Effectiveness of Early Intervention with Florfenicol on a
Streptococcus iniae Infection in Blue Tilapia

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Abstract.—A study was performed to assess the efficacy of feeding a florfenicol (FFC)-medicated diet to control experimental Streptococcus iniae infection in blue tilapia Oreochromis aureus. Tested doses of FFC were 0, 5, 10, 15, and 30 mg of active ingredient per kilogram of fish body weight (BW) per day. Fish were subjected to waterborne S. iniae exposure after skin scraping, and administration of medicated feed started at 6 h postchallenge and continued for 10 consecutive days, followed by a 12-d posttreatment observation period. Administration of FFC-medicated feed for 10 d significantly increased (P < 0.05) the survival of S. iniae-infected blue tilapia from 19% in the challenged, nonmedicated positive control group to 94, 96, 99, and 98% in the 5-, 10-, 15-, and 30-mg/kg FFC treatment groups, respectively. The survival rates of the different FFC-medicated treatment groups were not significantly different. At the conclusion of the experiment, no S. iniae carriers were detected in any challenged group receiving the FFC-medicated diet, while the bacterium was recovered from the S. iniae-challenged, nonmedicated survivors of the infection. This study suggests that using FFC at 10 mg/kg BW daily for 10 d is an effective treatment against S. iniae infection in blue tilapia.

Streptococcus iniae, an etiological agent of streptococcosis in farmed finfish, has emerged as an important fish pathogen in the past few decades. Streptococcosis affects more than 20 species of fish (Kitao 1993) and has a worldwide distribution (Agnew and Barnes 2007). Annual aquaculture losses due to S. iniae were estimated at US$10 million in the United States alone and at $100 million globally (Shoemaker et al. 2001). The disease represents a real danger to cultured warmwater fishes, particularly intensively cultured tilapias Oreochromis spp. Streptococcus iniae is a Gram-positive coccus that is nonmotile, catalase negative, fermentative in glucose, and non-spore-forming (Plumb, J. A. 1999). Fish infected with S. iniae commonly exhibit dark skin pigmentation, lethargy, erratic and spiral swimming, curved bodies, abdominal distention, exophthalmia, corneal opacity, hemorrhages, enlarged and nearly black spleens, and bloody mucoid fluid in the gastrointestinal tracts (Plumb, J. A. 1999; Darwish et al. 2002; Darwish and Hobbs 2005).

Proper fish husbandry and health management practices, such as providing good water quality, reducing levels of stress, and supplying vaccinations, are essential to successful fish farming; however, when an epizootic is in progress, antibiotics can be the only viable option for prevention of catastrophic mortalities. Florfenicol (FFC) is a broad-spectrum, primarily bacteriostatic antibiotic that acts by binding to 50S (large subunit) ribosomal RNA, thereby inhibiting bacterial protein synthesis (Plumb, D. C. 1999). Presently there are no approved antibacterial agents for use in tilapias cultured in the United States. Florfenicol has been approved for controlling susceptible bacterial infections of aquacultured fish in Japan (yellowtail Seriola quinquergiata, red sea bream Pagrus major, coho salmon Oncorhynchus kisutch, jack mackerels Trachurus spp., rainbow trout O. mykiss, sweetfish Plecoglossus altivelis, tilapias, and Japanese eel Anguilla japonica), South Korea (yellowtail and Japanese eel), Norway (Atlantic salmon Salmo salar), Canada (Atlantic salmon), and the United Kingdom (Atlantic salmon; Gaunt et al. 2004). Most recently, the U.S. Food and Drug Administration (FDA) approved FFC for treating enteric septicemia of catfish (ESC) and columnaris (conditional approval) in channel catfish Ictalurus punctatus. Ongoing studies are designed to gain FDA approval for use of FFC in all freshwater fishes (Pat Gaunt, Mississippi State University, personal communication). Expanding the approval of FFC to include tilapias is more feasible than exploring other antibiotics that have not been approved in the United States. There is currently no peer-reviewed literature on the efficacy of FFC for control of S. iniae infection in tilapias. The objective of the present study was to assess the efficacy of early intervention with FFC in controlling an S. iniae infection in blue tilapia Oreochromis aureus.

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Methods

Experimental Design

Groups of 10 blue tilapia each were weighed; using a randomization table generated by MINITAB version 13 (MINITAB, 2000), the fish were then randomly allocated to 30 tanks with 2 groups/tank for a total of 20 fish/tank. After a 7-d acclimation period, the 20 fish in each tank were collectively weighed. Six different treatments were equally and randomly assigned to the 30 tanks (5 tanks/treatment). In four of the treatments, fish were subjected to cutaneous scraping and then were challenged by waterborne exposure to *S. iniae* for 30 min; these fish were offered medicated feed at 5, 10, 15, or 30 mg of FFC/kg of fish body weight (BW) daily for 10 d. The fifth treatment group was also challenged but was offered nonmedicated feed (positive control). In the sixth treatment, fish were not challenged and were offered nonmedicated feed (negative control). Feeding of medicated or nonmedicated diets began 6 h after the bacterial challenge and continued daily for 10 d. Subsequently, all groups of fish were fed the control (nonmedicated) diet for an additional 12-d posttreatment observation period.

Fish and Culture Conditions

Blue tilapia of mixed sex (mean weight ± SE = 37.5 ± 0.8 g) were provided by the Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, Arkansas. Fish (20 fish/tank) were held in tanks containing 55 L of continuously aerated, flow-through well water. Flow rate in each tank was approximately 1 L/min, and temperature was maintained at 24.6 ± 0.03°C. During the 7-d acclimation period, the fish were fed the control (nonmedicated) diet at 2% BW daily. At the conclusion of the acclimation period, fish in each tank were collectively weighed to calculate the weight of diet (2% BW) to be administered after the bacterial challenge.

**Preparation of the florfenicol-medicated diet.**—Florfenicol (Schering Plough Animal Health Corp., Union, New Jersey; provided by Jim Bowker, U.S. Fish and Wildlife Service) was incorporated into a commercial production feed (Rangen Connatural Products, Angleton, Texas) to provide 5, 10, 15, or 30 mg of active ingredient (FFC)/kg BW daily at a ration level of 2% BW per day. The four FFC-medicated diets were formulated to contain 0.25, 0.50, 0.75, and 1.50 mg FFC/g of feed, respectively.

To incorporate antibiotic into the commercial diet, we pulverized the diet by grinding it to less than 0.5 mm in a hammer mill (Model F21M; W-W Grinder Corp., Wichita, Kansas). Pulverized rations were thoroughly blended with antibiotic in a V-mixer (Blendmaster laboratory blender; Patterson-Kelly, Stroudsburg, Pennsylvania) for 15 min. Dry ingredients were placed in a commercial food mixer (Model A-200; Hobart, Troy, Ohio), and water was subsequently added until a uniform mixture was obtained. The moistened mixture was passed through a meat grinder equipped with a 3-mm die to obtain uniform pellets. Pelleted diets were air-dried and then frozen in air-tight bags at −18°C until needed; the diets were stored at 4°C prior to feeding.

The FFC level in two 200-g samples of each FFC-medicated diet was analyzed with high-performance liquid chromatography to confirm the FFC concentration in the diet. The analysis was performed by Eurofins Scientific (Portage, Michigan) using Hayes’ (2005) method. The 5-, 10-, 15-, or 30-mg/kg FFC diets contained the following FFC concentrations (mean ± SE): 0.25 ± 0.001, 0.518 ± 0.003, 0.763 ± 0.005, and 1.48 ± 0.005 mg FFC/g feed, respectively.

**Challenge Protocol**

*Bacterium.—Streptococcus iniae* (isolate LA94–426; provided by John Hawke, Louisiana State University, Baton Rouge) was cultured on tryptic soy agar (TSA) supplemented with 5% sheep blood (5SB; Edge Biologicals, Inc., Memphis, Tennessee) for 24 h at 30°C. The bacterium was beta-hemolytic and was presumptively identified by biochemical testing (Pier and Madin 1976; Darwish and Hobbs 2005) using API 20 Strep (BioMerieux, Inc., St. Louis, Missouri) and confirmed by polymerase chain reaction (PCR) using the Roach et al. (2006) modification of the Zlotkin et al. (1998) method. The genomic DNA of the bacterium was extracted using Qiagen DNasy Blood and Tissue Kit (Qiagen, Valencia, California). The purity of the culture was monitored by streaking on TSA-5SB for isolated colonies and by Gram staining.

**Challenge.—**The virulent *S. iniae* isolate LA94–426 was passed four times in blue tilapia to maximize its virulence, and aliquots of the culture were stored at −80°C in brain–heart infusion broth (BHIB) containing 25% glycerin (Darwish and Hobbs 2005). The *S. iniae* isolate was grown for approximately 22 h in BHIB, and TSA-5SB plates were then streaked to confirm the culture’s purity. The challenge procedure was conducted according to the methods of Darwish and Hobbs (2005), with slight modifications. Briefly, the skin of the fish was scraped with a scalpel, which removed the scales and mucus unilaterally (on the right side) from an area located approximately 2 mm below the base of the dorsal fin, parallel to the longitudinal axis of the fish. The scraped area encompassed a width of approximately 5 mm and a length extending from the...
second to the fourth spines of the dorsal fin (anatomical markers).

In the challenge treatments, scraped fish in each tank were placed for 30 min in 10 L of aerated water containing \( S. \textit{iniae} \) at a concentration of \( 3.6 \times 10^7 \) colony-forming units/mL (Darwish et al. 2000) and then were returned to their respective tanks. Fish in the negative control group were similarly scraped and placed for 30 min in 10 L of aerated water but were not exposed to \( S. \textit{iniae} \). Positive control (PC) fish were challenged but received nonmedicated feed (i.e., 0-mg/kg dose). Each treatment had 100 fish equally divided among five tanks.

Dead and moribund fish were necropsied (external and internal pathology was recorded), and isolation of bacteria from the liver, trunk kidney, brain, and eye was attempted on TSA-5SB. At the conclusion of the experiment, four fish from each tank were sacrificed and necropsied, and the trunk kidney, brain, eye, and liver were cultured for \( S. \textit{iniae} \). When fewer than four fish were left in a tank, all fish in the tank were sampled. Recovered bacteria from infected fish were identified by biochemical testing and by PCR as previously described.

### Statistical Analysis

At the conclusion of the experiment, the survival percentages within tanks were arcsine transformed. Using MINITAB version 13, the data were subjected to one-way analysis of variance (Zar 1984; Sokal and Rohlf 1995), and the differences among treatment means were determined (Tukey 1953). Treatment effects were considered significant at \( P \)-values less than 0.05.

GraphPad Prism version 4 (GraphPad Software 2005) was used to conduct the following survival analyses: (1) Kaplan–Meier method to calculate the probability of survival at each day, (2) logrank tests to compare the survival curves, and (3) hazard ratio computation (Motulsky 1995). The hazard ratio compares the slope of the two survival curves and estimates the relative risk of death for fish in a given treatment compared with another treatment. For example, a hazard ratio of 2 indicates that the risk of death in one group is twice that in a second group. A Bonferroni correction wherein the \( \alpha \) level (in this case, 0.05) is divided by the number of pairwise comparisons was employed to account for increasing type I error with the number of comparisons made (Motulsky 1995).

### Results

#### Mortality, Clinical Signs, and Gross Pathology

At the conclusion of the experiment, FFC effectively reduced mortality in blue tilapia challenged with \( S. \textit{iniae} \). Treatment with FFC at 5, 10, 15, and 30 mg/kg BW daily for 10 d postchallenge significantly increased the final survival rates to 94 ± 2.5, 96 ± 1.9, 99 ± 1, and 98 ± 1.2%, respectively, compared with the 19 ± 2.5% survival in the positive control (challenged, nonmedicated) group (Figure 1). There was no significant difference among the survival rates of the challenged, medicated groups and the negative control group (Table 1). The hazard ratios calculated for the positive control group relative to the challenged, medicated treatment groups are provided in Table 1.

The logrank comparison indicated that the survival curves of the FFC-medicated treatment groups were significantly different from that of the positive control group. There was no significant difference among the survival rates of the FFC-medicated treatment groups and that of the negative control (nonchallenged, nonmedicated) group (92 ± 2.6%).

![Figure 1](image)
pathology were observed in the positive control group and the challenged, medicated treatment groups. During the course of the experiment, some of the sexually mature fish in different treatments expressed aggressive behavior that was associated with mortality; the negative control group had an 8% mortality rate, with no infectious pathology and a negative bacterial culture.

The feeding activities of the positive control fish declined at 2 d postchallenge; at 4 d postchallenge, feeding activities were virtually nonexistent. The negative control fish consumed all of the offered feed within minutes. The feeding activity of the challenged groups treated with FFC did not decline and was indistinguishable from that of the negative control.

Diseased fish were lethargic and later developed neurological signs. External signs included abdominal distension, eye lesions, and dark pigmentation, hemorrhages, and erythema of the skin. Internally, the organs exhibited petechial hemorrhaging, and there was serous ascitic fluid that became whitish and fibrinous as the infection progressed.

At the conclusion of the experiment, the remaining fish in all treatments, including the positive control, were feeding and did not exhibit clinical signs or gross pathology.

**Bacterial Isolation**

Severe and predominantly systemic infection was achieved in the positive control fish. Mortality of positive control fish was 81%, and 95% of the fish tested positive for *S. iniae*; the bacterium was isolated from the kidney (systemic infection) in 94% of positive control fish. *Streptococcus iniae* was also isolated from the dead and moribund fish in the challenged, medicated groups (5 of 13 fish, or 38%). *Streptococcus iniae* isolation from dead and moribund fish in all challenged groups is summarized in Table 2. Except for the positive control fish, no fish cultured positive for *S. iniae* after the 16-d postchallenge period. The eight mortalities from the negative control group did not culture positive for bacteria. At the conclusion of the experiments, attempts to isolate *S. iniae* from all negative control fish and challenged fish that received FFC-medicated diets were negative. *Streptococcus iniae* was isolated from 1 of the 18 positive control fish sacrificed at the conclusion of the experiment. The *S. iniae* cultures were presumptively identified by biochemical testing and were confirmed by PCR.

**Discussion**

The results of this experiment demonstrated the efficacy of FFC for control of blue tilapia mortalities caused by *S. iniae* infection. All FFC treatment levels (≥5 mg/kg BW daily for 10 consecutive days) significantly reduced mortality compared with that in the positive control group. The nonsignificant difference among the mortality levels of the FFC-treated fish is similar to that referenced in the literature. There was

Table 1.—Logrank comparison of survival curves and hazard ratios of blue tilapia subjected to cutaneous scraping, challenged by waterborne exposure to *Streptococcus iniae*, fed different florfenicol (FFC) levels in the diet for 10 d, and observed for a posttreatment period of 12 d. Challenged fish received 0, 5, 10, 15, or 30 mg of FFC/kg of body weight daily (0-mg/kg dose group was the positive control). Unchallenged, nonmedicated fish acted as the negative control (NC) group. Each comparison shows the *P*-value (left) and hazard ratio (right) of the two survival curves. The *P*-value is the probability that the two curves are identical in the overall population. The hazard ratio compares the slopes of the curves; for example, a hazard ratio of 2 indicates that the rate of death in one group is twice the rate in a second group.

<table>
<thead>
<tr>
<th>FFC treatment (mg/kg)</th>
<th>NC</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>P</em> = 0.001*</td>
<td>0.06</td>
<td><em>P</em> &lt; 0.0001</td>
<td>22.11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>P</em> = 0.5687 1.36</td>
<td><em>P</em> &lt; 0.0001</td>
<td>30.82</td>
<td><em>P</em> = 0.5192 1.51</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td><em>P</em> = 0.0166 8.33</td>
<td><em>P</em> &lt; 0.0001</td>
<td>130.9</td>
<td><em>P</em> = 0.0536 6.18</td>
<td><em>P</em> = 0.1727 4.07</td>
</tr>
<tr>
<td>30</td>
<td><em>P</em> = 0.0523 4.11</td>
<td><em>P</em> &lt; 0.0001</td>
<td>64.36</td>
<td><em>P</em> = 0.1523 3.04</td>
<td><em>P</em> = 0.4107 2.01</td>
</tr>
</tbody>
</table>

* Using the Bonferroni correction (see Methods), the *P*-value is significant at *P* ≤ 0.0033. Significant values are in bold text.

Table 2.—Bacterial isolation from kidney, brain, eye, and liver of dead and moribund blue tilapia (94 fish) challenged by waterborne exposure to *Streptococcus iniae* after skin scraping. The trial had challenged, nonmedicated treatment (i.e., positive control) and four challenged, medicated treatments at 5, 10, 15, and 30 mg of florfenicol/kg of body weight daily for 10 d (percent positive = [number positive/number tested] × 100).}

<table>
<thead>
<tr>
<th>Variable</th>
<th>Kidney</th>
<th>Brain</th>
<th>Eye</th>
<th>Liver</th>
</tr>
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<tbody>
<tr>
<td>Positive isolation</td>
<td>78</td>
<td>82</td>
<td>82</td>
<td>63</td>
</tr>
<tr>
<td>Negative isolation</td>
<td>14</td>
<td>12</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>No isolation attempteda</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>% positive</td>
<td>85</td>
<td>87</td>
<td>87</td>
<td>88</td>
</tr>
</tbody>
</table>

*a* Bacterial isolation was not attempted due to poor carcass condition resulting from postmortem changes.
no significant difference in mortality between the 5- and 10-mg FFC/kg BW treatments when FFC was used against *Edwardsiella ictaluri* in channel catfish (Gaunt et al. 2004) or against *S. iniae* in sunshine bass (female white bass *Morone chrysops* × male striped bass *M. saxatilis*; Darwish 2007). The current demonstrated efficacy of FFC at 10 mg/kg BW daily for 10 d is similar to other reports that show effectiveness of FFC against streptococcusiosis in sunshine bass (Darwish 2007) and against other diseases in different species, such as ESC in channel catfish (Gaunt et al. 2003, 2004, 2006), furunculosis in Atlantic salmon (Nordmo et al. 1994; Sheppard et al. 1994; Samuelsen et al. 1998), and vibriosis in Atlantic cod *Gadus morhua* (Samuelsen and Bergh 2004; Seljestokken et al. 2006). Increasing the FFC dose to 15 and 30 mg/kg BW daily in the present study did not improve the survival of challenged blue tilapia and was not grossly toxic; there were no notable clinical signs or gross pathology detected in the survivors of the two treatments. The observation of no toxic effect at 15 or 30 mg/kg BW daily in blue tilapia is similar to observations of channel catfish and sunshine bass (Gaikowski et al. 2003; Gaunt et al. 2003, 2004; Darwish 2007). Also, the feeding behavior of fish in the 30-mg/kg treatment suggests that the addition of FFC causes no palatability problems for blue tilapia. Florfenicol-medicated feed was palatable at doses as high as 100 mg/kg BW daily for channel catfish (Gaunt et al. 2003) and Atlantic salmon (Inglis et al. 1991).

Medicated feed would be ineffective once the fish become anorexic. The feeding activity of the positive control fish rapidly declined during early stages of the infection. The rapid decline highlights the importance of fish health monitoring and early intervention for minimizing losses. Gaunt et al. (2004) noted higher mortalities in channel catfish experimentally infected with *E. ictaluri* and fed an FFC-medicated diet at 2 d postchallenge compared with 1 d postchallenge. In the study by Darwish (2007), treatment of *S. iniae* infection in sunshine bass with FFC-medicated feed at 24 h postchallenge was crucial in preventing the explosive mortalities manifested in the challenged, nonmedicated group (38% at 2 d postchallenge and 89% at 3 d postchallenge) compared with 3, 1, 2, and 0% mortality in challenged fish treated with FFC-medicated feed at 5, 10, 15, and 30 mg/kg BW, respectively. In the current experiment and in previous efficacy experiments conducted with *S. iniae*-challenged blue tilapia or sunshine bass, dead and moribund fish in the medicated feed treatments had little or no food in their gastrointestinal tracts (Darwish and Hobbs 2005; Darwish 2007); outbreaks that have been identified later in the time course of the infection may respond less favorably to oral medications due to inappetence.

The results of the present study suggest that an FFC treatment regimen of 10 mg/kg BW daily for 10 consecutive days should be used, even with the nonsignificant difference in mortality between the 5- and 10-mg/kg treatments. Gaunt et al. (2004) conducted confirmatory efficacy testing using the 10-mg/kg FFC treatment; there was no statistically significant difference in mortality of *E. ictaluri*-infected channel catfish between 10- and 5-mg/kg FFC treatments. Gaunt et al. (2004) based the choice on the fish feeding hierarchies; more-aggressive feeders consumed higher doses of the medicated feed than did less-aggressive feeders (McCarthy et al. 1992). The higher dosage of FFC will probably ensure adequate intake of medication for all fish. This choice will be particularly appropriate in light of the decline in feeding during the early stage of the infection; a smaller consumed amount of the feed may have to provide effective therapeutic concentrations of FFC in fish tissues. In the present experiment, the efficacy of the 5-mg/kg dose might have been altered if the onset of the medicated diet had been delayed to 2 or 3 d postchallenge.

The reduction of mortality in the challenged, medicated fish suggests that the serum FFC concentration exceeded the minimum inhibitory concentration (MIC) of the pathogen by a factor of 2–4, which is the margin necessary for an antibacterial to be effective in controlling a systemic infection (Blood et al. 1979; Feng and Jia 2009). The *S. iniae* isolate used in the present study had an MIC value of 2 μg/mL (Darwish 2007). Darwish (2007) conducted an MIC assay of FFC against 19 isolates of *S. iniae*; 16 (84%) of the 19 isolates had a 2-μg/mL MIC, and the other three isolates had an MIC value of 4 μg/mL. This relative consistency should simplify the development of a treatment protocol for *S. iniae*.

The clinical signs and gross pathology observed in the present experiment are similar to those reported in natural and experimental infections (Plumb, J. A. 1999; Darwish et al. 2002; Darwish and Hobbs 2005). The infection was predominantly systemic, as indicated by the consistent isolation of *S. iniae* from the kidney. The re-isolation of the bacterium from positive control fish at 21 d postchallenge reinforces the suggestion that infected survivors could be carriers (Darwish et al. 2002; Darwish and Hobbs 2005), which would partly explain the difficulties encountered in controlling the infection once the bacterium is introduced into a tilapia culture operation.

The low number of mortalities (8 of 100 fish) in the negative control group could be explained by the aggressive behavior in sexually mature individuals,
which would result in limited mortalities (Steven Rawles and Ray Carter, Harry K. Dupree Stuttgart National Aquaculture Research Center, personal communication). The explanation is further supported by the negative bacterial isolation from all negative control fish and from eight fish in the FFC-medicated treatments. Blue tilapia will reach sexual maturity at 4–5 months of age and at a size as small as 28 g (Torrans 1977).

Although FFC appears to be effective against experimental *S. iniae* infection in blue tilapia, controlled field experiments and studies of target animal safety and tissue residues will be required by the FDA before FFC can be considered and approved for aquaculture use.

**Acknowledgments**

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