Nile tilapia, *Oreochromis niloticus*, blood agar and the culture of fish bacterial pathogens

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Abstract
Fish blood agar (FBA) was made using blood from Nile tilapia, *Oreochromis niloticus*. Four piscine bacterial isolates, *Streptococcus iniae*, *Acinetobacter calcoaceticus*, *Staphylococcus epidermidis*, and *Edwardsiella tarda*, were chosen according to their different Gram stain, morphologic, and haemolytic characteristics. The isolates were plated on FBA and sheep blood agar (SBA) to compare relative growth according to agar used. Bacteria counts and colony morphology were not significantly different between the same isolates cultured on FBA or SBA. When comparing zones of haemolysis, only the *S. iniae* zones of haemolysis were significantly smaller on FBA than on the SBA. The results of this study showed that FBA did not have any bacteriological benefits over SBA. Moreover, SBA is easily obtained commercially and may be relatively inexpensive given the labour required for the husbandry of healthy fish, harvest of fish blood, and production of sterile FBA plates. However, FBA may be a suitable alternative to SBA when SBA is not readily available.

Introduction
Blood agar plates are regularly used for the culture and subsequent isolation of bacterial pathogens of fish, and most studies have utilized mammalian blood agar plates for this purpose. Few studies have used agar made with fish or aquatic mammal blood or serum to culture piscine pathogens (Pier et al., 1978; Eldar et al., 1995a; Dhevendaran & Georgekutty, 1998; Lee et al., 1999), while some have used fish serum to supplement culture media for parasite culture (Isom & Hudson, 1982; Bienek & Belosevic, 1997; Ardelli & Woo, 2001). Different bacterial characteristics have been noted when using agar supplemented with fish or aquatic mammal blood or serum versus mammalian blood. Researchers have observed presumptive advantages (Pier et al., 1978; Dhevendaran & Georgekutty, 1998), disadvantages (Lee et al., 1999), or no apparent variations (Eldar et al., 1995a) in bacterial activity when using aquatic animal blood agar. Other studies have also noted differences in haemolytic bacterial characteristics when fish pathogens are grown on a variety of mammalian blood agar (Miyamoto et al., 1969; Pier et al., 1978; Eldar et al., 1995b).

The question remains whether fish blood agar (FBA) could confer benefits over mammalian blood agar when culturing and characterizing different piscine bacterial pathogens. Other studies generally assessed the growth of only one bacterial genus, such as *Streptococcus* (Pier et al., 1978; Eldar et al., 1995a; Dhevendaran...
In this study, we evaluated four different piscine bacterial genera and species: *Streptococcus iniae*, *Acinetobacter calcoaceticus*, *Staphylococcus epidermidis*, and *Edwardsiella tarda*, each chosen due to their different Gram stain, morphology, and haemolytic characteristics. The isolates were cultured using FBA or sheep blood agar (SBA), and multiple bacterial culture characteristics (relative growth, colony morphology, and haemolytic activity) were assessed to fully characterize growth of different genera and species of bacteria on both agar types. Based on our findings and those of other researchers, we assess the utility of FBA and indicate whether FBA can be useful culturing fish pathogens under conditions where SBA is not readily available.

**Materials and methods**

Nile tilapia, *Oreochromis niloticus*, were housed at the Aquatic Animal Health Research Laboratory (AAHRL), United States Department of Agriculture, Agriculture Research Service, Auburn, Alabama, USA. Tilapia were chosen as the source of the blood supplement because they are readily available in many aquaculture and research facilities throughout the world. In a non-lethal procedure, ten fish were bled from the caudal vein using 23 gauge needles and uncoated Vacutainer blood collection tubes™ (Becton Dickinson, Franklin Lakes, NJ, USA). Pooled blood had a packed cell volume of 30, and blood coagulation was prevented by adding an equal volume of Alsever’s Solution (Sigma, St. Louis, MO, USA). A tryptic soy agar (TSA) solution (Difco, Detroit, MI, USA) was autoclaved at 130°C and 22 psi for 20 min and then cooled to 50°C in a water bath before addition of 5% uncoagulated fish blood. The fish blood agar (FBA) was then poured into sterile polystyrene 100 x 15 mm petri dishes (Fisher Scientific, Pittsburgh, PA, USA) under sterile conditions. After the agar cooled and solidified, the plates were stored at 4°C until used in this study. Approximately 10% of the freshly-prepared FBA plates were incubated for 48 h at 35°C to check for sterility, and no contamination was observed. For comparison, the bacterial isolates were also grown on TSA containing 5% sheep blood agar (SBA; Remel, Lexena, KS, USA).

The following piscine bacteria used in this study were chosen largely based on their different Gram stain, morphology, nutritional requirements, and haemolytic characteristics: *S. epidermidis*, *S. iniae*, *E. tarda*, and *A. calcoaceticus* (Table 1). The respective bacteria were grown on TSA culture plates without blood at 35°C for 24 h and transferred into 10 mL of sterile saline. The inocula were standardized according to a Number 1 McFarland turbidity and diluted 1:100,000 to create final inocula of 3 x 10³ colony-forming units (CFU)/ml. Within 15 min, the diluted isolates were plated onto triplicate FBA and SBA plates using a spiral plater (Spiral Biotech 4000, Bethesda, MD, USA) to ensure even distribution of the isolate.

The plates were incubated at 35°C and colony growth, morphology, and zones of haemolysis were assessed after 24 and 48 h incubation. Mean bacteria CFU/ml were determined from three replicate plates of each isolate with the

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1 Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.
Q Count system (Spiral Biotech, Norwood, MA, USA) according to manufacturer’s instructions. Zones of haemolysis were assessed for *S. iniae* and *A. calcoaceticus* using calipers to measure from edge to edge of the clear zone of red cell haemolysis around an isolate colony. The plates were divided into quadrants and 10 colonies from each quadrant were measured. Mean measurements (mm) were determined for each plate, and then a mean was determined for each isolate from the triplicate plates.

All data was analyzed using the SAS program (SAS Institute, Cary, NC USA). Bacteria counts and zones of haemolysis for each bacterial pathogen grown on FBA and SBA were compared with one-way analysis of variance and Duncan’s multiple range test. Significant differences between groups were accepted at $p < 0.05$.

### Results and discussion

No contamination was observed on the FBA and SBA plates, and no other bacteria grew on the plates other than the ones tested. Pure colonies were observed within 24 h on both types of agar, and mean bacteria counts ranged from $1 \times 10^3$ to $9 \times 10^3$ CFU/ml (Figure 1). Three of the bacterial isolates, *E. tarda*, *S. iniae*, and *A. calcoaceticus*, grew well on the FBA and SBA. However, *S. epidermidis* produced considerably lower bacteria counts on both agars. Zones of β-haemolysis were observed around the *S. iniae* and *A. calcoaceticus* colonies, and the mean zones ranged from approximately 3 to 9 mm in size (Figure 2). No haemolysis was noted for the *S. epidermidis* or *E. tarda* isolates.

Though the bacteria counts and the zones of haemolysis varied considerably between the different bacterial genera and species, the differences were generally insignificant when comparing the growth, colony morphology, and haemolytic activity of each isolate on FBA and SBA plates. However, the zones of haemolysis of *S. iniae* grown on SBA plates were significantly increased above those grown on FBA ($p < 0.0001$). Dhevendaran and Georgekutty (1998) found that 90% of *Vibrio* spp. strains tested released haemolysin on fish blood agar versus 33% on calf blood agar. These results indicate that the *Vibrio* spp.

<table>
<thead>
<tr>
<th>Bacterial isolate and designation$^1$</th>
<th>Gram stain</th>
<th>Haemolysis</th>
<th>Fish species source</th>
<th>Organ source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em> ARS 01-TN-B</td>
<td>+</td>
<td>-</td>
<td>Nile tilapia (<em>Oreochromis niloticus</em>)</td>
<td>Brain</td>
</tr>
<tr>
<td><em>S. iniae</em> ARS 60</td>
<td>+</td>
<td>+</td>
<td>Hybrid striped bass (<em>Morone saxatilis</em> x <em>M. chrysops</em>)</td>
<td>Brain</td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em> ARS ET-04 Parent</td>
<td>-</td>
<td>-</td>
<td>Hybrid striped bass (<em>Morone saxatilis</em> x <em>M. chrysops</em>)</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em> ARS 00-DE-AM-133SL (bvalc)</td>
<td>-</td>
<td>+</td>
<td>Atlantic menhaden (<em>Brevoortia tyrannus</em>)</td>
<td>Skin</td>
</tr>
</tbody>
</table>

$^1$ All bacterial isolates were obtained from stocks maintained at the Aquatic Animal Health Research Laboratory, United States Department of Agriculture, Agricultural Research Service.

Table 1. Bacterial isolate and designation, Gram stain characteristic, haemolytic activity, and source.
Figure 1. Mean bacteria count (x 10^3 CFU/mL) ± standard error for *Staphylococcus epidermidis*, *Streptococcus iniae*, *Edwardsiella tarda*, and *Acinetobacter calcoaceticus* following culture on fish blood agar (FBA; solid black bars) or sheep blood agar (SBA; gray bars) for 24 or 48 h. No significant differences (p > 0.05) between any of the bacterial isolate bacteria counts on FBA and SBA were observed.

Figure 2. Mean zone of haemolysis (mm) ± standard error for *Streptococcus iniae* and *Acinetobacter calcoaceticus* following culture on fish blood agar (FBA; solid black bars) or sheep blood agar (SBA; gray bars) for 24 or 48 h. A significant difference (p < 0.0001) between the bacterial isolates zone of haemolysis on SBA and FBA is noted by asterisk.
haemolysin release or cellular activity might be upregulated in the presence of fish blood cells. Dhevendaran & Georgekutty (1998) do not state which species of fish were harvested for the blood. In the present study, the FBA did not produce increased haemolytic activity, though only 2 haemolytic bacterial isolates were tested.

Pier et al. (1978) cultured *S. iniae* isolated from an Amazon freshwater dolphin, *Inia geoffrensis*, on agar supplemented with 5% freshwater dolphin blood. These authors observed that the zones of haemolysis were three to five times greater than produced on agar supplemented with blood from other mammalian species. Further, clearer zones of β-haemolysis were produced with dolphin blood agar. In their paper, Pier et al. (1978) proposed that the dolphin red blood cells may be more susceptible to *S. iniae*-induced lysis or that *S. iniae* may have a greater affinity for dolphin cells. In contrast, the use of blood from certain species of organisms may have a negative effect on bacterial activity. Lee et al. (1999) observed that the addition of serum from grouper, *Epinephelus coioides*, to TSA reduced the protease production of *V. carchariae* isolated from grouper. The authors proposed that the grouper serum contained protease inhibitors, and this suggests that caution should be exercised when choosing the animal source of blood for blood agar plates.

Other researchers have found variability in bacterial isolate characteristics when cultured on different mammalian blood agars. For example, the haemolytic activity of streptococcal organisms may vary according to the animal blood and basal agar used for culture (Ruoff et al., 1999). Specifically, differences in *S. iniae* isolate haemolysis have been observed, presumably due to blood agar composition. One research group observed that *S. iniae* showed α-haemolysis or partial haemolysis (presumptively α-haemolysis) on 5% beef or human blood agar (Eldar et al., 1994; Eldar et al., 1995b), but complete haemolysis (presumptively β-haemolysis) on 5% SBA. Other haemolytic differences may also be attributed to the basal agar composition. Nguyen & Kanai (1999) observed β-haemolytic *S. iniae* isolates on heart-infusion agar (HIA) with 3% horse blood, but the same isolates were α-haemolytic when grown on Todd-Hewitt agar (THA) with 3% horse blood.

Facklam & Washington (1991) recommend the culture of streptococcal organisms on enriched infusion agar (TSA, HIA, THA), because they lack reducing sugars that would interfere with β-haemolytic activity. In addition, the blood concentration in the agar may influence the observed haemolysis; low concentrations may make α- or β-haemolysis difficult to determine, while high concentrations may cause β-haemolytic organisms appear as non-haemolytic organisms (Facklam & Washington, 1991). The blood source and basal agar composition must be considered when monitoring certain bacterial growth characteristics. In our study, TSA supplemented with either 5% sheep or tilapia blood allowed the observation of β-haemolytic *S. iniae*.

Overall, the tilapia blood agar failed to provide significant bacteriological benefits over SBA. Sheep blood agar displayed the only significant advantage, allowing for a
larger zone of haemolysis during S. iniae culture. Sheep blood agar may be more desirable, because it is widely available from commercial sources and is thus easily obtained. Furthermore, SBA may also be relatively inexpensive given the labor required for the husbandry of healthy fish, harvest of fish blood, and production of sterile FBA plates. However, SBA itself may not always be advantageous for financial reasons; Nguyen & Kanai (1999) used horse blood agar because it costs less than SBA. This study indicates that FBA is a useful alternative to SBA, especially in aquatic laboratories where fish blood is readily accessible, in areas where prepared media might be unavailable for purchase, or in labs that might not have the financial resources to purchase prepared media.

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References


