IN VITRO AND IN VIVO EFFICACY OF FLORFENICOL FOR TREATMENT OF Florence 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 warmwater fish. Francisellosis outbreaks have increased in frequency amongst aquaculture commercial operations, and have caused severe economical 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 minimal inhibitory concentration (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 reduced 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 untreated cells. In medicated feed trials, fish were fed 15 mg florfenicol/kg fish body weight for 10 days, and the feeding was initiated at either one, three, or six days post-challenge. Immersion challenges resulted in 30% mean percent survival in non-treated fish and fish receiving medicated feed administered at one and three days post infection showed higher mean percent survival (100% and 86.7%, respectively). A significant decrease (p<0.001) in bacterial numbers (cfu/g of spleen tissue) was observed in treated groups at both one and three days post-challenge, compared to non-treated infected fish. There were no differences in bacterial burden in the spleen between fish treated six days post-challenge and untreated controls. In conclusion, if administered during early stages of infection, florfenicol demonstrated the potential for effectively treating piscine francisellosis, including the capacity for intracellular penetration and bacterial clearance.
INTRODUCTION

Members of the genus Francisella are small, pleomorphic, Gram-negative bacteria, belonging to the gamma group of the class Proteobacteria (3, 7, 28). Many Francisella sp. are facultative intracellular pathogens, capable of replicating in macrophages and other various cell types in humans, rabbits, rodents, non-human 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 cause piscine francisellosis in a wide variety of fish species (19).

During the past five 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 USA, Taiwan, Costa Rica, Latin America, Hawaii, 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 nowadays 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 non-specific clinical signs and high mortality rates, or as a sub-acute to chronic syndrome with non-specific clinical signs like anorexia, exophthalmia, and anemia. The bacterium has a high infectivity rate in tilapia fingerlings. Low numbers (1-10 CFU) of the bacterium injected intra-peritoneally can cause colonization and significant damage to the head kidney and spleen, with a dose as low as 23 bacterium 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 *Francisella 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 United States Food and Drug Administration for use in United States aquaculture; oxytetracycline (TERRAMYCIN® 200 for Fish; Philbro 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 feed, florfenicol (AQUAFLOR®) 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*, amongst 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 chemotherapeutants 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 was presented that indicated 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 yet to demonstrate the potential use of any of these drugs for the treatment of francisellosis in fish utilizing medicated feed. Moreover, at this point is unknown if the antimicrobial susceptibility of *F. noatunensis* and *F. asiatica* is 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**

*Francisella asiatica* strain LADL 07-285A, isolated from cultured tilapia (*Oreochromis* sp.), was described in previous work (29). *Francisella* sp. isolates demonstrating >99% identity to *F. asiatica* 16S rDNA were recovered from moribund hybrid striped bass (20) and tilapia (15) and kindly donated by Dr. John Hansen, Interdisciplinary Program in Pathology, University of Washington, Seattle, Washington, USA. Polymerase chain reaction (PCR) and sequence comparison of the *iglABCD* operon and 16S rDNA 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 *F. victoria* (15) are in fact members of the recently described species *F. asiatica* (30, 32). *Francisella asiatica* isolates were grown on Cystine Heart Agar supplemented with bovine hemoglobin solution (CHAH) (Becton Dickenson (BD) BBL, Sparks, MD, USA) for 48 h at 28°C, or in a modified Mueller-
Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL, Sparks, MD, USA) and 0.1% glucose (MMH) (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 media 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 - 24 h at 28°C.

**Antimicrobial susceptibility testing**

The minimal inhibitory concentration (MIC) of florfenicol for *F. asiatica* isolates and *E. coli* were tested using Sensititre Just One® Strips (Trek Diagnostic Systems, West Sussex, UK), containing two fold dilutions of florfenicol (0.12-128 µg/ml), using slight modifications to the manufacturer’s suggested protocol. Briefly, bacterial isolates were grown on agar plates as described previously, and 3-5 colonies were dislodged with a sterile cotton swab, suspended in 4 ml of MMH, and adjusted to a 0.5 McFarland. For *F. asiatica*, 100 µl of this suspension was transferred to 11 ml of MMH broth, mixed, and 50 µl 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 24–48 h at 28°C. Bacterial growth was checked visually after removing the adhesive seal at 24 and 48h post inoculation. The MIC value 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 (16-27 g) were obtained from a source with no previous history of *Francisella* infection. A sub-sample of the population was confirmed negative for *F. asiatica* by complete clinical, bacteriological, and molecular analysis using previously published protocols to ensure they were negative for francisellosis (29). Fish were maintained at stocking densities of 10 fish per tank in 20 L flow through tanks at a temperature of 25°C. Fish were fed 2% fish body weight per day with a commercial tilapia feed (Burris Aquaculture Feeds, Franklinton, LA). Fish were acclimatized for at least four 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 drug for 10 d may be necessary to control infections of *Streptococcus* spp., another well know 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 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 was thoroughly mixed in a Twin Shell Dry Blender (Patterson-Killey Co, Division of the Harsco Corporation, East Strasburg, PA) for 15 m. 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 23-25°C and kept dry in bags maintained at 4°C until used. High-performance liquid chromatography (HPLC) was performed by Eurofins Scientific (Memphis, Tennessee) to analyze florfenicol concentrations in the experimental diet. Medicated feed samples analyzed by HPLC revealed that the florfenicol dose administered was 12.9 mg florfenicol/kg fish/d (86% of target).

**Infectivity challenge**

Six treatments were randomly assigned to 18 aquaria with three replicate tanks/group. Treatment groups were: three medicated, challenged treatments; a non-medicated, challenged treatment (positive control); a non-medicated, non-challenged treatment (negative control) and a medicated, non-challenged group to determine any detrimental effects the medication had on the fish. In all three medicated, challenged treatments, fish were fed medicated feed for a period of 10 days, starting at either one, three, or six days post challenge. Before and after the 10-d medication period, non-medicated 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). *Francisella 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 8x10⁷ CFU/ml was obtained by adding 0.5 L of a bacterial suspension in 1x phosphate buffered saline (PBS; pH 7.3) to 10 L 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 d of acclimation, and the 30 d post-challenge. 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 un-consumed feed in the tanks, the fish were allowed to consume the feed for 30 min, after which, non-consumed feed was removed out of the tanks and discarded.

**Bacterial load determinations**

Five fish from each medicated group and survivors from the non-medicated control group were sacrificed 30 d post-challenge, and spleens were harvested to determine approximate bacterial burdens. Organs were weighed, homogenized in 0.5 ml sterile PBS, plated in triplicate on CHAH and incubated at 25°C for three days prior to CFU determinations. CFU were expressed as 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 Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Invitrogen Corp., Carlsbad, CA) with 14mM Hepes Buffer (GIBCO, Invitrogen Corp.), 0.3% sodium bicarbonate (GIBCO, Invitrogen Corp.), 0.05 mM 2-beta mercaptoethanol (Sigma Chemical Co., St Louis, MO), and 5% heat-inactivated, pooled tilapia serum. Briefly, 96-well plates containing three day old cultures of THKDM at concentrations of 1-5 x 10^5 cells per well were used. *Francisella asiatica* was grown for 8 h in MMH at 25°C. Optical density (OD$_{600}$) of the culture was determined and the cells were adjusted to an estimated final concentration of 5 x
10^8 CFU/ml, based on an OD/CFU standard curve. One ml aliquots of the bacterial suspension were pelleted at 10,000 x g for 5 minutes in an Eppendorf 5415 D centrifuge (Eppendorf-Brinkman, Westbury, NY), and the pellet was resuspended in 1ml of normal autologous serum to opsonize the bacteria. Ten-fold serial dilutions were plated on CHAH plates after incubation to determine actual 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: 1 macrophage. The plates were centrifuged for 5 min at 400 x g to synchronize bacterial contact with macrophages. Following a two h incubation at 25°C with 5% CO₂, the cells were washed three times with warm CTMM (25°C), and further incubated with fresh CTMM containing 0 µg/ml, 1µg/ml, 10 µg/ml or 100 µg/ml of florfenicol for 0, 24, or 48 hours. 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, LDH (lactate dehydrogenase) cytotoxicity assay was performed using the colorimetric Cytotox 96 Kit (Promega, Madison, WI) according to the manufacturer’s instructions. Cytotoxicity assays were performed on both infected and non-infected THKDM exposed to 0, 1, 10 and 100 µg/ml of florfenicol in CTMM and for 0, 24 and 48 h. The percentage of cytotoxicity was calculated as 100 × [(experimental release – spontaneous release)] / [total release – spontaneous release)], where spontaneous release is the amount of LDH activity in the supernatant of uninfected cells and the total release is the activity in cell lysates (31).
Statistical analysis

The Statistical Analysis System (SAS Institute, Inc. 2003) was used with the general linear models 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 mean square means. For the mortality studies the percent mortalities were transformed with an arc-transformation to normalize the data. Colony forming units (CFU) recovered in the *in vitro* challenges were log$_{10}$ transformed for statistical analysis. All comparisons were considered significant at (P <0.05).

RESULTS

Susceptibility testing (MIC determination)

Minimal inhibitory concentrations as determined using the Trek diagnostic sensititre plates containing florfenicol indicated that all *F. asiatica* isolates were susceptible to a concentration of 2 µg/ml of florfenicol (Table 1). The *E. coli* control consistently showed an MIC of 4 µg/ml which is within the accepted quality control range for this organism in cation adjusted Mueller Hinton broth after 24-48 hr at 28°C (8).

*In-vivo* efficacy

The first mortalities occurred three days post-challenge in non-medicated tanks (non-medicated challenged group) and fish given medicated feed 6 days post-challenge. 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 (Figure 1). In both groups, fish became anorexic (non-consumption of feed after 30 min of feeding) beginning
at four days post-challenge. Excess feed was removed from the tanks 30 m after feeding to avoid water quality problems. Conversely, groups of fish that received medicated feed one or three days post-challenge ate all medicated feed within a few minutes and presented significantly higher survivability than the non-medicated challenged group (p<0.001). No mortality was observed in the group receiving medicated feed one day post-challenge, and only 13.3% of fish receiving medicated feed three days post-challenge died after 30 days (Figure 1). In the fish fed medicated feed one day post-challenge, 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 post-challenge. 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 to determine CFU/mg of organ weight. Only one fish from the group presented with medicated feed one day post-challenge had detectable CFU in the spleen. The counts were significantly lower (p<0.001) than in any other treatments (Figure 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 three days post-challenge (24.15±7.4 CFU/mg of spleen), were significantly lower compared with the non-medicated challenged group (310±77 CFU/mg of spleen) (p<0.01) (Figure 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 post-challenge (419±183 CFU/mg of spleen) and control (non-medicated and challenged) (P<0.05) (Figure 2). No mortality or lesions were observed in any of the medicated non-challenge controls or non-medicated non challenged controls.

**Efficacy of florfenicol to control intracellular *Francisella 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 µg/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 effects of florfenicol at any concentration between 0 and 100 µg/ml. At 24 h, the survival rates of THKDM were 99.96 ± 0.004% in the 1 µg/ml, 99.95 ± 0.005% in the 10 µg/ml, and 99.95 ± 0.012% in the 100 µg/ml florfenicol-treated macrophages compared with untreated macrophages (0 µg/ml of florfenicol). At 48 h, the survival rates were 99.91 ± 0.02 in the 1 µg/ml, 99.9 ± 0.01 in the 10 µg/ml, and 99.9 ± 0.01 in the 100 µg/ml of florfenicol-treated macrophages compared with 100% in untreated macrophages (0 µg/ml).

To investigate whether florfenicol affects *F. asiatica* survival within THKDM, cells were infected at a 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 µg/ml of florfenicol. The results showed efficient intracellular replication of *F. asiatica* within THKDM cultured with 0 and 1 µg/ml (Figure 3). At 24 and 48 h post-infection CFU counts of *F. asiatica* were greater (p<0.001) than those at 0 h in both the control and the THKDM cultured with media containing 1 µg/ml of florfenicol (Figure 3). On the other hand, infected macrophages that were cultured in
media with 10 and 100 μg/ml of florfenicol, showed no bacterial replication, and significantly (p<0.001) reduced numbers of bacteria at 48 h post-infection (Figure 3).

The lactate dehydrogenase (LDH) cytotoxicity assay performed in infected cells correlates with the results from the intracellular growth assays. The cytotoxicity observed in THKDM cultured with media 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 48h (Figure 4). Although the cytotoxicity observed in THKDM cultured with media containing 1µg/ml florfenicol was significantly lower (p<0.001) than that observed in the control wells at 24h, by 48 h the cytotoxicity had increased in this group (Figure 4).

DISCUSSION

The results obtained from the MIC determinations demonstrated that florfenicol is a suitable candidate for in vivo experimentation, since a low minimal inhibitory concentration (2μg/ml) was found. In mammalian pathogens like Pasteurella multocida, Actinobacillus pleuropneumoniae, Mannheimia haemolytica, and Histophilus somni, florfenicol has shown bactericidal activity at the respective MICs; whereas in others like Staphylococcus aureus MICs only have bacteriostatic activity (22). Therefore, maintaining concentrations in plasma above the MIC seem 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 florfenicol per kg fish body weight for 10 d, contain concentrations higher than the in vitro MIC (2μg/ml) for F. asiatica for the duration of the treatment (4, 16). In these studies, the mean T5 (midpoint of the 10-d treatment period) concentrations of florfenicol in serum (μg/mL) and
florfenicol residue in muscle–skin (µg/g) where 7.14 ± 2.49 and 9.98 ± 2.23, respectively, in 100 g tilapia fed the medicated feed (4).

The *in vivo* results showed that florfenicol treatment, initiated either one or three days post-challenge significantly decreased mortalities (p<0.001) to 0 and 13% (respectively), whereas 70% mortality was observed in the challenged non-medicated 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 post-infection, bacteria were largely cleared from the spleen of fish feed at one or three days post-infection. Bacteriological analysis of splenic tissue of survivors demonstrated significantly (p<0.001) reduced bacterial numbers in spleen from medicated fish 30 days post-challenge, as compared with the control group. No significant differences were observed between the challenged non-medicated group and the fish that were given medicated feed 6 days post-challenge. We speculate this can be attributed to disease-induced anorexia, which began around four days post-challenge. By six days post-challenge, 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 two days post-infection. 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 as 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* were obtained at florfenicol concentrations of 10 and 100 µg/ml in the extracellular environment (Figure 3). Moreover, significant reduction (p<0.001) in cytopathogenesis was observed 24 and 48 h post-infection in THKDM that contained 10 or 100 µg/ml of florfenicol in the extracellular environment compared to the control group containing no florfenicol (Figure 4). Similar results were found in murine macrophages, where significant reductions of viable intracellular non-typhoid *Salmonella* were observed at extracellular chloramphenicol concentrations equal to or 10 times greater than the MIC (6). Reduction of intracellular *Salmonella enterica* serovar *Typhimurium* PT99 was also observed in infected pigeon macrophages, although high concentrations of the antibiotic (>16 µg/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, ceftriaxine, thiampenicol, 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 suggests that *F. asiatica* is resistant to trimethoprim-sulfamethoxazole, and is susceptible to oxytetracycline *in vitro* (Soto et al. unpublished). Further research is necessary in order to evaluate the efficacy of oxytetracycline medicated 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 body weight, contained florfenicol concentrations greater than 10 µg/ml in tilapia serum, muscle and skin (4, 16), which is significantly greater than the 2 µg/ml MIC determined in this study. Moreover, in less than 24 h after a single oral dose of 10 mg/kg body weight of florfenicol, freshwater reared tilapia presented 5.21 µg/g, 5.27 µg/g, 4.59 µg/g and 5.50 µg/g concentrations of the antibiotic in the liver, gill, muscle and kidney, respectively (10). All these data suggests that florfenicol concentrations in medicated feed, as was 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 µg/ml of florfenicol in the cultured media, 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 30 days post-challenge. The effect of higher doses of drug in the feed on palatability is unknown.

In conclusion, florfenicol administered in medicated feed initiated at one day post-infection and fed daily for 10 days, significantly reduces mortalities in tilapia experimentally infected with *F. asiatica* and prevents dissemination of the bacterium to hematopoietic organs. No pathological changes occur and reduced numbers of bacteria remain in the spleen 30 days post challenge. Conversely, administration of medicated feed for 10 days beginning 3-6 days post-infection led to the development of a chronic, non-lethal infection suggesting *F. asiatica* may have the 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 diseases fish develop. The results from this study suggests that the infection could be contained or eliminated if early antibiotic treatment (<6 days post-infection) was initiated, preventing the bacterial load from
reaching a lethal level in the host. Recently, an iglC based Taq-Man 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 franciselliosis, 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 sub-acute to chronic diseases fish.

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noatunensis to species rank as Francisella noatunensis comb. nov., sp. nov. Int J Syst Evol Microbiol DOI 10.1099/ijs.0.002139-0.


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Table 1. Minimum inhibitory concentration (MIC) of florfenicol against *Francisella asiatica* and *Escherichia coli ATCC 25922* isolates obtained from culture fish.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Florfenicol MIC (µg/ml)</th>
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<tbody>
<tr>
<td><em>F. asiatica</em> LADL 07-285A</td>
<td>Tilapia</td>
<td>2</td>
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<tr>
<td><em>F. asiatica</em> LADL 07-285B</td>
<td>Tilapia</td>
<td>2</td>
</tr>
<tr>
<td><em>Francisella</em> sp. #1</td>
<td>Hybrid Striped bass</td>
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<tr>
<td><em>Francisella</em> sp. #2</td>
<td>Hybrid Striped bass</td>
<td>2</td>
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<tr>
<td><em>Francisella</em> sp. #3</td>
<td>Hybrid Striped bass</td>
<td>2</td>
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<tr>
<td><em>F. victoria</em></td>
<td>Tilapia</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> 25922</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Table 2. Histopathological lesions in selected tissues from medicated and non-medicated tilapia 30 days post-challenge with *F. asiatica*. The five treatments consisted of three medicated, challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge for a period of 10 days (1DPC). A second group was treated identically but medicated feed started at day three post-challenge (3DPC). The third medicated treatment started at day six post-challenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Spleen</th>
<th>Head kidney</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DPC</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3 DPC</td>
<td>Moderate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6 DPC</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderate</td>
</tr>
<tr>
<td>Control +</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderate</td>
</tr>
<tr>
<td>Control -</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**Legends:**

None: $X=0$

Mild: $X<7$

Moderate: $7>X<20$

Severe: $X>20$
Figure 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 active ingredient/kg body weight for 10 d. The five treatments consisted of three medicated, challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge for a period of 10 days (1DPC). A second group was treated identically but medicated feed started at day three post-challenge (3DPC). The third medicated treatment started at day six post-challenge (6DPC). Each treatment group had 30 fish equally divided among three tanks.
Figure 2. Reduced *Francisella asiatica* bacterial burden in the spleens of antibiotic fed tilapia.

The five treatments consisted of three medicated challenged treatments, a non-medicated challenged treatment (CON+), and a non-medicated non-challenged treatment (CON-). The three medicated challenged treatments consisted of one group given medicated feed at day one post-challenge (1DPC) for a period of 10 days. A second group was treated identically but medication feed started at day three post-challenge (3DPC) and in the third one medication started at day six post-challenge (6DPC). Five fish from each medicated group and survivors from the non-medicated control group were sacrificed 30 d post-challenge, and spleens were harvested to determine approximate bacterial burdens. Error bars represent mean ± SEM. Significantly different than CON+: * (P<0.005) and ** (P<0.001).
Figure 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 hours at 25°C with 5% CO2 followed by incubation with 0, 1, 10 or 100 µg/ml of florfenicol in complete tilapia macrophage media (CTMM). At 0, 24 and 48 h post-infection, cells were washed and lysed with 0.5% Saponin, followed by serial 10-fold dilutions plated on Cystein Heart Agar supplemented with hemoglobin (CHAH) plates and incubated at 25°C for 3 days for CFU determination. Experiment performed three times in triplicate. Error bars represent mean ± SEM. * Significantly different in counts than time 0; *P < 0.001.
Figure 4. Cytotoxicity of *Francisella asiatica* in tilapia head kidney derived macrophages incubated in florfenicol.

Bacteria were added to complete tilapia macrophage media (CTMM) at an MOI of 25:1 and incubated for 2 hours at 25°C with 5% CO₂. Zero, one, 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 standard error of triplicate samples and results shown are representative of three independent experiments. Significant differences between treatments and the zero µg/ml of florfenicol are marked; * (P<0.005) and ** (P<0.001).
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Florfenicol MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. asiatica</em> LADL 07-285A</td>
<td>Tilapia</td>
<td>2</td>
</tr>
<tr>
<td><em>F. asiatica</em> LADL 07-285B</td>
<td>Tilapia</td>
<td>2</td>
</tr>
<tr>
<td><em>Francisella</em> sp. #1</td>
<td>Hybrid Striped bass</td>
<td>2</td>
</tr>
<tr>
<td><em>Francisella</em> sp. #2</td>
<td>Hybrid Striped bass</td>
<td>2</td>
</tr>
<tr>
<td><em>Francisella</em> sp. #3</td>
<td>Hybrid Striped bass</td>
<td>2</td>
</tr>
<tr>
<td><em>F. victoria</em></td>
<td>Tilapia</td>
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<td><em>E. coli</em> 25922</td>
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### Mean values of granulomas in 10X microscopic field

<table>
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<tr>
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<td>Moderate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6 DPC</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderate</td>
</tr>
<tr>
<td>Control +</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderate</td>
</tr>
<tr>
<td>Control -</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**Legends:**

- None: $X = 0$
- Mild: $X < 7$
- Moderate: $7 > X < 20$
- Severe: $X > 20$