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Treatment of MAS caused by *Aeromonas sobria* in European seabass

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ABSTRACT

Aeromonas sobria is one of the most common causes of motile aeromonas septicemia affecting fishes which results in substantial mortality and economic losses in the aquaculture sector. In the present study, *Aeromonas sobria* was isolated from a diseased *Dicentrarchus labrax* earthen pond with a high mortality rate. Hemorrhagic ulceration of the skin, cataract, fins and tail erosions together, with gill hemorrhage in a few cases, were the common clinical signs reported for naturally and experimentally infected fish. Severe congestion in internal organs, particularly the elementary tract, liver and posterior kidney in both naturally and experimentally infected fish were the dominant postmortem lesions. Thirteen *A. sobria* isolates were biochemically identified by the Vitek 2 system and then confirmed by the phylogenetic analysis. Pathogenicity test revealed that *A. sobria* was virulent for *D. labrax* fingerlings with LD_{50} equals 2.64×10^7 CFU. $Fish^{-1}$. Histopathological examination indicated the presence of severe degenerative changes in the liver and kidney of experimentally infected *D. labrax* such as inflammation, necrosis, mononuclear cell infiltration, melano-macrophage centers activation. Doxycycline was the most effective antibiotic against all of the tested isolates, followed by flumequine (77%) and oxytetracycline (69%), while most *A. sobria* isolates were resistant to cephradine as only 15.3% of the isolates were susceptible while all isolates showed complete resistance to amoxicillin. The treatment trial indicated the efficacy of infeed administration of doxycycline at a dose of 20 mg. kg^{-1} bodyweight in decreasing fish mortality to half, but the higher dose (40 mg. kg^{-1} bodyweight) was more effective in termination of *A. sobria* infection in challenged *D. labrax* fingerlings.

INTRODUCTION

Aquaculture is one of the most important food sources and income for millions of people all over the world **(FAO, 2015)**. Since marine aquaculture represents about 14.5 % of the total aquaculture in Egypt **(Moustafa *et al.*, 2010; Wally, 2016)**, it is regarded as a radical source of the Egyptian national income **(Ali & Aboyadak, 2021)**.

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Sea Bass *(Dicentrarchus labrax)* is an economically significant species in the Mediterranean aquaculture **(Cardia & Lovatelli, 2007)**, with the top producers of this fish species located in Greece, Italy, Spain, Turkey and Egypt **(Gilchrist, 2017; FAO, 2017)**. The European seabass production in Egypt has increased from 19,027 tons in 2011 **(GAFRD, 2012)** to 30,313 tons, representing about (11.52) % of the total world production in 2019 **(FAO, 2021)**.

Fish diseases threaten the aquaculture industry by decreasing fish production **(Noga, 2010)**. This is widely obvious especially in the tropical countries including Egypt through the direct losses related to high mortalities and the indirect losses represented in disease management costs and decreased product value **(Athanassopoulou *et al*., 2009)**. Notably, bacteria are the most influential disease problem among the potential fish pathogens **(Meyer, 1991; Lafferty *et al*., 2015; Aboyadak *et al*., 2016)**.

In the Mediterranean Sea countries, Aeromonas is considered one of the most the dominant bacterial pathogens responsible for heavy losses in the European seabass production **(Toranzo *et al*., 2005; Ozturk & Altinok, 2014)**. Aeromonas is a normal inhabitant microflora in aquatic environments that may be turned into pathogenic and cause significant economic losses in the aquaculture industry worldwide **(Palu *et al*., 2006)**.

Aeromonas species are Gram-negative facultative anaerobic bacteria that have a wide host range, including fish, lower and higher vertebrates and humans **(Austin *et al.*, 2007; Rey *et al.*, 2009; Janda & Abbott, 2010)**. Aeromonas is responsible for severe septicemic diseases in both freshwater and marine fishes **(Austin *et al.*, 2007; Martinez-Murcia *et al.*, 2008)**. Several clinical signs including ulceration, fin and tail rot, abdominal distention, and exophthalmia were reported in Aeromonas infected fish **(Sreedharan *et al.*, 2011)**.

The virulence of Aeromonas is complex and involves multiple virulence factors, viz. hydrolytic enzymes, cytotoxic and cytotonic enterotoxins and haemolytic toxins **(Janda & Abbott, 2010)**. These virulence factors enable the bacteria to colonize, invade, replicate and damage the host tissues and impair the host defense system and spread to host blood **(Yu *et al.*, 2005)**. The release of extracellular hemolysin and aerolysin potentially contributes to septicaemia **(Chopra *et al.*, 1993; Nordmann & Poirel, 2002)**. Motile Aeromonads including *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae* are the major aetiological agents for MAS **(Wahli *et al.*, 2005)**.

Aeromonas sobria is a Gram-negative bacterium that presents in association with hemorrhagic septicemia in cold-blooded animals such as fish, reptiles and amphibians **(Austin & Austin, 2016)**. *A. sobria* is one of the most virulent Aeromonas species **(Janda & Kokka, 1991; Sreedharan *et al.*, 2011)**.

VITEK 2 compact system is a fully automated bacterial identification system that is based on the biochemical reactions specific to each bacterial species (**Ali *et al*., 2021a**). VITEK 2 system allows kinetic analysis by reading each test every 15min; the optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity, and colourimetric signals (**Ligozzi *et al.*, 2002**). **Ling *et al.* (2001)** and **Dar *et al.* (2016)** reported that, VITEK 2 system is effective in the identification of bacteria within the genus Aeromonas.

Tetracyclines, particularly oxytetracycline, are the most commonly used antibiotics in treating diseased cultured fish worldwide (**Aboyadak *et al*., 2016**). Eminently, doxycycline is a member of second-generation tetracyclines. Doxycycline has better chemical properties than the other tetracyclines, such as long plasma half-lives, high lipid solubility and extended antibacterial activity than older tetracycline members (**Arthur *et al.*, 2000**). In addition, doxycycline is effective against many bacterial fish diseases such as *A. hydrophila, Edwardsiella ictaluri, Fibrobacter columnaris*, *Pseudomonas fluorescens*, and *Vibrio vulnificus* (**Song *et al.*, 2014; Liu *et al.*, 2019**).

The present work aimed to isolate and identify the bacterial pathogens responsible for the disease outbreak affecting the cultured European seabass, ensure the pathogenicity of *Aeromonas sobria* and determine the effective doxycycline dose capable of treating this infection.

MATERIALS AND METHODS

Statement of compliance with the ethics guidelines:

Experimental fish used in the present research were handled, transported, examined and euthanized following the guidelines of the National Advisory Committee for Laboratory Animals Research **(NACLAR, 2004)** and **CCAC (2005)** for the care and use of fish in teaching and research.

Naturally infected fish samples:

Fifteen moribund fingerlings were collected alive from *Dicentrarchus labrax* monoculture earthen pond located at Eldeba triangle, Damietta province, Egypt. Samples were collected during the summer season of 2022; cultured fish were fed on 40% of protein floated marine fish ration produced by a private factory. Each fish was rinsed with sterile distilled water before packing in polyethylene bag as mentioned in the study of **Ali *et al*. (2021a)**. Collected fish were immediately transported to the Fish Diseases Lab, the National Institute of Oceanography and Fisheries in car refrigerator at 8ºC for bacterial isolation.

Antemortem and postmortem examination:

The case history, antemortem and postmortem examination of diseased fish was performed during the affected farm visit to avoid any nonspecific postmortem changes. Antemortem examination was performed as described by **Austin and Austin (2016)** and the postmortem examination was done following the method of **El-Bahar *et al.* (2019)**.

Initial bacterial isolation:

Using a sterile cotton swab, swaps were taken from the heart blood, hepatopancreas and posterior kidney; sampled swabs were placed in numbered tryptic soy broth tube, and tubes were incubated at 37°C for 24h. Brain heart infusion agar plate was streaked from the corresponding broth tube; after that, the plates were incubated at 37°C for 24h.

Identification of bacterial isolates using Vitek 2 Compact system:

The recovered bacterial isolates were biochemically identified using Gram-negative bacterial identification card as described in the study of **Ali *et al.*, (2021b)**. The biochemical profile of each isolate was monitored then automatically matched with the database for identification.

Molecular identification of *Aeromonas sobria*:

Genomic DNA extraction:

Genomic bacterial DNA was extracted using G-spin™ total DNA extraction kit, Intron, Korea.In a sterile Eppendorf tube, 0.5ml of three days bacterial culture broth (2 - 3 McFarland standard) was taken, and then 200µl of CL buffer was added and the tube was well mixed. Afterward, 20µl of Proteinase K and 5µl of RNase were added. Eppendorf tube was incubated at 56°C for 30min for a complete lyses, then the extraction's procedures were completed as described in **Ali *et al.* (2022)**.

Amplification of 16S rRNAgene:

Aeromonas sobria 16S rRNAgene amplification was performed using 27F (5´ AGAGTTTGATCCTGGCTCAG 3´) and 1492R (5´ GGTTACCTTGTTACGACTT 3´) universal bacterial primer according to **Tanrıkul and Dincturk (2021)**. PCR reaction mixture was prepared by adding 1.25µl of each primer to 3µl of genomic DNA; 12.5µl of 2X master mix and 7µl of nuclease-free water. PCR reaction was carried out in T100 Bio-Rad thermal cycler. PCR run started with initial denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 45sec, annealing at 57°C for 45sec and elongation at 72°C for 1min, finally run ended with one extension step at 72°C for 5min. Amplicon was separated by electrophoresis of PCR products in 1.5% w/v molecular grade agarose gel containing 0.5 µg/ml ethidium bromide **(Lee *et al.*, 2012)**. Briefly, in

ESH3350-SYS horizontal tanks, Consort, Belgum, agarose gel plate was added then covered with 1X tris borate EDTA buffer. PCR product (10 µl) was loaded to the agarose gel holes. EV3000 power supply Consort, Belgum was adjusted at 100V for one hour, and amplicon bands were visualized using gel documentation system, Azure C200, Azure Biosystems, USA.

Sequencing and phylogenetic analysis:

After the purification of the PCR product using MEGA quick-spin™ total fragment DNA purification kit, it was forward sequenced in the automated DNA Sequencer model 3130, Applied Biosystems, USA. The obtained sequence identity to the GenBank data was determined as described by **Bal and Hujol (2007)** using BLAST® (basic Local Alignment Search Tool). Then, the optimal phylogenetic tree was drown using MEGA 9 program with the neighbors-joining method **(Kumar *et al*., 2016)**.

Antibiogram tests:

Sensitivity test:

Antimicrobial susceptibility of all *A. sobria* isolates to some antimicrobial drugs was determined by agar disk diffusion according to the method of **Ali *et al*. (2021b)**. Amoxicillin (AMX 10 µg), cefradine (CE30 µg), oxytetracycline (OTC30 µg), doxycycline (DO 30 µg) and flumequine (UB30 µg) sensitivity disks, Oxoid® UK were used. Seeded Muller-Hinton agar plates were incubated at 37°C for 24h, then the inhibition zone was measured to the nearest mm and interpreted according to breakpoints mentioned by **CLSI (2016)**.

Minimum inhibitory concentration (MIC):

MIC of doxycycline was determined for all the recovered isolates according to the method of **Ali *et al*. (2019)**. Doxycycline solution was prepared at 1280µg. 100 $µl^{-1}$, then this solution was double-fold diluted for 15 successive times. The overnight cultured broth was adjusted to 0.5 McFarland standard then diluted to 0.5% with sterile broth. 2,3,5 tetrazolium chloride was added at 0.0001% with a sterile syringe. 9.8ml from broth was loaded in each sterile screw capped tubes, then 200µl from the antibiotic solution was added to achieve doxycycline concentration of 265, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 µg. ml^{-1}, respectively. Tubes were incubated at 37°C for 24h; the lowest antibiotic concentration that completely prevent bacterial growth (non-colored broth) is the MIC, but the red-colored broth is an indication of bacterial growth.

Experimental fish:

One hundred apparently healthy *D. labrax* fingerlings ranged between 25 - 38g in weight and 12 - 16cm in the total length were collected from the Mediterranean Sea on Alexandria coast; fish were transported to the wet fish diseases laboratory, NIOF, under continuous aeration using pure oxygen cylinder as mentioned by **Bosworth and Small (2004)**. Experimental fish were placed in $5m^3$ fiberglass aquaria, supplied with marine water with continuous water change at a rate of $1m^3$ per day. Fish were fed on 40% protein floated marine fish ration, Skretting co. at 3% of biomass and left for 15 days to acclimatize.

Pathogenicity test:

Pathogenicity test was performed according to **Saleh *et al.* (2021)** for (BX0815) *A. sobria* isolate. Single *A. sobria* colony was picked up from agar surface then incubated on brain heart infusion broth at $37^{\circ}C$ for 12h. Bacterial growth was collected and then adjusted to (5×10^6, 5×10^7 and 5×10^8) CFU ml^{-1}. Forty-eight *D. labrax* fingerlings were randomly divided into 4 equal groups in two replicates (12 fish/group & 6 fish/replicate); each fish in group 1, 2 and 3 was intrapretoneally inoculated with 0.2ml of bacterial suspension containing 10^6, 10^7 or 10^8 CFU.$fish^{-1}$, respectively, while group 1 (control negative group) was inoculated with 0.2ml of sterile normal saline. Feed was restricted at 24h before infection; fish were observed twice daily for 7 days for monitoring the clinical signs and mortality rate. Dead fish is considered only if challenged bacterial strain was re-isolated, and the lethal dose fifty (LD_{50}) was calculated as described in the work of **Reed and Munch (1938)**.

Histopathological investigation:

Hepatopancreas and posterior kidney tissue slices were collected from *A. sobria* experimentally infected fish, and tissues were fixed in 10% neutral formalin. Tissue slices were dehydrated in ascending grade ethyl alcohol, cleared in xylene then impeded in soft then hard Paraffin wax and cut to 5µm sections. Tissue sections were mounted over glass slides then stained with hematoxylin and eosin and photographed using Olympus microscope with digital camera **(Suvarna *et al.*, (2018)**.

Treatment trial:

The efficacy of doxycycline administration for controlling *A. sobria* infection was assessed. Briefly, forty-eight European seabass fingerlings were randomly divided into four groups, as shown in Table (٦). Group (5) remained as negative control, while group (6-8) were experimentally infected with *A. sobria* 2.64×10^7 CFU.$fish^{-1}$, in which group (6) served as positive control and group (7 & 8) received medicated feed containing doxycycline 20 and 40 $mg.kg^{-1}$ fish weight (770 and 1540 mg doxycycline hyclate per kg feed at feeding rate of 3% of biomass). Doxycycline powder (Doxyveto 50%, IMV co.,

Egypt) was mixed with 25ml of fish oil, and the mixture was evenly distributed to one kg of fish feed. The medicated feed was left for one day at room temperature to completely absorb the drugs, then it was preserved at 8°C. Medicated feed was administrated for 24h after the experimental infection; treatment was continued for seven successive days, and mortality rate was daily monitored for ten days.

RESULTS

Antemortem and postmortem examination:

Naturally infected fish showed increased mortality rate and signs of septicemia experimentally infected fish showed hemorrhagic ulceration of the skin, cataract, fins and tail erosions together with gill hemorrhage in few cases (Fig. 1). Internal organs particularly the elementary tract, liver and posterior kidney were severely congested in both experimental and naturally infected fish with *A. sobria*.

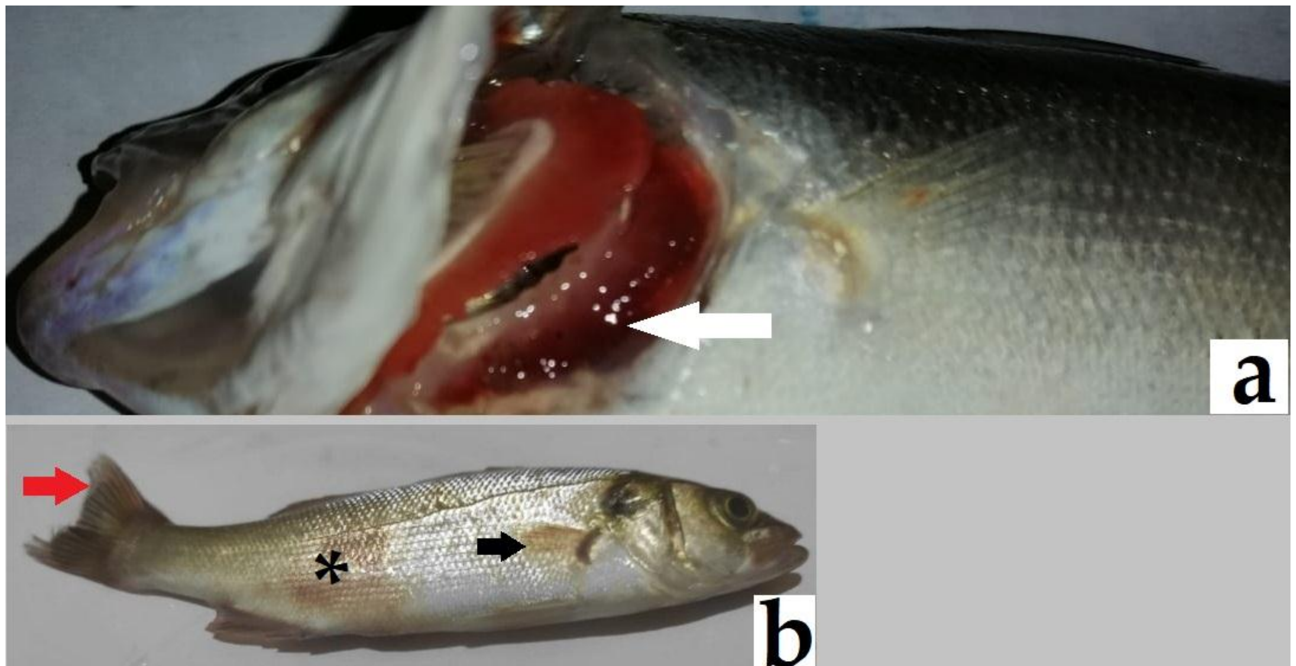

Fig. 1. A. Experimentally infected *Dicentrarchus labrax* showing congested gills (white arrow); **B.** Fin errosions (black arrow), tail errosions (red arrow) and congested skin (astrix).

Biochemical identification of bacterial isolates:

On brain heart infusion agar, *A. sobria* grew as small to medium white colonies; thirteen bacterial isolates were biochemically identified by Vitek 2 system as *A. sobria* with probability range between 89 and 93 %; the biochemical profile of *A. sobria* is shown in Table (1).

Table 1. Biochemical profile of *A. sobria* isolates

Biochemical reaction	Result	Biochemical reaction	Result
Ala-Phe-Pro-Arylamidase	+	Saccharose /Sucrose	-
Adonitol	-	D-Tagatose	-
L-Pyrrolydonyl-Arylamidase	-	D-Trehalose	+
L-Arabitol	-	Sodium Citrate	+
D-Cellobiose	-	Malonate	-
β–Galactosidase	+	5-Keto-D-Gluconate	-
H_2S production	-	L-Lactate alkalinization	+
β-N-Acetyl–Glucosaminidase	+	α–Glucosidase	-
Glutamyl ArylamidasepNA	-	Succinate Alkalinization	+
D-Glucose	+	β-N-Acetyl–Galactosaminidase	-
γ –Glutamyl–Transferase	-	α–Galactosidase	-
Glucose Fermentation	+	Phosphatase	-
β–Glucosidase	-	Glycine Arylamidase	-
D-Maltose	+	Ornithine Decarboxylase	-
D-Mannitol	+	Lysine Decarboxylase	+
D-Mannose	+	L-Histidine Assimilation	-
β–Xylosidase	-	Courmarate	+
β-alanine arylamidasepNA	-	β–Glucuronidase	-
L-Proline Arylamidase	+	O/129 Resistance (Comp. Vibrio)	+
Lipase	+	Glu-Gly-Arg- Arylamidase	+
Palatinose	-	L-Malate Assimilation	-
Tyrosine Arylamidase	+	Ellman	+
Urease	-	L-Lactate Assimilation	-
D-Sorbitol	-		
Probability	From 89% to 94%		

Molecular identification:

The phylogenetic analysis of the obtained 16SrRNA gene sequence indicated the presence of *A. sobria*, with 98.18 to 98.57% similarity to the available *A. sobria* deposited sequences in GenBank, including *A. sobria* strain JXYFP5 (accession no: KF569510.1, host *Neophocaena asiaeorientalis* fish), *A. sobria* strain hs-1 (accession no: FJ461353.1, host rice field eel fish), Aeromonas sp. strain AH1-6 F87NE (accession no. MZ490702.1 from river water) and *A. sobria* strain MTJW-9 (accession no KM516017.1, from South Korea). The phylogenetic tree is represented in Fig. (2); the obtained sequence was deposited in GenBank under OP252664 accession number for the *A. sobria* isolate named (BX0815).

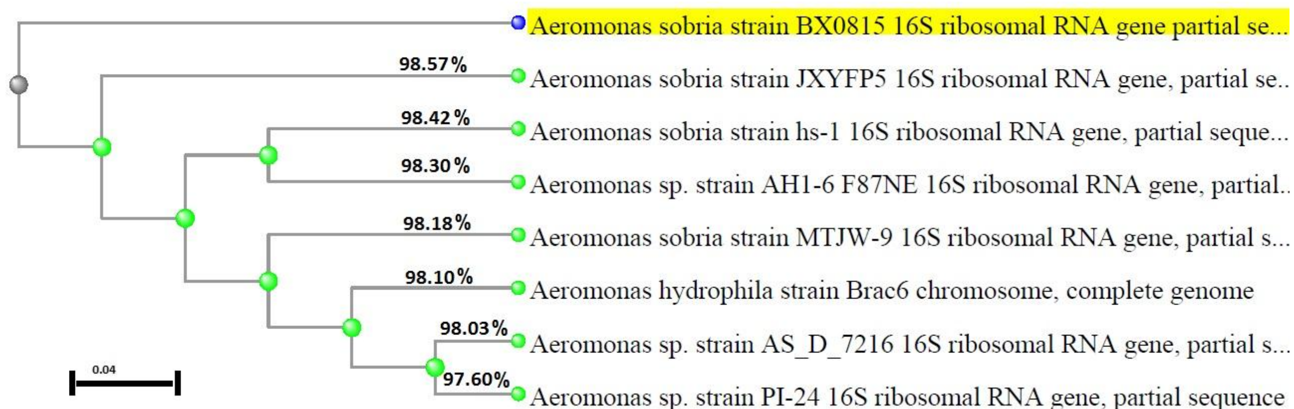

Fig. 2. A. Phylogenetic tree for 16S rRNA gene partial sequences of BX0815 isolate indicating 98.18 - 98.57 % similarity with *Aeromonas sobria* isolates registered in Gene bank.

Antibiogram tests:

Agar disc diffusion test:

All the tested *A. sobria* isolates (100%) were highly sensitive to doxycycline, followed by flumequine (77%) and oxytetracycline (69%); while, the vast majority of isolates was resistant to cephradine since only 2 isolates representing 15.3% were sensitive. All the *A. sobria* isolates were completely resistant to amoxicillin as represented in Tables (2, 3) and Figs. (3a, b).

Table 2. Agar disc diffusion test result for *A.sobria* isolates

Antibiotic	SB mm	NSI	%	ZD mm
Amoxicillin	**≥ 18**	**0**	**0**	**-**
Cephradine	**≥ 18**	**2**	**15.38**	**18**
Doxycycline	**≥ 16**	**13**	**100**	**21 - 25**
Flumequine	**≥ 22**	**10**	**76.92**	**22 - 24**
Oxytetracycline	**≥ 19**	**9**	**69.23**	**19 - 22**

SB: Sensitivity breakpoint in mm, **NSI:** Susceptible isolates No., **%:** Susceptible isolates %, **ZD:** Zone diameter range in mm.

Table 3. Antibiogram profile for each *A. sobria* isolate

Isolate No. / Antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	١٣
Amoxicillin	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephradine	R	R	S	R	R	R	R	R	R	S	R	R	R
Doxycycline	S	S	S	S	S	S	S	S	S	S	S	S	S
Flumequine	S	R	S	S	S	S	S	S	S	R	R	S	S
Oxytetracycline	S	S	R	R	S	S	S	R	S	S	S	S	R

Isolate No.: isolate number**, S:** Susceptible**, R:** Resistant

Minimum inhibitory concentration (MIC):

Minimum inhibitory concentration of doxycycline against *A. sobria* isolates ranged between 0.25 and 1µg. ml^{-1} as shown in in Table (4) and Fig. (3c).

Table 4. Minimum inhibitory concentration of all *A.sobria* isolates

MIC µg. ml^{-1}	1	0.5	0.25
NSI	2	7	4

MIC breakpoint for doxycycline is 4µg.ml^{-1},
NSI: Susceptible isolates No.,
MIC µg.ml^{-1}: Tested isolates MIC in µg.ml^{-1}.

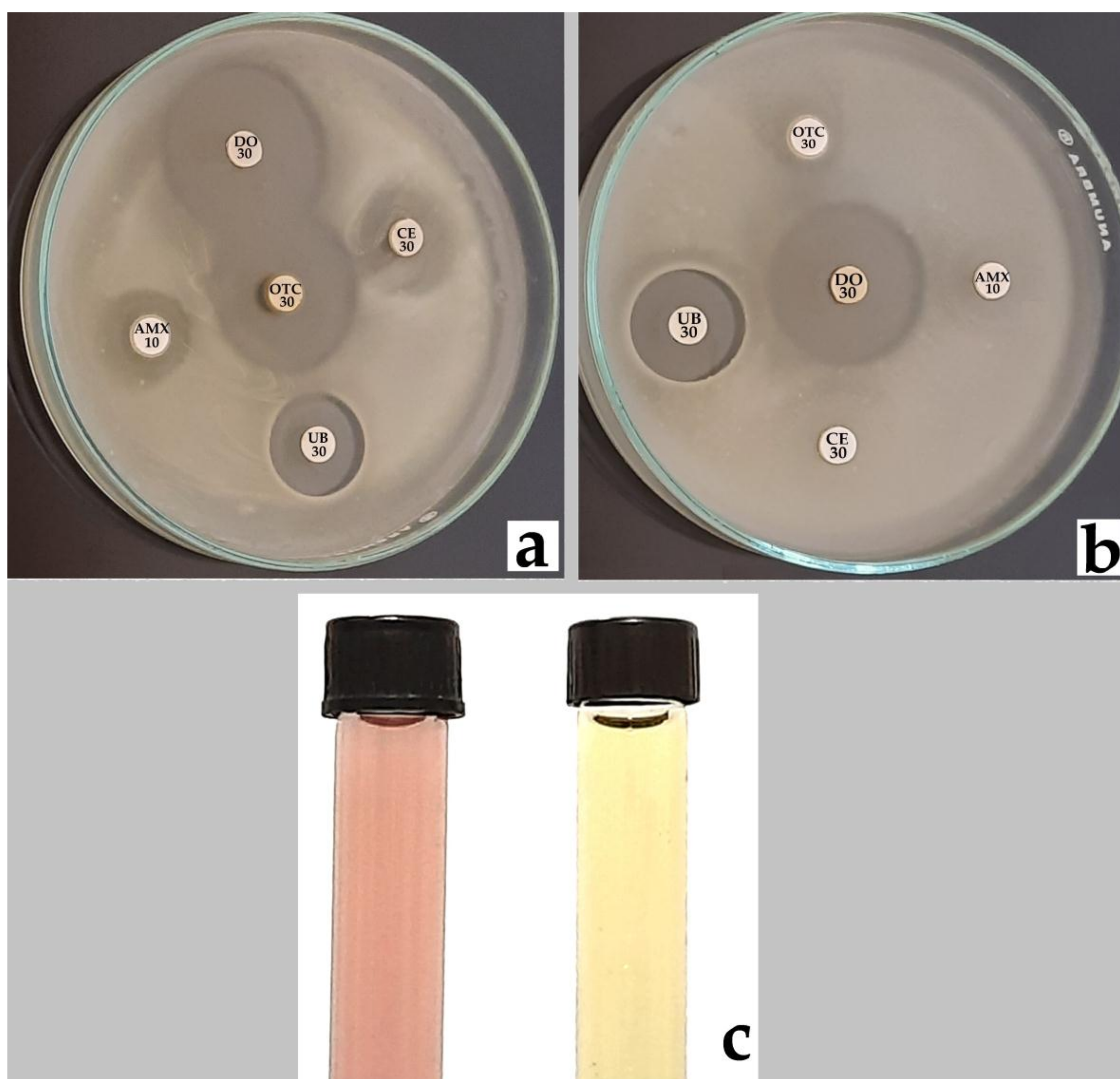

Fig. 3. (a) Sensitivity profile of BX0815 *A. sobria* isolate (the most sensitive isolate to antibiotics), it is sensitive todoxycycline, oxytetracycline, flumequine and cefradine. **(b)** Sensitivity profile of *A. sobria* isolate number 4 (the most resistant *A. sobria* isolate to antibiotics), it is sensitive to only todoxycycline and flumequine. **(c)** The MIC of doxycycline for *A. sobria* isolate number 5 estimated by 1µg. ml^{-1} as the right tube contain doxycycline (1 µg. ml^{-1}) prevents bacterial growth (yellow color), while the left tube containing doxycycline (0.5 µg. ml^{-1}) did not prevent bacterial growth (pink color).

OTC: Oxytetracycline 30 µg, **AMX:** Amoxicillin 10 µg, **DO:** Doxycycline 30 µg, **UB:** Flumequine 30 µg, and **CE:** Cefradine 30 µg.

Pathogenicity Test:

The cumulative mortality after seven days post infection is presented in Table (5); the calculated LD_{50} of *A. Sobria* for *D. labrax* fingerlings was 2.64×10^7 CFU. $Fish^{-1}$.

Table 5. Pathogenicity of *A. sobria* for *D. labrax* fingerlings

Group	Bact.Conc. CFU. $Fish^{-1}$	Dead fish	Mortality %
1 (Control)	Normal saline	0	0
2	10^6	1	8.3
3	10^7	5	41.67
4	10^8	11	91.67

Bact. Conc: Bacterial concentration, **Control:** Non-infected group, each group consists of 12 fish.

Histopathological Investigation:

Severe degenerative changes were observed in experimentally infected *D.labrax* liver such as the presence of hemorrhage and extraversion of RBCs in hepatic tissue. Inflammation, necrosis, mononuclear cell infiltration, diffused vacuolar degeneration and coagulative necrosis were also dominant (Fig. 4).

Moreover, the posterior kidney of infected fish was severely affected by the bacterial infections; the most abundant pathological lesions were melano-macrophage centers activation, inflammation represented in interstitial mononuclear cells infiltration, glomerular degeneration and glomerular hypertrophy. Renal tubules were also affected, and the proximal convoluted tubules epithelium was degenerated and sloughed off with interstitial aggregation of the inflammatory cells and detachment of the distal convoluted tubular epithelial (Fig. 5).

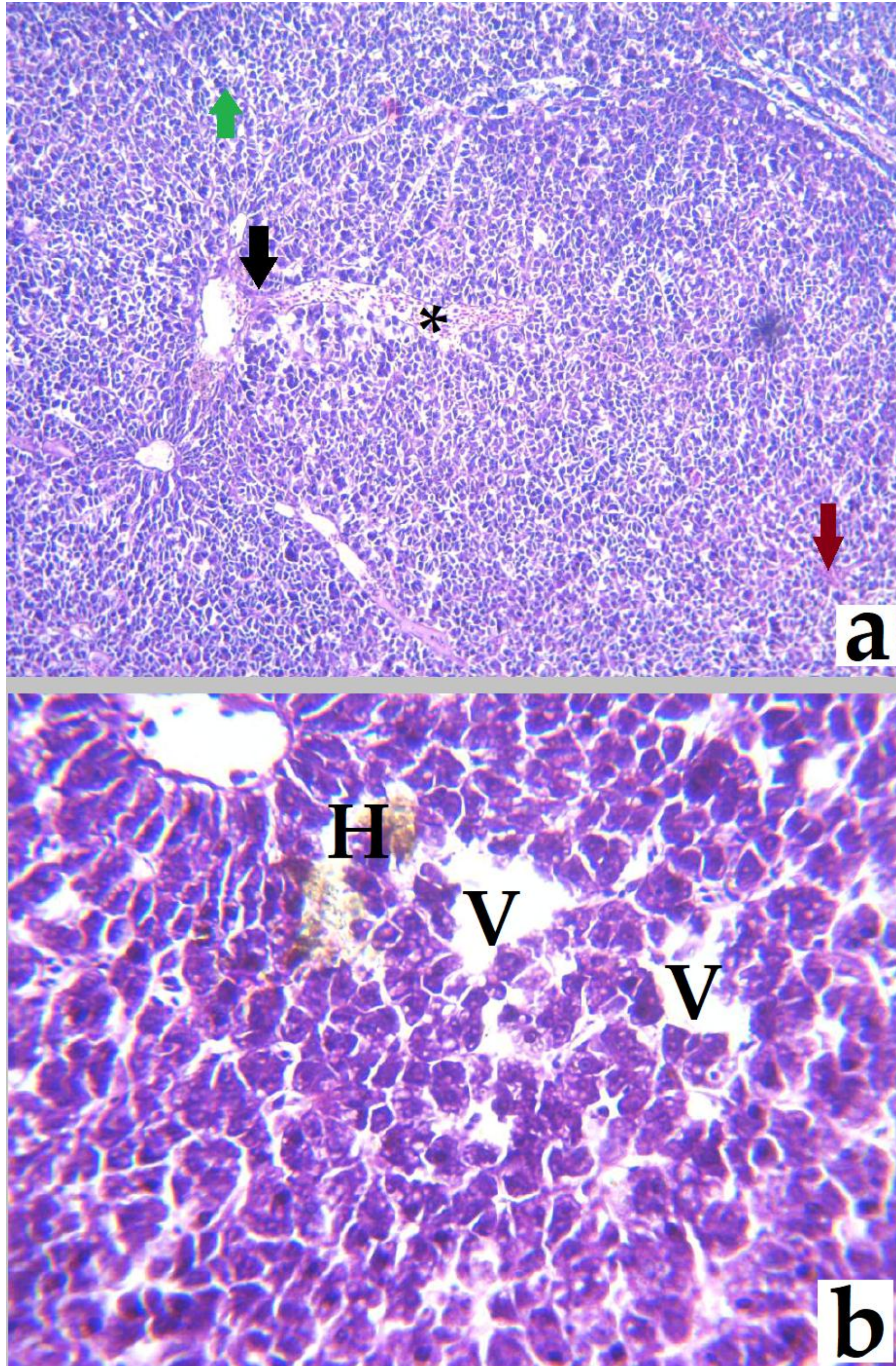

Fig. 4. Liver of *D.labrax* infected with *A. sobria* isolate **(a)** Extraversion of RBCs (astrix), necrosis (black arrow), mononuclear cell infiltration (green arrow), diffused vacuolar degeneration and coagulative necrosis (brown arrow), H & E, X = 100. **(b)** Hemosiderin deposition (H) and vacular degeneration (V), H & E, X = 400.

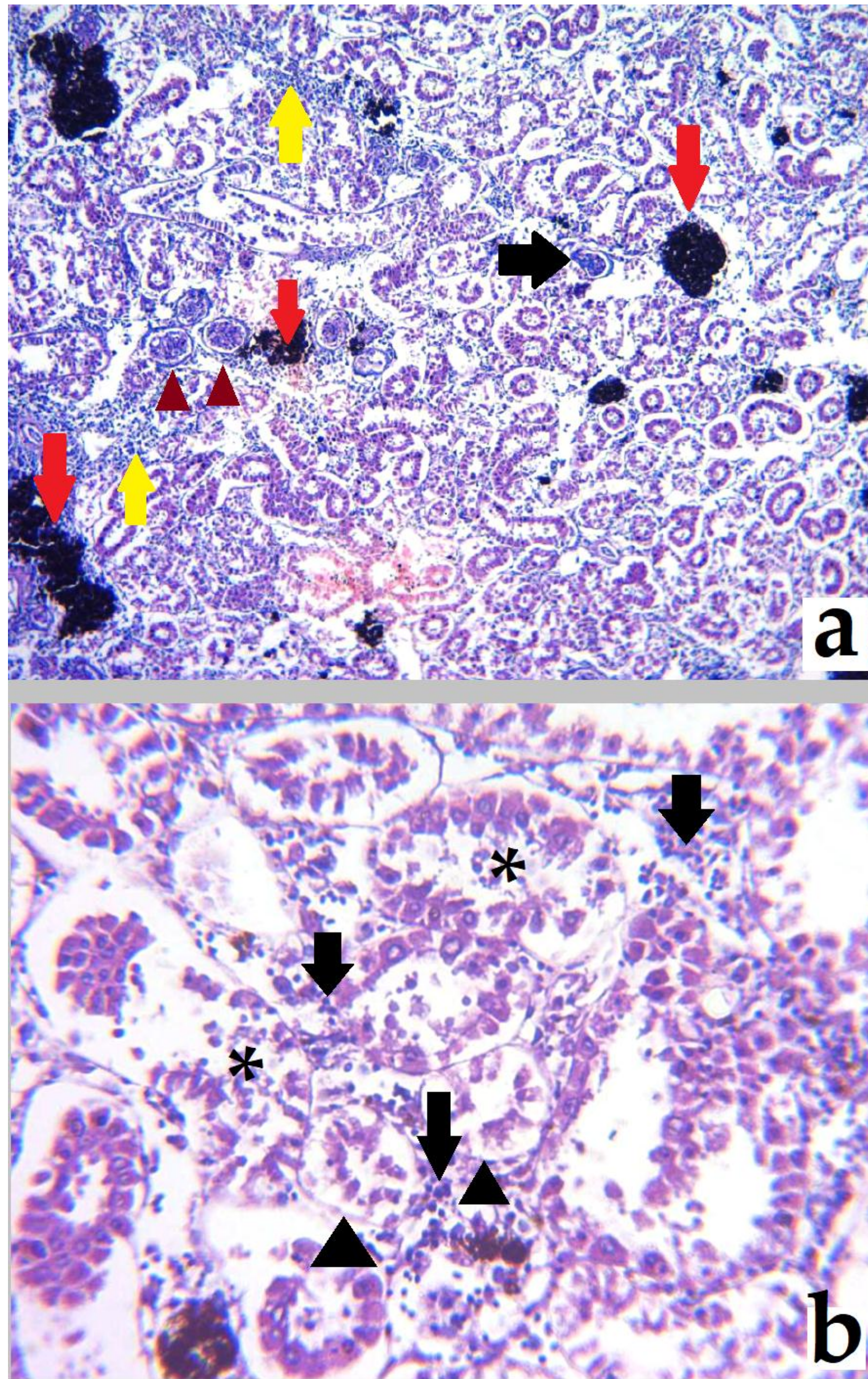

Fig. 5. Posterior kidney of *D.labrax* infected with *A. sobria* isolate **(a)** Melano-macrophage centers activation (red arrow), interstitial mononuclear cells infiltration (yellow arrow), glomerular degeneration (black arrow) and glomerular hypertrophy (brown arrow head), H & E, X = 100. **(b)** Degenerated proximal convoluted tubular epithelium (black arrow head), aggregation of inflammatory cells (black arrow) and detachment of distal convoluted tubular epithelial lining (astrix), H & E, X = 400.

Treatment trial:

Treatment trial indicated partial efficacy of doxycycline at a dose of 20mg. kg^{-1} of bodyweight in decreasing fish mortality, associated with *A. sobria* infection from 58% to 25%. While, the higher doxycycline dose (40 mg. kg^{-1} bodyweight) was more effective in treating fish from such infection by lowering fish mortality to only 8.33%, compared to the infected non-treated group (58%) as illustrated in Table (6).

Table 6. Efficacy of Doxycycline in treatment of *A. sobria* infection in *D. Labrax* fingerlings

Group	Doxycycline dose	Number of Dead fish	Mortality %
5 (control -ve)	**-**	**0**	**0**
6 (control +ve)	**-**	**7**	**58.33**
7	**20 mg. kg^{-1}**	**4**	**25**
8	**40 mg. kg^{-1}**	**1**	**8.33**

Group (5) is the control -ve group (non-infected non-treated); **Group (6)** is control +ve group (infected non-treated); **Group (7)** is infected treated with doxycycline 20 mg/kg^{-1}. BW, and **Group (8)** is infected treated with doxycycline 40 mg/kg^{-1}. BW, each group consists of 12 fish.

DISCUSSION

Motile aeromonas septicemia caused by *A. hydrophila,* and *A. sobria* has a significant economic impact on the global aquaculture **(Abd El-Hamid *et al.*, 2016)**، Aeromonas are considered as one of the most critical causes of mass mortalities in Egyptian aquaculture **(Eissa *et al.*, 2011)**. Seabass (*D. labrax*) is the most cultured marine fish species in Egypt, this work was conducted to investigate the cause of mass mortalities affecting cultured seabass farm in Egypt together with introducing an effective treatment against this disease.

This is the first document of *Aeromonas sobria* isolation from diseased cultured seabass in Egypt, while the first global report was in 2008 from *D.labrax* farm in central Greece **(Smyrli & Katharios, 2020)** accordingly, **Uzun and Ogut (2015)** isolated *A. sobria* from the European seabass in the Black Sea region of Turkey, and **Smyrli *et al.*, (2017, 2019)** have also isolated *A. sobria* from diseased seabass farmed in sea cages in

the Aegean Sea, so, Aeromonas is the most prevalent pathogenic bacteria affecting European seabass aquaculture, it is associated with high morbidity and mortality.

In the present study, diseased fish showed hemorrhagic skin ulcers and congested gills, internal organs particularly the elementary tract, liver and posterior kidney were severely congested. Identical clinical signs were reported by **(Uzun and Ogut (2015); Smyrli and Katharios, 2020)** in the naturally infected seabass with *A. sobria*. The recorded clinical signs and postmortem lesions are mainly attributed to circulation of the pathogenic bacteria and its toxic metabolites together with the expression of different virulence factors in the infected fish blood **(Aboyadak *et al*., 2016; El-Bahar *et al*., 2019; Abd El Tawab *et al*, 2022)**.

VITEK 2 automated microbial identification system is based on 47 different biochemical reactions for bacteria identification (each test results was automatically monitored every 15 min), this makes it as one of the most accurate, reliable and rapid techniques for identification of the pathogenic bacteria in human and veterinary medicine. In this study, the thirteen recovered bacterial isolates were identified as *A. sobria* with probability level ranged from 89% to 93% after 5.5 hour.

Molecular identification is the most trusted techniques for identifying bacteria **(Zrncic, 2020)**, VITEK 2 system result was verified by amplification and 16S rRNA gene of one selected isolate then sequencing of the resulted amplicon followed by phylogenetic analysis of the resulted sequence by comparing with the sequences present in the NCBI database. The obtained sequence was deposited in GenBank database under isolate name (BX0815) and accession number (OP252664). BX0815 isolate was identified as *A. sobria*, the similarity with previously deposited GenBank sequences for *A. sobria* was 97.60 - 98.57% for *A. sobria* strains isolated from diseased fish and water (JXYFP5, hs-1, AH1-6 F87NE and MTJW-9). The molecular identification avouches no doubt that *A. sobria* was responsible for the disease affecting the studied seabass farm.

Our results proved pathogenicity of *A. sobria* for *D. labrax* fingerlings with LD_{50} equals to 2.64×10^7 CFU. $fish^{-1}$, this value was quite similar to the LD_{50} recorded by **Yu *et al.*, (2015)** that was 2.1×10^7 CFU $fish^{-1}$ in farmed Mudloach (*Misgurnus mizolepis)*. Otherwise, it was lower than the LD_{50} value estimated by **Lim and Hong (2020)** that was 1.1×10^8 CFU. $fish^{-1}$ in rainbow trout (*Oncorhynchus mykiss*) but it was higher than the LD_{50} reported by **Smyrli *et al.*, (2019)** for *A. sobria* in *D. labrax* which was ranged between 3.3×10^5 to 1.4×10^6 CFU/fish. Variability in the pathogenicity may be governed by many factors such as environmental conditions, fish species and fish immune status، the virulence of challenging strain and activation of certain virulence genes (bacterial genotype).

Agar disc diffusion test and broth dilution test indicated the high susceptibility of the tested *A. sobria* isolates to doxycycline, this finding was identically reported by **Zhou *et al.*, (2019)** but only 69% of tested isolates was susceptible to oxytetracycline the same member in tetracyclines family. This variation in susceptibility to the same tetracyclines family members could be attributed to difference in antibiotic generations in this family, doxycycline is the most recent member of tetracyclines antibiotics for veterinary while oxytetracycline is the oldest and the most commonly used tetracycline for all animal species worldwide, so the bacterial resistance is most common for oxytetracycline but it is unfamiliar for doxycycline, resistance may arise from the previous exposure to sublethal antibiotic concentrations **(Ding *et al.*, 2012)**. The majority of *A. sobria* isolates were resistant to cephradine and all isolates are completely resistant to amoxicillin this elucidate high resistance for beta lactams antibiotics which could be due to secretion of beta-lactamase and cephalosporinase enzyme, similarly, **Lim and Hong, (2020)** reportedthe complete resistance of *A. sobria* to amoxicillin.

Many histopathological alterations were preeminent in the liver and posterior kidney tissues, including inflammation, necrosis and degenerations, this finding reflects the severity of infection and illustrated the cause of high mortality rate after septicemia, this finding are mainly attributed to bacterial invasion and production of different toxic metabolites that affected mainly the highly vascularized parenchymatous organs as liver and kidney. **(Miyazaki and Kaige, 1986; Wahli *et al.*, 2005; Yu *et al.*, 2015)** have also reported nearly similar findings in some fish species infected with *A. sobria*.

In the treatment trial oral administration of doxycycline at a dose of 40 mg. kg^{-1} bodyweight was very effective in termination of *A. sobria* infection in challenged *D. labrax* fingerlings which reflecting the high susceptibility of pathogen to this antibiotic, the lower antibiotic dose (20 mg. kg^{-1} bodyweight) was relatively effective in decreasing mortality but not completely cure the disease, this observation may be explained through difference in feed consumption from fish to another particularly under the disease condition (some fishes consumed more feed than another and so the taken drug dose may varied) that will affects the drug kinetics and subsequently drug concentration in the treated fish serum. Generally, this work recommended administration of doxycycline with feed at a dose of 40 mg. kg^{-1} bodyweight to treat *D. labrax* from the susceptible *A. sobria* infection.

CONCLUSION

A. sobria was isolated from diseased cultured seabass, for the first time in Egypt, signs of septicaemia were remarkable for both naturally and challenged fish. Oral administration of doxycycline at a dose of 40 mg. kg^{-1} bodyweight was very effective in the termination of *A. sobria* infection in challenged *D. labrax*.

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