboratory of the University of Applied Sciences and Arts Hannover (Germany) for microbiological analysis.

Collection of Environmental Samples

Based on a literature search, we selected locations in the cows’ environment that show a high presence of *Strep. uberis*. High cow traffic seems to be especially associated with the presence of *Strep. uberis*. The environmental samples were collected from different places or possible hotspots within the animals’ environment. These sites included the inner surface of the milking liner, drinking troughs (inside), the exit area of the milking parlor, bedding material from the lying area in the barn, the passageway to the pasture, soil or vegetation from the pasture lying area, and drinking troughs on pasture (outside) on those farms where cows had access to pasture (Cullen and Little, 1969; Bramley, 1982; Zadoks et al., 2003, 2005; Döpfer et al., 2005; Lopez-Benavides et al., 2005, 2007). The barn area in front of the milking parlor was also sampled because it had high cow traffic and because bacterial transmission to the teat skin immediately before milking, as well as milk leakage from individual animals, is considered a possible route of infection. In every farm, each spot was sampled once, although in farms with no pasture access, samples from the passageway to the pasture, lying area on pasture, and outside drinking trough were not available. Different techniques were used to sample the spots of interest (Table 1). The double-swab technique was performed according to DIN10113-1 (Deutsches Institut für Normung, 1997) to collect environmental samples from the surfaces of the liners and drinking troughs. The inner surfaces of the liners were sampled by swabbing the walls in 2 rounds. For drinking troughs, a sterile swab moistened with buffered peptone water (pH 7.0) was rolled over the surface (template area of

20 cm²) using moderate pressure in circular motions, followed by rolling of a dry cotton swab in the same manner. Both swabs were immersed in a sterile tube with 2 mL of buffered peptone water; then, the sticks of the swabs were broken off and discarded and the tube was immediately closed. A sample of the ground (minimum of 11 g) was collected from the barn area in front of the milking parlor, the exit of the milking parlor, passageway to the pasture, and lying areas using the hand in an inside-out sterile plastic bag. Further specifications for the sampling procedures are listed in Table 1. All environmental samples were transported at 5 to 8°C within 8 h to the laboratory (University of Applied Sciences and Arts Hannover, Germany) for microbiological analyses.

Analysis of Milk Samples

About 10 µL of well-mixed quarter foremilk sample was plated, using a sterile inoculation loop, on one quadrant of an esculin blood agar plate (Oxoid, Wesel, Germany) and incubated under aerobic conditions at 37°C for 48 h. The colonies that grew on esculin blood agar were primarily differentiated based on hemolysis patterns, esculin hydrolysis, Gram status, and cell morphology. The catalase reaction was also analyzed and gram-positive, catalase-negative cocci were differentiated by esculin hydrolysis. Esculin-positive colonies were further examined using modified Rambach agar, according to Watts et al. (1993), and phenol red agar medium supplemented with 1% D-sorbitol (Odierno et al., 2006). Gram-positive, esculin-positive, catalase-negative, β-D-galactosidase-positive, and D-sorbitol-positive cocci were identified as *Strep. uberis*.

Analysis of Environmental Samples

Surface samples were analyzed by mixing the tubes containing swabs for 1 min using a Vortex Genie 2 (Sci-

entific Industries, Bohemia, NY) and performing decimal dilutions with Ringer's solution (Merck, Darmstadt, Germany). Around 11 g of a solid or pasty sample was diluted with Ringer's solution and mixed by Stomacher (easyMIX, AES Chemunex/bioMérieux, Marcy l'Etoile, France) for 1 min. To determine the amount of streptococci and streptococci-like microorganisms, we used the spatula method according to DIN10192-5 (Deutsches Institut für Normung, 1995) to plate the diluted suspensions on Edwards modified medium supplemented with colistin sulfate and oxolinic acid (EMCO; Sawant et al., 2002). A maximum of 20 esculin-hydrolyzing colonies, if available, were isolated and checked for catalase-negative, gram-positive cocci, in accordance with Döpfer et al. (2005). The resulting colonies were subsequently cultured in parallel on modified Rambach agar medium to check for β -D-galactosidase activity (Watts et al., 1993) and on D-sorbitol agar to investigate D-sorbitol reduction ability (Odierno et al., 2006). The resulting presumptive *Strep. uberis* isolates were gram-positive, esculin-positive, catalase-negative, β -D-galactosidase-positive, and D-sorbitol-positive.

Storage of Isolates

The isolated microorganisms were cultured in brain heart infusion broth at 37°C for 24 h, mixed with 20% glycerin, and stored at -80°C until further investigation.

Molecular Analysis

The microbiologically identified *Strep. uberis* strains were further confirmed by PCR. The PFGE strain typing method was used to compare *Strep. uberis* strains of the environment with those from the mastitis milk samples.

PCR. DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands), and PCR for detection of *Strep. uberis* was performed using the primer and temperature profile described by Riffon et al. (2001). The reaction was carried out in a 25- μ L reaction mix, which consisted of 12.5 μ L of ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich, Munich, Germany), 20 pmol of primer mix, 5 μ L of template, and 5 μ L of pure H₂O (AppliChem, Darmstadt, Germany). Amplification reactions were carried out in an Mx3005P qPCR System thermocycler (Agilent Technologies, Santa Clara, CA). The PCR products were stained directly with Midori Green Direct (Nippon Genetics Europe GmbH, Dürren, Germany) and separated in a 2% agarose gel at 100 V for 2.5 h.

PFGE. DNA was extracted with Bio-Rad CHEF Genomic DNA Plug Kit (BioRad, Munich, Germany),

according to the manufacturer's instructions, and digested with 20 U of *Sma*I (New England Biolabs, Frankfurt am Main, Germany) at 25°C for 2 h. Finally, the plugs were washed with 1 mL of 0.5 $\times$ Tris-borate-EDTA (TBE) buffer (Roth, Karlsruhe, Germany) for 30 min at room temperature before being loaded on a 1% agarose gel. The fragments were separated using a Clamped Homogeneous Electric Fields Dynamic Regulation System, with a performing angle of 120° (CHEF-DR II System, Bio-Rad), filled with 0.5 $\times$ TBE buffer continuously chilled to a temperature of 14°C. Pulse timings of 1 to 15 s for 11.5 h and 15 to 45 s for 13.5 h at 5 V/cm (210 V), were applied. The PFGE gel was stained with Midori Green Advanced solution (Nippon Genetics Europe GmbH) according to the manufacturer's instruction and visualized by using the InGenius gel documentation system (Syngene, Cambridge, UK). Isolates that generated identical restriction patterns were identified as identical strains.

RESULTS

Four of the original 62 farms could not be used for evaluation because they either prematurely ceased to participate (3) or could not provide milk samples for examination (1). Seventeen farms had access to pasture during the main cycle of vegetation, 17 farms had access to half-day pasture (6 to 10 h), 13 farms used year-round indoor housing with short-time access to pasture (<6 h), and 15 farms used all-year indoor housing. A total of 1,895 mastitis milk samples were analyzed, out of which a minimum of 5 cows with clinical mastitis caused by *Strep. uberis* were found in each of 15 farms (farms A to O; Figure 1). A total of 134 mastitis isolates from the 15 farms were used for the comparisons. Six (farms B, D, K, L, M, and N) out of the 15 chosen farms had access to pasture during the main cycle of vegetation, 3 (farms A, G and O) were half-day pasture farms, 4 (farms C, H, I and J) used year-round indoor housing with short-time access to pasture, and 2 farms (farm E and F) had no access to pasture.

All of the isolates used for PFGE analysis were previously confirmed as *Strep. uberis* by PCR. Analysis of 103 environmental *Strep. uberis* isolates from 15 farms showed the following (Table 2). *Streptococcus uberis* isolates were detected in the barn area in front of the milking parlor in 8 farms (number of isolates): A (2), D (8), E (4), G (3), J (1), K (1), N (10) and O (4), and on the passageway to the pasture in 8 farms: B (1), D (4), G (4), H (1), K (4), L (1), M (2) and N (4). On 5 farms, *Strep. uberis* could be observed in the barn drinking troughs: C (1), D (2), F (1), G (1) and K (1); and 3 farms each had *Strep. uberis* isolates in the inner surface of liners: C (11), E (4), F (1); the exit area of

the milking parlor A (5), E (4), G (1); and the barn bedding material: E (6); G (1), N (6). In 2 farms, A (1), K (1), the isolates of *Strep. uberis* were found on pasture drinking troughs, and in 1 farm (farm I), *Strep. uberis* isolates were not detected in the cows' environment (Table 2). Overall, we collected 33 *Strep. uberis* isolates from 8 farms in front of the milking parlor, and PFGE results (Figure 2) indicated that 1 of the isolates was identical to a strain isolated from a clinical mastitis milk sample, both of them from the same farm. On the passageway to pasture, we found 19 isolates in 8 farms, where 2 isolates from 2 farms matched the mastitis milk isolates. On 1 of the farms (farm K), we observed that 1 isolate matched with 3 mastitis milk isolates from 3 cows. In another farm (farm B), 1 environmental isolate matched with 2 mastitis milk isolates from 2 cows. We compared 16 isolates from the liners on 3 farms and found 1 isolate that matched 1 mastitis case in 1 farm. One isolate from the drinking trough in the barn was identical to 1 mastitis case within the same farm (farm C). No matching strains were detected from other areas (e.g., pasture drinking troughs, exit area of the milking parlor, bedding material in the barn, and soil or ground vegetation of the grazing area). Identical strains within different environmental spots were also identified (Table 3). Strains from the waiting area in front of the milking parlor matched the strains found at the exit of the milking parlor (farms A and E), from the bedding material in the barn strain (farm N), and from the passageway to pasture (farms E and N). Strains from the passageway to pasture also matched those strains present in the exit of the milking parlor

(farm G) and in barn's bedding material (farm N). Two different strains isolated from the barn's bedding material matched with the strains from the passageway to pasture (farm N). We did not detect any matching isolates between the farms.

DISCUSSION

The aim of the present investigation was to describe the predominant transmission model of *Strep. uberis* infection at the farm level by comparing *Strep. uberis* strains from clinical mastitis samples and those from typical environmental sources. Our study was based on different sampling and diagnostic methods, which induces multiple methodical susceptibilities having an influence on the results. We only examined samples of clinical mastitis cases to improve the chances of finding relevant matching strains in the environment; a previous study by Jayarao et al. (1993) found less variety in strains from clinical mastitis than in those from subclinical mastitis. Clinical mastitis milk samples were collected over a period of 12 mo, resulting in a maximum of 1 yr between the first mastitis milk isolate and the first environmental isolate. Nevertheless, Todhunter et al. (1995) and Zadoks et al. (2003) previously reported the duration of infections to be as long as 370 and 309 d, respectively. We adopted 2 different techniques for the isolation of *Strep. uberis* from environmental samples and milk samples. The variety of different species in the environmental samples required the use of selective media to prevent contamination that could obscure essential colonies.

Figure 1. Distribution of *Streptococcus uberis* isolates from 15 farms for strain typing.

However, noncontaminated milk samples that usually harbor up to 2 different colony types were cultured on a nonselective medium. We chose the PFGE method for strain typing because it has higher discriminatory ability and can differentiate *Strep. uberis* strains better than other typing methods (Gillespie and Oliver, 2004). For the PFGE analysis, 1 cfu was taken out of one milk sample, because isolates from a single milk sample usually belong to the same strain (Oliver et al., 1998). Pryor et al. (2009) infected cows with a mixture of 5 *Strep. uberis* strains, and 1 strain predominated in 7 out of 10 quarters. The sampling spots in the dairy environment were selected based on some previous studies that found high numbers of *Strep. uberis* in the chosen spots (Table 1). In addition, the barn area in front of the milking parlor (i.e., the waiting area) was sampled. This spot was selected because it had high cow traffic but also that it could be involved in development of infection because of its spatial and temporal proximity to the zone of milking and potential for milk leakage

from animals passing by. Liners were sampled to prove the hypothesis of bacterial transmission during milking, indicating the cow-associated contagious mode of the pathogen (Smith and Hogan, 1993).

Streptococcus uberis isolates were detected in each of the 8 environmental sampling spots, and strains matching mastitis isolates were found in 4 spots (Table 2). This result supports the hypothesis that sources of *Strep. uberis* are areas with high cow traffic but also highlights the environmental character of *Strep. uberis*. It is possible that these spots are the source of the pathogens or, vice versa, were contaminated by mastitic milk. Nevertheless, some isolates were detected in liners, with one matching a mastitis milk strain (Table 3; farm C). This finding is in accordance with the results of Zadoks et al. (2003), where *Strep. uberis* was found in liners after *Strep. uberis*-infected animals had been milked. A high bacterial load on the inner surface of the liners increases the risk of transmission of *Strep. uberis* strains during milking, which suggests a host-adapted

Table 2. Origin of *Streptococcus uberis* environmental isolates and matches to mastitis strains

Farm	No. of mastitis isolates (matching environmental isolates)/ No. of strains isolated from milk samples ¹	No. of environmental isolates (matching isolate to milk isolate)	Sample origin
A	14/13	1 2 5	Drinking trough pasture Waiting area milking parlor Exit milking parlor
B	7 (2)/5	1 (1)	Passageway to pasture
C	9 (1)/7	11 (1)	Liner
D	7/4	1 (1) 2 8	Drinking trough barn Drinking trough barn Waiting area milking parlor
E	25 (1)/13	4 4 4 (1)	Passageway to pasture Liner Waiting area milking parlor
F	11/6	4 6 1	Exit milking parlor Bedding material barn Liner
G	6/4	1 1 3 1 4 1	Drinking trough barn Drinking trough barn Waiting area milking parlor Exit milking parlor Passageway to pasture Bedding material barn
H	9/5	1 2	Passageway to pasture Lying area pasture
I	10/7	0 ²	
J	6/3	1	Waiting area milking parlor
K	7 (3)/4	1 1 1 4 (1)	Drinking trough barn Drinking trough pasture Waiting area milking parlor Passageway to pasture
L	4/4	1	Passageway to pasture
M	5/4	2	Passageway to pasture
N	8/6	10 4 6	Waiting area milking parlor Passageway to pasture Bedding material barn
O	6/2	4	Waiting area milking parlor

¹Some isolates belong to the same strain.

²No environmental *Strep. uberis* isolates found.

Figure 2. Pulsed-field gel electrophoresis results. Lane 1: Lambda PFG Ladder (48.5–1,018 kb; New England Biolabs, Ipswich, MA); lanes 2–4: farm K, strains from mastitis milk; lane 5: farm K, strain from passageway to pasture; lanes 6, 7: farm B, strains from mastitis milk; lane 8: farm B, strain from passageway to pasture; lane 9: farm C, strain from mastitis milk; lanes 10, 11: farm C, strain from a liner and from a drinking trough; lane 12: farm E, strain from mastitis milk; lane 13: farm E, strain from the barn area in front of the milking parlor.

contagious mode of the strain. The limited variety of *Strep. uberis* mastitis strains within one farm could also indicate the contagious behavior of this pathogen. In our study, we found one farm (farm O) that showed a low variety of *Strep. uberis* strains in mastitis milk; 4 out of 6 isolates found on this farm were identical by PFGE. Davies et al. (2016) found a small subset of *Strep. uberis* sequence types, which is thought to be important for cow-to-cow transmission because they were found disproportionately frequently in clinical mastitis cases. Zadoks et al. (2001) analyzed an outbreak of *Strep. uberis* mastitis and suggested that contagious transmission played a role in the outbreak. A predominant strain could be contagious as well as environmentally transmitted from one hotspot in the environment. On farm O, *Strep. uberis* isolates were found in only one environmental spot (barn area in front of the milking parlor), and none of them matched the mastitis strains. This finding does not exclude the possibility of other spots harboring *Strep. uberis* within this farm. It can be deduced that single random sampling of each spot is not sufficient to find a matching strain. Oliver et al. (1998) reported *Strep. uberis* mastitis with identical strains in non-outbreak situations, and Zadoks et al. (2003) described *Strep. uberis* mastitis cases that were caused not by a variety of environmental strains but by

a predominant strain. Additionally, Pryor et al. (2009) showed the ability of a mastitis strain with pathogenic attributes to predominate within the mammary gland.

In 5 out of 14 farms, similar strains could be found in different environmental spots (Table 3). This confirmed that *Strep. uberis* has the ability to transmit between various spots and harbor different reservoirs of infection. Zadoks et al. (2005) hypothesized that fecal shedding plays a role in the maintenance of *Strep. uberis* in the farm ecosystem. Passageways and waiting areas are rich in manure and slurry, which may splatter onto the udder immediately before or directly after milking (while cows wait in front of the parlor or exit the parlor, respectively). Because the teat canals might be open during this time, fecal contamination of the udder and teat skin surface increases the possibility of *Strep. uberis* invading the udder through the teat canal (Krömker et al., 2014). We were able to find an isolate on the passageway to pasture that matched mastitis milk isolates on 2 farms of our study (B and K); on one of those farms (farm K), a matching isolate was found in 3 out of 7 mastitis cases.

In general, the highest number of *Strep. uberis* environmental isolates was detected in the waiting area in front of the milking parlor (Table 3). This might be due to milk leakage before milking and the presence of large amounts of feces that might contain mastitis strains and can splatter onto the udder from the ground or floor. Liquids such as waste milk and cleaning water, which are sometimes discharged from the parlor floor or pit via the waiting area, could also represent a mode of transmission of this pathogen. *Streptococcus uberis* strains were found in the milking parlor's waiting area and exit (farm A and E), the passageway to pasture (farm N), and the bedding material (farm E and N). This indicates the possible relevance of the milking parlor's waiting area at the front as a source of mastitis *Strep. uberis* strains and of the milking parlor for transmission of *Strep. uberis*. Moreover, the area in front of the milking parlor appeared to be the link between these spots. Another possible connection between the spots could be the passageway to pasture, which had matching isolates in the barn bedding material, milking parlor waiting area, and exit. We found no strains matching between the farms, which means each farm harbored its own unique strains, in agreement with the study by Douglas et al. (2000).

In summary, it is important to note that the constellation of *Strep. uberis*-positive spots, the number of matching strains in mastitis milk, and the variety of the *Strep. uberis* strains varied among farms, indicating that it might be useful to compare *Strep. uberis* mastitis strains at the farm level to obtain a better understanding of transmission pathways. For example, farm A (with a

Table 3. Identical strains of *Streptococcus uberis* within the environmental samples; some strains were found in different locations in the environment and some isolates originating from the same location were found to belong to the same strain

Farm	Isolates/strains (no.)	Locations of identical strains ¹
A	8/6	Waiting area milking parlor (a, a) Exit milking parlor (a)
B	1/1 ²	NP ³
C	12/7	Liner ⁴ (b) Drinking trough barn ⁴ (b) Liner (c, c) Liner (d, d, d, d)
D	14/11	Waiting area milking parlor (e, e) Waiting area milking parlor (f, f) Passageway to the pasture (g, g)
E	18/13 ⁵	Liner (h, h) Waiting area milking parlor (i) Exit milking parlor (i) Waiting area milking parlor (j) Bedding material barn (j) Bedding material barn (k, k, k)
F	2/2	NP
G	10/9	Exit milking parlor (l) Passageway to the pasture (l)
H	3/2	Bedding material pasture (m, m)
I		No environmental isolates
J	1/1	NP
K	7/6 ⁶	Passageway to the pasture (n, n)
L	1/1	NP
M	2/2	NP
N	20/12	Waiting area milking parlor (o, o, o, o, o, o) Waiting area milking parlor (p, p) Waiting area milking parlor (q) Passageway to the pasture (q) Bedding material barn (q) Waiting area milking parlor (r) Bedding material barn (r) Passageway to the pasture (s) Bedding material barn (s)
O	4/(4)	NP

¹Each strain is named with a different lowercase letter.²Matching with 2 milk isolates.³Not performed.⁴Matching with 1 milk isolate.⁵One isolate from barn area in front of the milking parlor matches with a milk isolate.⁶One isolate from the passageway to the parlor matches with 3 milk sample isolates.

high variety of mastitis strains) and farm O (with a low variety of mastitis strains) presented clearly different transmission of *Strep. uberis* infections. Farm O kept a more contagious and more adaptable strain than farm A. Furthermore, the data show that *Strep. uberis* could be found in each sampled spot but not in each farm at the same constellation. That means, that in addition to hygienic requirements for the farms, a more detailed investigation might be necessary for mastitis control. The importance of individual environmental locations for the origin of *Strep. uberis* infections of dairy cows also differs from farm to farm.

However, because of the high effort required, molecular biological investigations by strain typing in the context of mastitis control, except for research purposes, will probably remain an exception. In practice, based

on our frequent identifications of *Strep. uberis* mastitis strains, the hygiene of waiting areas in front of the milking parlor, drinking troughs, and passageway to the pasture should be the focus during udder health work.

CONCLUSIONS

Streptococcus uberis was frequently detected, along with some mastitis strains, in the dairy environment. We verified that both environmental *Strep. uberis* strains and mastitis strains could be transmitted to various spots in the environment of cows. This led to the conclusion that the modes of transmission of *Strep. uberis* include environmental sources in a contagious pattern, indicating that *Strep. uberis* control strategies

require approaches that address environmental as well as contagious pathogens. Moreover, an individualized *Strep. uberis* control strategy is needed for each farm. Molecular methods such as PFGE are useful to screen different *Strep. uberis* strains within a farm, thereby supporting the development of an appropriate control strategy.

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