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Subtyping of *Streptococcus uberis* by DNA Amplification Fingerprinting

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Total DNA of *Streptococcus uberis* from cows with mastitis was analyzed by DNA amplification fingerprinting (DAF) and compared with restriction endonuclease fingerprinting (REF). DAF grouped 22 strains into 15 distinct patterns, while REF grouped them into 12 patterns. These results suggest that DAF is a useful technique for subtyping strains of *S. uberis*.

Streptococcus uberis has emerged as an important mastitis-causing pathogen (11, 12, 15, 16). Conventional typing methods, including biochemical profiling, serotyping, use of antimicrobial resistance patterns, and plasmid profile analysis, are inadequate for delineating epidemiological information concerning bacterial reservoirs and transmission of this organism (10, 17, 18).

Restriction endonuclease fingerprinting (REF) has been used to subtype several *Streptococcus* species (4–6, 13, 14, 19) and was shown to be a useful technique for subtyping *S. uberis* of bovine origin (7, 10). More recently, DNA amplification directed by one or more short oligonucleotide primers of arbitrary sequence was used to produce a characteristic spectrum of amplified DNA fragments (2, 20, 21). Caetano-Anollés et al. (2) used one or more primers as short as 5 nucleotides to generate complex DNA fingerprints. This technique was termed DNA amplification fingerprinting (DAF). The objective of this study was to evaluate and compare DAF and REF methods for subtyping strains of *S. uberis*.

Bacteria. Strains of *S. uberis*, including 22 strains from mammary glands of 9 cows with subclinical and clinical mastitis and 2 reference strains (ATCC 27958, type I; ATCC 13386, type II) from the American Type Culture Collection (Rockville, Md.), were examined. Strains were identified as described previously (9). All strains isolated from cows were type I on the basis of restriction fragment length polymorphism analysis of 16S ribosomal DNA (8). Chromosomal DNA was isolated as described previously (10).

REF. *S. uberis* DNA samples were digested with *Hin*dIII (GIBCO BRL, Gaithersburg, Md.) and chromosomal DNA fragments were electrophoresed in 1% agarose gels as described previously (10).

DAF. DNA amplification was performed in a solution with a total volume of 25 μl containing 25 ng of template DNA, 0.25 μg of primer, 7.5 U of AmpliTaq DNA polymerase (Stoffel fragment) (Perkin-Elmer/Cetus, Norwalk, Conn.), 200 μM (each of the four) deoxynucleoside triphosphates (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), 6 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 10 mM KCl and was overlaid with 2 drops of mineral oil. Samples were amplified in an Ericomp thermocycler (Ericomp Inc., San Diego, Calif.) connected to a refrigerated water bath for 35 two-step cycles of 1 s at 96°C and 1 s at 30°C. Oligonucleotide primers were synthesized with >99% efficiency and the following sequences: 5'CGAGCTG3' (primer 7.7a), 5'GTAACGCC3' (primer 8.6d), 5'CGCGGCCA3' (primer 8.8a), and 5'GTGACGTAGG3' (primer 10.6e). Primer nomenclature defines the length of the primer, its GC content, and its order in the series. For example, primer 8.6d is an 8-mer with 60 to 69% GC, being fourth in the series.

Amplified DNA was separated by polyacrylamide gel electrophoresis (2) and visualized by silver staining (1). Biomarker low (Bioventures Inc., Murfreesboro, Tenn.) double-stranded DNA fragments were used as molecular weight markers.

Densitometric evaluation of DAF and REF patterns. DAF gels and the negative of Polaroid film type 55 of REF gels were scanned by using a computer-integrated laser densitometer (Ultroscan XL; LKB Produkter AB, Bromma, Sweden). Scans were evaluated by using Gelscan XL version 2.0 software (Pharmacia LKB Biotechnology, Uppsala, Sweden). Each DAF and REF pattern was examined for size (in kilobases) and number of DNA fragments. If differences were observed, then each DAF or REF was designated as a distinct pattern. Identical fingerprints were grouped into one pattern.

Densitometric evaluation of DAF patterns amplified with

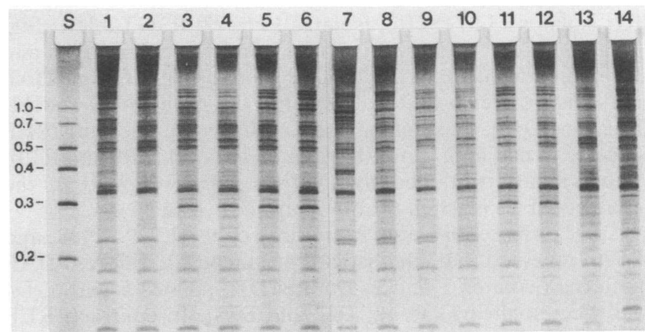

FIG. 1. DNA amplification fingerprints of genomic DNA from different *S. uberis* isolates amplified with primer 8.6d (5'GTAACGCC3'). Lanes 1 and 2, isolates 1 and 2 from cow L829, respectively; lanes 3 to 6, isolates 15 to 18 from cow K1449, respectively; lanes 7 to 10, isolates 19 to 22 from cow L1625, respectively; lanes 11 and 2, isolates 13 and 14 from cow K1420, respectively; lanes 13 and 14, isolates 11 and 12 from cow K1336, respectively; S, molecular size markers (in kilobases).

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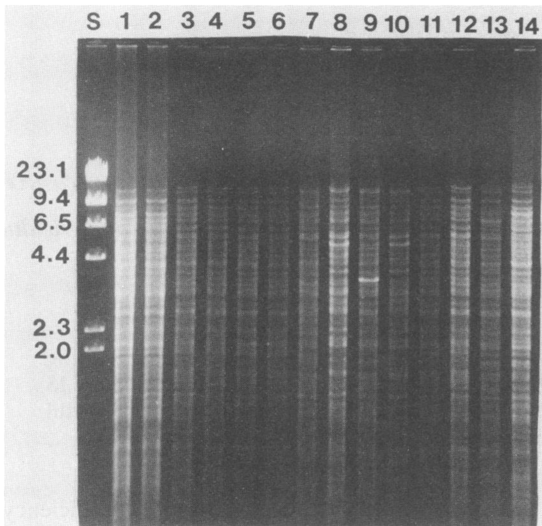

FIG. 2. Fourteen *S. uberis* isolates digested with *Hin*dIII and then electrophoresed on 1% agarose gels. Lanes 1 and 2, isolates 1 and 2 from cow L829, respectively; lanes 3 to 6, isolates 15 to 18 from cow K1449, respectively; lanes 7 to 10, isolates 19 to 22 from cow L1625, respectively; lanes 11 and 12, isolates 13 and 14 from cow K1420, respectively; lanes 13 and 14, isolates 11 and 12 from cow K1336, respectively; S, molecular size markers (in kilobases).

primer 8.6d revealed that the number of distinguishable DNA fragments from the genomic DNA template ranged from 11 to 18 (Fig. 1). The size of DNA fragments ranged from 0.12 to 1.4 kb. DNA fragments of 1.02, 0.33, 0.30, 0.23, 0.18, and 0.12 kb were present in all strains of *S. uberis* (Fig. 1; see Fig. 4). The 24 strains produced 15 distinct DAF patterns (Tables 1 and 2).

Densitometric analysis of REF patterns showed that fragments between 9 and 23 kb allowed discrimination between strains while fragments smaller than 5 kb showed a greater degree of homology (Fig. 2). The 24 strains produced 12 distinct REF patterns. Intense bands were observed in three plasmid-carrying strains (isolates 20 to 22). Twelve strains of *S. uberis* from four cows (K266, K1420, K1449, and L1625) belonged to three DAF subtypes (isolates 9 and 10, D1; isolates 13 to 18, D2; isolates 19 to 22, D3). The same set of isolates belonged to three REF subtypes (isolates 9 and 10, R1; isolates 13 to 18, R2; isolates 19 to 22, R3) (Table 1; Fig. 1 and 2). DAF of paired isolates from two cows (L988 and K1336) showed that each isolate produced a different DAF pattern (isolates 5 and 6, D10 and D11; isolates 10 and 11, D12 and D13). A similar observation was made with REF (isolates 5 and 6, R7 and R8; isolates 10 and 11, R9 and R10). Thus, results obtained by DAF and REF agreed in the analysis of 16 isolates (Table 2 and Fig. 1 and 2).

DAF of paired isolates from three cows (L829, L988, and L526) revealed that each isolate produced six different DAF patterns (isolates 1 and 2, D6 and D7; isolates 5 and 6, D4 and D5; isolates 11 to 12, D12 and D13). In contrast, REF classified these isolates into three subtypes, each set of paired isolates having the same REF pattern. The ability of DAF to identify subtypes that were indistinguishable by REF was confirmed by further analysis of isolates 1 and 2 from cow L829 (Fig. 3). Fingerprints obtained with primers 7.7a, 8.8a, and 10.6e showed that these isolates were distinguishable in every instance. Isolates 13 and 14 from cow K1420 that were similar by both DAF and REF were also

TABLE 1. *S. uberis* isolated from four cows having similar DAF and REF patterns

Isolate no.	Cow no.	Date of isolation (mo-day-yr)	Mammary gland[a]	Sample[b]	DAF pattern[c]	REF pattern[d]
9	K266	09-02-85	lf	D−7	D1	R1
10	K266	09-09-85	lf	D−0	D1	R1
13	K1420	01-05-91	lr	C+3	D2	R2
14	K1420	01-06-91	lr	MAS	D2	R2
15	K1449	12-17-90	lf	D+70	D2	R2
16	K1449	12-26-90	lf	D+77	D2	R2
17	K1449	01-02-91	lf	C−7	D2	R2
18	K1449	01-06-91	lf	MAS	D2	R2
19	L1625	12-29-90	rf	MAS	D3	R3
20	L1625	12-29-90	rr	MAS	D3	R3
21	L1625	12-29-90	lf	MAS	D3	R3
22	L1625	12-29-90	lr	MAS	D3	R3

[a] Relative position of the quarter of the mammary gland. Abbreviations: r, right; l, left; f, front; r, rear.
[b] Days relative to calving (C) or drying off (D). MAS, clinical mastitis.
[c] DNA amplification fingerprint pattern obtained with primer 8.6d.
[d] Restriction endonuclease fingerprint pattern obtained with restriction endonuclease *Hin*dIII.

examined with these primers to further confirm their common subtype (Fig. 3). *S. uberis* ATCC type I and type II strains were differentiated by both procedures (Table 2).

DAF has been used successfully to analyze genomic DNA of viral, bacterial, fungal, plant, and animal origin (2, 3), but the usefulness of DAF for subtyping bacteria has not been reported. The 24 strains of *S. uberis* examined resulted in 15 distinct DAF patterns and 12 REF patterns, supporting earlier evidence for clonal diversity among *S. uberis* of bovine origin (10). DAF and REF classified 16 of 22 isolates into the same subtypes. The remaining six isolates had different DAF but similar REF patterns. Relevant isolates were reexamined by DAF with three additional primers of 7, 8, and 10 nucleotides in length. Results obtained using these

TABLE 2. *S. uberis* isolated from five cows and two ATCC reference strains with dissimilar DAF and variable REF patterns

Isolate no.	Cow no. or ATCC no.	Date of isolation (mo-day-yr)	Quarter[a]	Sample[b]	DAF pattern[c]	REF pattern[d]
1	L829	07-25-89	rr	Hs	D6	R5
2	L829	07-27-89	rr	MAS	D7	R5
3	L988	11-27-89	rf	D−7	D8	R6
4	L988	12-04-89	lf	D−0	D9	R6
5	L277	11-24-89	lf	MAS	D10	R7
6	L277	11-28-89	lr	MAS	D11	R8
7	L526	01-29-86	rr	Hs	D4	R4
8	L526	02-06-86	rr	Hs	D5	R4
11	K1336	11-12-90	lr	Hs	D12	R9
12	K1336	11-14-90	lr	Hs	D13	R10
23	ATCC 27958[e]				D14	R11
24	ATCC 13386[f]				D15	R12

[a] Relative position of the quarter of the mammary gland. Abbreviations: r, right; l, left; f, front; r, rear.
[b] Days relative to drying off (D). Hs, herd survey; MAS, clinical mastitis.
[c] DNA amplification fingerprint pattern with primer 8.6d.
[d] Restriction endonuclease fingerprint pattern with restriction endonuclease *Hin*dIII.
[e] *S. uberis* (type I).
[f] *S. uberis* (type II).

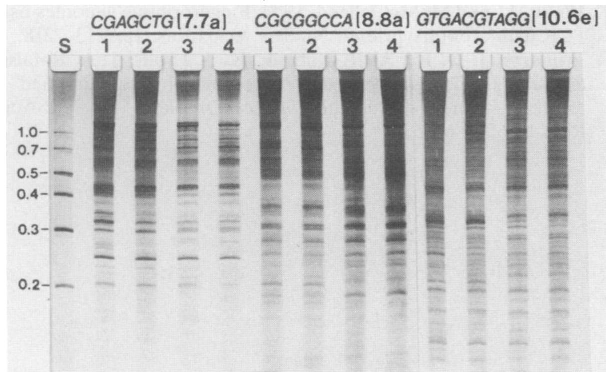

FIG. 3. DNA amplification fingerprints of genomic DNA of *S. uberis* amplified with primer 7.7a (5'CGAGCTG3'), primer 8.8a (5'CGCGGCCA3'), and primer 10.6e (5'GTGACGTAGG3'). Lanes 1 and 2, isolates 1 and 2 from cow L829; lanes 3 and 4, isolates 13 and 14 from cow K1420; lane S, Molecular size markers.

primers confirmed the particular usefulness of primer 8.6d to differentiate strains of *S. uberis*. Our results show that DAF can differentiate bacterial strains with a very high degree of resolution and offers an alternative approach for subtyping *S. uberis* of bovine origin (Fig. 4).

DAF is simple to execute. Pattern interpretation can be conducted easily, once a primer that produces an adequate distribution of DNA amplification fragments is identified. DAF also provides some advantages over existing typing methods in that it requires neither isotopic labeling nor the use of restriction endonucleases and provides better resolution of DNA amplification fragments than agarose gel separation methods. Computer-integrated laser densitometric analysis enabled storage, comparison, and simultaneous evaluation of several DAF patterns. In summary, results of this study indicate that DAF can be used to subtype microorganisms. This technique could be of value to reference and research laboratories involved in studies of bacteria isolated from humans, plants, and animals.

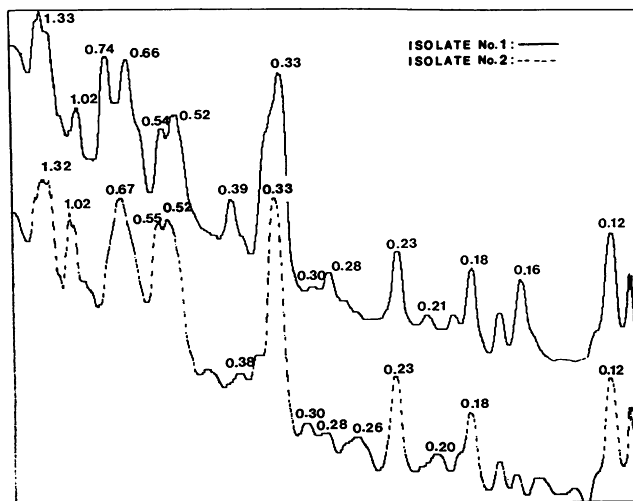

FIG. 4. Laser densitometric scan of genomic DNA of *S. uberis* strains amplified with primer 8.6d (5'GTAACGCC3'). Isolates 1 and 2 with dissimilar DAF patterns isolated from cow L829. The values are sizes (in kilobases) of DNA fragments.

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