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Abstract

A total of 56 *Staphylococcus aureus* isolates incubated for 2 h in the presence or absence of oxacillin were analyzed by flow cytometry after labeling with an *S. aureus*-specific peptide nucleic acid (PNA) probe. Two defined ratios, the paired signal count ratio (PSCR) and the gate signal count ratio (GSCR), differentiated methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) with sensitivities of 100% each and specificities of 96% and 100%, respectively.

Disciplines

Food Science | Human and Clinical Nutrition

Comments

Rapid Identification of *Staphylococcus aureus* and Methicillin Resistance by Flow Cytometry Using a Peptide Nucleic Acid Probe[▽]

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A total of 56 *Staphylococcus aureus* isolates incubated for 2 h in the presence or absence of oxacillin were analyzed by flow cytometry after labeling with an *S. aureus*-specific peptide nucleic acid (PNA) probe. Two defined ratios, the paired signal count ratio (PSCR) and the gate signal count ratio (GSCR), differentiated methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) with sensitivities of 100% each and specificities of 96% and 100%, respectively.

We have previously demonstrated that methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolates can be accurately and rapidly differentiated by flow cytometry based on fluorescence intensity and side scatter differences after brief exposure to oxacillin, using a nonspecific fluorescent nucleic acid dye (5). Flow cytometry to differentiate MRSA from MSSA would be even more useful if the method could incorporate *S. aureus*-specific probe labeling instead of using a nonspecific dye. *S. aureus* and other microorganisms can be accurately identified by labeling with specific peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) probes (3, 4).

Fresh overnight cultures of 56 clinical isolates of *S. aureus* (28 MRSA, 28 MSSA) were prepared on Columbia blood agar. These were isolates from blood, respiratory samples, tissue, and wounds of patients with various infections. All isolates came from separate patients. A 0.5 McFarland suspension in saline was diluted 1/20 in cation-adjusted Mueller-Hinton broth supplemented with 2% NaCl (CAMHB-NaCl) (1, 6). One hundred microliters of this diluted suspension was added to each of two tubes, one with 2 ml of CAMHB-NaCl containing 4 μg/ml of oxacillin and the other with 2 ml of CAMHB-NaCl without oxacillin, leaving an estimated inoculum size of 4×10^5 CFU/ml in the final suspensions. These two suspensions were incubated at 37°C for 2 h.

After 2 h of incubation, 200 μl was removed from each suspension, placed in a 1.5-ml microcentrifuge tube, and incubated for 3 min at 80°C. Then, 200 μl of Cy5-labeled *S. aureus*-specific PNA probe (AdvanDx, Woburn, MA) was added. After incubating at 55°C for 30 min, the suspension was centrifuged at 5,000 × *g* for 5 min, the supernatant was removed, and the pellet was resuspended in 500 μl of wash buffer. This was again incubated at 55°C for 10 min and centrifuged at 5,000 × *g* for 5 min; the supernatant was then removed and the pellet again resuspended in 500 μl of wash

buffer. This was again incubated at 55°C for 10 min. After allowing 5 to 10 min for cooling, 400 μl was removed and added to 2.6 ml of phosphate buffer, to yield a final 3-ml sample. This sample was analyzed in a MicroPRO flow cytometer (Advanced Analytical Technologies, Inc., Ames, IA) using a sampling volume of 0.25 ml, with cytometer settings as previously described (5).

Two analytic methods were evaluated to attempt to differentiate MRSA from MSSA. The first method sought to differentiate MRSA from MSSA based on differential growth rates in the presence of oxacillin. Cell counts at 2 h were compared for each isolate incubated in the presence or absence of oxacillin, and a paired signal count ratio (PSCR) was defined for each isolate as the ratio of signal event counts with and without oxacillin exposure. The second method sought to differentiate MRSA from MSSA based on differences in side scatter and fluorescence intensity. Dot plots of side scatter versus fluorescence intensity were examined at 2 h for the isolates exposed to oxacillin. Two gates were created and defined, with gate 1 containing most of the signal events corresponding to MSSA and gate 2 containing most of those corresponding to MRSA. A gate signal count ratio (GSCR) was then defined for each isolate as the ratio of signal event counts in gate 2 to those in gate 1. The PSCRs and GSCRs obtained were used to generate receiver-operating characteristic (ROC) curves. The Sigma-Plot 11.0 software (Systat Software Inc., San Jose, CA) was used to generate the ROC curves and calculate the associated areas, specificities, and sensitivities.

All 56 isolates were correctly identified as *S. aureus* by producing a signal with the *S. aureus*-specific PNA probe utilized. Additionally, MRSA and MSSA could be accurately differentiated from each other in 2 h, based on differential PNA probe uptake in the presence or absence of oxacillin (using the PSCR) and on fluorescence intensity and side scatter differences on exposure to oxacillin (using the GSCR). Representative dot plots of side scatter versus fluorescence intensity for MSSA and MRSA after 2 h of incubation in the presence or absence of oxacillin are shown in Fig. 1. The ROC curve describing the performance of the PSCR is shown in Fig. 2. The area under the ROC curve was 0.9987 (95% confidence

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MHB without oxacillin
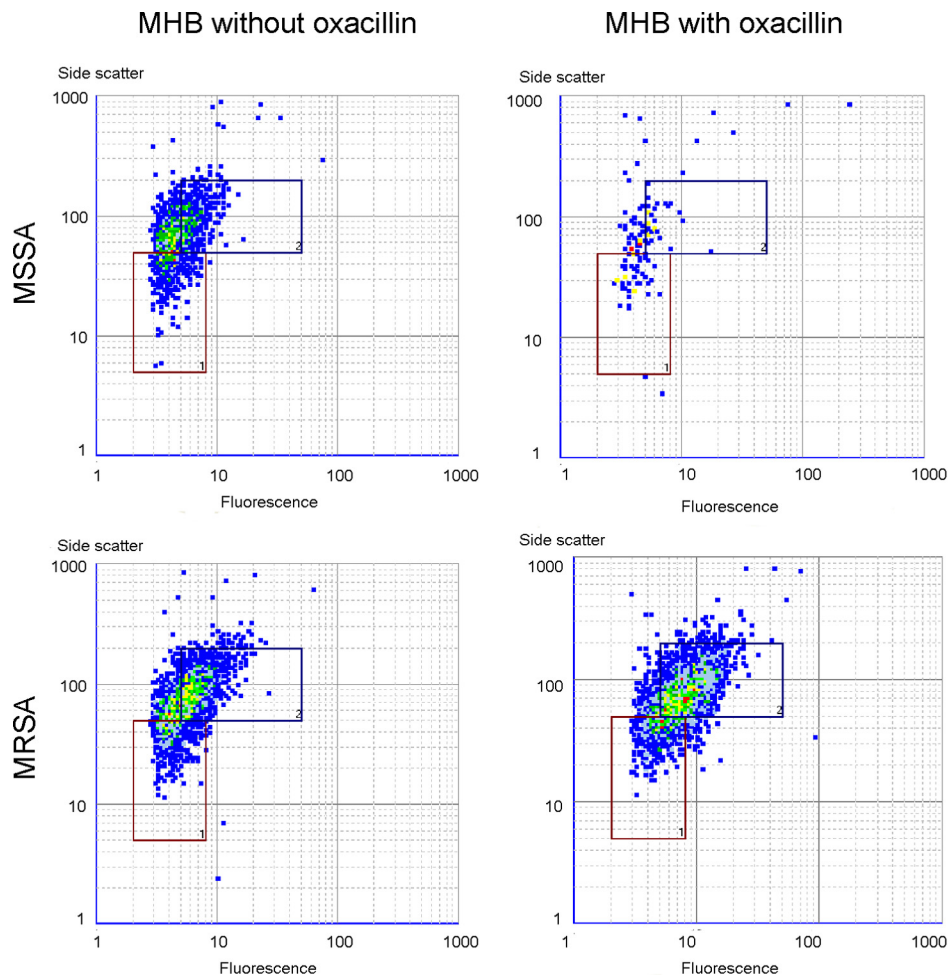
MHB with oxacillin

FIG. 1. Representative dot plots of MRSA and MSSA strains on flow cytometry after incubation in the presence or absence of oxacillin for 2 h, with gate 1 (red) and gate 2 (blue) shown, highlighting differences in signal events.

interval [CI], 0.9945 to 1.003) with a PSCR cutoff of 0.3725, providing 100% sensitivity and 96% specificity. The GSCR accurately differentiated MRSA from MSSA when the two gates were defined as follows: for gate 1, fluorescence intensity, 2 to 8; side scatter, 5 to 50; for gate 2, fluorescence intensity, 5 to 50; side scatter, 50 to 200. The ROC curve for the performance of the GSCR is outlined in Fig. 2. The area under the ROC curve was 1.000, with a GSCR cutoff of 0.8263, providing 100% sensitivity and 100% specificity.

Our previous study showed that given a suspension of *S. aureus*, one can differentiate MRSA from MSSA by flow cytometry using a dye that stains nucleic acid in a nonspecific manner (5). The present study shows that the same differentiation can be achieved using an *S. aureus*-specific dye instead of the nonspecific nucleic acid dye. This raises the possibility that this method could selectively identify *S. aureus* within a mix of microorganisms, thus making it possible to identify *S. aureus* as well as differentiate MRSA from MSSA directly in clinical samples, something that could not be done with nonspecific labeling of microorganisms. When considered in conjunction with our previous study, one can conclude that incubation in the presence of oxacillin rapidly (within 2 h) induces

changes in MRSA that allow differentiation from MSSA by flow cytometry using fluorescence intensity and side scatter readings using the defined GSCR. In this study, the ability to differentiate within 2 h using the PSCR, which depends purely on total signal counts, was an unexpected positive finding. Our previous study demonstrated that viable cell counts are not appreciably different at 2 h when MRSA and MSSA are incubated in oxacillin-containing media, when assessed using nonspecific bacterial dye. Thus, the difference in the PSCR noted in this study for MRSA and MSSA does not stem from a difference in the number of viable microorganisms. Fewer signal events are detected with MSSA after 2 h than at baseline (0 h) when incubated in the presence of oxacillin; this result is significantly different when the same comparison is made for MRSA. The underlying mechanism for the difference in the signal events is not clear. Nevertheless, the ability to differentiate MRSA from MSSA with great accuracy in this study in a reasonably sized cohort using methods similar to those in the previous study suggests the diagnostic utility of this approach.

Using *S. aureus*-specific PNA probes, it has been previously demonstrated that *S. aureus* can be identified rapidly and accurately by flow cytometry in blood culture bottles growing
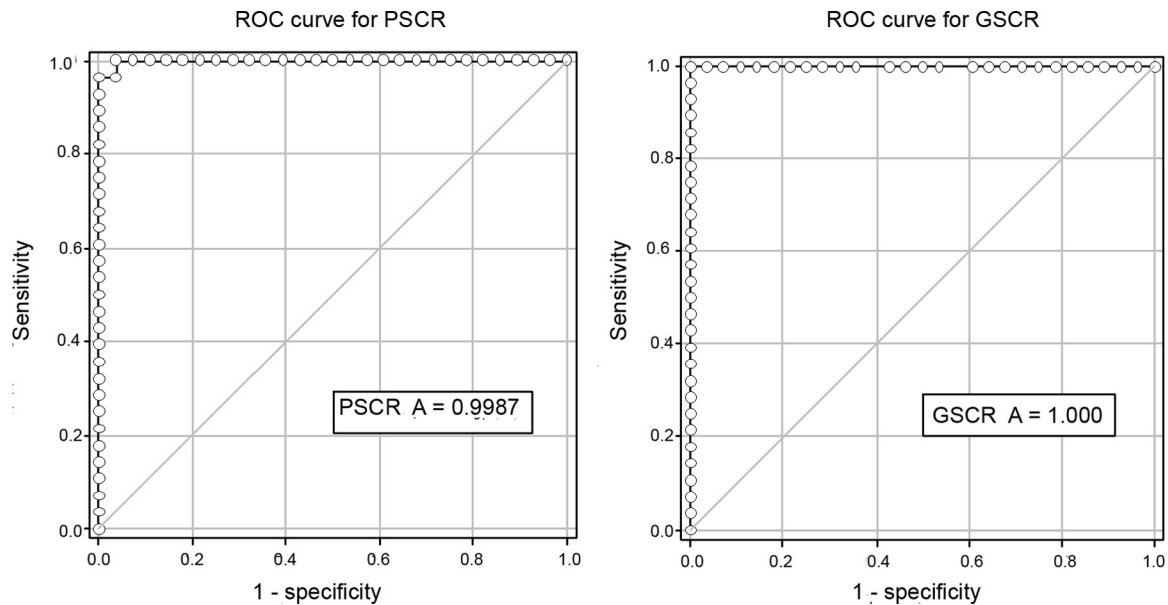
FIG. 2. ROC curves for the paired signal count ratio (PSCR) and gated signal count ratio (GSCR) for differentiating MRSA from MSSA by flow cytometry after 2 h of incubation in oxacillin-containing broth.

Gram-positive cocci (2). The ability to detect methicillin resistance in addition to the presence of *S. aureus* would provide additional clinically useful information in the same test. PNA probes are currently being used in clinical microbiology laboratories to rapidly identify *S. aureus* in blood culture bottles found to be growing Gram-positive cocci in clusters. This study demonstrates the potential to use flow cytometry to go one step further and to rapidly determine methicillin susceptibility in addition to identifying *S. aureus*. The method employed in this assay requires multiple steps. Even with the multiple manual steps involved, the total time to run a batch of 10 samples was about 3.5 h, including the 2 h of incubation time. If this test is considered one worthy of bringing to clinical use, many of these steps are amenable to automation. The design of the flow cytometer instrument we used and the nature of the test also allow for random access testing, another advantage for an assay under consideration for clinical application. The cost per sample of the flow cytometry test using this method is estimated to be about $24, compared with about $45 for the same identification using a commercially available real-time PCR assay. Rapid identification of *S. aureus* and differentiation of MRSA and MSSA can be accomplished by real-time PCR also, but the potential advantages of whole-cell analysis via flow cytometry include simplified sample preparation, the ability for random access testing, the potential for direct visual confirmation of target cell morphology by fluorescence microscopy, and the potentially lower cost per test.

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