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The Simultaneous Detection and Differentiation of *Staphylococcus* Species in Blood Cultures Using Fluorescence in situ Hybridization: A Comment

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Wang [1] reported a useful slide-based fluorescence in situ hybridization (FISH) protocol that involved applying two oligonucleotides with different fluorophores simultaneously. Compared to applying the *Staphylococcus aureus* probe [2] alone, the author demonstrated that using it in conjunction with the *Staphylococcus* spp. probe [3] increased the sensitivity and specificity of *S. aureus* detection and differentiation from coagulase-negative staphylococci (CoNS) [1]. We note that the multicolor dual-probe FISH method took at least 2 h to finish. However, it is possible to detect *S. aureus* with FISH in less than 1 h [4].

The author described difficulties in cell adhesion to the slides. We found that slides spotted with agarose [5] reduced cell loss and that *S. aureus* permeabilization responds better to methanol than ethanol [4]. Spotting specimens onto a 10-well diagnostic glass slide (Menzel-Gläser, X1XER308B) rather than smearing was also more effective. A greater concentration of cells was heat-fixed to a smaller slide area, less reagents were needed throughout the FISH protocol and more accurate comparisons could be made between specimens and FISH treatments on the same slide.

The lysis enzyme conditions stated may have resulted in the permeabilization time of at least 20 min. We observe that the optimal permeabilization temperature is not 30°C but approximately 35°C for lysozyme (Sigma, L6876) and 45°C for lysostaphin (Sigma, L4402). If specimens were fixed with methanol and then permeabilized at 46°C with a lysozyme and lysostaphin mixture, the permeabilization time can be shortened to 5 min [4].

We agree that using a *Staphylococcus* spp. and an *S. aureus* probe together may be the most robust probe option as the *S. aureus* and CoNS 16S rRNA are conserved, but we observe that an untested CoNS probe is available (CoNS 16S1442: 5'-CGACG-GCTAGCTCCAAATGGTTACT-3'). The EUB338 probe is a necessary control as it confirms if the protocol is effective, but we believe that the non-EUB338 probe is not necessary; we are not aware of any instances where it has failed as a negative control in the detection of staphylococci with FISH [2, 3, 5].

The author modified the commonly accepted FISH protocol [2–4]. It might have been simpler, however, to only adjust the hybridization buffer's formamide concentration and the washing buffer's NaCl concentrations rather than increasing the incubation temperature to 50°C as well [6]. Using 46°C with a 0.9 M NaCl hybridization buffer has the advantage of allowing stringency to be optimized to formamide concentrations between 0 and 60% for most probes [2–4, 6]. Similarly, a 48°C washing buffer step allows stringency to be optimized to NaCl concentrations between 0.014 and 0.9 M for most probes [3, 4, 6]. FISH protocol turnaround is more likely to be shortened by effective specimen preparation and permeabilization rather than stringency adjustments [4].

We found that the fluorescein isothiocyanate fluorophore, and to a lesser extent Cy3, suffers from poor signal, bleaching and spectral overlap. In our experience, Alexa Fluor® (Invitrogen) or DyLight Fluor® (Thermo Fisher) probes are superior in signal strength, photostability and spectral flexibility. For instance, fluorescence output could be doubled and potential photobleaching and bleed-through halved if fluorescein isothiocyanate and Cy3 were replaced with Alexa Fluor 488 and Alexa Fluor 555 fluorophores (Invitrogen).

We consider the dual-probe FISH technique described by Wang [1] a valuable addition to the detection of *S. aureus* which can be further enhanced.

References

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Reply

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I would like to thank Lawson et al. for providing an excellent opinion regarding the article 'Simultaneous detection and differentiation of *Staphylococcus* species in blood cultures using fluorescence in situ hybridization' [1]. I agree that heat fixation, and pretreatment at 46°C with a lysozyme and lysostaphin mixture is helpful for permeabilization.

As for the probe, it is difficult to design a single probe to cover all CoNS species. An alternative strategy could be to use two specific probes that target different conserved sequences (data not published). Non-EUB338 is necessary to detect nonspecific binding of oligonucleotides [2], although it seldom occurs to clinical specimens. The FISH protocol was a modification of that established by Jansen et al. [3], in which the cell wall of *Staphylococcus aureus* was rigid, such that an intensive hybridization signal could be taken into account as a priority rather than a turnaround time.

No doubt, Alexa Fluor® (Invitrogen) or DyLight Fluor® (Thermo Fisher) probes are superior to fluorescein isothiocyanate and

Cy3 in terms of signal strength; however, the expensive cost hinders its wide application in routine clinical laboratory.

There is room for enhancement in the permeabilization and hybridization protocol. I appreciate the effort made by the authors to share their experiences concerning the FISH protocol.

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