Duration of Infection and Strain Variation in *Streptococcus uberis* Isolated from Cows' Milk

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ABSTRACT

The duration of infection and pulsed-field gel electrophoresis (PFGE) types of bovine intramammary *Streptococcus uberis* isolates were examined. Milk samples were collected in duplicate from all 4 glands of 503 cows from 5 herds within 1 to 3 d of parturition and from 113 cows with clinical mastitis in the same herds throughout lactation. Glands from which *S. uberis* was isolated were resampled at 28-d intervals.

The prevalence of *S. uberis* was 12% for cows around parturition, and the median duration of infection was 16 d. Cows >2 yr old had a longer duration of infection than 2 yr old cows, and duration varied among herds. A total of 173 different PFGE types were identified from a total of 234 *S. uberis* isolates. Each farm had a unique set of PFGE types. Only 3 PFGE types were common to each of 3 pairs of cows, and these occurred on the same farm. Where *S. uberis* was isolated on more than one occasion from a gland, only 55% of the PFGE types were the same across time. For cows with multiple glands infected, only one-half (9 of 18) had the same PFGE type in more than one gland. No predominant PFGE type was identified in any herd.

It is concluded that there was wide heterogeneity of PFGE types, that the environment rather than other cows was the likely source of *S. uberis* infections, and that glands may be infected with multiple *S. uberis* PFGE types over a lactation.

(Key words: *Streptococcus uberis*, pulsed-field gel electrophoresis, mastitis, bovine)

Abbreviation key: PFGE = pulsed-field gel electrophoresis.

INTRODUCTION

Streptococcus uberis is the most common isolate from cases of clinical mastitis and is commonly found

in subclinical infections in early lactation (McDougall, 1998) and at the end of lactation (Williamson et al., 1995; Pankey et al., 1996) in New Zealand dairy cows. The prevalence of infection is related to the rate of new infection (the incidence rate) and the duration of infection (Bramley and Dodd, 1984). The duration of subclinical *S. uberis* infection has not been determined in the pasture-fed, seasonally calving New Zealand dairy industry. The duration of *S. uberis* infection has been reported as between 20 d and 9 mo in northern hemisphere studies (Todhunter et al., 1995; Watt, 1999; Zadoks, 2002).

Streptococcus uberis can be isolated from a number of sites on the cow including the vagina, tonsils, and escutcheon (Cullen, 1966) as well as from bedding and pasture (Bramley and Dodd, 1984; Todhunter et al., 1995). The main route of transmission appears to be from environmental sources. However, recent epidemiological and molecular typing studies have demonstrated that cow-to-cow transmission is likely to be occurring in some herds (Phuektes et al., 2001; Zadoks et al., 2001).

Variations in *S. uberis* strain type have been reported using restriction fragment length polymorphism (Hill and Leigh, 1989; Jayarao et al., 1992), randomly amplified polymorphic DNA (Gillespie et al., 1997; Gillespie et al., 1998; Zadoks, 2002), repetitive extragenic palindromic-polymerase chain reaction (Wieliczko et al., 2002), and pulsed-field gel electrophoresis (PFGE) procedures (Baseggio et al., 1997; Douglas et al., 2000; Phuektes et al., 2001). A large number of *S. uberis* types have been observed with all methods (Hill and Leigh, 1989; Jayarao et al., 1992; Douglas et al., 2000; Phuektes et al., 2001; Wieliczko et al., 2002; Zadoks et al., 2003). All *S. uberis* isolates from clinical cases of mastitis were in 2 of the 8 clusters defined from a group of subclinical and clinical *S. uberis* isolates when strains were analyzed with RFLP (Jayarao et al., 1992). Additionally, all *S. uberis* isolates from clinical cases had DNA fragments of >21 kb; only 21% of subclinical isolates had such large

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Table 1. Descriptive data for enrolled herds.

Bacteria isolated within 1 to 3 d of calving													
Herd	Breed ¹	Start of calving	Cows		<i>Streptococcus uberis</i>				Any bacteria				Clinical (no. ²)
			In herd	Sampled	Cows		Glands		Cows		Glands		
					(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	
			(no.)										
A	F	Jul 16	216	82	7	8.5	7	2.1	23	28.0	31	9.5	21
B	J	Jul 22	250	108	14	13.0	17	3.9	41	38.0	65	15.0	29
C	J	Jul 18	424	101	4	4.0	5	1.2	22	21.8	28	6.9	23
D	XB	Jul 5	236	103	5	4.9	7	1.7	25	24.3	42	10.2	22
E	F	Jul 31	350	109	29	26.6	35	8.0	91	83.5	224	51.4	6
Total			1126	503	59	11.7	71	3.9	202	40.2	390	19.4	101

¹F = Friesian, J = Jersey, and XB = Jersey-Friesian cross.

²Number of cows diagnosed by the herd owners with clinical mastitis in the entire lactation.

DNA fragments (Jayarao et al., 1992). Although multiple genotypes are invariably present, a predominant type has been found in some herds (Phuektes et al., 2001; Zadoks, 2002). The degree of homogeneity of *S. uberis* types isolated from a herd has been used to infer the predominant route of transmission. Where there is a predominant type present, it has been suggested that cow-to-cow transmission is likely; if a large number of types are present and no type predominates, then an environmental source of the pathogen is likely (Zadoks, 2002).

Variations in the pathogenicity of phenotypically different isolates of *S. uberis* have been reported following intramammary challenge (Hill, 1988). Additionally, one PFGE type was shown to be more likely associated with clinical mastitis than other types present in the same herd (Phuektes et al., 2001). Hence, genotypic differences, identifiable by molecular typing techniques, may be associated with differences in pathogenicity.

This study aimed to describe the duration of *S. uberis* infection under New Zealand dairy production systems and to re-assess duration with knowledge of the PFGE type.

MATERIALS AND METHODS

Sampling Protocol

Five herds were selected on the basis that they maintained a separate sub-herd containing those cows calved <8 milkings, maintained good records of all disease events, and where the herd managers were willing to collect milk samples from all cows with clinical mastitis. Four herds were in the Waikato region (Herds A to D), and the fifth herd was in the Manawatu region (Herd E) of New Zealand. All herds had a seasonal calving pattern that commenced in spring (July; Table 1), and the cows were fed predominantly on

rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture.

Each herd was visited 3 times weekly, and duplicate milk samples were collected from any cow that had calved in the previous 1 to 3 d (i.e., since the previous visit). These visits continued until approximately 100 cows from each herd had been sampled. Each teat end was scrubbed with a cotton wool ball dampened with 70% methanol, the first 3 strips of milk were discarded, and a 10-mL sample was collected from each gland into a factory-clean test tube. The teats were then recleaned and sampled a second time. Samples were transported at room temperature before being processed for bacteriology within 3 h of collection. An additional sample from each gland was collected at the same time for determination of SCC using a fluorometric cell counting technique (Fossomatic, Foss Electric, Hillerød, Denmark; operated by Livestock Improvement Corporation, Hamilton, New Zealand).

Any gland from which *S. uberis* was isolated (excluding contaminated samples and those with <3 colony forming units/10 μ L) was enrolled in the trial. Single milk samples were then collected from each enrolled gland at approximately 28-d intervals. Sampling ceased from a cow when *S. uberis* was not isolated from 2 sequential samples from any enrolled gland or when lactation ended. Cows detected with clinical mastitis by the herd owner using the criteria of swelling and/or heat of a gland or visible change in the milk (e.g., clots, strings, etc.) had a single milk sample collected from all glands at the time of diagnosis and 21 d later. These samples were labeled and frozen at -20°C until collection at the next routine farm visit. The glands with clinical signs were treated 3 times at 12-h intervals by intramammary infusions of 1 g of penicillin (Penalone, Schering-Plough, Upper Hutt, New Zealand), and the milk was discarded for 144 h following the last treatment.

Case Definitions

A gland was defined as being infected with *S. uberis* where ≥ 3 more colony forming units/10 μL were isolated from both samples (at initial enrolment) or a single sample (at resampling and from clinical cases) and where only one other colony type was present (which occurred on 15 occasions where coagulase-negative staphylococci, *Staphylococcus aureus*, or a yeast was co-isolated with *S. uberis*).

Bacteriological cure was defined as having occurred when 2 sequential samples were found not to contain *S. uberis*.

The duration of infection (days) was defined in 4 ways:

- as the interval from initial sampling to half way between the last *S. uberis*-positive sample and the first non-*S. uberis* positive sample for each gland, irrespective of PFGE type, where the gland was subsequently diagnosed with clinical mastitis and the duration was defined as the interval between initial sample and the day of clinical diagnosis;
- as in the first example, except that the duration included the period after diagnosis of clinical mastitis where clinical mastitis occurred;
- as in the first example except where the *S. uberis* PFGE type changed, in which case the duration was between initial isolation and half way between the final sampling from which the initial PFGE type was isolated and the first sample at which the new PFGE type was isolated; and
- as in the second example except the *S. uberis* PFGE type changed, in which case the duration was between initial isolation and half way between the final sampling from which the initial PFGE type was isolated and the first sample at which the new PFGE type was isolated

Where a gland was still infected at the end of lactation, the duration was defined as the interval from the initial sampling to the last *S. uberis*-positive sample that was collected at the routine sampling visit. Clinical failure was defined as having occurred where the herd owner chose to retreat a gland previously treated for clinical mastitis within 21 d of initial treatment.

Microbiology

Milk samples (10 μL) were streaked onto one quarter of a 5% blood agar plate containing 0.1% esculin (Fort Richard, Auckland, New Zealand) and incubated at 37°C for 48 h. The bacteria were provisionally speciated based on colony morphology, hemolysis pattern,

esculin reaction, and Gram stain. Gram-positive, catalase-negative cocci were defined as *Streptococcus* spp. A single colony of the presumptive *Streptococcus* spp isolate was streaked over the surface of a 0.1% esculin, 5% blood agar plate and grown for a further 24 h. A loopful of this growth was placed in each of 7 sugar reagents, and an isolate was defined as *S. uberis*, where the isolate did not ferment raffinose, but did ferment inulin, lactose, mannitol, salicin, sorbitol, and trehalose (Quinn et al., 1999). The remaining half plate of growth was collected onto a commercial transport swab (BBL Culture swab, Becton Dickinson, Sparks, MD) and held at 4°C for up to 30 d until transport to the Institute of Veterinary and Biomedical Sciences, Massey University for PFGE.

DNA Extraction

Genomic DNA for PFGE was prepared from the *S. uberis* strains according to the methods of Smith and Cantor (1997) and McEllistrem et al. (2000), with some modifications. Briefly, *S. uberis* isolates were grown on 5% sheep blood agar (Difco, Becton Dickinson) overnight at 37°C. The cells were harvested into 3 mL brain heart infusion broth (Difco) and the optical density of the broth was measured and adjusted to 1.4 at 610 nm. A 75- μL aliquot of cells was pelleted by centrifugation at 13,000 rpm for 5 min. Cells were washed once with 150 μL of Pett IV buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA; pH 8.0) then centrifuged and resuspended in 50 μL Pett IV buffer. The bacterial suspension was mixed with 100 μL of 1% low melt preparative grade agarose (Bio-Rad Laboratories (New Zealand) Pty Ltd, Auckland, New Zealand) and was dispensed into plug molds. After being solidified for 1 h on ice, the plugs were incubated overnight at 37°C in a lysis buffer solution (1M NaCl, 100 mM EDTA [pH 8.0] 6 mM Tris-HCl [pH 8.0], 0.5% Brij 58, 0.5% sodium deoxycholate, 0.5% sodium lauroyl sarcosine) containing 20 mg of lysozyme/mL (Roche Diagnostics, Auckland, New Zealand). The plugs were transferred into 1 mL ESP buffer (0.5 M EDTA, 1% sodium lauroyl sarcosine) containing 1 mg proteinase K/mL and were incubated overnight at 56°C. Following lysis, the plugs were washed 5 times for 1 h each time with 10 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) on ice.

Restriction Endonuclease Digestion

A 3-mm slice of each plug was equilibrated in 100 μL of 1.2 $\times$ restriction buffer (New England Biolabs, Beverly, MA) for 45 min on ice. The restriction buffer was removed and replaced with 100 μL of fresh one

Table 2. Duration of infection (d) of 69 glands with *Streptococcus uberis* initially diagnosed with infection around calving and sampled at approximately 28-d intervals. The duration is estimated as 1) from initial isolation to half way between the final isolation and first non-isolation, except where a gland was diagnosed with clinical mastitis in which case the duration was taken as the interval from initial isolation to day of clinical diagnosis; 2) from initial isolation to half way between the final isolation and first non-isolation but also including the time following a diagnosis of clinical mastitis; 3) as for 1 except that a change in pulsed-field gel electrophoresis (PFGE) type was defined as the end of an infection; and 4) as for 2 except that a change in PFGE type was interpreted as the end of infection.

	Duration excluding clinical period	Duration including clinical period	Duration of unique PFGE type	Duration including clinical period with unique PFGE type
Mean	31.1	32.2	24.5	24.5
SD	48.5	48.0	44.6	44.6
Minimum	0	0	0	0
Maximum	278	278	278	278
Percentile				
25th	10.75	12	10.75	10.75
50th	16	16	16	16
75th	21.5	21.5	19	19
Kurtosis	15.0	15.4	24.4	24.4
SE kurtosis	0.6	0.6	0.6	0.6
Skewness	3.6	3.7	4.8	4.8
SE skewness	0.3	0.3	0.3	0.3

× restriction buffer containing 30 units of *Sma*I (New England Biolabs). The plug slices were held on ice for an additional 45 min before an overnight incubation at 25°C.

PFGE

The restriction fragments were separated by PFGE on a CHEF-DRII system in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad Laboratories (New Zealand) Pty Ltd.) in 0.5 × TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA; pH 8.0) at 14°C. The gel was run at 6 V/cm for 23 h with an initial pulse time of 1 s and a final pulse time of 40 s. Lambda Ladder PFG Marker and Low Range PFG Marker (New England Biolabs) were included as molecular size standards. Twenty unknown samples were run on each gel in date order without regard to farm of origin or cow. Thus, repeated *S. uberis* isolations from the same cow might have been run and, hence, analyzed on different gels.

Analysis of PFGE Patterns

Gels were stained with ethidium bromide, and images were captured under ultraviolet illumination by the Gel Doc 2000 (Bio-Rad Laboratories (New Zealand) Pty Ltd.). The PFGE patterns were analyzed by Diversity Database software (Bio-Rad Laboratories (New Zealand) Pty Ltd). Similarities between the PFGE patterns were calculated by the Dice correlation coefficient with a maximum position tolerance of 1%. Dendrograms were constructed by the un-

weighted pair group method with arithmetic mean (UPGMA). Where the level of similarity between isolates was >60% on the dendrograms, the PFGE patterns were compared visually. Multiple isolates from the same cow were also visually assessed. Where the level of similarity was unclear, isolates were rerun on the same gel. Minor differences i.e., <3 band differences were recorded as subtypes, but treated as one PFGE type for analysis.

Statistical Analysis

The duration of infection of glands diagnosed with subclinical *S. uberis* infections were not normally distributed (Table 2) with most intervals being <20 d, but with a few longer intervals. Thus, data are presented as the median and percentiles (Table 2). The duration was further examined using Kaplan-Meier or Cox's proportion hazards models ("survival analysis"). Analysis was performed for each of the 4 definitions described previously. Initially, factors thought to affect the duration of infection (including age [coded as 2 yr old or >2 yr old], herd of origin, days postpartum at enrollment, whether the gland was a front or rear gland, number of glands infected within a cow, and the SCC [and log₁₀ SCC] of the gland at the time of enrollment) were tested in univariate models. Factors associated (i.e., $P < 0.2$) were then offered to a reverse stepwise model using likelihood ratios as the factor exclusion method. Glands were defined as right-censored when infection persisted to the end of lactation. The validity of the models was assessed by examination of the hazards ratio and log minus log plots.

Figure 1. Survival (i.e., probability of being infected) of any *Streptococcus uberis* (conventional; ∇) infection and of PFGE-specific *Streptococcus uberis* pulsed-field gel electrophoresis type ($\bullet$) of individual glands infected with *Streptococcus uberis*.

The proportion of glands diagnosed with clinical mastitis that were subsequently bacteriologically negative (i.e., the 'cure proportion') was analyzed using logistic regression. The age code (i.e., 2 yr old or >2 yr old), breed, and days postpartum (coded as 0 to 1, 2 to 3, 4 to 25, and >25 d postpartum at the time of diagnosis, which represent approximately quartiles of

the number of cases by days postpartum) were initially tested by univariate methods (i.e., χ^2). If these factors were associated with bacteriological cure proportion ($P < 0.2$), they were then offered to a reverse stepwise logistic regression model. The effect of age code (i.e., 2, 3, 4 to 8, and >8 yr old), herd of origin, and days postpartum at diagnosis (coded into quartiles as

Figure 2. Survival (i.e., probability of being infected) of individual glands infected with *Streptococcus uberis* in heifers (solid line) or cows (dashed line).

Figure 3. Survival (i.e., probability of being infected) of individual glands infected with *Streptococcus uberis* in 5 herds with each herd represented by a separate line type.

Table 3. Bacteria isolated from glands diagnosed with clinical mastitis by herd owners.

Isolate	n	All samples	Bacteria-positive isolates
			(%)
No growth	40	29.0	
<i>Staphylococcus aureus</i>	3	2.2	3.4
Coagulase-negative staphylococci	10	7.2	11.5
<i>Streptococcus uberis</i>	55	39.9	63.2
<i>Streptococcus agalactiae</i>	3	2.2	3.4
Other streptococci	2	1.4	2.3
<i>Escherichia coli</i>	4	2.9	4.6
Others	10	7.2	11.5
Contaminated ¹	11	8.0	
Total	138	100.0	100.0

¹>3 distinct colony types present.

above) on the proportion of clinical cases from which *S. uberis* was isolated was analyzed by χ^2 .

The trial was performed following approval by the Animal Ethics Committee of AgResearch, Ruakura and the Massey University Animal Ethics Committee.

RESULTS

Bacteriology

Forty percent of cows and 19% of glands had intramammary infections when sampled within 1 to 3 d of calving (Table 1). *Streptococcus uberis* was isolated from 12% of all cows and 4% of all glands (Table 1). A total of 59 cows and 71 glands were enrolled with subclinical *S. uberis* infection after this initial sampling. Forty-two cows had one *S. uberis*-infected gland, 13 had 2 *S. uberis*-infected glands, and one cow had 3 *S. uberis*-infected glands at enrolment.

Duration of Subclinical *S. uberis* Infection

The majority of *S. uberis*-infected glands underwent spontaneous bacteriological cure (55 of 71 [78%]), with a small number lost to follow up (2 of 71 [3%]), diagnosed with clinical mastitis (9 of 71 [13%]) or were still infected at the end of lactation (5 of 71 [7%]).

The median duration of infection was 16 d for all 4 definitions of duration (50th percentile; Table 2; Fig-

ure 1), but with numerically shorter 25th and 75th percentiles for the estimates that took account of changes in PFGE type (Table 2). Inclusion of the period of infection following diagnosis of clinical mastitis did not increase the estimated median duration of infection (Table 2). The duration of infection (Definition 1) was longer for cows than heifers (12 [95% CI = 7 to 17] vs. 11 [95% CI = 5 to 15] median d for cows and heifers, respectively; $P = 0.001$; Figure 2, hazards ratio = 6.0 [95% CI = 2.1 to 17.4]) and varied among herds ($P = 0.01$; Figure 3). Duration of infection was unaffected by whether a cow had only one or more than one gland infected with *S. uberis* ($P = 0.79$), the day postpartum at enrolment postpartum ($P = 0.32$), whether the gland was a front or rear gland ($P = 0.79$) or by the SCC or $\log_{10}$ SCC of the gland at enrolment ($P = 0.81$ and $P = 0.47$, respectively). The same variables were significant with similar hazards ratios when the other 3 methods of calculating duration were tested as the dependent variables (data not presented).

Clinical Mastitis

A total of 101 cows and 138 glands were diagnosed with clinical mastitis. Mastitis was diagnosed in one, 2, 3, and 4 glands in 79.6, 14.2, 1.8, and 4.4% of cows, respectively.

Streptococcus uberis was the most prevalent pathogen isolated from cases of clinical mastitis (63.2%; Table 3) with 'others' and coagulase-negative staphylococci the next most common isolates. The proportion of glands from which *S. uberis* was isolated did not differ among herds ($P = 0.57$), age codes (18 of 42 (43%), 2 of 8 (25%), 37 of 88 (42%), and 8 of 25 (32%) for 2, 3, 4 to 8, and >8 yr old cows, respectively; $P = 0.64$), or vary with time postpartum (20 of 43 [47%], 18 of 40 [45%], 13 of 30 [43%], and 13 of 48 [27%] for 0 to 1, 2 to 3, 4 to 25, and >25 d postpartum at diagnosis, respectively; $P = 0.18$). The proportion of glands that underwent bacteriological cure was 89.6%, and this proportion declined with increasing days postpartum at diagnosis (Table 4; $P = 0.03$).

PFGE Analyses

A total of 173 different PFGE types were identified from a total of 234 *S. uberis* isolates (Table 5). An example of a PFGE gel is presented in Figure 4, and the dendrogram constructed from isolates from one herd (Herd A) is presented in Figure 5.

Each farm had a unique set of PFGE types; that is, in no case was the same PFGE type isolated from more than one herd. In 4 of the 5 herds, each PFGE type

Table 4. Proportion of clinical mastitis cases bacteriologically cured (all pathogens) by DIM at diagnosis.

DIM at diagnosis	Total cows	Cured	
		(no.)	(%)
0 to 1 d	21	21	100.0
2 to 3 d	22	21	95.5
4 to 25 d	15	13	86.7
>25 d	19	14	73.7
Total	77	69	89.6

Table 5. Number and percentage of subclinical *Streptococcus uberis* pulsed-field gel electrophoresis (PFGE) types.

	Herd					Total
	A	B	C	D	E	
<i>Strep. uberis</i> isolates tested, n	32	67	52	19	64	234
PFGE types, n	18	54	34	17	50	173
Glands with multiple <i>S. uberis</i> isolates across time, ¹ no.	4	6	4	3	12	29
Glands with same PFGE type across time ² no.	4	5	4	1	2	16
%	100	83.3	100	33.3	16.7	55.2
Number of PFGE types in multiple cows ³ n	0	0	0	0	3	3
% ⁴	0	0	0	0	10.3	5.1
Cows with multiple glands infected with <i>S. uberis</i> , no.	2	11	6	4	12	35
Cows with the same PFGE type in multiple glands ⁵ no.	1	1	1	0	2	5
%	50	9.1	16.7	0	16.7	14.3
Cows with multiple <i>S. uberis</i> isolates across time, ⁶ no.	1	6	3	2	6	18
Cows with same PFGE type across time ⁷ no.	1	4	2	1	1	9
%	100	66.7	66.7	50.0	16.7	50.0

¹Glands from which *Strep. uberis* was isolated on more than one occasion over time.

²Glands from which the *Strep. uberis* PFGE type was isolated on more than one occasion over time.

³ Number of cows within a herd from which the same *Strep. uberis* PFGE type was isolated.

⁴The denominator is the total number of cows from which *Strep. uberis* was isolated.

⁵At least 2 glands with the same PFGE type within the same cow (either simultaneously or over time).

⁶Cows from which *Strep. uberis* was isolated from one or more glands on more than one occasion over time.

⁷Cows from which the same *Strep. uberis* PFGE type was isolated from one or more glands on more than one occasion over time.

was unique to one cow. In Herd E, 3 PFGE types were recovered from 2 cows each (Table 5).

For glands from which *S. uberis* was isolated on more than one occasion, 55% (16 of 29) had the same PFGE type (Table 5). However, there was a range among herds, with between 16% (Herd E) and 100% (Herds A and C) of glands having the same PFGE type on more than one occasion in the same gland (Table 5).

Only 14% of cows that had multiple glands infected with *S. uberis* simultaneously had the same PFGE type in ≥ 2 glands (Table 5).

Half of the cows (9 of 18) from which more than one *S. uberis* isolate was recovered over time had the same PFGE type within cow over time (Table 5).

Isolates from 2 glands had isolates showing minor variations in the PFGE banding pattern over time. For example, the PFGE pattern of isolates from one gland over a 4-mo period showed 3 closely related PFGE types (designated L2, L2a, and L2b; Figure 6).

PFGE Types of *S. uberis* Associated with Apparent Clinical Treatment Failures

Isolates of *S. uberis* recovered from 4 glands post-treatment of clinical mastitis were examined. The antibiotic sensitivity of each of the isolates was tested

using the Kirby-Bauer disc diffusion method, and all isolates were sensitive to penicillin. In 2 of the 4 cases, the PFGE type changed following treatment (Table 6). However, these changes were relatively minor as the PFGE types had 75 and 92% similarity with the original isolates (Table 6). In one case, the same PFGE type as that recovered pre-treatment was re-isolated following 2 samplings (at 28-d intervals) where no bacteria were isolated.

DISCUSSION

The median duration of *S. uberis* subclinical infection was 16 d in the present trial. This is shorter than the duration of 28 to 46 d reported from a Dutch study (Zadoks et al., 2001), a report that 62% of *S. uberis* cases have a duration of <31 d (Todhunter et al., 1995) and estimates of 95 d and 2.8 to 9 mo from other studies (Lam, 1996; Watt, 1999). The duration of infection in the current study might have been underestimated as some cows could have been infected for a significant period of time before the experiment commenced (i.e., left-censored) and some remained infected at the end of sampling (i.e., right-censored). This underestimation may be substantial, as approximately 32% of new *S. uberis* infections are reported to

Figure 4. Example of pulsed-field gel electrophoresis (PFGE) of *Sma*I macrorestriction fragments of *Streptococcus uberis* isolates of bovine origin. The top line above the gel indicates the PFGE type assigned to that isolate; the second line indicates the lane numbers with lane 1 = low range PFG marker and lane 12 = the Lambda Ladder PFG Marker (New England Biolabs).

occur in the dry period and 36% of *S. uberis* infections present at calving are acquired in the first half of the dry period (Todhunter et al., 1995). Other studies estimating duration might also have left censoring of some animals. The current study enrolled *S. uberis* infected cows around calving while other studies have enrolled cows throughout lactation (Zadoks et al., 2001, 2003). As the duration of infection has been shown to be shorter for infections detected at calving than later in lactation (e.g., 9 vs. 46 d for infections detected at calving compared with other times; Zadoks et al., 2003), the current estimate may be shorter than the average for infections across an entire lactation. A novel finding of this study was that cows (i.e., >2 yr old) had a longer duration of infection than heifers (i.e., 2 yr olds). A previous study did not find any effect of age on duration of *S. uberis* infection (Watt, 1999). The reason for the longer duration in older cows is not clear. The reason for the reported differences among herds in duration of infection is not known but may include factors related to milking management, nutrition, and/or cow genetics. It was assumed for the analysis of duration of infection that isolation of *S. uberis* at each sampling at 28-d intervals constituted continuous infection between samples. However, it is possi-

ble that spontaneous cure and re-infection with the same PFGE type could have occurred within the 28-d period, but with the high degree of heterology of *S. uberis* isolates within herds, this appears an unlikely possibility. A higher frequency of sampling and use of PFGE typing may help elucidate the extent that this occurs. In the initial analysis, changes in the PFGE type were ignored. However, as 45% of glands had changes in PFGE type over time, the duration was re-analyzed, taking account of the changes, and it was assumed that a change in PFGE type represented a new infection and that the initial infection had ended at the time of the change. The median duration of infection was unchanged when PFGE type and period of infection following clinical was taken into account. This was likely because only a small number of glands ($n = 9$; 13%) were diagnosed with clinical mastitis, and in each case the gland underwent bacteriological cure following treatment.

Multiple *S. uberis* PFGE types were identified both within and between farms in the present study. Previous studies have also identified a large number of *S. uberis* strains on farms in New Zealand (Douglas et al., 2000; Wieliczko et al., 2002) and elsewhere (Hill and Leigh, 1989; Jayarao et al., 1992; Phuektes et al., 2001; Zadoks, 2002). In the present study, the same PFGE type was only found in 2 different cows on 3 occasions, and all of these cows were in the same herd. Approximately only one-half of the cows and one-half of the glands with chronic infections had the same PFGE type over time. Additionally, in only 14% of cows in which multiple glands were infected did the same PFGE type occur in the same animal. These data suggest that in most cases infection occurs from a source other than an infected cow. In contrast to the current study, a predominant *S. uberis* type has been identified within some herds in other studies. For example, 50% (5 of 10) and 78% (22 of 28) of cows from which *S. uberis* were isolated had the same strain type within 2 herds in a Dutch study, respectively (Zadoks et al., 2003). The same strain type was found in 88% of infections from which multiple samples were collected (Zadoks, 2002). The same strain type was identified in each gland where a cow had multiple glands infected simultaneously (Zadoks et al., 2003). A predominant strain was also identified in 2 of 4 herds in an Australian study (Phuektes et al., 2001). It was concluded from these 2 studies that significant cow-to-cow transmission was occurring. The greater degree of heterogeneity of *S. uberis* types in the present study and a failure to find a dominant type may reflect differences in the epidemiology of *S. uberis* infection among production systems. Under the extensive pasture systems in New Zealand, it appears that

Figure 5. Dendrogram of *Streptococcus uberis* isolates from Herd A. The coding on the right-hand side of the diagram indicates the cow number (2 or 3 digits), the gland (i.e., LF = left front, LR = left rear, RF = right front, and RR = right rear), the calendar date of isolation (dd.mm.yy), and the clinical status of the gland (i.e., clinical [clin] or subclinical [sub]). The level of relatedness is indicated by the horizontal scale at the top of the diagram with 1 = indistinguishable, 0 = unrelated.

Figure 6. Diagrammatic representation of pulsed-field gel electrophoresis patterns of *Streptococcus uberis* isolates L2, L2a, and L2b all from cow 235, left rear gland, Herd C, isolated between January and May 2001.

a point source of *S. uberis* infection is uncommon and that transmission of *S. uberis* may be predominantly from environmental sources.

Where isolates differ by only 2 to 3 PFGE bands, it has been suggested that this may represent a single point change in the genome and that the types are closely related (Tenover et al., 1995). Two isolates differed by only 1 or 2 bands when isolated sequentially from the same glands over time in the present study. In one of these cases, the change occurred following treatment of clinical mastitis with an antibiotic that did not result in bacterial cure of the gland. It is plausible that treatment resulted in a genotype change that allowed the isolate to survive antibiotic treatment. However, no change in antibiotic sensitivity was demonstrated following testing using the Kirby Bauer disc diffusion method for these 2 PFGE types.

In the small number of glands treated for clinical mastitis and in which subsequent sampling suggested that treatment failure had occurred, different PFGE types were isolated post-treatment compared with

Table 6. Pulsed-field gel electrophoresis (PFGE) types of clinical *Streptococcus uberis* isolates before (pre-treatment) and after (Post 1 to Post 5) treatment where there was apparent failure of bacteriological cure.

Herd	Cow	Gland ¹	Date of treatment	PFGE type						Rel ²
				Pre-treatment	Post 1	Post 2	Post 3	Post 4	Post 5	
B	133	RR	Jan 2	K6	K6	no growth	no growth	K6		1.00
B	133	LR	Jan 2	K1	K1	K1	K1	K1		1.00
C	65	LR	Dec 14	L6	L4	L4	L4			0.75
C	235	RF	Jan 9	L2a	L2	L2	L2	L2	L2b	0.92

¹RR = Right rear, LR = left rear, and RF = right front.

²Degree of similarity between *S. uberis* PFGE types before and after treatment: 1 = indistinguishable and 0 = no relationship.

pre-treatment. However, it is not clear if bacteriological cure had indeed occurred and a new infection had subsequently become established, whether treatment itself resulted in a change in the PFGE type profile, or whether, in fact, multiple PFGE types were present pre-treatment but only one identified because of the laboratory techniques used (see subsequent).

In the present study, clinical mastitis was not associated with any particular strain type. In contrast, in a previous Australian study, one PFGE type was more commonly associated with clinical mastitis than other PFGE types (Phuektes et al., 2001). Those researchers suggested that the predominant strain had a virulence factor(s) not present in other strains endemic in the herd. In contrast, another study failed to find that different PFGE types had a different probability of being associated with clinical mastitis (Zadoks et al., 2001). Variations in virulence have been previously demonstrated among *S. uberis* strains (Hill, 1988). No one PFGE type or group of PFGE types were more likely to be associated with prolonged subclinical infection, diagnosis with clinical mastitis, or a failure to cure following treatment in the current study. Thus, cows in this population are likely to have been exposed to a range of *S. uberis* PFGE types, and the duration of infection and clinical outcome (i.e., diagnosed as clinical or not) may be related to cow factors or dose of *S. uberis* rather than to the specific PFGE type the gland was infected with.

A limitation of the present study, and many other studies, is that only one colony type from a number of colonies grown during initial culture was genotyped, because of the expense of PFGE. However, given the large number of *S. uberis* PFGE types identified, it is possible that multiple PFGE types might have been present concurrently within a single gland. Two PFGE types of *S. uberis* in the same gland at the same time have been previously reported as they were phenotypically different and readily distinguishable on the primary culture plate (Zadoks, 2002). Further studies are required to determine the prevalence of multiple

S. uberis PFGE type infections within the same gland. This is important as where only one colony type is tested and where multiple PFGE types are concurrently present, it may be falsely assumed that new infections are occurring over time as the PFGE type appears to change with time, when in fact it is just chance that one or other of the infecting strains is actually selected for typing at sequential sampling times.

It was concluded that there are many different PFGE types of *S. uberis* present within the New Zealand environment, that cow-to-cow transmission of *S. uberis* is likely uncommon relative to transmission from environmental sources, that changes in PFGE type within chronically infected glands are relatively common, that PFGE type is not correlated with the diagnosis of clinical mastitis, and that the duration of infection may be overestimated where the PFGE type of the infection is not known.

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