European Journal of Biotechnology and Bioscience
ISSN: 2321-9122; Impact Factor: RJIF 5.44
Received: 25-07-2020; Accepted: 10-08-2020; Published: 11-08-2020
www.biosciencejournals.com
Volume 8; Issue 3; 2020; Page No. 41-45

Development of oral prophylaxis against bacterial *haemorrhagic septicaemia* syndrome for aquaculture species

Nya EJ
Dept. of Biotechnology, Akwa Ibom State University, Mkpat Enin, P.M.B 1167, Uyo, Nigeria

Abstract
Aeromonas hydrophila is rated as one major causative agents of bacterial haemorrhagic septicaemia syndrome in aquatic species, but other etiological agents are Edwardsiella tarda, Pseudomonas fluorescens and Vibrio spp. The occurrence of bacterial *haemorrhagic septicaemia* disease in Rainbow trout experimentally challenged with virulent strain of *Aeromonas hydrophila* after being fed with Vitamin C and LPS supplemented prophylactic diets for 14 days was examined in this study. Polymerase chain reaction *PCR* was used to diagnose the causative agent and the present of this bacterium in the challenged fish. Mortalities from control group peaked at 70.6 % as compared to 17.6 % and 0 % in Vit. C and LPS treated groups respectively. Furthermore, surviving fish from the treated groups did not show any evidence of infections and the bacteria was not detected in them. However, using the methods of detection described, it would appear that the oral prophylaxis (supplemented diets) were able to protect the fish from the virulent *Aeromonas hydrophila* infections.

Keywords: Prophylaxis, Aeromonas hydrophila, bacterial haemorrhagic septicaemia, Rainbow trout

Introduction
Traditionally, *Aeromonas hydrophila* has been known as the causative agent of bacterial fish disease known as *haemorrhagic septicaemia* syndrome, motile *septicaemia*, ulcer, fin rot, or red sore disease, other etiological agents are *Edwardsiella tarda, Pseudomonas fluorescens and Vibrio spp* (Haley et al, 1967) [8]. Often, healthy fish suddenly developed swimming abnormalities, pale gills, bloat (abdominal distension) and skin ulcerations. The skin ulcers may occur at any site and are surrounded by a bright red rim of tissue (Randy, 1991). [18] Austin and Austin (1999) [2] described the *haemorrhagic septicaemia* disease as motile *Aeromonas septicaemia*, characterised by the presence of small surface lesions which lead to the sloughing off of scales. Internally, there may be the accumulation of ascitic fluid, and damage to the organs notably kidney and liver resulting to high mortality rate (Huizinga et al, 1979) [12].
Bacterial *haemorrhagic septicaemia*, *BHS*, has become recognised as being one of the most common disease problems that occur in fish farming operations on a worldwide basis. BHS is capable of producing mortalities ranging from five to 100 percent in farmed fish of fresh and marine water with temperature above 20ºC. Bacterial types isolated from outbreaks of *BHS* in tilapias include the following Gram-negative rods*: Aeromonas hydrophila* and other motile aeromonads (the "Aeromonas hydrophila complex", causing motile *Aeromonas septicaemia*, MAS), *Edwardsiella tarda, Pasteurella multocida, Pseudomonas fluorescens and Vibrio spp.* (Plumb, 1997) [16]. Most of the bacteria involved in cases of *BHS* are normal components of the bacterial flora of the fish themselves, and of the aquatic environment in which they are being farmed.
It follows from this that prevention must be based on good management practices involving the use of prophylaxis throughout the entire production process. Information on the control of this disease has focused on the successful use of suitable prophylaxis as a means of preventing massive outbreaks of *Aeromonas hydrophila*-associated *BHS* in fish farms (Plumb, 1997).
Fish prophylaxis technology is one of the least developed sectors of aquaculture particularly in Africa and other developing countries of the world (FAO, 2006) [7]. Oral prophylaxis is one of the major inputs in aquaculture production. It is one of the challenges facing the development and growth of aquaculture in the African continent. The oral prophylaxis developed so far has not made a significant progress as far as aquaculture industry is concern. According to Hetch (2000) [11], it is observed that research on Fish prophylactic compounds is abysmally inadequate and has not contributed greatly to aquaculture development and thus suggested that more research on prophylaxis and their uses in aquaculture production is required. Indeed, it is a major factor that can determines the profitability of aquaculture venture. Jamu and Ayinla (2003) [13] reported that oral prophylactic agents accounts for at least 60% of the total cost of fish production in sub-Sahara Africa, which to a large extent determines the viability and profitability of fish farming enterprise.
Principally, this necessitates the present study, to develop fish prophylaxis using non-conventional resources, determine the disease prevention and antimicrobial activity of the prophylactic agent for used in aquaculture production and for overall fish performance.

Materials and Methods
1. Diagnosis of *Aeromonas hydrophila* infections:
Diagnosis of infections caused by *A. hydrophila* was based on isolations and identification of *A. hydrophila* from infected rainbow trout fish (Austin and Austin, 1999) [2]. Briefly, Isolation was readily done from the surface lesions and kidney swab by the use of standard, non-selective bacteriological agar (nutrient agar / tryptone soya agar) with incubation at 20 - 25ºC for 24 – 48 h. Typically on non-selective media, cream, round, raised, shiny entire colonies

of 2 – 3 mm. diameter was seen developed within 24 h at 25ºC as indicative of the presence of *A. hydrophila* (Austin and Austin, 1999) [2]. Diagnosis was effectively done upon examination of key phenotypic traits of pure culture according to Austin and Austin, (1999) [2]. Serological methods such as slide agglutination, fluorescent antibody techniques (useful in laboratories) were also carried out to confirm the presence of the bacterial pathogen.

DNA Extraction from bacterial isolates

The bacterial culture were grown in TBS for 24 h at 28 ºC, and harvested by centrifugation at 3000 x g for 10 minutes at 4 ºC, washed twice and resuspended in 500 µL (0.5 ml) PBS. Then the bacterial cells were lysed, and the DNA extracted using a DNA extraction Kit (Qiagen) following the manufacturer's protocol for extraction of genomic DNA.

Agarose gel Electrophoresis of DNA

For the assessment of DNA extracted from bacterial isolates, *Aeromonas hydrophila* agarose gel electrophoresis were carried out using the Horizon 58 Gel Electrophoresis Apparatus (Gibco, BRL). 1.0 g of Agarose (Integra Bioscience) was dissolved in 100 ml of Tris- EDTA (TBE) buffer by heating in a microwave oven. After the dissolution, the solution was allowed to cool at 55 ºC, before 1.0 µl of Ethidium bromide (Sigma) was added. The gel solution was thoroughly mixed and poured into a taped gel casting tray with 14 wells integrated comb, and left to cool and solidified in a cool room for 4 h. Following electrophoresis, the DNA banding patterns on the agarose gel was immediately observed on a ultra violet (UV) transilluminator (UV products). Photographic records were taken for documentation purpose on a gel documentation unit (Amersham Bioscience).

Details of primers used in the 16S rRNA Sequencing

The DNA sequencing assay was carried out following the methodology of Brunt and Austin (2005) [3], with slight modifications. Briefly, the bacterial cells were lysed and the DNA extracted using a DNeasy tissue Kit (Qiagen), following the protocol for isolation of total DNA from animal tissues. DNA templates were amplified by the polymerase Chain Reactions (PCR) on a Perkin Elmer Gene APP 2400, using the universal primers.

27 F (5 ' AGAGTTTGATCMTGGCTCAG-3'

685 r [3] (5 ' TCTRCGCATTYCACCGCTAC-3') Lane, obtained from MWG Biotech,

Sequencing of purified PCR products

The purified PCR products were sequenced using 20 µl Reaction mixtures containing 4 µl of big dye deoxy Terminator Cycle Sequencing Kit (Applied Biosystems), 4 µl of x 5 sequencing buffer, 1 µl of either forward or reverse primer (5p mol μl^{-1}), 2 µl of purified PCR products and 9 µl of sterile MilliQ (Millipore) water were utilized. Cycle sequencing reactions were accomplished by initial denaturation at 98 ºC for 5 minutes, followed by 25 cycles of denaturation at 96 ºC for 10 s, annealing at 50 ºC for 5 s and extension at 60 ºC for 4 minutes.

Cycle sequenced templates were purified by the addition of 40 µl of 75 % (v/v) isopropanol (Sigma), then incubated at room temperature for 10 minutes to allow for precipitation of the DNA. Extractions were centrifuged at 10,000 x g for 20 minutes to pellet the DNA. The overlying supernatants were removed, and then the resultant pellets were washed thrice with 125 µl of 70 % (v/v) isopropanol to remove intemperate dyes and repeated thrice. The resultant samples were dehydrated at 90 ºC for 1 minute. The 16S rRNA gene sequences were compared in a BLAST search with those in the National Library of Medicine database.

Experimental Fish

Rainbow trout *Oncorhynchus mykiss* average wet weight 15 ± 0.2 g, obtained from commercial fish farms in Scotland, were stocked in a 500 L tank and kept for acclimatization and health check. The stocks were acclimatized under aerated condition for a period of 15 days to ameliorate the handling stress. The health status was examined upon the arrival for evidence of diseases after Austin and Austin (1999), by random sampling and aseptically streaking out their spleen and kidney on a TSA plate and incubated at 28 ºC for 48 h. One hundred (100) of them were randomly distributed in five experimental groups following a Complete Randomized design (CRD) and maintained in aerated, dechlorinated water tanks at 12 ºC. Facilities in the Aquarium guaranteed continuous aerations to all tanks to maintain optimum dissolved oxygen (DO) which was monitored at weekly intervals. Water temperature was 12 ± 1 ºC, Dissolved oxygen concentration 6.0 ± 0.3 mg L^{-1}, Ammonia-nitrogen concentration 0.14 ± 0.05 mg L^{-1}, Nitrite-nitrogen 0.04 ± 0.03 mg L^{-1}. Fish were fed their respective diets at the rate of 4 % body weight per day. The daily rations was divided into two parts and fed at between 9.00- 9.10 am and 16.00- 16.10 pm h. Faecal matters and leftover feeds were removed manually.

Preparation of Oral prophylaxis

Vitamin C were obtained from a local pharmaceutical store, crushed using a household press, sieved with wire mesh sifter of appropriate size and incorporated directly into commercial fish feed (Biomar, Bio-optimal Start) to achieve 0 g (control), 0.25g, 0.5g and 1.0 g 100 g^{-1} of feed. The modified feed was stored in screw cap bottles separately and stored at room temperature until needed.

Likewise, LPS isolated from E. coli 0111:B4 endotoxin, purified by phenol extraction, was obtained from Sigma-Aldrich (lot no. L2630-100MG). The LPS was incorporated directly into commercial fish feed (Biomar, Aarhus, Denmark) and shaken to mix well after appropriate measurement to achieve 0 mg (= control), 1.0 mg, 3.0 mg, 7.0 mg of LPS per 100 g^{-1} of feed. The modified feed was stored in screw-cap bottles at room temperature until use.

Feeding and Challenged Experiment

To study the effect of putative prophylaxis on the disease prevention activity against *Aeromonas hydrophila* caused infections (bacterial *haemorrhagic septicaemia* syndrome), five groups of 20 fish weighed average15 ± 0.5g each totaling one hundred (100) fish were fed twice daily to satiation for 14 days.

The experimental fish groups were fed twice daily with oral prophylaxis (the modified feed) and the Control to satiation for 14 days. Fish were fed with standard commercial diet after the administration of treated feeds used for the feeding trial..

For the challenge experiment, bacterial cultures were grown for 24 h at 28 ºC in 10 ml of TSB, harvested by centrifugation at 3000 x g for 10 minutes at 4 ºC, washed

once in 0.9 % (w/v) Normal Saline and the resultant suspension adjusted to 4.64 x 10^5 cells/ml after counting in a haemocytometer slide (Improved Neubauer) on a Kyowa light microscope at 100x magnifications.

The fish were subdivided into five (5) groups of twenty (20) each and injected intraperitoneally (i p) with 0.1 ml volumes of *A. hydrophila* cell suspensions. Behavioural alterations and mortality were monitored daily and examined for evidence of disease after Austin and Austin (2007) [1]. Bacterial were aseptically isolated from the spleen, kidney, muscles, eyes, gastrointestinal tracts and streak over plates of TSA with incubation at 28 ºC for 48 h. After the fourteenth day, the surviving fish were sacrificed and the internal organs examined and streaked out on TSA plates to confirm that they were free from infections.

The relative percentage survival (RPS) in different treatment groups were calculated following formula according to Burrells et al. (2001) [4].

RPS = 1 - (No. of mortality in treatment group / No. of mortality in Control group) x 100.

Determination of Inhibitory Activities to Pathogen:

A bacterial growth inhibition studies to test the production of antimicrobial properties of putative prophylaxis were performed, to determine the efficacy of candidate prophylaxis for in vivo studies in aquaculture production. Inhibitory activity was examined according to method described by Patrizia et al. (2003) [17] with modifications. Briefly, suspension of bacterial cultures were streaked out on plates of TSA and incubated for 24 h at 28 ºC. A drop (0.25 µl) or grains of the prophylaxis were spotted with a micro-dilutor handle on the streaked TSA plate, and further incubated for 24 h at 28ºC. Antimicrobial or inhibitory activity of the putative prophylaxis was indicated by a clear zone of inhibition in the lawn area around the spot of the producer. The experiments were perform in triplicates and mean values were used

Mode of action of the prophylaxis

Separate groups of 10 rainbow trout obtained from experimental groups (fed with oral prophylaxis (the modified feed) and the control, as before, were used to determine mode of action parameters. Thus, blood was collected by venepuncture, and transferred into vacuette tubes containing heparin as anticoagulant (Greiner) to prevent clotting. This blood was used for determination of haematocrit (Hct), haemoglobin (Hb) content, and total erythrocyte and leucocyte counts. For this, the blood was diluted to 10^2 and 10^3 in PBS, and the number of leucocytes and erythrocytes counted. Duplicate blood samples were also collected and allowed to clot at room temperature for 2 h and refrigerated overnight at 4 ºC before the clotted blood was centrifuged at 3000 x g for 10 min at 4 ºC, and the serum collected and stored at -70 ºC until used.

Immune parameters such as the lysozyme activity, respiratory burst and serum peroxidase activities were determined following methods previously described (Nya and Austin, 2009) [15]. Electrolytes, i.e. calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and ferrous iron (Fe) ppm /ml^{-1}, were determined by flame emission photometry (Rehulka, 2000) [19], using an automated system e Atomic Absorption Spectrometer (Perkin Elmer) with their appropriate standards. All experiments were perform in triplicates and mean values were used

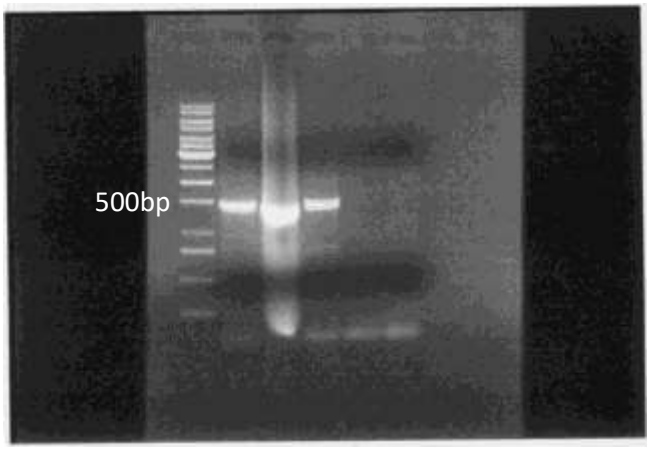
Statistical analyses Values for each parameter measured were expressed as arithmetic mean, standard error (SE). Hematological and biochemical parameters were tested using one-way ANOVA, and a comparison of the mean values was done by using Duncan's multiple range tests (Duncan, 1955) [6], at the 5% level of significance. The software programme SPSS (Version 14.0) for Windows was used.

Results

16S rRNA gene sequencing:

The pathogen *Aeromonas hydrophila* was further identified using PCR. Partial 16S rRNA was amplified and sequenced and the results of the sequences were analysed using 99% minimum similarity in the NCBI BLAST database. The forward and reverse sequences of *A. hydrophila* produced 100% significant alignment with *Aeromonas hydrophila* AN-1, AE55, AE53 with Accession No. AY 987735.1, AY987734.1, AY987733.1 and 987732.1 respectively.

500



bp

500Fig.3. 16S rRNA of *A. hydrophila* and *ORN2* DNA products

Determination of inhibitory activities of different Immunostimulants:

The production of inhibitory properties against the pathogenic bacteria *A. hydrophila* by different putative prophylaxis is shown in table 3.

Table 1: Production of inhibitory compounds against bacterial isolates

Tested prophylaxis	Bacterial isolates *A. hydrophila*
Vitamin C	S
Lipopolysacharides (LPS)	MS

S = sensitive with clearing zone diameter of ≤ 6mm, MS = moderate sensitive with faint clearing zone

Feeding and Challenge Experiments

The results of this experiment was indicated by the number of mortalities after intraperitoneal injection (i.p) with bacteria inoculums to group of Rainbow trout fed with prophylactic supplemented diets and the Control (non-supplemented diet) and are shown in table 3. All mortalities recorded during the trial were due to the Challenge infections with *A. hydrophila* and were observed from the second day post-challenge. The clinical signs observed in the fish were abdominal distension, muscle necrosis, gut ulcerations ascetic fluids, *haemorrhagic septicaemia*,

swollen spleen and exophthalmia. The mortalities were observed for 14 days post challenge.

Table 2: Mortalities of fish fed prophylaxis as compared to Control

Treatments	No. of fish challenged.	No. of mortality.	No. of survival.	Relative % Survival
Vitamin C				
0.25 g	17	8 (47.1%)	9	33.3
0.5g	17	5 (29.4%)	12	58.3
1.0 g	17	3 (17.6%)	14	75.0
Lipopolysacharides (LPS)				
1.0 mg	17	0 (0%)	17	100
3.0 mg	17	0 (0%)	17	100
7.0 mg	17	5 (29.4%)	12	58.3
Control	17	12 (70.6%)	5	00.0

Disease prevention mechanisms

Use of the modified diets for 14 days led to a marked reduction in mortality after challenge with *A. hydrophila.* Thus, 70.6% mortalities were recorded for the controls compared with 29.4% and 17.6% mortalities in the groups which were fed with 0.5 g and 1.0 g Vitamin C per 100 g feed, respectively (Table 2). Mortalities of only 0% were recorded in the groups fed with 1.0mg and 3.0 mg LPS per 100 g of feed. Survivors of the groups which received Vitamin C supplemented diet did not show any disease signs at the end of the experiments. However, the use of LPS supplemented diet for 14 days led to a marked reduction in mortalities after challenge with A. hydrophila. Thus, there was 70.6% mortality in the controls compared with 0% mortalities (RPS = 100%) in the groups which received 1.0mg and3.0mg of LPS per 100 g of feed, respectively. Moreover, the survivors of the treated groups did not show any sign of diseases at the end of the experiment. In all the groups treated with prophylactic diet, there was a significant increase in mean corpuscular Hb Concentration as was observed in the groups as compared with the controls. However, the use of prophylaxis led to significant decreases in the blood mean corpuscular volume and means corpuscular Hb values (data not shown).

Discussion

This study provides useful data for the control of disease and good management practices involving the use of prophylaxis throughout the entire aquaculture production process. Principally, it reinforces the view that prophylaxis is beneficial for the prevention and control of bacterial infections in aquaculture especially opportunistic bacteria like *A. hydrophila*. Previous work by Nya and Austin, (2009) [15] using rainbow trout which received supplemented dietary garlic for 14 day treatment periods revealed protection against challenge with *A. hydrophila*. This protection was said to be attributed to an enhanced innate defence mechanisms, such as high oxidative radical production by serum neutrophils, proliferation of lymphocytic cells and phagocytic activity of the head kidney macrophage (Nya and Austin, 2009) [15]. However, modulation of non-specific defence mechanisms in treated fish may have been chiefly by activation of the release of reactive oxygen species (ROS) by immune cells. This might explain the significant increase ($P < 0.05\%$) in the respiratory burst activity of the neutrophils, measured by the reduction of NBT to formazan as indicator of superoxide anion (O_2) production. This reactive oxygen species include superoxide radicals and hydrogen peroxide, which are known to be toxic to pathogenic bacteria (Hardie et al., 1996 [9] and 1991) [10]. Moreover, the significant difference between the treatment and control groups was similar to the finding of Choudhury et al., (2005) [5], who observed a high superoxide radicals and hydrogen peroxide, activity in Labeo rohita juveniles fed with 0.4% dietary yeast RNA. The disease prevention potential of oral prophylaxis is certainly supported by previous work of Sahu et al., (2007) [20], which noted the effectiveness of 5.0 g and 10.0 g of garlic kg) of feed for controlling *A. hydrophila* infection in L. rohita. Furthermore, Martins et al., (2002) [14] also reported the effect of Allium sativum on erythrocyte, leucocyte and Hct content.

In this study, Vitamin C and LPS supplementation induced significant changes in serum electrolytes in rainbow trout following 14 days administration. This is essential for maintaining the osmotic pressure needed for proper distribution of body fluids and acts as plasma carrier and non- specific ligand with many binding domains. Clearly, this study has highlighted the potential value of Vitamin C and LPS supplemented diet to aquaculture both in terms of prophylactic protection against specific bacterial disease and overall performance of fish.

Acknowledgement

The author is grateful to Sean McMenamy School of life Sciences Heriot Watt University, Edinburgh, UK, for technical assistance, and the Akwa Ibom state University (AKSU) Uyo, Nigeria for financial support.

References

1. Austin B, Austin DA. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. 4th ed. Godalming: SpringerePraxis; 2007.
2. Austin B, Austin DA. Bacterial fish pathogen – Disease in farmed and wild fish. 3rd Edition. Ellis Horwood series in Aquaculture and fisheries supp- ort, London, 1999.
3. Brunt J, Austin B. Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases. 2005; 28:693-701.
4. Burrells C, Williams PD, Forno PF. Dietary nucleotides, a novel Supplement in fish feeds and effect on resistance to disease in Salmonids. Aquaculture. 2001; 199:159-169.
5. Choudhury D, Pal AK, Sahu NP, Kumar S, Das S, Mukherjee SC. Dietary yeast RNA supplementation reduces mortality by Aeromonas hydrophila in Rohu, Labeo rohita L juveniles. Fish Shellfish Immunol. 2005; 19:281e91.
6. Duncan DB. Multiple range and multiple 'F' tests. Biometrics. 1955; 11:1e42.
7. FAO. Topics and issues fact sheets. State of World Aquaculture, fisheries Global information systems. Food and Agriculture organization of the United Nations, Rome, 2006.
8. Haley R, Davis SP, Hyde JM. Environmental stress and Aeromonas liquefaciens in American and thread fin Shad mortalities. Progressive Fish Culturist. 1967; 29:193.
9. Hardie LT, Ellis AE, Secombes CJ. *In vitro* activation

of rainbow trout macrophages stimulates inhibition of Renibacterium salmoninarum growth concomitant with augmented generation of respiratory burst products. Dis. Aquat Org. 1996; 25:175e83.
10. Hardie LJ, Fletcher TC, Secombes CJ. The effect of dietary Vitamin C on the immune response of Atlantic Salmon *Salmo salar*. Aquaculture. 1991; 95:201- 214.
11. Hetch T. Consideration on African Aquaculture. J. World Aquaculture. 2000; 31:12-19.
12. Huizinga HW, Esch GW, Hazen TC. Histopathology of red-sore disease of *Aeromonas hydrophila* in naturally and experimentally infected large-mouth bass, *Micropterus salmoides* (Lacepede). Journal of fish Diseases, 1979 2, 263- 277.
13. Jamu DM, Ayinla OA. Potential for the development of aquaculture in Africa NAGA. 2003; 26:9-13.
14. Martins ML, Moraes FR, Miyazaki DM, Drum CD, Onaka EM, Fenerick J, Bozzo FR. Alternative treatment for Anacanthorus penilabiatus, (Monogenea: Dactylogyridae) infection in cultivated pacu, Piaractus mesopotamicus (Osteichthyes: Characidae) in Brazil and its haematological effects. Parasite. 2002; 9:171-180.
15. Nya EJ, Austin B. of garlic (Allium sativum) to control Aeromonas hydrophila Infections in rainbow trout Oncorhynchus mykiss (Walbaum). J. Fish Dis. 2009; 32:963e70.
16. Plumb JA. Infectious Diseases of Tilapia. In: Tilapia Aquaculture in the Americas (Editors: BA Costa-Pierce and JE Rakocy), Vol. 1, World Aquaculture Society, Baton Rouge, USA, 1997, 212-228.
17. Patrizia M, Elisa G, Moreno B. Bacteriocin- like substances (BLS) Production in Aeromonas hydrophila water isolates. F E M S. Microbiol. Lett. 2003; 220(1):121-125.
18. Randy W. Diagnosis of *Aeromonas hydrophila* infections in fish. Animal Disease diagnostic Laboratory Newsletter, 1991, Pp. 1 of 1.
19. Rehulka J. Influence of astaxanthin on growth rate, condition and some blood indices of rainbow trout Oncorhynchus mykiss. Aquaculture 2000; 190:27e47.
20. Sahu S, Das BK, Mishra BK, Pradhan J, Sarangi N. Effects of Allium sativum on the immunity and survival of Labeo rohita infected with A. hydrophila. J Appl. Ichthyol 2007; 23:80e6.