RESEARCH ARTICLE

Antibacterial Activity of Doxycycline against *Aeromonas hydrophila* in Experimentally Challenged African Catfish *(Clarias gariepinus)*

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Abstract

Bacterial pathogens are the most serious agents causing diseases in both wild and cultured fish resulted in massive mortalities and economic losses. Motile Aeromonas Septicemia (MAS) is a prevalent bacterial disease caused by *Aeromonas hydrophila* (*A. hydrophila*) that impacts freshwater fish. This research aimed to evaluate doxycycline (DOX) antibacterial activity against *A. hydrophila* both *in vitro* and *in vivo*. The minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) of DOX against *A. hydrophila* previously isolated from African catfish (*Clarias gariepinus*) were determined to be 0.78μg/mL and 3.9μg/mL, respectively. For *in vivo* experiment, a total number of 80 apparently healthy African catfish, were distributed randomly into four equal groups. Group 1 (non-infected, non-treated) was kept as control, Group 2 (non-infected and treated) was non-infected and treated with 20 mg/Kg BW of DOX for 5 successive days in feed, Group 3 (infected) was inoculated intraperitoneally (IP) with *A. hydrophila* (2×10^8 CFU/ mL) and Group 4 (infected and treated) was infected with *A. hydrophila* then treated with 20 mg DOX/Kg BW. Our results revealed 70% mortality in African catfish experimentally challenged with *A. hydrophila* (Group 3). Moreover, significant elevation of serum alanine aminotransferase (ALT) (89±16.26, 54.67±6.44, 36±5.29 U/L, respectively), aspartate aminotransferase (AST) (195±7.64, 221.33±17.9, 211.33±12.72 U/L, respectively) and creatinine (0.68±0.098, 0.76±0.052, 0.58 ±0.023 mg/dL, respectively) was observed on 1st, 7th and 14th days post treatment. While treatment of the infected fish (Group 4) with DOX decreased the mortality rate to 30 %, improved the clinical signs and significantly reduced serum ALT (30.67±6.01, 22.67±1.86 U/L, respectively) and AST (153±7.57, 147.67±6.7 U/L, respectively) on 7th and 14th days post treatment. Also, it significantly decreased creatinine (0.21±0.026, 0.25±0.047, 0.21±0.053 mg/dL, respectively) levels at 1st, 7th and 14th days post treatment when compared with those of Group 3. The results showed that DOX could be used as an effective treatment against *A. hydrophila* infection in African catfish with little adverse effects.

Keywords: Doxycycline, *Aeromonas hydrophila*, African catfish, *In vivo, In vitro*, Antibacterial activity.

Introduction

Rapidly growing human populations in countries like Asia, Africa and South America drive the need for food fish, as a cheap high protein food source [1]. Aquaculture intensification has resulted in the promotion of circumstances that promote the growth of numerous illnesses and problems related to biofouling [2]. In aquaculture, antibacterial agents are used to manage bacterial diseases [3,4]. Antibacterial use in aquaculture needs veterinary prescription as in terrestrial animals [5-7].

Tetracyclines were commonly used in fish farming due to their wide-spectrum of activity

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and reduced price compared to other antimicrobials. However, resistance to the first generation of tetracyclines was developed by some bacterial agents affecting fish [8,9]. Doxycycline (DOX) is a long-acting / second-generation tetracycline antibiotic as well as being one of the most widely prescribed antibiotics in the world nowadays to treat a broad diversity of infectious microorganisms including sensitive intracellular or zoonotic microorganisms [10].

Motile Aeromonas Septicemia (MAS) that caused by *Aeromonas hydrophila (A. hydrophila)* has developed a serious problem to fish aquaculture and quality all over the world, resulting in serious production and marketing losses [11-13].

Hence, this study was carried out to study the *in vitro* antibacterial activity of DOX against *A. hydrophila*. Moreover, to elucidate the *in vivo* antibacterial activity of DOX in *A. hydrophila* challenged African catfish (*Clarias gariepinus)*.

Materials and Methods

Drugs

Doxycycline hyclate (Vibramycin®, capsule) was obtained from Pfizer, Egypt.

A. hydrophilla strain

The clinical *A. hydrophilla* pathogenic strain was previously isolated from African catfish with haemorrhagic septicemia and biochemically identified [14]. Briefly, naturally infected African catfish were sacrified by decapitation, the outer surface of the skin and fins were disinfected with 70% ethyl alcohol. Each fish was opened and samples were taken from the affected organs after heat sterilization of the exposed surface. All samples were transferred to be inoculated in tryptic soya broth (Oxoid, UK) and incubated at 25 ℃ for 18-24 h, then plated on tryptic soya agar (Oxoid, UK) and incubated at 25 ℃ for 18-24 h. The suspected colonies were picked up and sub cultured on Rimler-Shotts [Bioworld, USA] specific agar medium for further purification. Suspected colonies (yellow colour) were picked up, sub cultured on blood agar and incubated at 25 ℃ for 24 h for detection of the hemolytic activity. A loop full of pure culture was stabbed in semisolid tryptic soya agar for testing the motility.

Suspected colonies were firstly checked for oxidase test which was positive. Identification of the isolates was carried out using the routine study of the morphological characters, colonies and growth appearance as well as the biochemical reactions as described elsewhere [15,16].

Experimental fish

A total number of 80 apparently healthy African catfish of 100±5 g BW and did not exposed previously to antibiotics were purchased from fish hatchery, Central Laboratory for Aquaculture Research (CLAR), Abbassa, Egypt. They were reared at the wet laboratory of Fish Health and Management Department, CLAR in a fiberglass for 2 weeks to be acclimated with the experimental condition. Acclimated fish were randomly allocated at a rate of 5 fishes/100 L aquarium (100×40×50 cm). The different quality criteria of the water were checked daily. The pH was about 7.5, and the ammonia and dissolved O_2 levels were about 0.1 mg/L and >7 ppm, respectively [17]. Dissolved oxygen (DO) of water was determined by DO meter (YSI Model-58, USA) and pH was recorded by a portable pH meter (Jenway Model 3020, UK).The fish were fed daily on a drug-free pelleted diet. Fish were received formulated fish diet (fish feed manufacturing unit, CLAR) contained 35% protein once daily at a level of 3% of BW and extra feed was removed by siphoning. Water was partially changed every 3 days with chlorine free tap water and was continuously aerated using electric air pump (RINA, Italy). The experimental procedure was carried out in keeping with Zagazig University's Ethics of Animal Use in Research Committee (EAURC).

In vitro study

Minimum inhibitory concentration (MIC) determination

The MIC of DOX against *A .hydrophila* isolated from fish was estimated in triplicate by the Clinical and Laboratory Standards Institute (CLSI) broth macro-dilution method [18]. Briefly, 1 mL of 0.1 % DOX was dissolved in 1 mL freshly prepared Mueller-Hinton Broth (MHB) media (Bio-Merieux, France), in tube #1. Then, 1 mL from tube #1 was transferred to tube #2 containing 1 ml MHB media. The series of dilutions was

performed for a total of 12 tubes. After that, 20 µL (10^6 CFU/mL)) [19]. *A. hydrophila* suspension was separately inoculated into all 12 testing tubes along with 2 control tubes and incubated in a 30°C (the optimal growth temperature of *A. hydrophila* strain is 28 ~ 30°C) incubator for 24 h. The lowest drug concentration among tubes showing no bacterial growth would be the drug's MIC.

Mutant prevention concentration (MPC) determination

The mutant prevention concentration (MPC) stated the susceptibility of the small number of resistant mutant bacteria present before any drug therapy. The MPC was estimated by methods described previously [20]. Briefly, the *A. hydrophila* isolate was cultivated in MHB media for 24 h. Then the suspension was centrifuged at 4000 ×g for 10 min and the isolate was re-suspended to a concentration of 10^{10} CFU/mL using MHB media. A 300 µL of isolate suspension, having more than 10^{10} CFU/mL, were cultured on each of four Mueller-Hinton Agar (MHA) plates containing DOX at concentrations equivalent to 1×, 2×, 3×, 4×, 5×, 6×, 7×, 8×, 9× and 10× MIC. The plates were incubated at 30°C for 48 h, colonies were counted up and incubated again for further 72 h. The MPC was defined as the lowest drug concentration preventing the appearance of any mutant after 48 h and incubated again for an additional 72 h. Each experiment was performed three times.

In vivo study

Experimental design

Fish (n=80) were randomly distributed into 4 equal groups each one of 20. Group1 (non-infected, non-treated): it was kept as control, Group 2 (non- infected and treated): non-infected fish was treated with DOX (20 mg / Kg BW) [21] for 5 successive days in feed, Group 3 (infected): fish was inoculated intraperitoneally (IP) with *A. hydrophila* (2× 10^8 CFU/ mL) [22] and Group 4 (infected-treated): fish in this group was infected with *A. hydrophila* and treated with the previously mentioned dose and course of DOX. Clinical signs, mortalities and post mortem lesions were recorded over a period of 15 days.

Blood samples and serum preparation

Samples of blood were taken under anesthesia using 100mg/L of clove oil [23] from 5 fish every collecting time from the caudal blood vessels at 1st, 7th and 14th days after DOX administration. The serum samples were prepared after blood clotting by centrifugation at 3000 × g for 15 minutes. Serum samples were preserved at - 20° C till analyzed.

Biochemical analysis

Serum aspartate aminotransferase (AST) as well as alanine aminotransferase (ALT) were estimated colorimetrically by spectrophotometer (Spectronic 20 D, Milton Roy Company) using specific kits (Diamond, Australia) according to Reitman and Frankel [24]. Colorimetric determination of serum creatinine was carried out using spectrophotometer according to Henry [25].

Statistical analysis

The data have been displayed as mean ± SE. Statistically; data were assessed by one-way analysis of variance (ANOVA) and compared using Duncan's multiple range test at the 5% probability level. Significant difference between means was determined at probability levels of less than 0.05 [26].

Results

In vitro antibacterial activity of doxycycline against *A. hydrophila*

The MIC of DOX against *A. hydrophila* was 0.78µg/mL, whereas the MPC was estimated to be 5 MIC (3.9 µg/mL). The mutant selection window (MSW) was determined to be 0.78 – 3.9 µg/mL.

In vivo antibacterial activity of DOX against *A. hydrophila*-challenged fish

The infection of African catfish with *A. hydrophila* induced anorexia, dullness, loss of balance, sluggish movement, swimming near the water surface and progressive erosions allover fins and skin with erythema on the skin especially at the ventral abdominal area as showed in Figure 1. However, the treated group showed mild degree of clinical signs in comparison with the non-treated one (Figure 2).
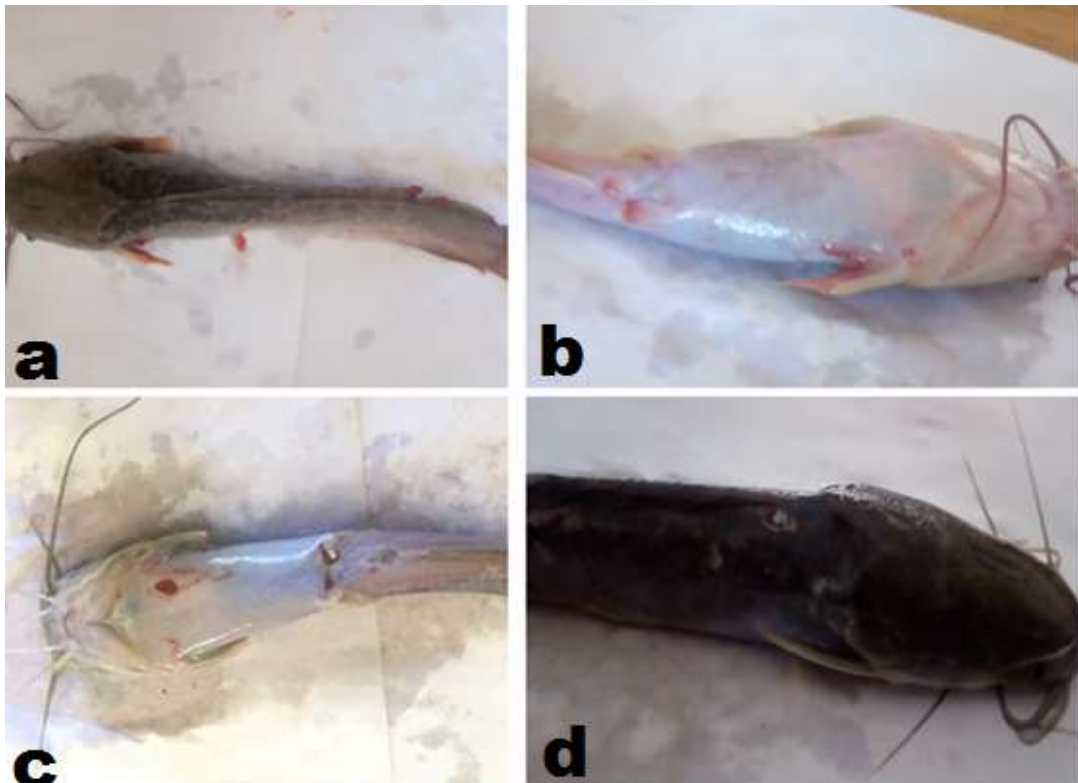
Figure 1: **Experimentally infected with *A. hydrophila* showing (a) redness and inflammation of fins, (b) redness and inflammation of anal opening and ventral fins, (c) deep ulcer formation at ventral body surface and (d) deep ulcer formation at dorsal body surface.**
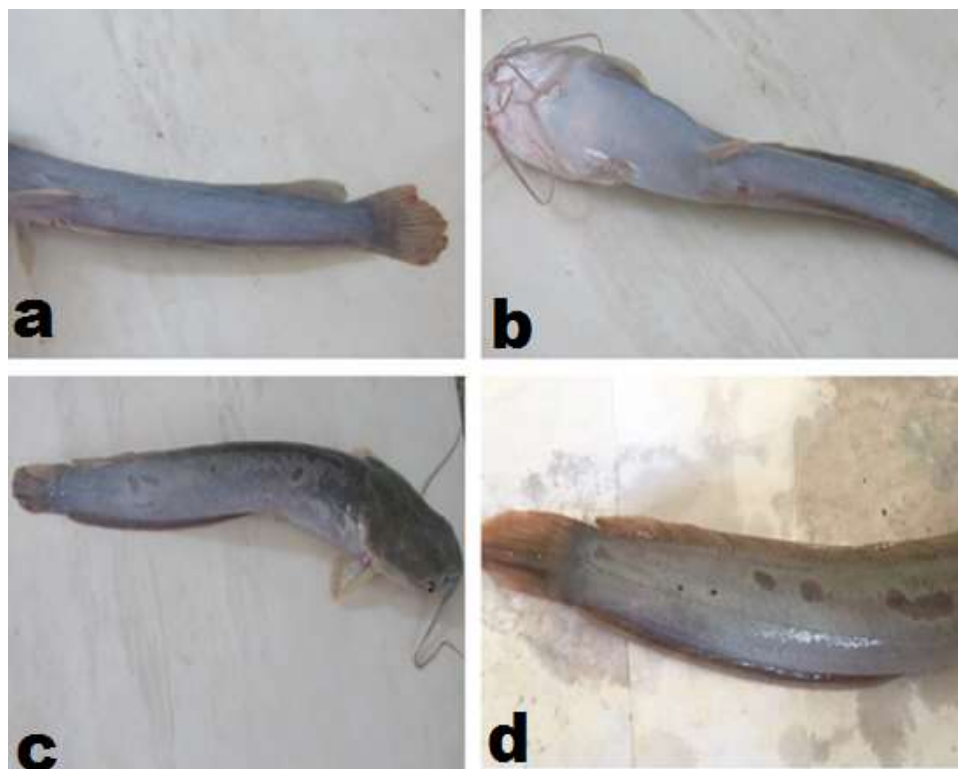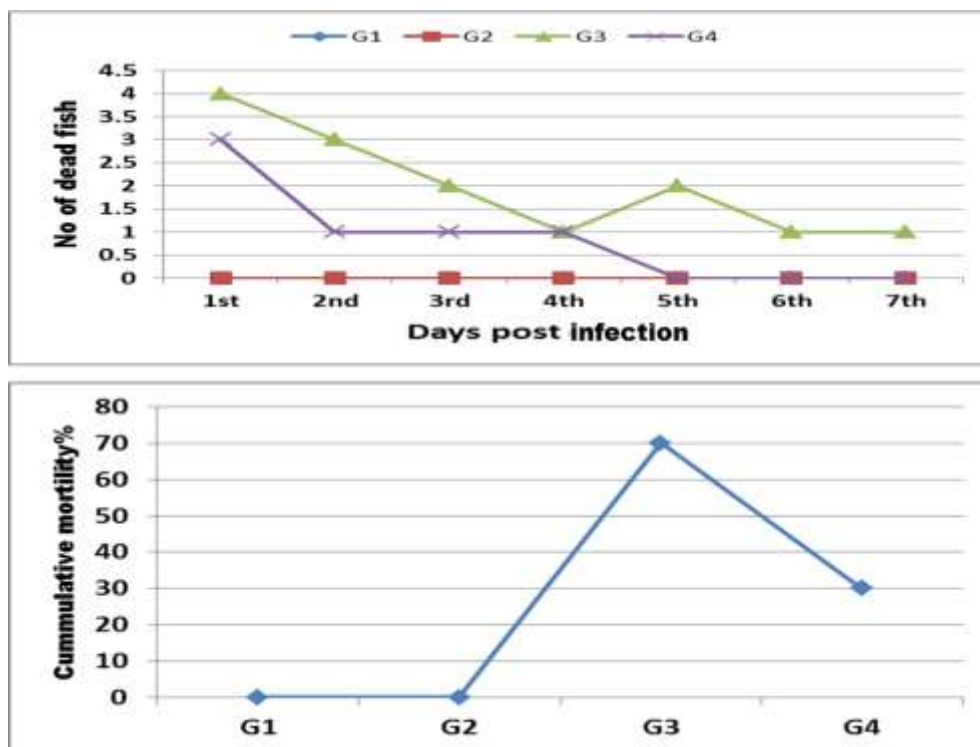

Figure 2: Clarias garipenus experimentally infected with A.hydrophila and treated with 20 mg DOX /Kg BW for 5 successive days in feed showing (a) slight redness of fins, (b) slight redness of anal openning and (c) &(d) shallow ulcers.

Mortality rate of experimentally infected fish

The mortality rate during the experiment is shown in Figure 3, as it was started at the 1st day post infection and reached 70% in the infected non-treated group. While treated group with the therapeutic dose of DOX (20 mg/ Kg BW for 5 successive days) showed reduction in mortality rate by 30 %. No mortalities were recorded in either non infected non treated group or treated non-infected group.

Figure 3: Effect of administration of 20 mg DOX/ Kg BW in feed for 5 successive days on number of dead fish (a) and mortality rate (%) (b) of *Claris gariepinus* experimentally infected with *A. hydrophilla*. G1 (control): non-infected, non-treated, G2 (non-infected and treated): was non-infected and treated with 20 DOX mg /Kg BW for 5 successive days in feed, G3 (infected): was inoculated intraperitoneally with *A. hydrophila* (2×10^8 CFU/ mL) and G4 (infected and treated): was infected with *A. hydrophila* and treated with DOX.

Biochemical analysis

Experimentally infected fish with *A. hydrophila* displayed a significant (P<0.05) increase in the level of serum ALT (89±16.26, 54.67±6.44, 36±5.29 U/L, respectively), AST (195±7.64, 221.33±17.9, 211.33±12.72 U/L, respectively) and creatinine (0.68±0.098, 0.76±0.052, 0.58±0.023 mg/dL, respectively) on 1st, 7th and 14th days post treatment compared with serum ALT (21±1.73, 23.33±2.6, 21.33±2.6 U/L, respectively), AST (143.33±3.53, 136.33±5.55, 136.33±5.55 U/L, respectively) and creatinine (0.24±0.0.072, 0.22±0.059, 0.23±0.067mg/dL, respectively) levels of the control group. Administration of DOX in feed for 5 succeeding days to *A. hydrophila*–challenged fish induced a significant (P<0.05) decrease in the level serum ALT (30.67±6.01, 22.67±1.86 U/L, respectively) and AST (153±7.57, 147.67±6.7 U/L, respectively) on the 7th and 14th days post treatment compared with serum ALT (54.67±6.44, 36±5.29 U/L, respectively) and AST (221.33±17.9, 211.33±12.72 U/L, respectively) of the infected-non treated group. Also treatment of challenged fish with DOX caused a significant (P<0.05) decrease in serum creatinine (0.21±0.026, 0.25±0.047, 0.21±0.053 mg/dL, respectively) levels on 1st, 7th and 14th days post treatment when compared with the creatinine levels (0.68±0.098, 0.76±0.052, 0.58±0.023, respectively) of the infected one (Table 1).

Table 1: Effect of doxycycline on some biochemical parameters of healthy and experimentally infected *Claris gariepinus* with *A. hydrophilla*

Group	ALT (U/L)			AST (U/L)			Creatinine (mg/dL)		
	Days post treatment								
	1st	7th	14th	1st	7th	14th	1st	7th	14th
G1	21± 1.73[b]	23.33±2.6[b]	21.33±2.6[b]	143.33±3.53[b]	136.33±5.55[b]	149.67±7.8[b]	0.24±0.0.072[b]	0.22±0.059[b]	0.23±0.067[b]
G1	30.4±3.9[b]	29.67±4.4[b]	22±2.31[b]	154±4.93[b]	143.67±4.41[b]	150±10.97[b]	0.23±0.058[b]	0.23±0.03[b]	0.23±0.027[b]
G3	89±16.26[a]	54.67±6.44[a]	36±5.29 [a]	195±7.64[a]	221.33±17.9[a]	211.33±12.72[a]	0.68±0.098[a]	0.76±0.052[a]	0.58±0.023[a]
G4	73.3±5.6[a]	30.67±6.01[b]	22.67±1.86[b]	183.33±14.53[a]	153±7.57[b]	147.67±6.7[b]	0.21±0.026[b]	0.25±0.047[b]	0.21±0.053[b]

Values are represented as the mean ± SE. The means within the same column carrying different superscripts are significantly different at P<0.05. G1 (control): non-infected, non-treated, G2 (non-infected and treated): was non-infected and treated with 20 mg DOX /Kg BW for 5 successive days in feed, G3 (infected): was inoculated intraperitoneally with *A. hydrophila* (2×10^8 CFU/ mL) and G4 (infected and treated): was infected with *A. hydrophila* and treated with DOX. G: Group.

Discussion

A. hydrophila causes substantial economic losses, due to high mortality rates and worsened production quality [27]. Due to their wide spectrum and reduced price compared to other antimicrobials, tetracyclines have been widely used in fish farming but some bacterial fish pathogens have demonstrated resistance to tetracyclines of the first generation [8,9]. Doxycycline, one of the second-generation tetracyclines, is effective in treating fish diseases [28], but its dosage schemes are extrapolated from other species of mammals that may not be suitable.

Dosage policies are presently based on number of factors, including MIC, susceptibility tests and achievable and sustainable levels of drugs. MIC testing became the acceptable method for susceptibility testing with its effectiveness, reproducibility, speed, simplicity and minimal cost. [30]. In the present study, the MIC of DOX against *A. hydrophila* isolated from African catfish was determined to be 0.78 μg/mL. As documented previously, 39 *A. hydrophila* isolates were studied for DOX susceptibility and the results revealed that 97.9 % of tested isolates were susceptible to DOX at a concentration ≤ 4 μg/mL [27]. This finding is in accordance to the MIC breakpoints described by the CLSI [31].

Mutant prevention concentration (MPC) is a new concept designed to address the enhanced incidence of antibiotic resistance by using antibiotic levels that can stop resistant bacterial populations from being selected [32].

It uses bacterial populations more than 10^9 CFU/mL [33-35]. One advantage of the MPC strategy over the MIC technique is that the MPC defines the drug concentration needed to eradicate all cells, including any spontaneously occurring resistant mutants (10^{-6}-10^{-8} frequency.) that are identified using such elevated inocula. Dosing approaches based on MPC testing can slow the emerging resistance substantially. The hypothesis of mutant selection window (MSW) is a novel concept that was described by Drlica and Zhao [36]. The lower border of the MSW is the lowermost concentration that prevents the growth of the majority of susceptible microorganisms. This is often the minimum inhibitory concentration (MIC) and the upper border is the minimum concentration that prevents growth of the least-susceptible single-step mutant subpopulation, the mutant prevention concentration (MPC). Using the notion of the mutant selection window (MSW), MPC values can assist to determine more powerful agents and agents that are less likely to pick for resistance [33]. Few MPC studies on agents other than quinolones have been conducted [37]. The findings of this research stated that the MPC of DOX on *A. hydrophila* isolated from African catfish was estimated to be 5 MIC (3.9 μg/mL). The MSW of DOX on *A. hydrophila* was estimated to be 0.78 − 3.9 μg/mL. Keeping plasma drug concentrations exceeding 3.9μg/mL is expected to limit the development of resistance [38].

Concerning the mortality rate in African catfish, infection with *A. hydrophila* produced

high mortality rate (70%), which may be attributed to the endotoxin excreted by the microorganism [39,40]. Our results agreed with that recorded by Anyanwu *et al*., [41] who found that the mortality rate in African catfish experimentally infected with *A. hydrophila* ranged from 60-90%. In this study, DOX administration at a dose of 20 mg /Kg BW in feed for 5 consecutive days was effective and resulted in an increased survival rate of fish infected with *A. hydrophila*, while the mortalities declined from 70 % in infected non-treated group to 30 % in treated group with DOX. Using of DOX in feeding regime after the infection of fish with *A. hydrophila* helped in clearance of disease signs and maintained the fish in a good condition. Similarly, Nasr [42] recorded a decline in the mortality rate of infected Nile tilapia with *A. hydrophila* to 20% after treatment with oxytetracycline.

The serum ALT and AST are considered sensitive indicators to evaluate the hepatocellular damage [43,44]. The infection of African catfish with *A. hydrophila* resulted in elevation in some biochemical parameters manifested by a significant increase in ALT, AST and creatinine compared to control. Halliwell [45] stated that the increase in enzymes activities was attributed to the liver damage that is caused by the effect of the infectious agent toxins which is followed by the escape of these enzymes into serum in high levels. Wells *et al.,* [46] stated that, high blood creatinine specifies a low glomerular filtration rate of the rear kidney, where creatinine is the product of muscle creatinine catabolism and is excreted by the trunk kidney [47].

These results are in agreement with those reported by Ahmed [48] and Amer *et al*., [49] who recorded an increase in the serum enzymatic activities in infected fish with *A. hydrophila.* Similar results were also detected in fish infected by *A. hydrophila* by Souza *et al.,* [50]; Dos Santos *et al*., [51] and Ahmad *et al.,* [52]. The significance increase in creatinine level was also reported by El-Barbary, [53] in *A. hydrophila* infected Nile tilapia (*Oreochromis niloticus).*

The treatment of *A. hydrophila–*challenged fish with DOX (20 mg/Kg BW) in feed for 5 successive days induced significant improvement in serum ALT, AST and creatinine levels on the 7th and 14th days post treatment compared to the infected non-treated one. This improvement might be because of the bacteriostatic action of the drug [54], which limits the destructive and toxic effects in the liver and kidney.

Conclusion

It was concluded that DOX could be one of the drugs of choice for treatment of the motile Aeromonas infection in African catfish with high therapeutic effect and minimal adverse effects.

Conflict of Interest

The authors declare no conflict of interest.

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الملخص العربي

النشاط المضاد للبكتيريا للدوكسيسيكلين فى سمك السلور الأفريقي المعدى بالايروموناس هيدروفيلا

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٢قسم بحوث صحة الأسماك ورعايتها - المعمل المركزى لبحوث الثروة السمكية (العباسة) - مركز البحوث الزراعية- مصر.

إن العوامل المسببة للأمراض البكتيرية هي من العوامل المسببة لأخطر مشاكل الأمراض في كل من الأسماك البرية والمستزرعة التي تسبب النفوق والخسائر المالية الفادحة. ويعد التسمم الدموى بميكروب الايروموناس هيدروفيلا احد أهم الأمراض البكتيرية التى تصيب أسماك المياه العذبة. لذا استهدف هذا البحث إلى تقييم قدرة المضاد الحيوي الدوكسيسيكلين في القضاء على ميكروب الإيروموناس هيدروفيلا سواء في المختبر أو في الجسم الحي. تم تحديد أقل تركيز مثبط للنمو وكذلك التركيز المانع للتحول (٠.٧٨ ميكروجرام/ملليلتر و ٣.٩ ميكروجرام /ملليلتر على الترتيب) لعقار الدوكسيسيكلين ضد ميكروب الإيروموناس هيدروفبلا المعزولة مسبقا من أسماك السلور (القرموط) الأفريقى. بالنسبة للتجربة الحية تم استخدام ٨٠ قرموطا أفريقيا سليم ظاهريا لم يتم معالجتهم بمضادات حيوية سابقا وتم توزيعهم عشوائيا إلى أربع مجموعات متساوية: المجموعة الأولى (مجموعه١)؛ ضابطة غير معداة وغير معالجة؛ (مجموعه٢)؛معالجة بعقار الدوكسيسيكللين(٢٠ ميلليجرام /كجم وزن حى فى العليقة لمدة خمسة أيام متتالية) وغير معداة؛(مجموعـه٣)؛معداة بميكروب الإيروموناس هيدروفيلا(٢×١٠٨ خلية فى الوحدة) وغير معالجة ؛(مجموعه٤)؛ معداة ومعالجة بعقار الدوكسيسيكللين. أدت العدوى بميكروب الإيروموناس إلى معدل النفوق بلغ ٧٠% فى المجموعة المعدية وغير المعالجة كما كانت هناك زيادة ملحوظة فى مستوى انزيمات الالانين أمينو ترانسفيراز (٨٩±١٦.٢٦، ٥٤.٦٧±٦.٤٤ و ٣٦±٥.٢٩ على التوالى) وأسبرتات أمينو ترانسفيراز (١٩٥±٧.٦٤، ٢٢١.٣٣±١٧.٩ و ٢١١.٣٣±٢.٧٢ على التوالى) وكذلك مستوى الكرياتنين (٠.٦٨±٠.٠٩٨، ٠.٧٦±٠.٠٥٢ و٠.٥٨±٠.٠٢٣ على التوالى) فى الدم عند مقارنتها بالمجموعه الضابطه.عند استخدام الدوكسيسيكللين لعلاج الاسماك المعداة بالإيروموناس انخفض معدل الوفيات إالى ٣٠% وتحسنت الأعراض الظاهرية وأنشطة انزيمات الألانين أمينو ترانسفيراز (٣٠.٦٧±٦.٠١ و ٢٢.٦٧±١.٨٦على التوالى) و أسبرتات أمينو ترانسفيراز (١٥٣±٧.٥٧ و ١٤٧.٦٧±٦.٧ على التوالى) فى اليومين السابع والرابع عشر بعد العلاج. كما تحسن مستوى الكرياتنين فى الدم (٠.٢١±٠.٠٢٦، ٠.٢٥±٠.٠٤٧ و ٠.٢١±٠.٠٥٣ على التوالى) فى الأيام الأول والسابع والرابع عشر بعد المعالجة عند مقارنتها بالمجموعه المعداه وغير معالجه وممما سبق نستنتج أن استخدام عقار الدوكسيسيكللين فى علاج التسمم الدموى الإيرومونـاسى فى أسماك القرموط الأفريقى يحقق نتائج علاجية عالية مع أثار جانبية قليلة.