ABSTRACT

Fifty plasmid-free strains of *Streptococcus uberis*, including 40 strains isolated from cows in two dairy herds in Tennessee with subclinical and clinical mastitis, 9 strains isolated from cows in the US, and 1 strain from Great Britain were examined for genomic DNA restriction fragment length polymorphisms. The 50 strains belonged to 35 DNA fingerprint patterns. Within a herd, fingerprint patterns from isolates obtained from cows with subclinical mastitis at different periods of the lactation cycle and from episodes of clinical mastitis were similar. Restriction fragment length polymorphism analysis revealed the presence of DNA fragments ≥21 kb in all 12 strains of *S. uberis* isolated from episodes of clinical mastitis and in only 8 of the 38 (21%) remaining strains. Cluster analysis placed the 50 strains into eight distinct clusters. Most strains from the northeastern US were confined to one cluster, whereas most of the isolates from episodes of clinical mastitis belonged to two different clusters. These data suggest that clonal diversity exists among strains of *S. uberis* isolated from bovine mammary secretions.

(Key words: bovine mastitis, clonal diversity, genomic deoxyribonucleic acid fingerprinting, *Streptococcus uberis*)

Abbreviation key: REF = restriction endonuclease fingerprints, RFLP = restriction fragment length polymorphism.

INTRODUCTION

Mastitis prevention measures, such as postmilking teat disinfection and dry cow antibiotic therapy, have had little influence in reducing the incidence of *Streptococcus uberis* IMI (14, 15). Over the last decade, the incidence of IMI caused by *S. uberis* in the US and in Great Britain has increased (1, 3, 9, 12, 13, 22).

Early attempts (16, 17, 21) to study the epidemiology of *S. uberis* were hampered because of the lack of suitable typing methods. Recently, application of restriction endonuclease fingerprinting (REF) (4, 8) and DNA amplification fingerprinting (5) techniques were used to draw epidemiological inferences concerning *S. uberis* IMI. Studies using REF (8) for subtyping of *S. uberis* revealed the occurrence of several clonal types within a herd. However, conclusive evidence could not be obtained; clonal diversity could have been due to strains of *Streptococcus parauberis* that could not be identified at the time of the study (8) because no suitable technique was available to differentiate between the two species. Differentiation of *S. uberis* from the newly described species *S. parauberis* (23) is now possible by restriction fragment length polymorphism (RFLP) analysis of 16S rDNA (6). In the present study, only strains of *S. uberis* identified by 16S rDNA analysis were examined for restriction fragments generated by digestion of genomic DNA with restriction endonuclease HindIII. Restriction fragments were analyzed to determine the diversity of *S. uberis* isolated from bovine mammary secretions.

MATERIALS AND METHODS

Bacteria

Fifty plasmid-free strains of *S. uberis* were evaluated. All strains were preserved in 10% skim milk and stored at -70°C. Strains were
subcultured from storage media onto 5% sheep blood agar plates and then maintained on brain-heart infusion agar slants (Difco Laboratories, Detroit, MI). The strains were examined for plasmids and RFLP for determination of type I strains as described previously (6, 8). Forty strains were isolated from subclinical and clinical mastitis from 40 cows on two of the University of Tennessee dairy research herds. One strain, NIRD 0140J from Great Britain, was provided by A. W. Hill at the Agricultural and Food Research Council, Institute for Animal Health, Compton, England. Nine strains isolated from different herds in the US were also evaluated. One strain from a herd in Vermont was provided by J. W. Pankey at the University of Vermont, 4 strains from a herd in New York by R. N. Gonzalez at Cornell University, 3 strains from a herd in Michigan by R. D. Walker at Michigan State University, and 1 strain was obtained from the University of Massachusetts.

All strains were identified by species by conventional biochemical tests, the Vitek Gram-Positive Identification System (Vitek Systems Inc., Hazelwood, MO) and API Rapid Strep System (Analytab Inc., Plainview, NY), and were serotyped as described previously (7).

Isolation of Chromosomal DNA

Chromosomal DNA of S. uberis was isolated by the method of Hill and Leigh (4) with modifications (8). All isolation steps were carried out in 1.5-ml Eppendorf tubes (Brinkmann Instruments Inc., Westbury, NY). Cells from 1.5 ml of overnight culture grown in brain-heart infusion broth (Difco) were washed once with 1.0 ml of Tris (10 mM) and EDTA (5 mM), pH 7.8, and then resuspended in 325 µl of the same buffer. Mutanolysin (25 µl, 5000 units/ml; Sigma Chemical Co., St Louis, MO) and freshly prepared lysozyme (25 µl, 10 mg/ml; Sigma) were added, and the mixture was incubated at 37°C for 30 min. Lysis of cells was achieved by addition of 20 µl of SDS (20% w/v in Tris (50 mM) and 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 addition of 200 µl of saturated NaCl (5 M), followed by agitation for 15 s, and removed by centrifugation (7000 × g at 4°C for 10 min). The pellet was discarded, and the supernatant was subjected to phenol:chloroform (1:1, vol/vol) followed by two chloroform:isoamylalcohol (24:1, vol/vol) extractions. The DNA was precipitated from the supernatant with 2.5 volumes of 95% ethanol and 30 µl of sodium acetate (1 M) overnight at −20°C. The resulting precipitate was collected by centrifugation (7000 × g at 4°C for 10 min), followed by 70% ethanol wash. The DNA pellet was dried under vacuum and rehydrated in 30 µl of buffer [Tris (10 mM) and EDTA (1.0 mM), pH 7.5].

Enzymatic Digestion of DNA

The DNA of S. uberis was quantified using a spectrophotometer (Spectronic 2000; Bausch and Lomb, Rochester, NY) at 260 nm. Five to eight microliters of chromosomal DNA equivalent to 3 µg were digested for 3 h at 37°C in 20-µl volumes containing restriction endonuclease HindIII [Bethesda Research Laboratories (BRL), Gaithersburg, MD]. The HindIII was used because DNA fragments were more clearly defined with this enzyme than with other restriction endonucleases (4). Digestion of S. uberis DNA samples was repeated to establish reproducibility. HindIII-digested λ DNA (BRL) was used as the molecular weight marker.

Agarose Gel Electrophoresis

The chromosomal DNA digested by HindIII was electrophoresed on 1% agarose gel in Tris acetate buffer (Tris acetate, 40 mM, and EDTA 1 mM) in 20 × 20-cm gels at 40 V for 16 h. Gels were stained with ethidium bromide (1 µg/ml), and DNA was visualized by UV transillumination (Fotodyne Inc., New Berlin, WI) and photographed with type 55 Polaroid film (Polaroid Corp., Cambridge, MA).

RFLP Analysis

The negative of the Polaroid film was scanned using a computer-integrated laser densitometer (Ultrascan XL; LKB Produkter AB, Bromma, Sweden). Scans were evaluated and compared using Gelscan XL version 2.0 software package (Pharamcia, LKB Biotechnology, Uppsala, Sweden). The number and size
of the DNA fragments in kilobase pairs relative to standard DNA molecular weight markers were determined. The 50 REF were compared with one another to identify similar and dissimilar REF patterns. When number of fragments and size (in kilobase pairs) differed,

Figure 1. HindIII digests of chromosomal DNA of four strains of Streptococcus uberis followed by electrophoresis on 1% agarose gel. All strains were isolated from one Tennessee herd. Lanes 1 and 2, strains 35 (p25) and 36 (p25), isolated from 2 dairy cows. Lane 3, strain 33 (p21), isolated from an episode of clinical mastitis. Lane 4, strain 34 (p22), isolated from an episode of clinical mastitis. Lane 5, HindIII-digested λ DNA.
each REF was designated as a distinct pattern. Identical REFS were grouped into one pattern. The similarity coefficient of Jaccard (20) was calculated between each pair of samples from REF phenotypes as

\[ S_j = \frac{n_{xy}}{n_x + n_y} \]

where \( n_{xy} \) is the number of restriction fragments shared between samples, and \( n_x \) and \( n_y \) are the total number of fragments observed in samples x and y, respectively. Unweighted pair group method (20) was used for phenetic analysis. Computations were performed using the NUMTAX program (18) at the University of Tennessee Computing Center.

RESULTS

Genomic DNA of S. uberis digested by HindIII was examined for DNA REF patterns and RFLP. Digestion of genomic DNA with HindIII produced DNA fragments ranging from 2 to 23 kb. Fragments <9 kb showed greater similarity among strains (Figures 1 and 2). The number of DNA fragments between 8 and 23 kb ranged from 6 to 16. The 50 strains of S. uberis belonged to 35 different DNA REF patterns (p1 to p35; Figure 3). The 40 strains from 40 cows from two dairy herds in Tennessee belonged to 28 DNA REF patterns. Eleven strains isolated from cows in one herd belonged to 7 DNA REF patterns. The remaining 29 strains isolated from cows in the second herd belonged to 21 DNA REF patterns (Figure 3). Densitometric evaluation of restriction fragments of the 40 strains isolated from cows in two dairy herds in Tennessee showed that all strains from episodes of clinical mastitis had restriction fragments ≥21 kb, whereas only 6 of 28 strains isolated from cows with subclinical mastitis at different periods of the lactation cycle had restriction fragments ≥21 kb.

Nine strains isolated from other dairy herds in the US and 1 strain from Great Britain belonged to 7 DNA REF patterns (Figure 3). Densitometric evaluation revealed that 2 strains isolated from a dairy herd in Michigan had restriction fragments ≥21 kb.

Occurrence of a fingerprint pattern appeared to be restricted to a herd. Within a herd, the same DNA fingerprint occurred at different periods of the lactation cycle. The DNA fingerprint pattern p21 was observed in strains isolated from different periods of the lactation cycle and from two episodes of clinical mastitis (Figure 2).

Cluster analysis was performed to estimate the relationship among strains. The 50 strains examined by cluster analysis could be classified into eight distinct clusters. Most strains from cows in two Tennessee herds isolated during different periods of the lactation cycle belonged to six of the eight clusters. Strains isolated from dairy herds in regions of the US and 1 strain from Great Britain belonged primarily to cluster 2, except for 2 strains from New York that belonged to cluster 1. Seven strains isolated from episodes of clinical mastitis from one Tennessee herd belonged exclusively to cluster 4, and strains isolated from episodes of clinical mastitis from the other Tennessee herd belonged to cluster 5.
DISCUSSION

Bacterial chromosomal DNA REF has been applied widely (2, 8, 10, 11, 19, 22) to identify similar and dissimilar strains within a species. Analysis by REF has advantages over methods that depend on phenotypic characterization because restriction sites in chromosomal DNA are not subject to culture conditions or to dramatic genetic instability (10).

Cluster analysis was performed to estimate the relationships among strains based on the occurrence of similar restriction fragments. Analysis of DNA fragments for RFLP revealed that all S. uberis strains isolated from episodes of clinical mastitis contained DNA fragments of ≥21 kb, suggesting that potential mastitis-causing strains of S. uberis in a herd can be identified by REF.

Five isolates from 5 different cows from one Tennessee herd collected during a herd survey, nonlactating period, parturition, and from two episodes of clinical mastitis all belonged to REF pattern p21. This fingerprint pattern was not found in the other Tennessee herd or in isolates from other regions in the US. These data suggest that prevalence of a particular DNA REF pattern could be confined to a herd. Within a herd, isolates with the same DNA fingerprint pattern were prevalent in mammary secretions of different cows when secretions were collected at different periods of the lactation cycle.

Cluster analysis revealed that S. uberis strains isolated from different periods of the lactation cycle from cows in two Tennessee dairy herds belonged to six of the eight distinct clusters. These data suggest heterogeneity among subclinical isolates of S. uberis strains. Heterogeneity was less in isolates from episodes of clinical mastitis. Streptococcus uberis isolated from episodes of clinical mastitis belonged primarily to clusters 4 and 5.

Observation was similar with isolates from other regions of the US. Strains with identical REF patterns were found within a herd, but not in other herds. Interestingly, all isolates from other regions of the US, except for 2 isolates from New York and the isolate from Great Britain, belonged to a single cluster (cluster 2). This finding suggests that clonal types may be confined to specific geographic areas. Except for S. uberis of clinical mastitis origin, within-herd similarity among strains ranged from 20 to 100%, suggesting the prevalence of different clonal types within a herd. Observations were similar for Enterococcus faecalis (11) strains of human origin. Results of the present study show that differences exist between S. uberis isolates obtained from cows with clinical and subclinical mastitis. A more extended study examining such differences by genomic DNA RFLP analysis could delineate potential virulence factors and help to identify pathogenic and nonpathogenic strains of S. uberis isolated from bovine mammary secretions.

CONCLUSIONS

The results of the present study suggest that clonal diversity exists among strains of S. uberis of bovine origin and that clonal types could be confined to a geographical region. Knowledge of the existing clonal types in a dairy herd could prove valuable in tracing the source of infection, especially during an outbreak of clinical mastitis or occurrence of antibiotic resistant strains on the farm.

ACKNOWLEDGMENTS

This investigation was supported by a Professional Development Award from The University of Tennessee, the Tennessee Agricultural Experiment Station, and The University of Tennessee College of Veterinary Medicine.
Medicine Center of Excellence Research Program in Livestock Diseases and Human Health. Authors express their appreciation to Teresa Herring for excellent clerical assistance.

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