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Application of small fragment restriction endonuclease analysis (SF-REA) to the epidemiological fingerprinting of *Staphylococcus aureus*

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Summary. Total cell DNA of 14 isolates of *Staphylococcus aureus* from patients of an intensive care unit (ICU) and 180 unrelated strains was examined by restriction endonuclease analysis (REA). *Eco*RI-generated DNA fragments were either subjected to conventional REA on agarose gels and stained with ethidium bromide or separated by polyacrylamide gel electrophoresis and visualised by silver staining (SF-REA). Both methods were compared for inter-strain discriminatory ability, reproducibility and handling. All DNA-cleavage patterns of unrelated strains clearly differed from each other when subjected to SF-REA. In contrast, all *S. aureus* isolates from the ICU gave identical restriction fragment patterns. These findings supported the suspicion of nosocomial infection in these patients. Conventional REA proved the identity of the ICU isolates, but it failed to differentiate between some of the unrelated strains. Therefore SF-REA of total cell DNA seemed to be superior. It has proved to be a very useful technique for studying the epidemiology of *S. aureus* in hospitals.

Introduction

Epidemiological investigations and analysis of nosocomial outbreaks of *Staphylococcus aureus* infection often are hampered by the relatively poor discriminatory properties of conventional marker systems such as antibiograms, phage typing, and plasmid profile analysis. Immunoblotting techniques seem promising, but they have some disadvantages: their reproducibility is linked to standardised antibody probes[1] and they recognise phenotypic rather than genomic characters.

We report the use of restriction enzyme digests of total cell DNA to recognise polymorphisms in restriction fragment lengths. In particular, the analysis of small fragments within the cleavage patterns may be a useful method for typing *S. aureus* isolates.

Restriction endonuclease analysis (REA) has been applied successfully to investigate phenotypically closely related strains of *Borrelia burgdorferi*,[2] *Mycobacterium* sp.,[3] *Pseudomonas aeruginosa*,[4] *Vibrio cholerae*,[5] *Legionella pneumophila*,[6] *Helicobacter pylori*,[7] *Neisseria gonorrhoeae*,[8] *N. meningitidis*,[9] *Clostridium difficile*,[10] group B streptococci,[11] *Staphylococcus epidermidis*[12] and even *S. aureus*.[13] Separation of restriction fragments is mostly performed by agarose gel techniques. These procedures are often time-consuming. Furthermore they depend on restriction enzymes which cut rarely and generate relatively large fragments, because small and very small fragments cannot be well resolved. It is difficult and sometimes impossible to evaluate small DNA-fragment patterns on agarose gels when outstanding markers or differences among the larger fragments cannot be detected. Employing polyacrylamide gel electrophoresis (PAGE) and silver staining we established a rapid and sensitive method for generating strain-specific, reproducible, well focused, and durable restriction patterns of small DNA fragments.

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Materials and methods

Bacterial strains

Staphylococci were isolated from clinical samples submitted routinely to our diagnostic laboratory. *S. aureus* was identified by conventional methods.[14] Of 194 strains examined, 14 were isolated from blood cultures, central venous catheters, tracheal aspirates and surgical wound swabs from seven patients in an ICU of one hospital (for details see table); 180 epidemiologically unrelated *S. aureus* isolates from patients of 15 different hospitals, including the one where the outbreak occurred, were collected over a period of 4 months. All strains were

Table. *S. aureus* isolates from the ICU

Sample no.	Date of isolation	Patient	Sample
1	05/22/89	A	Surgical wound
2	05/22/89	B	Penis swab
3	05/22/89	B	Tracheal aspirate
4	05/22/89	C	Tracheal aspirate
5	05/23/89	B	Central venous catheter
6	05/23/89	C	Central venous catheter
7	05/24/89	D	Central venous catheter
8	05/25/89	B	Tracheal aspirate
9	05/29/89	B	Surgical wound
10	05/29/89	B	Tracheal aspirate
11	06/05/89	C	Tracheal aspirate
12	06/27/89	E	Blood culture
13	06/27/89	F	Surgical wound
14	06/28/89	G	Blood culture

isolated by plating the samples on sheep blood agar. A single colony was grown overnight in 200 ml of Tryptone Soya Broth (Oxoid, CM129) at 36°C, harvested by centrifugation at 5000 *g*, washed twice in Tris-EDTA buffer, pH 8·0 (10 mM Tris-HCl, 1 mM disodium-EDTA), and stored at −20°C until tested.

Antibiograms

Antimicrobial susceptibility was determined by the disk diffusion method on Antibiotic-Sulphonamide-Sensitivity-Test Agar (Merck, 5392) with overnight incubation at 36°C.[15] The following antimicrobial agents were tested (μg/disk): penicillin (10), oxacillin (1), ampicillin (10), cephazolin (30), cefuroxime (30), cefoxitin (30), cefotaxime (30), cefaclor (30), gentamicin (10), amikacin (10), tetracycline (30), chloramphenicol (30), co-trimoxazole (25), ofloxacin (5), ciprofloxacin (5), erythromycin (15) and clindamycin (10).

DNA isolation

Total cell DNA was extracted from 300 μl of packed cells. Staphylococci were lysed by a modification of the procedure described by Harstein *et al.*[16] Briefly, cells were suspended in 2 ml of lysostaphin buffer (2·5 M NaCl, 10 mM disodium-EDTA, pH 8·0) and 50 μl of lysostaphin 1 mg/ml in Tris-EDTA buffer, pH 8·0 (Sigma, 7386) was added. After incubation for 60 min at 37°C, lysis was achieved by adding of 12 ml of lysing solution [dodecyltrimethylammonium bromide (Sigma, D-8638) 0·5%+ Triton X-100 (Serva, 37240) 0·25%]. The tubes were mixed gently and incubated for 15 min at 56°C. Mixtures were then extracted twice with 15 ml of phenol: chloroform (1:1, v:v). DNA from the upper phase was precipitated with an equal volume of isopropanol, washed in ethanol 70%, dissolved in 300 μl of Tris-EDTA buffer, pH 7·5, and stored at 4°C.

Restriction endonuclease digestions

DNA (5 μg) was completely digested in a reaction mixture containing a 10-fold excess of *Eco*RI (New England Biolabs, Inc., 101) in *Eco*RI buffer (50 mM NaCl, 100 mM Tris-HCl, pH 7·5, 5 mM $MgCl_2$, bovine serum albumin 100 μg/ml) at 37°C for 2 h. Digestions were terminated by adding an equal volume of gel loading buffer (Ficoll 400 15% w/v + Orange G 0·05% w/v in 10 mM disodium-EDTA, pH 8·0).

Polyacrylamide gel electrophoresis

PAGE was performed on an SE 250 Mighty Small II, 7-cm Vertical Dual Slab Unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The gels were cast in an SE 215 Mighty Small Multiple Gel Caster. Eight gels (0·75-mm thick) were prepared at once from an acrylamide 30%—bis-acrylamide 1·6% stock solution (Serva, 10675 and 29195) in TBE buffer (0·09 M Tris, 0·09 M boric acid, 0·002 M disodium-EDTA, pH 8·3). Before samples were loaded the slots were rinsed twice with tank buffer. Cells were then loaded with 1–20 μl of DNA digest. The gels were run at 18 mA constant current for 60 min in TBE buffer.

Agarose gel electrophoresis

DNA fragments were separated by electrophoresis on horizontal agarose 0·7% gels (Serva, 11404) for 20 h at 40 V in TBE buffer. Gels were stained with ethidium bromide 1 μg/ml for 30 min, briefly rinsed in water, and photographed under UV illumination. Phage λ DNA *Bst*EII digest (New England Biolabs, Inc., 301-4) served as a molecular size standard.

Silver staining

Polyacrylamide gels were stained by a modification of the silver staining procedure of Tsang *et al.*[17] The gels were treated successively with aqueous trichloroacetic acid 10% for 15 min and ethanol: acetic acid: distilled water (20:5:75 v:v:v) for 15 min. To clear up the background they were incubated for 5 min in aqueous potassium dichromate 0·5% solution and then rinsed three times with distilled water. Impregnation was performed in aqueous silver nitrate 0·1% solution for 10 min. After rinsing twice with water, colour was developed in an aqueous mixture of sodium carbonate 3% supplemented with paraformaldehyde 0·002%. The colour reaction was terminated by a short rinse with distilled water and addition of 100 ml of acetic acid 1%. Gels were stored in the same solution. They were stable for months without any loss of colour when kept in the dark.

Results

DNA extraction, restriction endonuclease digestion, and electrophoresis in polyacrylamide 5% gels

of 194 clinical isolates of *S. aureus* revealed clear gel patterns, consisting of multiple bands, varying in intensity and number. About 40 bands were identified after *Eco*RI digestion. All isolates were typable by this method. Unrelated *S. aureus* strains displayed a marked degree of polymorphism, particularly evident in the small fragments. Fig. 1 shows typical results of *Eco*RI DNA digests from epidemiologically unrelated *S. aureus* strains. Isolates 1–13 had identical antibiotic susceptibility profiles. They were resistant to penicillin and ampicillin, but sensitive to all other antimicrobial agents tested. Track 14 shows the DNA cleavage pattern of the epidemic strain from the ICU.

Fragments of 1000–4000 bp were well separated in polyacrylamide gels, yielding sufficient information for reliable identification of each isolate. The cleavage patterns of unrelated isolates clearly differed from each other. Each isolate produced its own unique "fingerprint". The cleavage patterns show a broad band in the upper third of the gel, containing large restriction fragments which cannot be resolved by polyacrylamide gel electrophoresis. They could be separated sufficiently, when subjected to electrophoresis in agarose 0·7% gels.

The method gave good reproducibility; three *Eco*RI digests performed on separate DNA extractions of the same strain produced identical restriction-fragment patterns, and even after 1, 20, 40 and 60 subcultures of a single strain, all *Eco*RI generated DNA cleavage patterns were identical, when electrophoresed on the same gel, confirming genetic stability of the clone examined.

These findings convinced us that SF-REA may be useful in investigating the transmission and acquisition of *S. aureus* within small human population groups and cohorts, such as hospitalised patients in wards and ICUs. Fourteen *S. aureus* isolates from clinical samples of seven patients from an ICU were collected over a 5-week period. In two cases *S. aureus* was isolated repeatedly from the same patients. All isolates were resistant to penicillin, ampicillin, gentamicin, amikacin, tetracycline, chloramphenicol, co-trimoxazole, ofloxacin, ciprofloxacin and erythromycin, but sensitive to oxacillin, cephazolin, cefuroxime, cefoxitin, cefotaxime and clindamycin; 10 isolates were resistant to cefaclor.

Fig. 2 shows the *Eco*RI-generated DNA-cleavage patterns of the 14 *S. aureus* isolates from the ICU. No differences between these isolates were detected, suggesting that all isolates were genetically identical and of common origin. With REA on agarose 0·7% gels, all isolates from the ICU also gave identical *Eco*RI restriction-fragment patterns, confirming the results of SF-REA, but considerable difficulties occurred in evaluating the profiles of unrelated strains. Because there were no outstanding markers within the relatively small number of well-focused bands of some profiles, restriction-fragment pat-

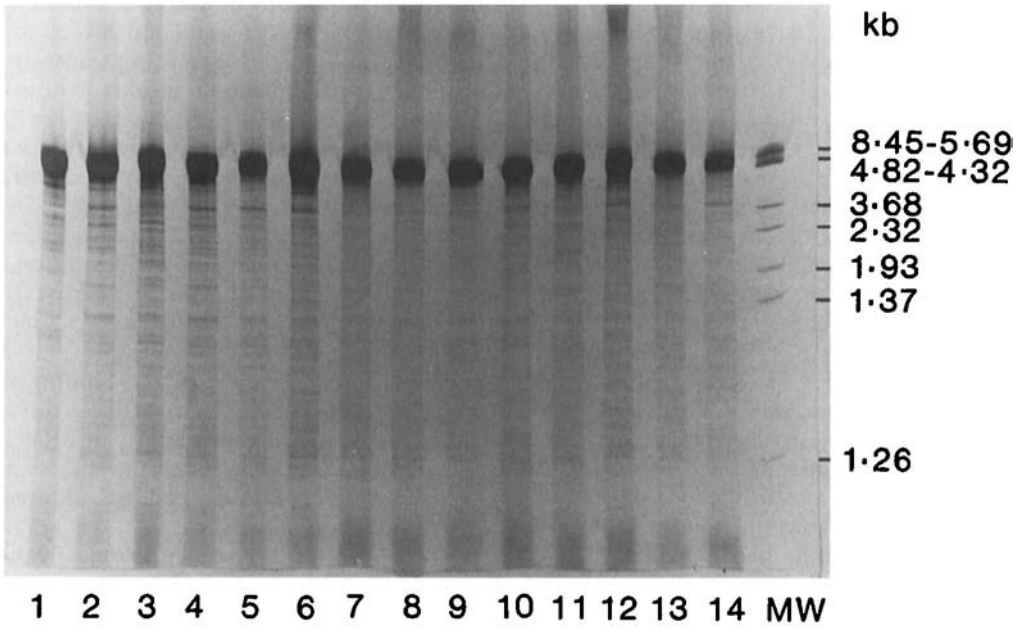

Fig. 1. *Eco*RI digested *S. aureus* DNA after electrophoresis in polyacrylamide 5% gel and silver staining. Tracks 1–13, unrelated strains; 14, epidemic strain from the ICU; MW, phage λ DNA, *Bst*EII digest.

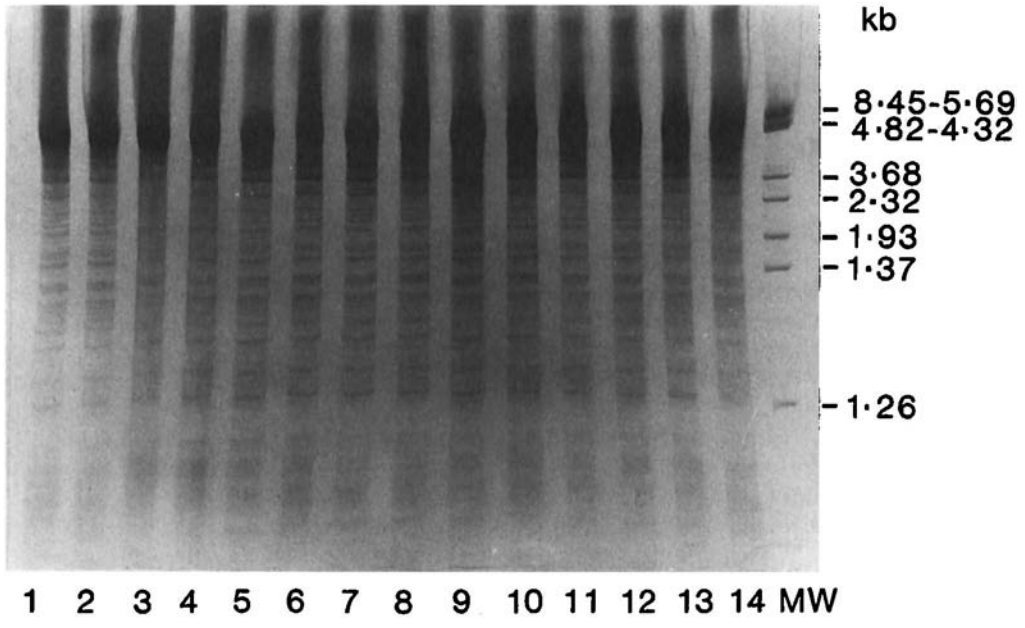

Fig. 2. *Eco*RI digested *S. aureus* DNA after electrophoresis in polyacrylamide 5% gel and silver staining. Tracks 1–14, isolates from the ICU (track number corresponds to sample number in table); MW, phage λ DNA, *Bst*EII digest.

terns looked rather similar. Therefore, reliable discrimination of these unrelated isolates by conventional REA was not possible.

Discussion

Antibiotic susceptibility testing, phage typing, and plasmid-pattern analysis have been standard methods for examining relatedness among *S. aureus* isolates. The antibiogram has proved to be useful in epidemiological studies, but it is often unstable and closely linked to the plasmid content.[18] Unfortunately, phage typing is available only at a few centres. Recourse to a reference laboratory takes time and, therefore, does not allow the rapid instigation of control measures. Even using the international set of phages as amended by the International Subcommittee of Phage-typing of *S. aureus* in 1975, 27–75% of epidemic *S. aureus* isolates remain untypable[19,20] at RTD and 100 RTD. Plasmid analysis seems to be superior in some cases,[21] but it depends on the presence of episomal DNA. Harstein *et al.*[16] reported that 34% of the *S. aureus* isolates examined in their study did not have any detectable plasmid DNA. Even immunoblot fingerprinting as a phenotype-based typing system has some disadvantages. It depends strongly on the antiserum used for the detection of staphylococcal antigens and cannot be easily standardised for general use. Antigenic variability also may render the interpretation of results more difficult.[22]

The results of this study clearly demonstrate that SF-REA of total cell DNA can be used as a reliable technique to identify *S. aureus* strains. The ability to distinguish between unrelated isolates makes SF-REA a useful tool for examining transmission and acquisition of *S. aureus* strains within human populations. Burnie *et al.*[22] reported the relatively low inter-strain discriminatory ability of whole cell REA performed in agarose gels in comparison with immunoblot fingerprinting. This finding was confirmed by our results. With polyacrylamide gel electrophoresis, a much higher resolution of small fragments is achieved than in agarose gels. The large number of well-focused bands can be used for more reliable strain discrimination that does not depend on the presence of outstanding markers, which may be absent. These qualities convinced us of the discriminatory superiority of SR-REA over conventional REA. We employed SF-REA to detect and confirm a nosocomial outbreak of *S. aureus* infection in an ICU.

As demonstrated, epidemiologically unrelated strains, with identical antibiograms, were clearly distinguished by this method. Although large fragments were not resolved in polyacrylamide gels, the analysis of small cleavage fragments of 1000–

4000 bp yielded sufficient information for reliable discrimination of all isolates examined. In contrast, all isolates from an ICU of one hospital exhibited identical *Eco*RI DNA-cleavage patterns. These findings strongly suggested that all isolates were genetically identical. The method was applied successfully to follow intra- and inter-individual spread. We demonstrated the transmission of one *S. aureus* strain between seven patients and its continuance for 1–2 weeks in two patients.

Reproducibility of the method and stability of the organisms examined are major factors for evaluating a typing system. We demonstrated genetic stability of *S. aureus* during 60 subcultures. Reproducibility of DNA extraction, restriction endonuclease digestion, electrophoretic separation on polyacrylamide gels, and silver staining was also good. SF-REA does not require expensive equipment, ultracentrifugation, time consuming agarose-gel electrophoretic techniques or blotting. Results can be achieved within 12 h from the point of cell harvest. Up to 20 samples can be processed easily in one working day. Therefore, SF-REA can support hospital infection control effectively.

Another advantage of this method is the stability of silver-stained polyacrylamide gels. They are stable for months. Original gels of different working periods can be compared directly. We conclude that SF-REA is a simple, rapid and accurate method for specifying the identity of *S. aureus* strains. It is especially useful for examining nosocomial outbreaks and person-to-person transmissions. The method is discriminatory, reproducible and suitable for routine laboratory use.

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