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Marine Flexibacteriosis (Tenacibaculosis), Infection In Red Sea Cultured Asian Sea Bass *Lates calcarifer* Barramundi In Saudi Arabia With Trials For Treatment Using Oxytetracycline And Florfenicol.

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ABSTRACT

The present study investigate pathogencity of *Flexibacter maritimus* (*Tenacibaculum maritimum)* in Red Sea cultured Asian Sea bass, *Lates calcalrifer* fish with a trial for treatment and control using antibiotics florfenicol and oxytetracycline. Naturally infected fish Sea bass, *Lates calcalrifer* fish was stocked in circular cement and earthen ponds along Red Sea coast , Fisheries Research center, Jeddah belonged to Agricultural ministry Saudi Arabia. Large number of Asian Sea bass fish suffering from discoloration, deep ulceration on the body especially back region, erosions and hemorrhages at the base of fins. A total number 180 naturally infected Asian Sea bass fish were divided into three groups each 20 with three replicates 1st group infested non treated, 2nd group treated with florfenicol 10 mg/kg body weight of fish in diet for 10 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, cortisol concentration of serum and phagocytic index were recorded along 3 weeks post treatment. The study concluded that *Flexibacter maritimus* infection in Asian Sea bass fish should be diagnosed and treated as quick as possible using florfenicole to avoid fish mortalities and economical losses.

Keywords: *Flexibacter maritimus* - Red Sea cultured Sea bass – Lates *calcalrifer* –florfenicol - oxytetracycline .

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INTRODUCTION

Marine *flexibacteriosis (tenacibaculosis*) , infection in fish are caused by the genus *Tenacibaculum* is part of the family Flavobacteriaceae and currently consists of 15 species. They are found as part of the environment in water, tidal flats and mud. Some species are pathogens of fish, some have been isolated from diseased fish and sea anemone, but virulence capacity is yet to be established, and some are part of the microflora of oysters [1].

Flavobacteriaceae are responsible for devastating losses in wild and farmed fish stocks around the world. In addition to directly imposing negative economic and ecological effects, flavobacterial disease outbreaks are also notoriously difficult to prevent and control despite nearly 100 years of scientific research [2]. This pathogen, also recognized as an important problem worldwide, has been first reported in Chile in 1993 and is currently affecting all three cultivated salmonid species: Atlantic salmon (*Salmo salar*), Coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*) [3]. The bacterium was described initially and recovered in 1948 from a die-off in coho salmon (*Oncorhynchus kisutch*; Borg, 1960) [4].

Tenacibaculum species are strictly aerobic, Gram-negative rods that do not have flagella but are motile by gliding. They may or may not adhere to agar. A yellow pigment is produced that is mainly zeaxanthin. Flexirubin-type pigment is not produced [5].

Tenacibaculum maritimum is the causative agent of *tenacibaculosis*, an ulcerative disease that affects a variety of marine fish species. Over the years a number of names have been used for the disease including salt water columnaris disease, bacterial stomatitis, black patch necrosis and eroded mouth syndrome. Clinical signs typically include ulcers and necrosis on the body surface, eroded mouth, frayed fins and tail rot, and the disease may progress to septicaemia [6].

The first isolation was in Japan from diseased cultured red seabream (Pagrus major) fry 15 to 60 mm in length, gilthead or black seabream (Acanthopagrus schlegeli) fry and rock bream (Oplegnathus fasciatus) [7,8]. Infected fish displayed eroded mouths, frayed fins, tail rot, and skin lesions that progressed to shallow ulcers. Mortality rates were 20–30%.

Florfenicol (FFC) is the broad-spectrum antibiotic. It is approved in the USA for control of mortality due to enteric septicemia associated with *Edwardsiella ictaluri* in catfish (USFDA 2009a), for coldwater disease associated with Flavobacterium psychrophilum, Florfenicol is efficacious against a number of fish pathogens [9. 10. 11. 12. 13. 14]. Worldwide, FFC is currently approved for selected aquaculture indications in 25 countries (R. Endris, Intervet/Schering-Plough Animal Health, personal communication). In 15 of these countries, FFC is specifically approved for use against all susceptible bacteria including *F. columnare* [15].

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 [16]. Thus the present study investigate pathogenesis of *Flexibacter maritimus* infection in Red Sea cultured Asian Sea bass *Lates calcalrifer* barramundi fish with a trial for treatment using antibiotics florfenicl (FFC) and oxytetracycline OTC.

MATERIAL AND METHODS

Naturally infected fish

Broadstock Asian Sea bass fish was stocked in cement and earthen ponds along Red Sea coast in Fisheries Research Center, Jeddah, Saudi Arabia. Large number of fish suffering from discoloration, ulcers on the skin, erosion in fins. Great numbers of fish died, body weight, clinical and postmortem changes were recorded [17]. Specimens of fish was taken alive for bacteriological examination in microbiology lab., Fisheries Research Center, Jeddah, Saudi Arabia.

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Bacteriological examination

Identification of *Tenacibaculum species*

Presumptive Diagnosis

The following criteria provide a basis for presumptive diagnosis: observation of characteristic clinical signs and the presence of slender Gram-negative rods in parallel in lesions; dry rhizoid, yellowish colonies are produced on [18] containing sea salts (AO-M) or *Flexibacter maritimus* FMM after three days incubation at 25 °C; cells are motile by gliding or flexing, but lack flagella. The tendency of the bacterium to form mounds (haystacks) or columns, as detected in wet mounts of diseased tissue, also aids diagnosis.

Confirmatory diagnosis

Inoculation of skin lesion on cytophaga medium prepared in 70% seawater, also known as Anacker-Ordals (AO) medium containing sea salts (AO-M). [18] or FMM (4 days at 25-28 ^{O}C).

Cellular morphology was determined by Gram stain and the bacterial cells were observed under a microscope (magnification, X100). Motility was determined on wet mounts under phase contrast microscopy (magnification, X40). The other tests used were: colony appearance and consistency, pigment production after exposing cultures to KOH (20%) solution. Colonies having a flexirubin type of pigment exhibit an immediate color shift from yellow or orange to red, purple or brown when flooded with 20% KOH and revert to their initial color when flooded by an acidic solution [19]**.** Gliding motility was checked under a microscope (magnification, x 100) by using a hanging drop preparation of a 48 hours liquid medium culture. The presence of flexirrubin type pigments, Congo red absorption, Congo red absorption was assessed by flooding plates containing colonies with a 0.01 % aqueous solution of Congo red for a few seconds, followed by rinsing with tap water. Catalase was determined with hydrogen peroxide. DNAse, Starch hydrolysis, The starch hydrolysis was determined using medium containing a 0.2% (w.v–1) solution of soluble starch on which colonies that hydrolyzed starch produced clear areas, Gelatin liquefaction were all as described by [20]**.** Gelatin degradation was tested with the liquid medium supplemented with 0.4% (w.v–1) gelatin which was flooded with ammonium oxalate. Gelatin degradation was indicated when a local clearing was observed in the medium, Production of acid from glucose was determined using liquid medium supplemented with 0.4% dextrose, Casein hydrolysis was determined with agar medium supplemented with 10% dry milk. The plates were flooded with 1% HCl five days after inoculation, in which clear areas were indicative of proteolysis, The ability to reduce nitrates was investigated in 0.1% potassium nitrate broth tubes.

Blood sampling

Five randomly chosen fish from each experimental and control group, were taken and anaesthetized with clove oil (0,1-0,06 mg/L) of water, and used for blood collection. Blood samples were collected from each group from the caudal vein before treatment ,7th, 14 day and 21 days after final feeding. Blood samples were divided into two portions, first portion with anticoagulant for determination phagocytic index , the other portion without anticoagulant for serum separation for determination of cortisole concentration in serum. Blood was collected from caudal vein, with 1 ml plastic syringe rinsed with anticoagulant, (tri sodium citrate) 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 after centrifugation and stored at - 20°C.

Determination of Cortisol concentration

Cortisol concentration was determined in serum according to [21]**.**

Phagocytosis assay

Phagocytosis was determined as follow: 50 µg of well identified *Candida albicans* strain culture (previously adjusted to be 100 mg/ml) were added to1ml of pooled samples collected from treated and control fish, then put in water bath at 23-25 ^{o}C for 3-5

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hours. Air dried blood smears fixed with methanol and stained with Giemsa stain, phagocytic index was calculated as follow:

Phagocytic index (PI)

Number of yeast cells phagocytized / Number of phagocytic cells according to [22].

Experimental design for treatment

A total number of 180 naturally infected Asian Sea bass 250-300 gm body weight were randomly collected from infected pond for treatment experiment. They were divided into three groups with three replicates 20 fish each, 1st group was kept as a control without treatment, 2nd group subjected for treatment with Florfenicol 10 mg/kgm body weight in diet (was sprinkled on the dry pellets to adsorb into it then dried in incubator at 25 °C) for 10 successive days [15], and 3rd group was subjected for treatment with oxytetracycline 75 mg/kg body weight of fish in diet for 10 successive days [23]. Each group was isolated in a separate fiber glass tank 500 liter containing sea water with continuous aeration at temperature 27 ± 2 °C, pH 7.55, dissolved oxygen 5±2 mg/l. and salinity (45 g/l).

Statistical Analysis

The statistical analysis was performed by one way ANOVA analysis of variance according to [24]. 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 signs of infected Asian Sea bass fish were represented as sluggish movement, loss of escape reflex (fig, 1) , sloughing of scales, ulceration of the skin at the region of dorsal of the body (saddleback disease) (fig. 2) . Fading of skin color, and eroded dorsal and caudal fins with protruded eyes (pop eyes) (fig. 2 and fig. 3), Off food and finally death. Postmortem examination revealed that congestion of gills, congestion and inflammation with enlargement of the liver, gall bladder, spleen and kidney with serous fluid accumulation in the peritoneal cavity (fig. 4).

Figure 1: Showing Asian Sea bass *Lates calcalrifer* live swimming in the Red Sea cultured earthen pond suffered from large and deep ulcers on the back (saddleback disease) and caudal fin growing on it green algae accompanied with destroyed opaque eyes.

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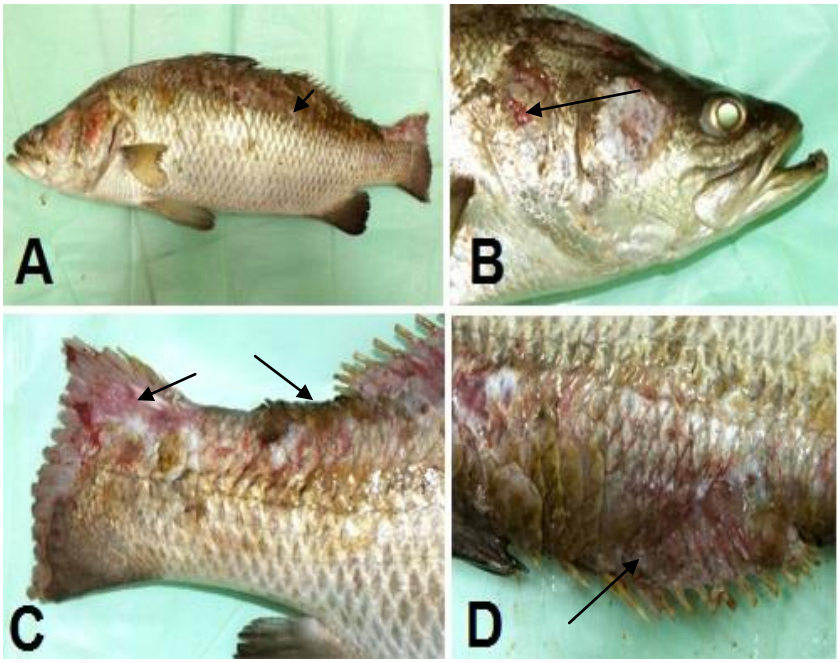

Figure 2 : Showing Asian Sea bass barramundi suffered from (A&B) sloughing of scales with deep ulcers on dorsal region, caudal peduncle and caudal fin (arrows) (C&D) Caudal fin and dorsal region (arrows)

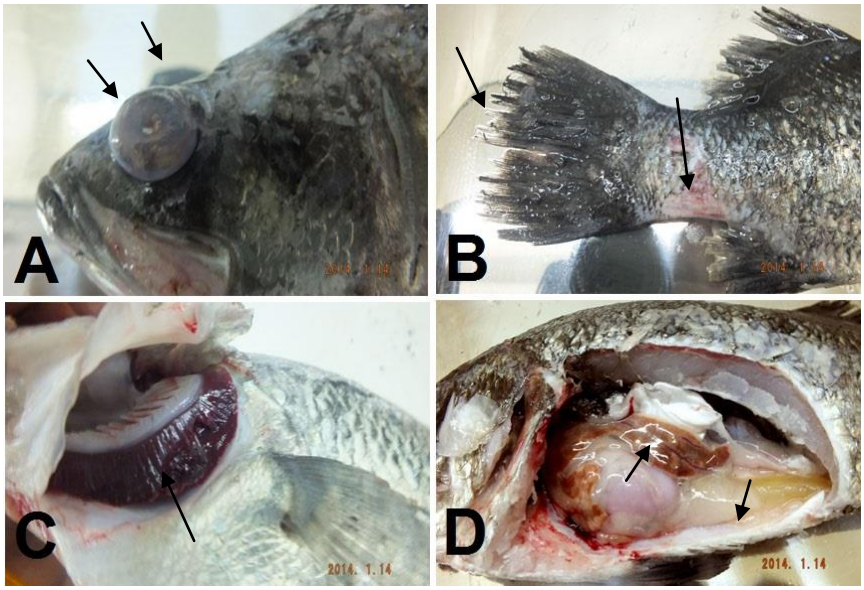

Figure 3: Showing Asian Sea bass barramundi suffered from (A) Bulged eyes (arrows) (B) Sloughing of scales with ulcers on caudal peduncle with eroded caudal and dorsal fins (arrow) (C) Congested slimy gills (arrow) (D) inflamed liver with ascetic fluids in the body cavity (arrow)

Bacteriological examination

The isolated bacteria is an aerobic, oxidase positive, Gram-negative, slender, flexible rod (0.5×2–30 µm. Cells from older cultures are shorter in length and spherical cells may be seen. The latter are not viable. Flagella are not present but cells have a gliding motility that can be seen in a wet preparation (hanging drop) made with seawater. Growth occurs on cytophaga medium containing 70 % sea water, also known as Anacker-Ordal's (AO) medium containing sea salts (AO-M). Colonies are slow growing, thin, flat, light yellow in colour and rhizoid and grow to about 5 mm after 4–5 days at 25°C. In liquid medium growth is seen as a thin layer of growth (pellicle) at the surface. Growth does not occur anaerobically. Growth occurs at 15–34°C with an optimum of 28°C. Growth does not occur when AO agar is supplemented with NaCl. At least 30% seawater is

required for growth. Growth also occurs on Flexibacter maritimus medium as light yellow, flat colonies with irregular edges. This medium contains the same ingredients as MA 2216 but without the chloride, phosphate and other ions contained in MA 2216. It also contains sodium acetate. Good growth occurs on MA 2216 . Glucose, galactose, fructose, cellobiose and rhamnose are not fermented. Agar, chitin and starch are not degraded but casein is degraded. Colonies absorb Congo red stain flexirubin-type pigments.

The isolated bacteria are biochemically characterized by conventional methods in plates and tubes. Biochemical characterization showed that the gliding bacteria belonged to the species *Flexibacter maritimus*, Criteria for presumptive identification of *F. maritimus* include Gram stain morphology, motility, oxidase and catalase production, agar hydrolysis, seawater requirement, Congo red reaction, and flexirubin pigment.

Table 1: Mortality rate during treatment of infected fish with Florfenicol and oxytetracycline

groups	Number of treated fish	Number of died fish	survival	Percentage of mortality %
Control group	20	14	6	70
Florfenicol group	20	0	20	0
Oxytetracycline	20	2	18	10

Results of mortality rate during treatment

Percentage of mortality rate in control group (without treatment) was 70% while in group treated with florfenicol was 0 % and 10% in group treated by oxytetracycline.

Table 2 : Determination of Cortisol concentration mg/ml in Asian Sea bass fish for 3 weeks after treatment

groups / day	Before treatment	7th day*	14th day*	21 day*
Group 1 (infected non treated)	32±1.6A	31±1.5A	37±1.8A	33±1.7A
Group 2 (Florfenicole)	37±1.8B	517±25.8b	211±10.5b	41±2.5B
Group 3 (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 raw (by LSD using ANOVA at p < 0.05).

Table 3 : Determination of phagocytic index % in Asian Sea bass for 3 weeks post treatment

groups / day	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 (Florfenicole)	113.5±5.6 B	114.0±7.2B	117.0±5.8B	125.3±6.2B
Group 3 (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 raw (by LSD using ANOVA at p < 0.05).

Cortisol concentration (mg/ml) and phagocytic index % in Asian Sea bass for 3 weeks post treatment:

Cortisole concentration in serum was significantly increased in group treated with Florfenicol 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 Florfenicol not significantly differ than those before treatment while after 3 weeks increased than those before treatment , in contrast the group treated with oxytetracyclin displayed significant decrease of phagocytic index for three weeks post treatment tables 2 and 3 .

DISCUSSION

The present study aimed to investigate the pathogenesis of *Flexibacter maritimus* in Red sea cultured Asian Sea bass with a trial of treatment using 2 common antibiotics oxytetracycline and Florfenicol to flavobactreria infection, in Asian Sea bass *L. calcalreifer*.

The isolated bacteria was identified as *Flexibacter maritimus* and the confirmation was carried out by biochemical tests.

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 skin at the region of dorsal of the body. Fading of skin color, and eroded dorsal fins [25]. Off food and death. Postmortem examination revealed that congestion of gills, inflammation and enlargement of the liver, spleen and kidney with serous bloody fluids accumulation in the peritoneal cavity the clinical sings coincide with the finding of [26.27].

The main clinical signs observed where it was clear from pathogencity of *Flexibacter maritimus*. 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. In adult fish it affects 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 [28]. Asphyxia and respiratory dysfunction may be attributed to damaged epithelium of gill filaments of secondary gill lamellae which caused by *Flexibacter* infection as it is the target organ for the pathogen [26.4].

Concerning the effect of oxytetracycline and florfenicole in treatment *Flexibacter maritimus* infection in Asian Sea bass, it is clear from the results that oxytetracycline was highly effective against *Flexibacter maritimus* infection at the dose of 75 mg/kg fish for 10 days. These results nearly agreed with finding given by [23] on the other hand the use of florfenicol at the dose of 10 mg/kg diet for 10 days was highly effective in treatment of flavobacteriosis in infected Sea bass, the results nearly agree with the results met by [15] who reported that In agreement with previously conducted studies with catfish fingerlings, there were no visible gross lesions associated with the FFC treatment [29. 30] **.** These comparative mortality data demonstrate that florfenicol administered to catfish infected with F. columnare is safe, significantly reduces mortality, and should be valuable for control of columnaris disease in farmed catfish. Romet and oxytetracyline are prescribed [31]. However, there are reports of resistance to Romet [31] and oxytetracyline [32]**.**

Regarding the comparison of the effect of the 2 antibiotics on *Flexibacter maritimus* we can make sensitivity test in vitro but in the present study we made experimental treatment on natural infected Sea bass in vivo to determine the effect of 2 antibiotics on the natural immune response and bacteria together at the same time. Now today, most pathogenic organisms are becoming resistant to antibiotics [33]**.** 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 [34] .

Concerning the effect of treatment with antibiotics on the level of cortisol in serum and phagocytic index, the present study revealed that Cortisol concentration in serum was significantly increased in group treated with Florfenicol up to 2 weeks after treatment but after 3 weeks it returned normally as before treatment. The concentration of cortisol 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 Florfenicol not significantly differ than those before treatment while after 3 weeks increased than those before treatment in contrast, the group treated with oxytetracyclin displayed significant decrease of phagocytic index for three weeks post treatment the results confirmed by [34] who reported that her study revealed significant

elevation of cortisol level during oxytetracycline treatment. and **[35]** who reported that OTC had a suppressive effect on specific and non-specific immune system parameters, such as leucocyte counts, oxidative radical production (nitrobluetetrazolium activity), total plasma protein and immunoglobulin levels, and phagocytic activity.

Increase cortisol concentration 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 [36 and 34].

Present study revealed significant elevation of cortisole level during oxytetracycline treatment. 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. 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 [37]. The 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 it [34]**.**

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 [38] who reported that Tetracycline causes depletion of the immune system, also [39] reported that some antibiotics prevented white blood cells from attacking and killing bacteria. The 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 [36] 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 [37]**,** 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 [36]**.** Also Consistent with those results [38. 39] 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. In addition of [32] who reported that OTC also had a suppressive effect on specific and non-specific immune system parameters.

The immune system in the body is natural defense mechanism against infection. It allows the body to fight against invasion by bacteria, viruses, yeast, fungus etc. Taking antibiotics reduces the level of bacterial infection, but the immune system still has to completely finish fighting the infection. Once the body has a particular infection, and the body fights it without the use of antibiotics, the immune system will develop 'memory T cells'. The next time if the body contract the same infection, these memory T cells "remember" the previous infection and mounts an immediate immune response to fight it [40].With the use of antibiotics we are giving the responsibility of fighting infection to the antibiotics instead of the body's immune system. So overtime, with the overuse of antibiotics the immune system can become less effective. With taking antibiotics, the immune system can become weakened, meaning that the body is more susceptible to infection than before taking the antibiotics.

In conclusion, *Flexibacter maritimus* infection in Red Sea cultured Asian Sea bass should be diagnosed and treated as quick as possible to avoid fish mortalities and great economical losses, using florfenicole which is recommended and preferable than oxytetracycline treatment (immune suppression) in fish bacterial infection as its application is effective, more safe, and of little side effects on fish.

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