A trial for treatment of Flavobacteriosis in Red Sea cultured Humor fish *Epinephelus polyphekadion* (Bleeker, 1849) using Humate substance and Oxytetracycline

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Abstract: Present study investigate pathogencity of falvobacteria on Red Sea cultured **Humor *Epinephelus polyphekadion*** with a trial for treatment and control it using hamate substance and oxytetracycline. Naturally infected **Humor** fish ***Epinephelus polyphekadion*** was stocked in cement ponds along Red sea private fish farm. Large number of fish suffering from discoloration, ulcers on the skin, erosion in fins and mouth. 90 naturally infected fish was divided into three groups with three replicates 1st group infested non treated ,2nd group treated with humate substances 15 mg/kg body weight in diet for 45 days, 3rd group treated with long acting oxytetracycline 75 mg/kg body weight in diet for 10 days . blood samples were taken at the end of every week for three weeks post treatment , mortality rate, RBCs count, total WBCs count, cortisol concentration in serum and phagocytic index were recorded along 3 weeks post treatment . the study concluded that Flavobacteria infection in Shahwan fish should be diagnosed and treated as quick as possible to avoid fish mortalities and economical losses, using natural treatment such as humate substances which is recommended and preferable than antibiotics (oxytetracycline) treatment in fish bacterial infection as their application as it is cheap, more safe, and of little side effects on fish.

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Key words: Falvobacteria- Red sea cultured **Humor** fish - ***Epinephelus polyphekadion*** - hamate substance- oxytetracycline- RBCs count- total WBCs count- cortisol - phagocytic index.

Introduction

Many bacterial taxa have at one time or another been associated with fish diseases. Many should be considered as opportunistic pathogens, which colonize and cause disease in already damaged hosts. The initial weakening process may involve pollution or a natural physiological state (e.g during reproductive phase) in the life cycle of fish, or any other stress factor represented as physical injury, nutritional imbalance, disturbance of water temperature where, the water temperature was elevated to 27- 28^{O}C the fish were listless, anorexic and showing respiratory distress (**Austin and Austin,2007**).

Falvobacteria spp. is gram –ve fish pathogen affecting young and adult fish. In young fish ,gills is usually the major site of damage typically, congestion of blood vessels supplying the gills with dissociation of surface epithelium of lamellae from the capillary bed. There may be scattered areas of haemorrhaging (**Pacha and Ordal,1967**). While in adult fish it affect gills, skin and/or musculature. Skin discoloration, fading and muscle lesion may be documented, superficial erosion of skin particularly on the posterior flank, and lower jaw with eroded pectoral and dorsal fins (**Michel *et al*.,2002**). Large number of bacteria infecting fish through the lesion and fish die within 48h. (**Shoemaker *et al*.,2008**).

Humic and fulvic acids are natural organic substances found in natural water systems and thus are compatible with all aquatic life forms. Prolonged stress and inadequate adaptation to stress in aquaculture could result in the reduction of the immune defense systems of fish which could lead to infection and disease. They can be found in peat, coal, many upland streams. It arise by the microbial degradation of biomolecules dispersed in the environment after death of the cells (**Mrinsky et al.,1995**).

The potential of humic substances in treatment fish diseases has scarcely been investigated, although previous experiments demonstrated as protective effect of humus extract against atypical *Aaeromonas salmonisida* infection in carp (ulcer disease) (**Kodama *et al*.,2007**).

Oxytetracycline is widely used in aquatic medicine to combat mainly gram-negative bacteria . Long-acting formulations of OTC (OTC-LA) are widely used in veterinary medicine to provide prolonged broad spectrum antibacterial activity at high therapeutic blood concentrations with minimal tissue reactions in infected animals (Rigosr et al., 2010). Thus present study investigate pathogencity of falvobacteria on Red sea cultured Hamor fish ***Epinephelus polyphekadion*** with a trial for treatment and control it using hamate substance and oxytetracycline

Material and methods:

Naturally infected fish:

Humor fish ***Epinephelus polyphekadion*** was stocked in cement ponds along Red sea private fish farm. Large number of fish suffering from discoloration, scales sloughing, ulcers on the skin, at

the caudal peduncle, erosion in fins. Large number of fish died, body weight, clinical and postmortem changes were recorded **(Innes 1966)**. Samples from fish was taken for bacteriological examination.

Bacteriological examination :

Under complete aseptic conditions swaps were taken from lesions, ulcers on the caudal peduncle and dorsal and pelvic fins. Also from internal organs (liver, spleen and kidney) to culture on nutrient broth and incubated at room temperature (27±2^{o}C) for 24 h.

Isolation and identification:

The etiological agent readily recovered from diseased tissues (ulcers and fins) on low nutrient media and incubated at 28^{o}C for 24 h. then re-isolated on tryptic soya agar TSA. The isolated bacteria was identified by API20E as rapid identification system. The results were recorded after incubation at 28^{o}C for 36 h. (**Austin and Austin, 1999).**

Blood sampling:

Five randomly chosen fish from each group and control group, were taken and anaesthetized with tricaine methane sulphonate (Ms 222, 50 mg/L) of water, and used for blood collection. Blood samples were taken from each group from the caudal vessels before treatment and 7th, 14 day 21 day, after final feeding. Blood samples were divided into two portions, first portion with anticoagulant for hematological examination, the other portion without anticoagulant for serum separation for biochemical and immunological analysis. Blood was collected from caudal vein, with 1 ml plastic syringe rinsed with anticoagulant, and a part of the blood was transferred immediately, added to an equal volume of 10% tri sodium citrate, and stored at 4°C. The remaining blood was kept at room temperature for 1 h without anticoagulant to collect the serum and stored at - 20°C.

For phagocytosis assay, 10^7 cells of formalin-killed Flvobacteria johansonae were added to 0.1 ml of pooled blood samples from two fishes in a sterile microplate and incubated for 30 min at 25^{O}C after mixing in the well. After incubation, the blood-bacteria suspension was mixed gently and 50 µL of this suspension was smeared on three glass slides. After air drying, the smears were fixed in 95% ethanol, re-dried, and stained with Giemsa. The phagocytic cells and phagocytosed bacteria were counted for phagocytic activity and phagocytic index (**Kawahara *et al*., 1991**).

Experimental design:

A total number of 90 naturally infested Humor fish ***Epinephelus polyphekadion*** were randomly collected from infected pond for treatment experiment. They were divided into three groups with three replicates 10 fish each, 1st group was kept as a control without treatment 2nd group subjected for treatment with humic acid 15 mg/kg body weight in diet was sprinkled on the dry pellets to adsorb into it then, dried in incubator at 25 ^{o}C for 45 day (**Kadoma *et al*.,2007**). 3rd group was subjected for treatment with oxytetracycline 75 mg/kg body weight in diet for 10 days (**Rigosr *et al.*, 2010**) . Each group was isolated in a separate fiber glass tank 500 cubic liter containing sea water with continuous aeration with automatic air pump at temperature 25 ± 2 ^{o}C pH 7.19 and dissolved oxygen 5 ppm., salinity (52 ppm).

Statistical Analysis :

The statistical analysis was performed by one way ANOVA analysis of variance according to (**Kachigan,1991**). The multiple tests were carried out to determined difference between treatments means at significance level $P<0.05$.

Results :

Clinical and postmortem signs :

The general clinical sings in infected Humor **fish** ***Epinephelus polyphekadion*** Fig 1 were represented as sluggish movement, loss of escape reflex, ulceration of the skin at the region of caudal peduncle Fig 2 . Fading of skin color, fins . Erosion and ulceration at the region of mouth, off food and finally death. Postmortem examination revealed that congestion of gills, inflammation and enlargement of the liver, spleen and kidney with serous fluid accumulation in the peritoneal cavity.

Figure 1 : **Humor fish *Epinephelus polyphekadion* showing deep ulcer on the caudal peduncle (arrow).**

Figure 2 : Humor fish *Epinephelus polyphekadion* showing ulcer at the caudal peduncle (arrows) (magnification).

Table 1: Mortality rate during treatment of infected fish with humates and oxytetracyclines

groups	Number of treated fish	Number of died fish	Percentage of mortality %
Control group	10	9	90
Humates group	10	1	10
Oxytetracycline	10	4	40

Results of bacteriological examination (isolation and identification):

The isolated bacteria on TSA revealed greenish yellow colonies, with staining with Giemsa stain revealed gram –ve bacilli. Identification was done using API20E as rapid identification system. From the data obtained by API20E the isolated bacteria was identified as *"Flavobacteria johansonae"*

Results of mortality rate during treatment:

Percentage of mortality rate in control group (without treatment) was 90% while in group treated by humic acid was 10% and 40% in group treated by oxytetracycline.

Table 2: Determination of RBCs count x 10^7/ml in Humor fish For 3 weeks post treatment

day / groups	Before treatment	7th day*	14 day*	21 day*
Group 1 (control)	104±5.2A	102±5.1A	105±5.3A	103±5.2A
Group2 (humic acid)	106±5.3B	104±5.2B	110.7±5.5B	113±5.4B
Group3(oxytetracycline)	99.6±4.9C	91.4±4.6C	89.3±4.5C	90.7±4.6C

*** post treatment**

Each value represents mean ± S.E.; N=5.

Small letters (a), (b), and (c) represent a significant change to capital letters A, B, and C respectively at the same row (by LSD using ANOVA at $p < 0.05$)

Table 3 : Determination of total WBCs count x 10^7/ml in Humor fish for 3 weeks post treatment

day / groups	Before treatment	7th day*	14 day*	21 day*
Group 1 (infected non treated)	4.45±0.22A	4.65±0.23A	4.33±0.21A	4.37±0.21A
Group2 (humic acid)	4.13±0.21B	6.55±0.33B	5.50±0.28B	4.90±0.25B
Group3(oxytetracycline)	4.35±0.21C	7.13±0.36c	7.99±0.40c	6.81±0.34C

*** post treatment**

Each value represents mean ± S.E.; N=5.

Small letters (a), (b), and (c) represent a significant change to capital letters A, B, and C respectively at the same row (by LSD using ANOVA at $p < 0.05$)

Table 4 : Determination of Cortisole concentration mg/ml in Humor fish for 3 weeks after treatment

Day / groups	Before treatment	7th day*	14 day*	21 day*
Group 1 (infected non treated)	32±1.6A	31±1.5A	37±1.8A	33±1.7A
Group2 (humic acid)	37±1.8B	517±25.8b	211±10.5b	41±2.5B
Group3(oxytetracycline)	35±1.7C	714±35.7c	607±30.4c	113±5.6c

*** post treatment**

Each value represents mean ± S.E.; N=5.

Small letters (a), (b), and (c) represent a significant change to capital letters A, B, and C respectively at the same row (by LSD using ANOVA at $p < 0.05$)

Table 5 : Determination of phagocytic index % in Humor fish for 3 weeks post treatment

day / groups	Before treatment	7 day*	14 day*	21 day*
Group 1 (infected non treated)	111.2±5.6 A	107.0±5.4A	112.0±5.6A	101.0±5.0A
Group 2 (humic acid)	113.5±5.6 B	114.0±7.2B	117.0±5.8B	125.3±6.2B
Group3 (oxytetracycline)	109.7±5.5C	33.7±2.7c	37.0±3.1c	73.9±5.0c

*** post treatment**

Each value represents mean ± S.E.; N=5.

Small letters (a), (b), and (c) represent a significant change to capital letters A, B, and C respectively at the same row (by LSD using ANOVA at $p < 0.05$)

Results of RBCs count (X 10^7/ml) ,Total WBCs count (X 10^7/ml), Cortisole concentration (mg/ml) and phagocytic index % in Humor fish for 3 weeks post treatment

The results of RBCs count not significantly effected in three groups, along the 3 weeks post treatment, while total WBCs count significantly increased in the group treated by oxytetracycline and these effect continued for 2 weeks then returned to be non significant after 3 weeks, cortisole concentration in serum was significantly increased in group treated with humic acid up to 2 weeks after treatment but after 3 weeks it returned normally as before treatment. The concentration of cortisole in group treated by oxytetracycline was significantly higher than those before treatment for 3 weeks after treatment. Regarding the percentage of phagocytic index in group treated by humic acid not significantly differ than those before treatment while after 3 weeks increased than those before treatment , in contrast in group treated with oxytetracyclin it was significant decrease for three weeks post treatment table 1,2,3,and 4.

Discussion

Treatment of diseases in fish is limited because of only a few chemicals approved for use in aquaculture and these are not effective against all pathogens. Chemical and antibiotics treatment may be linked to side effects such as toxic stress All chemicals approved for use in aquaculture are suspected of being mutagenic or carcinogenic with a high potential to non-target organisms (**Meinelt *et al*. 2001b, 2002**).

Present study aimed to investigate the comparison difference of by antibiotic treatment represented by oxytetracycline and natural product treatment represented by humic acid to flavobactreria infection, in Humor fish ***E. polyphekadion*** . The general clinical sings in infected fish were represented as sluggish movement, loss of escape reflex, slimy fading skin, asphyxia with aggregation of fish near the water surface. ulceration of the skin at the region of caudal peduncle . Fading of skin color, and eroded dorsal fins (**Austin and Austin, 2007**). Off food and finally death. Postmortem examination revealed that congestion of gills, inflammation and enlargement of the liver, spleen and kidney with serous fluid accumulation in the peritoneal cavity these clinical sings coincide with the finding of (**Noga, 2010**).

The main clinical signs observed where it was clear from pathogensis of flavobacteria spp. it is gram – ve fish pathogen affecting young and adult fish. Gills is usually the major site of damage typically, congestion of blood vessels supplying the gills with dissociation of surface epithelium of lamellae from the capillary bed . there may be scattered areas of hemorrhaging. while in adult fish it affect gills, skin and/or musculature. Skin discoloration fading and muscle lesions have been documented, superficial lesions erosion of the skin particularly on the posterior flank, eroded fins and lower jaws (**Michel *et al*., 2002**). Asphyxia and respiratory dysfunction may be attributed to damaged epithelium of gill filaments which caused by flavobacteria infection as it is the target organ for the pathogen **(Austin and Austin, 2007**).

Concerning the effect of oxytetracycline and humic acid on treatment flavobacteriosis, it appears from the results that oxytetracycline was highly effective against flavobacteria infection at the dose of 75 mg/kg diet for 10 days. these results nearly agree with finding given by (**Rigosr *et al*., 2010**)) on the other hand the use of humic acid at the dose of 15 mg/kg diet for 45 days was highly effective in treatment of flavobacteriosis in cultured Humor fish these results nearly agree with the results met by **Kadoma *et al*.,(2007)** who demonstrate that there is protective effect of humus extract against atypical *Aeromonas salmonisida* infection in carp (ulcer disease). The use of natural treatment for controlling bacterial diseases of fish is more safe than antibiotic treatment which have side effects on fish and environment as it is affect water, toxic to fish and of public health importance in food fish where there is residues in fish musculature. The application of antibiotics may lead to the development of drug resistance because of overuse or misuse. **(Gudding *et al*., 1999**). We must always remember that chemotherapy and antibiotics is the last trial for control fish diseases, as most chemicals used for treatment can affect pathogen as well as the fish.

Concerning the effects of flavobacteriosis in cultured Humor fish on red cells counts, the study revealed that there is decrease in the mean red cells counts, in infested fish in comparison to control group but not significant, these results may be due to hemorrhage and ulcers occur in damaged and destructed skin and gill filaments as the results of infection with flavobacteria also may be due to off food of infested fish. There were significant alteration in total WBCs count in infested fish and treated with oxytetracycline. These results may be attributed to stimulation of hemobiotic organs and immune system due to infection with flavobacteria in contrast, the group treated with humic acid there is no alteration in WBCs count these may be due to the effect of humic acid on the physiological status of cultured Humor fish improving it, stabilizing WBCs count near to the control group improving immunological status of fish **(Osman *et al*., 2009**).

Disturbance in serum constituents of infested fish was represented as increase cortisol level in group treated by oxytetracycline. It is hormone released by the adrenal tissue in response to stress. Studies on various fish indicate that cortisol levels increase rapidly and dramatically when fish are stressed. Cortisol, like epinephrine, can also produce many physiological changes. If prolonged, these changes can lead to metabolic imbalances, such as increases in protein breakdown and elevated thyroid hormones, which can further increase the demands on the body, leading to biochemical exhaustion **Martinez-Porchas (2009)**.

Results of the present study revealed significant

elevation of cortisole level during oxytetracycline treatment. The effect of cortisol on the immune system that is central to the investigation. Cortisol can directly interfere with the normal functioning of the immune system. It is this disruption of the system that leads to the diseases associated with stress, particularly bacterial infections. Specifically, cortisol is known to interfere with the immune process known as phagocytosis. Phagocytosis, which actually means "cell eating," refers to the actions of certain white blood cells that "consume" bacteria or other foreign materials that enter the body. This process is often the first line of defense when bacteria break through the skin and mucous membranes or through wounds **Patiño *et al.* (1987**). These white blood cells, called macrophages (meaning big eaters), are mobilized by chemical signals to move into the area where bacteria have managed to penetrate the body. Once these phagocytic cells arrive at the scene, they surround the invading bacteria and engulf them.

The immune response was depressed by administering oxytetracycline. That can suppress the immune response in carp and rainbow trout. Thus should also be limited in it's use because it increases bacterial resistance to treatment and interferes with the immune response, the results of the present study Agree with **Zagury (2006)** who reported that Tetracycline causes depletion of the immune system, also **Challem (1996)** reported that that some antibiotics prevented white blood cells from attacking and killing bacteria. This author described that tetracycline-class antibiotics may be the worst offenders in this regard. Other literature reinforces the negative impact of tetracycline on immune response. Also **Banck & Forsgreen (1979)** evaluated the effect of antibiotics on suppression of lymphocyte function in vitro.

They concluded that doxycycline caused a significant depression of the mitogenic response of both B and T lymphocytes. This effect was not reversible. Antibody production by lymphocytes incubated for 6 days with doxycycline was completely depressed. Tetracyclines acts by inhibition of protein synthesis and according to these researchers this same mechanism justifies the negative effect over antibody production. As indicated by **Korkelia (1971),** the catabolic effects in patients receiving normal doses of tetracycline could completely abolish the effect of parenteral nutrition by inhibiting the utilization of amino acids for protein synthesis. In addition chemotactic response is also inhibited by doxycycline (**Banck & Forsgreen, 1979).** Also Consistent with those results **Grondel *et al.* (1985a; 1985b**) demonstrated that low concentrations of tetracycline delay leukocytes mitosis, which means that these drugs impact the number of cells available to guarantee the cellular immune response. **Stetsenko (1981**) evaluated the impact of tetracycline administered orally in daily doses on the immune and hematopoietic systems of rabbits and concluded that this antibiotic causes the depletion of the immune system

In conclusion, Flavobacteria infection in cultured Humor fish should be diagnosed and treated as quick as possible to avoid fish mortalities and economical losses, using natural treatment such as humate substances which is recommended and preferable than antibiotics (oxytetracycline) treatment in fish bacterial infection as their application is cheap , more safe, and of little side effects on fish .

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