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Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds

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SUMMARY

A longitudinal observational study (18 months) was carried out in two Dutch dairy herds to explore clinical, epidemiological and molecular characteristics of *Streptococcus uberis* mastitis. Infections ($n=84$) were detected in 70 quarters of 46 cows. Bacterial isolates were characterized at strain level by random amplified polymorphic DNA (RAPD) fingerprinting. Persistent infections were usually attributable to one strain, while recurrent infections could be caused by different strains. When multiple quarters of a cow were infected, infections were mostly caused by one strain. In each herd, multiple strains were identified yet one strain predominated. The majority of all infections were subclinical, and infections attributed to predominant strains were more chronic than infections attributed to other strains. Epidemiological and molecular data suggest infection from environmental sources with a variety of *S. uberis* strains as well as within-cow and between-cow transmission of a limited number of *S. uberis* strains, with possible transfer of bacteria via the milking machine.

INTRODUCTION

Streptococcus uberis is an important udder pathogen in the modern dairy industry. It ranks among the main causes of mastitis in countries around the world, including Australia [1], Brazil [2], Canada [3], The Netherlands [4, 5], New Zealand [6], the United Kingdom [7] and the United States [8]. The pathogen is a major barrier to the control of bovine mastitis [7, 9], partly because the epidemiology of the disease is incompletely understood [1, 10].

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Streptococcus uberis is usually classified as 'environmental pathogen' [7, 11]. The primary reservoir of environmental pathogens is the dairy cow's environment, and exposure is not limited to the milking process [11]. Sources of *S. uberis* in the environment of the bovine udder include body sites, manure, pasture and bedding material [12–14]. New infections with *S. uberis* may occur in dry cows and in heifers before calving [15–17]. Because dry cows and preparturient heifers are not milked, infections in those animals cannot be the result of cow-to-cow transmission in the milking parlour. Therefore, the environment is most likely the source of infection. In recent years,

molecular techniques have contributed evidence for the environmental origin of *S. uberis*. Many strains of *S. uberis* can cause bovine mastitis, implying that mastitis does not result from cow-to-cow transmission of a limited number of strains within a herd [1, 6, 7, 18].

The emphasis on the environmental nature of *S. uberis* is partly a result of the failure to eradicate *S. uberis* mastitis through management that controls transmission of contagious mastitis [9]. However, indications that contagious or cow-to-cow transmission of *S. uberis* may be important are re-emerging from modern molecular and mathematical approaches to epidemiology. Molecular studies have yielded evidence for direct transmission and the predominance of particular strains in some herds [1, 19]. Mathematical studies, based on a modification of the standard Susceptible-Infectious-Recovered (SIR) model for infectious diseases [20], showed the prevalence of *S. uberis* infections to be a predictor for the incidence of new infections. This observation is in accordance with the hypothesis that infected cows may act as a source of infection for herd-mates [21].

Intramammary infections (IMI) caused by *S. uberis* may be clinical or subclinical, and can vary in duration. Subclinical infections may hamper the control of mastitis because they often go unnoticed and untreated, resulting in long duration of the infection. Chronic infections with *S. uberis* are known to occur [18, 22] and a role for such infections in the epidemiology of *S. uberis*, possibly through transmission at milking time, has been suggested [10, 23]. However, there are few longitudinal studies that combine molecular data and field observations in support of the claim that *S. uberis* mastitis can be contagious.

This study describes *S. uberis* infections that were observed in two commercial dairy herds in The Netherlands over a time period of 18 months. The objective of the study was to examine the clinical manifestation of naturally occurring *S. uberis* mastitis and the association between bacterial strains and epidemiological or clinical characteristics of infections, with the aim to enhance our understanding of the spread of *S. uberis* in dairy herds. As part of this effort, the role of the milking machine in transmission of *S. uberis* was probed.

HERDS, MATERIAL AND METHODS

Herds and animals

Data and isolates were obtained from two commercial dairy herds in The Netherlands. The herds participated in a longitudinal observational study (June 1997–December 1998) on the population dynamics of mastitis. Cows were mainly Holstein–Friesians, partly cross-bred with Dutch Friesian or Meuse–Rhine–Yssel cows. In winter, animals were housed in a free-stall barn with a concrete slatted floor, and cubicles with wood shavings as bedding material. Lactating cows were mostly on pasture during the summer. Herd 1 consisted of 95 ± 5 (mean $\pm$ S.D.) lactating cows with an average 305-day milk production of 8166 ± 459 kg, and had an arithmetic mean bulk milk somatic cell count of 235 ± 75 ($\times 10^3$) cells/ml in the year preceding the study. Herd 2 consisted of 41 ± 2 lactating cows, with average 305-day milk production of 8508 ± 165 kg, and had an arithmetic mean bulk milk somatic cell count of 205 ± 69 ($\times 10^3$) cells/ml in the year preceding the study.

In herd 1, cows were milked twice daily in a two-by-four open tandem parlour. Mastitis management included regular monitoring of milking machine function, use of individual paper towels, antibiotic treatment of all clinical cases of mastitis, antibiotic treatment of selected cows with subclinical mastitis, and antibiotic treatment of all cows at drying off. Post-milking teat disinfection was practised from June to September 1997 and again from April to June 1998. An outbreak of *S. uberis* mastitis occurred in this herd between November 1997 and April 1998 and has been described in detail elsewhere [21]. In herd 2, cows were milked twice daily in a two-by-five herringbone parlour. Mastitis management was similar to herd 1, but post-milking teat disinfection was practised throughout the study. Herds were considered to be illustrative of an intermediate level of management and bulk milk somatic cell count under current farming conditions in The Netherlands [24].

Collection and processing of samples

Quarter milk samples were collected every 3 weeks from all lactating animals, using aseptic technique [25]. Farmers collected additional quarter milk samples at calving (prior to first contact with the milking machine), at drying off, before culling, and when clinical mastitis was detected (any visual abnormality of milk or udder, with or without systemic signs of disease). A fraction of fresh quarter milk samples was used for determination of quarter milk somatic cell count (QMSCC) by a Fossomatic milk cell counter (Foss Electronic, Hillerød, Denmark), with the exception of samples that were collected by farmers.

All milk samples for microbiological analysis were stored at −20 °C until processing. Within 3 weeks of collection, 0·01 ml of milk were cultured and all bacterial species were identified according to National Mastitis Council standards [25]. The number of colony forming units (c.f.u.) was recorded for all samples. When ⩽10 c.f.u. of a pathogen were identified, individual colonies were counted. Higher c.f.u.-counts were categorized as 10–49 c.f.u./plate, 50–199 c.f.u./plate or ⩾200 c.f.u./plate. Preliminary identification of *S. uberis* was based on colony morphology and aesculin hydrolysis on Edward's medium. Isolates from herd 1 were confirmed as *S. uberis* using the API 20 Strep system [26]. For herd 2, only those isolates that were used for strain typing were confirmed as *S. uberis* using the API 20 Strep system.

Definition of infection

In accordance with current definitions of intramammary infection [21, 27, 28], a quarter was considered infected when ⩾1000 c.f.u./ml of *S. uberis* were cultured from a single sample, when ⩾500 c.f.u./ml were cultured from 2 of 3 consecutive milk samples, when ⩾100 c.f.u./ml were cultured from 3 consecutive milk samples, or when ⩾100 c.f.u./ml were cultured from a sample obtained from a quarter with clinical mastitis. Samples containing more than three bacterial species were considered contaminated, and were not regarded as informative of IMI status. Samples that were culture negative during antibiotic treatment for udder disease were also not considered informative of IMI status. A previously infected quarter was considered as having recovered from IMI if none of the above definitions were met and the sample was free of *S. uberis*. Quarters that had recovered from IMI were eligible for a new episode of infection.

For IMI that were first detected at calving or as clinical mastitis, the sample date was assumed to be the date of onset of infection. For IMI that were last detected at drying off or at culling, the sample date was taken as the endpoint of infection. For all other combinations the midpoint between the last negative and first positive sample was taken as starting point, and the midpoint between last positive and first negative sample was taken as endpoint of IMI. The terms 'positive' and 'negative' are used to describe the infection status of the quarter from which the sample was taken [21]. Infections that were not accompanied by any visual abnormalities of the milk and/or the udder were called subclinical infections. When clinical signs, such as clot formation in milk, developed in a quarter with subclinical infection, this was considered to be a clinical flare-up.

Liner swabs

In herd 1, swabs were taken from milking machine unit liners on two occasions (swab experiment 1 and 2) to determine the presence of *S. uberis* on liners before and after milking of *S. uberis* infected cows. Five cows that were chronically infected with *S. uberis* in one quarter were selected. Cows were led into the milking parlour so that the milking unit and teat cup that would be used for the infected quarters were known. Before milking started, designated teat cup liners were swabbed to check cleanliness. Next, cows with infected quarters were milked, using standard milking routine. In swab experiment 1, liners were swabbed in duplicate after infected quarters had been milked. In swab experiment 2, milking of cows with infected quarters was followed by milking of two cows that had no *S. uberis* infection in any quarter. Swabs were not taken after milking the infected cow, but after milking of the first uninfected cow following the infected cow, and again after milking of the second uninfected cow. Infection status of infected and uninfected cows was determined based on prior milk culture results, and confirmed with culture results of milk samples that were taken on the days that swabs were obtained.

Swabs consisted of sterile cotton wool, mounted on plastic rods and stored in a sterile container. Swabs were inserted into the teat cup to the point were the liner joined the short milking tube, and withdrawn in a spiraling motion while rotating the swab. Swabs were reinserted into containers together with 5 ml of cooled sterile peptone-saline solution (0·85% w/v sodium chloride and 0·1% w/v peptone water (Oxoid, CM9, Haarlem, The Netherlands) in sterile water). Swabs were cooled and transported to the laboratory within a few hours. In the laboratory, swabs were removed from the transport medium and inserted into 5 ml of serum broth (0·5% w/v Lab Lemco (Oxoid), 1·0% w/v Bacto peptone (Oxoid), 0·5% w/v sodium chloride and 0·1% glucose in sterile water, pH 7·4). After vigorous shaking and overnight incubation at 37±1 °C, an inoculum of 0·1 ml was plated on Edward's medium. Plates were incubated for 21±3 h at 37±1 °C. Isolates showing colony morphology suggestive of *S. uberis* were identified at the species

level using the API 20 Strep system. Results were expressed as absence or presence of *S. uberis*.

RAPD fingerprinting

DNA isolation was performed as described by Gillespie et al. [29]. Amplification of bacterial DNA using primer OPE-04 (5′-GTGACATGCC-3′; Operon Technologies, Alameda, CA, USA) was done as described by Jayarao et al. [30] with *S. uberis* strain ATCC 27958 as positive control. Amplified products and molecular size markers (pBR322 DNA digested with *Bst*NI and ΦX174 DNA digested with *Hae*III; New England BioLabs, Beverly, MA, USA) were electrophoresed in 2% agarose with TBE buffer (0·9 M Tris base, 0·09 M boric acid, 2·5 mM EDTA; pH 8·3). Gels were run at 150 V (6·25 V/cm) for 3·5 h and were stained with ethidium bromide (1·0 μg/ml; Sigma Chemical Co., St. Louis, MO, USA). The DNA was visualized by ultraviolet transillumination (Fotodyne Inc., New Berlin, WI, USA) and photographed with type 55 Polaroid® film (Polaroid Corp., Cambridge, MA, USA). Each DNA amplification pattern was examined for the number of DNA fragments and their size relative to molecular size standards [22]. Isolates with the same number and size of DNA fragments were considered to belong to the same strain, irrespective of band intensity of fragments. Strains were designated an arbitrary letter to identify a genotype.

As far as possible, isolates from every infected episode were used for strain typing. If more than one isolate was available from an infected episode, isolates from the early, intermediate and late stage of infection were used, to test the assumption that chronic infections are usually persistent infections caused by one strain type [1, 22]. For some milk samples, re-isolation of bacteria for RAPD fingerprinting after prolonged storage at -20 °C was not successful and strain types could not be determined.

Statistical analysis

Duration of infection was censored for infections that were observed at the start and the end of the study, and for infections in cows that were dried-off or culled. Therefore, duration of infection was represented using Kaplan–Meier survival curves (S-Plus version 6.0, Insightful Corp., Seattle, WA, USA, 2001) and compared between groups using a two-sample or multiple-sample Log Rank survival-test, as appropriate (Statistix version 7.0, Analytical Software Co., La Jolla, CA, USA, 2000). Association between median QMSCC and duration of infection was examined using Pearson's correlation coefficient (Statistix 7.0). For herd B, the association between bacterial strain (dominant strain *vs.* other strains) and time of onset of infection (non-lactating *vs.* lactating period) was examined using Fisher's exact test. Association between bacterial strains and clinical manifestation of the infection was also examined by Fisher's exact test. For QMSCC and c.f.u.-count, many observations were available for some infected quarters. Therefore, the associations between possible explanatory variables (herd, quarter position, strain) and outcome measures (QMSCC, c.f.u.-count) were analysed using ProcMixed, with the covariate of interest as fixed effect and quarter as repeated effect. For this analysis, QMSCC was normalized by log transformation. Compound symmetry was used to model the covariance structure of repeated observations within udder quarters [24]. Analyses were run in SAS version 8.01 (SAS Institute Inc., Cary, NC, USA, 1999). Significance was declared at $P<0·05$ for all statistical analyses.

RESULTS

Clinical and bacteriological observations

In herd 1, 54 infections were observed in 47 quarters of 31 cows. Four infected quarters were present at the start of the study and 50 new infections were detected during the 18-month observation period. Four *S. uberis* infections were detected in heifers at calving, one infection was detected in a multiparous cow at calving, one infection was detected as severe clinical mastitis in the early dry period (mixed infection with *Escherichia coli*), and 44 infections were first detected during lactation. In herd 2, 30 infections were observed in 23 quarters of 15 cows. Seven infected quarters were present at the start of the study, and 23 new infections were detected during the study. One infection was detected in a multiparous animal at calving, and all other infections were detected during lactation. Two quarters that were chronically infected with *S. uberis* showed mixed infection with *E. coli* or *Staphylococcus aureus* at one or two samplings, respectively. One cow showed multiple mixed infections of short duration (1 *Streptococcus dysgalactiae*; 1 *S. dysgalactiae* and *S. aureus*; 1 *E. coli*).

Out of 54 *S. uberis* infections in herd 1, 11 showed clinical signs and 43 (80%) were subclinical throughout the period of observation. Infections that were

Table 1. *Number of clinical, subclinical and combined clinical/subclinical intramammary infections with* Streptococcus uberis *observed over an 18-month period in two Dutch dairy herds*

Course of infected episode	Herd 1*	Herd 2*
Clinical onset		
Clinical only	3	3
Followed by subclinical only	2	2
Followed by subclinical with clinical flare-ups	0	1
Subtotal clinical onset	5	6
Subclinical onset		
Followed by subclinical only	40	16
Followed by subclinical with clinical flare-ups	6	3
Subtotal subclinical onset	46	19
Unknown onset		
Followed by subclinical only	3	4
Followed by subclinical with clinical flare-ups	0	1
Subtotal unknown onset	3	5
Total number of episodes	54	30

* Herd 1 consisted of 95 ± 5 (mean $\pm$ S.D.) lactating animals. Herd 2 consisted of 41 ± 2 lactating animals.

accompanied by clinical signs began as clinical mastitis in 5 cases and as subclinical infections in 6 cases. The number of clinical episodes per infection ranged from 1 to 5. In herd 2, 20 of 30 episodes were subclinical (67%) throughout the period of observation. Infections that resulted in clinical signs started as clinical mastitis in 6 of 10 cases. Per infection, 1 or 2 clinical episodes were observed (Table 1).

Duration of infection was estimated for 47 infections in herd 1 and for 26 infections in herd 2. Infections that were first detected at drying off or at the last sampling of the study were excluded from calculations of duration because there was no follow-up after onset of infection. Therefore, calculation of duration was not considered meaningful. The duration of infections ranged from 1 to 309 days in herd 1 (median = 46 days), and from 1 to 280 days in herd 2 (median = 29 days). The true duration of infections may have been underestimated, because the duration was truncated for infections that were present at the start or the end of the study and for infections in animals that were culled. Drying off was considered as the end of an infected episode because all quarters that were infected at drying off and that were examined again at the next calving had been cured during the dry period. Spontaneous cure of infection was observed in 14 and 10 quarters in herds 1 and 2, respectively, while cure after treatment was observed in 14 and 6 quarters for herds 1 and 2.

Infections that were detected at calving ($n = 5$, median duration = 9 days) were significantly shorter than infections that were first detected at a week or more in lactation ($n = 65$, median = 46; Log Rank Test, $P < 0{\cdot}01$; Fig. 1). Infections that were present at the start of the study were excluded from this analysis, because the lactation stage at onset was unknown. Two infections that were detected within a week post calving were also excluded from analysis. Samples at calving may be culture negative as a result of the presence of antibiotics used for dry cow treatment. Therefore, it was thought that infections that were first detected within a week after calving could have originated in the dry period or in the lactating period and they were not included in either category. Duration of infection did not differ between herds or between positions of udder quarters (left or right, front or rear) (Log Rank Test, $P = 0{\cdot}95$, $0{\cdot}55$ and $0{\cdot}47$, respectively).

Infections that started as clinical mastitis were significantly shorter ($n = 9$, median = 13 days) than infections that started subclinically ($n = 56$, median = 45 days; Log Rank Test, $P < 0{\cdot}02$). Infections with subclinical onset and clinical flare-ups were longer ($n = 9$, median = 90 days) than infections that were fully subclinical ($n = 47$, median = 42 days; Log Rank Test, $P < 0{\cdot}01$). Duration of subclinical infections, infections with clinical onset and infections with subclinical onset followed by clinical flare-ups is depicted in Figure 2.

QMSCC in infected quarters ranged from 5000 cells/ml to 9 999 000 cells/ml (upper limit of detection system). For 21 quarters, there were at least 4 observations of QMSCC during infection, spanning a period of at least 2 months. In herd 1, the lowest median QMSCC in such a chronically infected quarter was 215 000 cells/ml and the highest median QMSCC was 4 719 000 cells/ml. In herd 2, lowest median QMSCC was 65 000 cells/ml and the highest median QMSCC was 3 412 000 cells/ml. Examples of high and low median QMSCCs in quarters with chronic *S. uberis* infection are shown in Figure 3. Two cut-off values for somatic cell count that are commonly used as indicators of infection are included in Figure 3. Ten of 21 quarters had median QMSCC $\leqslant$ 500 000 cells/ml and 5 had a median QMSCC $\leqslant$ 250 000 cells/ml. The correlation between median QMSCC and duration was not significant (Pearson correlation = $0{\cdot}31$; $P = 0{\cdot}16$). QMSCC of chronically infected quarters was

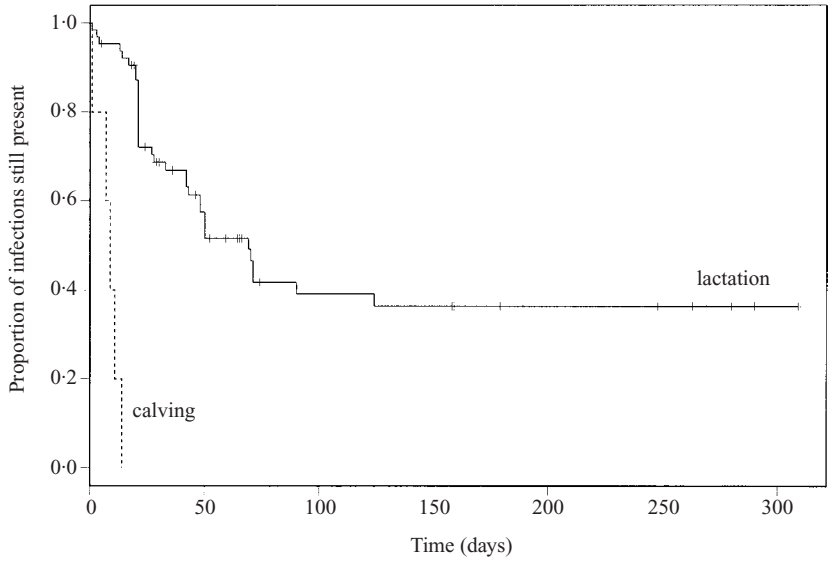

Fig. 1. Kaplan–Meier curve of duration of *Streptococcus uberis* infections for quarters that became infected during lactation (full line; infection detected at >7 days after calving, $n=65$) and for quarters that were infected at calving (dashed line; $n=5$). Tick marks indicate censoring of observations. Lines are significantly different (Log Rank Test, $P<0{\cdot}01$).

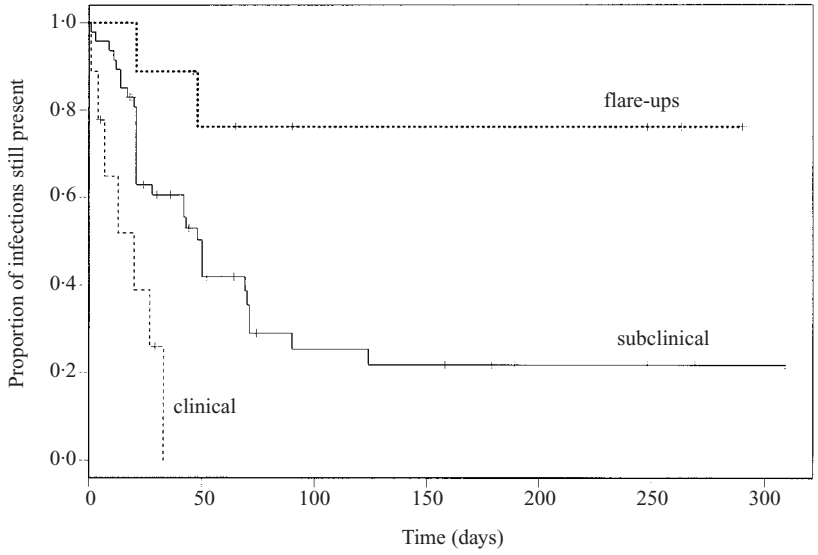

Fig. 2. Kaplan–Meier curve of duration of *Streptococcus uberis* infections with clinical onset (dashed line; $n=9$), subclinical onset and no clinical flare-ups (full line; $n=47$), or subclinical onset followed by clinical flare-ups (dotted line; $n=9$) observed in two dairy herds during 18 months. Tick marks indicate censoring of observations. Lines are significantly different (Log Rank test, $P<0{\cdot}02$).

not significantly associated with herd of origin or quarter position (front or rear, right or left) (Likelihood Ratio Test; $P=0{\cdot}05$, $0{\cdot}16$ and $0{\cdot}58$, respectively).

The number of bacteria in milk from infected quarters was generally high, with infected quarters shedding >1000 c.f.u./ml in 84·9% of 230 samples in herd 1, and in 88·3% of 139 samples in herd 2. Samples from infected quarters were culture negative for *S. uberis* in 5·7% of samples in herd 1, and in 5·8% of samples in herd 2, mostly during antibiotic treatment. Bacterial shedding did not differ between herds (Likelihood Ratio Test; $P=0{\cdot}83$).

Strain typing

In herd 1, 12 strains were identified among 111 isolates that were RAPD fingerprinted. In herd 2, 7 strains were identified among 41 isolates that were

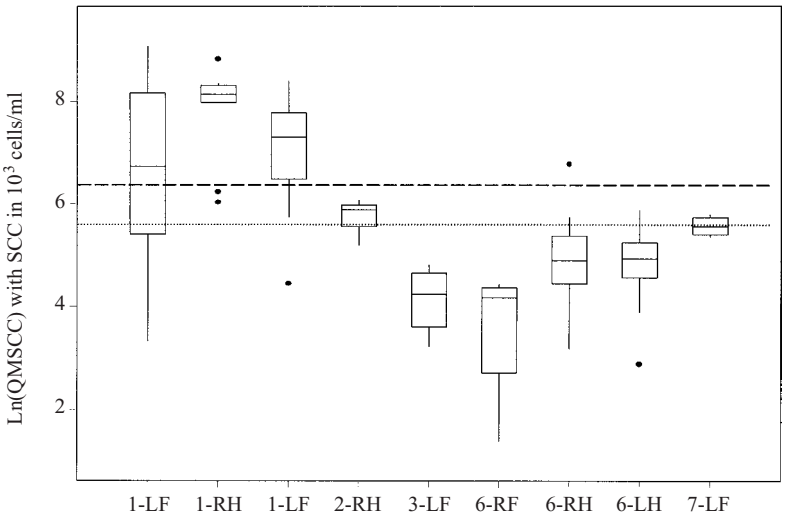

Fig. 3. Natural logarithm of somatic cell count (SCC) of quarters with chronic *Streptococcus uberis* infection. Boxes represent the middle half of SCC measurements for each quarter and are bisected by the median value. Whiskers represent a range of typical data, i.e. data that are at most 1.5 times the box width away from the box, while dots represent extreme values (Statistix version 7.0, Analytical Software Co., La Jolla, CA, USA, 2000). Numbers on the horizontal axis represent cows and letters represent quarter position (RF, right front; LF, left front; RH, right hind; LH, left hind). Dashed line indicates SCC of 500 000 cells/ml. Dotted line indicates SCC of 250 000 cells/ml.

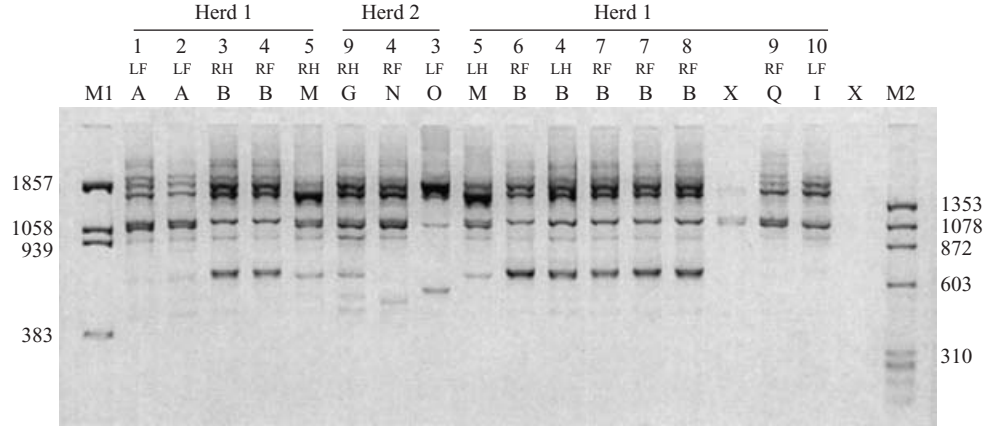

Fig. 4. Example of banding patterns obtained through random amplified polymorphic DNA (RAPD) typing of *Streptococcus uberis*. Herd, cow, and quarter are indicated for each sample. RF, right front; LF, left front; RH, right hind; LH, left hind. Letters represent *S. uberis* strains. X indicates empty lane. Lane M1 is pBR322 DNA digested with *Bst*NI, and lane M2 is ΦX174 DNA digested with *Hae*III. Molecular size of standards (kb) is indicated on either side of the figure.

RAPD fingerprinted. An example of strain typing results is shown in Figure 4. The number of isolates, quarters and cows associated with each RAPD type is summarized in Table 2. A dominant strain was identified in each herd. The dominant RAPD type from herd 2, strain A, was also detected in herd 1. The dominant RAPD type from herd 1, strain B, was not detected in herd 2. Of 17 RAPD types that were identified, 2 were obtained from both herds.

When multiple isolates were typed per IMI, isolates mostly belonged to one RAPD type, as exemplified in Table 3 (cows 1, 2, 5 and 6–8). For 40 infections, more than one isolate was typed (range = 2–8 isolates; median = 3 isolates). For 35 of 40 infections with multiple RAPD-typed isolates, every isolate belonged to the same type (87·5%). When infections were simultaneously present in multiple quarters of a cow, and strain typing results were available for each quarter,

Table 2. *Number of isolates, episodes of infection, infected quarters, and infected cows per* Streptococcus uberis *strain in two dairy herds. In herd 1, 111 out of 217* S. uberis *isolates were typed by random amplified polymorphic DNA (RAPD) fingerprinting. In herd 2, 41 out of 131* S. uberis *isolates were RAPD fingerprinted. Only infections with known strain types are included*

	Herd 1				Herd 2			
Strain	Isolates	Episodes	Quarters	Cows	Isolates	Episodes	Quarters	Cows
A	12*	4	4	4	30	9	8	5
B	83	29	29	22	—	—	—	—
C	1†	1	1	1	—	—	—	—
D	2	1	1	1	—	—	—	—
E	—	—	—	—	3	2	2	1
F	1	1	1	1	—	—	—	—
G	1	1	1	1	3	3	3	2
H	—	—	—	—	1	1	1	1
I	5	1	1	1	—	—	—	—
J	1	1	1	1	—	—	—	—
K	—	—	—	—	2	1	1	1
L	1	1	1	1	—	—	—	—
M	2	1	1	1	—	—	—	—
N	—	—	—	—	1	1	1	1
O	—	—	—	—	1	1	1	1
P	1	1	1	1	—	—	—	—
Q	1	1	1	1	—	—	—	
Total	111	39‡	37‡	28‡	41	18	15	10

* In one quarter, a mixed infection was detected (strain A and strain F were present in the same sample; colonies differed in aspect on culture plate). In one quarter, two different strains (A and B) were detected on two consecutive occasions. In one quarter, three different strains (A, B and Q) were detected on three consecutive occasions).

† Superinfection with strain C in a quarter that was chronically infected with strain B. Strain C was isolated from an extra sample taken during a clinical episode.

‡ Column does not add up to total, due to detection of multiple strains per infected episode in some quarters.

the same RAPD type was isolated from each quarter within a cow (7 cows in herd B, 3 cows in herd C). The onset of infection in multiple quarters of a cow could have been simultaneous (2 and 1 cow(s) in herds B and C, respectively, e.g. Table 3, cow 1). Alternatively, infection with a specific strain in one quarter could be followed by infection with the same strain in other quarters of the cow (5 and 1 cow(s) in herds B and C, respectively, e.g. Table 3, cow 8). Quarters within a cow that had simultaneous presence of infection could be adjacent (6 and 3 cows in herds B and C, respectively, e.g. Table 3, cows 1, 6 and 8) or diagonally opposed (1 cow in each herd, e.g. Table 3, cow 8). When quarters within a cow were infected during non-overlapping periods of time, isolates could belong to the same RAPD types or to different strain (Table 3, cow 8). Similarly, consecutive episodes of infection that were separated by periods of cure within quarters could be caused by the same RAPD type (Table 3, cow 6) or different strains (two quarters of 1 cow in herd B, one quarter in herd C; Table 3, cow 8). Because strain typing data are not available for all infected episodes from both herds, representation of the results in the above paragraph is qualitative, aimed at exemplifying different infection scenarios, rather than quantitative.

Associations between strains and clinical characteristics

In herd 1, 8 of 10 infections (80%) with clinical signs and known strain type were associated with the dominant strain (strain B), and two infections were associated with other strains (J and P). For infections without clinical signs and with known strain type, 21 of 27 (78%) were associated with the dominant strain, and 11 were associated with other strains (A, D, F, I, L, M and Q; due to infections with multiple strains total does not add up to 27). In herd 2, infections with clinical signs were associated with strain A or isolates of undetermined type, while there was considerable heterogeneity among isolates that caused subclinical infections (strains A, E, G, H, K, N, O and untyped

Table 3. *Strains of* Streptococcus uberis *isolated from selected quarters of dairy cows in herd 2 over an 18-month period with 3-weekly sampling* (*S*)*, illustrating infection scenarios at cow and herd-level*

	1*			2	3	4	5	6		7	8			9	10
S	LF	RH	LH	RH	LF	RF	RF	RH	LH	LF	LF	RH	LH	RH	LH
1	—	—	—	A	—	—	—	+	+	+	—	E	—	—	—
2	—	—	—	+	—	—	+	A	A	+	E	E	—	—	—
3	A	A	A	+	—	dp†	—	A	A	A	dp	dp	dp	—	—
4	A	A	A	A	—	—	dp	+	A	A	—	—	—	—	A‡
5	A	A‡	A	A	—	—	dp	+	+	—	—	—	—	—	—
6	+	+	+	+	—	—	dp	+	+	—	—	—	—	—	—
7	+	+	+	+	—	—	dp	+	+	—	—	—	—	—	—
8	A	A	+	+	—	—	dp	+	+	—	—	—	+	—	—
9	+	+	+	+‡	—	—	—	+	+	—	G	—	+	—	—
10	+	A	A	—	—	—	—	+	+	—	—	G	—	—	—
11	+‡	+	+	—	—	—	—	+	+	—	—	+	H	—	dp
12	+	+	A	—	—	—	—	A	+	dp	—	—	—	—	dp
13	+	+	+	—	dp	—	—	+	+	—	—	—	—	—	—
14	+	A	+	—	dp	—	—	+	A	—	dp	dp	dp	—	—
15	A	+	+	—	—	—	—	dp	dp	—				G	—
16	dp	dp	dp	—	—	—	—	dp	dp	—					—
17				—	—	—	—	dp	dp	—					—
18				—	—	—	—	—	—	—					—
19				dp	—	N	—	—	—	—					—
20					—	—	—	—	—	—					—
21					—	—	—	—	A‡	—					—
22					—	—	—	—	+‡	—					
23					—	—	—	—	—	—					
24					+	dp	—	—	—	—					
25					O	dp	K	—	+	—					
26					+	—	K	—	+	—					
27					+	—	+	+	+	—					

* Numbers identify cows. Letters identify quarters. RF, right front; LF, left front; RH, right hind; LH, left hind.
Infection with *S. uberis* is represented by RAPD type if the type was determined; +, *S. uberis* infection present, but strain typing data not available; —, no *S. uberis* infection present; † dp, dry period; ‡ clinical mastitis. Empty cells indicate absence of cows due to culling.

isolates). The association between strain (A, B, others, or unknown) and clinical signs (categorized as ever *vs.* never clinical, or as infection entirely subclinical, subclinical onset followed by clinical flare-ups or clinical onset of infection) was not significant (Fisher's Exact test, $P = 0{\cdot}19$ and $0{\cdot}07$, respectively).

Infections caused by strain A ($n = 9$, median duration = 248 days) tended to be longer than infections caused by strain B ($n = 27$, median duration = 50 days; Log Rank test, $P = 0{\cdot}06$). Infections caused by strain A were significantly longer than infections caused by strains C to Q ($n = 13$, median duration = 17 days; Log Rank test, $P = 0{\cdot}02$). Infections caused by strain B were also longer than infections caused by strain C to Q (Log Rank test, $P = 0{\cdot}03$). Duration of infections caused by strains A, B or other strains is depicted in Figure 5. Data from quarters for which no RAPD typing results were available or that yielded multiple RAPD types were excluded from this analysis.

No significant associations were identified between QMSCC and strain of *S. uberis* (strain A, B, other strains, or undetermined strain types; Likelihood ratio test, $P = 0{\cdot}42$). Similarly, bacterial numbers in milk samples (c.f.u./ml) were not significantly different between samples from quarters infected with strain A, B, other strains or undetermined strain types (Likelihood ratio test, $P = 0{\cdot}35$).

Association between strains and epidemiological characteristics

In herd 1, an outbreak of *S. uberis* mastitis occurred [21]. Isolates from infections observed in the period preceding the outbreak, from infections that occurred

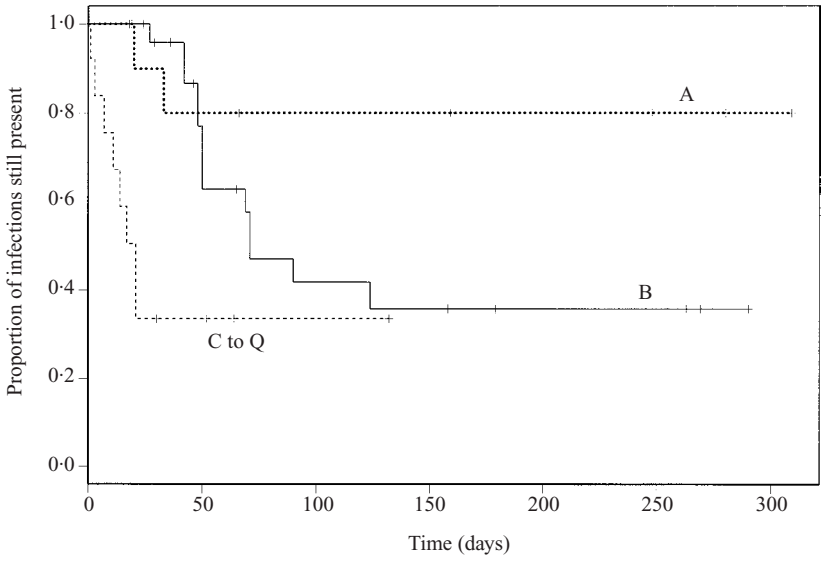

Fig. 5. Kaplan–Meier curve of duration of *Streptococcus uberis* infections caused by the dominant strain in herd 1 (RAPD type B, full line, $n=27$), the dominant strain in herd 2 (RAPD type A, dotted line, $n=10$), and by non-dominant strains in both herds (RAPD types C to Q, dashed line, $n=13$) during an 18 month observation period. Duration of infection was significantly different between dominant strains and non-dominant strains (Log Rank Test, $P<0{\cdot}05$).

during the mastitis outbreak and from infections that occurred after the outbreak were fingerprinted. The RAPD type of bacterial isolates was determined for 39 of 54 observed *S. uberis* infections. For each of the 27 consecutive herd visits, the number of quarters that were infected with a specific strain was determined. Numbers are shown in Figure 6. The majority of infections during the outbreak of *S. uberis* mastitis was attributable to strain B. Strain B had not been identified in the pre-outbreak period. Other strains were identified throughout the pre-outbreak, outbreak and post-outbreak period, but never in more than two quarters at any one time. Infections with strain B always started during lactation, while 6 out of 9 infections with other strains started during the non-lactating period (two-sided Fisher's Exact test, $P<0{\cdot}0001$).

Liner swabs

No *S. uberis* was cultured from any of the liner swabs taken from milking machine unit liners before milking. In swab experiment 1, *S. uberis* was isolated from 5 of 5 liner swabs immediately after milking of *S. uberis* infected cows. Of 5 *S. uberis* infected cows, 4 shed strain B in their milk, and 1 shed strain A. For each cow-liner combination, the same strain was obtained from the infected quarter and from the teat cup liner. In swab experiment 2, milking of an infected cow was followed by milking of 2 uninfected cows and swabs were taken after milking of the uninfected cows. Three out of 4 liner swabs taken after 1 uninfected cow had been milked harboured *S. uberis*, and 1 out of 4 liner swabs taken after 2 uninfected cows had been milked was positive for *S. uberis*. Infected cows in experiment 2 all shed strain B in milk and milk from uninfected cows was culture negative. All *S. uberis* isolates that were retrieved from liner swabs in experiment 2 were identified as type B.

DISCUSSION

Control of the mastitis pathogens *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Staphylococcus aureus* has reduced the number of clinical mastitis cases caused by those bacteria. As a result, there has been a relative (and possibly absolute) increase in the number of cases of clinical mastitis caused by *S. uberis* [7]. Together with studies that report the majority of *S. uberis* infections to be clinical [17], this has led to emphasis on *S. uberis* as a cause of clinical mastitis. Our study shows that *S. uberis* can be a major cause of subclinical mastitis in dairy herds, and that clinical cases constitute a small proportion of infections caused by *S. uberis*. This is in agreement with observations by Jayarao et al. [31] who report as many as 95% of cases within a herd to be subclinical. Furthermore, we showed that clinical cases of *S. uberis*

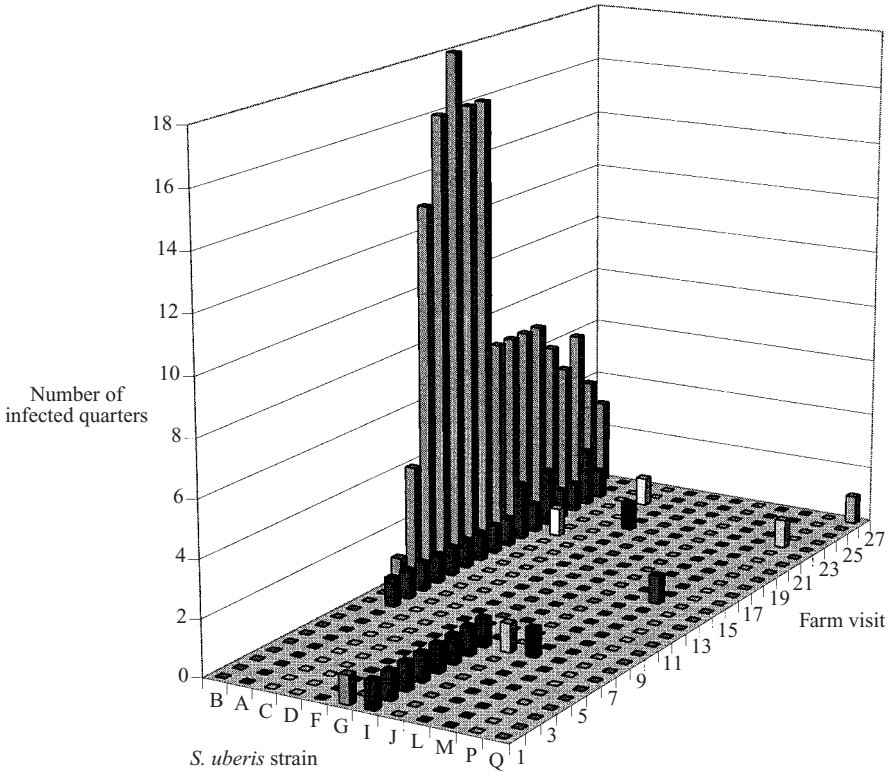

Fig. 6. Number of quarters that was infected with a specified strain of *Streptococcus uberis* during an 18-month observation period with 27 farm visits at 3-week intervals in a dairy herd with 95 ± 5 lactating animals (mean $\pm$ S.D.). Strains were specified by random amplified polymorphic DNA (RAPD) fingerprinting for 39 out of 54 observed infected periods.

mastitis are not necessarily of short duration, but may be indicative of a chronic and largely subclinical infection process. In The Netherlands, *S. uberis* is the second most frequently isolated pathogen from milk samples from cows with subclinical mastitis [5].

In both study herds, clinical infections in dry cows were rare. This is in contrast to reports from the United States [11, 15, 17] and may be a result of successful dry cow treatment in the study herds, and of differences between herds and countries in dry cow management or bacterial flora. Several new infections were detected at calving, most of them subclinical. Such infections probably originated in the non-lactating period. Infections that were present at calving were of shorter duration than infections that occurred during lactation and had limited impact on the incidence of clinical mastitis or the prevalence of subclinical mastitis in the study herds. The effect of infections at calving on production in the subsequent lactation was not examined.

The range of duration of *S. uberis* infections observed in our study [1–309 days] is similar to the range reported elsewhere (1–370 days) [17]. Mean duration in the study by Todhunter et al. [17] was 12 days. This is close to the median duration of infections with clinical onset in our study, but much shorter than the overall mean duration of 72 days. Because duration was not normally distributed, the median duration was thought to be a more appropriate measure for the central value than the mean. Half of the observed infections lasted more than an estimated 42 days and approximately one in four infected episodes lasted more than 72 days, emphasizing that chronic infections are no exception. Infections with subclinical onset were significantly longer than infections with clinical onset. Short duration of infections with clinical onset could theoretically be the result of a strong response of the cow's immune system resulting in clearance of the infection, or it could be the result of early detection by farmers. Early detection and treatment of mastitis may result in higher probability of cure compared to treatment of chronic infections, as described for *Staphylococcus aureus* [32]. It would be of interest for farmers to identify cow and quarter traits (e.g. parity, quarter position, duration of infection, QMSCC) and bacterial characteristics (e.g. antibiotic susceptibility, strain type) that influence the probability of cure of *S. uberis* infections with or

without treatment. If risk factors were known, the probability of cure for a specific individual could be determined and well-informed treatment decisions could be made. In the long term, this approach would contribute to prudent use of antibiotics.

For diagnosis of subclinical infections, bacteriological culture of milk samples is necessary. Whole herd bacteriological surveys of quarter milk samples are too expensive to be performed on a routine basis. Therefore, milk somatic cell count is used commonly as an indirect indicator of infection. In The Netherlands, 250 000 cells/ml is used as the cut-off value to discriminate between non-infected quarters and quarters that are suspect of infection [5]. This value is used at cow level, i.e. for pooled samples in which milk from infected quarters is mixed with milk from uninfected quarters that normally have lower QMSCC. At a quarter level, a cow-side test such as the California Mastitis Test (CMT) can be used. This screening test is positive for quarters with QMSCC 400 000–500 000 cells/ml or higher. In our study, a considerable proportion of infected quarters had median QMSCC below those detection limits. This means that chronic *S. uberis* infection can go unnoticed with standard screening strategies. This could pose an infection control problem, because infected quarters may be a source of infection for other animals in the herd, as will be discussed in the context of epidemiological findings. Furthermore, even at cell-counts below 250 000 cells/ml, milk quantity and milk quality are affected by subclinical mastitis [33].

Previous studies found that isolates from one milk sample usually belong to the same strain [1, 22]. Therefore, only one isolate per milk sample was RAPD fingerprinted in the present study. One exception was a milk sample from a heifer at calving, which yielded two morphologically distinct colony types and two strains. For several episodes of infection, multiple consecutive isolates were typed. Within an episode in a quarter, isolates mostly belonged to the same strain. This has been reported by others, and supports the idea that infections are persistent rather than recurrent with different strains [1, 22]. It also justifies our interpretation of strain typing results from the early, intermediate and late stages of an infection as indicative of the whole infected episode. When multiple episodes in a quarter occurred, episodes could be associated with the same strain or different strains, as has been shown by Oliver et al. [22] for isolates from different lactations within a quarter. When multiple quarters in a cow were infected, infections were usually caused by the same strain. This confirms results from Phuektes et al. [1] and suggests within-cow transmission of bacteria. Exposure of multiple udder quarters of a cow to the same environmental source cannot be ruled out, but infection of one quarter often preceded infection of other quarters within a cow and it is unlikely that different quarters would be exposed to the same environmental source at different points in time.

Although the total number of strains present in the study herds may have been underestimated because isolates could not be typed from all observed infections, multiple *S. uberis* strains were identified in each herd. This is in agreement with reports from Australia [18], New Zealand [6] and the United States [22]. The variety of strains isolated from milk is consistent with the hypothesis that the environment harbours a variety of *S. uberis* strains and acts as a source infection [7, 11, 17]. The detection of new infections in animals at calving observed in our study is probably the result of infections that occurred during the non-lactating period. This would also be explained by an environmental origin of the bacteria.

In addition to the variety of strains that occurred with low incidence and prevalence, a predominant RAPD type was identified in each herd. The predominance of a single RAPD may be the result of the inability of a typing system to discriminate between closely related strains, or it may reflect infection of multiple cows from a common environmental source. Alternatively, it could be the result of cow-to-cow transmission of bacteria. Occurrence of a limited number of strains is generally accepted as evidence for the contagious nature of pathogens such as *Streptococcus agalactiae* and *S. aureus* [18, 19, 34]. It seems plausible that contagious transmission also occurs for *S. uberis*, as has been suggested in other strain typing studies [1, 19].

Predominance of particular strains in a herd could be the result of differences between strains in pathogen virulence, i.e. the ability of a pathogen to cause disease. For intramammary infection, the outcome that is most easy to detect is the occurrence of clinical signs in infected quarters or animals. Hill [23] and Phuektes et al. [1] report that some strains are more likely to cause clinical mastitis than others. Jayarao et al. [35] found less heterogeneity among isolates from clinical mastitis than among isolates from subclinical infections, suggesting that the ability to cause clinical disease may be limited to specific strains. Most studies that address virulence of *S. uberis* [7, 36] are based on *in vitro* work, and the relevance of different virulence

factors *in vivo*, at cow level or at herd level is unknown. The *in vivo* study by Hill [23] is the only one to have been linked to *in vitro* virulence characteristics [37]. More work needs to be done to determine the cow-level and herd-level relevance of *in vitro* traits that are considered to be virulence factors.

In our study, the absence or presence of clinical signs, the bacterial content of milk samples, and the QMSCC of chronically infected quarters were not significantly associated with specific *S. uberis* strains. This could indicate lack of discriminatory power of the typing system or the statistical analyses, or true absence of a biological association. Lack of an association between strains and bacterial shedding in milk was also reported by Phuektes et al. [1]. It should be born in mind that the identification and definition of infection depends on culture techniques and the bacterial content of milk samples so that infections with very low levels of bacterial shedding may go undetected with current standard procedures. Duration of infection was the only characteristic that was significantly associated with specific *S. uberis* strains. Duration of infection can be considered a clinical characteristic, but it is also of epidemiological importance. Longer duration of infection implies a longer window of opportunity for spread of bacteria from cow to cow. Indeed, infections caused by the predominant strain in each herd, i.e. the strain with the highest incidence, had longer duration than infections attributed to other strains.

The outbreak of *S. uberis* mastitis that was observed in herd 1 was almost entirely attributable to *S. uberis* strain B. Outbreaks of *S. uberis* mastitis have been described before, and it has been suggested that cases of *S. uberis* mastitis that were left untreated and remained in the milking herd may have resulted in spread of the pathogen to other cows in the herd [38]. The notion that contagious spread in herd 1 in our study occurred during milking is strengthened by the fact that infections with strain B were only observed in lactating animals, and never in non-lactating animals. Furthermore, *S. uberis* could be isolated from teat cup liners, not only after milking of infected quarters, but also after subsequent milking of uninfected quarters. This shows that transmission via the milking machine, which has long been accepted to play an important role in spread of *S. aureus* infection [39], may also play a role in transmission of *S. uberis*. In an earlier report, mathematical analysis was used to determine that the prevalence of infections was a significant predictor for incidence of new infections in herd 1, as would be the case for contagious transmission [21]. Results from RAPD typing support the conclusion that cow-to-cow transmission played a role in the outbreak of *S. uberis* mastitis.

In summary, findings from our study suggest that two subpopulations can be distinguished within the bacterial species *S. uberis*. One subpopulation, exemplified by most of the RAPD types that we identified, comprises strains that infect cows from environmental sources. Infections may occur at any time during the lactating or non-lactating period, and are often of short duration. The second subpopulation, exemplified by RAPD types A and B in this study, consists of strains that cause predominantly subclinical and chronic infections, and spread from cow-to-cow during the milking process. The contagious spread of *S. uberis* can be controlled through implementation of management measures that reduce cow-to-cow transmission of bacteria. The success that can be achieved with such programs has been known for many decades [16, 40] and is still important today.

So far, molecular epidemiology has been used primarily to determine variability in *S. uberis* strains isolated from the bovine mammary gland. As a next step in *S. uberis* research, strains isolated from cattle should be compared to strains isolated from the environment, including skin, mucosa, rumen, manure, bedding, and pasture, to determine the relative importance of different environmental sources in the dynamics of *S. uberis* mastitis. In addition, the virulence and transmission mechanisms of different strains need to be studied, both *in vitro* and *in vivo*, at cow-level and at herd-level. Finally, to allow for comparison of result obtained by different research groups world wide, it would be desirable to have a reproducible typing method with enough discriminatory power and yet sufficient simplicity to allow easy storage and comparison of data. The need to develop such a method has been recognized before [19], but most molecular studies are based on pulsed-field gel electrophoresis [1, 18, 19] or RAPD-fingerprinting [22, 35]. Both methods require interpretation of complex banding patterns on gels that are often difficult to standardize, interpret, store and compare. It would be a great asset if a library typing system, such as the binary typing system for *S. aureus* [41] or multilocus sequence typing as used for *S. aureus* and *Streptococcus species* [42] could be developed for typing of *S. uberis*.

In conclusion, chronic subclinical intramammary infections with *S. uberis* occur frequently and may

serve as a source of infection for other cows in a herd. A contagious route of spread, possibly through transmission via the milking machine, plays a role in the dynamics of *S. uberis* mastitis in some dairy herds, and seems to be associated with specific *S. uberis* strains. When dealing with *S. uberis* problems, both environmental sources of infection and contagious routes of transmission should be given due consideration. To facilitate comparison of results from different studies, it would be desirable to have an internationally standardized typing system for *S. uberis*.

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