

Original Research Article

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Evaluation of Efficacy of Oxytetracycline Oral and Bath Therapies in Nile Tilapia, *Oreochromis niloticus* against *Aeromonas hydrophila* Infection

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ABSTRACT

Aeromonas hydrophila is a potent pathogen that causes mortalities in tilapia. The present study evaluated the effectiveness of oxytetracycline (OTC) oral and bath therapies on Nile tilapia *Oreochromis niloticus* against *A. hydrophila* infection. Medicated feed was prepared by admixing oxytetracycline dihydrate powder and fed to fish at 60 mg OTC kg/biomass/day for 10 days. Bath therapy was done by immersing the fries in OTC suspensions (20 mg/l) for 1 h for 4 days. *Aeromonas hydrophila* was moderately virulent, causing 20-55% mortalities at 2×10^6 - 2×10^7 cfu/ml level by abrasion-bath treatment. The LD₅₀ value for juvenile tilapia was 6×10^7 cfu/fish. Efficacy of OTC was evaluated by abrading the tilapia followed by immersion in *A. hydrophila* suspension containing 2×10^7 - 5×10^7 cfu/ml for an hour. After 3 days of disease progression, the fish were given OTC feed and/or bath treatment. The oral and bath therapies in fries with OTC recorded low mortalities (17 and 21%) than the respective control (37 and 24%). The juveniles appeared to be susceptible to *A. hydrophila* than the fries. It recorded 66.7% mortality in OTC fed group than in control (90%). The oral therapy was better than the bath therapy against *A. hydrophila* infection. Since OTC is one of the approved antibiotics for treating bacterial diseases of fish and effective in controlling *A. hydrophila*, judicious use of OTC feed is recommended for the control of *Aeromonas* infection in tilapia.

Keywords

Oreochromis niloticus,
Aeromonas hydrophila,
Oxytetracycline,
Medicated feed,
Bath treatment.

Article Info

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Introduction

Tilapia is one of the most important cultured fish and currently ranks second after carps in global production (FAO, 2012).

Tilapias have long been considered as an ideal species for use in aquaculture due to its hardy nature, fast growth, tolerance of suboptimal water quality and disease resistance (Little *et al.*, 2008). Nile tilapia *Oreochromis niloticus* considered as the most important farmed tilapia species, representing >73% of

total tilapia production in 2010 (FAO, 2012). Diseases are one of the major problems that affect the tilapia production. Bacterial diseases have become major barriers to aquaculture, especially when water temperature is warm. *Aeromonas hydrophila* is an opportunistic as well as primary pathogen of variety of aquatic and terrestrial animals including man. The presence of *A. hydrophila*, by itself, is not indicative of disease and, consequently, stress is often

considered to be a contributing factor in the outbreak of disease caused by this bacterium (Goharrizi *et al.*, 2015). *Aeromonas* septicemia was reported in tilapia culture (Noga, 2010; Austin and Austin, 2012; Noor El-Deen *et al.*, 2014). Elevated water temperature, decreased dissolved oxygen concentration, or increased ammonia and carbon-dioxide concentrations have been shown to promote stress in fish and trigger motile aero monads infection (Noga, 2010; Austin and Austin, 2012)

A wide variety of aquadrugs are used to control the diseases caused by bacteria, fungi, parasites and viruses (Hernandez, 2005; Noga, 2010; Austin and Austin, 2012). Oxytetracycline (OTC) is one of the USFDA (United States Food and Drug Administration) approved chemotherapeutics for treating specific bacterial diseases in temperate and warm water finfish (FDA-CVM, 2007). It is particularly effective against Gram-negative bacteria such as *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. (Austin and Austin, 2012). Reports on the efficacy of OTC to control specific bacterial diseases in temperate condition are available (Schmidt *et al.*, 2007; Austin and Austin, 2012; Romero *et al.*, 2012).

But, its efficacy on cultured fish under the tropical Indian condition is poorly documented. Often OTC is used in Indian aquaculture (Hatha *et al.*, 2005; Bharathkumar and Abraham, 2011) without veterinary prescriptions. The effectiveness and safety of a drug before approval requires a scientific evaluation by USFDA in USA. Also, the USEPA (United States Environmental Protection Agency) requires a scientific evaluation of a chemical's safety before it can be registered and sold (FDA-CVM, 2007). There appears to be no regulation on the use of OTC in Indian aquaculture. Also the efficacy and safety levels of the FDA approved antibiotics

including OTC on fish cultured in India are not established. This study was, therefore, aimed at to evaluate the effectiveness of OTC feed and bath treatments on *O. niloticus* against *A. hydrophila* infection.

Materials and Methods

Bacterial strain and experimental fish

The β -haemolytic bacterial strain *Aeromonas hydrophila* BBT₄K₃, isolated from the kidney of haemorrhagic septicemic Nile tilapia *Oreochromis niloticus*, was from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences (FFSc), West Bengal University of Animal and Fishery Sciences, Kolkata, India. The identity of this strain was confirmed phenotypically by Vitek 2 compact system (bioMérieux, France). Healthy monosex (all male) *O. niloticus* fries (weight: 1-2 g; length: 2.3-3.0 cm) and juveniles (weight: 8.2-18.0 g; length: 8.2-11.5 cm) were brought from Naihati, North 24 Parganas district, West Bengal, India in oxygen filled polythene bags to FFSc, Kolkata as and when required. The fish were acclimatized for 3 h followed by disinfection with 5 ppm potassium permanganate for 15 min. The fries and juveniles were stocked in 500 litre capacity fiberglass reinforced plastic (FRP) tanks respectively at 200 and 75 numbers/tank containing 400 litre clean bore well water. The fish were acclimatized for about two weeks and fed ad-labium with commercial pellet basal feed (CP Pvt. Ltd., Andhra Pradesh, India) at 2% body weight (bw).

Pathogenicity of *Aeromonas hydrophila* BBT₄K₃

Preparation of bacterial cell suspension

Aeromonas hydrophila, maintained on TSA slant, was streaked onto TSA plate and then incubated at 35°C for 24 h to get young

culture. One young discrete colony of this strain was aseptically picked, transferred to 10 ml TSB and incubated at 35°C for 24 h. This 24 h old culture was then transferred to 300 ml TSB and reincubated at 35°C for 30 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 25°C in a cooling centrifuge.

The cell pellets were washed thrice by centrifugation with sterile physiological saline (0.85% NaCl) and finally resuspended in 10 ml saline, and used immediately.

A portion of the cell suspension was suitably diluted up to 10⁻⁹ in sterile saline and the number of cells/ml of suspension was determined by spread plating on TSA after incubation at 35°C for 24 h (Collins *et al.*, 2004).

Pathogenicity on *Oreochromis niloticus* fries by abrasion-bath treatment

The healthy *O.niloticus* fries were selected and released into the glass aquaria (n=8) of size L60 x H30 x W30 cm @ 10 fish/aquarium and acclimatized for 5 days. All the aquaria were filled with 30L clean bore-well water and covered with nylon netting for adequate protection. Prior to challenge, the fish were anesthetized with clove oil (50 µl/l water). The scales of fish from aquaria 1-6 were scrapped off gently with a scalpel from caudal peduncle to the pectoral fin, *i.e.*, in the opposite direction (abraded) as described in Adikesavalu *et al.*, 2015). The abraded fish from aquaria 1-2 and 3-4 were then immersed in 1000ml suspension containing *A. hydrophila* at 2×10⁷cfu/ml and 2×10⁶cfu/ml for 1 h, respectively. The abraded fish of aquaria 5-6 were dipped in 0.85% saline instead of bacterial suspension for 1 h and served as positive control. The fish of aquaria 7-8 were neither abraded nor challenged and served as negative control.

Pathogenicity on *Oreochromis niloticus* juveniles by intraperitoneal injection

Pathogenicity of *A. hydrophila* BBT₄K₃ on *O. niloticus* juveniles (weight: 15.3±2.1 g; length: 9.2±1.1 cm) was tested by intraperitoneal injection method. Twelve numbers of glass aquaria (Size: L60 ×H30 ×W30 cm) were selected, disinfected, cleaned, filled with 30 litre clean bore-well water after 7 days of drying, and conditioned for 3 days. All the tanks were stocked with 10 fish each and covered with nylon netting for adequate protection. After 3 days of acclimatization, aliquots (0.1 ml) of *A. hydrophila* cell suspensions from 10⁰ to 10⁻⁴ dilutions were injected intraperitoneally, *i.e.*, in the abdomen at a 45° angle between the pelvic fins and anal vent, in such a way so as to get 10⁸-10⁴cfu/fish. Control fish received 0.1 ml of sterile saline.

The challenged fish were maintained in their respective tanks for 28 days and fed daily with commercial pellet basal feed on demand. Observations on mortality, external signs of infections and behavioural changes were recorded daily. The lethal dose at which 50% of the experimental populations die (LD₅₀) was calculated by standard method (Reed and Muench, 1938).

Antibiogram of *Aeromonas hydrophila* BBT₄K₃

Sensitivity of *A. hydrophila* against 12 antibiotics namely amoxyclav (30 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), clindamycin (2 µg/disc), cotrimoxazole (25 µg/disc), erythromycin (15 µg/disc), gatifloxacin (5 µg/disc), gentamicin (10 µg/disc), nitrofurantoin (300 µg/disc), oxytetracycline (30 µg/disc), sulphafurazole (300 µg/disc) and vancomycin (30 µg/disc) was tested by agar disc diffusion technique (CLSI, 2006a) on Mueller Hinton agar (MHA) at 35°C. Interpretation of sensitivity

was based on the zone size interpretation chart provided by the manufacturer of the discs (HiMedia, India).

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of OTC by broth dilution and tetracycline Ezy MIC™ strip

The MIC of OTC was determined against *A. hydrophila* BBT₄K₃ by broth dilution (CLSI, 2006b) and Tetracycline Ezy MIC™ strip (HiMedia, India) methods (CLSI, 2012).

Broth dilution method

The OTC dihydrate powder (HiMedia, India) was initially dissolved in 0.85% saline at concentrations 1000 times higher than the most concentrated level to be tested. Various concentrations (µg/ml) tried for MIC determination was 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.1 and 0.

The MHB with or without antibiotics at varying concentrations were inoculated with 2 µl (~10⁴ – 10⁵ cells) from 10-12 h old culture grown in MHB at 35⁰C. All the tubes were incubated at 35⁰C for 24 h and observed for turbidity by comparing them with the control tube, *i.e.*, growth or no growth.

The MIC was determined as the minimum concentration (µg/ml) showing no growth or turbidity at 24 h. To determine the MBC, the tubes that showed no growth or turbidity at 24 h were taken.

The inocula from these tubes were streaked onto MHA plates and incubated at 35⁰C for 24 h. The MBC was determined as the minimum concentration (µg/ml) showing no growth at 35⁰C when incubated overnight (CLSI, 2006b).

Tetracycline Ezy MIC™ strip method

The pure culture of *A. hydrophila* was inoculated into MHB and incubated at 35⁰C for 6 h. Inoculum from MHB was spread onto MHA plate using a sterile cotton swab. After 15 min, a single Tetracycline Ezy MIC™ strip was placed onto the seeded agar plate at a desired position. The plate was incubated at 35⁰C for 18-24 h and observed for the zone of inhibition in the form of an ellipse. If the ellipse intersects the strip in between two dilutions, the MIC is read as the value which is nearest to the intersection. When the growth occurred along the entire strip, the MIC value is greater than the highest values on the strip. When the inhibition ellipse is below the strip, the MIC value is less than the lowest value on the strip (CLSI, 2012).

Evaluation of efficacy of OTC therapy

Abrasion, bacterial challenge and OTC oral therapy in tilapia fries

As per FDA, the approved dose of OTC is 2.50-3.75 g/100 pounds body weight (bw)/day (or 55-83 mg/kg biomass/day) for 10 consecutive days (FDA-CVM, 2007). The OTC feed was prepared by admixing required quantity of OTC dihydrate powder (HiMedia, India) in 1 kg of basal feed with 5 ml of vegetable oil to get a desirable concentration of 60 mg/kg biomass/day (FDA-CVM, 2007) when fed at 3% bw. The top dressed feed was mixed thoroughly, dried under the fan for 24 h and then placed in a clean and dry container with a tight-fitting lid. The control feed was prepared as above without OTC. The experiment was carried out in plastic tanks of size (L58 cm × H45 cm × B45 cm) with mono sex *O. niloticus* fries (weight: 1.1±0.1 g; length: 3.0±0.3 cm). The fish were stocked @ 40 fish/tank and fed with basal feed at 3% bw. About 50% of the water was exchanged and waste feed and faecal materials were removed

daily. The water quality parameters were maintained optimally (Water temperature: 18.0-23.5°C; pH: 7.7-8.7; dissolved oxygen: 3.5-5.1ppm and nitrate and ammonia: <0.02 ppm) throughout the experiment. The experimental fish were divided into 4 groups, namely group 1: non-abraded and unchallenged (negative control), group 2: abraded, unchallenged and control feed fed (positive control), group 3: abraded, challenged and control feed fed, and group 4: abraded, challenged and OTC feed fed. After acclimatization for 5 days, the fish from groups 2, 3 and 4 were abraded as described earlier (Adikesavalu *et al.*, 2015). The abraded fish from groups 3 and 4 were then dipped in 1000 ml solution containing *A. hydrophila* at 5×10^7 cfu/ml for 1 h. The abraded fish of group 2 were dipped in 0.85% saline instead of bacterial suspension for 1 h and served as positive control. All the fish after the dip in bacterial cell suspension/saline were transferred to the respective tanks. The fish of groups 1-3 were fed with control feed at 3% bw throughout the experimental period twice daily. The fish of group 4 were fed with control feed during the pre-treatment (1-5 days) and disease progression (6-8 days) periods. During the treatment period for 10 days (9-18 days), they were fed with OTC feed at 3% bw twice daily, equivalent to 60 mg/kg biomass/day. During the post-treatment period for 21 days (19-39 days), the fish were fed with control feed. Observations on mortality, external signs of infections and behavioural changes were recorded daily.

Abrasion, bacterial challenge and OTC bath therapy in tilapia fries

The FDA-approved dose of OTC bath treatment (immersion therapy) is 20 mg/l of water for 1 h for 4 days to control the mortality caused by bacterial diseases (WGADCB, 2011). The experiment was carried out in plastic tanks of size (L58 × H45

× B45 cm) with mono sex *O. niloticus* fries (weight: 1.1 ± 0.1 g; length: 3.0 ± 0.3 cm). The fish were stocked @ 35 fish/tank and fed with basal feed at 3% bw. About 50% of the water was exchanged and waste feed and faecal materials were removed daily. The water quality parameters were maintained optimally throughout the experiment. The grouping of fish and treatment protocols were as described above, except the mode of OTC treatment. The abraded fish from groups 3 and 4 were then dipped in 1000 ml solution containing *A. hydrophila* at 2.0×10^7 cfu/ml for 1 h, and transferred to the respective tanks. Three days post-abrasion and challenge, the fish of group 4 (abraded and challenged) were given an OTC bath as below. In brief, the OTC bath suspension was prepared by dissolving appropriate quantities of OTC hydrochloride powder (Terramycin® 500 mg, Pfizer, Bangalore). The abraded fish of group 4 were transferred to the treatment troughs with 3 litre water containing 20 mg OTC/l and allowed to remain in the troughs for 1 h. After the bath treatment, the fish were transferred to the respective tanks. This treatment was continued for 4 consecutive days. The fish were fed with basal feed twice daily at 3% bw during the entire study period (1-33days). Observations on mortality, external signs of infections and behavioural changes were recorded daily.

Abrasion, bacterial challenge and OTC oral therapy in tilapia juveniles

The experiment was carried out in plastic tanks of size (L58 × H45 × B45 cm) with *O. niloticus* juveniles (weight: 7.8 ± 0.5 g; length: 8.2 ± 0.4 cm). The experimental protocol, grouping of fish and treatment protocols were the same as that of fries. The concentration of *A. hydrophila* used for the challenge was 2×10^7 cfu/ml for 1 h. The freshly dead tilapia fries and juveniles of pathogenicity and efficacy of OTC therapy

trials were subjected to necropsy and bacteriology. Inocula from the haemorrhagic area and kidney of freshly dead fish were streaked onto Rimler-Shotts agar (RSA) plates and incubated at 35⁰C for 24-48 h (Austin and Austin, 2012).

Relative percent survival (RPS)

The efficacy of OTC was evaluated by calculating the relative percent survival (RPS) for OTC feed and bath treatments as per (Amend, 1981)

$$RPS = 1 \left[\frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}} \right] \times 100$$

Statistical analysis

The results of each experiment are expressed as mean±standard deviation and analyzed by one-way analysis of variance (ANOVA) using Microsoft excel version 2010 to test the significance of difference between the control and experimental groups. Comparison of mean values was done by Duncan's Multiple Range Test (Duncan, 1955). Probability level of 0.05 was used to find out the significance in all cases.

Results and Discussion

Phenotypic characterization and antibiotic sensitivity of *Aeromonas hydrophila* BBT₄K₃

The Vitek 2 compact system identified the bacterial strain as *A. hydrophila* on the basis of phenotypic characterization (Table 1). This strain was resistant to amoxyclav, clindamycin, erythromycin and vancomycin, and sensitive to chloramphenicol, ciprofloxacin, co-trimoxazole, gatifloxacin, gentamicin, nitrofurantoin, oxytetracycline and sulphafurazole (Table 2).

MIC and MBC of OTC against *Aeromonas hydrophila* BBT₄K₃

The MIC and MBC of OTC against *A. hydrophila* by classical broth dilution method were 12.5 µg/ml and 25.0 µg/ml, respectively. The MIC of OTC by Tetracycline Ezy MICTM strip method was 8 µg/ml (Table 3).

Pathogenicity of *Aeromonas hydrophila* BBT₄K₃ on tilapiafries and juveniles

The abraded and challenged monosex *O. niloticus* fries were weak and lying at the tank bottom. They showed abnormal movement, loss of scales at the abraded area, skin peeling with haemorrhagic lesion, pale gills, haemorrhages in the opercular region, fin rot, tail rot, reddening and darkening of the body colour. *Aeromonas hydrophila* BBT₄K₃ caused 55±7% and 20% mortalities in *O. niloticus* fries at 2×10⁷ cfu/ml and 2×10⁶ cfu/ml, respectively (Fig. 1). The LD₅₀ value was close to 2×10⁷cfu/ml for fries. Five percent mortality was noticed in abraded control group. The abraded fish were lethargic initially and recovered within 7 days, except the wound healing.

In challenged *O. niloticus* juveniles, lethargy, abnormal behaviour, wandering around the corners, resting at the bottom and vertical swimming were observed. Cent percent mortality was noted within 12 h of injection at 1.2×10⁸ cells of *A. hydrophila*/fish.

No mortalities were observed in stocks injected with 1.2×10⁷, 1.2×10⁶, 1.2×10⁵ and 1.2×10⁴ cfu/fish, but the fish were weak and lying at the bottom of the tank. Inflammation and haemorrhages were seen at the injected site. No mortalities were noticed in saline injected control group. The LD₅₀ value of *A. hydrophila* BBT₄K₃ was estimated to be 6x10⁷ cfu/fish.

Efficacy of OTC therapies

The mortalities recorded during the different periods of treatment regimes in abraded monosex *O. niloticus* fries challenged with *A. hydrophila*, and subsequently fed OTC feed for 10 days are presented in figure 2. On day 1 post-abrasion and bacterial challenge, 2.8-6.7% mortalities were noticed in fish. During the disease progression period, *i.e.*, on day 8, 8.6-11.4% mortalities were noticed in challenged groups. At the end of 10 day OTC feed treatment regime, *i.e.*, on day 18, the mortalities noticed in abraded, challenged and fed with control feed (group 3); abraded, challenged and fed with OTC feed (group 4), and positive control groups were 30.0, 14.2, and 2.8%, respectively. At the end of the experimental period (day 39), the highest mortality (36.7%) was observed in abraded, challenged and control feed fed fish. In OTC fed group, the observed mortality was 17.1%. The RPS was found to be 53.3. Significant differences existed among all the treatment groups ($P<0.05$).

The results on the effect of OTC bath therapy are presented in figure 3. After the disease progression period, *i.e.*, on day 8, 18-19% mortalities were noticed challenged groups. On day 12, *i.e.*, at the end of OTC bath treatment regime, 23.8% and 20.9% mortalities were observed in untreated and OTC treated fish, respectively. No further mortalities were recorded during the post-treatment period (day 12-33). The RPS was 12. Significant differences existed among all the treatment groups ($P<0.05$).

The abraded and challenged *O. niloticus* juveniles were observed to be weak, hanging and/or lying at the bottom of the tank. They showed erratic movement, loss of scales, skin peeling with haemorrhagic lesion, pale gills, haemorrhages in the opercular region, reddening in the pectoral fin base region, tail

rot and darkened body colour. At the end of 10 day OTC treatment regime, 66.7% mortality was noticed compared 80% in control. The total mortalities at the end of experiment in control and OTC feed fed groups were 90 and 66.7%, respectively (Fig. 4). The RPS was 25.9. Significant differences existed among all the treatment groups ($P<0.05$).

The wound healing at the abraded area was faster in OTC treated groups compared to control. The mortalities recorded in OTC oral therapy (17.1%) were significantly lower than the bath therapy (23.8%) in tilapia fries. Samples from the affected haemorrhagic area as well as the kidney of freshly dead and moribund fish of the *A. hydrophila*BBT₄K₃ challenged fish yielded typical yellow colonies on RSA plates after 24 h of incubation at 35°C. The isolated bacteria were identified as *A. hydrophilaby* conventional biochemical tests. Internally, pale kidney, liquefaction, discoloration and haemorrhages of the internal organs were observed in the abraded and challenged fish stocks.

Aeromonas hydrophila has been consistently associated with diseases or multiple of infections among variety of food fish throughout the world (Noga, 2010; Austin and Austin, 2012). Antibiotics are the treatment of choice to control the bacterial diseases. Rey *et al.*, (2009) opined that determination of lethal dose of the bacterium before the experimental challenge is advantageous for successful experiment and induction of clinical signs and symptoms. Accordingly, in abrasion-bath treatment 20-55% mortalities in *A. hydrophila* challenged tilapia fries at 2×10^6 - 2×10^7 cfu/ml were recorded. The mortality rate in challenged tilapia varied in different trials possibly due the varying physiological status of the experimental fish. The results corroborate Schlotfeldt and Alderman (1995), who noted varying effects of *A. hydrophila*

infection on fish. According to the degree of virulence (Mittal *et al.*, 1980) and on the basis of lethal dose values (2×10^7 cfu/ml for fries and 6×10^7 cfu/fish for juveniles) the *A. hydrophila* BBT₄K₃ was found to be

moderately virulent to tilapia with an ability to cause systemic infection. Nevertheless, some strains of *A. hydrophila* clearly behave as primary pathogen (Noga, 2010; Austin and Austin, 2012).

Fig.1 Pathogenicity of *Aeromonas hydrophila* BBT₄K₃ on monosex *Oreochromis niloticus* fries by abrasion-bath treatment. Control: Abraded and unchallenged

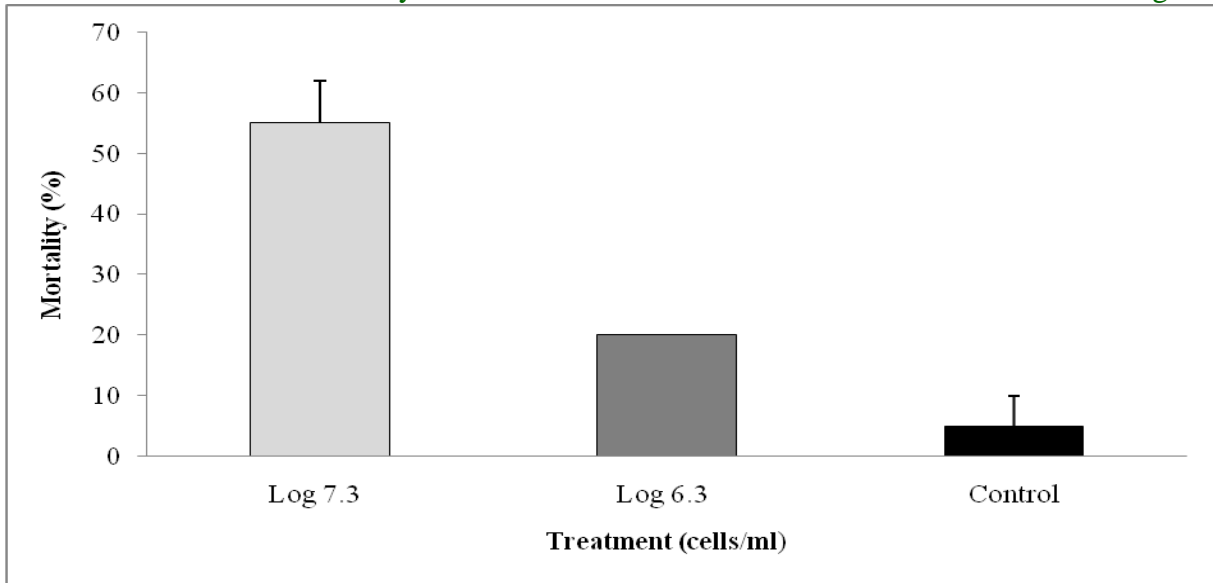


Fig.2 Effect of 10 days OTC feeding on the abraded (A) monosex *Oreochromis niloticus* fries challenged (C) with *Aeromonas hydrophila*. Note: No mortality was noted in negative control. Positive control: Abraded and unchallenged tilapia fries. *: Bars sharing asterisk (*) differ significantly ($P < 0.05$)

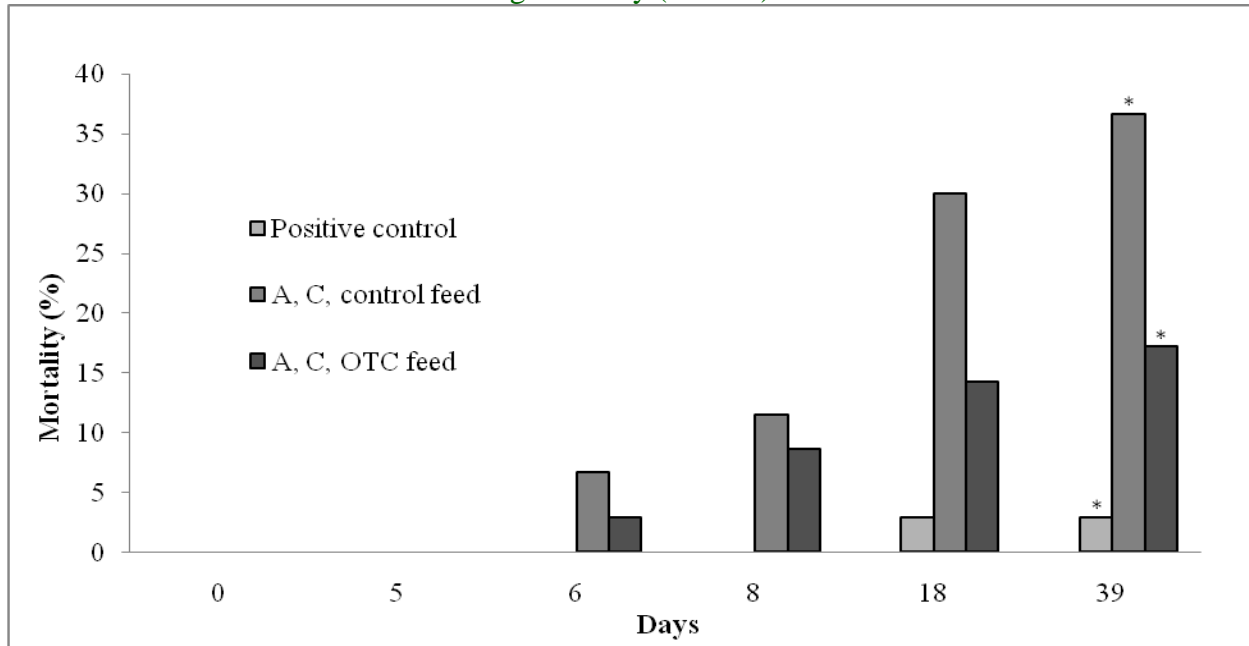


Fig.3 Effect of 4 days OTC (0 and 20 mg/l) bath treatment on the abraded (A) monosex *Oreochromis niloticus* fries challenged (C) with *Aeromonas hydrophila* BBT₄K₃. Note: No mortality was noted in negative control. Positive control: Abraded and unchallenged tilapia fries.
: Bars sharing asterisk () differ significantly ($P < 0.05$)

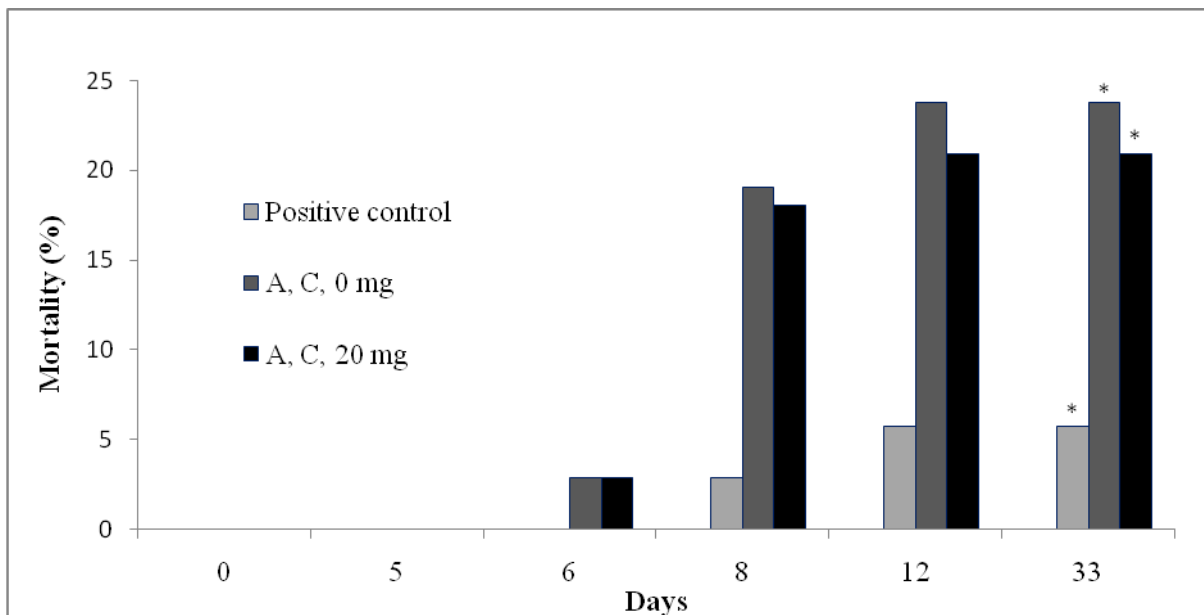


Fig.4 Effect of 10 days OTC feeding on the abraded (A) *Oreochromis niloticus* juveniles challenged (C) with *Aeromonas hydrophila* BBT₄K₃. Note: No mortality was noted in negative control. Positive control: Abraded and unchallenged tilapia juveniles. *: Bars sharing asterisk (*) differ significantly ($P < 0.05$)

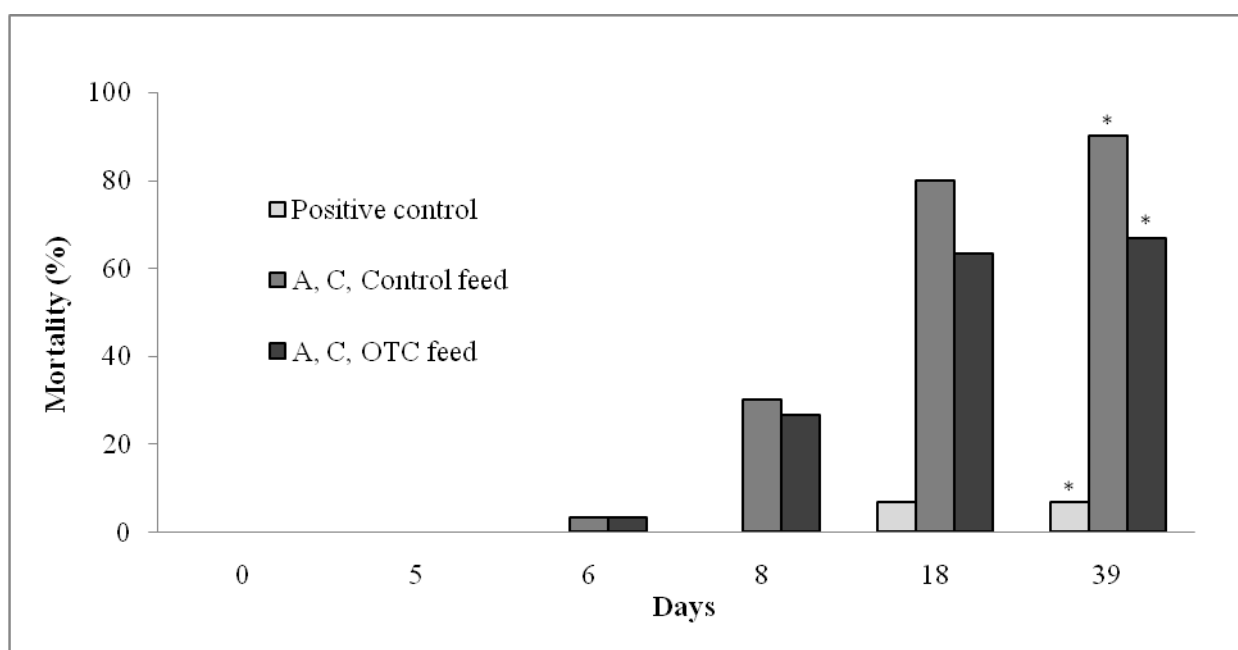


Table.1 Phenotypic characterization of fish pathogenic *Aeromonas hydrophila* BBT₄K₃ by Vitek 2 compact system

Phenotypic characteristics	<i>Aeromonas hydrophila</i> BBT ₄ K ₃
5-Keto D-gluconate (5KG)	-
Adonitol (ADO)	-
Ala-Phe-Pro-arylamidase (APPA)	-
Alpha-galactosidase (AGAL)	-
Alpha-glucosidase (AGLU)	-
Beta-alanine arylamidasepNA (BAlap)	-
Beta-galactosidase (BGAL)	+
Beta-glucuronidase (BGUR)	-
Beta-glucosidase (BGLU)	-
Beta-xylosidase (BXYL)	-
Citrate (sodium) (CIT)	-
Coumarate (CMT)	+
D-Cellobiose (dCEL)	-
D-Glucose (dGLU)	+
D-Maltose (dMAL)	+
D-Mannitol (dMAN)	+
D-Mannose (dMNE)	+
D-Sorbitol (dSOR)	-
D-Tagatose (dTAG)	-
D-Trehalose (dTRE)	+
Ellman (ELLM)	+
Fermentation/ glucose (OFF)	+
Gamma-glutamyltransferase (GGT)	-
Glu-Gly-Arg-arylamidase (GGAA)	+
GlutamylarylamidasepNA (AGLTp)	-
Glycine arylamidase (GlyA)	-
H ₂ S production (H ₂ S)	+
L Pyrrolydonyl-arylamidase (PyrA)	-
L-Arabitol (IARL)	-
L-Histidine assimilation (IHISa)	-
Lipase (LIP)	-
L-Lactate alkalisation (ILATk)	+
L-Lactate assimilation (ILATa)	-
L-Malate assimilation (IMLTa)	-
L-Prolinearylamidase (ProA)	+
Lysine decarboxylase (LDC)	-
Malonate (MNT)	-
O/129 Resistance (O129R)	+
Orinithine decarboxylase (ODC)	-
Palatinose (PLE)	+
Phosphatase (PHOS)	-
Saccharose/Sucrose (SAC)	+
Succinate alkalisation (SUCT)	+
Tyrosine arylamidase (TyrA)	+
Urease (URE)	-
β-N-acetyl-galactosaminidase (NAGA)	-
β-N-Acetyl-glucosaminidase (BNAG)	+

Table.2 Antibiotic sensitivity of fish pathogenic *Aeromonas hydrophila* BBT₄K₃

Antibiotics (µg/disc)	Zone of inhibition in mm	Antibiogram
Amoxyclav (30)	7	R
Chloramphenicol (30)	32	S
Ciprofloxacin (5)	26	S
Clindamycin (2)	7	R
Co-trimoxazole (25)	21	S
Erythromycin (15)	11	R
Gatifloxacin (5)	22	S
Gentamicin (10)	18	S
Nitrofurantoin (300)	21	S
Oxytetracycline (30)	18	S
Sulphafurazole (300)	28	S
Vancomycin (30)	9	R

Table.3 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of oxytetracycline against *Aeromonas hydrophila* BBT₄K₃

Methods	MIC (µg/ml)	MBC (µg/ml)
Broth dilution	12.50	25.00
Ezy MIC TM	8.00	-

The *in-vitro* drug susceptibility tests contribute to understand and evaluate the drug resistance of the pathogen, and help to take treatment decisions in controlling the disease. The β -haemolytic *A. hydrophila* strain isolated from the kidney of septicemic *O. niloticus* was sensitive to OTC and exhibited varying degrees of resistance to other antibiotics. The results of this study are in agreement with few earlier studies (Awan *et al.*, 2009; Stratev *et al.*, 2013) but contradictory to other observations (Dias *et al.*, 2012; Bharathkumar and Abraham, 2014). *Aeromonas* spp were, in general, sensitive to tetracycline, chloramphenicol, nitrofurantoin, amikacin, gentamicin, cefazolin, tobramycin, cephalixin, cefuroxime, ceftriaxone, cephalothin, cefoxitin, cefotaxime, colistinsulfate, ciprofloxacin and trimethoprim-sulfamethoxazole (Awan *et al.*, 2009). The MICs values of 12.5 µg/ml and 8 µg/ml recorded respectively by broth dilution and Ezy MICTM strip methods, and the MBC value of 25 µg/ml also advocated the susceptibility of *A. hydrophila* to OTC.

The variations in MIC values could be attributed to the use of solid and liquid media, which generate numerically different MIC values (CLSI, 2006a, b). The MIC for OTC has been recorded for several *A. hydrophila* and other motile aeromonads around the world. For example, *Aeromonas* strains exhibited MICs in the range of >0.06-256 mg OTC/ml (Guzand Kozinska, 2004). Also *Aeromonas* strains with high MIC values for OTC ranging from 50 µg/ml (Neowajh *et al.*, 2015) to 600 µg/ml (Singh *et al.*, 2009) have been reported. Conceivably, the high MIC values for OTC may cause problems for chemotherapy in the future.

The present study aimed at the control *A. hydrophila* infection at the recommended dosage of OTC by appropriate means (FDA-CVM, 2007; WGADCB, 2011). It was observed that the mortalities due to *A. hydrophila* challenge were more during the initial 3-5 days of infection in fries, which reduced drastically in the later phase. These findings corroborate the observations made in

tilapia hybrids (Rey *et al.*, 2009), where changes in liver, kidney, spleen, intestine, muscle, stomach and brain were noticed up to 3 day post-infection to *A. hydrophila*. At the end of 7 days post-challenge the bacteria got eliminated from the major tissues. Similar results were also noted in *A. hydrophila* challenged *Labeorohita* (Das *et al.*, 2014). During the OTC therapy, it was observed that the recovery rate of the experimentally infected fish varied with OTC dose and the method of application. In OTC feed trial, 14.2% mortalities were observed during the 10 days treatment regime, despite the poor intake of medicated feed initially by the abraded and challenged fish. On the other hand, 20.9% mortalities were noticed during the 4 days treatment regime of OTC bath treated group. The observations on the significantly high mortalities during the post-treatment regime in bath (23.8%) therapy (20 mg OTC/l) than in feed (17.1%) therapy (60 mg OTC/kg biomass/day) suggested that the treatment of fish with OTC feed is better than the bath treatment. The results were also justified from the calculations of RPS, which yielded the highest RPS (53.2) for oral therapy and the lowest (12.0) for bath therapy.

In the present study the *O. niloticus* juveniles were found to be susceptible to *A. hydrophila* infection than the fries. The abraded *O. niloticus* fries experienced significantly low mortalities (37%) and high RPS (53.2) compared to abraded juveniles (90%) and low RPS (25.9) when challenged with *A. hydrophila*. It has been demonstrated that the effects of *A. hydrophila* infection in fish can vary according to the resistance to infection (Schlotfeldt and Alderman, 1995) and with the size of the animal (Oliveira *et al.*, 2011). The present results corroborate the observations of Haque *et al.*, (2014), who demonstrated the effectiveness of 2 g OTC/kg diet in reducing the bacterial load of fish under artificial culture condition.

The main advantage of oral therapy is that it does not stress the fish. Nevertheless, Floyd (1993) documented that bath treatment was effective in controlling only the external infection such as fin rot, bacterial gill disease, superficial fungal infections and ectoparasitic infestations in fish. Bath treatment was reportedly not as effective as feed or other treatment methods, particularly for systemic infections, because of generally poor internal absorption of the antibiotic being used (Sekkinand Kum, 2011). The dosage required for treatment with a medicated feed depends on the original level of active ingredient/kg fish bw. It is obvious that the majority of the infected fish do not eat. Also the fish do not effectively metabolize antibiotics and will pass them largely unused back into the environment in the faeces. It has been estimated that 75% of the antibiotics fed to fish are then put into the water through excretion (Burrige *et al.*, 2010; Romero *et al.*, 2012). Hence, bath therapy should be considered only when the majority of the fish are not eating and fish should be switched to oral medication as soon as they resume eating.

In India antibiotic sales and usage are not regulated except the recent guidelines on the registration of antibiotic free aquadrugs by the Coastal Aquaculture Authority of India (<http://www.caa.gov.in/>). Till date, no veterinary prescriptions are required in India for the use of antibiotics in aquatic animals. The safety and efficacy of FDA approved antibiotics including OTC are not available or poorly understood on fish cultured in India. The present study demonstrated that OTC was effective in controlling the *A. hydrophila* infection in tilapia through oral and bath therapies under Indian conditions, with OTC oral therapy being the most effective. Since OTC is one of the FDA approved antibiotics for treating bacterial diseases of temperate and warm water finfish, the responsible use of

OTC is, therefore, recommended for the control of *Aeromonas* infection in tilapia. The results of the present study would provide some baseline data on the efficacy of OTC to the policy makers and regulatory authorities of Indian aquaculture.

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Declaration of Interest

The authors declare that there is no conflict of interest.

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