In Vitro and *In Vivo* Efficacy of Florfenicol for Treatment of *Francisella asiatica* Infection in Tilapia[▽]

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Francisella asiatica is a recently described, Gram-negative, facultative intracellular fish pathogen, known to be the causative agent of francisellosis in warm-water fish. Francisellosis outbreaks have increased in frequency among commercial aquaculture operations and have caused severe economic losses in every case reported. The lack of effective treatments for piscine francisellosis led us to investigate the potential efficacy of florfenicol for inhibition of *F. asiatica* *in vitro* and as an oral therapeutic agent *in vivo*. The MIC of florfenicol for *F. asiatica*, as determined by the broth dilution method, was 2 µg/ml, which indicates its potential efficacy as a therapeutic agent for treatment of francisellosis. The intracellular susceptibility of the bacterium to florfenicol in tilapia head kidney-derived macrophages (THKDM) was also investigated. Addition of florfenicol to the medium at 10 µg/ml was sufficient to significantly reduce bacterial loads in the THKDM *in vitro*. Cytotoxicity assays done in infected THKDM also demonstrated drug efficacy *in vivo*, as determined by lactate dehydrogenase (LDH) release. Levels of LDH released from infected THKDM were significantly lower in macrophages treated with florfenicol ($P < 0.001$) than in untreated cells. In medicated-feed trials, fish were fed 15 mg of florfenicol/kg of fish body weight for 10 days, and the feeding was initiated at either 1, 3, or 6 days postchallenge. Immersion challenges resulted in 30% mean percent survival in nontreated fish, and fish receiving medicated feed administered at 1 and 3 days postinfection showed higher mean percent survival (100% and 86.7%, respectively). A significant decrease ($P < 0.001$) in bacterial numbers (number of CFU/g of spleen tissue) was observed in treated groups compared to nontreated infected fish at both 1 and 3 days postchallenge. There were no differences in bacterial burden in the spleens between fish treated 6 days postchallenge and untreated controls. In conclusion, if florfenicol is administered during early stages of infection, it has the potential for effectively treating piscine francisellosis, including the capacity for intracellular penetration and bacterial clearance.

Members of the genus *Francisella* are small, pleomorphic, Gram-negative bacteria belonging to the *Gammaproteobacteria* (3, 7, 28). Many *Francisella* spp. are facultative intracellular pathogens, capable of replicating in macrophages and other various cell types in humans, rabbits, rodents, nonhuman primates, and fish. The bacteria may also exist as endosymbionts of amoebae and arthropods (1, 2, 7, 26, 31, 34). *Francisella asiatica* and *Francisella noatunensis* are two recently described members of the genus that causes piscine francisellosis in a wide variety of fish species (19).

During the past 5 years *F. asiatica* has been implicated as the causative agent of mortality in tilapia (*Oreochromis* sp.) and other important warm-water species cultured in the United States (including Hawaii), Taiwan, Latin America (particularly Costa Rica), and Japan (13, 14, 15, 17, 19, 20, 29, 33). Due to its increase in incidence, high infectivity rates, and wide range of fish hosts, francisellosis is now considered one of the most important emergent diseases in aquaculture (13, 14, 15, 17, 19, 20, 29, 33). In tilapia the disease can present as an acute syndrome with few nonspecific clinical signs and high mortality rates or as a subacute to chronic syndrome with nonspecific

clinical signs like anorexia, exophthalmia, and anemia. The bacterium has a high infectivity rate in tilapia fingerlings. Low numbers (1 to 10 CFU) of the bacterium injected intraperitoneally can cause colonization and significant damage to the head kidney and spleen, with a dose as low as 23 bacteria resulting in mortality (30). Macroscopic and microscopic examination often reveals enlarged internal organs containing widespread multifocal white nodules (13, 14, 15, 17, 19, 20, 29). Moreover, *F. asiatica* has been found to be resistant to serum killing and can penetrate, replicate, and survive in tilapia head kidney-derived macrophages (THKDM) (31).

Very limited data on fish pathogen susceptibility to antibiotics have been published. Only recently have guidelines been published for broth microdilution testing of fish pathogens (8); however, methods for fastidious organisms such as *F. asiatica* are not included in this publication. Clinical breakpoints are not available for this class of fish pathogens either. Currently, only three antibiotics have been approved by the U.S. Food and Drug Administration for use in U.S. aquaculture: oxytetracycline (Terramycin 200 for fish; Phibro Animal Health, Fairfield, NJ), ormetoprim-sulfadimethoxine (Romet-30 type A medicated article; Pharmaq AS, Oslo, Norway), and florfenicol (Aquaflor type A medicated article; Intervet/Schering-Plough Animal Health, Roseland, NJ). Florfenicol is a fluorinated derivative of thiamphenicol that blocks the peptidyltransferase at the 50S ribosome subunit and acts against a wide variety of both Gram-positive and Gram-negative bacteria (5). As a medicated

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feed, florfenicol has been used to treat a wide variety of fish diseases in various warm- and cold-water cultured fish species, including *Vibrio anguillarum*, *Aeromonas salmonicida*, *Streptococcus iniae*, *Listonella anguillarum*, and *Edwardsiella ictaluri*, among others (9, 11, 23, 24, 27).

Due to the emergent nature of francisellosis in fish and the fastidious characteristics of the bacteria, there is currently very little published data regarding antibiotic susceptibility of *F. asiatica* *in vivo* or *in vitro*, and at present there are no known efficacious chemotherapeutics or vaccines available (17, 20, 29, 30, 31, 32). Additionally, antimicrobial therapy in facultative intracellular bacteria is more complex than in extracellular bacteria since the efficacy of the drug depends on its ability to penetrate and accumulate within the cell, cellular metabolism, subcellular disposition, and bioavailability of the drug (25). For *F. noatunensis*, *in vitro* data were presented that indicated that strain GM2212T was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, and erythromycin yet susceptible to ceftazidime, tetracycline, gentamicin, and ciprofloxacin (21). No further research has been published to demonstrate the potential use of any of these drugs in medicated feed for the treatment of francisellosis in fish. Moreover, at this point it is unknown if the antimicrobial susceptibilities of *F. noatunensis* and *F. asiatica* are the same.

The goal of the present study was to determine the ability of florfenicol-medicated feed to control experimentally induced *F. asiatica* infection in tilapia. Additionally, we evaluated the capacity of florfenicol to eliminate intracellular *F. asiatica* from THKDM *in vitro*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *F. asiatica* strain LADL 07-285A, isolated from cultured tilapia (*Oreochromis* sp.), was described in previous work (29). *Francisella* sp. isolates demonstrating >99% identity to the *F. asiatica* 16S rRNA gene were recovered from moribund hybrid striped bass (20) and tilapia (15) and kindly donated by John Hansen, Interdisciplinary Program in Pathology, University of Washington, Seattle, WA. PCR and sequence comparison of the *iglABCD* operon and 16S rRNA gene sequences, as well as phenotypic characteristics, temperature requirements, and host range analysis, demonstrated that isolated *Francisella* sp. 1, *Francisella* sp. 2, *Francisella* sp. 3 (20), and *Francisella victoria* (15) are, in fact, members of the recently described species *F. asiatica* (30, 32). *F. asiatica* isolates were grown on cystine heart agar supplemented with bovine hemoglobin solution (CHAH) (BDBBL, Sparks, MD) for 48 h at 28°C or in a modified Mueller-Hinton II (MMH) cation-adjusted broth supplemented with 2% IsoVital X (BD BBL, Sparks, MD) and 0.1% glucose (29). Broth cultures were grown overnight at 25°C in a shaker at 175 rpm, and bacteria were frozen at -80°C in the broth medium containing 20% glycerol for later use. *Escherichia coli* ATCC 25922 used as a control organism for the MIC determinations was grown using Luria-Bertani broth or agar for 16 to 24 h at 28°C.

Antimicrobial susceptibility testing. The MICs of florfenicol for *F. asiatica* isolates and *E. coli* were tested using Sensititre Just One Strips (Trek Diagnostic Systems, West Sussex, United Kingdom), containing 2-fold dilutions of florfenicol (0.12 to 128 µg/ml), using slight modifications to the manufacturer's suggested protocol. Briefly, bacterial isolates were grown on agar plates as described previously, and three to five colonies were dislodged with a sterile cotton swab, suspended in 4 ml of MMH broth, and adjusted to a 0.5 McFarland standard. For *F. asiatica*, 100 µl of this suspension was transferred to 11 ml of MMH broth and mixed, and 50 µl was added to each well of the Sensititre plate containing florfenicol. For each plate, one well contained the bacterial inoculum without florfenicol (positive control), and one well contained the bacterial inoculum with an antibacterial agent to prevent bacterial growth (negative control). Test plates were covered with an adhesive seal, provided by the manufacturer, and incubated for 24 to 48 h at 28°C. Bacterial growth was checked visually after the adhesive seal was removed at 24 and 48 h postinoculation. The MIC was defined as the

lowest concentration exhibiting no visible growth. The MIC assay for the *E. coli* 25922 and *F. asiatica* isolates was replicated five times.

Fish. Tilapia fingerlings (16 to 27 g) were obtained from a source with no history of *Francisella* infection. A subsample of the population was confirmed negative for *F. asiatica* by complete clinical, bacteriological, and molecular analysis using previously published protocols to ensure that the fish were negative for francisellosis (29). Fish were maintained at stocking densities of 10 fish per tank in 20-liter flowthrough tanks at a temperature of 25°C. Fish were fed 2% of the fish body weight per day with a commercial tilapia feed (Burris Aquaculture Feeds, Franklinton, LA). Fish were acclimatized for at least 4 weeks prior to challenge.

Medicated feed. The medicated feed was produced by mixing 660 mg of florfenicol (Intervet/Schering-Plough Animal Health, Roseland, NJ) with 880 g of tilapia feed (Burris Aquaculture Feeds, Franklinton, LA). This medicated ration, when fed at 2% of the fish body weight per day for a period of 10 days, corresponded to a daily drug dosage of 15 mg/kg fish. We utilized this dose since it has been suggested by preliminary studies that a dose of 15 mg of drug for 10 days may be necessary to control infections of *Streptococcus* spp., another well-known pathogen of cultured tilapia (4). The commercial diet was ground to less than 600 µm in a Thomas-Wiley Laboratory Mill (model 4; Thomas Scientific, Swedesboro, NJ) and passed through a 600-µm-pore-size sieve (USA Standard Testing Sieve; VWR Scientific Aquaculture Supply). Florfenicol was added to the pulverized feed with 13 g of carboxymethyl cellulose (CMC) sodium salt (Sigma-Aldrich Corp., St. Louis, MO) and thoroughly mixed in a Twin Shell Dry Blender (Patterson-Killey Co., East Strasburg, PA) for 15 min. The dry ingredients were then placed in a commercial food mixer (model A-200; Hobart, Troy, OH), and an appropriate amount of distilled 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 for 24 h under forced air in a temperature-controlled room at 23 to 25°C and kept dry in bags maintained at 4°C until used. High-performance liquid chromatography (HPLC) was performed by Eurofins Scientific (Memphis, TN) to analyze florfenicol concentrations in the experimental diet. Medicated feed samples analyzed by HPLC revealed that the florfenicol dose administered was 12.9 mg of florfenicol/kg of fish/day (86% of target).

Infectivity challenge. Six treatments were randomly assigned to 18 aquaria with three replicate tanks/group. Treatment groups were as follows: three medicated challenge treatments, a nonmedicated challenge treatment (positive control), a nonmedicated nonchallenge treatment (negative control), and a medicated nonchallenge treatment to determine any detrimental effects the medication had on the fish. In all three medicated challenge treatments, fish were fed medicated feed for a period of 10 days, starting at either 1, 3, or 6 days postchallenge. Before and after the 10-day medication period, nonmedicated feed was administered at 2% of body weight per day. Infectivity challenges in tilapia fingerlings were carried out according to a bath challenge model previously described (30). *F. asiatica* isolate LADL 07-285A recovered from moribund tilapia (29) was utilized in all infectivity trials. Feed was restricted for 24 h prior to challenge, and the water supply was turned off immediately before the addition of bacteria. A challenge dose of 8×10^7 CFU/ml was obtained by adding 0.5 liters of a bacterial suspension in $1 \times$ phosphate-buffered saline (PBS; pH 7.3) to 10 liters of tank water. Fish were maintained in the bath for 3 h, after which time the water supply was restored. Tanks were oxygenated continuously, and water temperatures were maintained at 25°C for the duration of study. The negative controls were handled similarly but were not exposed to *F. asiatica*. Fish were observed twice daily during the 30 days of acclimation and the 30 days postchallenge. Mortality in each tank was recorded twice daily, and when dead fish were removed from a tank, the quantity of feed administered to the tank was reduced proportionally to the decrease in tank biomass (based on mean fish weight). In order to prevent water quality deterioration due to accumulation of unconsumed feed in the tanks, the fish were allowed to consume the feed for 30 min, after which nonconsumed feed was removed from the tanks and discarded.

Bacterial load determinations. Five fish from each medicated group and survivors from the nonmedicated control group were sacrificed at 30 days postchallenge, and spleens were harvested to determine approximate bacterial burdens. Organs were weighed, homogenized in 0.5 ml of sterile PBS, plated in triplicate on CHAH medium, and incubated at 25°C for 3 days prior to CFU determinations. The number of CFU were expressed as the mean $\pm$ standard error of the mean (SEM). Organs (head kidney, spleen, and liver) from additional remaining survivors were used for histological examinations.

Intramacrophage survival assays. To determine internalization and intracellular growth of bacteria, THKDM were infected with *F. asiatica* following previously published protocols (31). The complete tilapia macrophage medium (CTMM) consisted of RPMI 1640 medium (Gibco-Invitrogen Corp., Carlsbad,

TABLE 1. MIC of florfenicol for *F. asiatica* isolates obtained from culture fish and for *E. coli* ATCC 25922

Isolate	Source	Florfenicol MIC ($\mu\text{g/ml}$)
<i>F. asiatica</i> LADL 07-285A	Tilapia	2
<i>F. asiatica</i> LADL 07-285B	Tilapia	2
<i>Francisella</i> sp. 1	Hybrid striped bass	2
<i>Francisella</i> sp. 2	Hybrid striped bass	2
<i>Francisella</i> sp. 3	Hybrid striped bass	2
<i>F. victoria</i>	Tilapia	2
<i>E. coli</i> ATCC 25922 ^a		4

^a Control grown in LB broth or agar.

CA) with 14 mM HEPES buffer (Gibco-Invitrogen Corp.), 0.3% sodium bicarbonate (Gibco-Invitrogen Corp.), 0.05 mM 2- β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 5% heat-inactivated, pooled tilapia serum. Briefly, 96-well plates containing 3-day-old cultures of THKDM at concentrations of 1×10^5 to 5×10^5 cells per well were used. *F. asiatica* was grown for 8 h in MMH broth at 25°C. The optical density at 600 nm (OD_{600}) of the culture was determined, and the cells were adjusted to an estimated final concentration of 5×10^8 CFU/ml, based on an OD/CFU standard curve. Aliquots (1-ml) of the bacterial suspension were pelleted at $10,000 \times g$ for 5 min in an Eppendorf 5415 D centrifuge (Eppendorf-Brinkman, Westbury, NY), and the pellet was resuspended in 1 ml of normal autologous serum to opsonize the bacteria. Tenfold serial dilutions were plated on CHAH plates after incubation to determine the actual number of CFU/ml. Following a 30-min incubation at 25°C, the 96-well plate was inoculated with 5 μl of treated bacteria per well to achieve a multiplicity of infection (MOI) of 25 bacteria to 1 macrophage. The plates were centrifuged for 5 min at $400 \times g$ to synchronize bacterial contact with macrophages. Following a 2-h incubation at 25°C with 5% CO_2 , the cells were washed three times with warm CTMM (25°C) and further incubated with fresh CTMM containing 0 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, or 100 $\mu\text{g/ml}$ of florfenicol for 0, 24, or 48 h. Cells in five wells were lysed by the addition of 100 μl of 1% saponin in PBS at each time point. The lysates were serially diluted and spread onto CHAH plates to determine viable counts. Experiments were performed in triplicate on a minimum of three separate occasions to affirm the reliability of the results.

Detection of *F. asiatica*-mediated cytotoxicity. In order to monitor the THKDM cells during experiment, a lactate dehydrogenase (LDH) cytotoxicity assay was performed using a colorimetric Cytotox 96 Kit (Promega, Madison, WI) according to the manufacturer's instructions. Cytotoxicity assays were performed on both infected and noninfected THKDM exposed to 0, 1, 10, and 100 $\mu\text{g/ml}$ of florfenicol in CTMM and for 0, 24, and 48 h. The percentage of cytotoxicity was calculated as follows: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})]$, where experimental release is the amount of LDH activity in the supernatant of each different treatment being investigated, spontaneous release is the amount of LDH activity in the supernatant of uninfected cells, and total release is the activity in cell lysates (31).

Statistical analysis. SAS software (SAS Institute, Inc., Cary, NC) was used with the general linear model procedure (PROC GLM) to conduct analysis of variances (ANOVA) of factorial arrangements of treatment. When overall tests indicated significance, pairwise comparisons of main effects were calculated with Tukey's test. Interaction effects were examined by pairwise *t* test comparisons of least square means. For the mortality studies the percent mortalities were transformed with an arc transformation to normalize the data. The numbers of CFU recovered in the *in vitro* challenges were $\log_{10}$ transformed for statistical analysis. All comparisons were considered significant at a *P* value of < 0.05 .

RESULTS

Susceptibility testing (MIC determination). MICs as determined using the Trek diagnostic Sensititre plates containing florfenicol indicated that all *F. asiatica* isolates were susceptible to a concentration of 2 $\mu\text{g/ml}$ of florfenicol (Table 1). The MIC for the *E. coli* control was consistently 4 $\mu\text{g/ml}$, which is within the accepted quality control range for this

organism in cation-adjusted Mueller-Hinton broth after 24 to 48 h at 28°C (8).

***In vivo* efficacy.** The first mortalities occurred at 3 days postchallenge in tanks of nonmedicated fish (nonmedicated challenged group) and at 6 days postchallenge in fish given medicated feed. In both groups mortalities rose rapidly to ~40% by day 15, and final cumulative survivals of 30 and 50%, respectively, were recorded at day 30 when the experiment was terminated (Fig. 1). In both groups, fish became anorexic (nonconsumption of feed after 30 min of feeding) beginning at 4 days postchallenge. Excess feed was removed from the tanks 30 min after feeding to avoid water quality problems. Conversely, groups of fish that received medicated feed 1 or 3 days postchallenge ate all medicated feed within a few minutes and presented significantly higher survivability than the nonmedicated challenged group ($P < 0.001$). No mortality was observed in the group receiving medicated feed 1 day postchallenge, and only 13.3% of fish receiving medicated feed 3 days postchallenge died after 30 days (Fig. 1). In the fish fed medicated feed 1 day postchallenge, the spleen and head kidney contained multiple accumulations of melanomacrophages that surrounded or were adjacent to small, muscular splenic arterioles, and rare splenic arterioles contained luminal accumulations of melanomacrophages, as demonstrated by histological analysis performed in surviving fish 30 days postchallenge. In one fish, granulomatous inflammation was present in the spleen and kidneys, with well-delineated foci of necrosis and granuloma formation. No pathological changes were observed in any of the other fish in this group (Table 2).

Digests of harvested spleens from each group of fish challenged were plated on CHAH medium to determine the number of CFU/mg of organ weight. Only one fish from the group presented with medicated feed 1 day postchallenge had detectable CFU counts in the spleen. The counts were significantly lower ($P < 0.001$) than in any other treatments (Fig. 2). The spleen from this fish looked normal and was not enlarged, suggesting that infection was not progressing. Bacterial counts in the spleens from fish medicated 3 days postchallenge (24.15 ± 7.4 CFU/mg of spleen) were significantly lower than counts of the nonmedicated challenged group (310 ± 77 CFU/mg of spleen) ($P < 0.01$) (Fig. 2). Histopathological analysis in the former group showed widespread granulomas and mixed inflammatory infiltrates composed mainly of macrophages and lymphocytes in all the analyzed fish. The granulomas consisted of large, foamy, vacuolated macrophages encircled by thin fibrous capsules and small cuffs of lymphocytes with fewer neutrophils. The centers of the granulomas were often necrotic (Table 2). There were no significant differences between bacterial counts in the spleens of fish receiving medicated feed 6 days postchallenge (419 ± 183 CFU/mg of spleen) and controls (nonmedicated and challenged) ($P < 0.05$) (Fig. 2). No mortality or lesions were observed in any of the medicated nonchallenged controls or nonmedicated nonchallenged controls.

Efficacy of florfenicol to control intracellular *F. asiatica* *in vitro*. To evaluate the cytotoxic effects of florfenicol, THKDM were grown in 96-well plates in the presence of different concentrations of florfenicol (0, 1, 10, and 100 $\mu\text{g/ml}$) for 24 and 48 h. The LDH assay was used to assess the cytotoxicity of florfenicol to THKDM, and there were no detectable cytotoxic

FIG. 1. *In vivo* infectivity trial. Cumulative percent survival of tilapia challenged by immersion exposure to *F. asiatica* and subsequently administered florfenicol-treated feed daily at 15 mg of active ingredient/kg of body weight for 10 days. The five treatments consisted of three medicated challenge treatments, a nonmedicated challenge treatment (CON+), and a nonmedicated nonchallenge treatment (CON-). The three medicated challenge treatments consisted of one group given medicated feed at day 1 postchallenge for a period of 10 days (1DPC). A second group was treated identically, but medicated feed was started at day 3 postchallenge (3DPC). A third group was given medicated feed beginning at day 6 postchallenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.

effects of florfenicol at any concentration between 0 and 100 $\mu\text{g/ml}$. At 24 h, the survival rates of THKDM were $99.96\% \pm 0.004\%$ in the 1 $\mu\text{g/ml}$, $99.95\% \pm 0.005\%$ in the 10 $\mu\text{g/ml}$, and $99.95\% \pm 0.012\%$ in the 100 $\mu\text{g/ml}$ florfenicol-treated macrophages compared with untreated macrophages (0 $\mu\text{g/ml}$ of florfenicol). At 48 h, the survival rates were $99.91\% \pm 0.02\%$ in the 1 $\mu\text{g/ml}$, $99.9\% \pm 0.01\%$ in the 10 $\mu\text{g/ml}$, and $99.9\% \pm 0.01\%$ in the 100 $\mu\text{g/ml}$ florfenicol-treated macrophages compared with 100% in untreated macrophages (0 $\mu\text{g/ml}$).

To investigate whether florfenicol affects *F. asiatica* survival within THKDM, cells were infected at an MOI of 25 as described in Materials and Methods and incubated for 0, 24, and 48 h after bacterial infection in CTMM containing 0, 1, 10, or 100 $\mu\text{g/ml}$ florfenicol. The results showed efficient intracellular

replication of *F. asiatica* within THKDM cultured with 0 and 1 $\mu\text{g/ml}$ (Fig. 3). At 24 and 48 h postinfection, CFU counts of *F. asiatica* were greater ($P < 0.001$) than those at 0 h in both the control and the THKDM cultured with medium containing 1 $\mu\text{g/ml}$ of florfenicol (Fig. 3). On the other hand, infected macrophages that were cultured in medium with 10 and 100 $\mu\text{g/ml}$

FIG. 2. Reduced *F. asiatica* bacterial burden in the spleens of antibiotic-fed tilapia. The five treatments consisted of three medicated challenge treatments, a nonmedicated challenge treatment (CON+), and a nonmedicated nonchallenge treatment (CON-). The three medicated challenge treatments consisted of one group given medicated feed at day 1 postchallenge (1DPC) for a period of 10 days. A second group was treated identically, but medicated feed started at day 3 postchallenge (3DPC); in the third one medication started at day 6 postchallenge (6DPC). Five fish from each medicated group and survivors from the nonmedicated control group were sacrificed at 30 days postchallenge, and spleens were harvested to determine approximate bacterial burdens. Error bars represent mean $\pm$ SEM. Significant differences from results for the CON+ group are indicated as follows: *, $P < 0.005$; **, $P < 0.001$.

TABLE 2. Histopathological lesions in selected tissues from medicated and nonmedicated tilapia at 30 days postchallenge with *F. asiatica*.

Treatment ^a	Relative presence of granulomatous lesions in tissue ^b		
	Spleen	Head kidney	Liver
1 DPC	None	None	None
3 DPC	Moderate	None	None
6 DPC	Severe	Severe	Moderate
Control +	Severe	Severe	Moderate
Control -	None	None	None

^a The five treatments consisted of three medicated challenge treatments, a nonmedicated challenge treatment (Control +), and a nonmedicated nonchallenge treatment (Control -). The three medicated challenge treatments consisted of one group given medicated feed at day 1 postchallenge for a period of 10 days (1DPC). A second group was treated identically, but medicated feed started at day 3 postchallenge (3DPC). The third medicated treatment started at day 6 postchallenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.

^b Determinations were made in a $\times 10$ microscopic field. The scale in number of granulomas is as follows: none, 0; mild, <7 ; moderate, >7 to <20 ; severe, >20 .

FIG. 3. Florfenicol-mediated killing of intracellular *F. asiatica* in infected tilapia head kidney-derived macrophages. Bacteria were added at an MOI of 25:1 and incubated for 2 h at 25°C with 5% CO₂, followed by incubation with 0, 1, 10, or 100 µg/ml of florfenicol in CTMM. At 0, 24, and 48 h postinfection, cells were washed and lysed with 0.5% saponin, followed by serial 10-fold dilutions plated on CHAH medium; samples were then incubated at 25°C for 3 days for CFU count determination. The experiment was performed three times in triplicate. Error bars represent mean $\pm$ SEM. *, significantly different from value at time zero ($P < 0.001$).

of florfenicol showed no bacterial replication and significantly ($P < 0.001$) reduced numbers of bacteria at 48 h postinfection (Fig. 3).

The results of the LDH cytotoxicity assay performed in infected cells correlate with the results from the intracellular growth assays. The cytotoxicity observed in THKDM cultured with medium containing 10 and 100 µg/ml florfenicol was significantly lower ($P < 0.001$) than that observed in the control group (0 µg/ml) at both 24 and 48 h (Fig. 4). Although the cytotoxicity observed in THKDM cultured with medium containing 1 µg/ml florfenicol was significantly lower ($P < 0.001$) than that observed in the control wells at 24 h, by 48 h the cytotoxicity had increased in this group (Fig. 4).

DISCUSSION

The results obtained from the MIC determinations demonstrated that florfenicol is a suitable candidate for *in vivo* experimentation since a low MIC (2 µg/ml) was found. For mammalian pathogens like *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica*, and *Histophilus somni*, florfenicol has shown bactericidal activity at the respective MICs, whereas it has only bacteriostatic activity at the MIC for *Staphylococcus aureus* (22). Therefore, maintaining concentrations in plasma above the MIC seems advisable to control *F. asiatica* in tilapia. Recent pharmacokinetic work has shown that serum, skin, and muscle of tilapia medicated with florfenicol via a medicated ration at 15 mg of florfenicol per kg of fish body weight for 10 days contain concentrations higher than the *in vitro* MIC (2 µg/ml) for *F. asiatica* for the duration of the treatment (4, 16). In these studies, at the midpoint of the 10-day treatment period (T5), the mean concentrations of florfenicol in serum (µg/ml) and florfenicol residue in muscle-skin (µg/g) were 7.14 ± 2.49 and 9.98 ± 2.23 , respectively, in 100 g of tilapia fed the medicated feed (4).

The *in vivo* results showed that florfenicol treatment, initiated either 1 or 3 days postchallenge, significantly decreased

FIG. 4. Cytotoxicity of *F. asiatica* in tilapia head kidney-derived macrophages incubated in florfenicol. Bacteria were added to CTMM at an MOI of 25:1 and incubated for 2 h at 25°C with 5% CO₂. Zero, 1, 10, or 100 µg/ml of florfenicol was added to each culture. At 24 and 48 h cytotoxicity was assayed by the amount of lactate dehydrogenase released from infected cells. The error bars represent the standard error of triplicate samples, and results shown are representative of three independent experiments. Significant differences between treatment groups and the untreated (0 µg/ml) control are indicated: *, $P < 0.005$; **, $P < 0.001$.

mortalities ($P < 0.001$) to 0 and 13% (respectively), whereas 70% mortality was observed in the challenged nonmedicated group. Treatment of francisellosis with florfenicol-medicated feed prevented the development of an acute lethal form of disease but was unable to provide complete clearance of the bacterial infection. By 30 days postinfection, bacteria were largely cleared from the spleen of fish fed at 1 or 3 days postinfection. Bacteriological analysis of splenic tissue of survivors demonstrated significantly ($P < 0.001$) reduced bacterial numbers in spleen from medicated fish at 30 days postchallenge compared with the control group. No significant differences were observed between the challenged nonmedicated group and the fish that were given medicated feed 6 days postchallenge. We speculate that this can be attributed to disease-induced anorexia, which began around 4 days postchallenge. By 6 days postchallenge, fish were not eating enough to achieve the desired dose, resulting in an ineffective treatment. This is in agreement with previously published work, where sunshine bass infected with *Streptococcus iniae* became anorexic at 2 days postinfection. As a result, medicated feed administered after fish became anorexic was ineffective at treating the infection (9).

The pharmacokinetics of an antimicrobial in a given host species, at a given water temperature, relative to the *in vitro* MIC of the drug to the pathogen theoretically should determine the outcome of therapeutic intervention; however, in fish culture, other factors such as stressful environmental conditions may play a role. Also the ability of the bacteria to reside in privileged intracellular sites should be considered an important factor in determining effective treatments as these sites are not readily accessible to many antimicrobials. In *in vitro* experiments with THKDM, where macrophages were exposed to different concentrations of florfenicol for 48 h, a significant reduction ($P < 0.001$) in the number of intracellular *F. asiatica* bacteria was obtained at florfenicol concentrations of 10 and 100 µg/ml in the extracellular environment (Fig. 3). Moreover,

significant reduction ($P < 0.001$) in cytopathogenesis was observed at 24 and 48 h postinfection in THKDM that contained 10 or 100 $\mu\text{g/ml}$ of florfenicol in the extracellular environment compared to cytopathogenesis in the control group without extracellular florfenicol (Fig. 4). Similar results were found in murine macrophages, where significant reductions of viable intracellular nontyphoid *Salmonella* bacteria were observed at extracellular chloramphenicol concentrations equal to or 10 times greater than the MIC (6). A reduction of intracellular *Salmonella enterica* serovar *Typhimurium* PT99 was also observed in infected pigeon macrophages although high concentrations of the antibiotic ($>16 \mu\text{g/ml}$) were required in the extracellular environment (22). Various antibiotics like aminoglycosides, tetracyclines, fluoroquinolones, rifampin, and telithromycin have demonstrated bactericidal capacity and efficacy in killing intracellular *F. tularensis* in infected murine macrophage cells (18). On the other hand, penicillin G, amoxicillin, ceftriaxone, thiamphenicol, and erythromycin failed to display any significant activity against intracellular *F. tularensis* compared to drug-free controls (18). No report has been made concerning the efficacy of florfenicol in the treatment of *F. tularensis* in mammals. Recent data suggest that *F. asiatica* is resistant to trimethoprim-sulfamethoxazole and is susceptible to oxytetracycline *in vitro* (E. Soto et al., unpublished data). Further research is necessary in order to evaluate the efficacy of oxytetracycline-mediated feed to control fish francisellosis, as well as to evaluate the capacity of this drug to penetrate the THKDM and control intracellular bacteria.

As previously described, tilapia receiving medicated feed at 15 mg/kg of body weight contained florfenicol concentrations greater than 10 $\mu\text{g/ml}$ in tilapia serum, muscle, and skin (4, 16), which is significantly greater than the 2 $\mu\text{g/ml}$ MIC determined in this study. Moreover, in less than 24 h after a single oral dose of florfenicol at 10 mg/kg of body weight, freshwater-reared tilapia presented 5.21 $\mu\text{g/g}$, 5.27 $\mu\text{g/g}$, 4.59 $\mu\text{g/g}$, and 5.50 $\mu\text{g/g}$ concentrations of the antibiotic in the liver, gill, muscle, and kidney, respectively (10). All these data suggest that florfenicol concentrations in medicated feed, as used in this study, are sufficient to penetrate the intracellular environment and control infection in a dose-dependent manner. Interestingly, concentrations as high as 100 $\mu\text{g/ml}$ of florfenicol in the cultured medium did not affect survival of the THKDM, as demonstrated by the amount of LDH released in the medium (data not shown). Thus, a higher concentration of antibiotic in the feed could potentially eliminate the persistent bacteria found in splenic tissue at 30 days postchallenge. The effect of higher doses of drug in the feed on palatability is unknown.

In conclusion, florfenicol administered in medicated feed initiated at 1 day postinfection and fed daily for 10 days significantly reduced mortalities in tilapia experimentally infected with *F. asiatica* and prevented dissemination of the bacterium to hematopoietic organs. No pathological changes occurred, and reduced numbers of bacteria remained in the spleen at 30 days postchallenge. Conversely, administration of medicated feed for 10 days beginning 3 to 6 days postinfection led to the development of a chronic, nonlethal infection, suggesting that *F. asiatica* may have a propensity for latency. In these cases, the main reason for the ineffectiveness of the treatment seems to be associated with the anorexic condition that the diseased fish develop. The results from this study suggest that the infection

could be contained or eliminated if early antibiotic treatment (<6 days postinfection) was initiated, preventing the bacterial load from reaching a lethal level in the host. Recently, an *iglC*-based TaqMan real-time PCR assay with high sensitivity and specificity for the detection and quantification *F. asiatica* has been developed (32). The assay can potentially be used as a rapid diagnostic test for francisellosis, with the great benefit of fast turnaround of results (hours), which can aid the producer and diagnostician in starting medicated feed protocols early, thus preventing the anorexic manifestation of subacute to chronic diseases in fish.

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