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Analyzing Multiclonality of *Staphylococcus aureus* in Clinical Diagnostics Using *spa*-Based Denaturing Gradient Gel Electrophoresis▽

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We present a novel denaturing gradient gel electrophoresis (DGGE) method which characterizes multiclonal communities of *Staphylococcus aureus*. The *spa* PCR-based DGGE method simultaneously separates strains that differ in only one base, thereby revealing multiclonal colonization and infections.

Staphylococcus aureus causes a wide range of infections and is responsible for a considerable portion of hospital-acquired infections.

In 30% to 70% of healthy individuals, *S. aureus* is a transient or persisting part of the residential flora (5, 10).

Cespedes et al. investigated the frequency of simultaneous nasal carriage of multiple *S. aureus* strains by picking three bacterial colonies from plates derived from each colonized individual. Fewer than 7% of them were predicted to carry >1 strain (1). The simultaneous presence of an invasive and a carrier strain of methicillin-resistant *S. aureus* (MRSA) in one individual was reported by Soderquist and Berglund (12). The issue of multiclonal colonization is important, but conventional laboratory methods for detection of *S. aureus* are based on culture of a single colony. This might result in the identification of an antibiotic-susceptible commensal strain rather than a second, more resistant strain, which may impair the antibiotic treatment and bias epidemiological conclusions.

We have developed a species-specific denaturing gradient gel electrophoresis (DGGE) method for *S. aureus*, utilizing *spa*, to characterize multiclonal colonization and infection. The novel assay was used to investigate a MRSA outbreak, revealing colonization with two different strains in some of the individuals.

spa typing has been documented to be a useful tool in investigations of MRSA epidemiology (7) and for studies of *S. aureus* transmission (6). Thus, we based our DGGE method on *spa* and primer pairs, described by Kahl et al. (4), which were modified for DGGE analyses by the attachment of a GC clamp (11) at either the forward or the reverse primer. Annealing temperature and $MgCl_2$ concentrations for two primer combinations were optimized for PCR specificity and efficiency (data not shown).

Eight *S. aureus* isolates of known *spa* types were acquired from the Microbiology Laboratory, Ryhov County Hospital, Jönköping, Sweden. All isolates contained 10 *spa* repeats (Table 1). The isolates were suspended in 200 μl PCR-grade water (Sigma-Aldrich, St. Louis, MO) and lysed for 10 min at 95°C. DNA was purified using the MagAttract DNA Mini M48 kit on BioRobot M48 (Qiagen, Hilden, Germany).

The most stringent separation of the PCR products (not shown) was achieved when the GC clamp was attached to the forward primer spa-1113f-GC. The optimized PCR mixture contained 12.5 μl HotStar Mastermix (Qiagen), 1.5 mM $MgCl_2$ (Roche, Mannheim, Germany), 0.2 nmol forward primer spa-1113f-GC (5′-**CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCG**TAAAGACGATCCTTCGGTGAGC-3′; GC clamp indicated in bold), and 0.2 nmol reverse primer spa-1514r (5′-CAGCAGTAGTGCCGTTTGCTT-3′) (TIB Molbiol, Berlin, Germany). The reaction conditions were 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 61°C, and 60 s at 72°C, with a final extension for 10 min at 72°C.

The PCR products were analyzed using a DCode universal mutation detection system (Bio-Rad Laboratories Inc., Hercules, CA). Polyacrylamide gradient gels (160 by 160 by 1 mm) composed of 37.5:1 acrylamide-bisacrylamide (7%) and 1× TAE (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA [pH 8.3]) with 15% to 50% denaturant were cast with the aid of a gradient former (Bio-Rad Laboratories Inc.). The gels were polymerized with 10 μl of TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) and 173 μl of 10% ammonium persulfate. Two hundred forty nanograms of DNA was loaded into each well, and the gels were run in 1× TAE buffer at 62°C for 14 h at 130 V. Gels were stained in 1× TAE buffer containing SYBRGold (Invitrogen, Paisley, United Kingdom) for 40 min and visualized in UV transillumination using a charge-coupled device (CCD) camera (LAS-3000; FujiFilm, Tokyo, Japan).

The detection limit was 20 gene copies of *spa* per reaction (not shown), and the method could discriminate PCR products of the same length from a mixture of eight strains (Fig. 1). In the developed *spa*-DGGE assay, it was possible to simultaneously amplify DNA of two different *spa* types (t064 and t355, respectively) in a mixture with a concentration difference of 1:1,000 (not shown). By using conventional cultivation meth-

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TABLE 1. *spa* types and repeat succession of *S. aureus* strains used in the evaluation of the *spa*-DGGE method

spa type	Repeat succession
t015	08-16-02-16-34-13[a]-17-34-16-34
t050	08-16-02-16-34-34[a]-17-34-16-34
t008	11-19-12-21[a]-17-34-24-34-22-25
t064	11-19-12-05[a]-17-34-24-34-22-25
t002	26-23-17-34-17-20-17-12-17-16
t012	15-12-16-02-16-02-25-17-24-24
t355	07-56-12-17-16-16-33-31-57-12
t3061	07-21-17-34-13-34-34-13-33-13

[a] Repeat numbers 13 and 34 as well as 5 and 21 differ in only one base, respectively.

ods, an extreme number of colonies would be required to achieve a comparable sensitivity to detect multiclonality.

An outbreak of MRSA occurred at a nursing home in 2009. Screening for possible colonization of patients ($n = 24$), staff ($n = 50$), and family members of colonized staff ($n = 6$) was performed. Swab (Copan, Brescia, Italy) samples ($n = 229$) from throat, anterior nares, and groin were cultured in broth, and the presence of MRSA was verified in 37 broth samples from 12 individuals, by detection of *nuc* and *mecA* according to the method of Nilsson et al. (9). One MRSA isolate from each individual was *spa* typed as described previously (3, 4). Two different but closely related *spa* types were isolated from the 12 individuals (4 patients, 4 staff, and 4 relatives of staff) of the outbreak. Nine individuals were colonized with *S. aureus* of *spa* type t015, and three were colonized with t069 isolates.

One milliliter of broth samples was centrifuged (10,000 × *g*, 3 min), and DNA extraction was performed as described above. When 28 available broth samples positive by culture for MRSA were analyzed by *spa*-DGGE, all samples were confirmed to contain either *spa* type t015 or *spa* type t069. Besides, in broths from three individuals, *spa* types t015 and t069 were detected simultaneously (Fig. 2). Dual colonization of MRSA was indicated in samples from the groin and the throat in one of these individuals and in the throat of a second. The remaining samples from these two individuals contained only *spa* type t069. In the third individual, the samples from throat and nares contained t069 and t015, respectively.

S. aureus multiclonality is rarely studied, although multiclonal infections occur and such infections may indeed affect the selection of antimicrobial therapy and might impede the treatment of serious infections (2). Cespedes et al. showed by culture of three colonies from each sample that fewer than 7%

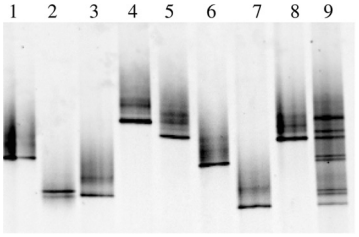

FIG. 1. *spa*-DGGE analysis of the eight different strains (lanes 1 to 8, t064, t355, t012, t3061, t050, t008, t002, and t015, respectively; lane 9, a mixture of equal amounts of eight PCR products).

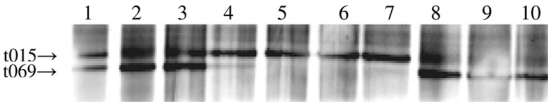

FIG. 2. Broth samples from the outbreak containing MRSA isolates of *spa* type t015 or t069 were analyzed by the *spa*-DGGE method. In samples from two individuals, both *spa* types, t015 and t069, were detected (lanes 2 and 3). In lanes 4 to 7, the samples contain *spa* type t015, and in lanes 8 to10, the samples contain *spa* type t069. In lane 1, PCR products from both *spa* types, t015 and t069, are mixed and analyzed as a control.

of the population was colonized by more than one strain in the anterior nares (1). However, their approach will probably reveal only major clones. Mongkolrattanothai et al. found two genetically distinct *S. aureus* strains in 25% of nasal and perianal swab samples from children when 4 to 15 colonies were picked from each culture (8). Thus, there is, using culture, still a risk of underestimating the diversity of *S. aureus*.

To conclude, we describe a sensitive molecular method with high discriminatory power useful in clinical samples for multiclonal characterization of *S. aureus* colonization and infection. The method offers the potential to become a valuable epidemiological tool as well as a tool for the investigation of *S. aureus* infections.

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