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Comparison of an Automated Ribotyping System, Pulsed-field Gel Electrophoresis and Randomly Amplified Polymorphic DNA Fingerprinting for Differentiation of *Streptococcus uberis* Strains

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Abstract: The discriminatory power of three molecular typing methods, automated Ribo printer®, pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA fingerprinting (RAPD), was compared by subtyping 46 *Streptococcus uberis* isolates. Forty-two *Streptococcus uberis* isolates were cultured from milk samples of dairy cows from various geographical regions and four isolates were reference strains. The automated Ribo printer® identified 29 different strain patterns of *S. uberis* using the restriction enzyme *Pvu* II. Thirty-five strain patterns of *S. uberis* were distinguished with PFGE following *Sma* I digestion. The RAPD fingerprinting technique distinguished 14 different *S. uberis* strain patterns. *Streptococcus uberis* isolates (K577 and K717, M413 and M419 and M526 and M671) were indistinguishable by all three methods of fingerprinting. The PFGE method detected more *S. uberis* strain pattern differences than the other two methods.

Key words: Fingerprinting, PFGE, RAPD, Ribo printer®, *Streptococcus uberis*

INTRODUCTION

Mastitis control programs including improved milking practices and hygiene, postmilking teat disinfection, therapeutic and prophylactic antimicrobial therapy and culling of chronically infected animals have effectively controlled intramammary infections by contagious mastitis pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus*. However, mastitis caused by environmental pathogens has become a major problem. It has been suggested that the niche vacated by contagious mastitis pathogens has become occupied by environmental mastitis pathogens, resulting in an increased prevalence of mastitis caused by environmental pathogens[1]. *Streptococcus uberis*, an environmental pathogen, has been implicated as a major cause of mastitis in dairy cows throughout the world. The primary reservoir of *S. uberis* is the environment of the dairy cow and includes: manure, pasture, bedding and various bovine body sites[2]. In addition, *S. uberis* has been isolated frequently from multiparous cows and primigravid heifers near parturition and from cows with clinical and subclinical mastitis during early lactation[3]. Epidemiological information concerning bacterial reservoirs and transmission of *S. uberis* would be beneficial for control and treatment of *S. uberis* mastitis in the dairy herd.

Numerous molecular-based subtyping methods have been developed that can help delineate epidemiological information concerning bacterial reservoirs and transmission of this important mastitis pathogen. The objective of this study was to evaluate and compare three methods; an automated Ribotyping system, pulsed-field gel electrophoresis (PFGE) and randomly amplified DNA fingerprinting (RAPD) for strain differentiation of *S. uberis* of bovine origin.

MATERIALS AND METHODS

Bacterial strains: *Streptococcus uberis* (n=46) was obtained from mammary secretions of cows from various regions of the United States and New Zealand or from the American Type Culture Collection (ATCC, Manassas, VA, USA). The *S. uberis* isolates were obtained from three dairy research herds in Tennessee (Middle Tennessee Experiment Station (MTES) n=6, Dairy Experiment Station (DES) n=6, Knoxville Experiment Station (KES) n=6), Colorado (n=6), Washington (n=6), New Zealand (n=7), ATCC (n=4) and The University of Tennessee Mastitis Laboratory reference strains (n=5) obtained from dairy cows with mastitis. *Streptococcus uberis* was identified based on colony morphology, Gram stain, catalase reaction, esculin hydrolysis and growth in 6.5% sodium chloride. All streptococcal isolates were identified to the species level by the API 20 Strep (bioMérieux, Hazelwood, MO, USA), were maintained in 10% skim milk and stored frozen at –80°C.

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Ribotyping: *Streptococcus uberis* was ribotyped using the Ribo printer® Microbial Characterization System (DuPont-Qualicon, Wilmington, DE, USA) following manufacturer's recommendations. Briefly, the automated system lysed bacterial cells and cut the released DNA into fragments with a restriction enzyme (*Pvu* II). Fragments were separated by size by 1% agarose gel electrophoresis. The DNA was transferred through hybridization to a nylon membrane and was probed with a 6.7 kb probe derived from *Escherichia coli* rRNA operon. The probe was detected with alkaline phosphatase-labeled anti-sulfonated anti-DNA antibodies. Each lane of sample was normalized to a standard marker set and band intensity. A digitizing camera captured the light emission as a data image. The system used the pattern to compare it to others in the database for characterization and identification.

Pulsed-field gel electrophoresis

Preparation of genomic DNA in agarose blocks: The method utilized for PFGE of *S. uberis* was a modification of the method described by Gautom[4] for typing Gram-negative organisms. Isolates of *S. uberis* were grown overnight on tryptic soy agar with 5% sheep blood (Becton Dickinson Microbiology System, Cockeysville, MD, USA) and were suspended in 2 to 3 mL of TE buffer (100 mM Tris-100 mM EDTA, pH 7.5). The cell suspension was adjusted with TE buffer to 20% transmittance using a colorimeter (bioMérieux). A 160 μL aliquot of the bacterial suspension was transferred to a 1.5 mL micro-centrifuge tube. To each tube, 40 μL of lysozyme (10 mg mL^{-1}, Sigma Chemical Co. St. Louis, Mo, USA) and 7 μL lysostaphin (Sigma Chemical Co., 1:10 dilution of 1 mg mL^{-1}) were added. The suspension was incubated at 37°C for 10 to 15 min. To each tube, 7 μL proteinase K (20 mg mL^{-1}; Roche Molecular Biochemicals, Indianapolis, IN, USA) were added and gently mixed. Added to this was 200 μL of 1.6% InCert/SDS agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) maintained at 55°C. This bacterium-agarose mixture was added immediately to plug molds (Bio-Rad Laboratories, Hercules, CA, USA). Plugs were allowed to solidify for 10 min at 4°C and then transferred to a 2 mL tube containing 1.5 ml ES buffer (0.5 M EDTA, pH 9.0: 1% sodium-lauroyl-sarcosine; Sigma Chemical Co.) and 40 μL of proteinase K (20 mg mL^{-1}; Roche Molecular Biochemicals). Plugs were incubated in a 55°C shaker water bath for 45 min. After incubation, ES buffer was removed and plugs were transferred to 50 mL tubes. Plugs were washed in 10 mL sterile distilled water that was preheated to 50°C for 15 min in a shaker water bath. Water was removed and replaced with 10 mL Plug Wash TE buffer (10 mM Tris pH 7.5 and 1 mM EDTA, pH 7.5) preheated to 50°C. This was incubated at 50°C in a shaker water bath for 15 min. This wash was repeated 2 times with Plug Wash TE buffer at 50°C in a shaker water bath for 15 min. Plugs were stored at 4°C in 1 mL Plug Wash TE until used.

Restriction endonuclease digestion of genomic DNA: For restriction endonuclease digestion, two 1 mm wide slices of plugs were incubated at 37°C for 1 to 1.5 h with 30 U *Sma* I (BioWhittaker Molecular Applications) restriction endonuclease enzyme in 100 μL of the appropriate restriction enzyme buffer.

PFGE: The DNA fragments were separated by clamped homogeneous electric field (CHEF) electrophoresis using a CHEF-Mapper (Bio-Rad Laboratories). Plug slices were loaded and electrophoresed in 1% SeaKem gold agarose (BioWhittaker Molecular Applications) with 2 L of 0.5X TBE (0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA pH 8.0) running buffer. Electrophoresis was performed with a CHEF-Mapper using the following conditions: initial switch time, 2.16 s; final switch time, 35.07 s; angle, 120°; gradient, 6.0V/cm; temperature, 14°C; ramping, linear; run time, 14 h. After electrophoresis, gels were stained in 500 mL distilled water with 50 μL ethidium bromide (10 mg mL^{-1}; Sigma Chemical Co.) for 20 min followed by two 15 min washes with distilled water. The DNA fragments were visualized by transillumination (Fotodyne Inc., New Berlin, Wl, USA) and photographed with type 55 Polaroid film (Polaroid Corp Cambridge, MA., USA).

Isolation of DNA for RAPD: Chromosomal DNA of *S. uberis* was isolated using methods described by Jayarao *et al.*[5]. Cells from an overnight culture grown in tryptic soy broth were pelleted by centrifugation at 7,000xg for 3 min at 4°C, washed once with 1 mL of TE buffer (1mM Tris-5 mM EDTA, pH 7.8) and resuspended in 200 μL of the same buffer. After addition of 25 μL of lysozyme (20 mg mL^{-1}: Sigma Chemical Co., St. Louis, MO, USA), bacteria were incubated at 37°C for 30 min. Cells were lysed by addition of 20 μL sodium dodecyl sulphate (SDS) buffer (20% SDS wt/vol in 50 mM Tris-20 mM EDT, pH 7.8) followed by addition of 3 μL of proteinase K (20 mg mL^{-1}: Sigma Chemical Co.) and further incubated at 37°C for 1 h. Protein was precipitated by addition of 200 μL of saturated NaCl (5M) followed by agitation for 15 s and removed by centrifugation at 700xg for 10 min at 4°C. The pellet was discarded and the supernatant was subjected to phenol: chloroform (1:1) extraction followed by two chloroform:

isoamyl alcohol (24:1) extractions. DNA was then precipitated from the supernatant with 2.5X volume of 95% ethanol and left overnight at -20°C. The resulting precipitate was collected by centrifugation at 7,000xg for 5 min at 4°C then washed with 70% ethanol. The DNA pellet was dried under vacuum and rehydrated in 30 μL of buffer (10mM Tris-1mM EDTA, pH 7.5).

RAPD fingerprinting: Amplification of *S. uberis* DNA using primer OPE-04 (Operon Technologies, Alameda, CA, USA), evaluation of DNA fingerprint patterns and analysis of subtypes were done as described by Jayarao *et al.*[6] and Gillespie *et al.* [7]The PCR was performed in a total volume of 25 μL. Bacterial DNA (30 ng/5μL) was used as the template. The PCR mixture contained 2.5 μL of 10X Mg-free buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0) and 1% Triton X-100], 2 μL of 25 mM $MgCl_2$, 1 μL of deoxribonucleoside triphosphate (100 mM of each deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate), 11.8 μL of TE buffer (pH 7.5), 2 μL (10 p mol) of primer OPE-04 (5'-GTGACATGCC-3'; Operon Technologies) and 3.5 U of *Taq* DNA polymerase (Promega, San Diego, CA, USA). Following an initial denaturation step at 94.5°C for 120 s, parameters of the thermocycler (iCycler, Bio-Rad Laboratories) were set at 94.5°C for 70 s, 30°C for 60 s and 72°C for 130 s. Thirty-five cycles of DNA amplification were performed. Ramping time from 94.5 to 33°C was 150 to 165 s. Amplified products were electrophoresed in 2% agarose with TBE buffer (0.9 M Tris base, 0.09 M boric acid, 2.5 mM EDTA, pH 8.3). Gels were run at 150 V for 3.5 h and stained with ethidium bromide (1.0 mg mL^{-1}; Sigma Chemical Co.). The DNA was visualized by transillumination (Fotodyne Inc.) and photographed with type 55 Polaroid film (Polaroid Corp). The gel was analyzed with the aid of the Molecular Analyst version 1.6 (Bio-Rad Laboratories).

Strain classification: Molecular Analyst Software version 1.6 (Bio-Rad Laboratories) was used to determine strain relatedness for each of the three typing methods. The Dice binary coefficient along with the UPGMA (unweighted pair group method using arithmetic averages) was used to construct dendrograms and to determine similarities. The dendrogram is visual illustration of the hierarchic representation of linkage levels between pairs of strains. Band position tolerance of 3% was used for comparison of DNA patterns. The Dice method only considers the presence or absence of bands. Strains that exhibited 93% similarity were considered to be the same subtype. A similarity of 93% was chosen to correspond with the Ribo printer® similarity index which is used under stringent quality control. The automated Ribo printer® System uses a similarity index of 90% as a cut-off for identical strains. The Ribo printer® Data Analysis System (DuPont-Qualicon) was also used for strain classification of *S. uberis* isolates that were analyzed using the automated Ribo printer®. Grouping of strains by the Ribo printer® Microbial Characterization Data Analysis System (DuPont-Qualicon) was compared to grouping of strains by the Molecular Analyst Software version 1.6 (Bio-Rad, Laboratories).

RESULTS

Strains of *S. uberis* were evaluated for discrimination of strain patterns using three molecular typing methods, automated Ribo printer®, pulsed-field gel electrophoresis (PFGE) and randomly amplified DNA fingerprinting (RAPD). All 46 *S. uberis* strains were evaluated with the Ribo printer® Microbial Characterization system, 43 strains were evaluated with the RAPD method and 45 strains were evaluated with PFGE. Some strains of *S. uberis* (NZ1557, NZ1635 and UT690) produced poor quality RAPD profiles and isolate C5283 produced a poor quality PFGE profile. These isolates produced a capsule that possibly interfered with DNA isolation and subsequent analysis of the sample. The automated Ribo printer® yielded 4 to 8 fragments in the 0.75 to 0.45 kb size range and identified 29 different strain patterns of *S. uberis* using the enzyme *Pvu* II. The PFGE of chromosomal DNA digested with *Sma* I yielded 10 to 20 fragments in the 436.5 to 48.5 kb size range and distinguished 35 different *S. uberis* strain patterns. The RAPD procedure yielded 4 to 8 fragments in the 1.2 to 0.40 kb size range and 14 different strain patterns were identified with the RAPD procedure. Dendrograms (Fig. 1, 2 and 3) illustrate similarities between strains of *S. uberis* using the Ribo printer®, PFGE and RAPD, respectively. Strains that exhibited 93% similarity were considered to be the same subtype. This similarity was chosen to correspond with the Ribo printer® Data Analysis System and to align standard control strains.

Six isolates (K577 and K717, M413 and M419, M526 and M671) were indistinguishable by all three methods of fingerprinting. Some strains that were identical by the Ribo printer® were also identical by the RAPD method but were different with the PFGE method. The PFGE method also identified identical strains that concurred with the Ribo printer®, but were different from the RAPD method.

The two most common strain patterns identified with the automated Ribo printer® system was Ribo 1 and

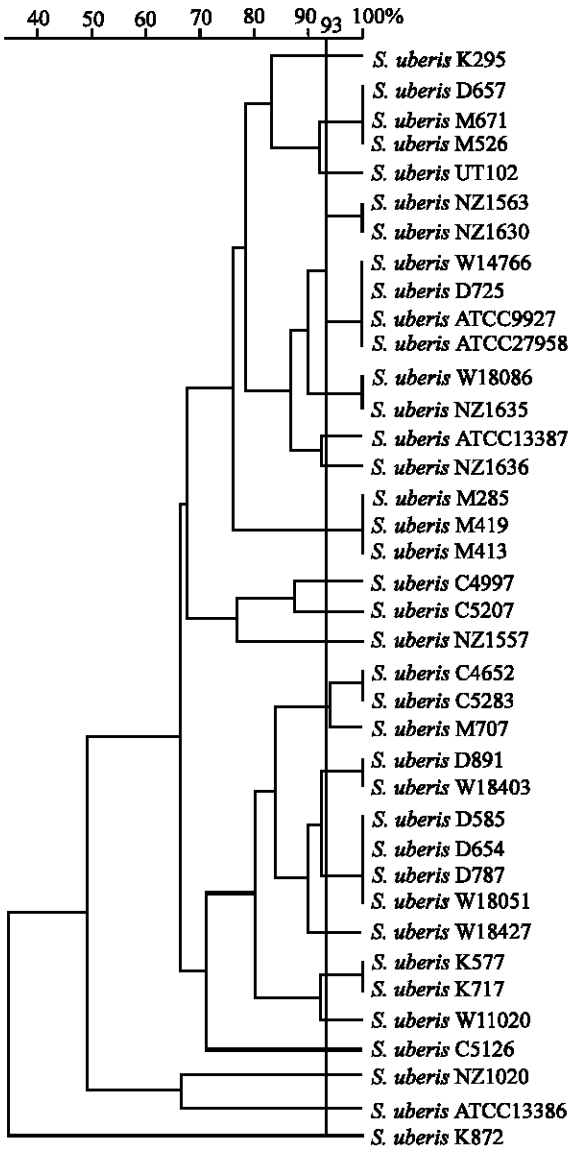

Fig. 1: Dendrogram generated from analysis of automated Ribo printer® fingerprints of *Streptococcus uberis*. The scale represents similarity values of banding patterns of *S. uberis* strains

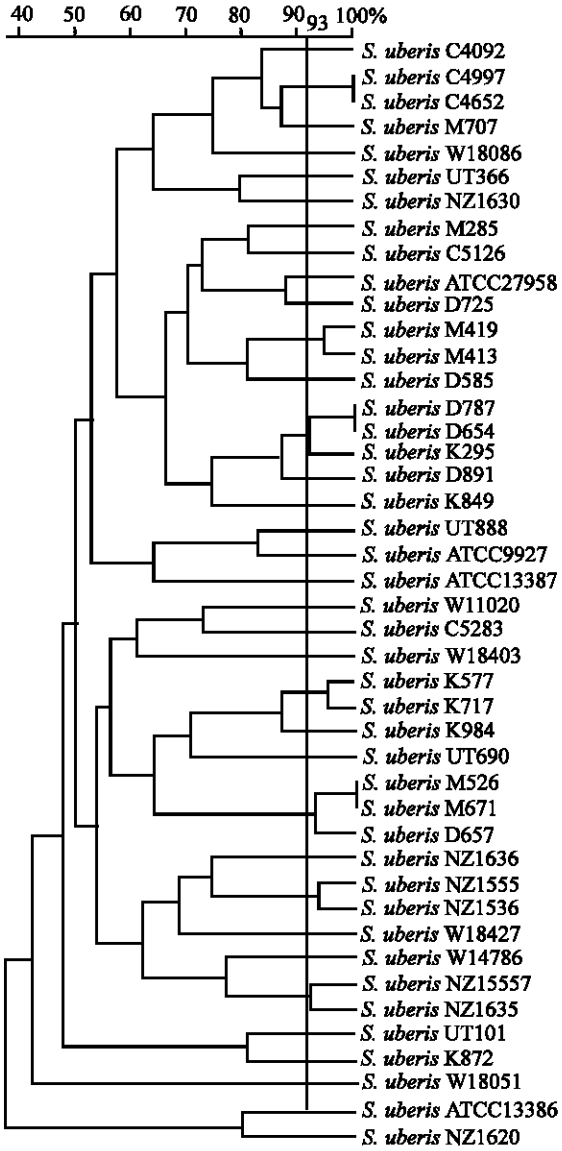

Fig. 2: Dendrogram generated from analysis of PFGE fingerprints of *Streptococcus uberis*. The scale represents similarity values of banding patterns of *S. uberis* strains

Ribo 6. Five strains of *S. uberis* identified as Ribo 1, 3 were from the DES farm and 2 were from Washington. Of those isolates, 2 (D654 and D787) were indistinguishable by PFGE (Table 1). Strains of *S. uberis* identified as Ribo 6 contained three ATCC reference strains, one isolate from DES and one isolate from Washington (Table 1). The ATCC reference strains 27958 and 9927 were identical with the Ribo printer® and RAPD method, but different with the PFGE method (Table 1).

Of the 45 *S. uberis* isolates evaluated by PFGE, 35 different strain patterns were identified (Table 1). Three isolates each were grouped in PFGE 13 and PFGE 25 of which M526 and M671 were indistinguishable by both the Ribo printer® and RAPD methods, while isolates D787 and D654 were identical with the Ribo printer® method, but they were differentiated with the RAPD method (Table 1). Isolates NZ1555 and NZ1563 had similar PFGE and RAPD profiles but different profiles with the Ribo printer®.

The most common pattern with the RAPD method was RAPD 5, which characterized 13 isolates as identical and was seen in isolates from all geographical locations

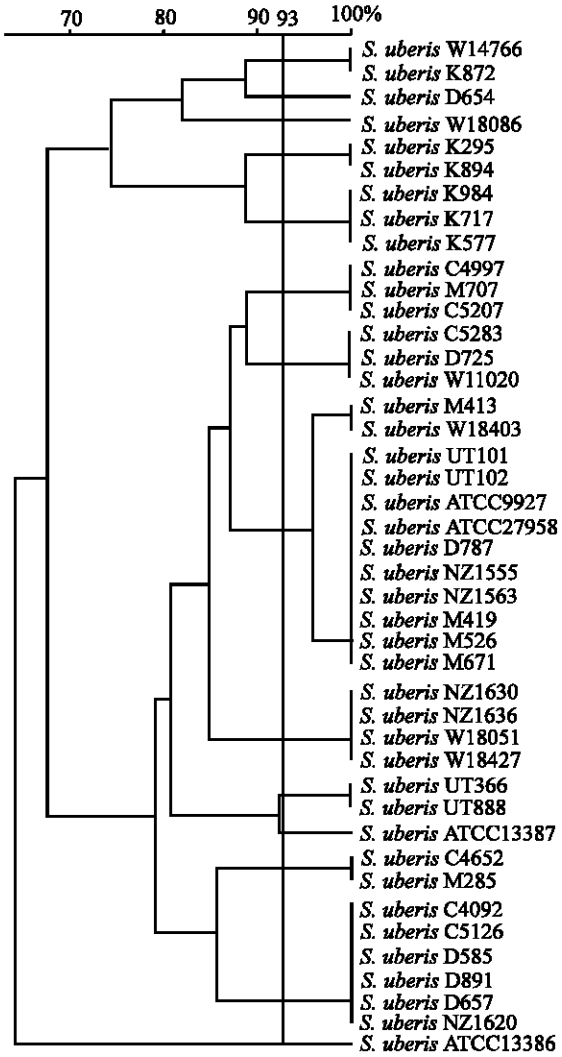

Fig. 3: Dendrogram generated from analysis of RAPD fingerprints of *Streptococcus uberis*. The scale represents similarity values of banding patterns of *S. uberis* strains

except Colorado and also from the ATCC reference strains (Table 1). Two sets of isolates (M526 and M671) and (M413 and M419) were also indistinguishable by PFGE and automated Ribo printer® (Table 1). Two strains from New Zealand (NZ1555 and NZ1563) were indistinguishable by both the RAPD and PFGE methods, but different with the automated Ribo printer® (Table 1). Two isolates (M285 and M413) were from different quarters of the same cow sampled 25 days apart, however, these two isolates had different RAPD and PFGE profiles, but automated Ribo printer® patterns were the same (Fig. 4).

All three methods were able to discriminate *S. parauberis* (ATCC 13386) from *S. uberis*. Degree of similarity between *S. parauberis* (ATCC 13386) and other *S. uberis* strains was 39, 49 and 64% for the PFGE, Ribo printer® and RAPD methods respectively. This degree of similarity is proportional to the number of different subgroups found with each method; 35, 29 and 14 different strains identified with the PFGE, Ribo printer® and RAPD methods, respectively.

Images generated with the automated Ribo printer® were saved as tiff images and imported into the Molecular Analyst software to compare results of the two different analysis programs. Eight of the tiff images generated by the automated Ribo printer® system were not suitable for analysis with the Molecular Analyst software. Analysis of the Ribo printer® tiff images by the Molecular Analyst software closely matched analysis of the Ribo printer® Microbial Characterization system (Table 1).

DISCUSSION

The use of molecular typing methods for differentiation of closely related bacterial strains has many advantages over conventional bacterial typing methods based on biochemical utilization, enzymatic profiles, serotyping and antibiotic resistance patterns. One advantage of molecular typing methods is decreased variability associated with cultural conditions that lead to atypical biochemical characteristics associated with phenotypic characterization. Secondly, molecular typing methods focus on the unique nucleic acid composition of the bacterium rather than phenotypic expression of products encoded by nucleic acids. The use of molecular typing methods allows for differentiation of closely related bacterial strains.

The Ribo printer® is an automated system that lyses bacterial cells, digests ribosomal DNA into fragments with a restriction enzyme and separates fragments by gel electrophoresis. The gel is transferred to a membrane where it is hybridized with a chemiluminescent DNA probe containing the rRNA operon from *E. coli*. A digitizing camera captures the light emission as a data image and banding patterns are stored in a computerized database.

Pulsed-field gel electrophoresis is often considered the "gold standard" of molecular typing methods[8]. The PFGE method involves embedding bacteria in agarose followed by lysis and digestion of chromosomal DNA with a restriction endonuclease. Small slices of the agarose containing chromosomal DNA fragments are added to an agarose gel and restriction fragments are resolved into discrete banding patterns.

The RAPD method is based on use of short random sequence primers, 9 to 10 bases in length, which bind to

Table 1: Comparison of automated Ribotyping, pulsed-field gel electrophoresis and randomly amplified polymorphic DNA fingerprinting for genotyping *Streptococcus uberis*

Isolate	Source	Ribo printer [a]data analysis	Ribo printer [b]molecular analyst	RAPD [b]molecular analyst	PFGE [b]molecular analyst	Isolate	Source	Ribo printer [a]data analysis	Ribo printer [b]molecular analyst	RAPD [b]molecular analyst	PFGE [b]molecular analyst
13386	ATCC	29	21	14	35	M413	MTES	8	8	5	11
13387	ATCC	6	6	10	18	M419	MTES	8	8	5	11
27958	ATCC	6	4	5	10	M526	MTES	2	2	5	25
9927	ATCC	6	4	5	17	M671	MTES	2	2	5	25
C4092	Colorado	15	[c]NS	11	1	M707	MTES	9	13	8	3
C4652	Colorado	16	12	13	2	NZ1555	New Zealand	27	[c]NS	5	27
C4997	Colorado	17	9	8	2	NZ1557	New Zealand	20	11	[d]NT	30
C5126	Colorado	18	19	11	7	NZ1563	New Zealand	19	4	5	27
C5207	Colorado	17	10	8	19	NZ1620	New Zealand	26	20	11	34
C5283	Colorado	16	12	7	[d]NT	NZ1630	New Zealand	19	4	6	5
D585	DES	1	15	11	12	NZ1635	New Zealand	21	5	[d]NT	30
D654	DES	1	15	12	13	NZ1636	New Zealand	19	7	6	26
D657	DES	2	2	11	25	UT101	UT	22	[c]NS	5	31
D725	DES	6	4	7	9	UT102	UT	25	3	5	[d]NT
D787	DES	1	15	5	13	UT366	UT	23	[c]NS	9	6
D891	DES	3	14	11	14	UT690	UT	28	[c]NS	[d]NT	24
K295	KES	14	1	3	13	UT888	UT	24	[c]NS	9	16
K577	KES	10	17	4	22	W11020	Washington	5	18	6	20
K717	KES	10	17	4	22	W14766	Washington	6	4	1	29
K849	KES	11	[c]NS	5	15	W18051	Washington	1	15	6	33
K872	KES	12	22	1	32	W18086	Washington	4	6	2	4
K984	KES	13	[c]NS	4	23	W18403	Washington	1	14	5	21
M285	MTES	8	8	13	8	W18427	Washington	7	16	6	28

[a] Ribo printer microbial characterization system data analysis software.
[b] Molecular analyst software v 1.6.
[c] Photo from Ribo printer not suitable for analysis with molecular analyst software.
[d] Not tested.

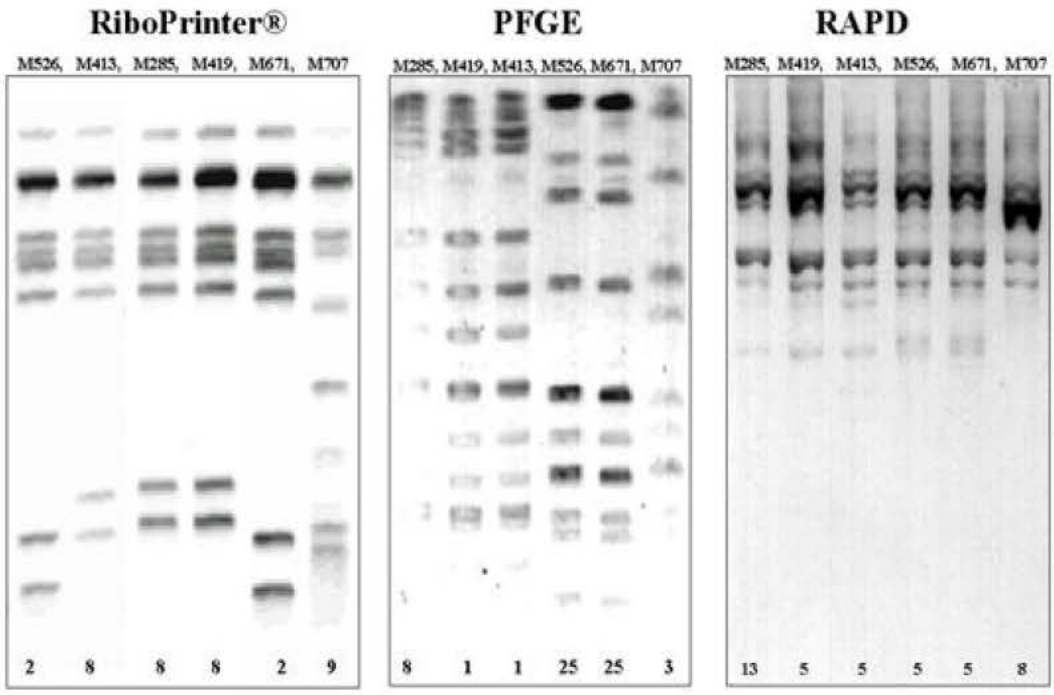

Fig. 4: Comparison of representative DNA fingerprint patterns using an automated Ribo printer® system, PFGE and RAPD for genotyping *Streptococcus uberis*. Isolates are identified above each lane and RiboPrint, PFGE and RAPD pattern groups are identified at the bottom of each lane

chromosomal DNA sequences at low annealing temperatures and are used to initiate amplification of regions of the bacterial genome. When two RAPD primers anneal within a few kilo-bases of each other, a PCR product with a molecular length corresponding to the distance between the two primers results. The number and location of these random primer sites vary for different strains of a bacterial species. During PCR, DNA fragments corresponding to the location of the random primer sites are amplified. These amplified DNA fragments are separated by agarose gel electrophoresis and produce a pattern of bands, which is characteristic of the particular bacterial strain.

The PFGE method appears to be more discriminatory for detecting differences in strains of *S. uberis* than either the automated Ribo printer® system or the RAPD method and was able to resolve isolates that were indistinguishable by either of the other two methods. The PFGE was shown in previous studies[9-12] to be a highly discriminatory method for detecting strain differences of streptococci isolates from bovine origin. Phuektes *et al.*[13] evaluated PFGE and antibiogram patterns and found the discriminatory power of antibiogram patterns to be relatively low as compared to PFGE.

In the present study, *Pvu* II was chosen for restriction endonuclease digestion for the Ribo printer® based on recommendations by technical experts from Dupont-Qualicon. Utilization of another enzyme could possibly produce patterns with more discriminatory power. In a study by Quale *et al.*[14] ribotype profiles of *Streptococcus pneumoniae* with *Pvu* II digestion were less discriminatory than those obtained with *Hind* III. Ribotype patterns obtained after digestion with *Hind* III were comparable to those obtained by PFGE for *S. pneumoniae* strains[14].

The RAPD method has been used to differentiate new and persistent *S. uberis* intramammary infections in dairy cows[15]. Oliver *et al.*[15] were able to detect new *S. uberis* intramammary infections using the RAPD method that would never have been detected using phenotypic methods. In the present study, 2 isolates (M285 and M413) from different mammary glands of the same cow sampled 25 days apart exhibited different RAPD and PFGE patterns suggesting a new intramammary infection by a different strain of *S. uberis*.

When evaluating which molecular typing method to use, one needs to consider purpose for molecular typing, convenience, price and ease of use. The Ribo printer® is marketed as a bacterial identification and genetic characterization system. Based on the number of bands seen with each method, the RAPD method may be more suited for bacterial species identification, while the PFGE method may be more suited for differentiation of closely related strains. The PFGE method is more time consuming than the other methods and requires an expensive electrophoresis apparatus and control system. While the automated Ribo printer® system is the most expensive method, the automated system is very user friendly with limited hands on. If expense and time are important factors, the RAPD method could be developed on any thermocycler and gel electrophoresis apparatus. All three methods can be used to discriminate between closely related strains of *S. uberis*. However, based on the results of this study, PFGE appears to be more discriminatory for detecting differences in strains than either the Ribo printer® Characterization System or the RAPD method. Continued studies using the PFGE method could help delineate important epidemiological information concerning reservoirs and transmission of *S. uberis,* a significant mastitis pathogen.

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