JOURNAL OF THE
ARABIAN AQUACULTURE SOCIETY
Vol. 9 No 1 Arabian Aquaculture Conference June 2014

Effect of *Tenispeculam maritimum* Infection on Cultured *Mugil capito*

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ABSTRACT

A total number of (60) Mugil Capito (45 ± 5 gm) obtained from floating cages at Rashid area, El-Behera Governorate, Egypt, were transported to the laboratory of fish diseases Department, Faculty of Veterinary Medicine, Alexandria University in oxygenated water, and acclimated for two weeks . The fish divided into 6 groups (10 fish / group), 2 groups used as control, while other groups infected with ***T. maritimum*** by half LD_{50} dose equal 0.5 (1.5×10^5 CF/ml) of water by prolonged immersion of fish for 18 hours at 18-20 C, then clinical signs, PM lesions and mortalities were recorded . Weekly sample were taken for histopathological examination for four weeks . Re-isolation of the infected bacteria was determined for verification of death . The clinical signs was offood, sluggish movement and respiratory distress fish showed erythemia on body surface and around fins, darkness of skin coloration, opaqueness on the eye in some cases as well as fin and tail rot . The most predominant clinical signs were ulcers with different degrees . PM lesions showed sever congestion in kidney, gills, liver and the abdominal cavity filled with hemorrhagic ascetic fluids . Histopathlogical findings were lamellar telangiectasis of gills developed to multifocal fusion of secondary gill lamellae, congestion in hepatic sinusoids and vacular and hydropic degeneration of hepatic cell . In spleen the lymphoid cell depletion accompanied by telangiegiectosis of the red pulp and white pulps and depletion of blood elements in posterior kidney. PCR used for detection of ***T. maritimum*** which used in experimental infection . The results revealed that the primer sequence showed maximum identify with the sequence of ***T. maritimum*** with 100% hemology .

Keywords:

INTRODUCTION

The cage culture of finfish, especially marine cage farming is becoming more popular because of the many economic advantages. Disease outbreaks can occur more often when fish are raised under intensive culture conditions due to high density, crowding, handling, temperature, pollution and biofouling ***(****Sobhana, 2009).*

Fish diseases due to bacterial infections are the major problems in aquaculture as they found naturally in the fish environment and under certain stress condition cause severe economic losses to fish farms (*Olsson et al., 1998*).

In Egypt, Seabass (*Dicentrarchus Labrax L*) is one of the most popular marine fish; it is intensively cultured in floating cages. Since cage culture is an open system exposed to cross dissemination of infection between cultured and wild fish (*Palleroni, 1984).* While, Gilthead Seabream (*Sparus*

aurata L) is a marine fish with high economic value in the aquaculture . Only a few bacteriological surveys have involved the marine fish species diseases outbreaks (***Toranzo et al., 2005***) .

Therefore, the present study was focused and aimed to selection of *T. maritimum* to apply the pathogenicity test in Mugil capito, application of PCR for accurate identification of the isolated *Tenacibaculum maritimum* (*T. maritimum*).

MATERIALS AND METHODS

A- Material

1- Fish

A total number of (60) apparently healthy Mugil capito were used for the experimental work . They were collected from floating cage at Rashid area, Al-Behera governorate with average body weight (45 ± 5g) in large plastic bags containing water enriched by oxygen (2/3), then transported to the laboratory of fish disease, Fac. of Veterinary Medicine, Alexandria University as soon as possible.

B- Methods

1. Gross clinical examination :

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

2. Postmortem (PM) examination :

according to ***Conroy and Herman (1981***).

3. Identification of bacterial isolates :

according to the methods described by Austin and Austin (1999).

4. Biochemical characters using conventional tests :

The biochemical tests used in our study were : Cytochrome oxidase, Citrate utilization, Indole production, **Voges–Proskauer,** H2S production, Hydrolysis of urea, Sugar fermentation, **Vibrostatic agent test .** ***Amlacher (1970***).

5. Serotyping of the bacterial isolates :

Bacterial strains

All bacterial isolates were used as antigens in the agglutination tests. The strains were isolated from epizootics occurring in the Wadi-Mariut area at Alexandria governorate. Stock cultures of the isolates were maintained by periodic transfer on tryptic soy agar **(*Oxoid Manual, 1982*)** slants with the appropriate salt concentration. For long term storage, cultures were kept at - 80 C^0 in medium with 15% glycerol.

6- PCR for detection of virulent strain of *T. maritimum* :

Detection of T. maritimum DNA by PCR (Bader and Shotts, 1998)

Enrich samples were 4- 6 hours on broth culture or use them from slope agar directly.

Extraction of DNA of T. maritimum

One ml of distilled water was added to *T. maritimum* growth on the slope agar then shaken well. The bacterial suspension was centrifuged and the pellet was re-suspended in distilled water by using vortex. The genomic DNA was extracted by boiling of the suspension for 10 - 15 minutes in water bath to ensure lysis of the bacterial cells and complete denaturation of DNA.

The PCR Programming of thermal cycler for detection of *T. maritimum* rRNA was adjusted as shown in this Table :

No. of cycles	Temperature (C)	Time	Target
1 Cycle	94	5 min	Initial denaturation
	a) 94	1 min	Denaturation
30 cycles	b) 65	1 min	Annealing
	c) 72	1.5 min	Extension
1 cycle	72	10 min	Final extension
1 cycle	- 20	Until used	Preservation

Detection of PCR products (Sambrook et al., 1989)

7. Experimental infection :

Route of infection

The bacterial isolates of ***T. maritimum*** were selected for laboratory inducing the Tenacibaculosis. By using prolonged immersion of fish for 18 hrs with the pathogen at water temperature 18±20 °C, the disease could be easily reproduced with the fish showing the classical signs of Tenacibaculosis according to (Avendano-Herrera et al., 2006).

8. Experimental design :

After the period of acclimation about two weeks of *Mugil capito (M. capito)*, a total number of 60 apparently healthy fish, each one weighted (45 ± 5 g) were selected, and then divided to 6 equal groups . Two groups used as a control, while other four groups were experimentally infected by *T. maritimum* suspension containing 0.5 ($1.5x10^5$ cell mL^{-1} water) of immersion bath equal to half LC_{50} of *T. maritimum* described by *Abd El-Galil and Hashiem (2012b)* . All groups were observed for four weeks .

The clinical signs and mortalities were recorded daily and the dead fish was postmortem examined. A weekly sample was taken from infected and control groups, scarified and examined for post mortem changes, then collected specimens for histopathological studies for four weeks.

The Specificity of death was determined by re-isolation of tested bacterium, *T. maritimum* from freshly dead fish during the period of observation according to the method described by *Soliman (1988)* for verification of deaths.

9. Histopathological Studies :

Following complete necropsy of the sacrificed and freshly dead fish, specimens were collected from laboratory treated ***M. capito***. The sample collected from kidney, liver, gills, spleen, heart, skin and muscles for histopathological examination. Thereafter, these specimens were rapidly fixed in 10% natural formalin buffered phosphate for at

least 24 hours, after that the specimens washed by running tap water then dehydrated through ascending grads of ethanol, cleaning in by chloroform and embedded in paraffin wax at 60 C . Paraffin block were prepared from which 5 microns thick sections were obtained by microtome. These sections were stained by hematoxylin and eosin stain (H & E) according to method described by ***Culling, (1983)*** .

RESULTS

1- Results of clinical examination

a. Clinical signs

The clinical signs of the experimentally infected *M. capito* with *T. maritimum* were off food, lethargy, some 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 whereas appeared at the base of all fins especially the pectoral and anal fins (Photo 2) . Some fish showed hemorrhagic inflamed swollen vent (Photo 1), opaqueness on the eye (Photo 4), darkness of the skin coloration and tail and fin rot (Photo 3) and in other cases, there was extensive hemorrhagic ulceration on the skin of the experimentally infected *M. capito* (Photo 5 and 6) (arrows). The mortality started after 48 hours from infection with *T. maritimum*. The rate of mortality was higher at 1st week than other weeks.

Photo (1): ***Experimentally infected M. capito showed hemorrhagic inflamed swollen vent.***

Photo (2): ***Experimentally infected M. capito showed severe hemorrhage at the base of anal and pectoral fin.***

Photo (3) : ***Experimentally infected M. capito showed darkness of the skin coloration and tail and fin rot.***

Photo (4) : ***Experimentally infected M. capito showed opaqueness on the eye.***

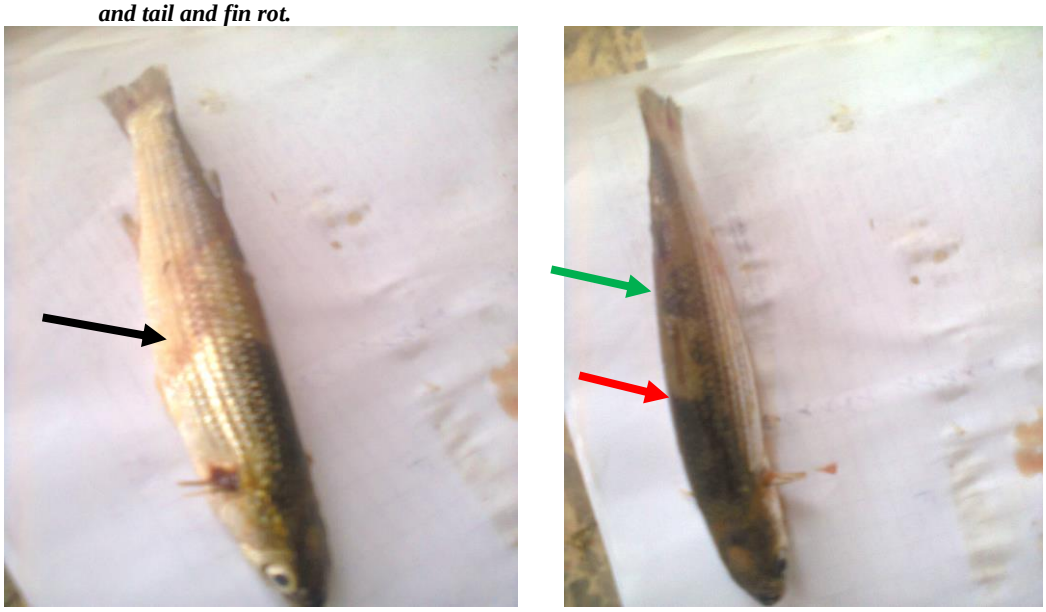

Photo (5) and (6): ***Experimentally infected M. capito showed extensive hemorrhagic ulceration on the skin***

b. Postmortem lesions

The PM lesions of the experimentally infected *M. capito* with *T. maritimum* showed; severe congestion in kidneys (Photo 8), in the gills, liver, intestine and the abdominal cavity had filled with bloody hemorrhagic ascetic fluids that was noticed by opening the fish (Photo 7).

Photo (7) : ***Experimentally infected M. capito showed severe congestion in the gills, liver and intestine and the abdominal cavity had filled with bloody hemorrhagic ascetic fluids.***

Photo (8) : ***Experimentally infected M. capito showed severe congestion in the kidney.***

2. Results of re-isolation of the T. maritimum from the experimentally infected M. capito :

Re-isolation of *T. maritimum* 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 bacterial isolate used in immersion bath . The control group remained clinically health and showed neither pathological lesions nor bacterial isolation and none of the control group died.

3. *Result of Histopathological findings of the experimentally infected M. capito with T. maritimum :*

The histopathological changes differed from week to another among different organs. In gills, lammeller telangictasis and oedematous separation of secondary lammeller epithelium (Photo 9) in the firest week while in the fourth week gills suffered from multifocal fusion of secondary gill lamellae (Photo 10).

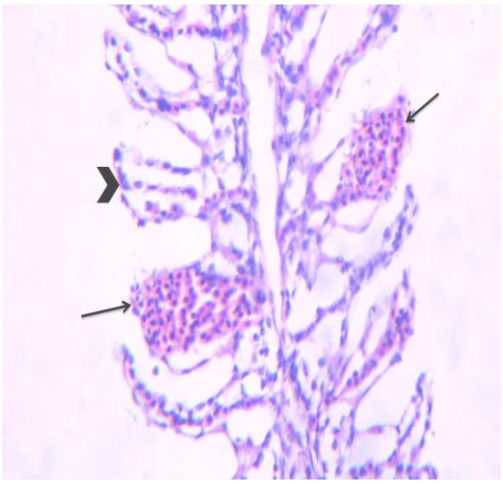

Photo (9) ***Gills of experimentally infected M. capito during 1st week of infection showing lamellar telangiectasis (arrow) and edematous separation of surface epithelium of secondary lamellae from capillary beds. (head arrow) H&E. (X 250).***

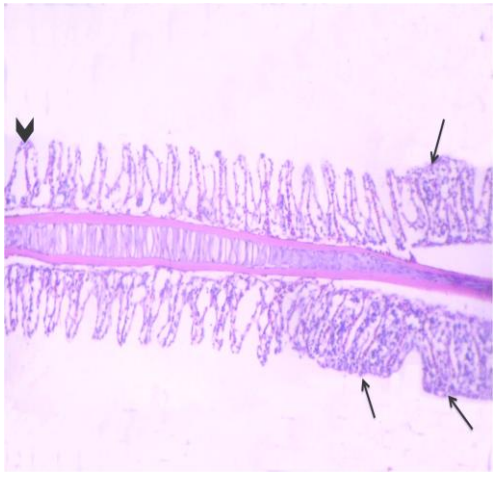

Photo (10) ***Gills of experimentally infected M. capito during 4th week showing multifocal fusion of secondary gill lamellae (arrows) and epithelial lifting (head arrow). H&E. (X 250).***

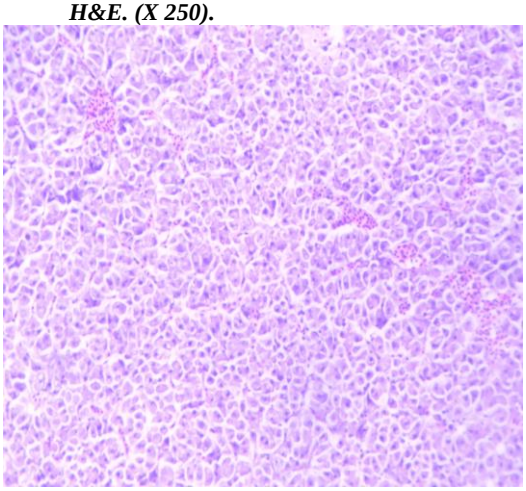

Photo (11) ***Hepatopancreas of experimentally infected M. capito during 1st week of infection showing congestion of hepatic sinusoids. H&E. (X 160).***

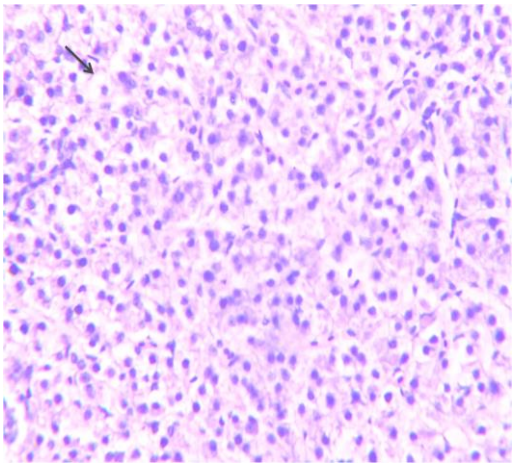

Photo (12) ***Hepatopancreas of experimentally infected M. capito during 4th week of infection showing vacuolar and hydropic degeneration. H&E. (X 250).***

In hepatopancrease, congestion of hepatic sinusoid (photo 11) in the first week, while in the 4th week vacuolar and hydropic degeneration (photo 12) and activation of melanomacrophage center (photo 15) were observed. The spleen showed lymphoid cells depletion accompanied by telangectasis of red pulp sinusoid (photos 13 and 14) . In addition to depletion of blood element of posterior kidney (photo 16).

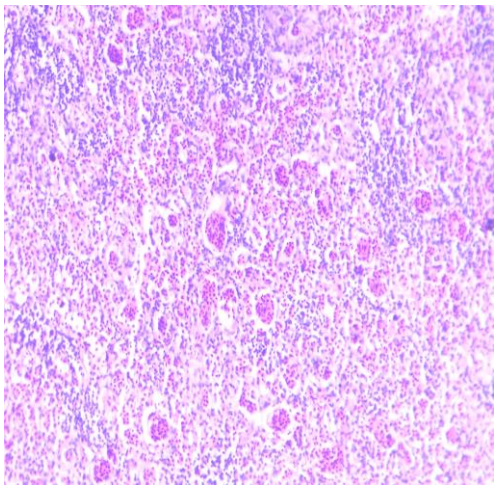

Photo (13) ***Spleen of experimentally infected M. capito during 1st week showing lymphoid cell depletion accompanied by telangiectasis of the red pulp sinusoids at the expense of white pulps. H&E. (X 160).***

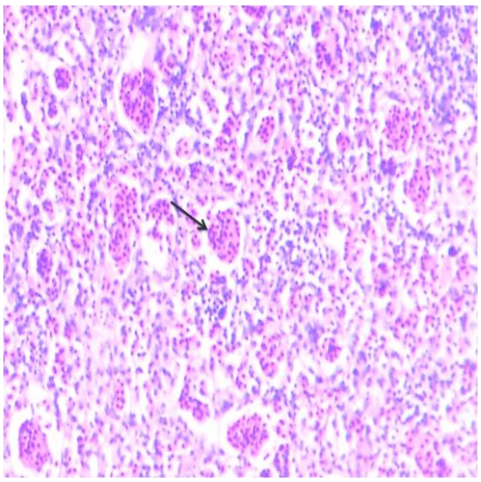

Photo (14) ***Spleen of experimentally infected M. capito during 4th week showing extensive lymphoid cell depletion accompanied by telangiectasis of the red pulp sinusoids (arrow) at the expense of white pulps. H&E. (X 250).***

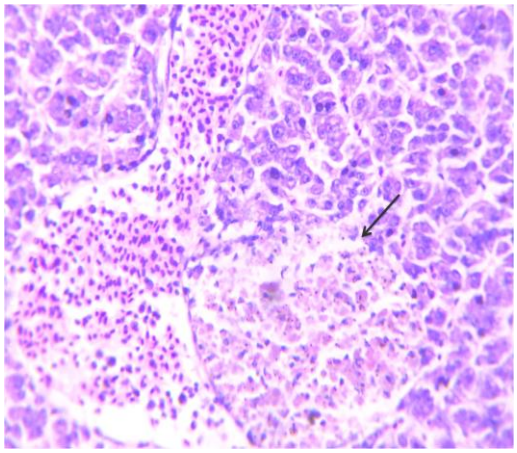

Photo (15) ***Hepatopancreas of experimentally infected M. capito during 4th week showing activation of the MMCs. H&E. (X 160).***

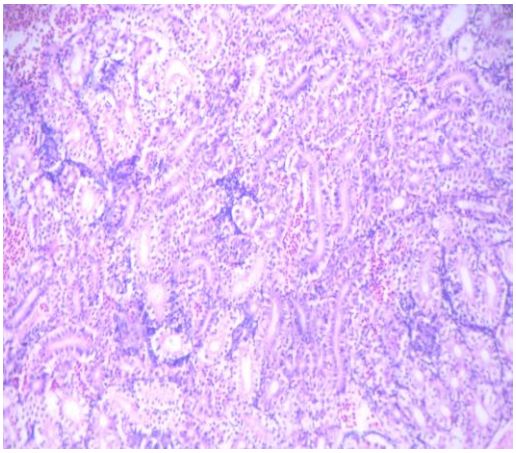

Photo (16) ***Posterior kidney of experimentally infected M. capito during 4th week showing depletion of blood element. H&E. (X 250).***

3. Result of PCR of T. maritimum :

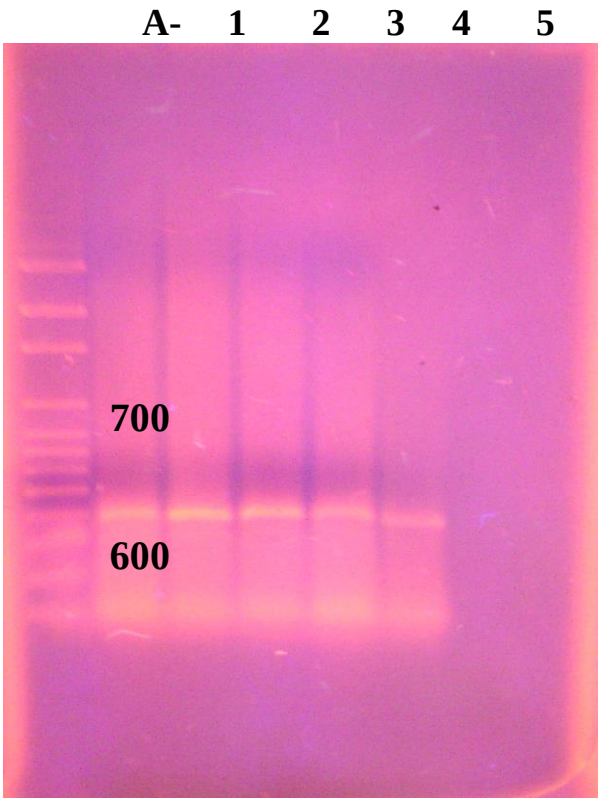

Photo (17) ***shows : Gel photo of amplified products when utilizing polymerase chain reaction (PCR); whereas lane A is, 100-bp DNA ladder (Biotools; B & M laboratories S. A., Madrid, Spain) while lanes 1 to 5 are the rRNA gene (400 bp) of 5 isolates of T. maritimum.***

DISCUSSION

In the present work, we spot the light on studying the pathogenicity of the bacterial isolate (*Tenacibaculum maritimum*) (*T. maritimum*) in Mugil capito (M. capito) .

In concern to experimental infection of *M. capito* by *T. maritimum*, our results showed that the most predominant clinical signs were ulcers, which developed until reaching the under lining musculature also tail and fin rot as well as necrosis in gill and eroded mouth were observed in some infected fish .

These results were in agreement with these recorded by (*Santos et al., 1991*), where they mentioned that due to some variation in external pathological signs of the disease, there are different names to designate this ulcerative condition for examples salt water columnaris disease, Gliding bacterial disease of sea fish, bacterial stomatitis, eroded mouth syndrome and Black patch necrosis.

In the same manner, *Cepeda and Santos (2002*) mentioned that, the pathology of the disease caused by *T. maritimum* has mainly been associated with necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on gills and eyes. However, some authors tried to recorded the virulence mechanisms of *T. maritimum*, a first prerequisite for the successful colonization of the host tissue is the ability to adhere, this may lead to a specific or non-specific form of attachment (*Ofek and Doyle, 1994*). Specific adhesion is mediated through specific compounds on the surface of the bacterium which bind to receptors present on the host tissue. Non specific adhesion depends on hydrophobic or ionic interactions between certain structure on the surface of the bacterium and the supporting substrates.

Hemagglutinating activity being a hydrophobic surface property is sometime associated with virulence.

Pazos (1997) showed that *T. maritimum* cells agglutinate abroad spectrum of erythrocytes. Additional structures such as pilli, fimbriae and flagella, known to be involved in adhesion and colonization of other bacteria (*Toranzo et al., 2005*).

With regard to the production of toxins of *T. maritimum*, *Baxa et al. (1988 a)* conducted in vitro experiments in red and black Seabream, and concluded that, the pathogenicity of this pathogen can be attributed to the synergistic interaction of the toxins and enzymes present in the extra-cellular product (ECP), which could facilitate the alteration and erosion of the host tissue and contribute to colonization and invasion. Another putative virulence factor examined in *T. maritimum* is its capacity to express high-affinity iron-uptake mechanisms, which can complete with the host iron-binding proteins. In addition, it has also been reported that intraperitoneal injection of hemin before experimental infection increased the lethality of this pathogen (*Avendano-Herrera, 2005*).

In regard to histopathological finding of experimentally infected *Mugil capito* by *T. maritimum* this results revealed that gills showing lammeller telangictasis and oedematous separation of secondary lammeller epithelium in the firest week while in the fourth week gills suffer from multifocal fusion of secondary gill lamellae .

In hepatopancrease congestion of hepatic sinusoid in the first week, while in the 4^{th} week vacuolar and hydropic degeneration and activation of melanomacrophage center were observed. The spleen showed lymphoid cells depletion accompanied by telangectasis of red pulp sinusoid . In addition to depletion of blood element of posterior kidney .

This results were agreement with that obtained by *Abd El-Galil and Hasheim (2012b),* where they reported that *T. maritimum* isolated from kidneys and liver .

Moreover *Powell et al. (2004)* reported that *T. maritimum* produce variable mortalities of fish and they noted that respiratory damage as consequence of gills abrasion . In the same manner *Avendano-Herrera et al. (2006)* reviewed that *T. maritimum* cause extensive skin damage and gills abrasion with subsequent systemic infection.

In concern to PCR of *T. maritimum*, the results of our study indicated that the primers sequence of 16S rRNA gene as target in *E. coli* numbering system showed complete identify with the sequence of *T. maritimum* gene. The PCR amplified the 16S rRNA genes at the correct size products at 400 bp with 100% homology of *T. maritimum*. These results were in complete agreement with that obtained by (*Bader and Shotts, 1998*).

Moreover, *Avendano-Herrera et al. (2004)* reviewed that it was necessary to support the diagnostic methods of *T. maritimum* by use specific molecular DNA based methods due to difficult isolation of bacterium from diseased fish and a definitive diagnosis must be supported by isolation of *T. maritimum* on appropriate specific media.

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دراسة تأثير العدوى بميكروب التنيسبيكيولم ماريتيم على أسماك الطوبار المستزرعة

رياض حسن خليل ـ يونس على عطا

قسم أمراض الدواجن والأسماك ـ طب بيطرى اسكندرية

التجربة المعملية لإحداث العدوى : استخدم فى هذه التجربة ٦٠ سمكة من أسماك الطوبار تم تجميعها من الأقفاص العائمة من منطقة رشيد بمحافظة البحيرة بمصر ثم نقلها فى أكياس بها ماء وأكسجين إلى معمل قسم أمراض الدواجن والأسماك بكلية الطب البيطرى ـ جامعة الإسكندرية ووضعها فى أحواض بها مصدر أوكسجين وتركها لمدة أسبوعين للأقلمة على ظروف المعمل .

تم تقسيم السمك إلى ٦ مجموعات كل مجموعة بها ١٠ سمكات بواقع ٢ مجموعة مقارنة وأربعة مجموعات للعدوى الصناعية بعترة التنيسيبكولم ماريتيم لمدة ١٨ ساعة عند درجة حرارة (٢٠ ± ٢) درجة بمعدل ($0.5x\ 1.5x10^5$ CFU/ml water) وهى تساوى نصف الجرعة المميتة ومن ثم ملاحظة الأعراض المرضية والتغيرات التشريحية وكذلك النفوق وأخذ عينات أسبوعياً من أحواض العدوى الصناعية وأحواض المقارنة للفحص المجهرى وكذلك إعادة عزل البكتريا لتحديد سبب النفوق والأعراض وكانت النتائج كالآتى :

أ- نتائج الأعراض المرضية كانت عبارة من عدم إقبال الأسماك على العلف والحركة الغير متزنة وأعراض تنفسية بالإضافة إلى الاحتقان على السطح الخارجى للسمكة وبجوار الزعانف وكانت فى بعض الأسماك عتامة بالعين وتغير لون الجلد إلى الأسود وكان أكثر الأعراض هى وجود قرح بالسطح الخارجى للجسم بمراحلها المختلفة .

ب- الصفة التشريحية للأسماك النافقة والمريضة ظهرت فى شكل احتقان شديد فى الأعضاء الداخلية (الكبد ، الكلى ، الطحال و الخياشيم) ووجود سوائل دموية فى التجويف البطنى للسمكة عند فتحها .

ج- نتائج الفحص المجهرى للعينات التى تم تجميعها كانت توضح وجود تغيرات فى أنسجة الخياشيم مع التحام فى هذه الأنسجة نتيجة العدوى أما بالنسبة لأنسجة الكبد كان بها احتقان شديد مع تآكل وتنكرز فى الخلايا الكبدية وأما أنسجة الطحال كان بها تجمع من الخلايا المناعية بالإضافة إلى نقص حاد فى الخلايا الدموية فى أنسجة الكلى .

تم عمل اختبار البلمرة المتسلسل (PCR) لميكروب تنيسيبكولم ماريتيم بعد عزله من سمك الطوبارة وذلك بعد الحقن بأسبوع والتى تم استخدامها فى التجربة المعملية لإحداث العدوى . وأوضحت النتائج التطابق الكامل بين العترات المختبرة والبادى الذى تم استخدامه بما يوضح أن هذه العترات هى عترات لبكتيريا التينوسبكليم ماريتيم .