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Role of Some Antibacterial Drugs in Control *Streptococcus iniae* Infection in *Oreochromis niloticus*

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Abstract

Streptococcus iniae infection is considered one of the most important diseases affecting cultured fish causing severe economic losses. This work was conducted to determine the efficacy of some antibacterial drugs in treatment of Nile tilapia (*Oreochromis niloticus*) fish experimentally infected by *Streptococcus iniae* which were done through; determine the sensitivity of *Streptococcus iniae* to different antibiotics, detect the lethal dose fifty (LD_{50})of tested *Streptococcus iniae* strain in *Oreochromis niloticus* followed by treatment of the experimentally infected fish with florfenicol, norfloxacin and oxytetracycline at a dose of 50, 100 and 100mg/kg body weight respectively by oral gavage. AST, ALT, uric acid and creatinine in fish serum was studied together with histopathological examination of liver and posterior kidney tissues.

Tested *Streptococcus iniae* strain was sensitive to Florfenicol, Norfloxacin and Oxytetracycline with MIC equal to 8, 0.25 and 2µg/ml respectively. The LD_{50} of *Streptococcus iniae* in *Oreochromis niloticus* weighting 80 ± 5g was 0.2 ml of (6×10^8) CFU/ml by intraperitoneal inoculation. There were no mortalities in infected florfenicol and norfloxacin treated groups while it was 20% in infected oxytetracycline treated group. Florfenicol, norfloxacin and oxytetracycline can be considered an effective treatment for control of susceptible *Streptococcus iniae* infection in *Oreochromis niloticus* at a dose level of 50, 100 and 100 mg/kg body weight respectively for seven successive days.

Keywords: *Streptococcus iniae; Oreochromis niloticus*; Florfenicol; Norfloxacin; Oxytetracycline; LD_{50}; AST; ALT; Uric acid; Creatinine; Hepatopancreas; Posterior kidney; Histopathology

Abbreviations: NIOF: National Institute of Oceanography and Fisheries; LD_{50}: Lethal Dose; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase

Introduction

According to FAO, tilapias *(Oreochromis sp.*) are among the most cultured fish worldwide as it rose commercially in more than 100 countries [1]. Egypt is producing about 71.38% of total cultured fish in Africa and occupies the eighth level all over the world as it is producing about 919585 tons of cultured fish that represents 1.54% of total cultured fish all over the world according to [2] report. Streptococcal infection in fish is a septicemic disease that has been reported worldwide causing severe economic losses in fish production [3]. It has been reported in many other fish species throughout the world, contributing to an annual loss of approximately 250 million USD annually in 2008 [4]. *Streptococcus iniae* is an encapsulated, non-Lancefield group, beta-hemolytic, gram-positive cocci [5]. *Streptococcus iniae* produces a cytolysin with haemolytic traits, which is a functional homologue of streptolysin S. Expression of this cytolysin is necessary for local tissue necrosis [6].

Increased AST and ALT as a result of hepatic cells destruction together with increased creatinine due to posterior kidney destruction in infected fish was recorded by [7]. Presence of degenerative changes in liver and posterior kidney tissues of experimentally infected *Oreochromis niloticus* was recorded by [8]. Antibiotics are very useful tool to any fish-health manager's to help in elimination of bacterial fish diseases [9]. Oxytetracycline was found to be the antibiotic having the highest

frequency of application in aquaculture, while the second most widely used antibiotic was florfenicol followed by norfloxacin [10]. The aim of the present work is to determine the efficacy of Florfenicol, Norfloxacin and Oxytetracycline in treatment of Nile tilapia (*Oreochromis niloticus*) experimentally infected by *Streptococcus iniae* from the survival percent, together with detects improvement of liver and posterior kidney organ function can result by termination of infection.

Materials and methods

Drugs

Floricol® 10% florfenicol oral solution and Oxyvet® 20% Oxytetracycline Hcl water soluble powder from Pharma Swede co, Egypt. Epinor® 400mg Norfloxacin tablets EIPICO, Egypt were used.

Fish diet

Fish were fed on commercial extruded pellet ration containing 35% total protein. Feeding was performed once daily as 3% of fish body weight according to [11].

Bacterial strain

A virulent strain of *Streptococcus iniae* was obtained kindly from the fish disease lab, National Institute of Oceanography and Fisheries (NIOF), Alexandria branch, Egypt.

Bacteriological Media

Tryptic Soy broth, Tryptic Soy agar, Muller - Hinton broth and Muller - Hinton agar manufactured by Oxoid, U. K were used in growth and sensitivity tests for *Streptococcus iniae*.

Sensitivity discs

Sensitivity discs of florfenicol, norfloxacin and oxytetracycline, (Bioanalyse) ®, Turkey are used.

Anesthetic

Quinaldine (Argent), USA was used as a fish anesthetic during all experiments at a dose of 25mg/l as described by [12].

Kits

Kits for determination of ALT, AST, creatinine and uric acid were manufactured by Bio Diagnostic, Egypt.

Antimicrobial sensitivity tests

Agar disc diffusion test was performed as described by [13] and broth micro dilution test was performed as described by [14].

Experimental design

I. Experimental animals: A total number of 110 apparently healthy Nile tilapia (*Oreochromis niloticus*) weighing 80 ± 5g were used. They were maintained 15 days prior to the experimental infection for acclimatization according to [15]. Fish were mentioned in 400 litter fiberglass tank supplied with dechlorinated tape water, with continues aeration, 20% water change was done each other day.

II. Pathogenicity test: Sixty apparently healthy Nile tilapia (*Oreochromis niloticus*) fish weighting 80 ± 5g were used in the pathogenicity test to determine the median lethal dose (LD_{50}) of *Streptococcus iniae* according to method described by [16]. Six equal groups each contain 10 fish, the first group (control) injected intraperitoneal with 0.2 ml of sterile (PBS), the second group injected intraperitoneal with 0.2 ml of Streptococcus iniae contain 6×10^8 CFU / ml sterile (PBS), and each following group inoculated with 10 fold serial dilution. Each group were maintained in 100 litter glass aquarium supplied with dechlorinated tape water, with continues aeration, 20% water change was done each other day.

III. Determination of in vivo efficacy of antibiotics in treatment of experimentally infected fish with *Streptococcus iniae*: Fifty apparent healthy O*reochromis niloticus* fish weighting 80 ± 5g were grouped into 5 groups 10 fish in each group. Group 1 was negative control inoculated with 0.2ml each fish with sterile (PBS) by intraperitoneal injection and group 2, 3, 4 and 5 were inoculated with 0.2ml PBS containing (6×10^8) CFU (LD_{50}) for induction of experimental infection. Drugs were administrated 24 hours post experimental infection by oral gavage daily for 7 days as shown in (Tables 1).

Table 1: Used drug doses and duration of treatment.

Group	Treatment
Group 1: Non-infected Non-treated	-
Group 2: Infected non-treated	-
Group 3: Infected Florfenicol treated	Florfenicol 50mg/kg B.W. once daily by gavage for 7 days
Group 4: Infected Norfloxacin treated	Norfloxacin 100mg/kg B.W. once daily by gavage for 7 days
Group 5: Infected Oxytetracycline treated)	Oxytetracycline 100mg/kg B.W. once daily by gavage for 7 days

IV. **Blood samples:** Blood was collected via heart puncture according to the method described by [17]. Samples were collected from each group one day after end of treatment. Serum was separated by centrifugation at 3000 rpm for 5 min and kept at -20°C for biochemical analysis.

V. **Serum biochemical analysis:** The activity of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) was determined according to [18]. Serum creatinine was determined according to the method described by [19] and Serum uric acid was determined according to the method described by [20].

VI. Histopathological examination: Hepatopancreas (liver) and posterior kidney tissue specimens were taken

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from all groups one day after end of treatment then were immediately fixed in 10% neutral buffered formalin, and then dehydrated, in ascending grades ethyl alcohol. Samples were cleared in three changes of xylol, and then were impregnated in paraffin wax cubes sectioned 4-5µ thickness. Sections were mounted on slides and stained with haematoxylin and eosin according to the method described by [21].

VII. Statistical analysis: The data were statistically analyzed for variance (ANOVA) and least significant difference as described by [22] using (SPSS version 16) computer statistical software. Data were evaluated as significant at $P \leq 0.05$.

Results

Agar disc diffusion test and broth micro dilution test revealed sensitivity of *Streptococcus iniae* to florfenicol, norfloxacin and oxytetracycline with MIC equal to 8, 0.25 and 2µg/ml respectively (Table 2). Pathogenicity test revealed that the LD_{50} of *Streptococcus iniae* in *Oreochromis niloticus* fish weighting 80 ± 5g was 0.2ml of (6×10^8) CFU / ml by intraperitoneal inoculation (Table 3) and (Figure 1). The mortality rate at the end of experiment was shown in (Table 4) and (Figure 2) in which infected florfenicol and infected norfloxacin treated groups showed no mortalities while oxytetracycline group showed 20% mortality. Serum biochemical analysis of liver function tests in (Table 5) and (Figures 3 & 4) and kidney functions tests results were shown in (Table 6) and (Figures 5 & 6). The histopathological examination of hepatopancreas of different groups was shown in (Figures 7A -7E) and that of posterior kidney tissue shown in (Figures 8A -8E).

Table 2: Antimicrobial sensitivity and Broth micro dilution tests results.

Antibiotic	Disc concentration (µg / disc)	Inhibition Zone diameter mm	MIC (µg / ml)
Norfloxacin	NOR (10)	22.15	0.25
Florfenicol	KF (10)	22.55	8
Oxytetracycline	OT (30)	19.35	2

Table 3: Pathogenicity test result.

Group	Inoculums	Mortality %
Group (1)	0.2 ml of sterile PBS.	0%
Group (2)	0.2 ml of (6×10^8) CFU/ml	50%
Group (3)	0.2 ml of (6×10^7) CFU/ml	20%
Group (4)	0.2 ml of (6×10^6) CFU/ml	0%
Group (5)	0.2 ml of (6×10^5) CFU/ml	0%
Group (6)	0.2 ml of (6×10^4) CFU/ml	0%

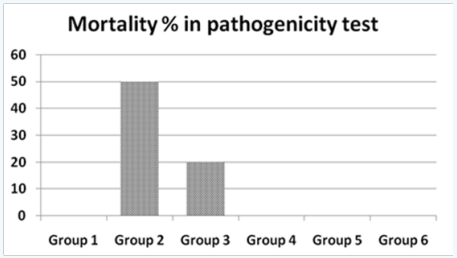

Figure 1: LD_{50} of *Streptococcus innae* to *Oreochromis nilocticus*.

Table 4: The mortalities rate at the end of treatment.

Group	Treatment	Mortality %
Group (1)	Non-infected non-treated	0%
Group (2)	Infected non-treated	60%
Group (3)	Infected florfenicol treated	0%
Group (4)	Infected norfloxacin treated	0%
Group (5)	Infected oxytetracycline treated	20%

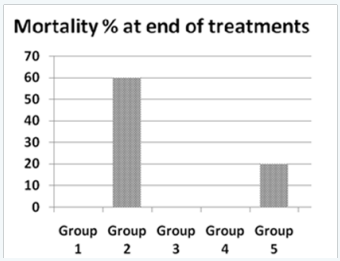

Figure 2: Mortality rate at end of treatment trial.

Table 5: Serum ALT and AST level of different groups.

Group	ALT U/L	AST U/L	No. of samples
Group (1)	39 ± 2.19^{b}	141.12 ± 5.09^{b}	8
Group (2)	55 ± 2.58^{a}	172.33 ± 4.77^{a}	6
Group (3)	46.42 ± 2.04^{bc}	154.57 ± 5.82^{bc}	7
Group (4)	45.37 ± 3.12^{bc}	149 ± 5.23^{b}	8
Group (5)	51.83 ± 3.21^{ac}	167 ± 5.68^{ac}	6

Values are mean ± S.E. data were compared by ANOVA, values at the same column and has the same letter are non-significant at $P \leq 0.05$.

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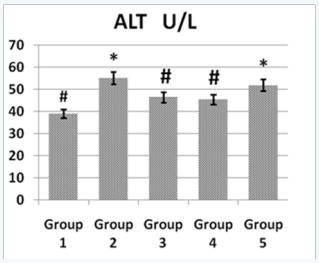

Figure 3: Serum alanine aminotransferase (ALT) level.
*Significant change with Non-infected non-treated group.

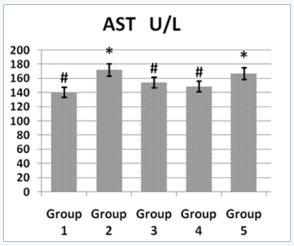

Figure 4: Serum aspartate aminotranferase (AST) level.
#Significant change with infected non-treated group.

Table 6: Serum creatinine and uric acid level of different groups.

Group	Creatinine mg/dl	Uric acid mg/ dl	No. of samples
Group (1)	0.22 ± 0.015^{b}	1.65 ± 0.079^{b}	8
Group (2)	0.35 ± 0.012^{a}	2.27 ± 0.083^{a}	6
Group (3)	0.28 ± 0.020^{bc}	1.83 ± 0.053^{bc}	7
Group (4)	0.26 ± 0.025^{bc}	1.77 ± 0.096^{bc}	8
Group (5)	0.31 ± 0.022^{ac}	1.98 ± 0.183^{bc}	6

Values are mean ± S.E. data were compared by ANOVA, values at the same column and has the same letter are non-significant at P ≤ 0.05.

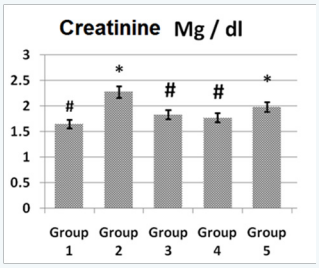

Figure 5: Serum creatinine level.
*Significant change with Non-infected non-treated group.

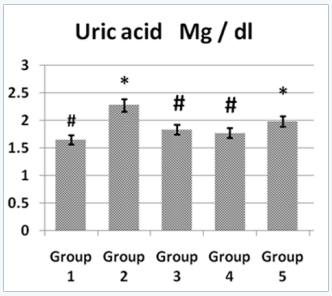

Figure 6: Serum uric acid level.
#Significant change with infected non-treated group.

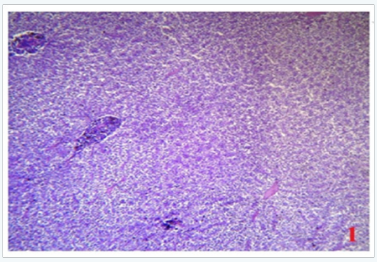

Figure 7(A): Hepatopancreas of normal fish showing normal tissue architecture, normal hepatocytes with absence of inflammatory reaction and normal blood sinusoids (X = 200).

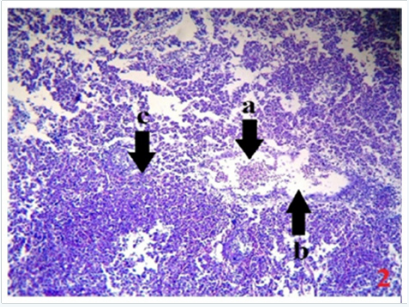

Figure 7(B): Hepatopancreas of infected non treated fish showing haemorrhage (a), necrosis (b) and mononuclear cell infiltration (c). (X = 200).

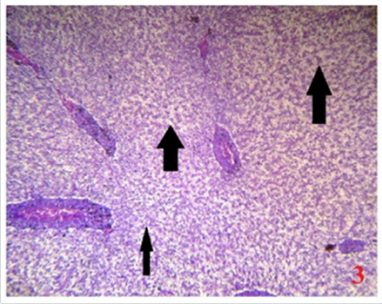

Figure 7(C): Infected florfenicol treated group hepatopancreas showing presence of mild to moderate hepatic cell vacuolation (arrow) with absence of inflammatory reaction (X = 200).

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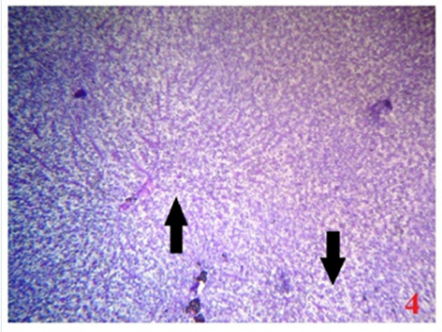

Figure 7(D): Infected norfloxacin treated group hepatopancreas showing presence of mild hepatic cell vacuolation (arrow) with absence of inflammatory reaction (X = 200).

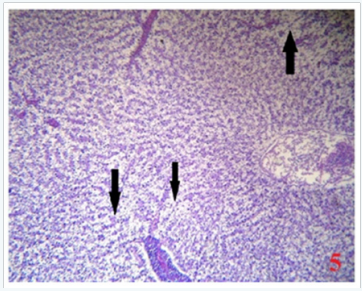

Figure 7(E): Infected oxytetracycline treated group hepatopancreas showing presence of moderate to severe hepatic cell vacuolation (arrow) with absence of inflammatory reaction. (X = 200).

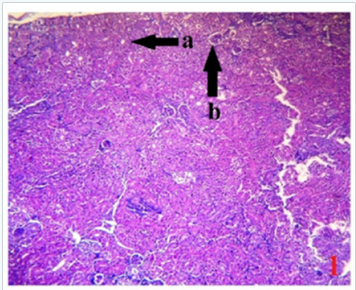

Figure 8(A): Posterior kidney of normal fish showing normal tissue architecture, normal convoluted tubules (a) normal glomeruli and Bowman's space (b) absence of inflammatory reaction or congestion (X = 200).

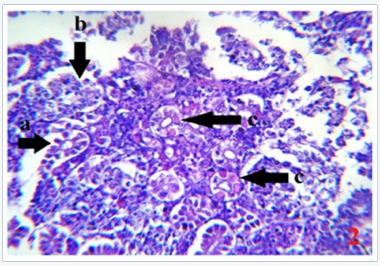

Figure 8(B): Posterior kidney of infected non treated fish showing degenerated renal tubules showing sloughing of tubular epithelium (a), degeneration of glomerular tuft with presence of hyaline droplet degeneration (c) of tubular epithelium (X = 800).

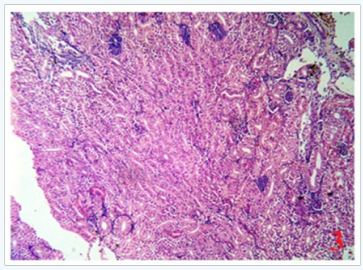

Figure 8(C): Infected florfenicol treated group posterior kidney showing normal tissue architecture, normal convoluted tubules, normal glomeruli and Bowman's space with absence of inflammatory reaction or congestion (X = 200).

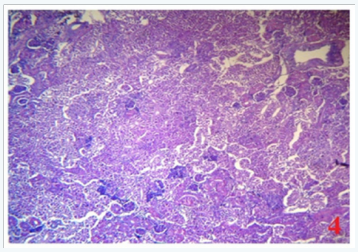

Figure 8(D): Infected norfloxacin treated group posterior kidney showing normal tissue architecture, normal convoluted tubules, normal glomeruli and Bowman's space with absence of inflammatory reaction or congestion (X = 200).

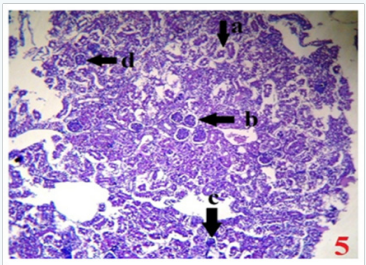

Figure 8(E): Infected oxytetracycline treated group posterior kidney showing presence of degeneration and detachment of tubular epithelium of some renal tubules (a), increase of Bowman's space (b), some glomeruli shows degeneration (c) and other showing hypertrophy (d) with absence of inflammatory reaction (X = 200).

Discussion

It was evident from the present work that LD_{50} of *Streptococcus iniae* in *Oreochromis niloticus* was 0.2 ml of 6×10^8 CFU by intraperitoneal injection. Nearly similar result was recorded by [23] where LD_{50} was 1.14×10^7 CFU. This minor difference is probably due to difference in fish weight (40-45 g). Meanwhile, [24] found that the LD_{50} of *Streptococcus iniae* in *Oreochromis niloticus* was 1×10^5 to 1×10^6. That was almost to same difference in the weight of fish used (15-25 g).

The antimicrobial sensitivity tests indicated sensitivity of the tested *Streptococcus iniae* strain to florfenicol. That was described by [24-26]. They recorded high sensitivity of *Streptococcus iniae* to florfenicol. Our results showed that the

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MIC of florfenicol against tested *Streptococcus iniae* strain was 8µg/ml. That was the same MIC of florfenicol as described by [26] while it was slightly higher than that recorded by [24] which was 2µg/ml. This difference could be attributed to the difference in the tested isolates. Results revealed a high sensitivity of tested *Streptococcus iniae* strain to norfloxacin. That was in agreement with results described by [23,27,28]. They recorded the sensitivity of *Streptococcus iniae* isolated from fish to norfloxacin. MIC of norfloxacin against tested Streptococcus iniae strain was 0.25µg/ml which was matching MIC against most aquatic pathogenic bacteria (0.244µg/ml) as described by [29]. The agar disc diffusion and broth micro dilution tests also, revealed sensitivity of tested *Streptococcus iniae* strain to oxytetracycline. That was in agreement with results described by [23,30-32]. They recorded the sensitivity of *Streptococcus iniae* isolated from diseased fish to oxytetracycline. MIC of oxytetracycline against tested *Streptococcus iniae* strain was 2µg/ml. That was higher than that recorded by [33] as they found MIC of oxytetracycline against *Streptococcus iniae* to be 0.5µg/ml. This variation may be due to the difference in the tested isolates.

It was clear that using florfenicol, norfloxacin and oxytetracycline at a dosage level of 50, 100 and 100mg/kg body weight daily by oral gavage for seven successive days respectively was effective in termination of experimental *Streptococcus iniae* infection in *Oreochromis niloticus*. There was not any recorded mortalities in infected florfenicol and norfloxacin treated groups and the survival rate was 100%. It was 80% in oxytetracycline treated group which was greatly decreased than that of infected non treated group as its survival rate was 40%. This greater increase in survival rate and absence or decreased mortalities resulted mainly by inhibition or destruction of the causative agent under the antibacterial effect of used antibiotics. This result was in agreement with that described by [8,24,34] who recorded a decreased mortality of Oreochromis niloticus and *Oreochromis aureus* experimentally infected with *Streptococcus iniae* and treated with florfenicol. This result was also, in agreement with that described by [35-37] as they recorded decreased mortality of flounder, carp and grass carp infected with bacterial fish pathogens and treated with norfloxacin. In keeping with our results [33] recorded the efficacy of oxytetracycline in controlling *Streptococcus iniae* infection in blue tilapia *Tilapia aurea* as the group received oxytetracycline at a dose of 100mg/kg body weight for 14 days which showed a great increase in survivals which reached 98% in comparison with infected non treated group which showed a 7% survival rate.

Regarding the liver and kidney functions (AST, ALT, creatinine and uric acid) there was a significant decrease in AST, ALT, creatinine and uric acid in infected florfenicol or norfloxacin treated groups in comparison with infected non treated group. That indicated the efficacy of both drugs in termination of infection and protection of hepatopancreas and posterior kidney from destructive effect caused by the causative agent and its toxins (*streptolysin S*). In infected oxytetracycline treated group there was non-significant decrease in AST, ALT and creatinine from infected non treated group pointing to the presence of some adverse effect on both hepatopancreas and posterior kidney induced by oxytetracycline. In the same manner [38] recorded an increased AST, ALT, creatinine and uric acid of healthy nurse sharks *Ginglymostoma cirratum* inoculated with oxytetracycline at a dose level of 25mg/kg body weight.

In accordance with biochemical results of liver functions the histopathological examination of hepatopancreas of infected florfenicol or norfloxacin treated groups indicated the presence of mild hepatic cell vacuolation with absence of inflammatory reaction. Hepatic cell vacuolation could be due to the response to toxins produced by the causative agent. Meanwhile, the absence of inflammatory reaction was probably due to destruction of the invading organism under the antibacterial effect of both drugs. The histopathological examination of hepatopancreas in infected Oxytetracycline treated group indicated the presence of moderate to severe hepatic cell vacuolation with absence of inflammatory reaction. Hepatic cell vacuolation could occur as a response to both effects of exotoxins produced by the causative agent and oxytetracycline. Meanwhile, the absence of any inflammatory reaction was mainly due to destruction of invading organism under the bacteriostatic effect of oxytetracycline. In agreement with our results, [39] recorded fatty changes and vacuolations in the hepatocytes of healthy *Oreochromis niloticus* treated with oxytetracycline at a dose of 100mg/kg ration for 12 weeks.

Concomitantly with results of serum biochemical determination of creatinine and uric acid, the histopathological examination of posterior kidney tissue of infected florfenicol or norfloxacin treated groups indicated the presence of normal kidney tissue architecture, normal convoluted tubules normal glomeruli and Bowman's space with absence of inflammatory reactions or congestion. Absence of inflammatory reaction and degenerative changes was probably due to absence of destructive effect of invading organism under antibacterial effect of both drugs. The histopathological examination of posterior kidney tissue of infected oxytetracycline treated group indicated the presence of degeneration and detachment of tubular epithelium of some renal tubules, increased Bowman's space and degeneration of some glomeruli together with absence of inflammatory reaction. Degenerative changes present could be due to both effects of exotoxins released from *Streptococcus iniae* and oxytetracycline on posterior kidney tissue. Meanwhile, the absence of inflammatory reaction is mainly due to the fading of the destructive effect of the invading organism under the bacteriostatic effect of oxytetracycline. Confirming to our results [39] recorded the presence of degenerative changes of posterior kidney tissue with periglomerular lymphocytic aggregation in healthy treated *Oreochromis niloticus* treated

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with oxytetracycline. [40] also, recorded the presence of diffuse cytoplasmic vacuolization of the renal duct epithelium in kidney of *Cyprinus carpio* administrated medicated feed containing oxytetracycline at a dose level of 15mg/kg body weight.

Conclusion

It could be concluded that florfenicol and norfloxacin can be considered an effective treatment for control of susceptible *Streptococcus iniae* infection in *Oreochromis niloticus* at a dose level of 50 and 100mg/kg body weight respectively for seven successive days by oral route without any adverse effect on treated fish. With vigilance, oxytetracycline can be used in treatment and control of susceptible *Streptococcus iniae* infection in *Oreochromis niloticus* at a dose level of 100mg/kg body weight for seven successive days by oral route considering its adverse effects on hepatopancreas and posterior kidney tissue.

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