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Effect of Vibrio Alginolyticus on Mugil Capito

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ABSTRACT

Isolation and identification of the bacterial isolates affecting cultured marine fishes in Egypt as well as studying the relationship between heavy metal concentrations in water and fish tissues with the incidence of these bacterial diseases . Experimental infection : A total number of (110) *Mugil capito* (*M. capito*) (50 gm ± 20) obtained from earthen pond culture in same area of Wadi-mariuot were transported to the laboratory of Poultry and Fish diseases department at faculty of Veterinary medicine - Alexandria university in plastic bags supplied with oxygen (2/3) and were left for 2 weeks for acclimation. (a) For calculation of the LD_{50} 0.2 ml of *V. alginolyticus* (10^6 cells / ml) with different dilutions from 10^{-1} to 10^{-6} was I/P injected in 6 groups of fish (10 fish / group) while the seventh group was also I/P injected with 0.2 ml sterile saline solution 0.9 % and used as control group, then mortalities, clinical signs and PM lesions were recorded after the injection by one week. Then samples for histopathological examination were taken. Re-isolation of the injected bacteria was determined for verification of death. (b) Chronic experiment : by IM injection of 0.2 ml of 1/10 of the LD_{50} in 20 fish (in 2 equal groups) and also IM injection of 0.2 ml of sterile saline solution 0.9 % in other 20 fish (in 2 equal groups; control +ve and Control –ve), then mortalities, clinical signs and PM lesions were recorded for one month after injection and also samples taken for histopathological examination. Results revealed that : 1) The LD_{50} was ($10^{-2.5}$) and there were a generalized signs of septicemia appear as: hemorrhagic spots on external body surface, on fins especially tail fin and on gill cover while in some fish have hemorrhagic swollen protruded anus. Moreover, some fish have nervous manifestations. 2) The PM lesions appear as severe congestion in gills, liver, kidneys and intestine while the most characteristic sign was copious amount of bloody stained ascetic fluids drops from the fish upon opening. 3) The re-isolation of the bacterium causing these signs and PM lesions found to have the same culture and biochemical characteristics of the injected bacterium and this confirm the cause of mortalities, signs and PM lesions. 4) No mortalities, signs or PM lesions were determined during the chronic experiment and this confirms that *V. alginolyticus* has acute and sub acute forms and not has a chronic form of infection. Histopathological results : In the LD_{50} experiment; there was congestion in blood capillaries of gills and blood sinusoids of liver with severe fatty changes in between hepatocytes, severe necrosis in posterior kidney and increase in melanomacrophage centers (MMCs) in Spleen. Moreover, in chronic experiment; there was hyperplasia of gill tissue, fatty degeneration and necrotic foci in hepatocytes, necrosis in posterior kidney till depletion of its haematopiotic tissues and also there was depletion of the white pulp of the spleen.

INTRODUCTION

Wedemeyer (1996) and Ellis (1999) Marine fishes represent the major investment choices for the national fishermen and also they are liable to variable number of environmental stressors, including chemical, natural and biological invaders. Such stressors are the main predisposing factors for the chronic immune

suppression which ends for stimulating the bacterial invasion.

Bacterial diseases are responsible for heavy mortality in both wild and cultured fish and one of the most important factors of economic loss since the beginning of marine fish culture (Anderson and Conroy, 1970).

The prevalence of diseases and the number and types of bacterial pathogens have been well documented in several cultured and wild freshwater fish species, however, only a few bacteriological surveys on the prevalence of bacterial pathogens responsible for outbreaks in marine fishes. Therefore, we have to explore this field to know how we can protect the Egyptian Mari culture against these pathogens.

The aim of current investigation was throw light an pathogencity of *V. alginolyticus* in *Mugil Capito* .

MATERIALS AND METHODS

1. Fish

Fish for experimental work

A total number of 110 apparently healthy *Mugil capito* (*M. capito*) with an average body weight of (50 ± 20 grams) were collected from earthen pond culture from the same area of Wadi-mariout, in large plastic bags containing water enriched by oxygen (2/3) and transported to the laboratory of the department of Poultry and Fish diseases - Faculty of Veterinary Medicine- Alexandria University as soon as possible. The fish were acclimated to the laboratory conditions for two weeks prior to the experiment. Random specimens from fish were taken for bacteriological examination to ensure that fish was non infected.

2. Aquaria

Fish were kept in prepared glass aquaria (90 x 50 x 35 Cm). These aquaria were used for holding the experimental fish throughout the period of the present study; the continuous aeration was maintained in each aquarium using an electric air pumping compressors.

3. Fish diets

Fish were fed on a commercial fish diet containing 45% crude protein. The diet was daily provided at a fixed feeding ratio of 3 % of body weight of fish as described by ***Eurell et al. (1978***).

1. Gross clinical examination

According to the method described by ***Amlacher (1970***).

2. Postmortem (PM) examination

According to Conroy and Herman (1981).

3. Experimental infection

3.1. Lethal dose fifty (LD_{50}) determinations

3.1.a. Preparation of the inoculum

The inocula were selected as the most predominant bacterial isolate which was ***V. alginolyticus*** and prepared as I/P injections according to Austin and Austin (1999).

The bacterial isolate was subcultured on trypticase soya agar plates and incubated at 25 ^{0}C for 24hr. A typical isolated colony was picked up and inoculated into trypticase soya broth and incubated at 25 ^{0}C for 24hr. The broth culture was centrifuged and supernatant decanted out. The sediment was re suspended in sterile saline and standardized for the optical density of MacCforland's No. 2 (each ml contain approximately 10^6 bacterial cells).

3.1. b. Experimental design of LD_{50} :

A total number of 70 apparently health ***Mugil capito*** (***M. capito***), weighting 50 ± 20 grams were selected after the period of acclimation about two weeks and then divided into 7 equal groups; each group contained of 10 fish. The first six groups were consistently inoculated I/P with bacterial suspension of ***V. alginolyticus*** suspension at a dose rate of 0.2 ml

while the control group (group 7) was injected I/P with 0.2 ml of sterile saline and act as a control group according to (Reed and Munch, 1938) to determine the LD_{50} (Table 1).

The LD_{50} was determined according to the method described by ***Reed and Munch (1938)*** using the following formula :

$$LD_{50} = \frac{\text{Mortalities above 50\% - 50}}{\text{Mortalities above 50\% - Mortalities below 50\%}}$$

The clinical signs and PM lesions were recorded daily for about one week and specimens for histopathological studies were collected.

The Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation (One week) according to ***(Soliman, 1988)*** .

3.2. Chronic experiment

Another 40 apparently healthy ***M. capito*** were divided into 2 groups; each group contains 2 replicates (10 fish / each replicate). First group was inoculated I/M with 0.2 ml of 1/10 LD_{50} of tested bacteria. The second group was inoculated I/M with 0.2 ml / fish of normal saline and served as a control group. All infected and control fish were observed daily to record their general health condition, clinical signs and mortalities. Experimental period was 30 days. Postmortem examination was performed on dead fish. The survivors at the end of the observation were sacrificed and examined for postmortem changes and specimens for histopathological studies were collected.

3.3. Histopathological examination

Specimens for histopathological techniques were freshly taken from infected organs and tissues of the experimentally infected ***M. capito*** during both LD_{50} and chronic experiments. Samples were trimmed and fixed in 10 % phosphate buffered formalin. Then washed in running tap water for 24 hours then dehydrated in different concentration gradients of alcohol and cleared in xylol. Samples then embedded in paraffin wax and sectioned into thin sections of 5 microns thickness. Sections were stained with H & E stain and examined microscopically according to (***Roberts, 2001***).

4. Statistical analysis

The data of bacteriological examinations and heavy metal concentrations were statistically analyzed using Chi-square and ANOVA tests according to (***SAS, 1987***). After that the results presented in the form of figures according to Harvard graphics 4 computer programs.

RESULTS

1. Results of bacteriological examinations

1.1. Results of experimentally infected fish

1.1.a. Result of lethal dose fifty (LD_{50})

Results of LD_{50} of ***V. alginolyticus*** were summarized in Table (2) . The obtained results showed that the LD_{50} of ***V. alginolyticus*** were **($10^{-2.5}$).**

Table (1) : ***Experimental design of LD_{50} of V. alginolyticus in M. capito :***

Group number	***V. alginolyticus*** dilution	Number of ***Mugil capito***
1	10^{-1}	10
2	10^{-2}	10
3	10^{-3}	10
4	10^{-4}	10
5	10^{-5}	10
6	10^{-6}	10
7	Sterile saline	10

Table (2) : Results of LD_{50} of ***V. alginolyticus*** in ***M. capito*** :

Conc. Of bacteria	No. of injected fish	Mortality ratio of *M. capito*
10^{-1}	10	7/10
10^{-2}	10	6/10
10^{-3}	10	**5/10**
10^{-4}	10	4/10
10^{-5}	10	4/10
10^{-6}	10	3/10
Control	10	0/10

LD50 in M. capito :

Proportionate distance (P.D.) = $\frac{60-50}{60-40} = 0.5$

$LD_{50} = 2 + 0.5 = 2.5 = (10^{-2.5})$

2. Results of clinical examination

2.1. Clinical signs

The clinical signs during the LD_{50} determination and it started to appear at the 2nd day post infection and continued until the end of the experimental period (one week). The clinical signs of the experimentally infected ***M. capito*** with ***V. alginolyticus*** were characterized by septicemic lesions where as fish exhibit sluggish movement and other showed nervous manifestations represented by listlessness. Fish showed generalized erythematic hemorrhages distributed on different parts of the body surface where as appear at the base of all fins especially the anal fins and at the operculum (Fig. 1) . Some cases showed hemorrhagic inflamed swollen vent (Fig. 2) .

2.1.a. Postmortem (PM) lesions

The PM lesions of I/P experimentally infected ***M. capito*** with ***V. alginolyticus*** showed; severe congestion in the gills, liver, kidney and intestine (Fig 3). Gall bladder was distended. The abdominal cavity had filled with copious amount of bloody hemorrhagic ascetic fluids (Fig 4) that was noticed upon opening the fish and drop-down on the paper under the fish sample (Fig 5) and (Fig 6).

2.1.b. Chronic experiment

Clinical signs of chronic infection started in the 1st week of infection and continued until the end of the experiment (4th week).

The clinical signs appeared as sluggish movement and loss of water reflexes with no external or PM lesions appear on the experimentally infected *M. capito*.

3. Results of re-isolation of the injected V. alginolyticus from the experimentally infected M. capito

Re-isolation of the injected bacterial isolate was obtained from freshly dead and sacrificed experimentally infected fish. Moreover, the results of culture and biochemical characteristics of the re-isolated bacterial isolate revealed the same morphochemical characteristics of the injected bacterial isolate.

The control group remained clinically health and showed neither pathological lesions nor bacterial isolation and none of the control group died.

Fig. (1) : Experimentally infected M. capito showing generalized erythema of the body and hemorrhages at operculum & base of pelvic and anal fins

Fig. (2) : Experimentally infected M. capito showing inflamed hemorrhagic anus.

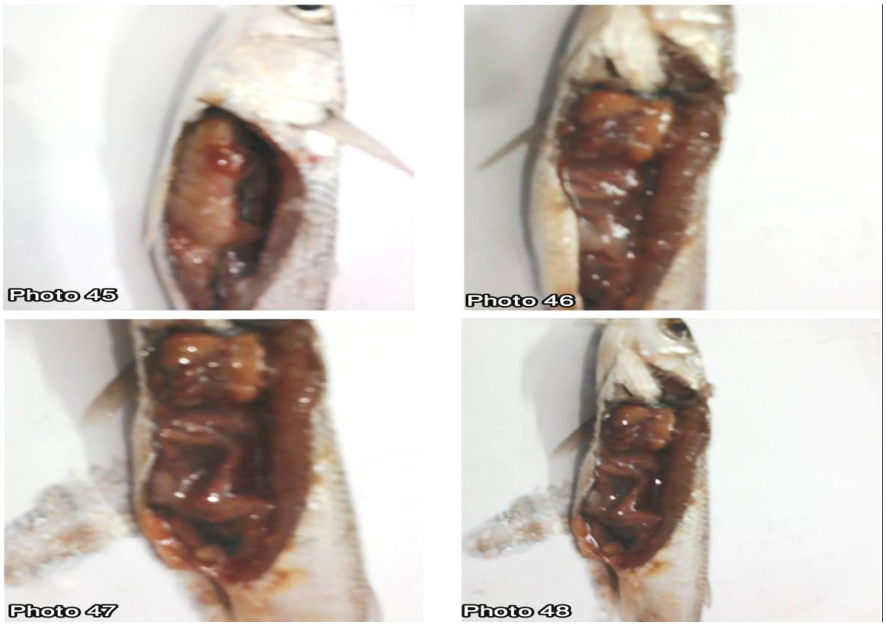

Fig. (3, 4, 5 , 6) : Bloody hemorrhagic ascetic fluid shown upon opening the fish and drops down on the behind paper.

4. Histopathological findings of experimentally infected M. capito with V. alginolyticus

4.1. Acute experiment (LD_{50})

Gills

Gills of *M. capito* infected with *V. alginolyticus* showing congestion of branchial blood vessels with separation of surface epithelium of secondary lamellae from the capillary beds H & E (X 250) (Fig. 7) .

Liver

Liver of *M. capito* infected with *V. alginolyticus* showing severe dilatation with congestion of hepatic sinusoids in between hepatocytes which suffered from severe fatty changes H & E (X 400) (Fig. 8) .

Kidney

Posterior kidney of *M. capito* infected with *V. alginolyticus* showing acute tubular necrosis characterized by epithelial necrosis with preservation of the tubular basement membrane H & E (X 250) (Fig. 9).

Spleen

Spleen of *M. capito* infected with *V. alginolyticus* showing hyper activation of melano macrophage centers (MMCs) where the melanophores over laden with melanin pigment H & E (X 250) (Fig. 10) .

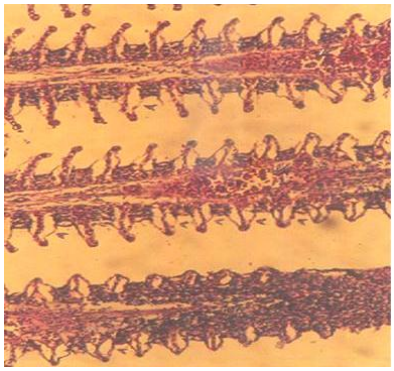

Fig. (7) : Gill of experimentally infected M. capito showing congestion of branchial blood vessels with separation of surface epithelium of secondary lamellae from the capillary beds. H & E (X 250).

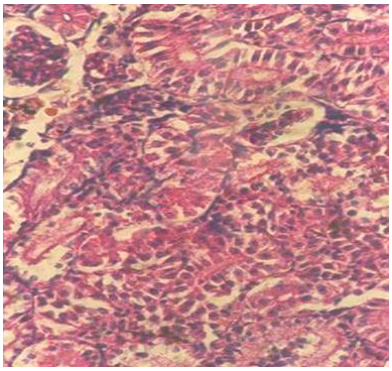

Fig. (8) : Posterior kidney of experimentally infected M. capito showing acute tubular necrosis characterized by epithelial necrosis with preservation of the tubular basement membrane. H & E (X 250).

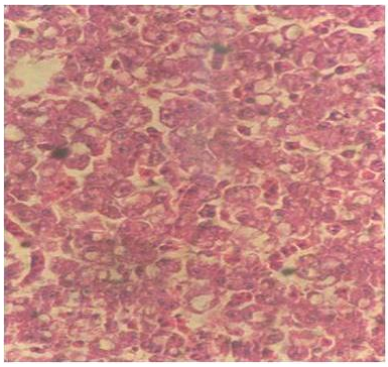

Fig. (9) : Liver of experimentally infected M. capito showing severe dilatation with congestion of hepatic sinusoids in between hepatocytes which suffered from severe fatty changes. H & E (X 400).

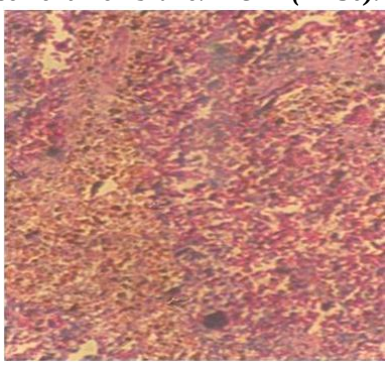

Fig. (10) : Spleen of experimentally infected M. capito - hyperactivation of MMCs where the melanophores over laden with melanin pigment. H & E (X 250).

5. Chronic experiment

Gills

Gills of *M. capito* infected with *V. alginolyticus* showing filamentous clubbing as the result of severe epithelial hyperplasia at the base of secondary lamellae at the apex of gill filaments H & E (X 250) (Fig. 11) .

Liver

Liver of *M. capito* infected with *V. alginolyticus* showing fatty degeneration of the hepatocytes and focal necrotic foci H & E (X 400) (Photo 12).

Kidney

Posterior kidney of *M. capito* infected with *V. alginolyticus* showing necrotic renal tubules with necrosis and depletion of inter tubular haemopiotic elements H & E (X 400) (Fig. 13) .

Spleen

Spleen of *M. capito* infected with *V. alginolyticus* showing moderate lymphocytic cells depletion where the splenic parenchyma suffered from depletion of white pulps H & E (X 250) (Fig. 14) .

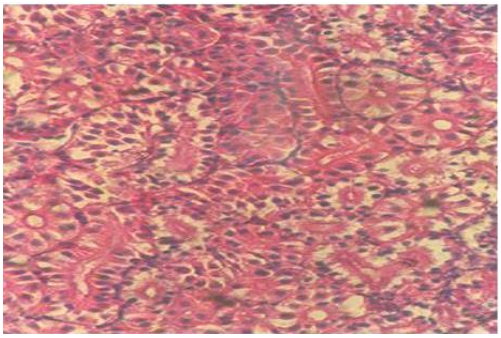

Fig. (11) : Posterior kidney of experimentally infected M. capito showing necrotic renal tubules with necrosis and depletion of inter tubular haemopiotic elements. H & E (X 400).

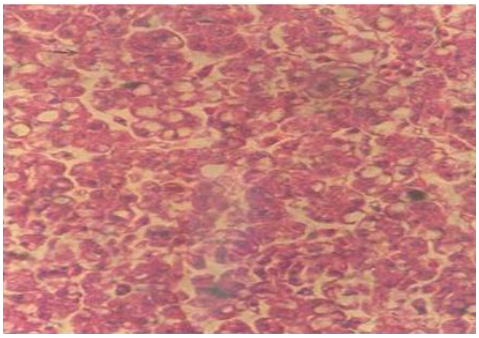

Fig. (12) : Liver of experimentally infected M. capito showing fatty degeneration of the hepatocytes and focal necrotic foci H & E (X 400).

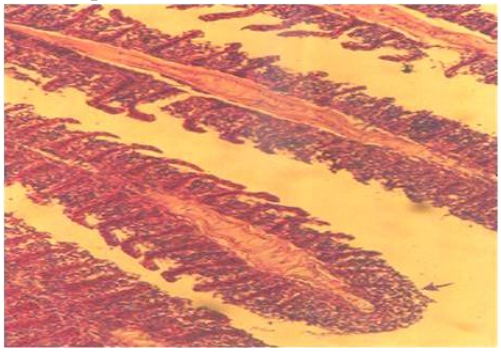

Fig. (13) : Gills of experimentally infected M. capito showing filamentous clubbing as the result of severe epithelial hyperplasia at the base of secondary lamellae at the apex of gill filaments. H & E (X 250).

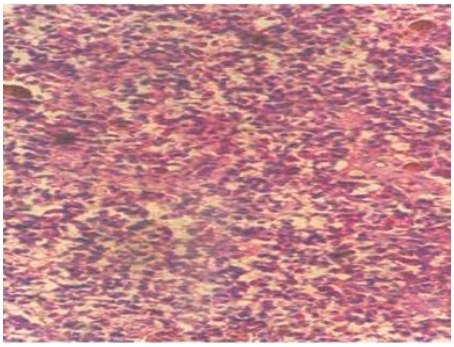

Fig. (14) : Spleen of experimentally infected M. capito showing moderate lymphocytic cells depletion where the splenic parenchyma suffered from depletion of white pulps. H & E (X 250).

DISCUSSION

Bacterial diseases, from the epizootiological point of view, affect nearly all cultured, wild marine and freshwater fish to a limit that exceeds all other disease causes combined. Moreover, bacterial diseases ranked first one among all the causative agents causing mariculture problems (Meyer, 1991).

In the present work, we spot light on the clinical picture and PM lesions of the most predominant bacterial pathogens affecting some marine fishes native to Wadi-mariut region, Borg El-Arab city, Alexandria governorate. Moreover, isolation and identification of these bacterial infections by both biochemical traditional methods and studying the pathogenicity of the most prevalent bacterial isolate (***Vibrio alginolyticus***) in ***Mugil capito.***

Concerning the clinical signs and Postmortem (PM) lesions of some examined marine fishes; the clinical picture and gross lesions of Vibriosis (Listonellosis) in naturally infected some marine fishes; our results were in concordance to that obtained by Actis et al. (1999); where they stated that fish affected by ***Listonella anguillarum*** (***L. anguillarum***) showed typical signs of a generalized septicemia with hemorrhage on the base of fins, exophthalmia and corneal opacity. Also, Austin and Austin (2007) who reported that the typical external clinical signs of ***V. anguillarum*** infection include red spots on the ventral and lateral areas of the fish and swollen and dark skin lesions that can ulcerate and bleed. The eyes were also infected, resulting in opacity at first, and later in ulceration and exophthalmia.

On the other hand; ***V. harveyi*** have several virulence factors such as the ability to attach and form biofilms (Karunasagar et al., 1994); Capacity to bind iron (Owens et al., 1996); Lipopolysaccharide (Montero and Austin, 1999); extra-cellular products (cysteine protease, phospholipase, haemolysin) (***Soto***-Rodriguez et al., 2003) and Bacteriocin-like substance (Prasad et al., 2005).

In regard to the results of clinical signs and PM lesions of Streptococcosis in naturally infected fishes; our results were in improvement with that obtained by (Colorni et al., 2002); where they mentioned that S. iniae affecting Dicentrarchus labrax causing severe bilateral exophthalmia and internally, hemorrhages were conspicuous in the abdominal cavity. Furthermore; Lactococcosis infected fishes have erratic swimming, uni- or bilateral exophthalmia, hemorrhages in the ocular zone, perianal area, fins and anal prolapsus (Eldar et al., 1999) and (Vendrell et al., 2004) while internally, there was enlargement of the spleen, focal areas of necrosis in the liver and spleen and hemorrhagic fluid in the intestine (Mu´zquiz et al., 1999) as well as there was accumulation of ascetic fluid in the peritoneal cavity, which may be purulent or may contain blood (Afonso et al., 2003).

These results may be attributed to *phosphoglucomutase enzyme as the virulence factor for* **S. iniae** *which inter-converts glucose-6-phosphate and glucose-1-phosphate which play important role in the production of* **S. iniae** *polysaccharide capsules* (Buchanan et al., 2005).

Furthermore, Fuller et al. (2002) found that the virulence factor could also be ca*used by the gene that is associated with β-hemolysis which gives the ability of* **S. iniae** *to hemolyse erythrocytes and damage host cell membranes results from the activity of cytolysins and the cytolysins possessed by* **S. iniae** *is homologous to streptolysin S (SLS). Other virulence factors of* **S. iniae** *were the immunoglobulin-binding* Proteins (Barnes et al., 2003) and the capsular structure of S. iniae (Lowe et al., 2007).

Septicemic bacterial infections such as Vibrios, Aeromonads, Pseudomonads, Photobacteria, Streptococci and Staphylococci have been observed in several fingerlings,

juveniles, adults and brood stocks of some marine fish species (Samuelsson et al., 2006).

The role of bacteria varies from their effect as primary pathogen to that of secondary invader in the presence of other disease agents; they may also serve as a stress factor and predispose fish to other diseases (Badran and Eissa, 1991).

In regards to the experimental infection of *Mugil capito* with *V. alginolyticus*; the results of LD_{50} differs from that obtained by (Balebona et al. 1998b) where they evaluated the in vivo and in vitro pathogenic activities of whole cells and extra-cellular products of ***V. alginolyticus*** for cultured gilthead Seabream and they found that the 50% lethal doses ranged from (5.4 x 10^4) to (1.0 x 10^6) CFU/g of body weight. Furthermore; Ben Kahla-Nakbi et al. (2006) tested several strains of ***V. alginolyticus*** isolated from diseased gilthead Seabream and Seabass for the virulence in both fish species by intraperitoneal injection and they found that LD_{50} values ranged from (5.01 x 10^4) to (6.20 x 10^7) CFU / fish. The difference in LD_{50} may be attributed to the susceptibility of different fish species, locality and the route of infectivity.

Clinical signs, PM lesions as well as the histopathological findings of ***M. capito*** experimentally infected with ***V. alginolyticus*** may be attributed to the virulence factors produced by ***V. alginolyticus*** such as Adherence to fish mucosa and ECP (Balebona et al., 1998b); hemolytic activity, hemoagglutination, protease production and the adherence capacities to epithelial cells (Zanetti et al., 2000); growth in iron-limiting conditions (Ben Kahla-Nakbi et al., 2009) and virulence genes such as *Collagenase* gene, *OmpK* gene and *toxR* gene (Cai et al., 2009).

Results of Clinical signs and PM lesions were in concordance with the results of (***Safinaz et al., 2011)*** where they mentioned that the clinical signs in ***M. capito*** infected with ***V. alginolyticus*** were hemorrhagic patches on the caudal peduncle area and superficial hemorrhagic ulcers at the abdominal wall while the PM changes in ***M. capito*** were characterized by deep seated muscle lesions, enlargement and congestion of the spleen which became cherry red in color and losses its sharp edges. Internally, fish accumulate fluid in the peritoneal cavity and in some cases have hemorrhagic livers (Balebona et al., 1998a). Moreover; Korun (2007) isolated ***V. alginolyticus*** from European Seabass (***Dicentrarchus labrax, L.***) and they found that all affected fish showed ulcers on the dorsal part of the body and hemorrhages on the operculum. Internal lesions included enlargement of the spleen, paleness of the liver and kidney, hemorrhages in the intestine.

The results of histopathological findings were in agreement with the results of (Safinaz et al., 2011) and (Sharma et al., 2011) where they stated that upon infection with ***V. alginolyticus,*** there were hepatic lesions included congestion, hemorrhage, swollen hepatocytes with vacuolation and perivascular hepatocytes showing degeneration and necrosis with the presence of bacteria.

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AUTHER

تأثير بكتيريا الفبريو الجينوليتكس على أسماك الطوبار

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تم التعرف وتحديد العترات الأصلية لبكتيريا الفبريو التى تصيب أسماك المياه المالحة فى مصر حيث تم إجراء تجربة عدوى لأسماك الطوبار فى المعمل الخاص بقسم أمراض الدواجن كلية الطب البيطرى جامعة الإسكندرية حيث تم استخدام (110) من أسماك الطوبار بوزن 50 جرام تم الحصول عليها من مزارع ترابية بمنطقة وادى مريوط بمحافظة الإسكندرية وتم نقلها إلى كلية الطب البيطرى معمل قسم أمراض الدواجن والأسماك جامعة الإسكندرية بطريقة صحية وعلمية وتم إجراء الأقلمة لمدة أسبوعين وتم الحصول على العترة المصنفة لبكتيريا الفبريو الجينوليتكس من قسم أمراض الدواجن والأسماك **وتـم إجـراء الآتـى :**

1- عمل اختبار الجرعة نصف المميتة عن طريق حقن 0.2 مم من بكتيريا الفبريو بتخفيفات من 10^{-1} إلى 10^{-6} فى البروتنيوم .

2- تم عمل مجموعة ضابطة تم حقنها بسلاين .

3- استمرت التجربة لمدة (96) ساعة تم تسجيل النفوق والأعراض الداخلية والخارجية وتم عمل عزل للبكتيريا مرة أخرى من الأسماك الميتة .

4- تم عمل التجربة المزمنة عن طريق حقن 0.2 مم من 0.1 من الجرعة نصف المميتة فى (20) سمكة
فى مجموعتين وتم الحقن فى العضلات .

5- تم عمل أيضا مجموعة ضابطة مكونة من حوضين يحتوى على (20) سمكة وتم تسجيل نسب النفوق والأعراض الداخلية والخارجية فى خلال شهر من الحقن .

تم عمل اختبار الهستوباثولوجى للعينات المصابة وتم تسجيل النتائج الآتية :

1- تجربة الجرعة نصف المميتة والتجربة المزمنة كان بها الأعراض (بقعة نزفية على السطح الخارجى والزعنفة الذيلية والغطاء الخيشومى بينما بعض الأسماك كان بها احتقانات فى منطقة فتحة المجمع بالإضافة إلى أعراض عصبية) .

2- الأعراض الداخلية فكانت فى صورة احتقانات فى الخياشيم والكبد والكلى والأمعاء بينما كانت أغلب وأهم الأعراض فى صورة سائل دموى بالتجويف الداخلى للبطن .

3- تم عزل البكتيريا من الأسماك المصابة وتم تصنيفها مرة أخرى بعد تسجيل النفوق والأعراض الداخلية والخارجية .

أما نتائج الهستوباثولوجى فكانت فى صورة : (تغيرات فى الخلايا الدهنية الخاصة بالكبد وتنكرز فى خلايا الكلى بالإضافة إلى زيادة فى خلايا إنتاج الميلانين وجد أيضا زيادة فى خلايا الخياشيم وتنكرز خلايا الكبد والكلى الخلفية بالإضافة إلى وجود تسريبات وتنكرزات فى خلايا الطحال .