

DNA fingerprinting of *Streptococcus uberis*: a useful tool for epidemiology of bovine mastitis

A. W. HILL AND J. A. LEIGH

AFRC Institute for Animal Health, Compton Laboratory, Compton, Newbury,
Berks RG16 0NN, UK

(Accepted 1 February 1989)

SUMMARY

A simple and reproducible typing system based on restriction fragment size of chromosomal DNA was developed to compare isolates of *Streptococcus uberis* obtained from the bovine mammary gland. The endonuclease giving the most useful restriction patterns was *Hind* III, although seven other endonucleases (*Bgl* I, *Eco* RI, *Not* I, *Pst* I, *Sfi* I, *Sma* I, *Xba* I) were also tested in the system. An image analyser was used to obtain a densitometric scan and a graphic display of the restriction patterns. Such a system will allow large scale data storage for future computer-aided comparison.

INTRODUCTION

Streptococcus uberis remains an important and inadequately controlled cause of mastitis in the dairy cow, resulting in clinical and subclinical disease in the lactating cow, and is the predominant organism isolated from the non-lactating udder (1). The lack of a reliable method of distinguishing the fine differences between isolates within a dairy herd hampers epidemiological studies. The identification of bacterial reservoirs responsible for new infections and understanding the importance of cross-infection as a means of transmission, will finally lead to better control.

In a recent study using lytic phage specific for *S. uberis* (Hill, 1988, unpublished observation), clinical isolates from a single dairy herd showed a low level of susceptibility to the phage and hence low typability. However, it did emphasise that, for a technique to be successful, it must detect fine differences between strains which are biochemically similar and, only distinguished by weak, and often variable reactions using phage.

DNA or genome fingerprinting has already been applied to a wide range of bacteria, including leptospira (2), *Vibrio cholerae* (3), campylobacter (4) and moraxella (5). Recently, Skjold & Cleary (6) described a method of DNA fingerprinting which was sufficiently discriminating to distinguish between strains of the M49 serotype of Group A streptococci.

This paper describes the rapid, small-scale isolation of chromosomal DNA and its digestion with endonucleases and assesses its potential as a repeatable and practical technique to distinguish between isolates of *S. uberis*.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were isolates obtained from the mammary gland of dairy cows within the Institute herds. They were either organisms isolated from clinical mastitis or obtained during routine milk sampling of cattle at calving, drying off or during the non-lactating period. The organisms were retained if they were Gram-positive cocci capable of hydrolysing aesculin. It is likely that from this source at least 80% of these isolates would be *S. uberis* (7). The others will include *S. bovis*, *S. faecalis*, *S. faecium* and possibly *S. viridans*. The isolates used here were subjected to five biochemical tests which are sufficient to distinguish *S. uberis* from the other species (8). *S. uberis* produces acid from inulin, fails to produce acid from raffinose, hydrolyses hippurate, produces ammonia from arginine and does not neutralize or produce gas from acid citrate medium. All media were prepared by the methods described by Garvie & Bramley (9).

Organisms were stored in Todd-Hewitt broth (Oxoid) at -20°C in the presence of 25% glycerol. For the isolation of DNA, bacteria were subcultured from the storage media into Todd-Hewitt broth (37°C 18 h) containing 10 units/ml hyaluronidase (Calbiochem).

Isolation of chromosomal DNA

All steps in the isolation were carried out in 1.5 ml bullet vials (Alpha Labs). Cells from 1.5 ml of overnight culture were washed once in 1.0 ml Tris (10 mM) EDTA (5 mM) pH 7.8, and resuspended in 350 μl of the same buffer. After the addition of 25 μl Mutanolysin (Sigma, 5000 units/ml) bacteria were incubated at 37°C for 30 min, and lysed by the addition of 20 μl SDS (20% SDS w/v in Tris [50 mM] EDTA [20 mM] pH 7.8) followed by 3 μl Proteinase K (20 mg/ml, Sigma) and a further incubation at 37°C for 1 h. Protein was precipitated by the addition of 200 μl saturated sodium chloride (approx. 6.0 M) followed by agitation for 15 s (10), and removed by centrifugation (7000 g) for 10 min. The pellet was discarded and DNA was precipitated from the supernatant with 2.5 volumes of ethanol and 30 μl sodium acetate (1.0 M). The resulting precipitate was collected by centrifugation at 7000 g for 5 min, dried under vacuum, and rehydrated in 30 μl buffer (Tris [10 mM] EDTA [1.0 mM] pH 7.5).

Enzymic digestion of DNA

After at least 24 h rehydration, 5 μl of DNA solution was digested with the appropriate restriction endonuclease according to the manufacturers instructions.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in Tris acetate buffer (Tris-acetate 0.04 M, EDTA 0.001 M) in 20 cm \times 20 cm gels containing 1.0% agarose at 40 V for 16 h. Gels were stained with ethidium bromide (1.0 $\mu\text{g}/\text{ml}$) and DNA visualized by transillumination (UVP Inc, Cambridge, UK) and photographed on Polaroid film (Type 667 professional).

Graphic representation of data using an image analyser

The polaroid photograph was scanned along each track using an image analyser (Analytical Measuring Systems, Optimax V Image Analyser), and a Television camera (Ikegami ITC S10), with a computer controlling a Line densitometry software package (Optimax). The data was then plotted as intensity (y axis) against migration distance (x axis).

RESULTS

During the development of the isolation procedure electrophoresis of material from in excess of 50 isolates failed to reveal the presence of plasmid DNA. The extraction produced genomic DNA > 25 Kb in size and sufficiently pure for digestion by endonucleases. In all, eight restriction enzymes were used; *Bgl* 1, *Eco* R1, *Hind* III, *Not* 1, *Pst* 1, *Sfi* 1, *Sma* 1, *Xba* 1. The effect of three enzymes (*Hind* III, *Bgl* 1 and *Eco* R1) on seven samples of DNA from *S. uberis* is shown in Fig. 1. *Bgl* 1 cleaved the DNA very infrequently, leaving many fragments with a size above 23 Kb. *Eco* R1 digestion resulted in more cleavage with the largest fragment forming a distinct band at about 20 Kb. Although some difference could be seen in the restriction patterns discrimination between the isolates was very poor. In contrast, the DNA cleaved during digestion with *Hind* III resulted in several strain specific fragments between 23 Kb and 9.4 Kb. At best, the other endonucleases produced results similar to *Bgl* 1, but in the case of *Not* 1 and *Sfi* 1 no digestion could be detected.

Since *Hind* III gave restriction patterns which allowed discrimination between strains, it was used to digest DNA from 24 isolates of aesculin hydrolysing streptococci obtained from the Institute herd over a 4-week period. The restriction patterns are shown in Fig. 2. The three isolates (A, Fig. 2) and four isolates (B, Fig. 2) were obtained from the same quarter of the same cow on different occasions. The biochemical tests carried out on the isolates suggests that strains 5 and 6 (Fig. 2) which do not have typical restriction patterns after digestion were *S. bovis* and *S. faecium* respectively.

During this work, digested DNA from one strain showed an unusually intense band at about 7.5 Kb., (Fig. 2, track 7). When the undigested DNA from this strain was electrophoresed it revealed a plasmid in excess of 40 Kb. The band at 7.5 Kb would appear to be the largest fragment of this plasmid following digestion. No other plasmid was found.

Graphical representations of some of the tracks after scanning with the image analyser are shown in Fig. 3. The graphs 1–4 represent the results of scanning the numbered tracks in Fig. 2.

DISCUSSION

Although limited success in the discrimination of isolates of *S. uberis* has been achieved using bacteriocins (11) and lytic phage (Hill, unpublished observations), there is still a need for a more practical and precise method of typing this organism. The inability to detect plasmids in *S. uberis* isolates (T. R. Field, unpublished observations, and present report) rule out the use of plasmid profiles

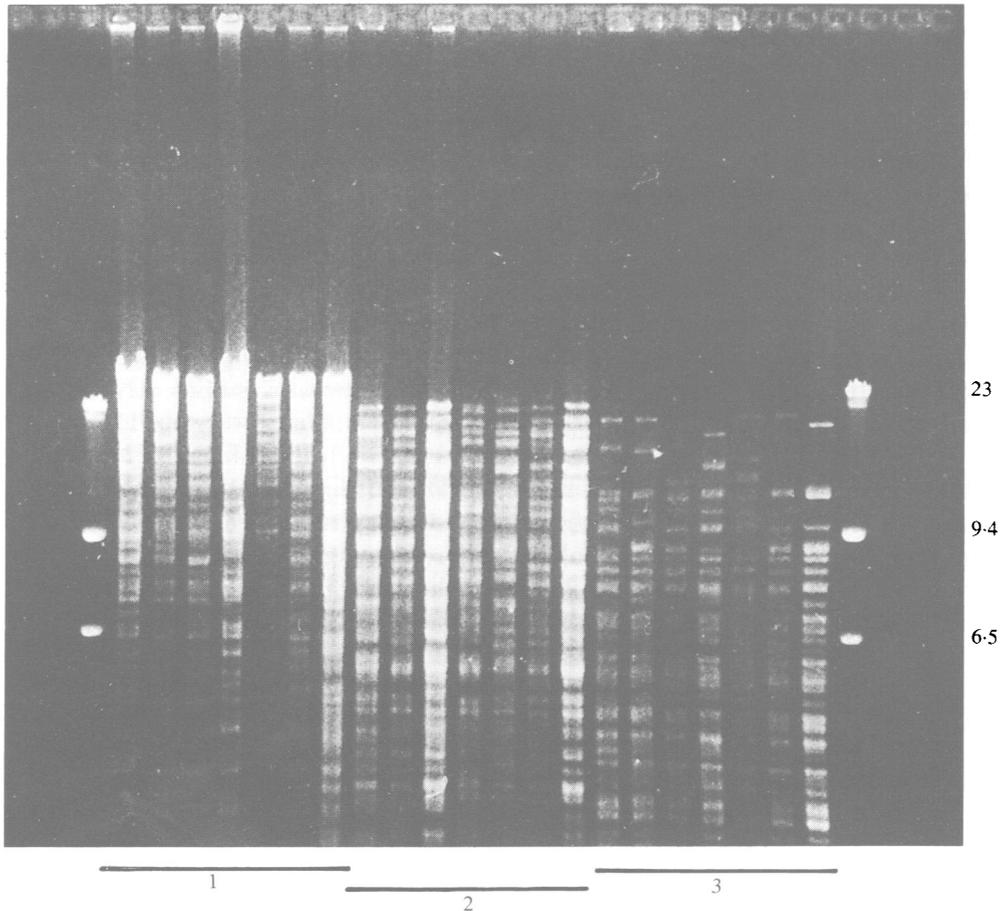


Fig. 1. Electrophoresis of chromosomal DNA from seven isolates of *Streptococcus uberis* digested with three endonucleases; 1: *Bgl* I, 2: *Eco* R1, 3: *Hind* III. All chromosomal digest are in the same order. Molecular weight markers are unboiled *Hind* III digested λ phage DNA.

as a typing method. Many reports have shown the value of endonuclease digestion of bacterial DNA for typing isolates (2-6, 12-14). However, the technique was used for fine discrimination or comparison of groupings obtained by other typing methods. In the case of *S. uberis* it will be necessary to develop a typing system based entirely upon the DNA restriction patterns.

The technique described in this report is clearly very reproducible. The isolates from the same mammary quarters during a recurrent infection, (Fig. 2, A and B), can easily be identified as the same bacterial type. The differences between the other DNA samples demonstrates the power of the technique for discriminating and identifying isolates. This is even more significant when one considers that in a recent finding using a typing system based on lytic phage it was concluded that the differences between the typable bacteria depended on very weak and variable reactions, (Hill, unpublished observations).

Comparison of the restriction patterns from *S. uberis* shows that the fragment

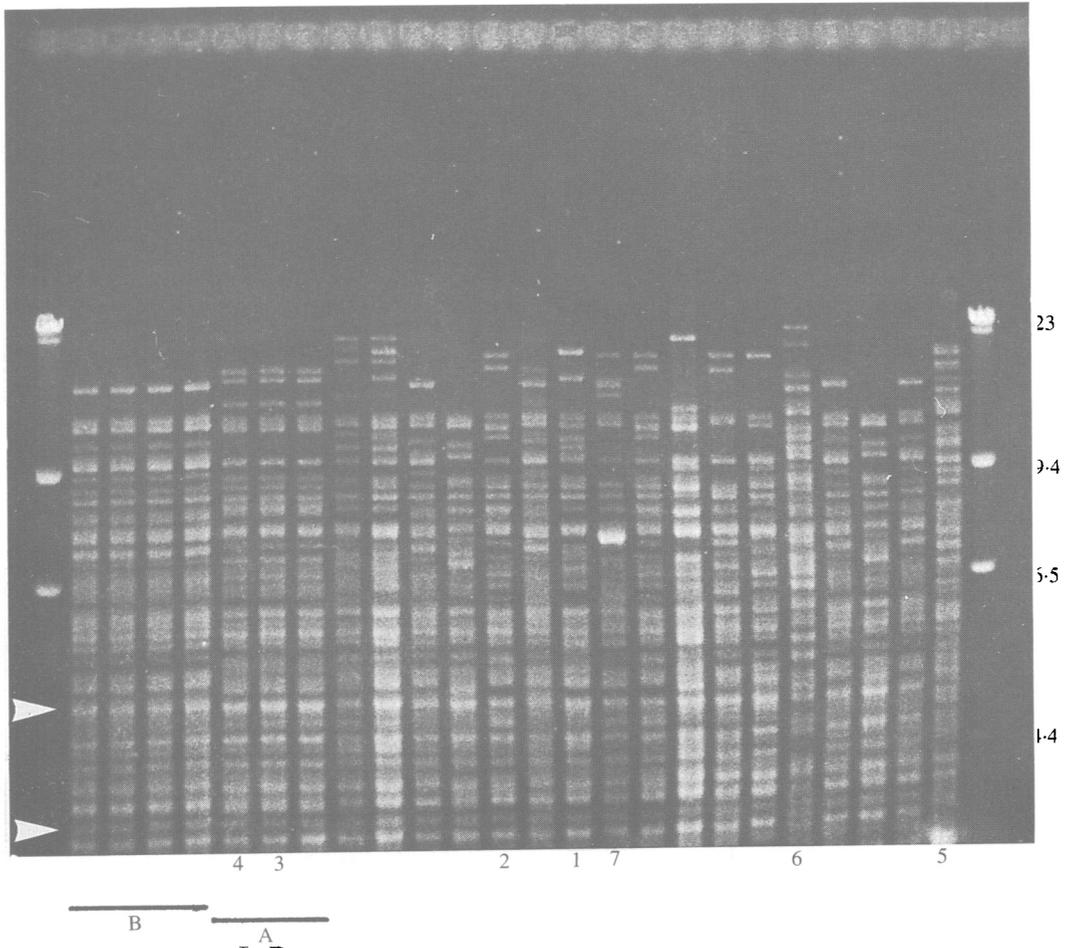


Fig. 2. Electrophoresis of chromosomal DNA from 24 isolates of aesculin hydrolysing streptococci, following digestion with *Hind* III.

A and B, three and four strains respectively from the same mammary gland of two cows on separate occasions during recurrent infections.

1-4 represent tracks scanned with an image analyser and plotted in Fig. 3; 5, DNA from an organism which was shown biochemically to be *S. bovis*; 6, DNA from an organism which was shown biochemically to be *S. faecium*; 7, DNA from *S. uberis* showing fragment of large plasmid at 7.5 Kb.

Restriction patterns between arrows demonstrate a high degree of homology within the species. All other tracks are DNA from strains of *S. uberis*. Molecular weight markers are unboiled *Hind* III digest of λ phage DNA.

length of the discriminating region is between 9 and 23 Kb., (Fig. 2). In contrast, the fragments smaller than 5.0 Kb (between arrows, Fig. 3) show a great degree of homology within the species. The patterns obtained with the 22 isolates of *S. uberis*, a single *S. bovis* (track 5) and *S. faecium* (track 6) demonstrates a marked difference between the small fragments (< 9 Kb.) obtained with *S. uberis* and the two other streptococci. This difference may prove useful for the species determination of aesculin hydrolysing streptococci.

Although the present method gives sufficient separation of fragments for image

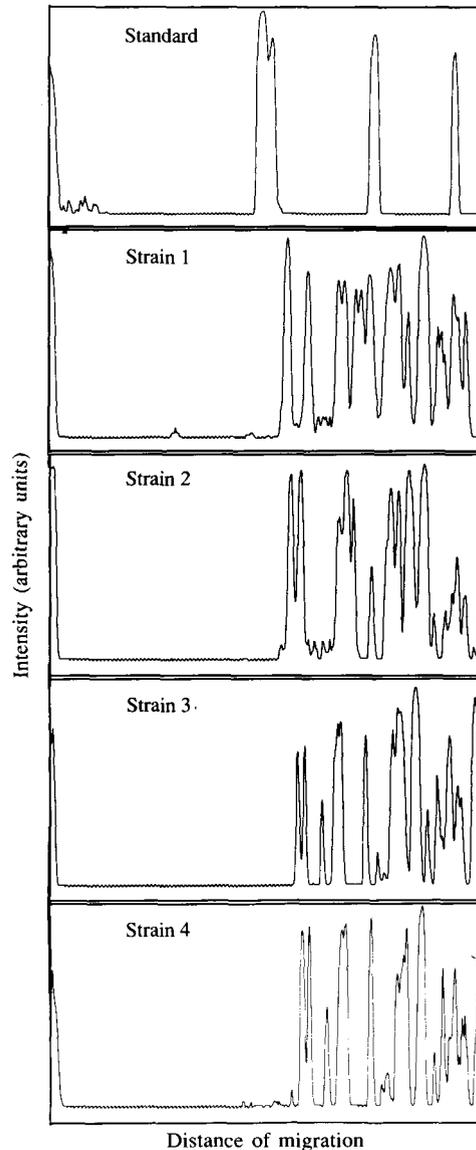


Fig. 3. Graphic plots of five electrophoresis tracks after scanning with an image analyser. Strains 1-4 represent the corresponding tracks on Fig. 2, together with a scan of molecular weight markers.

analysis scanning, the typing would benefit from greater separation within the 23-9 Kb range making computer comparison more precise. We are currently trying to achieve this using pulse field inversion gel electrophoresis (PFGE). Such a system has already been used to separate larger chromosomal fragments obtained during DNA fingerprinting of *Pseudomonas aeruginosa* (12).

The availability of image analysis of restriction patterns and hence data storage will prove invaluable in the comparison of isolates. This will be particularly so when comparison of patterns from different electrophoresis gels is required.

Now that a practical method of typing *S. uberis* and other aesculin hydrolysing streptococci has been developed, a detailed investigation of the epidemiology of the organisms can be performed.

ACKNOWLEDGEMENTS

The authors thank William Ditcham and Amanda Ashton for their technical assistance and Dr Richard Sellwood for his help during the early part of this work.

REFERENCES

1. Bramley AJ, Dodd FH. Reviews of the progress of dairy science: Mastitis control-progress and prospects. *J Dairy Res* 1984; **51**: 481–512.
2. Marshall RB, Wilton BE, Robinson AJ. Identification of leptospira serovars by restriction endonuclease analysis. *J Med Microbiol* 1981; **14**: 163–6.
3. Kaper JB, Bradford HB, Roberts NC, Falkow S. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. *J Clin Microbiol* 1982; **16**: 129–34.
4. Kakoyiannis CK, Winter PJ, Marshall RB. Identification of isolates of *Campylobacter coli* from animals and humans by bacterial restriction endonuclease DNA analysis (BRENDA). *Appl Environ Microbiol* 1984; **48**: 545–9.
5. Marshall RB, Winter PJ, Cooper BS, Robinson AJ. Subspecies differentiation of *Moraxella bovis* by restriction endonuclease analysis (BRENDA). *N Z Vet J* 1985; **33**: 67–70.
6. Skjold SA, Cleary PP. DNA fingerprints of Group A, serotype M49 streptococci. In: Ferretti JJ, Curtiss R, eds. *Streptococcal genetics*. Washington, DC: American Society for Microbiology, 1987: 127–9.
7. Bramley AJ. *Streptococcus uberis* mastitis. Epidemiology and pathogenesis. *Anim Health Nutr* 1987; **42**: 12–6.
8. Kruze J. The aetiology of bovine mastitis caused by *Streptococcus uberis* [Ph.D. Thesis]. Reading, UK: University of Reading, 1983. 256 pp.
9. Garvie EI, Bramley AJ. *Streptococcus uberis*: an approach to its classification. *J Appl Bacteriol* 1979; **46**: 295–304.
10. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleate cells. *Nucleic Acids Res* 1988; **16**: 1215.
11. Tagg JR, Vugler LG. An inhibitor typing system for *Streptococcus uberis*. *J Dairy Res* 1986; **53**: 451–6.
12. Bautsch W, Grothues D, Tummler B. Genome fingerprinting of *Pseudomonas aeruginosa* by two-dimensional field inversion gel electrophoresis. *FEMS Microbiol Lett* 1988; **52**: 255–8.
13. Burnie JP, Lee W. A comparison of DNA and immunoblot fingerprinting of the SII biotype of coagulase negative staphylococci. *Epidemiol Infect* 1988; **101**: 203–12.
14. Kakoyiannis CK, Winter PJ, Marshall RB. The relationship between intestinal *Campylobacter* species isolated from animals and humans as determined by BRENDA. *Epidemiol Infect* 1988; **100**: 379–87.