Potentiality of Selected Commercial Antibiotics Challenged with *Aeromonas* sp.

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Abstract This study was intended to address, the effect of oxytetracycline, erythromycin, and streptomycin on the antimicrobial resistance of *Aeromonas* sp. isolated during the production process, from infected common carp (*Cyprinus carpio*). Antimicrobial resistance patterns were defined by determination of minimal inhibitory concentrations (MIC) of three antimicrobial drugs, and bacteria. Results indicated that *In vitro* bactericidal activity, oxytetracycline showed lowest number of bacterial colonies (1×10^{-4} c.f.u./ml) than erythromycin (3×10^{-4}) and streptomycin (4.5×10^{-4}) compared to the control (9×10^{-4}). Oxytetracycline showed the highest maximum zone of inhibition (20 mm) than erythromycin (18 mm) and streptomycin (15 mm) in antibiotic susceptibility test. In infection inhibition assay, serum from oxytetracycline fed diets inhibited *Aeromonas* infection to common carp. The susceptibility of the fish fed antibiotics (50 µg/mg feed) and challenge with *Aeromonas* sp. (1×10^{-4}) was examined for 30 days. There was high relative percent of survivability (RPS) in oxytetracycline was 73%. Very low RPS 63% and 47.37% was found in erythromycin and streptomycin against *Aeromonas* sp. infection. These results demonstrated that the oxytetracycline might be a potential for *Aeromonas* sp. infection to common carp (*Cyprinus carpio*).

Keywords *Aeromonas* sp. Potentiality, Antibiotics, Antimicrobial Resistance, Minimal Inhibitory Concentrations (MIC) and Disease Resistance

1. Introduction

Fish production through aquaculture is increasing day by day. But the total production is still behind the demand. Disease is one of the major limiting factors to obtain the production target. Among various diseases bacterial disease in fish is a serious threat to aquaculture system in Bangladesh. Various types of disease such as ulcer type disease including epizootic ulcerative syndrome, bacterial hemorrhagic

septicemia, tail and fin rot, bacterial gill rot, dropsy, columnaris disease, fungal disease and parasitic disease are important limiting factors for sustainable fish production [1]. Infections caused by motile aeromonads are probably the most common bacterial disease of freshwater fish [2]. Resistance of *Aeromonas* sp. to commonly used antibiotics is an emerging problem in the farmed fish. Therefore, the present work attempted to evaluate the potentiality of antibiotics to inhibition assay, bactericidal activity and susceptibility against *Aeromonas* sp. infection.

2. Materials and Methods

2.1. Fish and Management

Common carp, *Cyprinus carpio* (weight 270.7 ± 10.4 g, N= 100) was obtained from fish farm located at Chachra, Jessore and were transported to the laboratory of Fisheries and Marine Bioscience (FMB), Jessore University of Science and Technology (JUST), on July to December 2013. The fish were immediately examined by their movement, eye condition and external lesion to find out their health status and acclimatized in the indoor aquarium (100 L) with recirculating aerated water. Continuous aeration was provided to maintain dissolved oxygen level at 7.5 ± 0.5 mg/l⁻¹ and one-third of the aquarium water was exchanged daily and by siphoning the waste materials was removed. During the experimental period water temperature, pH and TDS (total dissolved solid) were $22 \pm 0.8^\circ\text{C}$, 5.94 ± 0.21 and 434 ± 0.29 mg/l⁻¹ respectively. Fishes were provided with normal feed (without herbal extract) at the rate of 5% of their body weight twice a day at 09:00 and 16:00 h for 3 days but at the first day of their arrival no feed was provided.

2.2. *Aeromonas* *Hydrophila* Isolation

A. hydrophila isolated from diseased common carp (*Cyprinus carpio*), was used in FMB laboratory for the study. Stocks were grown in brain heart infusion (BHI,

Hi-media, Indian) and nutrient broth for 24 hrs at 37 °C and then kept in -20°C until use. The subculture was taken and centrifuged (4000 rpm for 15 min), after centrifugation the supernatant was discarded and the pellet was resuspended in sterile phosphate buffer saline (PBS). The culture was adjusted at 3.5×10^{-7} colony forming units (CFU) ml⁻¹ by 10 time's serial dilution and incubated at 37°C for 24 hours. The bacterium was confirmed by some biochemical test (Table 1).

Table 1. Identifying characteristics of fish pathogenic strain *Aeromonas hydrophila*

Identifying characteristics	<i>Aeromonas hydrophila</i>
Colony	Yellowish
Morphology	Small rods
Gram strain	-
Catalase	+
Oxidase	+
Gelatin liquefaction	+
Indole production	+
OF test	F
Arabinose	+
Manitol	+
Sucrose	+
Inositol	+
Esculin hydrolysis	+
Voges-proskauer reaction	+
Ammonium production	-
Glucose	G

Note: + = positive reaction; - = negative reaction; O = oxidation; F = fermentation; G = gas.

2.2. Antibiotic Treatment Conditions

The study was carried out during a disease outbreak (caused by the bacteria *Aeromonas hydrophila*) occurring in a common carp, *Cyprinus carpio* lot. The fish were treated on decision of the veterinarian with antibiotics at 12 mg per kg of bodyweight per day during eight consecutive days. The trout were fed the antibiotics-supplemented food at a rate of 0.5% of body weight per day. At the onset of treatment, mean fish weight was approximately 270 g and water temperature was about 30 °C. This treatment was successful in curing the affected common carp lot. At the end of the study period, water temperature in the farm environment was about 25-30 °C.

2.3. Bleeding, Serum Separation and Bactericidal Activity Test

Blood from the randomly selected fish were drawn directly from the caudal vein with the help of a sterilized 1 ml hypodermal syringe containing EDTA (Ethylene-Diamine-Tetra-Acetic Acid) as an anticoagulant using 24 gauge needles. For serum separation blood was

collected without anticoagulant in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 4500 g for 10 min. The collected serum was stored in sterile serum tubes at -20 °C until used for assays. All the procedures were carried out in the sterilized condition. *A. hydrophila* was used as a model to examine the effectiveness of antibiotics to kill the bacterial infection. 100 µl of serum was added to 100 µl bacterial suspension and mixed before incubated for 1 hours at 37°C. A blank control was prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile 0.05 M sodium phosphate buffer, PBS (pH 6.2) at a ratio of 1:10. The serum-bacterial mixture (100 µl) was plated onto the nutrient agar plates with incubation for 24 hours at 37°C before the number of colonies was counted.

2.4. Antibiotic Susceptibility Test

Antibiotic susceptibility tests were carried out against *A. hydrophila* with three antibiotics by disc diffusion method [3]. Antibiotics disc of oxytetracycline (30µg), erythromycine (30µg) and streptomycin (30µg) were used for the study [4]. NA and BHI agar was poured into the plates and allowed to solidify. The standard inoculum (10^6 cfu/ml) was swabbed over the surface of the media using sterile cotton swab to ensure the confluent growth. The plates with incubation for 24 hours at 37°C before the number of colonies were counted. All the experiments were carried out in triplicate.

2.5. Agglutination Titer Assay

The test for agglutination titer was realized individually for bacterium *Aeromonas* sp. according to Yildirim, et al. 2003 [5]. The bacterial cell suspension was centrifuged at $300 \times g$ for 15 min and supernatant was discarded. The resulting pellets were washed twice with 0.85% phosphate buffer saline (PBS) solution and pellets were resuspended in PBS to an optical density of 0.8 at 540 nm. Starting with a dilution of 1:10 (10- µl serum and 90 µl PBS), two-fold serial serum dilutions were made in 96-well round bottom microtiter plates by adding 50 µl of diluted serum into the reaming wells plated with 50 µl of PBS. Thereafter, 50 µl of bacterial cell suspension was added to each well; thus, the initial serum dilution was 1:20. The plates were covered with plastic film and incubated at room temperature for 2 hours at 4°C for 24 hours. The agglutination end point was estimated as the last serum dilution where cell agglutination was visible after incubation. Agglutination titers were reported as log₁₀ of the reciprocal of the highest serum dilution showing visible agglutination as compared to the positive control.

2.6. Assessment of *Aeromonas* infection inhibition

Aeromonas infection inhibition assay was carried out as follows: *Aeromonas* cells were grown in 96-well plates at 37°C until the density reached to 1×10^6 cfu/ml. The cells were washed gently with PBS and then incubated for 4 hours

at 37°C with 100 µl serum from oxytetracycline fed diets against the target bacterium diluted at 1:50, 1:100 or 1:200 with maintenance medium. After incubation, the cells were washed twice with PBS and then infected with *Aeromonas* sp. at LD₅₀ at 37°C for 1 hour. After bacterial adsorption, the unattached bacteria were removed by washing with PBS and 100 µl maintenance media were added. The infection was allowed for 5 days at 37°C and the cytopathic effect (CPE) was monitored by photography microscope, serum (1:50 diluted) was used as negative control. The experiment was performed twice in duplicate.

2.7. Challenge Test

To evaluate the effects of antibiotics in the diet on fish disease resistance, *Aeromonas* sp. was used as the pathogenic agent. The susceptibility of the fish fed with different antibiotics and challenged with *Aeromonas* sp. (1×10^{-4} c.f.u./ml) was examined for 30 days. The relative percent survival (RPS) was calculated by following formula [6]:

$$RPS = 1 - \left(\frac{\text{percent mortality in treated group}}{\text{percent mortality in control group}} \right) \times 100$$

2.8. Statistical Analysis

All the data are expressed as mean±SE. Statistical analysis of data involved one way analysis of variance (ANOVA) followed by the comparison of means following Stat Professional Software and SPSS windows 15.0 version. The level of significance was expressed as p-value at 0.05 levels.

3. Results

3.1. Bactericidal Activity

Figure 1. Bactericidal activity of serum of common carp, *Cyprinus carpio* fed with different doses of antibiotics supplemented diets against *Aeromonas* sp.

The lowest number of bacterial colonies indicated the efficiency of antibiotics to kill the pathogen. With oxytetracycline the lowest number of colonies i.e. 1×10^{-4} on

media with the 30 µg/ml dose with highly significant differences to the control, 9×10^{-4} . The 30 µg/ml dose recorded a highest number of colonies; 3×10^{-4} and 4.5×10^{-4} for erythromycin and streptomycin respectively in Figure 1.

3.2. Antibiotic Susceptibility Test

In the drug susceptibility assays, *Aeromonas* sp. was susceptible against oxytetracycline (20 mm), erythromycin (18 mm) and streptomycin (15 mm). In the antibiotic susceptibility test, oxytetracycline exhibited maximum activity than that of erythromycin and streptomycin in Figure 2.

Figure 2. Antibiotic susceptibility test showed zone of inhibition of some antibiotics against *Aeromonas* sp.

3.5. Bacterial Agglutination Test

The agglutination antibody titers were quantified following the standard micro-agglutination procedure using serial two fold dilution of each antibiotic pool. Using highest antibiotic dilution showed visible agglutination as compared to the positive control. Negative agglutinations were found usually lowest antibiotic dilution.

3.6. Infection Inhibition Assay

Table 3. *Aeromonas* sp. infection inhibition assay using fish antiserum in vitro.

Serum	Sera dilution	Days post infection				
		1 day	2 nd day	3 rd day	4 th day	5 th day
Pre-serum	1:50	-	+	++	+++	+++
	1:50	-	-	-	-	+
Post-serum	1:100	-	-	-	+	++
	1:200	-	-	+	++	+++

Note: +++, 50%–75% cells showing CPE; ++, 25%–50% cells showing CPE; +, few cells showing CPE; –, No CPE.

To determine the role of bacterium during *Aeromonas* sp. at LD₅₀ infection, serum against the molecule were tested for the ability of inhibiting *Aeromonas* sp. infection. While *Aeromonas* sp. cells were incubated with serum and infected

with *Aeromonas* sp., less than 25% of cells showed obvious CPE at the second day after infection, and more than 50% CPE occurred at the fifth day (Table 3). By contrast, the CPE didn't appear until the fifth day in the presence of 1:50 diluted post-immune sera, but the CPE was gradually aggravated by pre-incubation with lower concentrations of post-immune sera at 1:100 or 1:200 dilutions (Table 3).

3.7. Challenge Test

The highest survivability was 75 % and 65% in fish fed with 50 µg/mg of oxytetracycline and erythromycin for 30 days against *Aeromonas* sp. The lowest survivability of 5% was observed in fish fed with 0% dose diet. The survivability decreased to 50% with 50 µg/mg of streptomycin dose diet (Fig. 3). The lowest survivability of 5% was noted in the control while 50% survivability was observed for streptomycin 50 µg/mg dose applications (Fig. 3). The highest RPS; 73% was found for oxytetracycline, 63% and 47.37% for erythromycin and streptomycin respectively.

Figure 3. The survivability of common carp fed with different doses of antibiotic supplemented diets against *Aeromonas* sp.

4. Discussion

The motile *Aeromonas* group is responsible for severe fish diseases, such as hemorrhagic septicemia [7,8,9] and ulcerative disease of fish [10]. The worldwide distribution of this group is a consequence of its great adaptability to different aquatic environments [11]. Infections by *Aeromonas* had been observed in various freshwater fish species and eventually in marine fish, reptile, bovine, and man [7,12]. MIC has been recorded for several *Aeromonas hydrophila* strains around the world [13,14,]. In the present study *Aeromonas* sp. was isolated from hemorrhagic septicemia and ulcerative disease affected common carp.

In the present study, Studied on pure cultures of *Aeromonas* sp. were isolated from the skin, kidney, heart, and liver of the common carp. The isolates were found to be sensitive to and oxytetracycline, erythromycin and streptomycin. Oxytetracycline showed the better result than the erythromycin and streptomycin. Pure cultures of *Aeromonas hydrophila* were isolated from the skin, kidney, heart, and liver of the gold fish. All of the isolates were found to be susceptible to oxytetracycline, neomycin, streptomycin and resistant to ampicillin, amphotericin, bacitracin,

cephalexin and moderately sensitive to Erythromycin, Kanamycin, [15] which support the present study. The isolates of *Aeromonas hydrophila* were tested for their susceptibility to florephekol, flumequine, rithromycin olaquinox and oxitetracycline [16], which support the present study. Antibiotic susceptibility assay with commercial antibiotics and oxytetracycline showed the better result than erythromycin and streptomycin [4], which supports the present study.

In the present study bacterial agglutination was performed and oxytetracycline showed the better result than the erythromycin and streptomycin at the same dose in 96 wells plate. In the present study assessment of MIC was also performed and result showed that, 50 µg/ml for oxytetracycline and 55 µg/ml, 60 µg/ml for erythromycin and streptomycin respectively. Miranda and Zemelman, (2002) studied on bacterial resistance to oxytetracycline in Chilean salmon farming by spread plate method and agar plate dilution method. They recommended that 30 to 100 µg/ml oxytetracycline is helpful for bacterial inhabitation [17], which supports the present study. Singh et al., (2009) isolated twelve isolates of oxytetracycline resistant bacteria were mainly dominated in adult fishes. Their selected strains exhibited high levels of oxytetracycline resistance with minimum inhibitory concentrations (MICs) ranging from 50 to 600µg/ml [18], which also support the present study. Guz and Kozinska, (2004), isolated twenty one *Aeromonas* pathogen from carp (*Cyprinus carpio*) and tested susceptibility to 22 antimicrobial agents by disc diffusion method. They resulted that, 100% were resistant to ampicillin and penicillin, and sensitive to trimethoprim-sulphamides, oxolinic acid, flumequine, chloramphenicol, norfloxacin, linkomycin, pefloxacin. Most isolates were resistant to cephalothin (57%) and erythromycin (52%) [19].

Haque et al., (2014) observed the effectiveness of antibiotic, oxytetracycline reducing the bacterial load in rohu fish under artificial culture condition in the laboratory. They used live fish method for experiment and resulted that 2 gm/kg oxytetracycline in diet, twice daily is potential antibiotic to reduce bacterial load in fish and can be used commercially for maintaining the fish health in aquarium condition [20], dose did not support the present study because fishes may be treated prior to study time and bacteria gained resistancy against minimum dose or dose may be varied species to species. In the present study challenge test was performed with oxytetracycline, erythromycin, streptomycin and control against *Aeromonas* sp. affected fish. Oxytetracycline treated fish showed highest RPS value than the other two antibiotics and resulted that antibiotics have the potentiality to cure the fishes, which support various workers in this field.

Aeromonas sp. is very common bacterial pathogen which affects fish species frequently and can cause severe disease. At the end of present study it can be concluded that, *Aeromonas* sp. was responsible for hemorrhagic septicemia and ulcerative disease of fish. The *Aeromonas* sp. was found to be sensitive to oxytetracycline, erythromycin and streptomycin.

5. Conclusions

In vitro bactericidal activity, oxytetracycline showed lowest number of bacterial colonies (1×10^{-4} c.f.u./ml) and highest maximum zone of inhibition (20 mm) in antibiotic susceptibility test. In infection inhibition assay, serum from oxytetracycline fed diets inhibited *Aeromonas* infection to common carp, which suggested that the serum from oxytetracycline fed diets played an important role in the infection of *Aeromonas* sp. to common carps. There was high relative percent of survivability (RPS); 73% in oxytetracycline against *Aeromonas* sp. infection, so the oxytetracycline than other antibiotic tested in this study might be a potential for *Aeromonas* sp. infection to common carp (*Cyprinus carpio*).

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