NOTES

Binary Typing of *Staphylococcus aureus* Strains through Reversed Hybridization Using Digoxigenin-Universal Linkage System-Labeled Bacterial Genomic DNA

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A novel binary typing (BT) procedure, based on reversed hybridization of digoxigenin-universal linkage system-labeled bacterial DNA to strip-immobilized probes, is presented. Chromogenic detection of hybrids was performed. *Staphylococcus aureus* isolates (n = 20) were analyzed to establish the feasibility of BT. A technically simple and fast procedure has been developed for application in routine microbiology laboratories.

Reliable probe-based microbial typing systems are not yet commonplace in microbiological practice (1, 4, 7, 8, 12). In the past we identified domains that are differentially present within the staphylococcal genome on the basis of randomly amplified polymorphic DNA analysis. These probes were used to develop a DNA probe-based typing approach. The strain-specific DNA probes provide a simple binary output and have been presented before (13–15). We describe here the development of a new format for the binary typing technique. In the newly described procedure DNA is extracted from overnight *Staph-

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* Data are from reference 10. Abbreviations: MRSA, methicillin-resistant *S. aureus*; PFGE, pulsed-field gel electrophoresis; TAR916 SHIDA, Transposon 916, Shine Dalgarno; AP PCR, arbitarily primed PCR; RAPD, randomly amplified polymorphic DNA analysis (ERIC2, AP1, and AP2 are primers); NT, not typeable.

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Staphylococcus aureus cultures, labeled with the digoxigenin (DIG)-
universal linkage system (ULS) (9), and reversibly hybridized
to strips containing the immobilized probes. Signal is gener-
ated by chromogenic staining, and binary types can be read
visually. This novel binary typing system will be introduced,
and its versatility will be discussed.

Well-characterized strains of S. aureus (n = 20) were ob-
tained from a reference collection (10). Isolates were cultured
onto blood agar plates at least once, and single colonies were
used for further testing.

DNA was isolated (i) by the protocol described by Boom et
al. (3), (ii) with a miniprep of bacterial genomic DNA with a
cetyltrimethylammonium bromide-NaCl solution (2), (iii) by
extraction with phenol-chloroform (5), and (iv) by proteinase
K-sodium dodecyl sulfate (SDS)-treatment and boiling.
Another method (method vi) consisted of simple DNA isolation by
10 min of boiling only. An alkaline method (method vii) for
DNA extraction was used as well. Finally, a method (method
vii) used the Wizard genomic DNA purification kit. The ex-
traction of staphylococcal DNA was performed according to
the manufacturer’s instructions. The DNA size distribution
and the concentrations of all preparations were estimated by
gel electrophoresis and were compared to those of a reference
series of bacteriophage λ DNA (5). DNA was stored at −20°C
for labeling and hybridization experiments.

For the optimization of DNA labeling, purified S. aureus
DNA was labeled by using three different ratios (micrograms
of DNA versus units of ULS), namely, 1:1, 1:2, and 1:4 (digox-
genin-ULYSIS nucleotide labeling kit; KREATECH Diag-
nostics, Amsterdam, The Netherlands). A serial dilution of
labeled DNA from all strains (1 μl) was spotted onto a mem-
brane to check the labeling efficiency. The procedures for the
generation, validation, and application of the 12 strain-specific
DNA probes have been described in detail elsewhere (13–15).

Purified DNA probes (n = 12) were spotted (1 μl) onto a
membrane strip (5 by 80 mm; Hybond N+; Amersham Life
Science, Little Chalfont, United Kingdom). For hybridization
quality control, 10 ng of petunia DNA (LMC 1322) and 0.25 ng
of the S. aureus amplified nuc gene (6) were spotted as negative
and positive hybridization controls, respectively. The DNA
probes were denatured by 0.5 M NaOH–1.5 M NaCl treatment
for 15 min at room temperature. Subsequently, the strips were
neutralized with 0.5 M Tris-HCl–1.5 M NaCl (pH 7.4) for 15
min at room temperature (RT). The strips were washed with
2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate
(5), and the denatured DNA was cross-linked on the strip with
UV light (280 nm). Each strip was transferred into a Greiner
tube (15 ml), and 1 ml of DIG Easy Hyb buffer (Roche Mo-
olecular Biochemicals, Almere, The Netherlands) was added.
The labeled DNA was denatured for 5 min at 100°C and
quenched on ice. The sample was centrifuged to collect con-
densate, and 1 ml of DIG Easy Hyb buffer was added. The
labeled DNA was then added to the hybridization mixture, and
the mixture was incubated overnight at 42°C in a rotation oven.
After hybridization, the strips were washed twice in 2× SSC–
0.1% SDS for 5 min each time at RT and twice in 0.5× SSC–0.1%
SDS for 15 min each time at 68°C. The strips were
processed by the protocol accompanying the Roche Molecular
Biochemicals hybridization kit. After posthybridization wash
steps, the strips were equilibrated in 1× maleic buffer for 1
min. The strips were blocked for 30 to 60 min at RT with 1×
blocking buffer under slight agitation. The strips were incu-
bated with conjugate (anti-DIG alkaline phosphatase conju-
gate, 150 mU/ml) for 10 min at RT. The strips were washed
twice for 15 min each time in 1× wash buffer at RT and were
equilibrated twice for 5 min each time in detection buffer.
Finally, the strips were incubated in freshly prepared color
substrate (nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-
phosphate ready-to-use tablets; Roche Molecular Biochemi-
cals) for a maximum of 3 h at RT in the dark. Hybridization of
labeled genomic DNA with each of the 12 different DNA
probes (probes AW-1 through AW-12) was scored with a 1 or
a 0 according to the presence or absence of the hybridization
signal, respectively. The combination of hybridization results
forms the binary type.
The current conditions for the binary typing procedure are summarized below and are outlined in Fig. 1. Genomic staphylococcal DNA was purified with the Wizard genomic DNA purification kit and labeled with DIG-ULS (target DNA versus label at a ratio of 1:1). A concentration of 200 ng of labeled DNA per ml of hybridization buffer was used.

The hybridization results for the *S. aureus* strain collection, determined by the currently developed reversed hybridization procedure, are depicted in Fig. 2 and are specified in Table 1. Strains 1 to 5 (Fig. 2, lanes 1 to 5, respectively) showed identical binary codes. Strains 6 to 10 (Fig. 2, lanes 6 to 10, respectively), representing genetically related strains, displayed binary patterns that varied for no more than 2 of the 12 probes. Of the 10 unique strains (Fig. 2, lanes 11 to 20, respectively), all except strains 18 and 19 exhibited unique binary codes.

Here we present a new format of binary typing as an example of a single-species typing test developed for the characterization of *S. aureus* strains. In previous studies, we defined the resolution, reproducibility, and stability of the epidemiological markers of the binary typing technique (11, 13–15). Technically speaking, the initial method, which involves repeated hybridization of the probe to digested staphylococcal DNA, was complex and time-consuming. We therefore developed a simple and fast format for the characterization of *S. aureus* strains based on reversed hybridization with 12 strip-immobilized DNA probes. The major advantage of ULS labeling for this application is the direct labeling of culture-amplified total genomic *S. aureus* DNA (target labeling). The additional value of binary typing is its simplicity, speed, reproducibility, and lack of need for expensive peripheral equipment. The efficiency of the labeling reaction with DIG-ULS (9) depends on the time, the temperature, the label-to-DNA ratio, and the purity of the DNA. A simple and standardized procedure resulted in optimal labeling and hybridization results. Hybridization conditions and probe and target concentrations were optimized.

The data obtained by the novel binary typing protocol are identical to those that were obtained by the conventional binary typing method. Epidemiologically linked strains were again identified as clusters, whereas unique isolates were well differentiated. The reversed hybridization data are corroborated by those obtained by other typing techniques such as pulsed-field gel electrophoresis and randomly amplified polymorphic DNA analysis (Table 1).

The new binary typing protocol described here provides a simple and fast probe-based molecular typing strategy for the characterization of *S. aureus* strains and generates easily interpretable results, which can be compiled in a database. This culture-amplified nucleic acid probe technology can be developed for the characterization of other species and can be easily expanded with probes that directly detect genes associated with virulence factors and resistance determinants. It may be concluded that this technique comprises a compact, user-friendly *S. aureus* typing system suitable for use in the development of an international database for both methicillin-susceptible and methicillin-resistant *S. aureus* strains. In addition, the binary typing system has the clear potential to be useful in peripheral laboratories as well. The interlaboratory reproducibility of the assay is currently the subject of a multicenter study.

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REFERENCES


FIG. 2. Binary typing results for the methicillin-resistant *S. aureus* strain collection obtained after hybridization on the strip-immobilized DNA probe panel (probes AW-1 through AW-15).