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Detection of *Staphylococcus aureus* With a Fluorescence In Situ Hybridization That Does Not Require Lysostaphin

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To detect with whole-cell fluorescence in situ hybridization (FISH), *Staphylococcus aureus* is typically permeabilized with lyozyme and lysostaphin. We tested whether it was feasible to detect *S. aureus* and differentiate it from *Staphylococcus epidermidis* with lysozyme-only permeabilization. We compared lysozyme permeabilization to *S. aureus* permeabilized with lysozyme in combination with lysostaphin. It was determined that *S. aureus* treated with agarose, methanol, and lysozyme could be detected with FISH. The 1 hr protocol is a useful alternative to conventional FISH. J. Clin. Lab. Anal. 25:142–147, 2011. © 2011 Wiley-Liss, Inc.

Key words: early diagnosis; fluorescent in situ hybridization; gram-positive bacteria; molecular diagnostic; *Staphylococcus aureus*; lysostaphin; lysozyme; techniques

INTRODUCTION

Slide-based fluorescence in situ hybridization (FISH) is a reliable method for detecting pathogenic *Staphylococcus aureus* and distinguishing it from the relatively benign *Staphylococcus epidermidis* (1–3). If DNA rather than the costly Peptide Nucleic Acid probes (Panagene) are applied, permeabilization is necessary to ensure access of probes to in situ ribosomal RNA (rRNA) (4,5). Usually, permeabilization is conducted with the enzymes lysozyme (Sigma, L6876; Sigma-Aldrich, St. Louis, MO) and lysostaphin (Sigma, L4402), either mixed together (6,7) or in two steps (2,8). Other permeabilization treatments, such as hydrochloric acid (9), nisin (10), proteinase K (9), staphylolysin (11) or Triton X–100 (12) are only sometimes adopted (2,3,5,6,13).

Permeabilization can complicate the application of FISH in routine laboratory diagnostics, as it has to be conducted precisely (2). Underpermeabilization can result in a low FISH signal and overpermeabilization in lysis and cell loss (4). A simplification of this step leading to more consistent outcomes is desirable. Lysozyme applied on its own for the detection of *S. aureus* was previously reported, but the assays described took a number of hours (14,15). We report here the efficacy of applying a single enzyme (lysozyme) instead of two, to rapidly detect *S. aureus* with FISH.

MATERIALS AND METHODS

Preparation

To reduce cell loss (16), an agarose (Bio–Rad, 162–0102; Bio-Rad Laboratories, CA) bed was applied to diagnostic glass slides (Menzel–Gläser, X1XER308B; Menzel Gläser, Braunschweig, DE). The bed was prepared by adding 0.02% (w/v) agarose and 0.01% (w/v) sodium azide (Sigma, S2002) to Milli-Q water® (MQ) (Millipore, Billerica, MA) and dissolving it by heating without boiling in a microwave oven. The agarose dilute was spotted 10 µl to each slide well and dried on an 80°C hotplate. Blood agar plates of clinical isolates

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positive for *S. aureus* and *S. epidermidis* were randomly collected from a major hospital. Isolate identity was confirmed via polymerase chain reaction (17). For safe handling, the first ten isolates of *S. aureus* negative for the mecA gene and the first ten isolates identified as *S. epidermidis* were selected for further testing. Isolates were deidentified at collection and labeled numerically to ensure their identity was blinded when assessed. Isolates were cultured in 50 ml sterilized tubes of nutrient broth (Oxoid, CM0001; Oxoid, Hampshire, UK) and incubated at 37°C with a gentle rotation until turbid. Broth dilutions for blood cultures were used, as it allowed shortened incubation times of 1–2 hr from frozen isolates as opposed to day or overnight incubations. To enhance probe signal and further reduce cell loss, prewarmed 0.4% (w/v) agarose and the broth culture of the isolates was diluted 1:1 (16). The agarose–isolate dilute was then spotted 10 µl to each slide well and fixed with an 80°C hotplate until dry.

Permeabilization

To further fix and partially permeabilize the isolates, the slides were washed in 50 ml sterile tubes of absolute methanol for 3 min (6). Slides were removed and dried on a hotplate. The slides were cooled and 10 µl of freshly prepared 15 mg/ml lysozyme (18) in unbuffered MQ water (12,19,20) was pipetted to each well. Typically, lysozyme is buffered with Tris–HCl at pH 8.0 (2,3,5,6,13), but for simplicity and to attain a more intense signal (21), we followed reports where buffering was omitted (12,19,20). Slides were fitted in 50 ml tubes to prevent evaporation and then placed in a 37°C (21) incubator for 30 min (12). The lysozyme action was stopped by immersion in absolute methanol for 1 min and then dried on a hotplate (6).

The isolates were also permeabilized with a lysozyme–lysostaphin mixture. The protocol was identical to the lysozyme-only treatment, but with the following modifications. Lysostaphin (Sigma, L4402) at 100 µg/ml (3) was added to the lysozyme in MQ water (19) and incubated at 40°C (21,22) for 3 (6) instead of 30 min. As a control, lysozyme–lysostaphin was kept unbuffered, but we observed cell morphology was better preserved if it was buffered at pH 8.0 (2,3,5,6,13). As before, slides were immersed for 1 min in absolute methanol (6).

Additional tests were performed to compare the quality and applicability of other reagents. Fixatives and permeabilizers were selected on the basis of previous reports and the signal intensity, cells stained with FISH, cell loss after FISH, time taken for the assay, and costs were compared (Table 1), for different permeabilization treatments. The lysozyme and lysostaphin and lysozyme-only treatments are already described. The treatment with lysostaphin excluded lysozyme (23). The treatment without agarose excluded agarose spotting to the slides or agarose in dilution with the isolates (16). The treatment with lysozyme after ethanol replaced the methanol fixation step with absolute ethanol (2). The proteinase K treatment replaced the 30 min lysozyme step with 10 min incubation in 1 mg/ml proteinase K (P4850, Sigma) at 40°C, a methanol rinse for inactivation, and 10 min incubation with 1 mg/ml lysozyme at 40°C. The lysozyme after HCl acid treatment was the same as the proteinase K treatment, except proteinase K was replaced with 1 M HCL at 37°C (24). The treatment with Tween 20 (P7949, Sigma) after lysozyme added a 5 min incubation step at room temperature with Tween followed by a water rinse. The treatment with Triton X–100 (T8787, Sigma) after lysozyme (12) was the same as Tween, expect with Triton. The no permeabilization treatment omitted the personalization step. If not listed, other FISH steps were the same as the lysozyme-only treatment.

FISH

A hybridization buffer was prepared with 0.9 M NaCl (Sigma, S6191), 20 mM Tris–HCl (Sigma, T1503, T3253), and 0.02% (w/v) SDS (Sigma, L4390) in MQ water (25). Either 15% (v/v) deionized formamide

TABLE 1. Comparison of Different *S. aureus* Permeabilization Treatments Concerning Quality and Robustness

Permeabilization treatment	Signal intensity	Cells stained	Cell adhesion	Time (min)	Cost ($)
Lysozyme and lysostaphin	++++	++++	+++	7	10
Lysostaphin	++++	+++	+++	7	9
Lysozyme	++++	++++	+++	34	5
Lysozyme without agarose	++	++	++	33	5
Lysozyme after ethanol	+++++	+++	+++	34	5
Lysozyme without alcohol fixation	+++	++	+++	31	5
Lysozyme after proteinase K	++++	++++	+	21	6
Lysozyme after HCl acid	–	–	++	21	5
Tween 20 after lysozyme	+++++	++++	+++	39	5
Triton X-100 after lysozyme	++++	++++	+++	39	5
No permeabilization	+	+	+++	4	4

(Applichem, A2156; Applichem, Darmstadt, DE) and 2 μM of Sau probe (Sau 16S69: 5′-GAAGCAAGC-TTCTCGTCCG-3′) specific for *S. aureus* or 30% (v/v) formamide and 2 μM of EUB338 probe (EUB338 16S337: 5′-GCTGCCTCCCGTAGGAGT-3′) specific for bacteria was added (Invitrogen, Carlsbad, CA). Both oligonucleotide (DNA) probes were conjugated to the flurophore Alexa Fluor® 488 (Invitrogen). The buffer was spotted 10 μl to each well and the slides were fitted in 50 ml tubes and placed in a 47°C incubator for 20 min.

After hybridization, slides were immediately fitted in 50 ml tubes of prewarmed washing buffer containing 5 mM EDTA (Sigma, EDS), 0.64 M NaCl, 20 mM Tris–HCl, and 0.02% (w/v) SDS in MQ water (25). Tubes were then placed in a 47°C water bath for 3 min (6). Washing action was stopped by rinsing in a 50 ml tube of phosphate buffered saline (PBS) (Sigma, P4417) at room temperature and followed by drying with pressurized air (6). If required, isolates were counterstained with 15 μl of 1 μg/ml DAPI for 1 min and then rinsed with PBS (16). Cells were visualized with a fluorescence microscope (Olympus, BX51; Olympus, Tokyo, Japan) equipped with a fluorescein filter.

Different permeabilization treatments are listed in Table 1. The ratios are indicated by "++++" for all, "+++" for three quarters, "++" for half, and "+" for a quarter or less. A negative result is indicated by "–". The signal intensity (+) was measured relative to the lysozyme and lysostaphin FISH treatment. Cells stained (+) with FISH was measured from the ratio of cells with FISH to DAPI (Sigma, D9564) signal. Cell adhesion (+) was measured from the ratio of cells remaining after FISH to cells observed with DAPI before FISH. Time (Min) taken for each treatment included the sum of the agarose, fixation, and permeabilization steps. The cost ($) was rounded to the nearest dollar for a daily run of four FISH experiments, each with two slides (Sigma, USD). All treatments were adjusted so that cell lysis was minimal. The treatment was repeated in its final form three times. For each experimental variable, two wells were tested and three fields of view with an objective of X60 were assessed. Two independent, blinded observers analyzed the images. Slight variation was observed between slide wells, but not between experimental runs.

RESULTS

Table 1 summarizes the results of different treatments in terms of quality and robustness. Both lysozyme-only and lysozyme–lysostaphin permeabilization detected *S. aureus* and differentiated it from *S. epidermidis* with the Sau probe. For the initial tests, all enzymes were left unbuffered. Lysozyme–lysostaphin had a brighter signal than lysozyme-only treated *S. aureus*. However, the lysozyme–lysostaphin left cells overpermeabilized and lysed. Once a buffer at pH 8.0 was added, the lysis was controlled, and *S. aureus* treated for 3 min with lysozyme–lysostaphin, which had a result equivalent to that of a 30 min lysozyme-only treatment. Figure 1 illustrates the ability to detect *S. aureus* with the Sau probe for both treatments. In addition, no cross-reactivity was noted for the Sau probe; it was positive for *S. aureus* and negative for the *S. epidermidis* isolates. Likewise, both treatments detected *S. aureus* and *S. epidermidis* with the universal EUB338 probe.

We could not obtain a signal rapidly with lysozyme alone unless the *S. aureus* isolates were diluted in agarose. This lengthened the assay, but it was only a slight encumbrance as the step was performed in 1 min. Agarose doubled the signal intensity, the ratio of cells with signal, and increased cell adhesion. Without the agarose dilution, similar signal intensity was realized if the cells were hybridized for 70 instead of 20 min. We tested cell loss of isolates in agarose spotted to slides prepared and unprepared with an agarose bed. We observed that agarose spotted slides further reduced cell

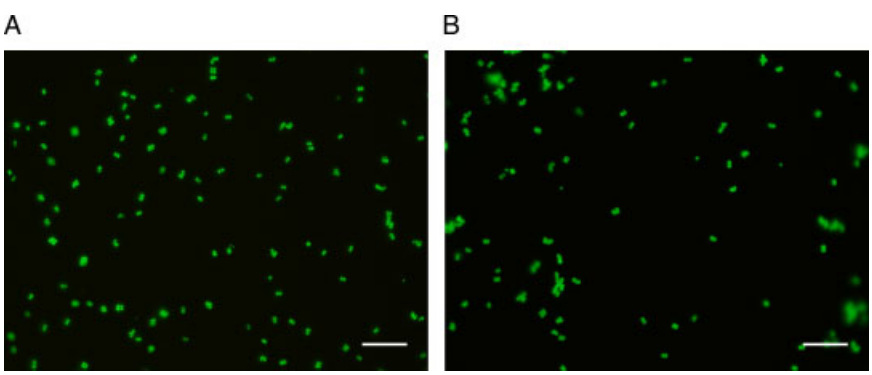

Fig. 1. *S. aureus* permeabilized with lysozyme (**A**), and *S. aureus* permeabilized with lysozyme–lysostaphin (**B**). *S. aureus* were labeled with the Sau probe conjugated to the fluorophore Alexa Fluor® 488. Bar is 10 μm. (A) *S. aureus* permeabilized with lysozyme. (B) *S. aureus* permeabilized with lysozyme and lysostaphin.

loss. This might be an advantage if the number of target cells is low. As the bed can be applied before a FISH procedure, its preparation does not complicate or lengthen FISH.

A number of different fixation procedures were tested (Table 1). Applying 100% methanol to the slides was observed to be the most effective and rapid fixation (6). Ethanol fixation of slides produced a brighter signal, but was less consistent than with methanol. Omitting the alcohol fixation step reduced both signal intensity and consistency. Tween 20 enhanced the signal, but it involved an additional assay step. Yet, when we tested lysozyme diluted with Tween, the signal did not differ from lysozyme-only permeabilization. In contradiction to previous reports (12), we saw no improvement with Triton X–100. Permeabilization with hydrochloric acid produced poor results; it seemed to inhibit the action of the conjugated probe itself.

As a control, FISH was performed with the permeabilization step omitted. Less than one quarter of the *S. aureus* cells had sufficient signal. As a further control, FISH was performed with only lysostaphin (19). High signal strength was observed, but the signal was less consistent than that of *S. aureus* treated with lysozyme–lysostaphin or the proposed lysozyme-only method. Isolates were tested directly from blood agar plates with lysozyme-only and in combination with lysostaphin. The results were consistent with tests of *S. aureus* cultured in nutrient broth. Poor results were obtained with lysozyme-only permeabilization if agarose was omitted. Preliminary testing (data not shown) with a healthcare-associated meticillin-resistant *S. aureus*, (HA)–MRSA isolate and a community-associated (CA)–MRSA isolate, was comparable to the mecA-negative isolates (26).

Lysoyme and lysostaphin are commonly applied at a pH of 8.0 (2,3,5,6,13). Buffering at pH 8.0 was found to reduce the loss of cell morphology with lysostaphin. However, we observed that lysozyme-only assay produced poor results unless the pH was reduced to 7.0. The lysozyme-only assay was tested and found to be effective without buffering, and so for simplicity, Tris-HCl buffer was omitted from the final tests. We experienced some difficulty applying Proteinase K. The precise concentration, incubation temperature, and time necessary for permeabilization but not overlysis was difficult to manage. Washing with 100% methanol reduced over-permeabilization, but an agarose bed and dilution in agarose did not stop the loss of up to half the cells.

To minimize thickness and visual aberration, we tested the lowest concentration of agarose necessary to maintain cell adhesion and signal intensity (27). We found that an agarose concentration of 0.02% (w/v) was sufficient for the slide bed and 0.2% sufficient for the isolate dilution. For simplicity, we diluted 0.4% (w/v) agarose 1:1 with the isolates. This may, however, have a negative effect on the assay's sensitivity if microbe numbers are low. To reduce overdilution of cells, we trialled one part agarose at 0.8% to three parts of nutrient broth with isolates, without signal loss. An additional benefit of agarose was that the probe concentration could be reduced by a factor of five without loss of signal. Initially, experiments were performed at 5 μM probe concentrations, but after the addition of agarose, this was reduced to 1 μM. As a safety margin, the final experiments were performed at 2 μM.

DISCUSSION

We set out to validate whether lysostaphin was necessary for detecting *S. aureus* with FISH. We demonstrated that *S. aureus* can be successfully permeabilized rapidly without lysostaphin. The ability of lysozyme-only to permeabilize *S. aureus* is likely owing to how the isolates were prepared after culturing and how they were fixed and permeabilized. Isolates were diluted in agarose to enhance signal intensity (16,27); fixed in mid-log phase when rRNA numbers were high (1,13); permeabilized by heat, methanol (6) and lysozyme (12); treated with a relatively high concentration of unbuffered lysozyme (12,18–20); and finally incubated for an extended period of time (12) at an optimal temperature for lytic activity (21).

There were some drawbacks to using an agarose bed and an agarose isolate dilution. For agarose stock dilution to mix properly with isolates in nutrient buffer, it needed to be prewarmed. When viewed with a fluorescence microscope, the agarose did create visual aberrations and thickening of the specimen. To see all the cells in focus, it was necessary to adjust the microscope stage Z-axis up and down while viewing. Figure 1 illustrates FISH-labeled *S. aureus* inside and outside the focal plane. However, these encumbrances were offset by the doubling in signal intensity and cell adhesion. Rapid and effective FISH with only lysozyme was possible with this signal enhancement. When using bacteria from pure culture, cell loss was not a problem. However, it was felt that this study would have a wider utility if this parameter was optimized as well.

Handling of lysostaphin was not straightforward. Minute amounts were involved (28) and upon weighing, the lyophilized powder (Sigma, L4402) readily absorbed moisture from the atmosphere, making exact measurement difficult. When diluted in water, its decline in activity was noticeable after 1 week. We saw some variation in *S. aureus* strain response to lysostaphin. These variables made the titration of lysostaphin necessary before each experiment to ensure that isolates

were permeabilized optimally. Furthermore, lysostaphin was approximately 40 times more expensive by volume spotted than lysozyme (Sigma, L6876, L4402). In contrast, if only lysozyme was applied, the permeabilization step was more robust, less sensitive to variation in bacteria strains, less likely to overpermeabilize, and titration was unnecessary. Dilutions can be stored at 4°C for 2 months before activity loss was noticeable. The weighing was relatively simple and did not require a microbalance scale housed in a draft-free enclosure. If preparation mistakes are made, the enzyme was reformulated quickly and without significant expense.

A limitation of the lysozyme-only FISH protocol was its turnaround time. At 1 hr, it was twice as long as the fastest reported lysozyme–lysostaphin protocol (6). However, this was still half the time of other presumptive tests for *S. aureus* (29,30). In conclusion, this study detected and differentiated *S. aureus* from *S. epidermidis* with a 1 hr FISH method that did not require lysostaphin. The procedure worked with Staphylococci taken directly from agar plates (data not shown), but further testing is required to assess the sensitivity and specificity of this practical method on blood cultures.

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