



Standardization of Dose and Delivery of Oxytetracycline against *Streptococcus agalactiae* Infection in Genetically Improved Farmed Tilapia (GIFT)

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10.18805/IJAR.B-4398

ABSTRACT

Background: Tilapia (*Oreochromis niloticus*) is one of the most important aquaculture species in India. *Streptococcus agalactiae* is a highly debilitating pathogen of farmed tilapia. In this study, oxytetracycline (OTC) dose and delivery were standardized against *S. agalactiae* infection in genetically improved farmed tilapia (GIFT) and pharmacokinetics after oral administration was evaluated.

Methods: LD₅₀ value of *S. agalactiae* was found to be 6.2×10^6 CFU/fish. Two methods of drug delivery were investigated at three different doses of 50, 70 and 90 mg/kg against the *S. agalactiae* infection. Pharmacokinetics of OTC was studied after *per os* administration of 100 mg/kg body weight per day and analysing the residues in kidney, liver and muscle at different time intervals.

Result: *Per os* delivery of OTC 90 mg/kg controlled *S. agalactiae* infection and the withdrawal time of OTC to fall below MRL of 0.05 µg/g at 30°C was 21 days in kidney and liver and 14 days in muscle. The study indicated that the *S. agalactiae* infection in tilapia could be controlled by OTC and fish can be marketed after providing a withdrawal period of 14 days.

Key words: GIFT, LD₅₀, MRL (Maximum residue level), Oxytetracycline (OTC), Pharmacokinetics, *Streptococcus agalactiae*, Tilapia, Withdrawal time.

INTRODUCTION

Aquaculture is one of the fast-growing food production sectors in the world that contributed to 47% of the total fish production of 171 million tonnes in 2016 (FAO, 2018). Increased fishing pressure and biological alterations associated with climate change dwindled capture fisheries in India and worldwide. *Oreochromis niloticus* is an important species belonging to the *Cichlidae* family, native to Africa and Middle East regions with an estimated production of 5.3 million tonnes in 2016 that contributed to 10% of the global aquaculture production (FAO, 2018). In India, the production of tilapia is estimated to be 18,000 tons in 2016 with a 10% projected annual growth rate.

Of the several infectious agents that severely impede the economic development of aquaculture sector, *Streptococcus agalactiae* is one of the major pathogens responsible for the huge economic losses with an annual estimated to loss of about \$ 40 million in China (Chen *et al.* 2012). *S. agalactiae* known as Group B *Streptococcus* (GBS) is a Gram-positive coccus, recognized as a pathogen of humans and animals including fish (Bowater *et al.* 2018). GBS infections are reported in Nile tilapia (*Oreochromis niloticus*) (Li *et al.* 2014) and barcoo grunter (*Scortum barcoo*) (Liu *et al.* 2014). It causes septicaemia and meningitis in farmed and wild fish. GBS is first reported in hatchery-reared freshwater fish in the United States in 1966 (Robinson and Meyer 1966). Clinical signs of *S. agalactiae* infection include lethargy, anorexia, "C" shaped body posturing, erratic swimming and whirling (Evans *et al.* 2006).

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How to cite this article: Kalaria, K.K., John, K.R., George, M.R. (2021). Standardization of Dose and Delivery of Oxytetracycline against *Streptococcus agalactiae* Infection in Genetically Improved Farmed Tilapia (GIFT). Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4398.

Submitted: 09-01-2021 **Accepted:** 23-08-2021 **Online:** 24-09-2021

S. agalactiae isolated from different geographic locations were found to be sensitive to ofloxacin, cephalosporin and tetracycline in Taiwan (Huang *et al.* 2014) and gentamicin, ciprofloxacin, chloramphenicol, erythromycin, sulfamethoxazole/ trimethoprim and tetracycline in Malaysia (Aisyhah *et al.* 2015). Among these, oxytetracycline (OTC) is an approved broad-spectrum antibiotic in aquaculture (Rigos and Troisi 2005), belonging to the tetracyclines class, a protein synthesis inhibitor. It is used for the treatment of fish bacterial diseases such as furunculosis, aeromonosis, pseudomonosis, lactococcosis and vibriosis (AliAbadi and MacNeil 2002; Cenavisa, 2016). Withdrawal time of OTC in Nile tilapia at different doses and by different route of

administration has been reported previously (Chen *et al.* 2004; Paschoal *et al.* 2012; Soltan *et al.* 2013). But there are no reports regarding genetically improved farmed tilapia (GIFT) strain developed by the World Fish Centre, which exhibits higher growth performance compared to local strains of Nile tilapia. Hence the present study was conducted to standardize the dose and delivery route of OTC against *S. agalactiae* infection in GIFT and investigate the withdrawal time of OTC in tilapia (GIFT).

MATERIALS AND METHODS

Fish and bacterial strain

Four hundred fifty juvenile tilapia (GIFT), averaging 22 ± 5 g were procured from a local fish farm in 2019 and acclimatized for a period of 10 days in 20 m² rectangular cement nursery tank after disinfection with 2 mg/L potassium permanganate. The fish were fed twice a day and water quality parameters were observed daily. *Streptococcus agalactiae* (ATCC-12386) culture was purchased from ATCC (Hi-media, Mumbai)

Antibiotic sensitivity test

Antimicrobial susceptibility testing was carried out by disk diffusion method on Mueller-Hinton agar. OTC discs with 30 µg per disc were obtained commercially (HiMedia, Mumbai). Florfenicol disc at the same concentration was prepared after obtaining the drug from Meteoric biopharmaceuticals (Lalitha, 2004). The discs were placed on the agar plates after addition of 100 µl *S. agalactiae* culture and incubated at $30 \pm 2^\circ\text{C}$ for 24 h and the diameter of zone of inhibition in mm was measured.

Estimation of LD₅₀

Juvenile tilapia (GIFT) were introduced into 100 L tanks having 60 L water with eight fish per tank. Tilapia were injected 0.1 ml of serially diluted (10^{-1} , 10^{-3} , 10^{-5}) *S. agalactiae* having a bacterial count of 6.2×10^9 CFU/ml intraperitoneally under anaesthesia by benzocaine (10 mg/L). First group received 6.2×10^8 CFU/ml, second group 6.2×10^6 and third 6.2×10^4 CFU/ml (6.2×10^7 , 6.2×10^5 , 6.2×10^3 CFU/fish respectively). Control groups were injected with 0.1 ml of sterile saline. Fish were fed once daily and water quality

parameters were continuously observed. Samples of brain and kidney were collected from moribund fish for histopathology and dead fish were subjected to bacterial isolation. LD₅₀ value was calculated by formula of Reed and Muench (1938).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of OTC was found out by slightly modified macrodilution method of CLSI (2012). The inoculum of 100 µl *S. agalactiae* culture was added to 5ml TSB tubes prepared with different concentrations of OTC viz. 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml along with a control without antibiotic. Tubes were incubated for 24 h at $(30 \pm 2)^\circ\text{C}$ and bacterial growth (CFU/ml) was calculated by serial dilution and incubation on TSA for 24 h at $(30 \pm 2)^\circ\text{C}$.

Experimental design for standardization of dose and delivery

The fish (GIFT) (20 ± 5 g) were first challenged with 0.1 ml of *S. agalactiae* intraperitoneally after anesthetization with benzocaine (10 mg/L) and negative control with 0.1 ml of sterile PBS. OTC was then administered at different concentrations by intraperitoneal injection and orally through feed (Table 1). The water quality parameters such as pH, water temperature and dissolved oxygen, feeding behaviour, clinical signs of diseases and mortality were monitored. OTC by injection was administered at three doses at 24 h, 4th day and 7th day post-challenge in Groups 1, 2 and 3. For groups 4, 5 and 6 OTC feed was prepared by mixing OTC solution into a commercial feed along with 10 µl of soybean oil per gram to avoid antibiotic loss by hydro-solubilization during feeding. Fish were fed once a day at 2% of their body weight. The OTC treated groups were medicated for 10 consecutive days. During the observation period, samples of brain and kidney were taken from negative control, positive control and medicated groups for histopathology and bacteriology. The mortality data of OTC treated and control groups were compared by two-way Analysis of Variance (ANOVA). *P* values <0.05 were considered significant.

Histopathology

Kidney and brain of moribund fish were collected from normal

Table 1: Experimental design for standardization of dose and delivery of oxytetracycline against *S. agalactiae*.

Groups*	Dose (mg/Kg of body weight)	Route of delivery
Group 1	50	Intraperitoneal injection
Group 2	70	
Group 3	90	
Group 4	50	Oral through feed
Group 5	70	
Group 6	90	
Group 7	Nil	No drug, control feed (positive control)
Group 8	Nil	No drug, no challenge control feed (negative control)

*All groups were run in duplicate and had 15 fish each except 7 and 8 which had 10 fish each. All groups except 8 were challenged with *S. agalactiae* intraperitoneally.

and infected fishes of LD₅₀ analysis and medicated groups and fixed using neutral buffered formalin. Tissue sections were cut using standard procedures and stained in haematoxylin and eosin. The sections were examined and photographs taken under bright field in a trinocular microscope (Nikon, Japan).

Pharmacokinetics of OTC in GIFT

Healthy juvenile tilapia (GIFT), averaging 25 ± 5 g were procured from a local fish farm. Fish were acclimatized for a period of 10 days in 500 L capacity tanks. The fish were fed once a day and water quality parameters were observed daily. The water temperature was 30 ± 2°C during the course of the experiment. Two days before the experiment, the fish were starved until medication. OTC medicated feed was given to the fish for 7 days at the rate of 2% average body weight per day that was adjusted to a dose of 100 mg OTC/kg body weight per day. Medicated feed was prepared by dissolving OTC capsule (Pfizer limited) in distilled water and a mixture of 1.120 g of OTC in 336 g of feed and 10 µl of soybean oil, which was used as a binder, was added to per gram commercial feed.

After 7 days of feeding the medicated diet, five fish were randomly selected from the tank at each sampling time at 3, 6, 12, 18, 24, 36 h and 2, 3, 4, 7, 14 and 21-day intervals. Fish were decapitated and muscle, liver and kidney were collected. For each sampling, tissues were collected from 5 fishes and stored at - 80°C. Control fish were sampled before the initiation of the study.

RIDASCREEN, Europroxima oxytetracycline kit (R-biopharmneugen Pvt. Ltd Netherlands) was used for monitoring the oxytetracycline residues in fish. Samples were analysed after 21 days of sampling by the oxytetracycline ELISA kit having a limit of detection (LOD) of 2 ppb (µg/L) according to the manufacturer's instructions. Briefly, samples were homogenized and transferred into 15 ml plastic tubes and vortexed for 10 min after adding 3 ml of McIlvaine buffer. The mixture was then centrifuged at 2000 g for 10 min. The supernatant (50 µl) was pipetted into new tube and 200 µl of dilution buffer was added and absorbance values were measured in an ELISA reader at 450 nm. The results were used for calculating the concentration of antibiotic in tissues from the calibration curve plotted for the concentration standards (Fig 1).

RESULTS AND DISCUSSION

Antibiotic sensitivity of *S. agalactiae*

Streptococcus agalactiae is a major problem in tilapia farming responsible for high mortality. The antibiotic susceptibility test of *S. agalactiae* showed that the bacteria was sensitive to both oxytetracycline and florfenicol with higher sensitivity to OTC. Zone of inhibition of OTC was 38 mm while florfenicol was 27 mm. This result was similar to the earlier reports on the pathogen sensitivity to OTC (Ali *et al.* 2010; Najiah *et al.* 2012; Reyes *et al.* 2019). The antibiotic was reported to be effective in controlling mortality in Nile

tilapia infected with *S. agalactiae* (Faria *et al.* 2014; Mariotto *et al.* 2018).

LD₅₀ of *S. agalactiae* in GIFT and histopathology

Infected tilapia injected with serially diluted *S. agalactiae* showed clinical signs such as spiral swimming, decayed caudal fin, swollen abdomen, exophthalmia and lesions on body (Fig 2). No clinical signs or mortality was recorded in the control group. Mortality started after 30 h of injection of *S. agalactiae* at 6.2×10^7 CFU /fish and continued up to 5 days. Cumulative mortality is given in Fig 3. *S. agalactiae* was also re-isolated from the dead and moribund fish challenged by i.p. injection and identified by 16s r RNA sequence analysis indicating that the death/morbidity was due to *S. agalactiae*. The LD₅₀ of *S. agalactiae* in tilapia was found to be 6.2×10^6 CFU/fish, which is similar to an earlier study of two bacterial strains from Nile tilapia that had the LD₅₀ value of 6.8×10^6 and 5.3×10^6 CFU/fish (Wang *et al.* 2013). However, it was lower to the LD₅₀ range of 1.72×10^7

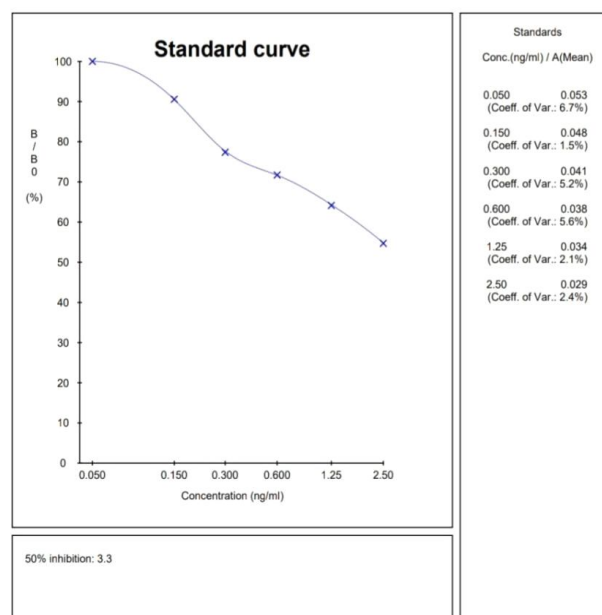


Fig 1: Standard curve for estimation of OTC by using ELISA.



Fig 2: *S. agalactiae* infected fish with decayed caudal fin and exophthalmia.

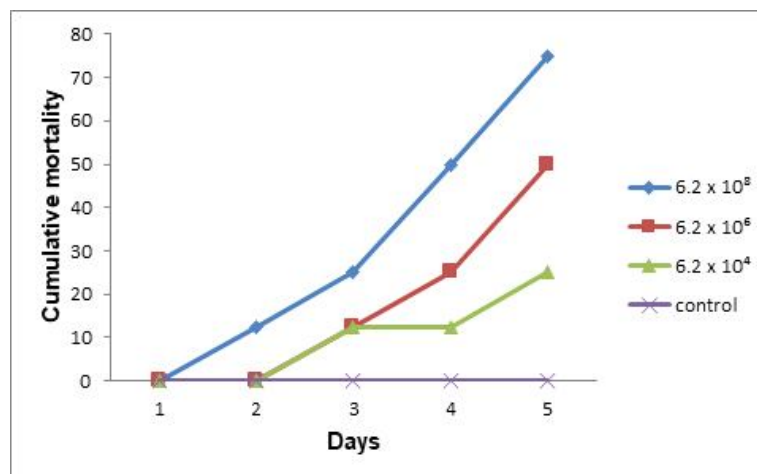


Fig 3: Cumulative mortality of tilapia (GIFT) infected with *S. agalactiae*.

CFU to 6.3×10^9 CFU/ml reported for adult tilapia (Nur-Nazifah *et al.* 2011) indicating the strain in this study is more virulent. Clinical signs such as lethargy, anorexia, erratic swimming and whirling, decayed caudal fin, swollen abdomen, exophthalmia and lesions on body observed during the study were similar to those reported earlier in tilapia for *S. agalactiae* infections (Evans *et al.* 2006).

Histopathological lesions such as severe tubulonephrosis, congested glomerulus and sharply decreased hemopoietic tissue observed in the kidney (Fig 4, 5) of the infected fish and meningeal congestion and vacuolar degeneration of the neuron in the brain (Fig 6, 7) were similar to the observation of He *et al.* (2017).

Minimum inhibitory concentration (MIC) of OTC against *S. agalactiae*

MIC of OTC noticed in the present study was 80 µg/ml (Table 2), which was higher than the earlier report of 0.5 - 8.0 µg/ml for *Streptococcus* isolates obtained from the diseased tilapia (Maisak *et al.* 1995) and 0.6 to 31.25 µg/ml of the 29 strains of *S. agalactiae* isolated from Nile tilapia (Faria *et al.* 2014).

Standardization of dose and delivery

No mortality or clinical signs of disease were observed in the negative control groups during the experimental period. All control groups were euthanized by benzocaine overdose and subjected to bacteriological assessment, which produced negative results for bacterial pathogens. All positive control groups challenged with *S. agalactiae* at 6.2×10^7 CFU/fish exhibited a cumulative mortality of 70% during the treatment period. The main clinical signs observed were spiral swimming, decayed caudal fin, swollen abdomen, exophthalmia and lesions on the body. Treatment by injection was very stressful for fish as seen from the mortality in all three doses that ranged from 30 to 63.3%. The cumulative mortality recorded in the both types of drug delivery is shown in the Fig 8 and 9. Treatment by medicated feed at an effective dose of 50 mg/kg body weight was unable to control the mortality and had a cumulative mortality of

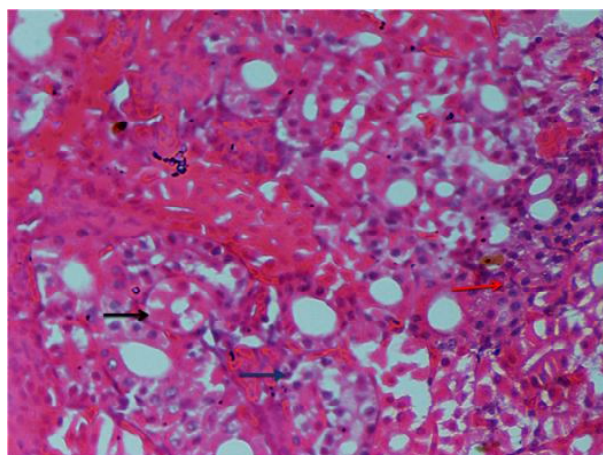


Fig 4: Kidney from uninfected fish stained with H&E showing renal tubules (black arrow), hemopoietic tissue (red arrow) and normal renal corpuscles, showing glomerulus (blue arrow).

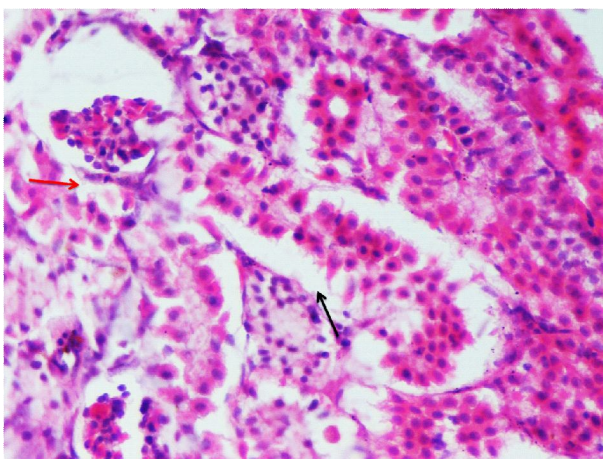


Fig 5: Kidney from fish injected with 10^8 cells of *S. agalactiae* showing severe tubulonephrosis (black arrow) and a sharp decrease of hemopoietic tissue, with congested glomerulus and necrotic debris in the Bowman's capsule (red arrow).

40%. The dose of 70 mg/kg and 90 mg/kg body weight were efficient to control mortality with low cumulative mortality of 26.6% and 20%. The high survival rate of infected tilapia treated with 70 mg/kg OTC incorporated feed showed that

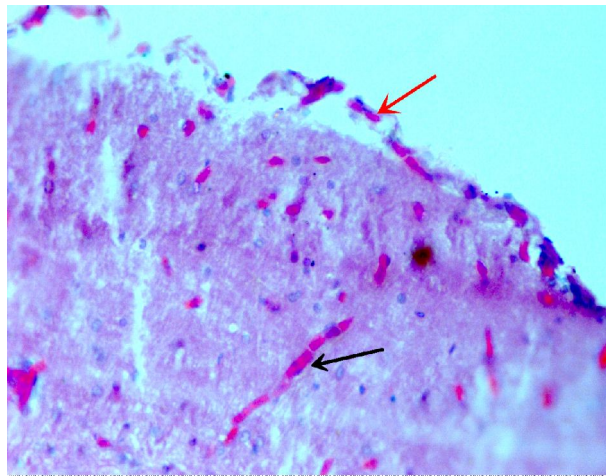


Fig 6: Brain from uninfected fish (600 X) showing brain microvessels (black arrow) and thin meninges (red arrow).

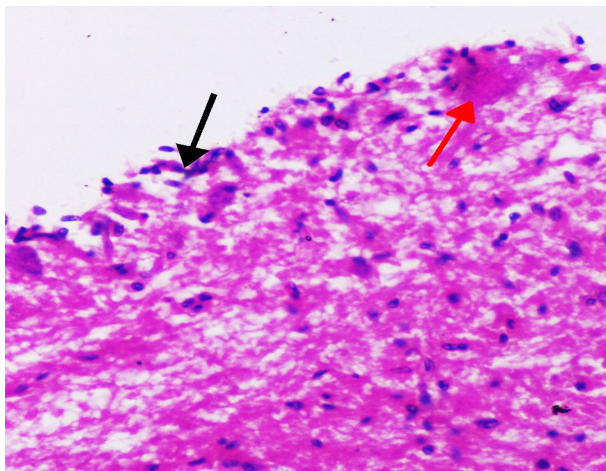


Fig 7: Brain from fish (600 X) injected with 10^8 cells of *S. agalactiae* showing meningeal congestion (black arrow) and vacuolar degeneration (red arrow).

Table 2: MIC of OTC against *Streptococcus agalactiae*.

No.	Concentration of OTC ($\mu\text{g/ml}$)	Growth
1	5	+
2	10	+
3	20	+
4	30	+
5	40	+
6	50	+
7	60	+
8	70	+
9	80	-
10	90	-
11	100	-

Table 3: OTC concentration ($\mu\text{g/g}$) in the tissues of tilapia (GIFT) held at $30 \pm 2^\circ\text{C}$.

Sampling time	Kidney	Liver	Muscle
3 h	0.332	0.208	0.293
6 h	0.312	0.187	0.216
12 h	0.264	0.175	0.195
18 h	0.253	0.166	0.138
24 h	0.227	0.142	0.130
36 h	0.175	0.125	0.125
48 h	0.142	0.115	0.118
3 d	0.133	0.106	0.113
4 d	0.112	0.096	0.100
7 D	0.073	0.077	0.088
14 d	0.094	0.062	0.046
21 d	0.049	0.046	0.038
Control	ND	ND	ND

ND* - Not detected.

the drug and delivery method was better than earlier reports where the survival was only 56% (Swastika and Niezha, 2016). The histological changes noticed in positive control were similar to those found in LD₅₀ studies. There were minimal changes compared to control tissues in the samples collected from medicated fish (90 mg/kg) (Fig 10 and 11). Two-way ANOVA revealed that there is no significant difference ($p > 0.05$) in treated groups.

Pharmacokinetics of OTC

No mortality was recorded during the experiment and water quality parameters were within normal culture conditions with the temperature at $30 \pm 2^\circ\text{C}$, dissolved oxygen $> 6 \text{ mg/L}$ and pH 7.38 ± 0.03 . In control fish tissues, OTC was not detected. Concentration of OTC in different tissue samples is given in Table 3. In liver, OTC concentration was under the maximum residue level (MRL) value on 21 day. In kidney on 21 day, OTC was under MRL and in muscle OTC was under MRL on 14th day. According to food and drug administration, the maximum residual level of antibiotics in fish before human consumption is $0.05 \mu\text{g/g}$. According to Aquatic Animal Drug Approval Partnership (FDA 2001), the withdrawal time of oxytetracycline dihydrate for disease control in salmonids and catfish is 21 days. Reports from the temperate countries on the withdrawal time of OTC from tissues indicate wide variations in the time required for the antibiotic to fall below MRL depending on the fish species and the temperature of rearing. Withdrawal time of OTC was 21 days in *Cyprinus carpio* at a water temperature 20°C (Grondel *et al.* 1987); 60 days in rainbow trout above 12°C (Jacobsen, 1989) and 25 days in grass carp at $21 \pm 1^\circ\text{C}$ after 7 days oral administration of OTC at 100 mg/kg per day (Zhang and Li, 2007). Following a single dose of 100 mg/kg by oral administration, the OTC concentrations in muscle of perch and black seabream was reported below $0.05 \mu\text{g/g}$ after 30 days at $17.3 \pm 0.8^\circ\text{C}$ (Wang *et al.* 2004). In the present study, OTC concentrations in the kidney and

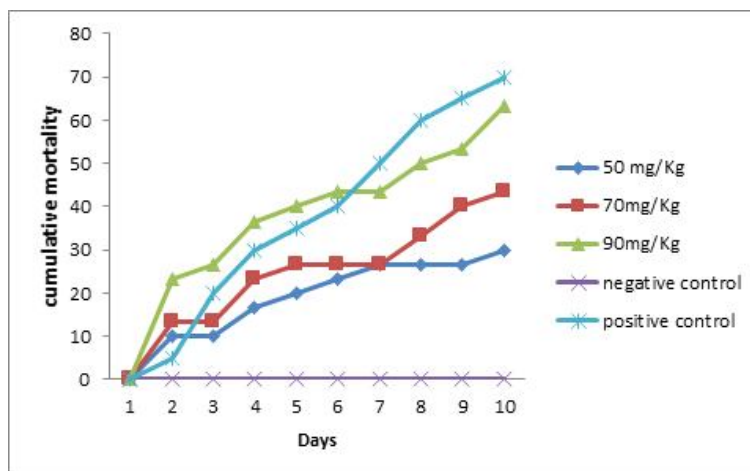


Fig 8: Cumulative mortality of fish observed in treatment with oxytetracycline by intraperitoneal injection.

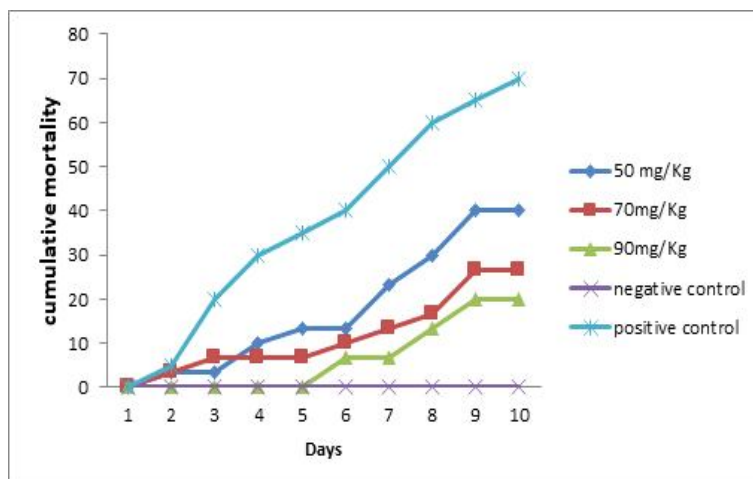


Fig 9: Cumulative mortality of fish observed in treatment with OTC through medicated feed.

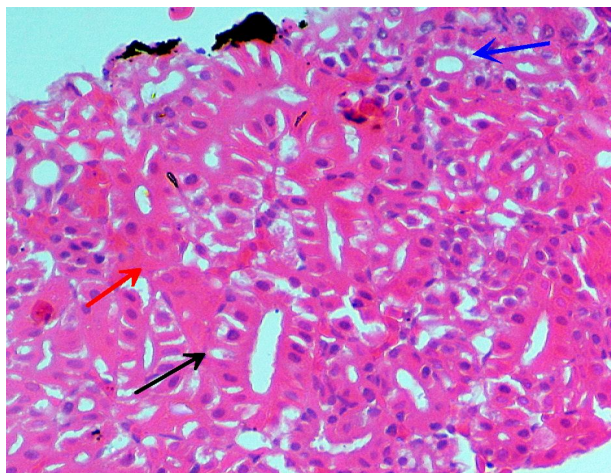


Fig 10: Kidney of fish (600 X) fed on 90 mg/kg OTC treated medicated feed with normal renal tubules (black arrow) and hemopoietic tissue (red arrow).

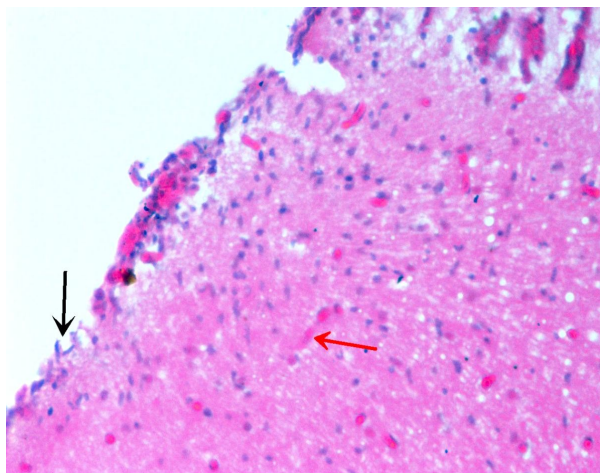


Fig 11: Brain of fish (60X) fed on 90 mg/kg OTC treated medicated feed with thin meninges (black arrow) and brain microvessels (red arrow).

liver fell below the stipulated MRL of 0.05 µg/g in 21 days and in muscle it fell below the MRL in 14 days at 30 ± 2°C after oral administration of OTC at the rate of 100 mg/kg for 7 days. In earlier studies conducted in *Oreochromis niloticus* with OTC administered at 40, 80, 120 mg/kg at water temperature 29.17°C, the drug was eliminated from the tissues after 21 days (Soltan *et al.* 2013).

CONCLUSION

Thus, the current study indicated that the *S. agalactiae* infection in GIFT can be controlled by the administration of OTC at 70 mg/kg applied through feed and the fish fillets would be ready for consumption after 14 days of the last dose at the tropical water temperatures of about 30°C. Though the application of the antibiotics in aquaculture is restricted widely, controlled and cautious application of the safe quantity of the antibiotic orally could be taken up for controlling the *S. agalactiae* infection in tilapia (GIFT).

ACKNOWLEDGEMENT

Funding support for the National Surveillance Programme for Aquatic Animal Diseases (sub project no - 21) by NFDB-ICAR-NBFGR, Lucknow under which the investigation was carried out is gratefully acknowledged.

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