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Epidemiological typing of bovine streptococci by pulsed-field gel electrophoresis

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SUMMARY

Pulsed-field gel electrophoresis (PFGE) was used to investigate the epidemiology of streptococcal mastitis in dairy cattle. The most prevalent streptococcal species, *Streptococcus uberis* (60–80% of streptococcal isolates), was highly heterogeneous, with different cows only rarely sharing the same pulsotype. *S. agalactiae* was rarely encountered, however all eight isolates from one farm generated identical PFGE profiles, which differed from those of all other isolates examined, confirming cow-to-cow transmission. Fifty-two isolates of *S. dysgalactiae* from 27 cows on 5 farms generated 6 different profiles. However, on individual farms, only one or two pulsotypes usually predominated. This species is generally regarded as an environmental pathogen but our data suggest that cow-to-cow transmission of *S. dysgalactiae* may occur. In spite of the variation in PFGE profiles of isolates from different cows, persistent infections in individual cows were usually caused by the same pulsotype of *S. uberis* or *S. dysgalactiae*.

INTRODUCTION

Mastitis is a disease of major economic importance to the dairy industry, causing reduced milk quality and leading to loss in production and increased use of drugs and veterinary services. Estimates of economic loss for each case of intramammary infection are US $130–320, with the reported prevalence in different countries 40–60% [1]. Although mastitis control programmes have been partially successful in reducing the prevalence of intramammary infections [1], control is hampered because most infections are subclinical, and not recognized unless laboratory investigations are undertaken [2–4]. Moreover precise knowledge of the mechanisms of transmission and pathogenicity of different subtypes of the various species of mastitis pathogens is currently lacking.

Staphylococcus aureus and three species of streptococci (*Streptococcus uberis*, *S. agalactiae*, *S. dysgalactiae*) are the predominant causes of mastitis in dairy cattle in most countries [1, 5]. *S. uberis*, the major streptococcal species isolated from bovine intramammary infections, is commonly found in older cows during the non-lactation period and may contribute significantly to clinical mastitis in early lactation [2, 6]. *S. uberis* is not controlled by routine hygiene procedures in the milking shed, supporting the assumption that this organism is acquired from the cow's environment [2]. *S. agalactiae* is a primary udder pathogen that responds to therapy with antimicrobial agents, hence eradication from herds is possible if good hygiene is practised at milking and infected cows are treated promptly with antimicrobial

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agents [3]. The prevalence of mastitis due to *S. agalactiae* has decreased in recent years, although it remains a significant cause of mastitis in herds that are not well managed [1]. *S. dysgalactiae* is usually classified as an environmental pathogen, but may behave as a contagious pathogen at times [6]. Most infections due to *S. dysgalactiae* occur during the dry period and in early lactation [2, 6].

Classical epidemiological tools have provided only limited information on the relative importance and transmission of subtypes within the three species of bovine streptococci. However, with the availability of molecular typing tools to differentiate between strains of bacteria, it is now possible to determine the distribution and persistence of specific molecular types and to identify clones with possible enhanced virulence or transmissibility. Few studies have used molecular fingerprinting techniques for subtyping bovine streptococci. Two early studies used restriction fragment length polymorphisms (RFLP) to study the epidemiology of *S. uberis* [7, 8]. However, differentiation between closely related strains appeared to be difficult due to the large number of bands generated. Ribotyping produces more manageable profiles and has been used to subtype *S. agalactiae* [9, 10] and *S. dysgalactiae* [11], while PCR fingerprinting has been used to subtype *S. uberis* and *S. dysgalactiae* [12, 13]. Pulsed-field gel electrophoresis (PFGE) is regarded as the reference typing method for strain differentiation within bacterial species because it provides highly reproducible and manageable restriction profiles representing the entire bacterial genome and has high discriminatory power [14]. However, we are aware of only two studies, which used 18 and 46 isolates respectively, that have applied PFGE to subtype bovine streptococci [15, 16].

This paper describes the application of PFGE to investigate the distribution, transmission and persistence of bovine streptococcal subtypes among dairy cattle in Victoria, Australia, using a larger number of isolates than in previous studies.

METHODS

Study farms

This study was conducted on six commercial dairy farms in Victoria, Australia. Farms R, B, W, H and O milk 100–150 cows using a herringbone system, while farm J milks 800 cows and uses a rotary system. Management systems, breed of dairy cow and distribution of calving dates differ between farms.

Streptococcal isolates

The bovine streptococci were selected from a culture collection consisting of over 600 isolates that were recovered from composite milk samples (pooled from 4 quarters) collected from 6 dairy farms in Victoria, Australia at intervals of approx. 2–4 weeks during the entire 1997–8 lactation period. The isolates were representative of the species distribution of streptococcal mastitis pathogens on the 6 dairy farms. On the study farms, *S. uberis* was the most prevalent streptococcus species (60–80% of streptococcal isolates), followed by *S. dysgalactiae* which was present on 5 of the 6 farms (0–25% of streptococcal isolates). *S. agalactiae* was isolated from only 2 of the 6 farms. The isolates had previously been identified as *Streptococcus* spp. and speciated using selected cultural, biochemical and serological tests [17]. Those selected included (i) all isolates recovered during a period of high prevalence on each farm and (ii) isolates from milk of cows with chronic infection (two or more samples yielding *Streptococcus* spp.). They consisted of 130 isolates of *S. uberis* from 3 of the farms (after great diversity was demonstrated on 3 farms, isolates from the remaining farms were not examined), 9 isolates of *S. agalactiae* and 52 isolates of *S. dysgalactiae*. The reference strains used in the study were *S. agalactiae* ATCC 27956, *S. dysgalactiae* ATCC 27957, and *S. uberis* ATCC 13387. Additional reference strains used were *S. agalactiae* RMIT B439, *S. dysgalactiae* RMIT W816, and *S. uberis* RMIT W674.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed by a modification of methods described by Smith and Cantor [18], Fasola et al. [19] and Bert et al. [16]. In brief, cell pellets were suspended in Pett IV buffer (10 mM Tris–HCl pH 7·6, 1 M NaCl), mixed with an equal volume of 2·4% low melting point agarose (BioRad, Hercules, CA, USA) and lysed at 37 °C overnight in buffer consisting of 6 mM Tris–HCl pH 7·6, 1 M NaCl, 0·1 M ethylenediaminetetraacetic acid EDTA pH 7·6, 0·2% (w/v) sodium desoxycholate (Oxoid), 0·5% (w/v) N-lauryl sarcosine (Sigma, St Louis, MO, USA), and 1 mg/ml of lysozyme (Boehringer–Mannheim, Mannheim, Germany) added prior to use. Preliminary experiments showed that the addition of mutanolysin

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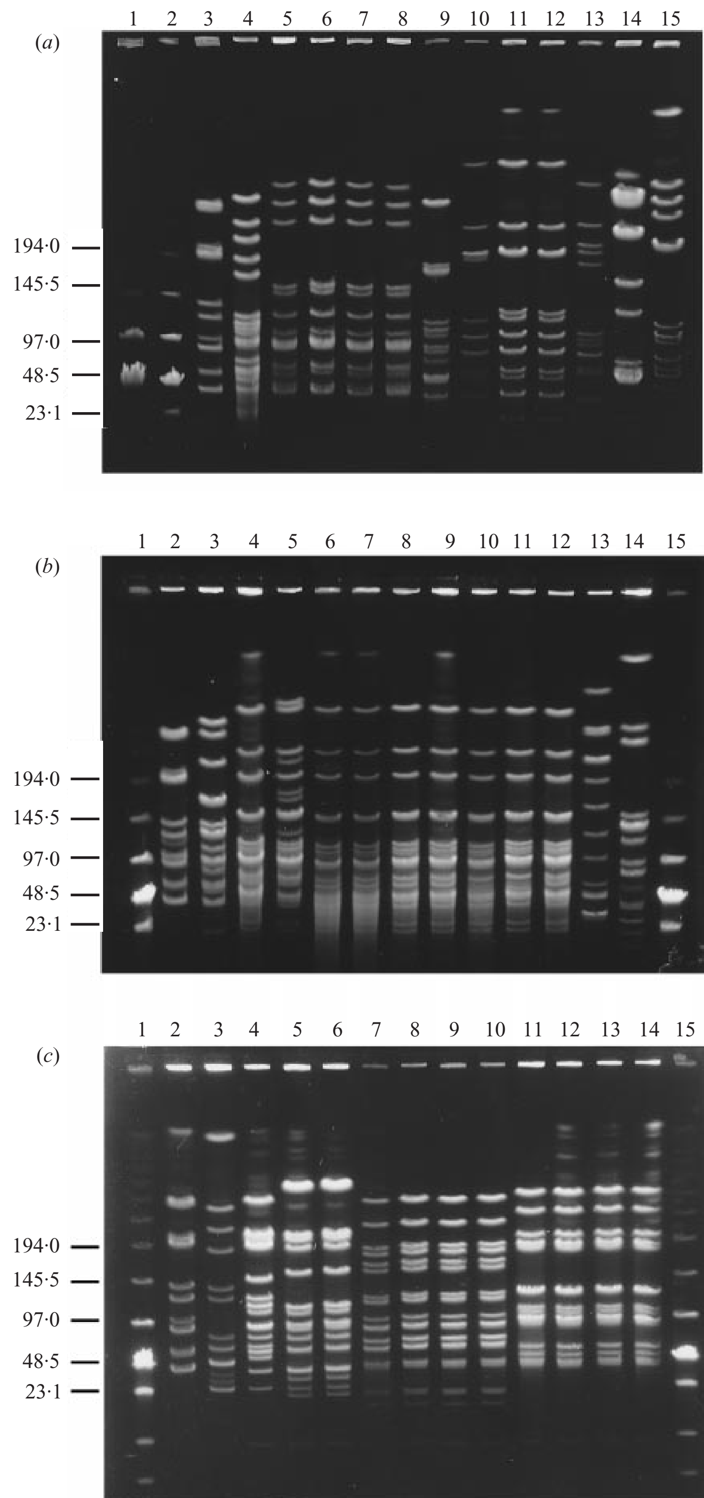

Fig. 1. *Sma*1 restriction profiles of a representative set of *S. uberis* isolates from three farms. (*a*) Farm R. Lane 1, lambda ladder PFG marker (Boehringer–Mannheim); lane 2, low range PFG marker (Boehringer–Mannheim); lanes 3–15, isolates from cows R269 (6 isolates), R271, R349 (3 isolates), R357, R358 (2 isolates). (*b*) Farm W. Lane 1, low range PFG marker (Boehringer–Mannheim); lane 2, *S. uberis* ATCC 13387; lanes 3–14, isolates from cows W203, W344 (9 isolates), W367, W390. (*c*) Farm H. Lanes 1 and 15, low range PFG marker (Boehringer–Mannheim); lane 2, *S. uberis* ATCC 13387; lanes 3–14, isolates from cows H18, H19, H23 (2 isolates), H29 (4 isolates), H38 (4 isolates). Molecular size standards are given in kb.

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Table 1. *Patterns of new and persistent infections with* S. uberis *from farms R, W and H**

Cow	Pulsed-field gel electrophoresis (PFGE) type isolated from milk samples									
	19/8/97	9/9/97	22/9/97	14/10/97	25/11/97	6/1/98				
R1				U1	U2					
R171			U3a	U3a	U7					
R239			U9	U9		U9				
R269	U10	U7	U7	U7	U7					
R349		U14a		U14	U14					
R358					U16	U17				
R454			U18	U18a		U18				
R469		U19	U20	U19	U19					
R501		U21	U22	U22		U23				
R508	U25	U25	U25	U25	U25					
R562				U28	U28					
	26/5/97	9/6/97	16/6/97	1/7/97	14/7/97	28/7/97	11/8/97	26/8/97	15/9/97	6/10/97
W303	U36				U35	U35	U35	U35		U35
W344		U37	U38	U37	U37	U37	U37	U37	U37	U37
W674	U46a	U46	U46	U46	U46	U46	U46a			
	13/10/97	4/11/97	15/12/97	5/1/98	16/2/98	31/3/98				
H5			U60	U60						
H23	U64	U64								
H29	U65	U65	U65	U65						
H38	U66			U66	U66	U66				
H42	U67	U67								
H43	U68	U68	U68	U68						

* The pulsotype of the *S. uberis* isolates is represented by U, followed by the type number.

(Sigma) to the lysis buffer [16, 19], provided no additional benefit. After purification, the DNA was digested with 20 U of *Sma*1 (Boehringer–Mannheim) at 25 °C overnight. Approx. 1·22 μg DNA was loaded into wells in a 1% agarose gel. The DNA fragments were separated using a contour-clamped homogenous electric field device (CHEF-DR 11, BioRad) with pulse times of 5–15 s for 11 h and 15–45 s for 13·5 h at 180 V. PFGE patterns were interpreted according to the criteria described by Tenover et al. [20]. Each isolate was given a profile number. *S. uberis*, *S. agalactiae* and *S. dysgalactiae* were designated as U, A and D respectively. Unrelated isolates (> 3 band differences) were assigned to different profile numbers (e.g. U1 and U2), isolates generating identical restriction patterns were given the same profile numbers and those with up to 3 band differences were assessed as probably related and assigned as subtypes (e.g. U1a, U1b). Reproducibility of PFGE patterns was examined by analysing 4 independent sets of DNA samples prepared from the 3 ATCC reference strains.

RESULTS AND DISCUSSION

All isolates of *S. uberis*, *S. agalactiae* and *S. dysgalactiae*, when analysed by PFGE, produced 10–16 well-resolved fragments of 10–500 kb. Patterns were identical for all samples of DNA isolated from the same strain. There was no overlap between profiles generated by the three different species examined.

The PFGE patterns for *S. uberis* showed great variation. Among the 130 isolates (collected from 73 cows on 3 farms), 74 distinct PFGE profiles were observed. Isolates from different cows almost invariably had distinct DNA patterns (Fig. 1). This high level of heterogeneity supports classical epidemiological studies, suggesting an environmental reservoir with limited, if any, transmission from cow-to-cow during the milking process.

We found only five examples of the same pulsotype of *S. uberis* being isolated from different cows on the same farm. These were type U3 (includes U3 and subtype U3a), isolated from four cows and types U7,

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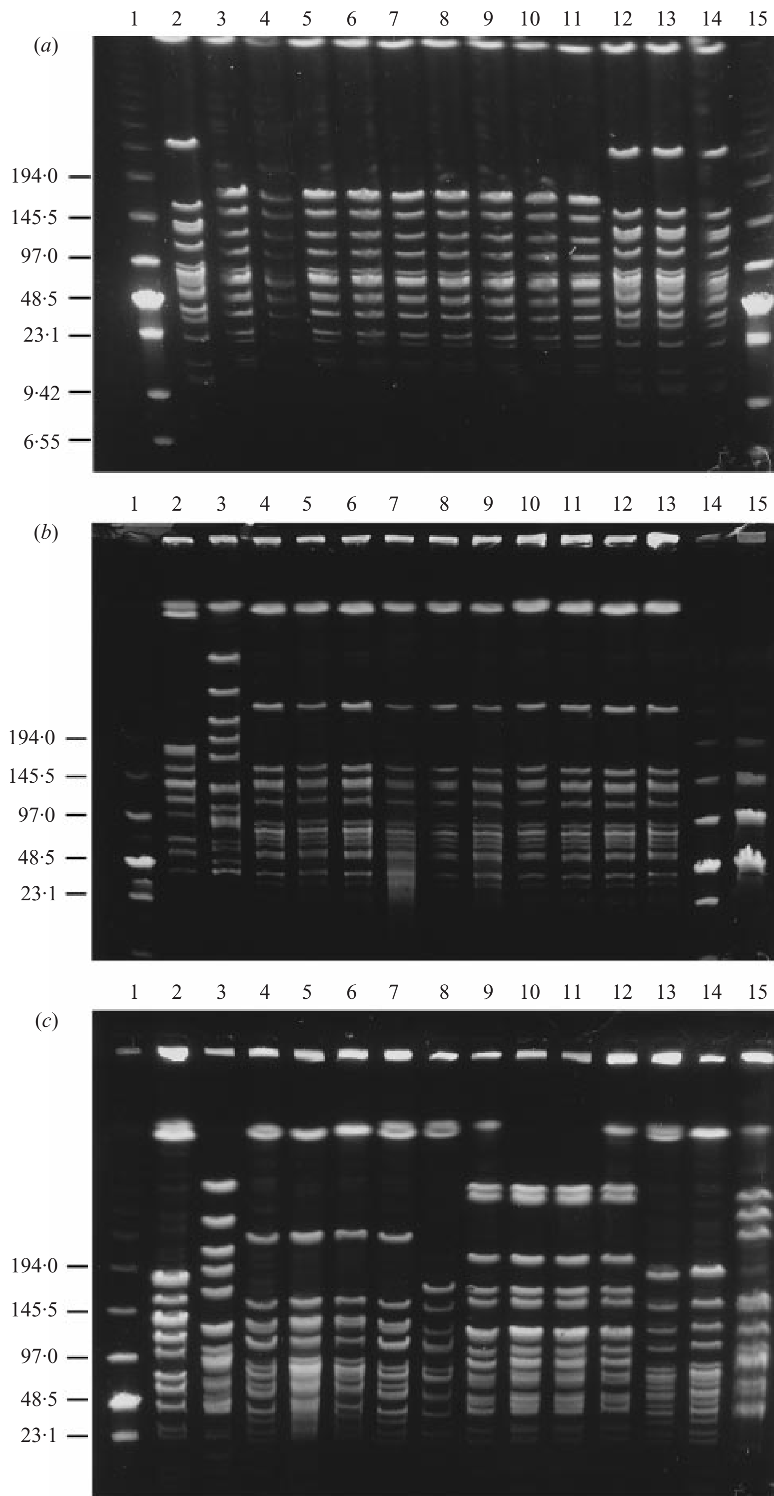

Fig. 2. *Sma*1 restriction profiles for a representative selection of *S. dysgalactiae* isolates from four farms. (*a*) Farm R. Lanes 1 and 15, low range PFG marker (Boehringer–Mannheim); lanes 2–14, isolates from cows R2, R17 (2 isolates), R128 (4 isolates), R230 (3 isolates), R478 (3 isolates). (*b*) Farm B. Lanes 1 and 14, low range PFG marker (Boehringer–Mannheim); lane 2, *S. dysgalactiae* ATCC 27957; lane 3, *S. dysgalactiae* RMIT W816; lanes 4–13, isolates from cows B39, B460, B516, B532 (2 isolates), B533 (3 isolates), B644, B750; lane 15, lambda ladder PFG marker (Boehringer–Mannheim). (*c*) Farms H and J. Lane 1, low range PFG marker (Boehringer–Mannheim); lane 2, *S. dysgalactiae* ATCC 27957; lane 3, *S. dysgalactiae* RMIT W816; lanes 4–15, isolates from cows H3, H10 (2 isolates), H16, H19, H75 (4 isolates), J962 (2 isolates), J616. Molecular size standards are given in kb.

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U20, U35 and U50 that were each isolated from two cows. In contrast, 18 of 20 cows examined because of persistent streptococcal infections harboured the same pulsotype for periods up to 5 months (Table 1, Fig. 1). Transmission of streptococci from these cows was apparently rare, however, as only two pulsotypes associated with persistent infections (U3a and U7) were recovered from another cow in the same herd. Eight of the 20 cows with prolonged infections harboured more than one pulsotype of *S. uberis* during the course of the infection, although in most instances, one pulsotype was predominant (Table 1). As we used composite milk samples and our procedure for culturing milk did not include picking multiple colonies of the same phenotype, we could not determine whether these cows experienced new infections or mixed infections involving the same or different quarters. We also identified cows that had two or more infections during lactation, caused by *S. uberis* and *S. dysgalactiae* respectively.

Diversity among *S. uberis* isolates was also found in earlier epidemiological investigations using different molecular techniques, i.e. restriction fragment length polymorphisms (17 RFLP types, 42 isolates, 17 cows, 2 farms) and PCR respectively (12 PCR types, 22 isolates, 9 cows) [8, 12]. More recently, Basseggio et al. [15] reported diversity of *S. uberis* in a study of 21 isolates from milk samples collected on 10 farms in Victoria, Australia. These molecular studies support the use of control measures aimed at providing dairy cows with a clean uncrowded environment to limit survival and multiplication of the *S. uberis* at extra-mammary sites.

Eight isolates *S. agalactiae* from different cows on the same farm were identical to each other but different from the one isolate from another farm and from the two reference strains (data not shown). Although the number of isolates of *S. agalactiae* examined was too small to draw firm conclusions, these data suggest the presence of a single clone that was transmitted between cows. They support the findings of Basseggio et al. [15], that isolates within each of three herds were similar or identical, but isolates from different farms had different PFGE profiles. Ribotyping of *S. agalactiae* also demonstrated greater diversity between farms than within farms [10]. These findings confirm data from classical epidemiological studies and support the continued use of hygienic practices during milking as a measure to control infections with *S. agalactiae*.

PFGE restriction patterns of *S. dysgalactiae* were more diverse than those of *S. agalactiae* but less so than *S. uberis*. Fifty-two isolates from 5 farms (27 cows) generated 6 different DNA patterns, which were designated D1 to D6 (Figs 2, 3). Type D1 was the most common pulsotype, comprising 44% of all isolates and was represented on three farms. Pulsotypes D2 and D3 comprised 27 and 10% of isolates respectively and were each represented on 2 farms. The remaining pulsotypes were less prevalent and appeared to be confined to specific farms (Fig. 4). However, 12 cows with infections for periods of up to 5 months were continuously infected with the same pulsotype. There was only one example of two different pulsotypes being isolated from one cow (Table 2).

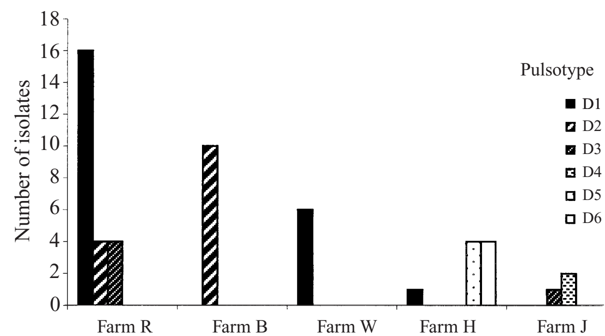

Fig. 3. Number of isolates of *S. dysgalactiae*, types D1–D6 from five farms.

Considering that other investigators have demonstrated a high degree of polymorphism among PFGE profiles of *S. dysgalactiae* [16], our study provides strong support for cow-to-cow transmission. Acquisition from a common environmental source or the presence of a limited number of clones within the species cannot, however, be ruled out. These results are similar to those of two recent studies, using PFGE and ribotyping respectively, in which similar pulsotypes were found both between and within herds [11, 15].

Some reports have stated that approx. 60% of streptococcal infections in dairy cattle are present for 30 days or less [2], but our findings indicate that at least some streptococcal infections persist in the same cow for periods of up to 5 months. While it could be argued that these represent multiple acquisitions from the environment, this interpretation seems unlikely, particularly for *S. uberis*, in view of the diversity of isolates found within herds. Mixed or new infections caused by different pulsotypes of *S. uberis* were also demonstrated. These findings are similar to those of Oliver and colleagues [13] who demonstrated both

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Table 2. *Patterns of new and persistent infections with* S. dysgalactiae *from farms R, B, W, H and J**

Cow	Pulsed field-electrophoresis (PFGE) type isolated from milk samples					
	19/8/97	9/9/97	22/9/97	14/10/97	25/11/97	6/1/98
R17		D1	D1			
R128	D1			D1	D1	D1
R230			D1		D1	D1
R276			D1	D1	D1	
R478				D2	D2	D2
R502		D3	D3	D1	D3	D3
	26/5/97	16/6/97	14/7/97	11/8/97	15/9/97	6/10/97
B532				D2		D2
B533	D2	D2	D2			
	26/5/97	9/6/97	16/6/97	1/7/97	14/7/97	28/7/97
W308		D1	D1	D1	D1	D1
	13/10/97	4/11/97	15/12/97	5/1/98		
H10			D2	D2		
H75	D5	D5	D5	D5		
	12/8/97	26/8/97				
J962	D4	D4				

* The pulsotype of the *S. dysgalactiae* isolates is represented by D, followed by the type number.

persistent and new infections with *S. dysgalactiae* and *S. uberis* using PCR-based DNA fingerprinting.

This study has not specifically addressed the possible existence of clones with enhanced virulence or transmissibility. The great diversity of *S. uberis* indicates that virulence is not associated with any specific molecular type. More work is required to determine whether a limited number of clones of *S. dysgalactiae* is specifically adapted to the bovine mammary gland and whether subspecies exist that are specific to different animal species.

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