Optimization of a Two-Step Permeabilization Fluorescence In Situ Hybridization (FISH) Assay for the Detection of *Staphylococcus aureus*

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Background: Aspects of the fluorescence in situ hybridization (FISH) method for the detection of clinically important bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli*, were investigated for optimization. **Methods:** Various approaches to optimizing the FISH procedure were taken and different methods were compared. To save time, hybridization and washing buffers were prepared beforehand and stored at –20°C and mixed to their final formamide and NaCl concentrations just before use. The use of 50-ml tubes for hybridization

incubation reduced drying out, reagent wastage, and reaction times. **Results:** A two-step permeabilization FISH assay was developed that used phosphate-buffered saline as a buffer for lysostaphin. It could detect bacteria with DNA probes conjugated to fluorophores with a higher signal intensity and the less expensive biotinylated DNA probes with minimal cell lysis in 1 hr. **Conclusions:** The two-step assay might be used when the FISH signal is weak, bacterial numbers are low or if there is a need to use other reporter molecules. J. Clin. Lab. Anal. 25:359–365, 2011. © 2011 Wiley-Liss, Inc.

Key words: fluorescence in situ hybridization; FISH; Gram-positive bacteria; molecular diagnostic techniques; *Staphylococcus aureus*; *Staphylococcus epidermidis*; Staphylococci

INTRODUCTION

Following a positive blood-culture and Gram-stain, fluorescence in situ hybridization (FISH) can be used to identify the bacteria present such as the clinically important *Staphylococcus aureus* (1–4). The FISH procedure typically uses a single permeabilization step (hereafter referred to as the one-step FISH assay) and DNA probes (also called oligonucleotides or oligos) conjugated to fluorophores (1,5–8). To permeabilize *S. aureus*, the one-step FISH assay applies a lytic enzyme mixture of lysozyme and lysostaphin. As it can be more robust, FISH can also use a two-step permeabilization (two-step FISH assay) to detect *S. aureus* (2,4,9–14). To permeabilize *S. aureus*, two-step FISH assay applies a lysozyme step, and a quick water rinse followed by a lysostaphin step.

The DNA probes conjugated to fluorophores (hereafter, oligo-f) are relatively small in molecular weight and so can gain rapid access to in situ rRNA targets. The detection of *S. aureus* with FISH and biotinylated probes (hereafter, oligo-b) is rarely reported (15–17).

Greater permeabilization is needed for streptavidin to gain in situ access to acteria as it has a high molecular weight. This can lengthen the assay time (15–17) and lead to over-permeabilization or cell lysis. A rapid oligo-b FISH assay, however could offer cost savings.

As far as we are aware, there are no reports of the detection of *S. aureus* with a two-step FISH assay and oligo-b in 1 hr or less. *S. aureus* was chosen for testing with oligo-b and FISH as it is an important pathogen and its permeabilization for DNA probes is more involved. Since *S. epidermidis* is phylogenetically similar to *S. aureus* and its rRNA nearly identical (7), it was included as a negative

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control. *E. coli* was also tested to ensure that the FISH assays developed could also detect Gram-negative bacteria (11). DNA rather than peptide nucleic acid (PNA) were used as they are less expensive.

MATERIALS AND METHODS

Two FISH assays were developed and tested. These are listed and compared in their optimized form in Table 1. The one-step permeabilization FISH assay is a modified version of the FISH assay described by Poppert et al. (1). The two-step permeabilization FISH assay extends the one-step assay by the addition of an extra permeabilization step and a streptavidin incubation step (15). Details for the optimized one-step and two-step FISH assays are not included in Table 1.

Specimen Preparation

So that isolates could be tested at a nonclinical location, clinical patient isolates of *S. aureus*, *Staphylococcus epidermidis* and *Escherichia coli* were collected on agar plates from a major hospital. Only penicillin

binding protein (PBP2)-negative *S. aureus* were selected. Isolate identity was confirmed with polymerase chain reaction (18) and was then de-identified for experimentation. To control for potential differences between strains, ten isolates of each type of bacteria were collected. To enable the isolates to be compared over a number of FISH experiments, the collected isolates were cultured in nutrient broth (CM0001; Oxoid, Hampshire, UK), centrifuged at 4,000 rcf, aliquoted and frozen as described by Baldrich et al. (19). An aliquot of *S. aureus*, *S. epidermidis*, and *E. coli* was thawed and recultured in nutrient broth for testing with FISH. As a control, isolates were tested directly from the plates and no difference in signal was observed. To test with FISH, cultures of clinical isolates in nutrient broth were spotted (10 µl) onto the slides (X1XER308B; Menzel Glaser, Braunschweig, DE), dried at 80°C for 3 min, and then fixed with absolute (m)ethanol for 1 min.

Cell Permeabilization

To reduce preparation time, stock solution of lysozyme (L6876; Sigma-Aldrich, St. Louis, MO) and lysostaphin (L4402; Sigma) were prepared up to a week in advance and stored as 1.5-ml aliquots in sterile plastic tubes. For the one-step permeabilization FISH assay, 15 mg/ml lysozyme and 0.1 mg/ml lysostaphin were applied in one-step as described by Poppert et al. (1). For the two-step permeabilization assay, 10 µl of lysozyme at 15 mg/ml in Milli-Q[®] (MQ) water (Millipore, Billerica, MA) (20,21) was spotted onto the slide wells and incubated in 50-ml tubes (210–261; Greiner, Frickenhausen, Germany) for 6 min at 38°C (22). The lysozyme was rinsed off with, PBS (P4417; Sigma), and the slides were rapidly dried with pressurized air (1) or by centrifuging in 50-ml tubes for 1 min at 100 rcf. Lysostaphin at 0.1 mg/ml in PBS was spotted (10 µl) onto the slides and incubated in 50-ml tubes at 47°C for 6 min. Lysostaphin was removed by rinsing the slides in absolute (m)ethanol for 1 min and then drying at 80°C for 1 min. When *E. coli* isolates were tested, permeabilization was omitted.

Hybridization

To save time, the hybridization buffer and washing buffer were prepared in advance. Hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% (w/v) SDS, and 1 µg/ml DAPI) with no formamide or with 60% (v/v) deionized formamide were prepared and stored for up to a year at –20°C in 5-ml sterile plastic screw-top tubes. When needed, the buffers were thawed and mixed to the desired target formamide concentration. The concentration of formamide used in this study was 30% (v/v), about 5% higher than the lowest probe formamide dissociation concentration estimated with mathFISH

TABLE 1. Comparison of the Optimized One-Step or Two-Step Permeabilization FISH Assays

One-step (1)	Two-step (This study)
<i>Preparation:</i> Cultures of clinical isolates were diluted with PBS, spotted, fixed to slides at 80°C, fixed in methanol, and air-dried	
<i>Permeabilization:</i> Slides were spotted with lysis reagent (15 mg/ml lysozyme, 0.1 mg/ml lysostaphin, 20 mM Tris-HCl at pH 7.0 in MQ water) and incubated at 40°C, rinsed with methanol, and air-dried	<i>Permeabilization:</i> Slides were spotted with 15 mg/ml lysozyme with 20 mM Tris-HCl at pH 7.0 in MQ water and incubated at 38°C, rinsed with PBS, and dried with pressurized air <i>Permeabilization:</i> Slides were spotted with 0.1 mg/ml lysostaphin, 20 mM Tris-HCl diluted in PBS and incubated at 47°C, rinsed with methanol, and air-dried
<i>Hybridization:</i> Slides were spotted with hybridization buffer (30% formamide, 0.9 M NaCl, 20 mM Tris-HCl at pH 8.0, 0.01% SDS, a 1-µM probe, and MQ water), and incubated at 47°C	
<i>Washing:</i> Slides were incubated with washing buffer (0.3–0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS, 10 mM EDTA, and MQ water) at 47°C, rinsed with PBS	
<i>Mounting:</i> Slides were mounted with a cover-slip while wet	<i>Streptavidin/Mounting:</i> Slides were spotted with streptavidin-f and incubated at 38°C, rinsed with PBS, and mounted with a cover-slip while wet

(mathfish.cee.wisc.edu) (47°C hybridization incubation, 0.9 M NaCl and 1 µM of probe) (23). The oligo (1 µM) could be added to the buffer mix and stored at 4°C in 1.5-ml sterile plastic aliquots for a week before performing FISH.

For hybridization, 10 µl of buffer with oligo (1 µM) was spotted onto the slides and incubated at 47°C for 10 min. Oligos tested were STAAUR specific for *S. aureus* (STAAUR-16S69: 5'-GAAGCAAGCTTCTCGTCCG-3') (12) (Invitrogen, Carlsbad, CA), STAPHY specific for *Staphylococcus* (STAPHY-16S697 5'-TCCTCCA-TATCTCTGCGC-3') (12) (Invitrogen), and EUB338 specific for eubacteria (EUB338 16S337: 5'-GCTGC-CTCCCGTAGGAGT-3') (Sigma) (24) (Invitrogen). These oligos were biotinylated (oligo-b) or directly conjugated to Alexa Fluor® 488 (Invitrogen), Alexa Fluor® 555, FITC or Cy3 (Genworks, Adelaide, Australia) labeled on the 5' end (oligo-f).

Specimen Washing

In a similar fashion to the hybridization buffer, the washing buffer without salt (20 mM Tris-HCl, 5 mM EDTA and 0.01% (w/v) SDS) or with 1.8 M NaCl were prepared and stored for up to a year at 4°C in 1 l bottles (GL45; Schott, Mainz, Germany). When needed, the buffers were mixed to the target NaCl concentration in a 50-ml tube, typically 1:4, to make approximately 0.3 M NaCl. So that the washing buffer could be reused multiple times, before immersing the slide in the 50-ml tubes of washing buffer for 3 min at 47°C, the hybridization buffer was rinsed off with washing buffer.

Streptavidin Conjugation

The slides were removed from the washing buffer and rinsed in PBS. For the one-step FISH assay, the slides were mounted wet with PBS and cover-slips for microscopy. For the two-step FISH assay, the slides were dried with pressurized air and spotted with 10 µl of streptavidin conjugated to Alexa Fluor® 488 (S-32354; Invitrogen) (hereafter, streptavidin-f), DyLight® 488 (21832; Thermo Fisher, Waltham, MA) or Alexa Fluor® 555 (S-32355; Invitrogen) at 10 µg/ml in PBS. Slides were incubated at 47°C for 10 min, rinsed with PBS, and mounted as before, for microscopy.

Microscopy and Statistical Analysis

The FISH signal was observed with an epifluorescence microscope (BX51; Olympus, Tokyo, Japan) fitted with a 60× dry objective (UPLFLN; Olympus) and FITC/DAPI filters (U-MWU2, U-MWIB2; Olympus). Images were acquired at a resolution of 1,360 × 1,024 with an Olympus DP72 camera and software (DP2-BSW v2.2; Olympus) set to a gain of 200 ISO and an exposure of

400 msec. A representative image with a cell count of at least 100 was selected for each treatment from three experiment runs. Images were analyzed with ImageJ using its standard segmentation algorithms (v1.43u; NIH, Bethesda, MD). Cell numbers, morphology, and permeabilization were assessed with the FISH signal and 4',6-diamidino-2-phenylindole (DAPI) (D9564; Sigma). The mean signal intensity (8-bit gray-scale) and standard deviation for each FISH method were calculated. Summary statistics were compared with one-way analysis of variance (ANOVA) and a *P* value of <0.05 was considered significant.

RESULTS

Effect of Different FISH Assays and Probe Types on Signal

Two FISH assays were tested: the assay by Poppert et al. (1), which included a one-step permeabilization treatment and a modified version of that assay that included a two-step permeabilization treatment (Table 1). Each FISH assay was tested with two types of probes: Oligo-f probes that had DNA sequences conjugated to fluorophores and oligo-b probes that had DNA sequences conjugated to biotin and visualized with streptavidin-f. Each probe type was tested with three probe sequences: the STAAUR probe that was specific for *S. aureus* and the STAPHY probe specific for *Staphylococcus* (12), and the EUB338 probe specific for eubacteria (24).

As reported by Poppert et al. (1), the one-step permeabilization FISH assay successfully detected *S. aureus* and differentiated it from *S. epidermidis* with oligo-f probes in 45 min (Fig. 1A). The one-step assay (1), however produced a poor signal for *S. aureus* with the STAAUR oligo-b probe (Table 2) and a weak signal for *S. epidermidis* with the STAPHY oligo-b probe and streptavidin-f (data not shown). In contrast, the two-step permeabilization FISH assay successfully detected *S. aureus* with oligo-f, oligo-b probes, and streptavidin-f in 1 hr (Fig. 1B and D). Furthermore, the two-step assay produced a higher FISH signal with oligo-f probes than the one-step FISH assay (Table 2). The difference in oligo-f signal intensity was found to be significant (One-way ANOVA; *P* < 0.05).

Effect of Different Slide and Fixation Preparation

Other aspects of the FISH method were also investigated for optimization (data not shown). Heat fixing the bacteria on the slides at 80°C rather than air-drying shortened drying time from 10 to 3 min. Cell loss from the slides was observed after processing with FISH. Rinsing the slides with molten 0.2% (w/v) agarose (162-0102; Bio-Rad Laboratories, CA)

Fig. 1. *Staphylococcus aureus* treated with (A) the one-step permeabilization FISH assay or (B) the two-step permeabilization FISH assay and then labeled with 1 μ M/ml STAAUR oligo directly conjugated to Alexa Fluor[®] 488. *S. aureus* treated with the two-step permeabilization FISH assay and (C) not washed with PBS before lysostaphin, and (D) washed with PBS before lysostaphin and then labeled with 1 μ M/ml oligo-b STAAUR and 10 μ g/ml streptavidin Alexa Fluor[®] 488. (A) *S. aureus* treated with the one-step FISH assay. (B) *S. aureus* treated with the two-step FISH assay. (C) *S. aureus* not washed with PBS before lysostaphin. (D) *S. aureus* washed with PBS before lysostaphin. PBS, phosphate-buffered saline; FISH, fluorescence in situ hybridization.

TABLE 2. Comparison of the *Staphylococcus aureus* SI and CI for One- and Two-Step Permeabilization FISH Methods Using a STAAUR Oligo

Treatment	SI ^a	CI ^b
One-step FISH assay with oligo-f ^c	23.44	0.55
One-step FISH assay with oligo-f and Tween 20 [®] ^d	24.85	0.71
One-step FISH assay with oligo-b ^c	17.19	0.19
Two-step FISH assay with oligo-b ^c	23.27	0.48
Two-step FISH assay with oligo-f ^c	27.31	0.45

SI, signal intensity; CI, confidence interval; FISH, fluorescence in situ hybridization. Exposure time was the same for each image acquisition. The results were consistent across the ten isolates tested.

^aMean signal intensity in 8-bit Gray-scale.

^bConfidence interval was calculated at 95%.

^cAs described in Table 1.

^dThe one-step FISH assay with a 5-min, 1% Tween 20[®] step at room temperature before hybridization.

^eThe two-step FISH assay using an oligo-f, without the streptavidin incubation step.

minimized this loss (25,26). A number of specimen fixation techniques were tested. Alcohol fixation of isolates during dilution or after drying onto a slide was found to be necessary for a consistent FISH signal or if the isolates were stored for later testing. No difference in

the signal was observed between isolates fixed for 3 and 10 min. If fixed for 1 min, the FISH signal was less than when fixed for 3 min, but was used to shorten the assay. Ethanol fixation was observed to produce a higher signal intensity, whereas methanol was observed to produce a more consistent signal. Diluting isolates in absolute (m)ethanol 1:1 and heating to 80°C for 10 min did not improve the signal (27). For *S. aureus*, paraformaldehyde at 1% produced a weak signal (7,11).

Effect of Lysozyme and Lysostaphin Permeabilization Treatments

As reported by Poppert et al. (1), when 2 mg/ml lysozyme and 0.1 mg/ml lysostaphin in 10 mM Tris/HCl (pH 8) were combined and applied in a one 5-min step at 46°C, the FISH assay was simple and rapid for oligo-f probes. This treatment was further shortened to 3 min by combining 15 mg/ml lysozyme with 0.1 mg/ml lysostaphin in 10 mM Tris/HCl (pH 7) and incubating for 3 min at 40°C. Other variations of lysozyme and lysostaphin permeabilization were tested with oligo-f probes. If lysozyme was applied without lysostaphin, a 30-min incubation at 38°C was necessary for STAAUR signal (21). If lysostaphin was applied without lysozyme, a 10-min incubation at

47°C was sufficient for *S. aureus*, but the *S. epidermidis* EUB338 signal was poor. If oligo-b probes and streptavidin-f was used, the one-step permeabilization treatment produced a weak signal (Table 2).

To produce a satisfactory signal for oligo-b probes and streptavidin-f, lysozyme and lysostaphin were applied separately. The sequence of the lysozyme and lysostaphin steps was found to be important. As reported by Tavares et al. (2), lysozyme treatment before lysostaphin produced a higher and more consistent signal than vice versa. Buffering of the enzymes was also important. No difference was observed in STAAUR signal if lysozyme was buffered at pH 7.0, 8.0, or left unbuffered. Lysostaphin when unbuffered, however, led to over-permeabilization and cell lysis. When lysostaphin buffered in Tris-HCl pH 7.0 or 8.0 was applied, cell lysis was reduced but not completely abrogated (Fig. 1C). Cell lysis, however was minimized if a 1-min PBS wash step was added between the lysozyme and lysostaphin steps (Fig. 1D). The PBS treatment was further optimized by omitting the PBS wash step and instead diluting lysostaphin in PBS. Since the Tris-HCl buffering was not needed in the lysozyme step and lysostaphin was buffered with PBS, the assay preparation was simplified. For the different permeabilization treatments tested, no differences were observed among the ten isolates of each of the three bacteria (*S. aureus*, *S. epidermidis*, and *E. coli*).

Optimizing Hybridization

A surfactant step before hybridization was not necessary, however, a 1% (v/v) Tween 20[®] or 0.1% (v/v) Triton X-100[®] with 1% (w/v) bovine serum albumin in MQ water spotted (10 µl) onto the slides and incubated for 5 min at room temperature increased the signal intensity by 6% (Table 2). A number of different oligo and stain treatments were tested with the hybridization buffer. No difference was observed in the signal if the oligos were tested at concentrations of 0.25–3 µM. Since it was easier to prepare, 1 µM was chosen for further FISH testing. If Alexa Fluor[®] 488 and Alexa Fluor[®] 555 rather than FITC and Cy3 fluorophores were used, photo-stability increased from 10 sec to 1 min with a 100-W Olympus U-RFL-T burner. The use of 50-ml tubes for hybridization incubation reduced drying out, reagent wastage, and reaction times. By using 30% (v/v) formamide for all the oligos tested in this study, preparation was simpler and multiple oligos could be applied at the same time.

Optimizing Specimen Washing and Streptavidin Conjugation

The washing step could be substantially shortened if the NaCl concentration was increased from 0.3 to 0.9 M.

A 1-min PBS wash step at room temperature was also tested. This rapid and simple wash produced a high signal intensity, but also some nonspecific staining. If the nonspecific signal was unacceptable with a PBS wash, the wash could be repeated with regular washing buffer until the nonspecific staining was removed without adversely affecting the FISH signal. When oligo-b probes were used, an additional streptavidin-f incubation step was needed. Its signal was highest when streptavidin-f diluted in PBS was incubated at 38°C for 10 min. A number of measures were taken to counteract the nonspecific background signal associated with streptavidin-f. NaCl concentration in the previous washing buffer was increased from 0.3 to 0.9 M and the washing time lengthened from 3 to 10 min. Before applying, streptavidin-f was centrifuged at 10,000 *rcf* for 1 min. Finally, the streptavidin concentration was minimized without losing signal by diluting to a range of 1–10 µg/ml.

DISCUSSION

The study set out to optimize FISH for the detection of *S. aureus* and differentiation from *S. epidermidis*. *E. coli* was also tested to ensure that the FISH assays developed would work for Gram-negative bacteria as well. A merit of the study is that it addresses some aspects of the FISH procedure concerning fixation, permeabilization, buffers and fluorescent dyes that were previously unquestioned. Although some of the study experiments did not result in major improvements to the assay, they may still be valuable for further studies, especially those that aim to optimize FISH.

Preparing Buffers

To test a range of FISH treatments, hybridization and washing buffers were prepared beforehand and stored long-term at –20°C and mixed to their final formamide and NaCl concentrations just before use. It was relatively straightforward to prepare large volumes of buffer and store. As far as the authors are aware, this approach to preparing FISH buffers has not been reported elsewhere and is useful and applicable to routine diagnostics where labor costs are important. Since it was not necessary to prepare the reagents for each batch of FISH experiments, time-savings were made. Preparing and storing buffers for up to an year did not affect their application in the FISH assay or its signal. Hybridization buffer prepared with oligos could also be used for up to a week without signal loss or nonspecific binding (data not shown).

One-Step vs. Two-Step Permeabilization

The biotin-streptavidin system is rarely used in clinical FISH studies. The oligo-b assays are more

involved and take longer than those that use oligo-f. Multiple oligo-b probes applied to the same specimen for different microbes cannot be distinguished by streptavidin-f as it binds to all of them. This can be a major disadvantage since it is necessary in diagnostic FISH to combine the use of a species-specific probe with a eubacterial probe as an internal control. In addition, nonspecific background staining is higher when streptavidin-f is used. The study found, however that it is possible to detect bacteria quickly with a relatively simple oligo-b FISH assay. It was not possible to apply and distinguish between multiple oligo-b probes simultaneously with streptavidin-f. As a simple work-around, the same specimen was spotted to more than one slide well and a different oligo-b probe was applied to each well. The flip-side of this biotin-streptavidin system limitation is that only one streptavidin-f is required for visualization and the cost of an oligo-b probe is about a quarter of its oligo-f counterpart. Nonspecific background staining was controlled by more stringent washes, and minimizing the amount of streptavidin-f.

An unexpected outcome of the study was that the 1-hr two-step FISH assay produced a higher signal intensity than the one-step assay when oligo-f probes were used. This suggests that permeabilization is a key factor in hybridization. The time-to-result is 15 min longer, but if the FISH signal is low or the background nonspecific signal is high, the two-step FISH assay might be a more practical choice.

Study Limitations

Cultures of clinical samples rather than clinical isolates were tested. Reference strains and other frequently encountered microbes were not tested. Since it was difficult to control for the variation when comparing the FISH signal intensity between treatments, representative images were used. Each treatment was repeated three times, an image was taken and if the variation between the three images was not significant, one image was chosen as representative. The two-step FISH assay that was developed could not be shortened to less than 1-hr without loss of *S. epidermidis* EUB338 signal. The optimized protocols listed in Table 1 were a compromise to capture the varied responses of the microbes tested. *S. aureus* produced a higher and more consistent signal when treated with methanol and lysostaphin, whereas *S. epidermidis* produced a higher and more consistent signal with ethanol and lysozyme.

CONCLUSION

The study found that a FISH assay that used a lysozyme step followed by a PBS-lysostaphin step had a higher STAAUR signal and could be applied almost as

rapidly as the FISH assay that combined lysozyme and lysostaphin into one-step. The two permeabilization steps lengthen the assay, but provided optimal conditions for lysozyme and lysostaphin enzyme activity, better control over the process of permeabilization as well as a higher level of permeabilization. The two-step assay might be used when the FISH signal is weak, bacterial numbers are low or if there is a need to use other reporter molecules such as catalyzed reporter deposition (CARD)-FISH (26). Further testing of the findings is warranted in a clinical scenario.

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