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Rapid Method for Epidemiological Evaluation of Gram-Positive Cocci by Field Inversion Gel Electrophoresis

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We report a rapid method for the isolation of intact chromosomal DNA from gram-positive cocci that is suitable for in situ restriction endonuclease digestion in agarose blocks. When combined with a rapid field inversion gel electrophoresis protocol, this approach allows the preparation and electrophoretic analysis of chromosomal restriction fragments produced by rare-cutting enzymes in a total time period of 2 days from start to finish. The utility of the method is demonstrated in the epidemiological evaluation of *Staphylococcus epidermidis* clusters from two hospitals as well as of additional representative staphylococci and enterococci.

Some of the newest tools of molecular epidemiology are alternative electrophoretic methods such as pulsed-field electrophoresis (15), contour-clamped homogeneous electric field electrophoresis (4), and field inversion gel electrophoresis (FIGE) (3) in the comparative analysis of chromosomal restriction fragment length polymorphisms (RFLPs) generated by rare-cutting enzymes. While this approach has seen application with a variety of microorganisms (1, 2, 10–12, 14), its chief drawback has been the considerable period of time required for DNA preparation and electrophoretic analysis. We recently demonstrated the rapid electrophoretic separation of staphylococcal chromosomal restriction fragments by FIGE (7, 8). Others have published methods for the rapid preparation of chromosomal DNA from *Escherichia coli*, *Bradyrhizobium japonicum*, and *Bacillus subtilis* (5, 17) suitable for such electrophoretic analysis. We address both procedures here, reporting a 1-day protocol for the preparation of chromosomal DNA from gram-positive cocci (staphylococci and enterococci) coupled with a revised procedure for the rapid analysis of restriction fragments by FIGE. The method is demonstrated in the epidemiological analysis of two clusters (12 isolates) of *Staphylococcus epidermidis* previously described by Hébert et al. (9), but whose RFLPs were not analyzed, as well as unrelated isolates of other *Staphylococcus* species and enterococci.

MATERIALS AND METHODS

Bacterial strains. The 12 *S. epidermidis* isolates examined were previously identified and characterized as clusters from two different hospitals by Hébert et al. (9). From hospital A, four of five isolates (A1, A3, A4, and A5) were cultured from the pump blood of heart-lung bypass machines following cardiac surgery on different patients. The fifth hospital A isolate (A2) was obtained six days postoperation from a blood culture of the patient associated with the bypass machine yielding isolate A1. The hospital B isolates (B1 through B7) were from sequential blood cultures of one patient. Reference cultures included in this study were the type strains of *Staphylococcus aureus* (ATCC 12600), *Staphylococcus warneri* (ATCC 27836), and *Staphylococcus haemolyticus* (ATCC 29970). Clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium* (tested by the API Rapid STREP system [API Analytab Products]) were provided by the Clinical Microbiology Laboratory, St. Joseph Hospital, Omaha, Nebr.

Preparation of chromosomal DNA. For staphylococci, overnight agar cultures were inoculated (initial optical density at 540 nm [OD_{540}] = 0.1) into 10 ml of Trypticase soy broth (BBL Microbiology Systems) and incubated with shaking at 37°C to a final OD_{540} of ca. 0.7 (ca. 3×10^8 CFU/ml) over a time period of ca. 2 h, depending on the strains examined. The cells were harvested by centrifugation, washed with 10 ml of TEN buffer (0.1 M Tris [pH 7.5], 0.15 M NaCl, 0.1 M EDTA [Sigma]), and suspended in 20 ml of EC buffer (6 mM Tris HCl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij-58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine) (16). A 1.0-ml aliquot of the suspension was mixed with an equal volume of 2.0% SeaPlaque agarose (FMC Corp.) in EC buffer. Lysostaphin (1-mg/ml stock in water [Applied Microbiology, Inc.]) was then added (40 to 50 μl for coagulase-negative staphylococci, 10 μl for *S. aureus*) to the suspension, which was quickly mixed and immediately cast in blocks ca. 10 by 5 by 1 mm in size. For each strain, four to six blocks were incubated for 1 h at 37°C in 10 ml of EC buffer, during which time the blocks became clear in appearance. The liquid was decanted, and the blocks were suspended in TE buffer (13) and incubated with gentle shaking for 1 h at 55°C. The blocks were then transferred to fresh TE buffer for storage at 4°C until further analysis.

For *E. faecalis*, the procedure was as described above except that 100-μl aliquots each of lysozyme (20-mg/ml stock in water [Sigma]) and mutanolysin (10,000-unit/ml stock in water [Sigma]) were used instead of lysostaphin. *E. faecium* was generally handled in the same manner as *E. faecalis*, except that (i) Trypticase soy broth cultures were inoculated at an initial OD_{540} of ca. 0.25 to 0.3, (ii) Trypticase soy broth cultures were grown with rapid shaking to a final OD_{540} of 0.7 to 0.8, (iii) TEN and EC buffers were supplemented with $MgCl_2$ (final concentration = 1 mM), and (iv) lysozyme and mutanolysin were added to 1 ml of cell suspension in EC buffer, which was then incubated for 4 min at 37°C, followed by the addition of 1 ml of agar and casting in blocks.

Restriction endonuclease digestion and analysis by FIGE. Restriction endonuclease digestion was performed by transferring a single agarose block to a microcentrifuge tube containing 20 U of *Sma*I in 250 μl of restriction buffer. After 2 h of incubation at 27°C, the block was transferred to

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TABLE 1. Combined data on clusters of *S. epidermidis* isolates[a]

Hospital isolate no.	Biotype code	Plasmid profile	Antimicrobial profile
A1[b]	A3	A	A
A3[b]	A3	A	A
A4[b]	A1b	C1	C
A5[b]	A1b	C2	D
A2[c]	F2a	B	B
B1	A2a	A	A
B2	A2a	A	A
B4	A2a	A	A
B3	A2a	B	A
B7	A2a	E	A
B5	F1a	C	A
B6	F1a	D	A

[a] Adapted from Table 6 of Hébert et al. (9). See that study for a detailed explanation of antibiogram, plasmid profile, and biotype codes.
[b] Cultured from pump blood in heart-lung bypass machines.
[c] Isolated six days postoperatively from a blood culture of the patient associated with isolate A1.

modified TE buffer (10 mM Tris [pH 7.6], 20 mM EDTA) for storage at 4°C.

Chromosomal restriction fragment patterns were analyzed by field inversion gel electrophoresis (FIGE) (16 V/cm, 13°C) by using agarose minigels (0.8% SeaKem HGT, 15 by 10 cm [FMC Corp.]) in 0.5× TBE buffer as previously described (7, 8) except for the switching protocols. For restriction fragments >50 kb in size, initial 1.2- and 0.4-s forward and reverse pulses were linearly increased over 3 h to 12 and 4 s, respectively, followed by 0.75- and 0.25-s forward and reverse pulses, respectively, for 0.5 h. Restriction fragments <50 kb in size were separated by 0.4- and 0.2-s forward and reverse pulses, respectively, over a 3.25-h period.

RESULTS AND DISCUSSION

The utility of this method is demonstrated in the epidemiological analysis of the two *S. epidermidis* clusters described by Hébert et al. (9). From hospital A, five isolates (four from the pump blood of heart-lung bypass machines and one from a blood culture six days postoperation) were associated with four patients. In hospital B, seven isolates were obtained from multiple blood cultures of a single patient. Data from that study are summarized in Table 1 only to illustrate the previously proposed strain interrelationships. Thus, the reader is referred to Hébert et al. (9) for a detailed explanation of the antibiogram, plasmid profile, and biotype codes. It should be noted, however, that the antibiogram and plasmid profile codes (Table 1) were not originally intended for use in comparing isolates in different hospital groups (e.g., the type A plasmid profile of isolates from hospital A was different from that of isolates from hospital B).

As shown in Fig. 1, chromosomal RFLPs for isolates A1 and A3 (lanes 2 and 3) appeared identical, in agreement with the biotype, plasmid profile, and antibiogram data (Table 1). Different RFLPs were observed for isolates A4, A5, and A2 (Fig. 1, lanes 4 through 6), again in agreement with the previously reported data (although biotyping did not distinguish between isolates A4 and A5 [Table 1]). The chromosomal RFLPs of these five isolates thus support the interpretation of Hébert et al. (9) that, while the same *S. epidermidis* strain was cultured from pump blood on two occasions (isolates A1 and A3 [Fig. 1, lanes 2 and 3]), pump blood was not implicated as the source of postoperative infection occurring in one patient (isolate A1 versus A2 [Fig. 1, lanes 1 and 6]).

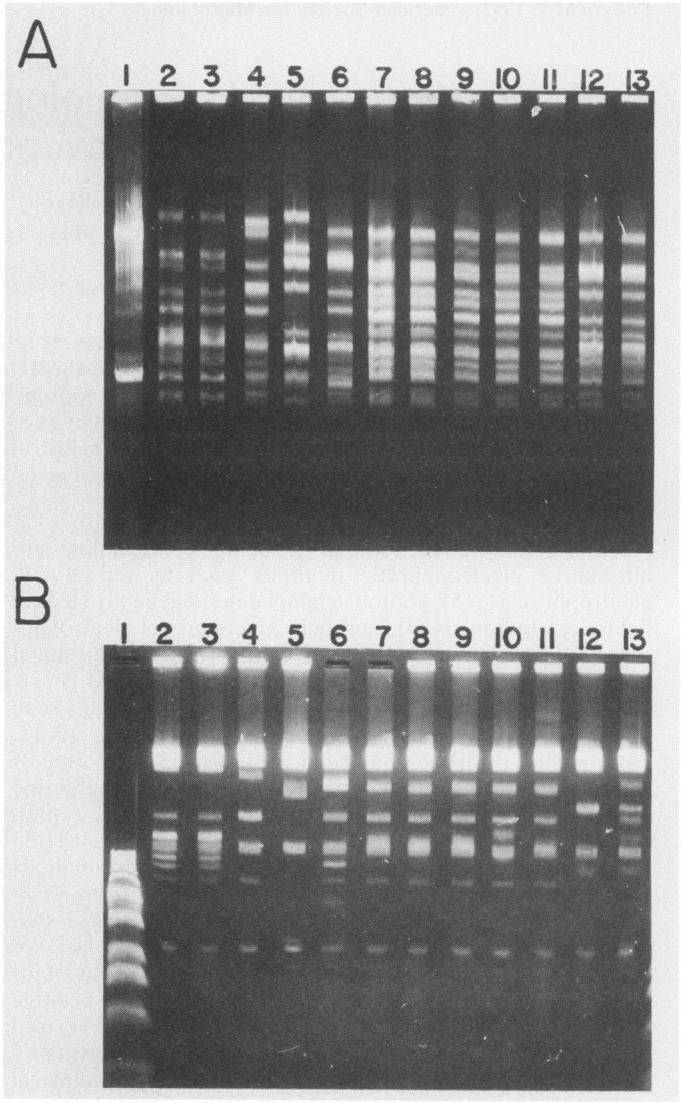

FIG. 1. FIGE of *Sma*I-digested chromosomal DNA >50 kb in size (A) or <50 kb (B) from *S. epidermidis* isolates A1 (lanes 2), A3 (lanes 3), A4 (lanes 4), A5 (lanes 5), A2 (lanes 6), B1 (lanes 7), B2 (lanes 8), B4 (lanes 9), B3 (lanes 10), B7 (lanes 11), B5 (lanes 12), and B6 (lanes 13). Molecular size standards are lambda (48.5-kb) oligomers (lane A1) and a 1-kb ladder (BRL) (lane B1). Isolates were arranged on the gels according to the interrelationships previously described within each hospital group (see the text and reference 9).

While antibiograms were not discriminatory for isolates from hospital B (Table 1), chromosomal RFLPs separated these isolates into two general relatedness groups, in good agreement with the biotype and plasmid data. In the first group, isolates B1, B2, and B4 (Fig. 1, lanes 7 through 9) appeared to be identical and highly related to B3 and B7 (Fig. 1, lanes 10 and 11). The latter two isolates differed only in chromosomal fragments <50 kb in size (Fig. 1B, lane 10) and in the absence of a ca. 400-kb fragment (Fig. 1A, lane 11), respectively. Biotyping identified, but did not discriminate within, this group of five isolates (Table 1). Plasmid profiles agreed with RFLPs in interrelating isolates B1, B2, and B4,

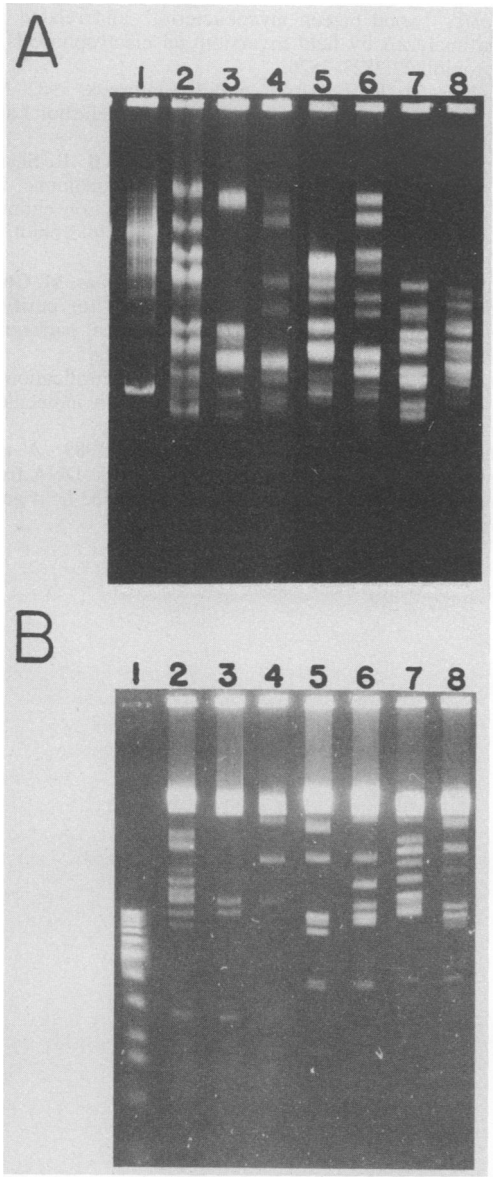

FIG. 2. FIGE of *Sma*I-digested chromosomal DNA >50 kb in size (A) or <50 kb (B) from reference strains of *S. aureus* (lanes 2), *S. warneri* (lanes 3), and *S. haemolyticus* (lanes 4) and from clinical isolates of *E. faecalis* (lanes 5 and 6) and *E. faecium* (lanes 7 and 8). Size standards are lambda (48.5-kb) oligomers (lane A1) and a 1-kb ladder (BRL) (lane B1).

while B3 and B7 were different (Table 1). Although we did not reconfirm plasmid profiles, the published data (9) suggest the presence of common plasmid bands in the type A, B, and E plasmid profiles of these isolates, thus supporting their potential relatedness. Chromosomal banding patterns for the second group of hospital B isolates, B5 and B6 (Fig. 1, lanes 12 and 13), appeared identical except for a minor difference in fragments <50 kb in size (Fig. 1B, lanes 12 and 13). Again, different but potentially related plasmid profiles support the interrelationship of these isolates (9) (Table 1). As with the previous hospital B isolates, biotyping identified but did not discriminate within this relatedness group. Taken together, these data suggest that the seven *S. epidermidis* isolates from this patient could represent two strains, exhibiting the dynamics of plasmid carriage and minor chromosomal variation over time.

Figure 2 illustrates the applicability of the method described above to the analysis of other species of gram-positive cocci as demonstrated by chromosomal banding patterns for the type strains of *S. aureus* (lanes 2), *S. warneri* (lanes 3), and *S. haemolyticus* (lanes 4) and the clinical isolates of *E. faecalis* (lanes 5 and 6) and *E. faecium* (lanes 7 and 8), data typical of those obtained from the analysis of hundreds of isolates.

Side-by-side FIGE comparisons of chromosomal restriction fragment patterns produced by this method versus those produced by the longer conventional procedure showed that the patterns were indistinguishable from one another (data not shown). In general, *Sma*I-digested DNA preparations were stable for ca. 1 week, depending on the organism, their ultimate deterioration owing perhaps to a combination of residual nuclease activity (despite the elevated concentration of EDTA in the modified TE holding buffer) and/or diffusion of restriction fragments out of the agarose blocks (6). However, this was not viewed as a serious disadvantage given the ease and low cost of the method and the stability of whole, undigested, chromosomal DNA in the agarose blocks, which yielded reproducible *Sma*I banding patterns over a period of several weeks.

When performed as described above, chromosomal DNA preparation and restriction endonuclease digestion may be accomplished in a single day. FIGE analysis may be similarly completed (3.5 and 3.25 h for restriction fragments >50 kb and <50 kb in size, respectively), often with a good preliminary indication of strain relatedness after only the 3.5 h (>50 kb fragment size) gel. Thus, the combined procedure allows isolates of interest to be comparatively analyzed with an overall time investment of ca. 2 days from start to finish. It is our hope that this result will serve to further stimulate interest in applying the power of FIGE and other alternative electrophoretic methods to the epidemiological analysis of problem microorganisms, which most certainly include the gram-positive cocci.

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