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[Article]

Efficacy of Florfenicol for Control of Mortality Caused by *Flavobacterium columnare* Infection in Channel Catfish

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Abstract.—The efficacy of florfenicol against infection by the bacterium *Flavobacterium columnare* was studied in channel catfish *Ictalurus punctatus* fingerlings held in 80-L aquaria. Nonabraded fish were challenged by immersion on day 0. Thirty 80-L tanks were randomly assigned in equal numbers to two treatment groups, one in which fish were fed a commercial diet without florfenicol (unmedicated feed) and one in which they were fed a diet containing 10 mg of florfenicol/kg of body weight (medicated feed) for ten consecutive days. Mortality was monitored during the treatment period and a 14-d posttreatment observation period. At the end of the posttreatment period, all fish were euthanized, examined for gross lesions, and cultured for *F. columnare*. Significantly fewer fish fed the medicated diet died (8.0%) than fish fed the unmedicated diet (54.2%). *Flavobacterium columnare* was cultured from 15.0% of the medicated fish, compared with 68.9% of the unmedicated fish. The gross lesions in the fish were consistent with columnaris disease, and *F. columnare* was cultured from 99.5% of the dead fish. No differences were observed in weight gain and appetence between the medicated and unmedicated groups. For the *F. columnare* strain used in this study, the minimal inhibitory concentration of florfenicol ranged from 0.5 to 1.0 mg/mL in the 30 bacterial cultures obtained from infected fish, and the mean disk diffusion zone of inhibition was 40 mm. There were no adverse effects among the medicated fish. Administration of florfenicol at a dosage of 10 mg/kg body weight for 10 d was efficacious and safe for the control of mortality from *F. columnare* infection in channel catfish.

Traditionally regarded as an important bacterial pathogen in farmed catfish, *Flavobacterium columnare* is second in occurrence to *Edwardsiella ictaluri* (Jack et al. 1992; Durborow et al. 1998; Plumb 1999; Soto et al. 2008). However, Mississippi State University, College of Veterinary Medicine, Aquatic Diagnostic Laboratory (hereafter, the ADL) case reports from 1997 to 2005 show *F. columnare*, the causative agent of columnaris disease, to be the most commonly diagnosed pathogen of catfish (MSU 2006). Before 2007, no antibiotics were approved in the USA for treatment of columnaris disease, but under the extra-label provision of the Animal Medicinal Drug Use Clarification Act of 1994 (USFDA 2008), Romet and oxytetracyline are prescribed (Hawke and Thune 1992). However, there are reports of resistance to Romet (Hawke and Thune 1992) and oxytetracyline (Farmer 2004).

Aquaflor contains the broad-spectrum antibiotic florfenicol (FFC) and is approved in the USA for control of mortality due to enteric septicemia associated with *Edwardsiella ictaluri* in catfish (USFDA 2009a), for coldwater disease associated with *Flavobacterium psychrophilum*, and for furunculosis associated with *Aeromonas salmonicida* in freshwater-reared salmonids (USFDA 2007). The U.S. Food and Drug Administration (USFDA) conditionally approved FFC in 2007 under the Minor Use Minor Species Health Act of 2004 for the control of mortality associated with *Flavobacterium columnare* in catfish (USFDA 2009b). The conditional approval allowed the sponsor (Intervet/Schering-Plough Animal Health [ISP]) to market the drug for columnaris disease before completing the efficacy part of the dossier because the required safety studies for FFC use in catfish were previously accepted by USFDA when the drug was first approved. Under the act the sponsor has 5 years to complete the efficacy studies.

Florfenicol is efficacious against a number of fish pathogens (Fukui et al. 1987; Inglis and Richards 1991; Samuelson et al. 1998; Nordmo et al. 1998; Bruun et al. 2000; Schmidt et al. 2000). Worldwide, FFC is currently approved for selected aquaculture indications in 25 countries (R. Endris, Intervet/Schering-Plough

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Animal Health, personal communication). In 15 of these countries, FFC is specifically approved for use against all susceptible bacteria including *F. columnare*, and 9 of the remaining countries allow FFC off-label use, not including the U.S. conditional approval to treat *F. columnare* infection in catfish.

In support of a New Animal Drug Application to use FFC to control mortality in channel catfish caused by *F. columnare*, a tank-dose confirmation study was undertaken. Research protocols for this study were prepared according to specific guidelines previously approved by the USFDA for use in the treatment of enteric septicemia: an FFC dose rate of 10 mg/kg of fish body weight (FBW) for 10 d . Herein, we report the results of the tank efficacy study conducted with an immersion challenge.

Methods

Fish and experimental design.—We obtained 600 laboratory-reared channel catfish fingerlings (90–150 d old; mean weight = 8.1 g, SD = 0.47) with no known history of exposure to *F. columnare* or *E. ictaluri* from the U.S. Department of Agriculture, Agricultural Research Service, Genetics Compound, Stoneville, Mississippi. In each of 30 tanks (80 L), 20 of those fingerlings were acclimated to experimental conditions for 8 d and fed a commercial diet at 2% FBW once per day. Challenge with *F. columnare* occurred on day 0, during which 100 fish at a time were removed from the acclimation tanks, placed in six 80-L aerated challenge tanks with 30 L of static water (27.4–29.2°C), and exposed by immersion to *F. columnare* for 4 h.

Postchallenge, the fish were indiscriminately netted from the challenge tanks and, in groups of 20 (undetermined ratio of males and females), were weighed and placed in each of 30 experimental aquaria (80 L). The total fish weight per tank ranged from 0.135 to 0.183 kg. The 30 tanks were randomly assigned in equal numbers to one of two treatment types in which they were fed either (1) an unmedicated diet (control) or (2) a diet containing FFC at 10 mg/kg FBW for 10 consecutive days. Treatment began the day after challenge (day 1).

Preparation and analysis of feed.—Catfish feed containing 36% crude protein (Land O' Lakes, Shoreview, Minnesota) was used for the study. The feed was ground and repelleted as previously described (Li et al. 1993). For medicated feed, FFC 50% premix (Aquaflor 50% Type A Medicated Article, Intervet/Schering Plough Animal Health, Roseland, New Jersey) was mixed with the dry ground feed to obtain a nominal concentration of 500 mg/kg, then repelleted. A batch of unmedicated control diet was manufactured in a similar fashion to the medicated feed, except no drug was added. Both batches of feed were stored in a monitored refrigerator at 4°C.

Feed concentrations of FFC were determined by Eurofins Scientific, Inc. (Kalamazoo, Michigan) following the method of Hayes (2005). The concentration of FFC in the medicated feed was 493 mg/kg (98.6% of nominal), and FFC was not detected in the unmedicated feed above the limit of quantitation. The unmedicated control feed sample was also assayed for oxytetracycline contamination analysis by Eurofins AvTech Laboratories, Inc. (Kalamazoo, Michigan) and for Romet contamination analysis by Minnesota Valley Testing Laboratories, Inc (New Ulm, Minnesota).

Société Générale de Surveillance (SGS) Agricultural Services (Memphis, Tennessee) analyzed all feeds for organophosphorus and organochlorine compounds. None of these potential contaminants were found at concentrations that would adversely affect the outcome of the study.

Fish environment, handling, and feeding.—We placed 30 L of unfiltered water from a 0.10-ha outdoor earthen pond at the Delta Research and Extension Center (Stoneville, Mississippi) into each of the 30 aquaria. The aquaria were plumbed into two separate water recirculating systems according to the study randomization code. Bacterial culture of the pond water failed to grow *E. ictaluri* and *F. columnare* colonies.

Two weeks before acclimating the test fish, five sentinel fish were placed in each of eight aquaria (four aquaria per recirculating system) to seed biofilters with denitrifying bacteria. At the beginning of acclimation, the sentinel fish were removed from the aquaria and were not used for the study. Aeration was achieved utilizing compressed air from air stones. Water temperatures during treatment and posttreatment observation periods ranged from 25.4°C to 31.6°C, and water flow rates ranged from 220 to 260 mL/min. The total ammonia nitrogen, pH, nitrite, chloride, and temperature were measured at least once weekly. The mean (SD) water quality values for both the control and FFC-treated systems were, respectively, nitrite = 0.25 mg/L (0.24) and 0.14 mg/L (0.05), ammonia = 0.49 mg/L (0.23) and 0.51 mg/L (0.25), and chloride = 28.4 mg/L (6.9) and 28.9 mg/L (6.1). Total alkalinity as $CaCO_3$ (205–291 mg/L) and total hardness as $CaCO_3$ (273–307 mg/L) for each aquarium were measured once before study initiation. Water for the tanks was analyzed for the presence of lead, copper, organochlorines, and organophosphates by Mississippi State Chemical Laboratory at Mississippi State University, and no residues were found in the water that would interfere with the husbandry of the fish. Water quality was within acceptable limits for the maintenance of channel catfish fingerlings throughout the studies

(Tucker and Hargreaves 2004). The photoperiod was 12 h light and 12 h dark.

During acclimation, the fingerlings received the above-described catfish feed at a rate of 2% FBW/d. No feed was provided on the day of stocking or the day of challenge. Following acclimation, fish were fed their assigned medicated or unmedicated treatments from day 1–10, and after treatment (days 11–24) both groups were fed the unmedicated commercial feed, all daily rates (days 1–24) being at 2% FBW. Throughout all phases of the study, feeding behavior and feed consumption of fish in each aquarium were numerically scored as follows: 3 = about 75–100% of feed consumed, 2 = about 50–75% of feed consumed, 1 = about 25–50% of feed consumed, and 0 = no feed consumption was detected.

Fish were observed twice daily for mortality. Any fish that died during the study were necropsied, and cultured for isolation of *F. columnare*. Dead or moribund fish were netted from tanks, and the quantity of feed administered to the tank was decreased proportionally to the reduced tank biomass based on mean fish weight. At the end of the study, all surviving fish were removed from the aquaria, counted, and weighed as a group. Surviving fish were euthanatized on day 25 and submitted for microbiologic and pathologic evaluation.

Preparation of inoculum and challenge of fish.—The *F. columnare* isolate was obtained from a channel catfish that became infected during a natural epidemic and was submitted to the ADL, and it was identified by polymerase chain reaction as *F. columnare* by Micro Technologies, Inc., Richmond, Maine. The sequence identified showed 99% homology to *F. columnare* strains listed in GenBank. The bacteria recovered from fish during the experiment were identified visually by phenotypic traits. The minimal inhibitory concentration (MIC) of florfenicol for the inoculum isolate, as determined using Sensititer plates (Trek Diagnostic Systems), was 0.5 μg/mL.

The archived *Flavobacterium columnare* isolate was inoculated into 2,000 mL Shieh broth (Shieh 1980) and incubated at 27°C (SD, 1) for about 70 h to prepare the challenge inoculum. During incubation, the culture was agitated with a stirring bar. After incubation, the Shieh broth was sampled for plate counts to calculate bacteria numbers by serial dilutions, and 250 mL of the resulting *F. columnare* inoculum was poured into each challenge tank. A bacteria plate count of an inoculum sample indicated that the challenge fingerlings were exposed to a calculated concentration of about 10^6 colony forming units (cfu) of *F. columnare*/mL.

Microbial methods.—The brains, posterior kidneys, skin, gills, and mouths of all dead fish were cultured for bacterial pathogens on Shieh plates (Shieh 1980; Song et al. 1988). Plates were incubated at 27°C for 2 d. Cultures that yielded yellow, strongly adherent, gliding colonies characteristic of *F. columnare* (Shamsudin and Plumb 1996) were considered positive. Measurement of the disk diffusion zones was conducted on *F. columnare* recovered in accordance with the recommended guidelines (CLSI 2006a; CLSI 2006b) outlined by the Clinical and Laboratory Standards Institute (note, these guidelines for in vitro testing for *F. columnare* by disk diffusion and MIC are provisional). Briefly, bacteria were inoculated in Shieh broth and after incubation the serial absorbances at an optical density of 600 nm were measured on a certified spectrophotometer and correlated with the bacterial concentration (cfu/mL) to ensure an approximate plate count of about 10^8cfu/mL The bacteria were uniformly streaked on dilute Mueller Hinton plates and were overlaid with an FFC-impregnated disk (Difco Laboratories, Detroit, Michigan) containing 30 μg FFC/disk. The plates were incubated at 27°C (SD, 1) until the zones of inhibition were clearly defined to facilitate accurate measurement. The zone of bacterial growth inhibition was measured to the nearest millimeter with a certified caliper. Control plates swabbed with *Escherichia coli* (American Type Culture Collection [ATCC] 25922) were concurrently incubated and zones were measured.

For the MIC assays, selected *F. columnare* cultures recovered from dead study fish were each uniformly streaked on Shieh plates and incubated at 27°C (SD, 1). After incubation, colonies from each plate were inoculated into Shieh broth. The broth concentration was standardized in a similar fashion to the disk diffusion assays to ensure that the media were uniformly inoculated. The MIC values were determined for 30 selected *F. columnare* cultured from fish that died during the study; this was done using Sensititer plates (Trek Diagnostic Systems) containing concentrations of FFC in twofold dilutions from 0.12 to 128 μg/mL. No MIC assays were performed on cultures from surviving fish. Sensititer plates were examined after about 48 h of incubation at 28°C (SD, 2). The reference standard was *E. coli* (ATCC 22922).

Statistical analysis.—At the termination of the study, data were examined for statistically significant ($\alpha = 0.05$) differences in fish mortality, positive tissue cultures, and weight change between the unmedicated control group and the FFC-medicated group. Daily mortality and cumulative mortality data were analyzed by a generalized linear mixed model with binomial errors and a default logit link. The model included fixed effect of treatment and random effect of block; the random effect was excluded from the model for the

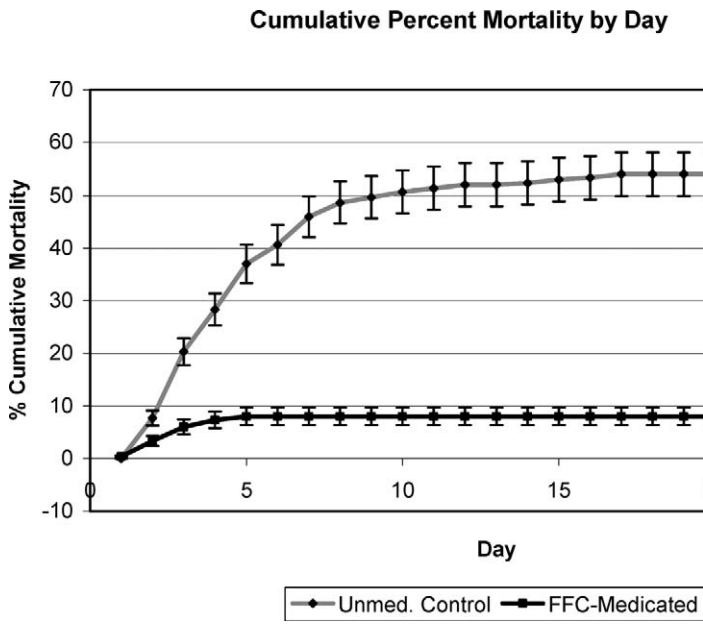

FIGURE 1.—Cumulative percent mortality ± SE of channel catfish that were challenged with *F. columnare* and then fed either an unmedicated diet or a medicated diet (florfenicol [FFC] at 10 mg/kg of fish body weight) for 10 d. The feeding regimes were initiated 24 h after exposure to *F. columnare*.

analysis of daily mortality because the variation of block was close to zero and the procedure did not converge (SAS PROC GLIMMIX was used for the analysis; SAS Institute 1999).

Fish were weighed by tank, and that weight was divided by the number of fish in the tank to yield a tank average fish weight. Average fish weight gain was analyzed by a mixed linear model. The model includes fixed effect of treatment and random effect of block (SAS PROC MIXED was used for the analysis).

Results

Among the 299 fish in the untreated control group (1 of the 300 fish was unaccounted for at the study's end and was excluded from analysis), mortalities occurred from days 2 to 21 (Figure 1), the majority occurring on day 3 (12.7% [SD = 2.16], 38/299 fish), day 4 (8% [1.65], 24/299), and day 5 (8.7% [2.04], 26/299). The control group cumulative mortality was 162 of 299 fish (54.2% [3.51]; Figure 2). The percent mortality in individual tanks ranged from 30% (6/20 fish) to 90% (18/20).

The first deaths occurred on day 1 among the treated fish, the majority of mortalities occurring on day 2 (3% [0.92], 9/300), day 3 (2.7% [1.02], 8/300) and day 4 (1.3% [0.59], 4/300). The cumulative mortality in the treated group was 24 of 300 fish (8.0% [1.87]). The percent mortality in individual tanks ranging from 0% (0/20 fish) to 20% (4/20 fish). The percent mortality for the challenged, unmedicated control group was significantly greater than that of the medicated group ($t = 9.58$, df = 14.12, $P < 0.0001$).

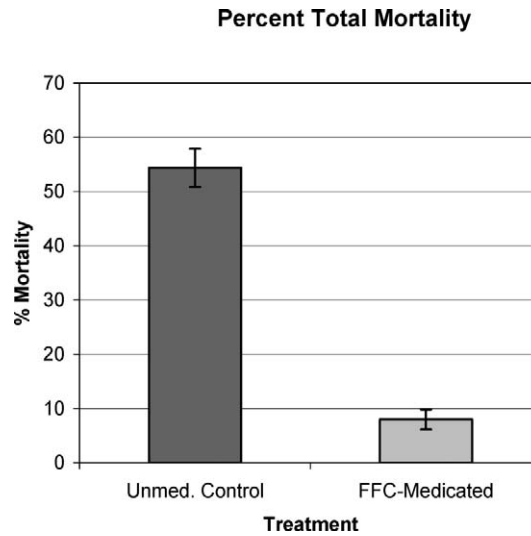

FIGURE 2.—Total mortality of channel catfish that were challenged with *F. columnare* and then fed either an unmedicated or a medicated diet. See Figure 1 for additional information.

F. columnare was recovered from 68.9% (206/299) of the fish in the unmedicated group compared with 15.0% (45/300) of the FFC-treated fish. Bacterial colonies morphologically characteristic of *F. columnare* were cultured from 99.5% (185/186) of fish mortalities. No deaths occurred in four FFC-treated tanks, and no surviving fish in these tanks were positive for *F. columnare*. These organisms were confirmed as *F. columnare* on the basis of gross morphology. Bacteria cultured from survivors included 43 *F. columnare* positive fish from the unmedicated group and 18 positive fish from the FFC medicated group.

The disk diffusion zones for *F. columnare* isolated from the study against FFC ranged from 35 to 43 mm. The 50% MIC was 0.5 μg/mL for both the control and medicated fish. The 90% MIC was 0.5 μg/mL for the control and 1.0 μg/mL for the medicated fish.

External lesions were present in mortalities from day 1 onward and included skin, fin, gill, and mouth ulceration, and necrosis. Swollen kidneys were seen in 36 unmedicated and 2 medicated fish. In fish with positive cultures for *F. columnare*, lesions were present in 197 of 251 fish (78.5%). These lesions were more prevalent in the unmedicated group compared with the medicated group.

Mean fish weight at the time of challenge ranged from 6.8 to 9.2 g, and the mean fish weight for survivors at the end of the study (day 25) ranged from 8.58 to 12.0 g. There was no significant difference in weight gain between the unmedicated and medicated groups ($P < 0.9046$). Feeding activity in both the unmedicated and medicated fish was graded as 3 throughout the study, indicating that 75–100% of the feed was consumed.

Discussion

Dead fish in this study had gross lesions in the gills, skin, and fins similar to those previously reported for *F. columnare* infections (Hawke and Thune 1992), and 99.5% were culture positive for *F. columnare*. Initiation of FCC treatment (10 mg FFC/kg FBW) at 24 h after exposure to *F. columnare* resulted in significantly higher survival rates in the medicated group than the unmedicated group. Cumulative mortality within tanks ranged from 0% to 20% in medicated tanks compared with a mean cumulative mortality of 53% in the unmedicated groups.

F. columnare was previously recognized only as an opportunistic pathogen with columnaris disease ensuing secondarily to stressors (Durborow et al. 1998; Plumb 1999). However, two genetic divisions of *F. columnare* are described that determine virulence and whether it is likely to be a primary or secondary pathogen (Soto et al. 2008; Decostere et al. 1999a).

Although virulent strains of *F. columnare* are described as primary pathogens (Soto et al. 2008), researchers utilizing these strains also incorporate stressful conditions in their challenge models. Reported experimental stressors used include: starvation (Soto et al. 2008), skin abrasion (Bader et al. 2006; Bader et al. 2003), static water (Thomas-Jinu and Goodwin 2004), high stocking density (Wakabayashi 1991; Durborow et al. 1998; Igutchi et al. 2004; Suomalainen et al. 2005; Lewbart 1998), and high water temperatures (Wakabayashi 1991; Durborow et al. 1998; Decostere et al. 1999b; Suomalainen et al. 2005). Mortalitly rates associated with *F. columnare* challenges in previous studies vary when the above factors are incorporated in columnaris diseases models. As examples, Bader et al. (2003) achieved an 80% mortality rate with abrasion, Soto et al. (2008) achieved a 60% mortality rate with starvation, and Thomas-Jinu and Goodwin (2004) achieved a 100% rate with a nonabraided fish in a static challenge; other variables, however, existed among these studies (e.g., variation in catfish strains, *F. columnare* strains, bacterial challenge [cfu/mL], immersion times, use of MS-222, and water quality). The *F. columnare* challenge model in our study followed the FDA requirements for the use of nonabraded fish and a 25-d study duration to demonstrate FFC efficacy against *F. columnare* in channel catfish. Use of a static system during this long period would have required impractical daily water testing and water changes. Therefore, we used a static system only for the 4-h immersion challenge. Additionally we incorporated only two more of the aforementioned stress factors in our model—high stocking density and higher water temperatures—because we could neither starve nor abrade our fish. Static water and high stocking density increase the chances of bacteria being transmitted from fish to fish (Wakabayashi 1991; Suomalainen et al. 2005). Warm temperatures (>20°C) are conducive to the growth of *F. columnare* (Suomalainen et al. 2005), and the number of field cases increases with warmer temperatures (20–30°C; MSU 2006). Although we utilized aerated static water for the challenge bath, a slow flow rate in the recirculation system was used for the remainder of the study. Additionally, a prestudy experiment determined that pond water produced higher infection rates with *F. columnare* in catfish than the aquifer water that is routinely used in tank studies at our institution (unpublished data). Although we did not explore the specific cause of enhanced infection using pond water, others suggest that the presence of organic matter found in pond water

enhances *F. columnare* infection (Fijan 1968; Wakabayashi 1991; Bernardet and Bowman 2006).

The mean feeding scores and the weight gain in all medicated and unmedicated surviving fish in this study indicated that neither *F. columnare* infection nor FFC in the diet adversely affected feed intake. Rates of feeding medicated feed to fish during bacterial disease outbreaks are often decreased to 1% FBW because of depressed appetite of diseased fish (Johnson and Smith 1994). Because our fish were appetent during this study, the 2% FBW feeding rate we used may have contributed to the high survival rate in FFC-medicated fish. This distributed medication over a larger number of feed pellets and increased FFC intake. In response to fingerlings' appetence in the wake of columnaris disease in the Mississippi Delta region, catfish farmers requested that local feed mills blend FFC at a higher rate of 3% FBW (Bob Harris, Fishbelt Feeds, Inc., Moorhead. Mississippi, personal communication).

In contrast, decreased feeding was previously suggested as a method to decrease bacterial infection in fish (Shoemaker et al. 2003). However, withholding of feed for seven or more days during an experimental *F. columnare* challenge in channel catfish fingerlings resulted in significantly more mortalities and decreased growth compared with fed fish (Klesius et al. 1999; Shoemaker et al. 2003). In addition, overwinter feeding studies showed significant decreased weight gains in nonfed catfish (Kim and Lovell 1995).

Of the survivors, there were significantly fewer culture-positive fish in the medicated fish than in the unmedicated fish. Carriers represent a potential source of reinfection in aquatic environments (Welker et al. 2005). A previous study with *Streptococcus iniae* in Nile tilapia *Oreochromis niloticus* demonstrated increased FFC efficacy and no culture-positive survivors treated with FFC at 15 versus 10 mg/kg FBW (Gaunt et al., in press). Ongoing studies will investigate whether FFC at 15 mg/kg FBW would eliminate the culture-positive state noted with the 10-mg group.

The disk diffusion zones for *F. columnare* isolated from the study against FFC ranged from 35 to 43 mm and the MIC values ranged from 0.5 to 1.0 μg/mL. Although epidemiological cutoff values are not established for *F. columnare* against FFC (Miller and Reimschuessel 2006), our procedures followed repeatable methods, which were verified against plate counts.

The MIC values from this study are in agreement with published values ranging from 0.3 to 1.6 μg/mL for other fish pathogenic bacteria (Fukui et al. 1987; Inglis and Richards 1991; Martinsen et al. 1993; Bruun et al. 2000; Schmidt et al. 2000), supporting the concept that FFC would be expected to be efficacious against *F. columnare* infections in catfish. To have clinical application the MIC and disk diffusion data must be correlated with FFC efficacy, dosage, and pharmacokinetic analysis (Miller and Reimschuessel 2006). Reports on FFC efficacy against *F. columnare* from farmers using the medicated feed and data from studies such as ours will be important to determine the clinical breakpoints for FFC in aquatic animal medicine.

In agreement with previously conducted studies with catfish fingerlings, there were no visible gross lesions associated with the FFC treatment (Gaunt et al. 2003; Gaunt et al. 2004). These comparative mortality data demonstrate that florfenicol administered to catfish infected with *F. columnare* is safe, significantly reduces mortality, and should be valuable for control of columnaris disease in farmed catfish.

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