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# In-Vivo and In-Vitro Evaluation on the Efficacy of Chemotherapeutic Agents against Important and Pathogenic Bacteria of Fishes under Constant Condition

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**Abstract:** An experiment was conducted to compare the efficacies of some selected antibiotics against common bacterial fish pathogens *viz., Aeromonas hydrophila*, *Edwardsiella tarda, E.coli*, *Staphylococcus aureus* and *Salmonella.* The test was conducted by infecting fish with selected bacterial pathogen which were then subjected to treatment. Four different antibiotics *viz.,* CFCIN (ciprofloxacin), Renamycin (oxytetracycline), AMYN (amoxicillin) and Sulfatrim (sulphadiazine + trimethoprim) were exposed in different doses against common fish bacteria. After ten days of observation blood sample were taken and bacterial analysis were made. Colony forming unit were then enumerated. After 3 days of exposure to ciprofloxacin and oxytetracycline Colony forming unit count was reduced significantly than amoxicillin. Ciprofloxacin showed the best result with 100% recoveries of challenged fish in prolonged treatment. Comparing with the amoxicillin, the CFU count of the oxytetracycline was significant ($p<0.05$). In conclusion, Ciprofloxacin, and oxytetracycline show better antimicrobial activity than amoxicillin and Sulfatrim in removing test bacteria from the blood.

Key words: Fish, Treatment, Antimicrobial efficacy, Bacterial pathogen, CFU

**Introduction:** Fish are the most diverse group of vertebrate occupying a variety of marine and fresh water habitat. They are cold-blooded or poikilothermic animals. Their body temperature varies passively in accordance with the temperature of the surrounding water. Although fish as a group are tolerant of a wide range of temperatures, from just below $0^0$C up to $45^0$C individual species generally have a preferred optimum, as well as a more restricted temperature range. Each species of fish has preferred ranges for the various parameters of water quality, such as temperature, dissolved oxygen and salinity and ideally the fish farm should operate at the optimum levels of each parameter to achieve fast growth and efficient performance (DACA, 2006)

One of the problems of the fishery sector in the capture fisheries and wild population is parasite and disease condition of fish parasite which reduces fish production by affecting the normal physiology of fish and if left uncontrolled, it can results in mass mortalities or in some cases, can be serve as source of infection for human and other vertebrates that consumed fish (Bush *et. al.,* 2001).

A common mistake of fish culturists is misdiagnosing disease problems and treating their sick fish with the wrong medication or chemical. When the chemical doesn't work, they will try another, then another. Selecting the wrong treatment because of misdiagnosis is a waste of time and money and may be more detrimental to the fish than no treatment at all. In addition it enhances development of drug resistance to already available limited number of antimicrobial and other chemical agent that used to treat fish (Frederick and Jerome, 1987).

Understanding the etiology of the disease is of crucial importance as it determines the choice

of potential treatment. Hence, the knowledge of fish bacteria and their relationship with the host as well as their effective treatments will help to select fish species for importation or for introduction from one locality to another and for introduction to different pond (Frederick and Jerome, 1987).

One of the main emphases in Ethiopia is to develop aquaculture to its full potential making a big contribution to national food availability, food security, economic growth, and trade and improved living standards. However, along with the growing interest in the development of fish industries in the different sites of the area, there will be an increasing awareness of importance of fish disease as one of the major detrimental factors in culturing fish in the coming future.

**Objectives of the study**

***General Objective*:** The main objective of this study was to identify the most important treatments of fish Pathogenic bacteria under constant condition without causing danger to the host.

***Specific objectives***

- ✓ To identify appropriate dose of Chemotherapeutic Agents in Constant water parameter.
- ✓ To compare the efficacy of different drug of the same property.

**MATERIALS AND METHODS**

**Study Area:** The study was conducted on fish collected from Lake koka, lake langanno, and Ziway lake.

**Fish Selection:** Fishes samples were collected using different centimeter mesh sizes of gillnets which were set at certain study sites of the lake during day time and throughout the night. Immediately after capture, total length (TL) and total weight (TW) of each specimen was measured to the nearest 0.1 cm and 0.1g, respectively. Then after, fish was adapted to the environment.

**Fish examination**: The codes of each fish, species, TL, TW, name of the site, date of sampling, types of parasites observed and different health related notes were recorded for each fish on separate field protocol. Any abnormalities on the fish were record. A hand lens was used for quick identification of any lesion on the skin and fins of the fish sample. Skin will also checked if there were capsules with metacercariae of trematodes in black dots and yellowish cysts which were sliced off the skin for further investigation and bacterial examination.

**Fish husbandry:** After examination, the relatively free fish was kept in separated plastic tank and was fed with different locally available fish meal at the rate of 5% of body weight of fish per day. Fish was randomly divided into 5 groups, four for fish to be treated with antibiotic and remain as a control for treatment. Fish was held indoors in circular tanks (150 1it) and oxygen was supplied with aerator.

**Diet preparation:** Both medicated and control diets were prepared. To prepare the control diet, locally available fish meal was pelleted to 4.5 mm diameter. Whereas medicated diets was prepared from locally available fish meal which was powdered and four drug of ant-biotic property (Ciprofloxacin, Renamycin, amoxicillin and Sulfatrim) in powder form was directly added to wet weight food with different doses. After thorough mixing, medicated diets were pelleted to a size similar to that of the control diet.

**Selection of Pathogens:** The common fish pathogenic bacteria were taken from the Ethiopian Health and Nutrition Research Institute, Microbiology Laboratory. All the bacterial cultures were maintained in nutrient broth. Serial dilutions of all the bacterial cultures were prepared in nutrient medium and used for further studies. Bacterial isolation, their characterization and pathogenicity test were completed according to the method described by Barrow and Feltham (1993) and Chowdhury and Muniruzzaman (2002). Finally, high virulent

species *viz., Aeromonas hydrophila*, *E.coli*, *staphylococcus aureus, salmonella spp* and *Edwardsiella tarda* were used for the experiment.

**Selection of antibiotics:** Locally available veterinary grade antibiotics used for the prophylaxis and treatment of livestock disease problems were used for this experiment. Based on preliminary investigation and availability in markets four available antibiotics *viz.,* CFCIN (Ciprofloxacin 10%; FnF) Renamycin (Oxytetracyclin, USP 200 mg; Renata Ltd.), Amoxicillin and Sulfatrim (Sulphadiazine BP 40% + Trimethoprim BP 8%; Techno Drugs) were selected for this study.

**In-vitro Efficacy Test**

**Disc preparation**: Suspensions of cultured bacterial isolates were prepared and 0.1 ml of each bacterial suspension was spread over Tryptone Soya Agar (TSA, Oxoid) plates using a sterilized glass rod. Fifty µl of testing agents were inoculated separately at pre-fixed doses on the sterile disc of blotting paper (3 mm diameter), dispensed earlier on the culture plates.

**Antibiotic sensitivity test**: Effects of selected antibiotics were determined by antibiotic sensitivity test using drug disc (paper disc) method against the most virulent bacterial isolates. Due to unavailability of different antibiotic discs, selected antibiotics were diluted in four different concentrations *viz.,* 25, 50, 75, 100 ppm and 50 µl was dropped on each blotting paper (3 mm in diameter) disc and incubated for 5 days at 20$^{o}$C. Sensitivity was recognized with clear zone surrounding the disc. The diameters of the restricted halos around the paper disc were measured time to time for determining the minimum inhibitory dose (MID).

Bacterial cultures were spread on nutrient agar plates and these plates incubated at 32±1$^{0}$C for 24 hrs. Three to four colonies were selected and transferred into 5ml nutrient broth medium and further incubated at 32±1$^{0}$C for 6-8hr. Sterile cotton swab was dipped into the bacterial suspension and pressed along the walls of tubes to remove excess of culture The entire agar surfaces were streaked with the swab. The inoculum was allowed to dry for 10-15 min with closed lid. The discs were placed inside culture plates under aseptic conditions and incubated at 32±1$^{0}$C for 24hr. After incubation the plates were observed and the diameter of inhibition zone was measured. The diameter of zone was calculated by using the following formula.

π (R1- R2) (R1+R2) where R1 = Radius of zone of inhibition + Radius of test bacteria zone. R2 = Radius of test bacteria zone (Well)

**In-vivo Efficacy Test:** After preparation of bacterial suspension, injections of selected bacterial isolates were performed on free *Oreochromis niloticus*.

Healthy young *Oreochromis niloticus* weighing 60 to 70gm were injected smoothly and carefully with 1.0 ml disposable syringes at a dose of 0.1 ml/fish comprising the suspensions of pre-selected pathogenic bacterial isolates. The experimental infection of the injected fish was expressed as lesion on fins, skin, head or body surface.

**Treatment and sampling:** After experimental infection with virulent isolates, diets medicated with different four ant-microbial properties were administered for each of four separated trial groups, and each group was feed for 10 days twice per day to observe their effects. Temperature was maintained at room temperature and dissolved oxygen was ensured by regular aeration. The date that the test diet first administered was labeled as study day 0. On Days 1 and 10 after the last exposure to the antimicrobial diet, randomly selected 5 fish of each group was sampled for bacteriological investigation. The treatments selected for this purpose were T1 with CFCIN, T2 with Renamycin, T3 with Amoxacilin, T4 with Sulfatrim and T5 as control. Sampling was performed at midmorning to avoid diurnal variation. The fifth group was served as a control which was received the same diet with

the same rate of feeding without the medication being added.

***Bacteriological Examination***: Blood can be serves as the ideal non-lethal tissues for detection of systemic infection. Blood was collected only from large enough fish that withstand the procedure. No more than 1ml of blood was collected from a 100g fish not to cause lethal result. Vacutainer tube with anti-coagulant was used for collection of blood sample.

***Culturing and examination of the sample*:** After blood collection, the media was prepared for enumeration of bacterial by dissolving the required amount of media in distilled water and autoclaved together with petri dish to have sterile required media.

One milliliter (1 ml) of homogenized blood sample was serially diluted in 9 ml of peptone water (ratio of 1:10) up to six dilutions. Sterile duplicate glass Petri dishes were labeled according to the dilution index. One ml of the dilutions will be aseptically withdrawn using a sterile 1 ml Pasteur pipette and was delivered into an open and sterile petri dish and then closed. The same was done for a duplicate petri dish. This was repeated till all the dilutions were pipetted into their corresponding plates. This was followed by pouring about 15 ml of standard plate count agar. The sample and the agar were then gently mixed by alternate clock and anti-clockwise rotations and left to solidify on the bench for about 30 min. The plates were inverted and incubated at 37°C for 48hr. After incubation, plates inoculated with sample dilution yielding between 30 and 300 colonies were counted. Colony count was made by using colony counter and expressed as CFU/ml of blood.

**Data management and Stastical Analysis:** Statistically data was analyzed using descriptive statistics and mean comparison procedure of the Statistical Package for Social Science Software (SPSS 20.0).

**Result and discussions:** The antibiotics giving zone in range 0-1.4cm were resistant, 1.4-2.0cm were intermediate and 2.1cm or above were sensitive. Effective inhibition was observed in ciprofloxacin and Renamycin against fish bacterial pathogens and the zone of inhibition ranges from 3.0-3.8cm and 2.6-3.6cm respectively. Ciprofloxacin and Renamycin has given the maximum zone of inhibition 3.8 cm and 3.6 cm against *S. aureus* and *A. hydrophila*, respectively. Few bacterial species were resistant to this antibiotic. All the pathogenic species were resistant to amoxicillin at low concentration.

Effect of different antibiotics on the fish pathogenic bacteria under laboratory condition provided useful information for treatment of bacterial fish diseases. Antibiotic sensitivity test of each pathogenic species was performed under *in-vitro* condition to determine minimum inhibitory dose (MID). All tested isolates (100%) were sensitive to CFCIN followed by Renamycin (84%) at 75 ppm. salfatrim was not so effective in lower concentration but sensitive for 80% isolates at 100 ppm. Moreover, only 53.33% pathogens were sensitive in case of Amoxacilin at 100 ppm. After investigation on 106 (out of 132) mesophilic aeromonads Yucel, *et al.,* (2005) found that all strains (*A. hydrophila, A. veroni bv. sobria, A. caviae*) were susceptible to ciprofloxacin. Wolska *et al.,* (1999) also reported that 99% of *Pseudomonas aeruginosa* strains were susceptible to ciprofloxacin. Sarker *et al.* (2000) performed drug sensitivity test and found that 50% of the *Aeromonas sobria* isolates were highly sensitive to oxytetracycline, oxolinic acid and chloramphonical and resistant to erythromycin and sulphamethoxazole.

Therapeutic effects of the antibiotics tested were examined through experimental infection. Best result was obtained with 100% recovery (Table 2) of infected fish when the antibiotic, CFCIN was used for prolonged fed treatment in laboratory condition. Renamycin was also found to be effective in healing bacterial infection (90.00 ± 2.89%) followed by Sulfatrim

(80.00 ± 5.78%). Amoxacilin was detected as less effective with 60.00 ± 4.62% recoveries of challenged fish. Kou *et al.* (1988) and Liao *et al.* (1996) used oxytetracycline in aquaculture as bactericide. Lio-Po and Sanvictores (1987) found positive effect of oxytetracycline in controlling *Pseudomonas* sp. in Tilapia fry. According to Shariff *et al.,* (1996) oxytetracycline (about 20 ppm) in a dip or bath solution is used against bacterial disease in Malaysia and Singapore. Chowdhury *et al.* (2003) found positive effect of Renamycin (oxytetracycline) against bacterial infection.

Antibiotics and chemotherapy have been used to prevent disease outbreaks and control proliferation of pathogens for a long time, causing the emergence of drug-resistant bacteria. Presently, although a good number of antibiotics such as norfloxacin, ciprofloxacin, oxytetracycline, gentamicin, chloramphenicol (Sahoo and Mukherjee, 1997), cefazolin (Zhang *et al.,* 2005) and aztreonam (Zhu *et al.,* 2006) etc. for tetracycline-resistant strains have been proven to be successful in controlling the infection.

In this study, the sensitivity of five pathogens was checked against different antibiotics. Ciprofloxacin and Renamycin possessed effective inhibition against bacterial growth. The zone of inhibition in range of 3.0-3.8 cm was observed for *Aeromonas hydrophila*, *E.coli*, *staphylococcus aureus, salmonella spp* and *Edwardsiella tarda* and these were sensitive to ciprofloxacin whereas *A. hydrophila, and Salmonella* sp. were sensitive to Sulfatrim whereas Most of the bacterial species were resistant to amoxycillin, Amabile *et al*., (1995) also observed zone of inhibition in range less than 1.4cm for these antibiotics. But there has been risk of using antibiotics as control agents in fish farming due to spread of antibiotic resistance to fish pathogens. (Austin *et al*., 1995; Moriarty, 1998). Therefore, different researchers suggested the alternative to the non-pathogenic strains of bacteria in the form of probiotics can be applied in fish disease prevention and control.

**Conclusion and recommendations:** The antibiotic compounds although potent against various pathogenic organisms in medicine but in aquaculture, different studies indicated the chances of development of antibiotic resistance among the fish pathogens. Keeping this in view, the probiotics (with single and multiple strains of non-pathogenic bacteria and / fungi), plant extracts, different oils, and more potent the bacteriophage therapy can be used to control fish pathogens. In this study, we have used different antibiotic to check their effectiveness and antimicrobial potency against the five fish pathogens and results validated through statistical analysis. However, further *in vitro* as well as *in vivo* studies need to be conducted to know more specifically about the effect and doses of probiotics and plant based extracts compounds that prove to be used in fish farming and management.

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**Table 1.** Selected antibiotics and their varied doses applied on bacterial isolates and inhibition zone

| No. | Name of antibiotics | | Doses | Inhibition zone(cm) |
|---|---|---|---|---|
| | Trade name | Type of antibiotics | | |
| **1** | CFCIN | Ciprofloxacin | 25 ppm | 3 |
| | | | 50 ppm | 3.3 |
| | | | 75 ppm | 3.5 |
| | | | 100 ppm | 3.8 |
| **2** | Renamycin | Oxytetracyclin | 25 ppm | 2.6 |
| | | | 50 ppm | 3.2 |
| | | | 75 ppm | 3.4 |
| | | | 100 ppm | 3.6 |
| **3** | AMYN | Amoxicillin | 25 ppm | 0.9 |
| | | | 50 ppm | 1.1 |
| | | | 75 ppm | 1.4 |
| | | | 100 ppm | 2.2 |
| **4** | Sulfatrim | Sulphadiazine + Trimethoprim | 25 ppm | 1.4 |
| | | | 50 ppm | 1.9 |
| | | | 75 ppm | 2.3 |
| | | | 100 ppm | 2.5 |

Table 2. The effect of antibiotics on experimentally infected fish with bacterial pathogens

| Treatment | Dose (g/kg diet) | Recovery (%) |
|---|---|---|
| CFCIN | 5 | 100.00 ± 0.00 a |
| Renamycin | 5 | 90.00 ± 2.89 b |
| Amoxacilin | 10 | 60.00 ± 4.62 c |
| Sulfatrim | 10 | 80.00 ± 5.78 c |
| Control | No dose | 0 d |

**Note**: The same letter in the same column is not significantly different at CI of 95%.