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Prevalence of Septicemia and Red Mouth Disease Caused by *Aeromonas sobria* at Sahl El-Housinia Fish Farm

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Abstract

The present study was carried out to isolate, identify and perform a trial for treatment of *Aeromonas sobria*; the etiological agent responsible for signs of septicemia with redness and hemorrhage of mouth in cultured *Oreochromis niloticus* and mullet species. A total of 312 *Oreochromis niloticus* (30-65 g) and 158 mullet species (200-300 g) were collected a live from a private fish farm at Sahl El-Housinia, Sharkia Governorate, Egypt. Bacteriological examination of samples from gills, kidney, intestine, liver and spleen was carried out. Molecular identification, pathogenicity and *in vitro* antibiotic sensitivity of the isolated strains as well as *in vivo* trials of treatment were performed. The naturally infected fishes were characterized by signs of septicemia with redness of mouth and different parts of the body. *Aeromonas sobria* prevalence in *Oreochromis niloticus* (35.89%) was higher than that in mullet species (20.88%). Antibiotic sensitivity test on PCR confirmed isolates (n=3) that were highly pathogenic revealed that *Aeromonas sobria* was highly sensitive to enrofloxacin. The trial treatment of experimentally infected *Oreochromis niloticus* with 0.2 ml ($9x10^8$ CFU/ml) of 24 hrs virulent *Aeromonas sobria* broth culture using enrofloxacin (2 mg/l for 5 days as medical water bath) revealed better health condition and improvement in the signs of infection and levels of the alanine aminotransferase, aspartate aminotransferase and creatinine. Histopathological findings of liver, kidney and intestine confirmed the results of serum biochemical analysis where lesions were alleviated to normal indicating improvement in the health condition due to the efficacy of enrofloxacin treatment. In conclusion, the use of enrofloxacin as a medical water bath by 2 mg/l for five days revealed good results but must be used under restrictions to avoid bacterial resistance to the antibiotic.

Keywords: *Aeromonas sobria, Oreochromis niloticus,* mullet species, Enrofloxacin

Introduction

Aquaculture is one of the most important distribution activities in Egypt. Fish is a cheap and valuable source of animal protein for the Egyptian citizens. Hence, there is a trend to intensify fish in fish farms to meet the consumer's needs appeared in Egypt. Such intensification resulted in increased chance of disease spread as a consequence of bad water quality and high stocking densities especially bacterial diseases which cause high loss in fish farms [1].

In particular *Aeromonas* species (namely, *Aeromonas hydrophila, Aeromonas sobria, Aeromonas aquarium, Aeromonas caviae*) are highly distributed in the aquatic environment and are considered as the major cause of bacterial infections affecting fish [2]. *Aeromonas* species are Gram negative, non-spore forming, rod shaped, facultative anaerobic bacteria [3]. The motile aeromonas are responsible for high economic losses in fish farms due to Motile Aeromonas Septicemia (MAS) or red sore disease [4].

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Aeromonas sobria (*A. sobria*) causes many problems in fish farms all over the world. It was reported as a pathogenic agent of tail rot in tilapia [5], and a fatal outbreak caused by *A. sobria* was recorded in gizzard shad (*Dorosoma cepedianum*) [6]. *A. sobria* caused epizootic ulcerative syndrome (EUS) in fish farms of Southeast Asia, particularly in Bangladesh and India. It also caused economic losses in perch fish farms in Switzerland [7, 8].

The aim of this study was to isolate and identify *A. sobria* from infected fish with signs of septicemia and redness of mouth at a private fish farm at Sahl El-Housinia, Sharkia Governorate, Egypt, in addition to attempts for treatment.

Materials and methods

Sampling

A total of 312 *Oreochromis niloticus* (*O. niloticus*) with variable sizes (30-65 gm) and 158 mullet species, with variable sizes (200-300 gm) were collected a live from a private fish farm at Sahl El-Housinia, Sharkia Governorate, Egypt. The samples were then transported to Fish Disease and Management Department, Faculty of Veterinary Medicine, Zagazig University during the period from April, 2014 till May, 2015.

The fish were kept in well-aerated glass aquaria at 27°C for clinical and postmortem examination.

Bacteriological examination

The surfaces of gills and internal organs were sterilized with heated spatula, sterile bacteriological loop was inserted through the sterilized area and then inoculated into trypticase soya broth. The inoculated tubes were incubated at 24-25ºC for 24 hrs. A loopfull from the broth was streaked onto tryptic soya agar and Aeromonas agar media (Lab 176) then incubated at 24-25ºC for 24-48 hrs [9]. Bacterial isolates were identified using conventional biochemical testing (in particular, Gram staining, motility and catalase tests) and using VITEK 2 system (Bio-Me´rieux, NC). Results were interpreted at 24–48 hours according to the manufacturer's instructions.

Molecular identification

The DNA was extracted by QIAamp DNA mini kit according to manufacturer's instructions. The extracted DNA was subjected to PCR for the amplification of 16S rRNA of *A. sobria* (Forward primer: 5′-GAA AGGTTGATGCCTAATACGTA-3′ and Reverse primer: 5′-CACAGCCAATATGTCGGTGAAG-3′) [10] and aerolysin gene (Forward primer: 5′-CACAGCCAATATGTCGGTGAAG-3′; Reverse primer: 5′-GTCACCTTCTCGCTCAGGC-3′) [11]. The PCR was performed in 25-µl volume consisting of 12.5-µl Emerald Amp GT PCR mastermix (Takara, Code No. RR310A kit), 1 µl for each forward and reverse primers, 6 µl Template DNA and PCR grade water up to 25 µl. The PCR cycling conditions for both genes were performed in PTC- 100 ^{TM} programmable thermal cycler [11].

Pathogenicity test of the isolated A. sobria

A total of 120 apparently healthy *O. niloticus* (45 ± 5 g) were obtained a live from Abbassa Fish Hatchery, Sharkia Governorate, Egypt. Fish were equally divided into four groups. Each group consisted of triplicates of 10 fish, each. The fish were kept in glass aquaria (80 X 40 X 30 cm) filled with 60 L of dechlorinated tap water. The fish were acclimated to their new environmental conditions for two weeks before the initiation of the experiment. During the experiment; water quality criteria were maintained at acceptable levels for dissolved oxygen (5.6-6.4 mg/L), temperature (26-28°C), pH (7.2-8.3), nitrite (<0.05 mg/L), ammonia (<0.05 mg/L) and salinity (0.5%). Water change was performed daily at a rate of 25%. The pathogenicity test was performed using three different isolates confirmed as *A. sobria* by PCR. Fish of groups 1, 2 and 3 were intraprotenially inoculated with 0.2 ml of 24 hrs old culture (9×10^8 CFU/ml) of the three isolates, respectively. Fish of group 4 were intraprotenially injected with sterile tryptic soya broth and kept as a

control. Clinical signs, postmortem lesions and mortalities were noticed. Re-isolation of the inoculated *A. sobria* was performed. Mortalities were recorded daily for two weeks. The test was considered positive when more than 50% of the fish died showing the characteristic signs and lesions of the disease [12].

Antibiotic Sensitivity Test (in vitro)

The susceptibility of PCR confirmed, highly pathogenic *Aeromonas sobria* isolates (n=3) against ten different antimicrobial agents was investigated using the disc diffusion method [13]. The antimicrobial agents used were Amoxicillin (AX, 25 mcg), Ampicillin (AM, 10 mcg), Chloramphenicol (C, 30 mcg), Ciprofloxacin (CIP, 5 mcg), Enrofloxacin (ENR, 5mcg), Erythromycin (E, 15 mcg), Gentamicin (CN, 10 mcg), oxolinic acid (OA, 2 mcg), Oxytetracycline (Ox, 30 mcg) and Trimethoprim- Sulfamethoxazole (SXT, 1.25/ 23.75 mcg) (Oxoid, England).

Table 1: Effect of treatment with enrofloxacin on serum biochemical parameters of *Oreochromis niloticus* experimentally infected with *Aeromonas sobria*

Parameters	G1	G2	G3
AST (µ /L)	4 ± 1^{c}	19.33 ± 1.52^{a}	12.66 ± 1.52^{b}
ALT (µ /L)	6.33 ± 1.52^{b}	26.33 ± 1^{a}	8 ± 1^{b}
Creatinine (mg / dL)	0.12 ± 0.015^{c}	0.29 ± 0.005^{a}	0.19 ± 0.005^{b}

ALT: Serum alanine aminotransferase, AST:aspartate aminotransferase, Means carrying different superscripts are significantly different at (P-value <0 .05), while means carrying similar superscripts are insignificantly different

In vivo trials for treatment

A total of sixty apparently healthy *O. niloticus* with an average body weight of 45 ± 5 g (mean ± SE) were obtained from the Abbassa Fish Hatchery, Sharkia Governorate, Egypt. The fish were acclimated in glass aquaria (80 × 40 × 30 cm) for two weeks and fed on a basal diet before being randomly divided into three equal groups; each with triplicates (10 fish replicate $^{-1}$). The first group (G1) served as control negative (non-infected and non-treated fish). The second (G2) served as control positive; fish were intraperitoneally inoculated with 0.2 ml ($9x10^{8}$ CFU/ml) of 24 hrs broth culture of virulent *A. sobria* without treatment.

Third group (G3); fish were intraperitoneally inoculated with 0.2 ml ($9x10^{8}$ CFU/ml) of 24 hrs broth culture of virulent *A. sobria* and treated with medical water bath using enrofloxacin (2 mg/l for 5 days) [14].

Fifteen days after the end of the treatment period, blood samples were collected from the caudal blood vessels without heparin for serum separation by centrifugation at 3,000 rpm for 15 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine levels were determined [15,16]. Specimens from the liver, intestine and kidney were also collected and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained by hematoxylin and eosin then they were examined microscopically for histopathology [17].

Statistical analysis

The results of serum biochemical analysis were statistically analyzed using analysis of variance using` one-way ANOVA; Post hoc: Duncan's multiple comparisons using SPSS version 14 (SPSS, Chicago, IL, USA). P value of <0.05 was considered statistically significant.

Results

Clinical findings of naturally infected fishes

Externally, the naturally infected *O. niloticus* showed signs of septicemia represented by hemorrhage and erythema on the external body surface including skin, fins, mouth and operculum. Loss of scales and skin erosions were also noticed. In postmortem examination, the internal organs including

liver, kidney and spleen were congested. In addition, the gall bladder was congested and distended with bile, while the intestine contained yellowish mucoid fluid (Figure 1A, B). Naturally, infected mullet species showed ascites, erythema, hemorrhage at the mouth and fins particularly the caudal fin. The postmortem examination revealed pale liver with focally congested area, the kidneys were severely congested and the intestine was filled with blood-tinged fluid (Figure 1C).

Bacterial isolates

A. sobria isolates were retrieved from only 102 *O. niloticus* (32.6%) and 33 mullet species (20.8%). A total number of 184 isolates were recovered from gills, kidney, intestine, liver and spleen samples; comprising of 134 isolates from *O.niloticus* and 50 isolates from mullet species as shown in Figure 2A. In case of *O. niloticus*; the highest prevalence of *A. sobria* was noticed in summer (45.9%) and spring (44.9%) seasons followed by autumn (42.5%) and winter (40%). Concerning mullet species; *A. sobria* showed the highest prevalence in autumn (42.4%) followed by spring (10.5%) and summer (10.2%) seasons while it was not detected in winter (Figure 2B).

Bacterial isolates were presumptively identified as *A. sobria* through biochemical identification; PCR was then performed on three biochemically expected isolates. The PCR amplified products of *A. sobria* 16S rRNA and aerolysin genes gave characteristic bands at 685 bp and 326 bp, respectively (Figure 3A and B).

Pathogenicity assay

Out of the three isolates used for the pathogenicity assay, isolates No. 2 and 3 were found to be highly virulent because they caused 90 and 80% mortality rates, respectively, of the inoculated fish during 48 hours post inoculation. Development of clinical symptoms in the form of ulceration of skin, hemorrhage at pectoral fin and fin rot especially at caudal fin were observed. Pathogenicity was confirmed through re-isolation of *A. sobria* from the internal organs of the inoculated fish.

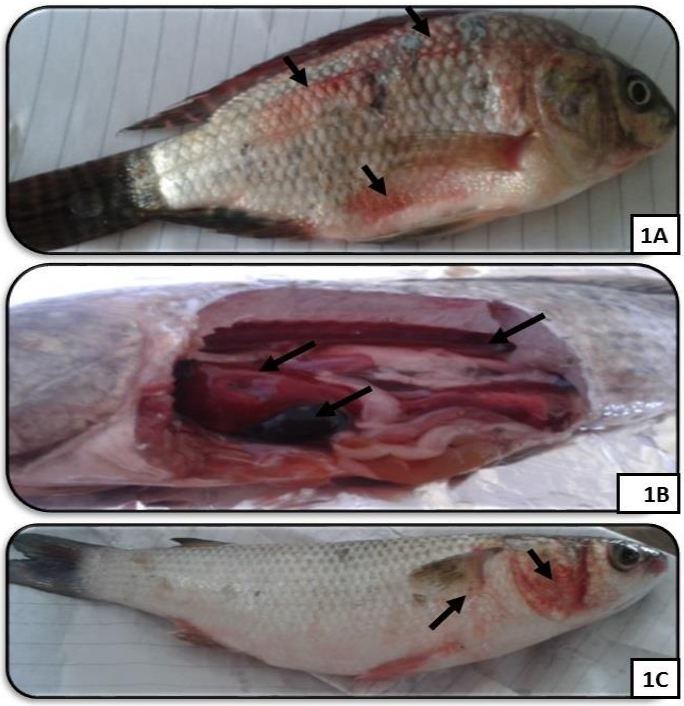

Figure 1: Naturally infected fishes with *Aeromonas sobria* (1A) *O. niloticus* showed hemorrhage and erythema on skin and fins. (1B) *O. niloticus* showed congestion of liver, kidney and spleen with moderate enlargement of liver. (1C) Mullet species showed ascites and hemorrhage at operculum and base of the fins.

Antibiotic sensitivity assay

The results of antibiotic sensitivity test showed that the three *A. sobria* were highly sensitive to enrofloxacin followed by ciprofloxacin, gentamicin, oxytetracyclin, oxolinic acid and chloramphenicol while it was resistant to amoxicillin, ampicillin, erythromycin and Trimethoprim-Sulfamethoxazole.

Treatment trials

The results of the *in vivo* treatment trail with enrofloxacin revealed that G2 (infected with *A. sobria* without treatment) exhibited high mortality rate (90%) compared to G3 that was infected with *A. sobria* and treated with enrofloxacin (20%). The moribund fish in G2 slightly responded to external stimuli and they showed restlessness, erythema and hemorrhage at different parts of the body particularly at the base of fins, operculum, mouth, around the anus and at the site of injection. Abdominal distention and irregular ulcers on the lateral surface of the trunk were also observed in some infected fish. The postmortem examination revealed congested gills and internal organs including liver and kidney with dark, enlarged spleen and distended gall bladder.

Compared with G2; the fish in G3 that were treated with enrofloxacin, appeared in better health condition and responded actively to external stimuli with improvement in the signs of infection and subsiding of the inflammation at the site of injection. The levels of serum ALT, AST and creatinine were significantly increased in case of fish experimentally infected with *A. Sobria* without treatment (G2) compared with non-infected and non-treated fish (G1).

In contrast, the levels of serum ALT, AST and creatinine were significantly decreased in case of experimentally infected *O. niloticus* treated with enrofloxacin (G 3) (Table, 1). Histopathological findings of *O. niloticus* experimentally infected with *A. sobri*a without treatment (G2) revealed severe congestion and hemorrhages in the liver especially around the affected portal areas with diffuse mononuclear cell infiltration.

The hepatocytes revealed vacuolar and hydropic degeneration besides focal vacuolation (Figure 4A). The kidney showed severe congestion of the renal blood vessels and extravasated erythrocytes among the renal tubules. Cystic dilatation of some renal tubules was also detected (Figure 4B). The intestine revealed round cells infiltrating the submucosa with edema and mild mucinous degeneration in the lining epithelium with hemorrhage submucosal (Figure 4C). In contrast, fish of G3 showed normal tissue architecture and cellular details of liver and kidney (Figure 4D and E).

Intestine of the same fish group showed moderate degree of edema beneath the submucosa together with few degenerative changes (Figure 4F).

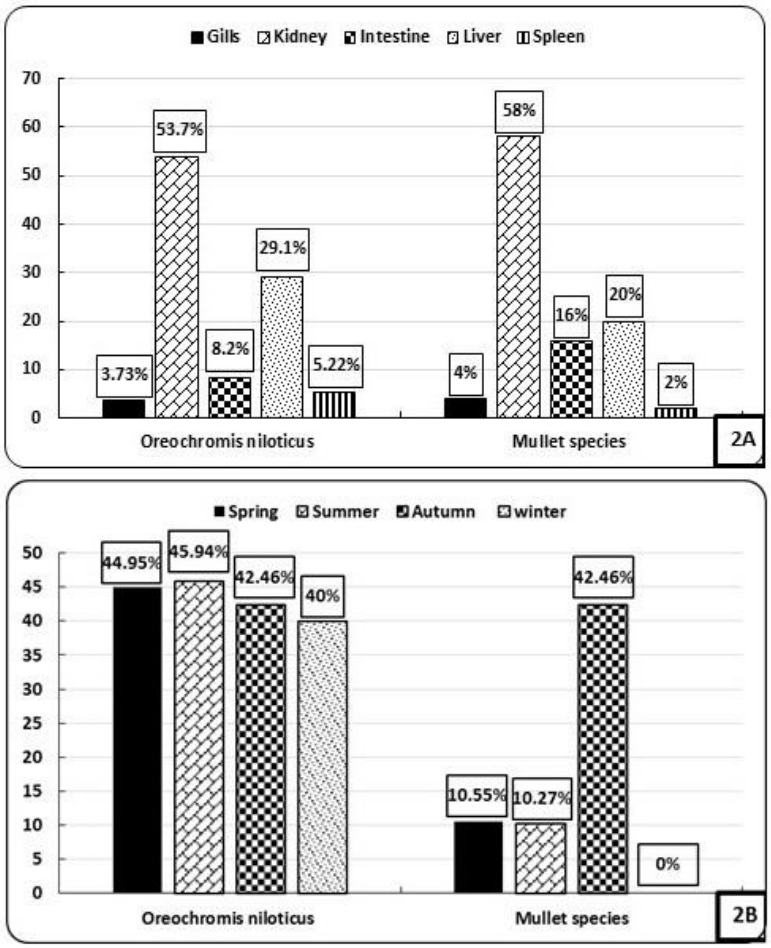

Figure 2: Percentage of *Aeromonas sobria* isolates isolated from *Oerochromis niloticus* and Mullet species (2A) in different organs and (2B) in different seasons.

Discussion

In the present study, most of the naturally infected fish with *A. sobria* were characterized by the presence of general signs of septicemia including hemorrhage at different parts of the body and the base of fins. Moreover, redness of mouth in some cases with congestion and enlargement of internal organs were observed.

Similar clinical signs as fin rot, local hemorrhages and clear ascites have been found in perch infected with *A. sobria* [8]. Also, *A. sobria* was the main causes of skin lesions, erosion and hemorrhagic ulceration in African catfish farms in Southeast Nigeria [18].

A mass mortality with signs of septicemia (exophthalmos, bleeding in eye and fins in addition to skin ulceration) were recorded at a farm in Slovakia in *Garra rufa* fish infected with *A. sobria* [19]. The pathogenicity and clinical signs caused by aeromonads were mainly related to its exotoxins (cytolytic) and enterotoxin (hemolysin, aerolysin, lipase and protease) [20]. The highest isolation rate of *A. sobria* (32.6%) was obtained from *O. niloticus* with 134 isolates from kidney (53.7%), liver (29.1%), intestine (8.2%), spleen (5.2%) and gills (3.7%). In addition, the highest prevalence of this strain was noticed in summer (45.9%) for *O. niloticus* and autumn (42.6%) for mullet species.

A. sobria was represented as 6% between the isolates of motile aeromonade that cause skin lesions of African catfish cultured in Southeast of Nigeria [18]. Aquatic organisms are poikilothermic which their body metabolism, immunity and resistance to diseases are affected by any increase or decrease in water temperature. The changes of water temperature are one of the main reasons for aeromonads infection in fish [3].

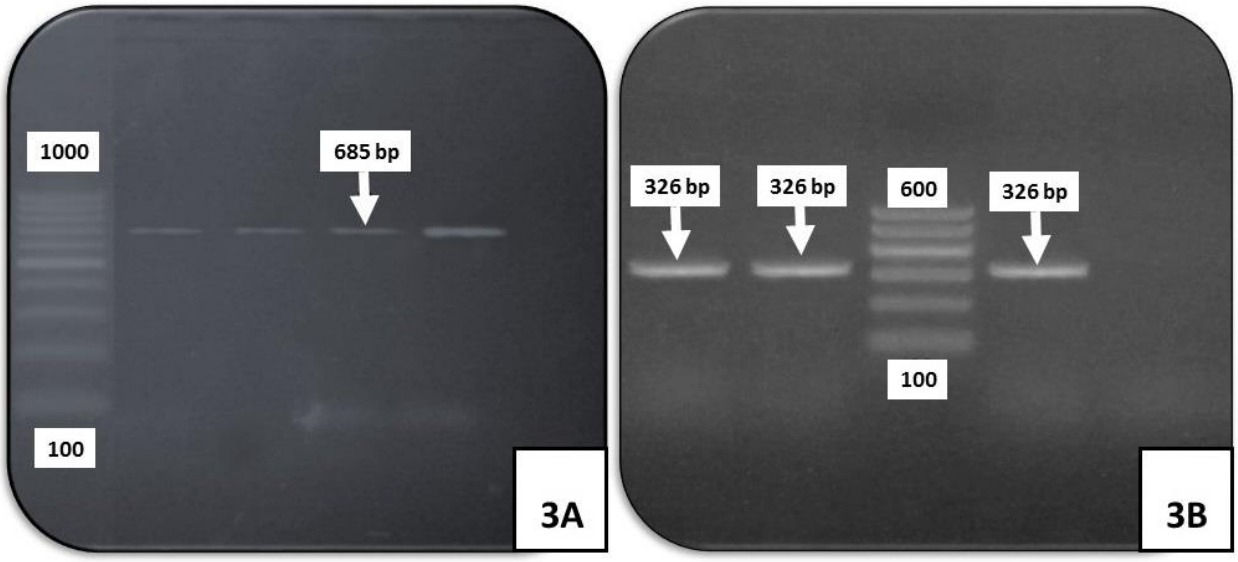

Figure 3: Agarose gel electrophoresis: (3A) PCR amplification of 685-bp 16S rRNA gene of *Aeromonas sobria* isolates. L: 100bp DNA ladder"Marker". (3B) PCR amplification of 326-bp aerolysin gene of *Aeromonas sobria* isolates.

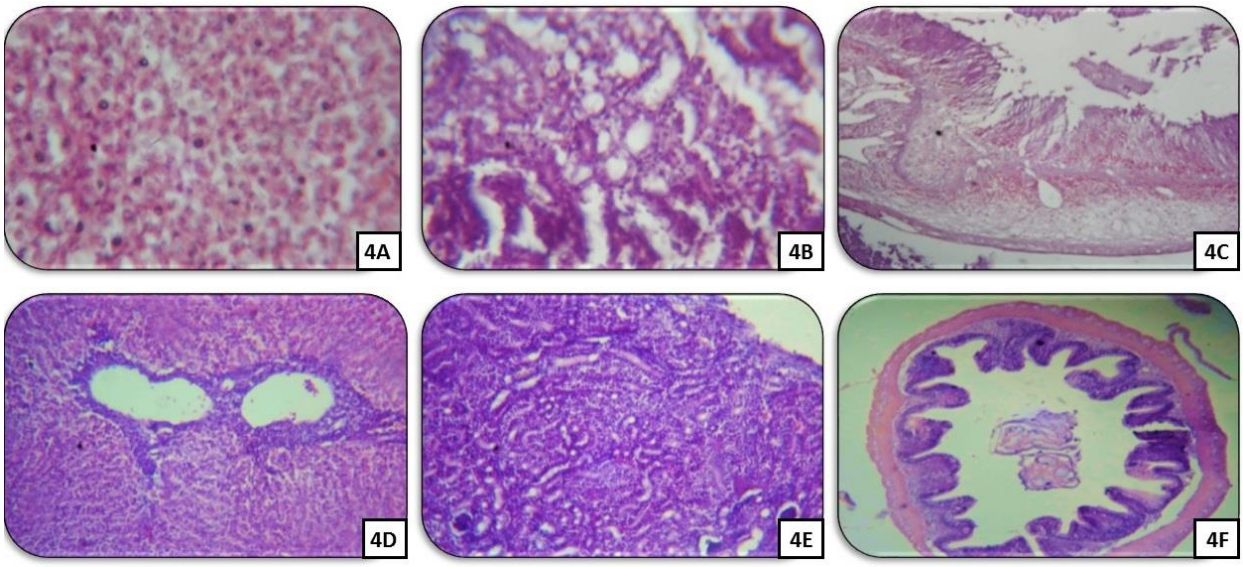

Figure 4: Histopathological findings of *Oreochromis niloticus* groups 2 and 3. (4A) The lesions of liver of *O. niloticus* from G2 served as control positive; fish were intraperitoneally inoculated with 0.2 ml ($9x10^8$ CFU/ml) of 24 hrs. broth culture of virulent *A.sobria* without treatment which represented by severe congestion and hemorrhages with diffuse mononuclear cell infiltration and hepatocytes vacuolar and hydropic degeneration besides focal vacuolation. (4B) Kidney of fish from G2 showed severe congestion of the renal blood vessels and extravasated erythrocytes among the renal tubules with cystic dilatation of some renal tubules. (4C) Intestine of fish from G2 revealed round cells infiltrating the submucosa with edema and mild mucinous degeneration in the lining epithelium. (4D, E) Liver and kidney of *O. niloticus* from G3 that fish were intraperitoneally inoculated with 0.2 ml ($9x10^8$ CFU/ml) of 24 hrs. broth culture of virulent *A.sobria* and treated with enrofloxacin (2 mg/l for 5 days) showed normal tissue architecture and cellular details. (4F). Intestine of fish from G3 showed moderate degree of edema beneath the sub mucosa together with few degenerative changes in the sub mucosal tissue (H&E × 200).

The biochemical characters of *A. sobria* isolated during the current work were in harmony with the characters of these bacteria isolated in outbreaks of fish disease in other studies [18,21,22]. *A. sobria* is one of single heterogeneous species, which has multiple hybridization groups (HGs) within other mesophilic aeromonas [23,24]. Therefore, the conventional methods of identification of *A. sobria* by phenotypic methods through the biochemical characters are not enough. For accurate identification, it is preferable to use molecular methods. The identification of the causative bacterial agents in freshwater fishes mainly depend on the toxin gene detection by PCR [25].

The identification of pathogenic aeromonas strain was newly determined by PCR detection to virulence genes namely, aerolysins, hemolysins, cytolytic [20]. In the present study, three isolates were used for the pathogenicity test, isolates No 2 and 3 showed high virulence by induction of 90 and 80% mortality rates, respectively. The pathogenicity of these isolates could be related to the detected aerolysins virulence gene.

Kozińska [26] recorded that the pathogenicity of the isolated aeromonas species in particular, *A. veronii bt. sobria*, *A. bestiarum* and *A. salmonicida* in common carp and *A. hydrophila* in rainbow trout was related to haemolytic and proteolytic activity of these isolates.

The results of antibiotic sensitivity test revealed that the examined three *A. sobria* isolates were highly sensitive to enrofloxacin while they were resistant to amoxicillin, ampicillin, erythromycin and Trimethoprim-Sulfamethoxazole. Consequently, enrofloxacin was the best drug choice for treatment. Several studies reported that *A. sobria* isolated from human, food, environmental samples, diseased fish, fresh water fish, frozen fish were resistant to ampicillin [27-30].

A. sobria isolated from farmed carp were sensitive to oxolinic acid [28]. On the other hand, only 61.1% of *A. sobria* strains were resistant to ampicillin [31]. In contrast to the results of the present study, *A. sobria* isolated from farmed carp were sensitive to trimethoprim-sulphamides [28]. In addition, *A. sobria* strains isolated from rainbow trout were resistant against oxolinic acid by 97.2% and oxytetracycline by 69.5% [31].

The *in vivo* treatment trial of fish experimentally infected with pathogenic *A. sobria* isolates using enrofloxacin was performed. The levels of serum AST and ALT, showed significant increase in G2 (infected group without treatment) compared with G1 (non infected and non-treated group). This could be attributed to the hepatic damage caused by the effect of different exotoxins released by *A. sobria* which lead to extensive release of these enzymes from the affected hepatic cells to the serum [26,32]. In contrary, the level of serum aminotransferases and creatinine showed significant decrease in enrofloxacin treated group, compared with the infected, non-treated group. Such results indicated an improvement in the liver and kidney functions. This result might be attributed to efficacy of enrofloxacin in the treatment of aeromoniasis which limit the destructive and toxic effects of *A. sobria* on the liver and kidney [14].

Similar results were also found in experimentally infected *O. niloticus* with *A. hydrophila* (1.5×10^8 CFU ml^{-1}) and treated with enrofloxacin (10 mg/ kg Bwt /day) for 10 successive days [33]. The results showed a significant decrease in the serum levels of urea, creatinine, ALT and AST compared with infected non-treated group. Histopathological findings in liver and kidney confirmed the results of serum biochemical analysis where the liver and kidney showed normal tissue architecture and cellular details.

The lesions of the intestine were reduced to moderate degree of edema beneath the submucosa together with few degenerative changes. These results support our findings, as enrofloxacin is able to limit the destructive effect of the bacteria on the liver and kidney tissues.

Conclusion

In conclusion, *Aeromonas sobria* is considered one of the main causes of economic losses and fish mortalities at Sahl El-Housinia, Sharkia Governorate, Egypt, especially with the climate change in Egypt and the change in water temperature as well as bad water quality. The use of enrofloxacin as a medical water bath by 2 mg/l for five days revealed good results but must be used under restrictions to avoid bacterial resistance to the antibiotic.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

معدل انتشار مرض التسمم الدموي والفم الاحمر الناجمة عن الايروموناس سوبريا في المزارع السمكية في سهل الحسنية

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اجريت هذه الدراسة بهدف عزل وتصنيف الايروموناس سوبريا المسبب لأعراض التسمم الدموي المصحوب باحمرار وأنزفه دموية على الفم في كلا من اسماك البلطي النيلي والبوري المستزرعة في احدى المزارع الخاصة في منطقة سهل الحسينية بمحافظة الشرقية مع محاولات لعلاج هذا المرض في الأسماك المصابة معمليا. وقد تم فحص عدد ٣١٢ سمكة من اسماك البلطي و١٥٨ سمكة من اسماك البوري. تم تجميع هذه الاسماك حية ثم نقلها الى معمل أمراض ورعاية الاسماك بكلية الطب البيطري – جامعة الزقازيق وذلك في الفترة من ابريل ٢٠١٤ حتى مايو ٢٠١٥. وقد تم فحص هذه الاسماك اكلينيكيا وبكتيريا من خلال العزل البكتيري من الاعضاء الداخلية للأسماك المصابة (الخياشيم-الكلى –الكبد-الامعاء-الطحال). تم التعرف على عترات الايروموناس سوبريا المعزولة باستخدام الاختبارات الكيميائية الحيوية وجهاز الفيتك ٢ وكذلك باستخدام تفاعل البلمرة المتسلسل. تم اجراء الاختبارات لمعرفة قدرة الميكروب على احداث المرض ومدى حساسية هذا الميكروب للمضادات الحيوية المختلفة بالإضافة الى محاولات لعلاج الأسماك المصابة معمليا. تمثلت الاعراض الظاهرية على الأسماك المصابة طبيعيا بوجود علامات التسمم الدموي مثل احمرار الفم والجلد والزعانف. تم عزل ميكروب الايروموناس سوبريا بنسبة اعلى في اسماك البلطي النيلي (٣٥.٨٩%) منها في اسماك البوري (٢٠.٨٨%). واوضحت النتائج المعملية حساسية ميكروب الايروموناس سوبريا للانروفلوكساسينن. تم تأكيد هذه النتائج من خلال علاج اسماك البلطي التي تم حقنها بجرعة ٠.٢ مل من بكتيريا الايروموناس سوبريا المحضنة لمدة ٢٤ ساعة بتركيز ٢ مجم/لتر كحمام مائي علاجي لمدة ٥ ايام من الانروفلوكساسين. حيث اظهرت النتائج استجابة علاجية عالية وذلك مع تحسن معنوي في مستوى انزيمات الكبد والكلى وايضا التغيرات الهستوباثولوجية في انسجة الكبد والكلى والامعاء للأسماك المصابة المعالجة.