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Genotypic and phenotypic analysis of *Streptococcus uberis* isolated from bovine mammary secretions

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SUMMARY

Genotypic and phenotypic analysis of 42 strains of *Streptococcus uberis* isolated from mammary secretions of 17 cows collected at different periods of the lactation cycle and from episodes of clinical mastitis were performed. Seventeen restriction endonuclease fingerprint (REF) patterns and 12 bacteriocin-like inhibitory substance (BLIS) fingerprints were observed. REF identified and differentiated closely related strains of *S. uberis* isolated from mammary secretions collected from the same cow at different periods of the lactation cycle and from episodes of clinical mastitis. BLIS fingerprinting of *S. uberis* complemented REF results. REF and BLIS fingerprinting provided evidence concerning persistence of infection in the same quarter or different quarters of the mammary gland over different periods of the lactation cycle, and occurrence of infection with similar and dissimilar strains of *S. uberis*. Biochemical profiles could not identify closely related strains nor did they complement REF results. Antibiotic resistance patterns alone were of little value in differentiating closely related strains, but were identical with isolates having same REF pattern. None of the *S. uberis* strains was found to carry plasmids. REF and BLIS fingerprinting can be utilized effectively to differentiate closely related and unrelated strains of *S. uberis* isolated from bovine mammary secretions.

INTRODUCTION

Widespread application of mastitis control measures have reduced the prevalence of *Streptococcus agalactiae* and *Staphylococcus aureus* intramammary infections. However, the proportion of intramammary infections caused by other *Streptococcus* species, *S. uberis* in particular, has increased markedly [1–4]. *Streptococcus uberis* is widely distributed over various sites of the cow and the environment [5], and it has been suggested that extramammary sources are responsible for the relative ineffectiveness of post-milking teat disinfection to control mastitis caused by *S. uberis*. This organism has also been isolated frequently from non-lactating mammary glands and from cases of clinical mastitis following parturition [2].

Epidemiologic studies of *S. uberis* have been limited by the lack of a convenient,

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reliable and reproducible method for comparing strains. This has resulted in lack of information concerning bacterial reservoirs responsible for new infections and hindered the understanding of cross infection as a means of transmission which could lead to better control of *S. uberis* intramammary infections [6].

Conventional typing methods based upon biochemical profiles, serotyping and antibiotic resistance patterns are inadequate for identifying closely related strains. Bacteriophage typing [7] and inhibitor typing [8], although promising, need further extensive study on their practical applicability for epidemiological studies of *S. uberis*. In recent years, restriction endonuclease fingerprinting (REF) of bacterial chromosomal DNA has been used successfully as a technique for subtyping organisms. This procedure provides information on the organization of the microbial genotype and has been used successfully to compare *S. mutans* [9], *S. pyogenes* [10], group B streptococci [11], *S. zooepidemicus* [12], *S. suis* [13], *Enterococcus faecalis* [14], group G streptococci [15] and *S. uberis* [6].

In this study, an attempt was made to identify a complement of tests which could be utilized for epidemiological studies on *S. uberis* isolated from dairy herds.

MATERIALS AND METHODS

Bacterial strains

A total of 42 *S. uberis* strains isolated from mammary secretions and from cases of clinical and subclinical mastitis from 17 cows in two University of Tennessee dairy research herds was used. Isolates were preserved in 10% skim milk and stored frozen at −70 °C. Isolates were subcultured from storage media onto 5% blood agar plates and maintained on Brain Heart Infusion agar slants.

Identification by biochemical profiles

Streptococcal isolates were identified to the species level by the Vitek Gram-Positive Identification System (GPI, Vitek Systems Inc., Hazelwood, MO, USA), API Rapid Strep System (Analytab Inc., Plainview, NY, USA) and conventional biochemical tests as described previously [16]. Organisms showing variable test reactions were re-evaluated to confirm atypical characteristics.

Serotyping

S. uberis isolates were serotyped using a streptococcal agglutination system (Streptex, Wellcome Diagnostics, Research Triangle Park, NC, USA) containing A, B, C, D, F, and G group-specific antibodies.

Resistance to antibiotics

Resistance or sensitivity to 10 antibiotics including penicillin, ampicillin, novobiocin, erythromycin, tetracycline, cephalothin, streptomycin, kanamycin, gentamicin and chloramphenicol was examined. Antibiotic impregnated disks (BBL Microbiology Systems, Cockeysville, MD, USA) were used in performing the standard diffusion assay as described by Bauer and colleagues [17].

Isolation of plasmid DNA

Isolation of plasmids was performed by techniques described by LeBlanc and Lee [18] and Anderson and McKay [19]. Both methods employed lysozyme

treatment of cells. Lysis was achieved with 1% sodium dodecyl sulphate (SDS) in Tris buffer, pH 8.0. Cellular proteins were precipitated with 1 M-NaCl followed by phenol extraction. The lysate was treated with RNAse (Sigma, St Louis, MO, USA) and DNA was precipitated with 95% ethanol. Plasmid DNA was detected by agarose gel electrophoresis [20].

Isolation of chromosomal DNA

Chromosomal DNA of *S. uberis* was isolated by the method described by Hill and Leigh [6] with modifications. All isolation steps were carried out in 1·5 ml Eppendorf tubes (Brinkmann Instruments Inc., Westbury, NY, USA). Cells from 1·5 ml of overnight culture grown in Brain Heart Infusion broth were washed once with 1.0 ml of Tris (10 mM) EDTA (5 mM), pH 7.8, and resuspended in 325 μl of the same buffer. After addition of 25 μl of mutanolysin (5000 units/ml, Sigma) and 25 μl of freshly prepared lysozyme (10 mg/ml, Sigma), bacteria were incubated at 37 °C for 30 min. Lysis of cells was achieved by addition of 20 μl of SDS (20% SDS w/v in Tris (50 mM), EDTA (20 mM), pH 7·8) followed by 3 μl of proteinase K (20 mg/ml, Sigma) and further incubated at 37 °C for 1 h. Protein was precipitated by the addition of 200 μl saturated NaCl (5 M) followed by agitation for 15 s and removed by centrifugation (7000 *g*) for 10 min. The pellet was discarded and the supernatant was subjected to phenol:chloroform (1:1) followed by chloroform: isoamyl-alcohol (24:1) extraction. DNA was precipitated from the supernatant with 2·5 volumes of 95% ethanol and 30 μl sodium acetate (1 M) overnight at −20 °C. The resulting precipitate was collected by centrifugation at 7000 *g* for 5 min followed by a 70% ethanol wash. The DNA pellet was dried under vacuum and rehydrated in 30 μl of buffer (Tris (10 mM), EDTA (1·0 mM), pH 7·5).

Enzymatic digestion of DNA

S. uberis DNA samples (5–8 μl) were digested for 3 h at 37 °C in 20 μl volumes containing restriction endonuclease *Hin*d III (Gibco BRL, Gaithersburg, MD, USA). Digestion of *S. uberis* DNA samples was repeated to establish reproducibility of the method. *Hin*d III-digested λ-DNA (Gibco BRL) was used as the molecular-weight marker.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in Tris acetate buffer (Tris acetate 0·04 M, EDTA 0·001 M) in 20 × 20 cm gels containing 1·0% agarose at 40 V for 16 h. Gels were stained with ethidium bromide (1·0 μg/ml) and DNA was visualized by u.v. transillumination (Fotodyne Inc., New Berlin, WI, USA) and photographed with type 55 Polaroid film.

Densitometric evaluation of REF

The negative of the Polaroid film was scanned using a computer integrated laser densitometer (Ultroscan XL, LKB Produkter AB, Bromma, Sweden). Scans were stored and retrieved for comparison and evaluation using Gelscan XL version 2.0 software package (Pharmacia, LKB Biotechnology, Uppsala, Sweden). Each of the 42 REF was compared to each other which resulted in identification of similar or dissimilar REF patterns. Confirmation of similarities and dissimilarities was

done by evaluating two REFs at one time. If differences in number of fragments and size (in kilobase pairs) were observed, then each REF was designated as a distinct pattern. Identical REFs were grouped into one pattern. Visual comparison of REF photographs was also undertaken to verify results obtained by densitometric scanning.

BLIS fingerprinting

Bacteriocin-like inhibitory substance (BLIS) fingerprinting was performed based upon the scheme devised originally by Tagg and Bannister [8] for haemolytic streptococci. The typing procedure involved testing a strain for BLIS production (P-type) and BLIS sensitivity (S-type). The combined P- and S-type designations represented the BLIS fingerprint of the strain. Columbia agar base (Gibco Ltd, Paisley, Scotland) containing 5% (v/v) human blood plus 0·5% calcium carbonate was used as the test medium. For P-typing, the test strain was grown as a 1 cm wide diametric streak culture for 18 h at 37 °C in an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2). Following incubation, growth was removed with a glass slide. The agar surface was sterilized with chloroform and nine indicator strains from 18 h Todd Hewitt broth cultures were inoculated across the line of the original test culture. After a further 18 h period of aerobic incubation at 37 °C, presence of strong inhibition of growth of the indicator streaks was recorded and results expressed in code form. This was done by considering the nine indicators as triplets (e.g., In1, In2, In3; In4, In5, In6; In7, In8, In9) and by scoring inhibition of an indicator as either 4, 2, or 1 depending on whether the indicator was the first, second, or third member of the triplet. Negative reactions were scored zero. Total scores for each triplet were written as a sequence of three numbers which represented the P-type of the test strain.

For S-typing, six BLIS-producing strains (Pr1–Pr6) were grown, sterilized and cross-inoculated with test organisms. After reincubation, inhibition patterns of test organisms were recorded as described for P-typing. The overall BLIS fingerprint was expressed as P-type/S-type, the first set of values indicative of the P-type and the second set of values indicative of the S-type. Details of the indicator and producer strains used for P- and S-typing, respectively, are presented in Table 1.

RESULTS

The Vitek Gram-Positive Identification System and the API Rapid Strep System identified correctly all 42 strains of *S. uberis*. This was confirmed by conventional biochemical tests. Twenty-seven of 42 (64·3%) strains possessed biochemical profiles characteristic to that of *S. uberis*. Atypical test reactions were observed in 15 strains of *S. uberis* for hydrolysis of hippurate (7%), arginine (7%), utilization of lactose (4·8%), inulin (4·8%), raffinose (4·8%) and ribose (9·5%). Four of 42 (9·5%) *S. uberis* strains belonged to serotype group G, while the remainder were ungroupable by the streptococcal agglutination system utilized in this study. Biochemical profiles did not complement results obtained by other typing methods (Tables 2–4).

None of the 42 strains of *S. uberis* was found to carry plasmids by either of the two plasmid isolation techniques employed. Presence of intense bands in REF patterns which are indicative of plasmids were also not observed.

Table 1. *Indicator and producer strains used in BLIS fingerprinting*

	Strain specification	Source
	Indicator (P-typing) strains	
In1	*S. uberis* (ATCC 27958)	McDonald (Tagg and Vugler [28])
In2	*S. uberis* D618	
In3	*S. uberis* D531	
In4	*S. uberis* 2	Jayarao and Oliver (present study)
In5	*S. uberis* 9	
In6	*S. uberis* 29	
In7	*S. uberis* 4	
In8	*S. uberis* D528	McDonald (Tagg and Vugler [28])
In9	group N streptococcus T-21	Tagg and Bannister [8]
	Producer (S-typing) strains	
Pr-1	*S. uberis* (ATCC 27958)	McDonald (Tagg and Vugler [28])
Pr-2	*S. uberis* 5	Jayarao and Oliver (present study)
Pr-3	*S. uberis* 12	
Pr-4	*S. uberis* 29	
Pr-5	*S. uberis* D528	McDonald (Tagg and Vugler [28])
Pr-6	*S. anginosus* T-29	Tagg and Bannister [8]

Table 2. S. uberis *strains with similar genotypic and variable phenotypic characteristics isolated from mammary secretions of seven cows*

Cow no.	Date of sampling	Q*	S†	BLIS fingerprint‡	Anti-biogram§	REF pattern‖	Biochemical profile¶
L380	12 Feb. 90	rf	d-0	000/77	Sm	p9	A
	19 Feb. 90	rf	d-0	000/77	Sm	p9	A
L988	27 Nov. 89	rf	d-7	001/77	Sm	p7	A/inu−
	4 Dec. 89	lf	d-0	001/77	Sm	p7	A
L792	25 Oct. 88	rf	c-7	000/77	Sm	p8	A
	25 Oct. 88	lf	c-7	000/77	Sm	p8	A
L114	11 Jan. 90	lr	c-7	000/77	Sm	p10	A/hip−
	29 Jan. 90	lr	c+10	000/77	Sm	p10	A/arg−
L451	2 Oct. 89	rf	c-7	024/34	Sm Km Gm	p6	A
	2 Oct. 89	rr	c-7	024/34	Sm Km Gm	p6	A
L100	18 Dec. 89	rr	d-0	733/52	Sm	p2	A/inu−
	11 Feb. 90	rf	c-7	733/52	Sm	p2	A
L315	1 Feb. 90	lf	d-0	001/77	Sm	p8	A
	5 Apr. 90	rf	c-0	001/77	Sm	p8	A

* Q: relative position of the quarter of the mammary gland; r = right, l = left; f = front, r = rear.

† S: sample – days relative to c = calving or d = drying off; hs = herd survey; MAS = mastitis.

‡ BLIS fingerprint: values indicate P-type/S-type.

§ Antibiogram: resistance to Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Tc, tetracycline; Er, erythromycin.

‖ REF pattern: restriction endonuclease fingerprint pattern.

¶ Biochemical profile. A: positive reactions for acetyl methyl carbinol, hippurate, esculin, arginine, optochin, dextrose, ribose, mannitol, sucrose, trehalose, lactose, salicin, sorbitol, inulin, cellobiose and 10% bile. Negative reactions for arabinose, xylose, raffinose 6·5% NaCl and 40% bile.

A/: same as A except negative (−) or positive reaction (+) for rib, ribose; arg, arginine; lac, lactose; hip, hippurate; raf, raffinose; and inu, inulin.

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Table 3. S. uberis *strains with different genotypic and phenotypic characteristics isolated from mammary secretions of six cows*

Cow no.	Date of sampling	Q*	S†	BLIS fingerprint‡	Anti-biogram§	REF pattern‖	Biochemical profile¶
L561	31 Oct. 88	rf	c-10	777/42	Sm Km	p2	A/rib−
	31 Oct. 88	lr	c-10	457/77	Sm Km	p13	A/raf+
L139	19 Feb. 90	rr	c-7	001/77	Sm	p8	A/rib−
	19 Feb. 90	lf	c-7	273/77	Sm	p11	A
	19 Feb. 90	rf	c-7	253/76	Sm	p11	A
L294	2 Sep. 88	rr	d-7	733/52	Sm Km	p1	A/rib−, hip−
	1 Feb. 90	lr	d-21	677/35	Sm Km	p10	A
L526	29 Jan. 86	rr	d-35	413/77	Sm Km Gm Tc	p3	A
	23 Feb. 90	rr	c-7	723/52	Sm Km	p14	A/lac−
	23 Feb. 90	lr	c-7	664/75	Sm Km	p15	A
L829	2 Apr. 86	lr	d-0	723/72	Sm Km	p16	A
	25 July 89	rr	hs	001/77	Sm Tc	p5	A/arg−
	27 July 89	rr	MAS	000/77	Sm Tc	p5	A/arg−
K266	2 Sep. 85	rf	d-7	723/42	Sm Km Gm Tc	p5	A
	2 Sep. 85	lf	d-7	733/42	Sm Km Gm Tc Er	p4	A
	2 Sep. 85	lf	d-0	733/42	Sm Km Gm Tc Er	p4	A

Footnotes as in Table 2.

Table 4. *Genotypic and phenotypic characteristics of* S. uberis *isolated from cows with clinical mastitis and different periods of the lactation cycle*

Cow no.	Date of sampling	Q*	S†	BLIS fingerprint‡	Anti-biogram§	REF pattern‖	Biochemical profile¶
L905	27 Sep. 88	lf	hs	000/77	Sm	p8	A
	17 Oct. 88	rr	d-0	000/77	Sm	p8	A
	13 Mar. 90	lr	MAS	723/52	Sm	p12	A
L124	14 Aug. 89	rr	d-7	000/76	Sm	p10	A
	14 Aug. 89	rf	d-7	001/77	Sm	p10	A/rib−
	21 Aug. 89	rr	d-0	001/77	Sm Km	p2	A/hip−
	18 Dec. 89	lr	MAS	000/77	Sm	p10	A
L188	22 Nov. 89	rf	MAS	000/42	Sm	p17	A
	17 Mar. 90	lf	MAS	001/77	Sm	p10	A/raf+
	20 Mar. 90	lf	MAS	001/77	Sm	p10	A
L16	27 Sep. 88	rf	hs	723/52	Sm	p4	A/lac−
	6 Oct. 88	rr	MAS	000/77	Sm	p9	A

Footnotes as in Table 2.

Densitometric evaluation of REF patterns of *S. uberis* showed that fragment length of the discriminating region was between 9 and 23 kb. Fragments smaller than 5 kb showed a greater degree of homology between strains (Figs. 1–3). The 42 strains of *S. uberis* isolated from mammary secretions and from episodes of clinical mastitis from 17 cows belonged to 17 distinct REF patterns. Subtyping of *S. uberis* was possible based upon strains having similar and dissimilar REF patterns (Tables 2–4). Paired isolates from seven cows had similar REF patterns (L380 – p9.p9; L988 – p7.p7; L792 – p8.p8; L114 – p10.p10; L451 – p6.p6; L100

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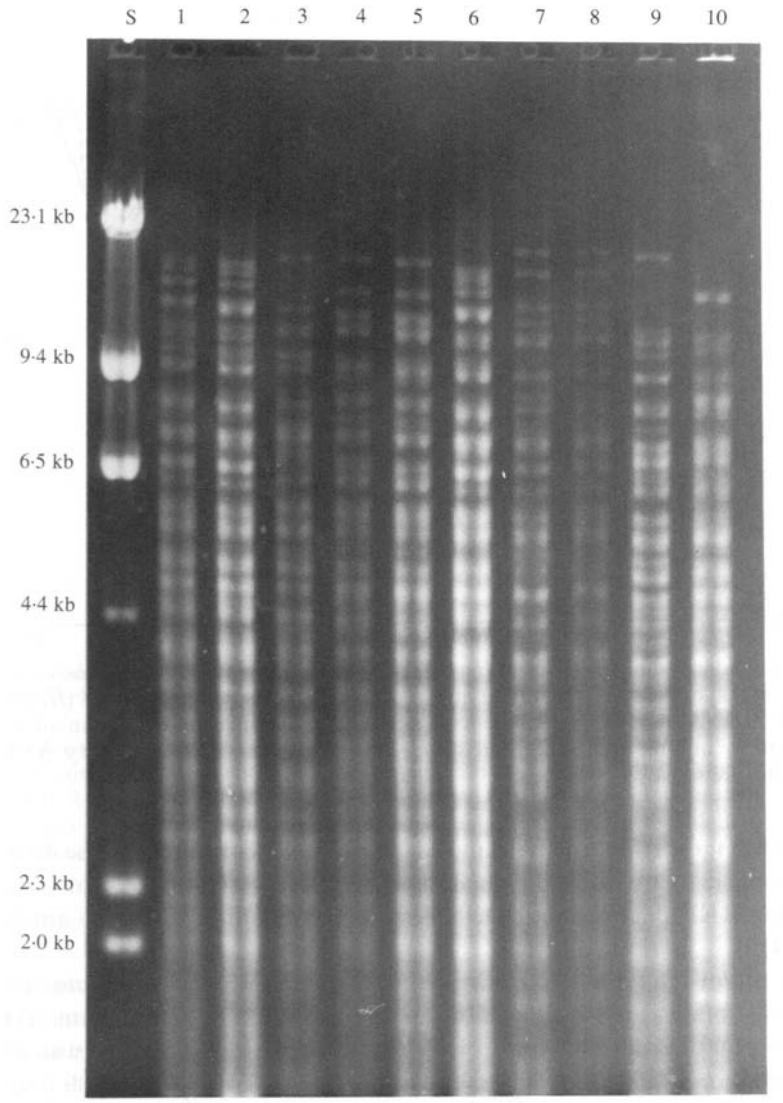

Fig. 1. *Hin*d III digests of 10 *S. uberis* isolates followed by electrophoresis on 1% agarose gel. Lanes 1 and 2 are isolates a and b from cow 792 (p8,p8); lanes 3–5 are isolates a, b, c from cow 266 (p5,p4,p4); lanes 6–8 are isolates a, b, c from cow 139 (p8,p11,p11) and lanes 9 and 10 are isolates a and b from cow 561 (p2,p13). Lane S is *Hin*d III-digested λ-DNA.

– p2,p2; L315 – p8,p8; Table 2). Paired isolates from three cows (K561 – p2,p13; L294 – p1,p10; L16 – p4,p9) had dissimilar REF patterns (Tables 3 and 4). In 5 of 6 cows from which 3 isolates from each cow were examined, 2 of the 3 isolates had similar REF patterns (K266 – p5,p4,p4; L139 – p8,p11,p11; L829 – p16,p5,p5; L905 – p8,p8,p12), while in one cow (L526 – p3,p14,p15) all three isolates had different REF patterns (Tables 3 and 4). Three of four isolates from cow L124 had similar REF patterns (p2,p10,p10,p10; Table 4).

The 42 strains of *S. uberis* belonged to 12 BLIS fingerprint patterns. On most occasions, isolates from the same cow which had identical REF patterns also had

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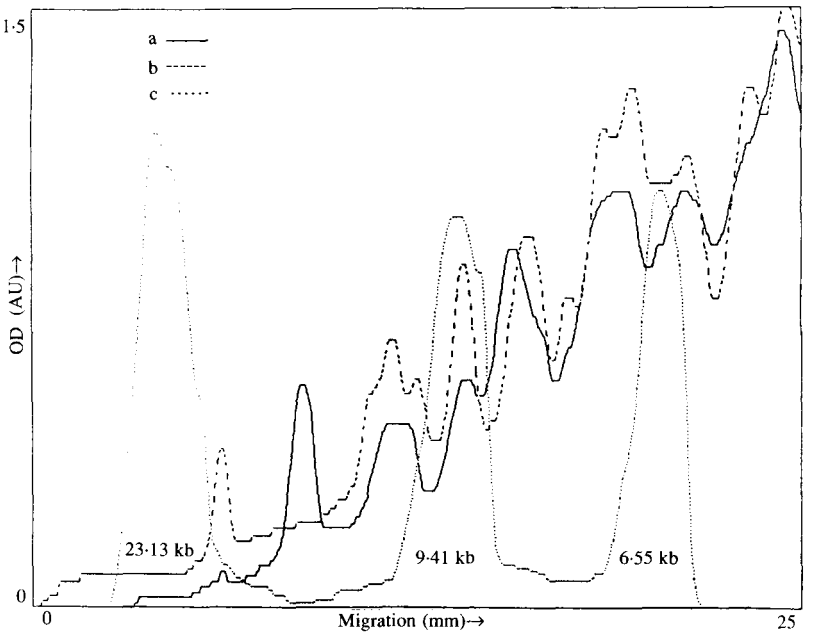

Fig. 2. Laser densitometric scans of *Hind* III-digested genomic DNA of *S. uberis*. Scans a and b correspond to lanes 9 and 10 in Fig. 1. Scan c corresponds to lane S (*Hind* III-digested λ-DNA) in Fig. 1. The *x*-axis shows the distance of migration of DNA fragments in millimetres (mm) and the *y*-axis shows the optical density (OD) in absorbance units (AU).

similar BLIS fingerprints for the same set of isolates (Tables 2–4). The 42 *S. uberis* isolates belonged to six different antibiograms. Isolates from the same cow which had a similar REF pattern and BLIS fingerprint also had the same antibiogram (Tables 2–4).

With the aid of REF and BLIS fingerprinting, preliminary inferences from this study were as follows: (1) Isolation of similar strains of *S. uberis* (same REF and BLIS fingerprint) in different quarters of the same mammary gland was observed in mammary secretions collected on the same day (L380, L792, L139 and L451). (2) Similar *S. uberis* strains were detected in the same quarter of a mammary gland from samples collected over a period of time (L988, L114, K266). (3) Similar *S. uberis* strains were detected in different quarters of the same mammary gland from samples collected over a period of time (L100, L315). (4) Dissimilar strains of *S. uberis* were isolated from different quarters of the same mammary gland on the same day (K266, L561, L139, L526). (5) Dissimilar strains of *S. uberis* were isolated in the same quarter of the mammary gland over a period of time (L526).

Three of six strains of *S. uberis* isolated from episodes of clinical mastitis belonged to REF pattern p10 (BLIS fingerprint type 001/77, 001/77, 000/77) and one isolate each to p5 (000/77), p12 (723/52), and p9 (000/77, Table 4). Isolates with similar REF patterns and BLIS fingerprints were also isolated from mammary secretions collected during the nonlactating and calving periods. This was observed in cow L114 (p10 – 000/77) and cow L380 (p9 – 000/77) (Tables 2 and 4).

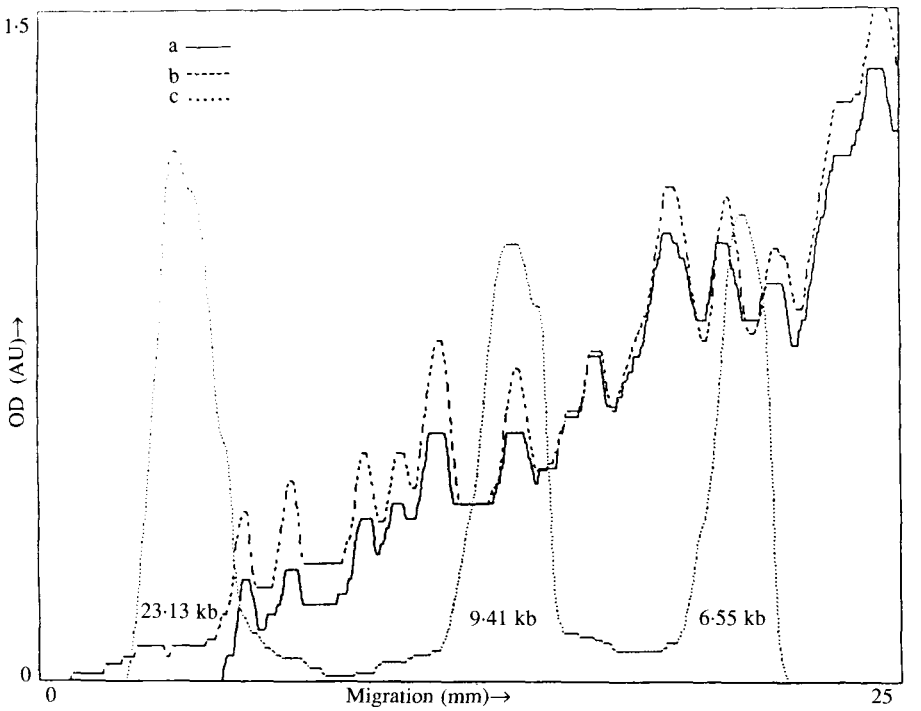

Fig. 3. Laser densitometric scans of *Hin*d III-digested genomic DNA of *S. uberis*. Scans a and b correspond to lanes 7 and 8 of Fig. 1. Scan c corresponds to lane S (*Hin*d III-digested λ-DNA) in Fig. 1. The *x*-axis shows the distance of migration of DNA fragments in millimetres (mm) and the *y*-axis shows the optical density (OD) in absorbance units (AU).

DISCUSSION

In the present study, REFs of *S. uberis* isolated from bovine mammary secretions and typing methods including BLIS fingerprinting, plasmid profile analysis, antibiotic resistance patterns, serotyping and biochemical profiling were performed. Application of these typing systems was undertaken with the objective of identifying a suitable typing system and other complementary tests which could be utilized in epidemiological investigations of *S. uberis*.

The Vitek Gram-Positive Identification System is an automated technique for identification of Gram-positive bacteria. The API Rapid Strep System is comprised of a set of enzyme profiles and other biochemical tests. Both systems identify bacteria based upon the profile index generated from test reactions. The profile index consists of a set of numbers specific for each biochemical profile. Both rapid identification systems were examined in this study with the purpose of utilizing the profile index numbers generated to aid in subtyping *S. uberis* based upon biochemical profiles. The profile index numbers generated by both systems proved inadequate for subtyping or even to complement REF or BLIS fingerprint results. Biochemical reactions that were atypical as observed by conventional biochemical tests are presented in this study. A similar observation was made by Skjold and co-workers [12] on the use of API Rapid Strep system for subtyping

of *S. zooepidemicus*. They [12] concluded that the API Rapid Strep system could not differentiate between strains that were clearly distinct based upon DNA fingerprint results.

Absence of plasmids in all strains of *S. uberis* evaluated indicated that plasmid profile analysis cannot be utilized for typing *S. uberis*. Similar observations were made for *S. uberis* [6], and for B group streptococci [11].

Inability to serotype *S. uberis* has been reported [21–23]. Serology was of little assistance in classifying *S. uberis* since strains fell into at least six serological groups, all of which include a variety of other *Streptococcus* species [24]. Facklam [25] reported that serotyping of 'viridans' streptococci was generally unsatisfactory which agrees with observations of the present study.

REF has been used successfully to compare strains of streptococcal species [9–13] including *S. uberis* [6]. In the present study, chromosomal DNA of *S. uberis* was digested with restriction endonuclease *Hin*d III and fragments produced were separated by gel electrophoresis. Strains were characterized on the basis of fragment patterns with the aid of a scanning laser densitometer. Restriction patterns of *S. uberis* showed that fragment length of the discriminating region was between 9 and 23 kb. Fragments smaller than 5·0 kb showed a greater degree of homology between strains. Similar observations were made by Hill and Leigh [6]. Similarity or dissimilarity between DNA fingerprints was the basis by which strains were differentiated. REF was easy to perform, reproducible and proved to be a reliable method for subtyping of *S. uberis*. The 42 *S. uberis* strains examined belonged to 17 subtypes which suggests the prevalence of a large variety of *S. uberis* strains on the two dairy herds. Similar observations were made with *S. zooepidemicus* [12], *S. suis* [13] and B group streptococci [11].

Inhibitor typing, now referred to as bacteriocin-like inhibitory substance or BLIS fingerprinting, has been utilized for differentiating strains of *S. agalactiae* [26], *S. dysgalactiae* [27] and *S. uberis* [28, 29] isolated from cows with mastitis. The first report on the inhibitor typing scheme for *S. uberis* [28] indicated that the scheme possessed considerable potential for typing *S. uberis* isolates and could aid in epidemiological investigations. While inhibitor typing showed considerable potential to differentiate isolates, its application for epidemiological studies has not been used extensively, probably because results could not be confirmed by any other complementary typing system. In the present study, a modified and improved version of this typing system was examined. The 42 *S. uberis* isolates examined belonged to 12 different BLIS fingerprints. Prevalence of a large number of BLIS fingerprints suggests diversity amongst *S. uberis* strains. A similar observation was made by Buddle, Tagg and Ralston [29] who reported the prevalence of 18 inhibitor profiles amongst 38 isolates obtained from dairy cows in New Zealand. Interestingly, eight of ten *S. uberis* strains from episodes of clinical mastitis had different inhibitor profiles.

Garvie and Bramley [24] suggested the existence of two genotypes of *S. uberis*, designated as type I and type II, based on DNA–DNA hybridization techniques. Conclusive evidence for the existence of two genotypes of *S. uberis* was reported recently by Williams and Collins [31] based on differences in *S. uberis* type I and type II 16S ribosomal RNA primary sequences. Based on this difference, *S. uberis* type II has been proposed as a new species designated *S. parauberis* [31]. Although

the two species are genotypically different, differentiation of *S. uberis* from *S. parauberis* based on biochemical, physiological and serological characteristics has not been defined and phenotypically both species are apparently very similar.

Currently, differentiation of *S. parauberis* from *S. uberis* can only be accomplished by use of DNA specific probes to identify variability in the V2 region which accounts for genotypic differences or by performing 16S ribosomal RNA sequencing. Thus, at this time we cannot exclude the possibility that *S. parauberis* could have accounted for some of the different REF and BLIS fingerprint patterns observed in this study. Until a reliable scheme for differentiating *S. parauberis* from *S. uberis* is available, isolates that are actually *S. parauberis* will likely be described as *S. uberis*.

There was a close association between REF and BLIS fingerprints which confirms the specificity of BLIS fingerprints. Strains with identical REF patterns and BLIS fingerprint types also had identical antibiotic resistance patterns. This strengthens the degree of interpretation as to ascribing strains to be of the same clonal type. A similar observation was made with antibiotic resistant *Legionella* species [30].

REF and BLIS fingerprinting could precisely identify a subtype. This when interpreted in an epidemiological setting provided the following preliminary inferences: (1) a particular subtype was prevalent in one or more quarters of a mammary gland; (2) a particular subtype persisted in the same quarter or different quarters of a mammary gland when examined over a period of time, and (3) *S. uberis* subtypes isolated from episodes of clinical mastitis were also found in mammary secretions of other cows collected during the nonlactating and calving periods.

At this stage, preliminary results using REF and BLIS fingerprinting are encouraging. A large heterogeneous group of *S. uberis* strains were observed on the two dairy herds. Examination of *S. uberis* isolates from extramammary sources on the dairy farm could provide a more detailed epidemiological picture. Use of a computer-integrated scanning laser densitometer proved to be an effective method to store, retrieve, compare and evaluate REF patterns. Although the rapid identification systems did not complement REF and BLIS fingerprint results, these systems can be utilized to identify *S. uberis* isolates. Examination of antibiotic resistance patterns would aid in strengthening REF and BLIS fingerprinting results. Identification of *S. uberis* by either of the rapid identification systems, tests for antibiotic resistance, REF and BLIS fingerprinting of *S. uberis* strains could be accomplished within 72–96 h.

Results of the present study suggest that REF and BLIS fingerprinting can be utilized effectively in identification of *S. uberis* subtypes, and can also be used to draw epidemiological inferences. We recommend use of these techniques to study the epidemiology and diversity of *S. uberis*.

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REFERENCES

1. Oliver SP, Mitchell BA. Prevalence of mastitis pathogens in herds participating in a mastitis control program. J Dairy Sci 1984; **67**: 2436–40.
2. Oliver SP. Frequency of isolation of environmental mastitis causing pathogens and incidence of new intramammary infection during the nonlactating period. Am J Vet Res 1988; **49**: 1789–93.
3. Oliver SP, Sordillo LM. Udder health in the periparturient period. J Dairy Sci 1988; **71**: 2584–606.
4. Smith KL, Todhunter DA, Schoenberger PS. Environmental pathogens and intramammary infection during the dry period. J Dairy Sci 1985; **68**: 402–17.
5. Bramley AJ, King JS, Higgs TM. The isolation of *Streptococcus uberis* from cows in two dairy herds. Br Vet J 1979; **135**: 262–70.
6. Hill AW, Leigh JA. DNA fingerprinting of *Streptococcus uberis*: a useful tool for epidemiology of bovine mastitis. Epidemiol Infect 1989; **103**: 165–71.
7. Hill AW, Brady CA. A note on the isolation and propagation of lytic phages from *Streptococcus uberis* and their potential for strain typing. J Appl Bact 1989; **67**: 425–31.
8. Tagg JR, Bannister LV. Fingerprinting of β hemolytic streptococci by their production of and sensitivity to bacteriocin like inhibitors. J Med Microbiol 1979; **12**: 397–411.
9. Caufield PW, Walker TM. Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. J Clin Microbiol 1989; **27**: 274–8.
10. Cleary PP, Kalpan EL, Livdhal C, Skjold S. DNA fingerprints of *Streptococcus pyogenes* are M type specific. J Infect Dis 1988; **158**: 1317–23.
11. Denning DW, Baker CJ, Troup N, Tompkins LS. Restriction endonuclease analysis of human and bovine group B streptococci for epidemiologic study. J Clin Microbiol 1989; **27**: 1352–6.
12. Skjold SA, Quie PG, Fries LA, Barnham M, Cleary PP. DNA fingerprinting of *Streptococcus zooepidemicus* (Lancefield Group C) as an aid to epidemiological study. J Infect Dis 1987; **155**: 1145–50.
13. Mogollon JD, Pijoan C, Murtaugh MP, Kalpan EL, Collins, JE, Cleary PP. Characterization of prototype and clinically defined strains of *Streptococcus suis* by genomic fingerprinting. J Clin Microbiol 1990; **28**: 2462–6.
14. Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J Clin Microbiol 1990; **28**: 2059–63.
15. Martin NJ, Kalpan EL, Gerber MA, et al. Comparison of epidemic and endemic group G streptococci by restriction enzyme analysis. J Clin Microbiol 1990; **28**: 1881–6.
16. Jayarao BM, Oliver SP, Matthews KR, King SH. Comparative evaluation of Vitek Gram-Positive Identification and API Rapid Strep Systems for identification of *Streptococcus* species of bovine origin. Vet Microbiol 1991; **26**: 301–8.
17. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility by standardized single disk method. Am J Clin Pathol 1966; **45**: 493–6.
18. LeBlanc DJ, Lee LN. Rapid screening procedure for detection of plasmids of streptococci. J Bacteriol 1979; **140**: 1112–15.
19. Anderson DG, McKay LL. Simple and rapid method for isolating plasmid DNA from lactic streptococci. Appl Environ Microbiol 1983; **46**: 549–52.
20. Meyers JA, Sanchez D, Elwell LP, Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 1976; **127**: 1529–37.
21. Stableforth AW. Streptococcal diseases of animals. In: Stableforth AW, Galloway IE, eds. Diseases due to bacteria, vol. 2. London: Butterworths Scientific Publications, 1959: 589–650.

22. Roguinsky M. Reaction de *Streptococcus uberis* avec les serums G et P. Annales de I'nstitut Pasteur 1969; **117**: 529–32.
23. Roguinsky M. Comparaison de *Streptococcus uberis* et de Streptocoques voisins. Ouest Med 1972; **25**: 983–5.
24. Garvie EI, Bramley AJ. *Streptococcus uberis*: an approach to its classification. J Appl Bacteriol 1979; **46**: 295–304.
25. Facklam RR. Physiological differentiation of viridans streptococci. J Clin Microbiol 1977; **5**: 184–201.
26. Tagg JR, Martin DR. Bacteriocin 'fingerprinting' of group B *Streptococcus* strains of bovine and human origin. In: Proceedings of the University of Otago Medical School. 1980; **58**: 22–3.
27. Schofield CR, Tagg JR. Bacteriocin-like activity of group B and C streptococci of human and of animal origin. J Hyg 1983; **90**: 7–18.
28. Tagg JR, Vugler LG. An inhibitor typing scheme for *Streptococcus uberis*. *J Dairy Res* 1986; **53**: 451–6.
29. Buddle BM, Tagg JR, Ralston MJ. Use of inhibitor typing scheme to study the epidemiology of *Streptococcus uberis* mastitis. NZ Vet J 1988; **36**: 115–19.
30. Tompkins LS, Troup NJ, Woods T, Bibb W, McKinney RM. Molecular epidemiology of *Legionella* species by restriction endonuclease and alloenzyme analysis. J Clin Microbiol 1987; **25**: 1875–80.
31. Williams AM, Collins MD. Molecular taxonomic studies on *Streptococcus uberis* types I and II. Description of *Streptococcus parauberis* sp. nov. J Appl Bacteriol 1990; **68**: 485–90.