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Assessment of Potential Pathogenicity of Emergent marine bacterium, *Tenacibaculum maritimum* to Thin lipped grey mullet (*Mugil capito*) farmed in Egypt

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ABSTRACT
The pathogenicity of marine bacterium, *Tenacibaculum maritimum* (*T. maritimum)* in cultured *Mugil capito* (*M. capito*) was determined through conducting experimental infection using immersion bath for 18 hours only with different concentrations of the bacterium and observed for one week to determine the median lethal dose (LD50). Another experiment was operated to determine the clinicopathological picture of *M. capito* when experimentally infected with suspension containing 1/10 of the LD50 of tested bacterium and then observed for another four weeks. The clinical signs postmortem (PM) lesions and mortalities were recorded daily.
Kidney, gills, liver and spleen were sampled and examined for any histopathological alterations. Results indicated that the LD50 of *T. maritimum* for *M. capito* was (1.5 × 105 CFUs mL-1). The infected fish has dark skin color, hemorrhages with different degrees at the base of all fins especially the pectoral and pelvic ones, hemorrhagic inflamed swollen vent, extensive hemorrhagic skin ulceration and severe congestion in visceral organs with copious amounts of bloody hemorrhagic ascetic fluids in the abdomen. The lesions concluded that *T. maritimum* is a serious pathogenic bacterium and can cause septicemic lesions in cultured fish with high economic losses.

Keywords: Tenacibaculum maritimum - Pathogenicity - Clinicopathological signs - hemorrhages - Mugil capito - LD50.

1. Introduction
Thin lipped grey mullet (*Liza ramada* is the scientific name) that belongs to the family Mugillidae, have many important characters; they can be introduced successfully in a poly culture conditions and being well distributed throughout fresh, brackish and marine waters in the tropical and subtropical regions of the world [1]. In Egypt, Mugil culture is one of the most important aquaculture activities, but the major obstacle which hinders its successful development and maintained sustainability is the diseases [2]. Bacterial diseases are the most important causes for heavy mortalities in both wild and cultured fish either to be a primary direct causative agent or to be an opportunist invader of a host that rendered moribund by some other disease [3]. From the bacterial diseases, Tenacibaculosis is considered to be the most serious bacterial disease that affecting a wide range of marine fish especially cultured ones [4] including Sole, *Solea solea L.*[5]; Senegalese sole (*Solea enegalensis*) [6]; Japanese lounder (*Paralichthys olivaceous*) [7] and turbot (*Psettamaxima*) [8]. Also, it has been recorded in Atlantic salmon (*Salmo salar L.*), Rainbow trout (*Oncorhynchus mykiss*) [9], cultured seabream and seabass in Egypt [10] and Picasso tigger fish (*rhinecanthus assasi*) and Black damsel fish (*Neoglyphieodon meles*) [11]. This disease is characterized by heavy mortalities and severe economic losses in mariculture worldwide including many countries like Japan, Scotland, Spain, France, and North America [12]. *Tenacibaculum maritimum* (formerly known as *lexibacter maritimus*) is the etiological agent of Tenacibaculosis and it has a specific tropism to certain parts of fish body especially skin, mouth, fins and tail of fish, causing eroded mouth, frayed fin, and tail rots and sometime necrosis on the gills and eye [6, 13] with severe necrotic and ulcerative lesions on the bodysurface with systemic signs [4].

This marine bacterium was isolated before from diseased samples of cultured marine fishes at Wadi-Mariout Lake at west Alexandria governorate, Egypt [10] and there is a potential probabilities for infecting cultured *M. capito* at the same region so that, this study was aimed to investigate the clinical signs, PM lesions and the histopathological alterations in cultured *M. capito* if experimentally infected with that bacterial isolate to identify whether this bacterium can cause a clinical disease or not in cultured *M. capito*.

2. Material and Methods:-

2.1. Fish and culture conditions:-

A total number of 90 apparently healthy *M. capito*, with average body weight of (50 ± 5 g /fish) were obtained from a private fish farm at Wadi-Mariout lake, Alexandria governorate and transported a live to the laboratory of the department of poultry and fish diseases, Faculty of veterinary medicine, Alexandria University in large plastic bags containing water enriched by oxygen (2/3). All fish were placed in aquaria and left acclimated for 2 weeks prior to the experiments. Experiments were conducted in prepared glass aquaria (90 x 50 x 35 Cm), supplied with chlorine free tap water [14]. The continuous aeration was maintained in each aquarium using an electric air pumping compressors. Settled fish wastes were cleaned daily by siphoned with three quarters of the aquarium's water, which was replaced by aeratedwater from the water storage tank. Water temperature was kept at 22 ± 1 °C and pH 8.5 during the experimental period.

2. 2. *Tenacibaculum maritimum* inoculum:-

T. maritimum strain was obtained from a previous work [10] and then subcultivated on plates of *Flexibacter maritimus* medium (FMM) [15]. Pure colonies of the *T. maritimum* isolates.

Were picked up and the strain was passed in small group of *M. capito* for reactivation, and re-isolated and identified again then used for other studies.

2. 3. Experimental infection:-

2. 3. 1. Experiment (1) [Determination of median lethal dose (LD50)]:-

Fifty of the acclimated *M. capito* were subdivided into five groups each of 10 fish and overnight cultures of *T. maritimum* were adjusted to densities (1.5×106, 1.5×105, 1.5×104 and 1.5×103 CFUs ml-1). The 1st, 2nd, 3rd and 4th fish groups were subjected to 18 hours immersion baths in the previous dilutions respectively while using a sterile saline solution for the 5thgroup that served as control group. The fish groups were closely observed for one week andortalities were recorded daily and the internal organs (Livers and kidneys) were asepticallystreaked on FMM for *T. maritimum* re-isolation.

Table 1: Design for determination of median lethal dose (LD50) of *T. maritimum*:-

Fish groups	No. of fish	Dose / fish (CFU / ml)	Route of injection
Group (1)	10	1.5×106	Immersion bath for 18 hours
Group (2)	10	1.5×105	
Group (3)	10	1.5×104	
Group (4)	10	1.5×103	
Group (5) (Control)	10	Sterile saline	

The LD50 was determined [16] using the following formula:-

LD50 = [Mortalities above 50% - 50] / [Mortalities above 50% - Mortalities below 50%]

The Specificity of death was determined by re-isolation of injected bacteria from freshly dead fish during the period of observation (One week) [17].

2.3.2. Experiment (2):-

Forty of the acclimated M. capito were subdivided into 2 equal groups each of 20 fish and each group was reared in a separate aquarium. The fish of the first group were infected by maritimum suspension containing 1/10 of the LD50 of tested bacteria in a bath immersion [18]. The second group was submitted to the same procedure without bacteria and used as control (using sterile saline). Fish groups were observed for four weeks, the clinical signs and mortalities were recorded daily and the dead fish were taken for any post mortem examination and histopathological changes.

2.3.3. Histopathological studies:-

Following complete necropsy of the freshly dead fish, specimens were collected from kidney, liver, gills and spleen 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 and from which 5 microns thick sections were obtained by microtome. These sections were stained by hematoxylin and eosin stain (H & E) [19].

3. Results and 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 have ranked the first one among all the causative agents causing serious problems in fish [20].

3. 1. Results of the median lethal dose (LD50):-

The mortality of the experimentally infected *M. capito* was reported for one week after immersion bath infection with different concentrations of *T. maritimum.* The fish mortality occurred during the 1st week of the experiment. The LD50 of *T. maritimum* for *M. capito* was (1.5 × 105 CFU mL-1) (**Table 2**).

Table 2: Design for determination of median lethal dose (LD50) of *T. maritimum*:-

Fish groups (10 fish for each)	Bath conc. ml -1	No. of dead fish / day							Total number of dead fish	Mortality Rate %
		1	2	3	4	5	6	7		
Group (1)	1.5×106	2	1	1	1	1	-	-	6	60
Group (2)	1.5×105	2	1	-	1	1	-	-	5	50
Group (3)	1.5×104	1	1	-	1	-	-	-	3	30
Group (4)	1.5×103	-	1	-	-	1	-	-	2	20
Control group	Sterile saline	-	-	-	-	-	-	-	-	-

By the end of the observation time (7 days), the mortalities within the experimentally infected fish reached to (60%) comparing to zero % mortality in the control group.

In regards to the LD50, results were similar to LD_{50} of *T. maritimum* when infected to in Surge Wrasses Fish (*Thalassoma Purpureum*) in immersion bath [21].

3. 2. Clinical signs of experimentally infected *M. capito*:-

The experimentally infected *M. capito* were off food, lethargic, some of them exhibit sluggish movement and other showed nervous manifestations (listlessness).

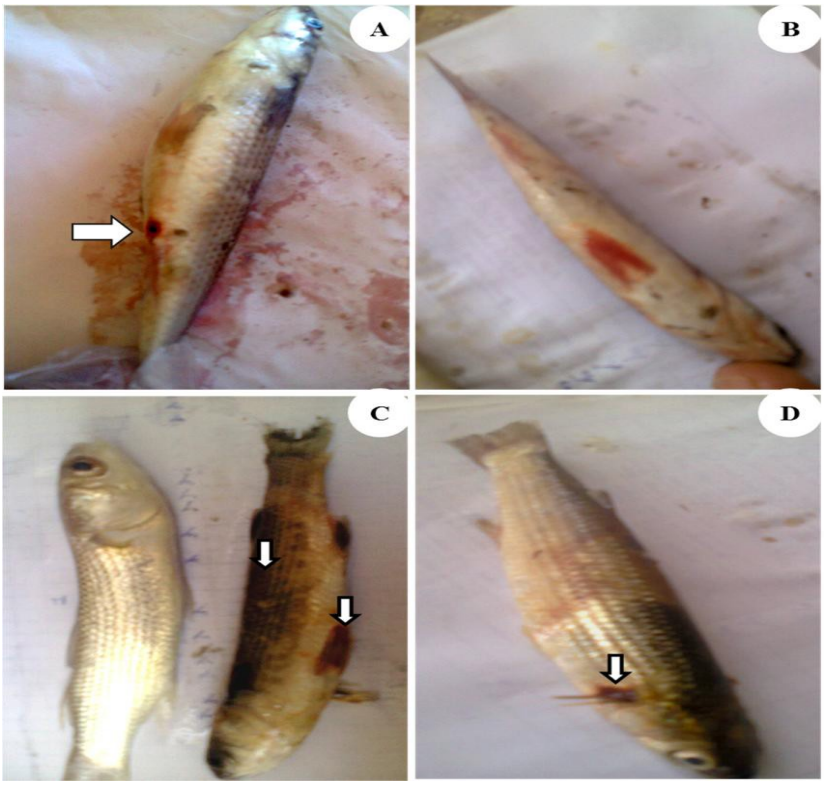

Fig 1: Experimentally infected M. capito has hemorrhagic inflamed swollen vent (arrow) (Photo A), severe hemorrhage at the base of pelvic fins (Photo B), darkness of skin color (arrow) with moderate hemorrhage at bas of pelvic fins (arrow) and fin erosions (Photo C) and severe hemorrhage at the base of pectoral fins (arrow) with extensive skin ulceration (Photo D).

3. 3. PM lesions of experimentally infected M. capito

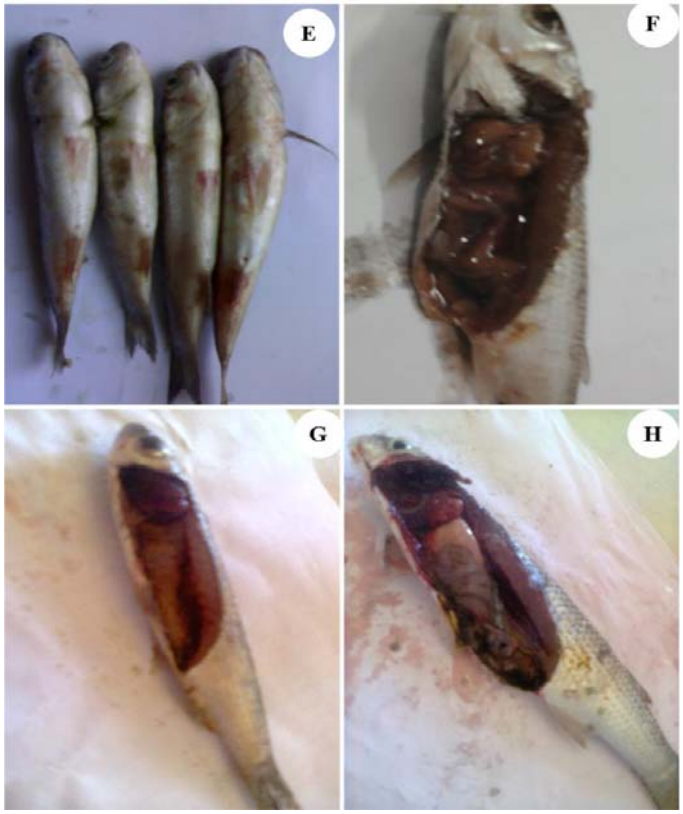

Fig 2: Experimentally infected *M. capito* showed moderate 150 erythematic hemorrhage at base of pelvic and anal fins **(Photo E),** septicemic lesions appeared as severe congestion in the gills and visceral organs with bloody hemorrhagic ascetic fluids that were noticed upon opening the fish (**Photo F & H)** and congestive kidneys (**Photo G).**

3. 4. Results of re-isolation of the *T. maritimum* from the experimentally infected fish:

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.

3.5. Histopathological findings of the experimentally infected fish with *T. maritimum*:-

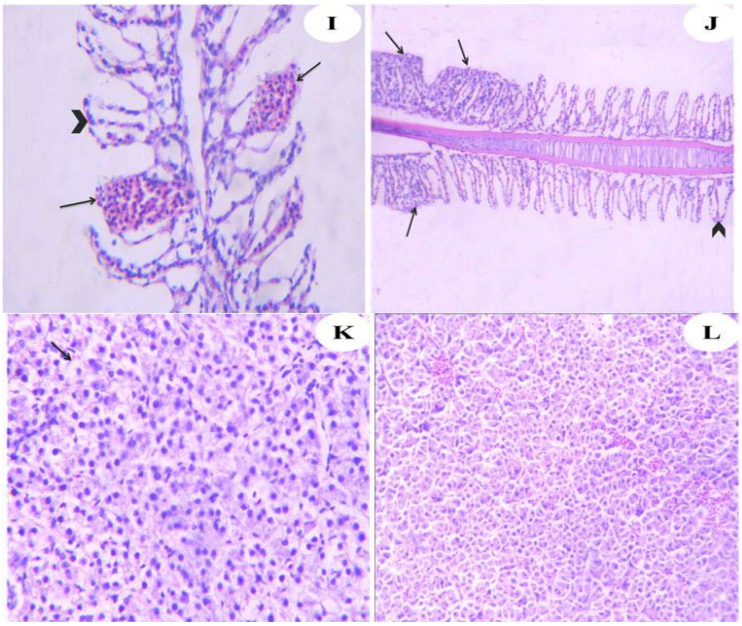

Fig 3: The gills of experimentally infected *M. capito* has lamellar 162 telangiectasis (arrow), edematous separation of surface epithelium of secondary lamellae from capillary beds (head arrow) **(Photo I)** and multifocal fusion of secondary gill lamellae (arrows) and epithelial lifting (head arrow), H & E (X 250) **(Photo J**). While, the hepatopancreas showing congestion of hepatic sinusoids, H & E (X 160) (**Photo K)** with vacuolar and hydropic degeneration, H & E (X 250) (**Photo L).**

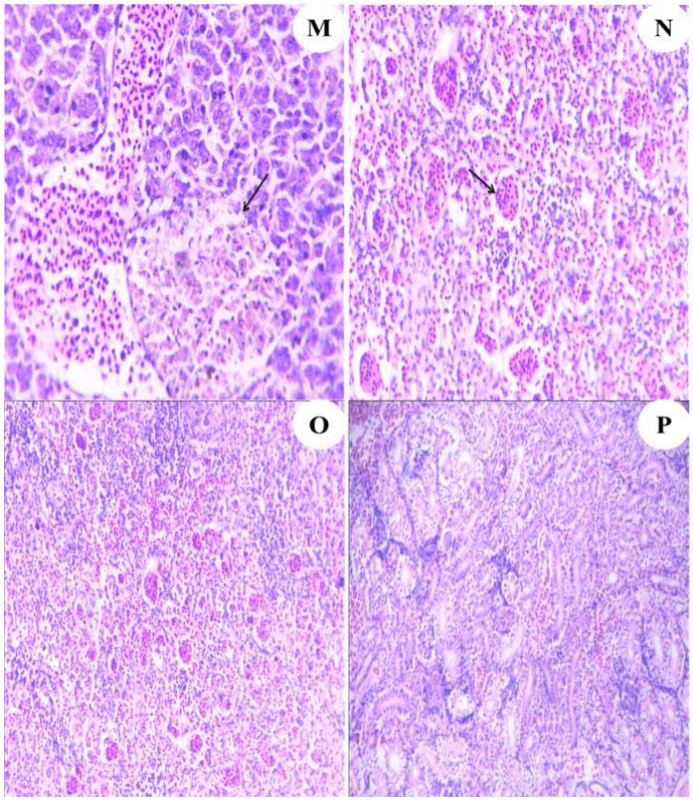

Fig. (4):- The hepatopancreas of experimentally infected *M. capito* has activation of the MMCs, H & E (X 160) **(Photo M);** the spleen has extensive lymphoid cell depletion accompanied by telangiectasis of the red pulp sinusoids (arrow) at the expense of white pulps, H & E (X 250) **(Photo N)** and lymphoid cell depletion accompanied by telangiectasis of the red pulp sinusoids at the expense of white pulps, H 173 & E (X 160) **(Photo O)** while the posterior kidneys showing depletion of blood element, H & E (X 250) (**Photo P).**

The results of clinical signs, PM lesions and histopathological findings of Tenacibaculosis of experimentally infected *M. capito* were similar to the results of Seabass ***(Dicentrarchus labrax* L.)** and gilthead Seabream ***(Sparus aurata* L.)** [22], where their results showed that ***T. maritimum*** caused fin erosions and necrotic ulcers of skin and muscle. Bacterial pathogens do their pathological effects through the production of several destructive weapons that usually known as virulence factors; therefore, the results of Clinical picture, PM lesions and the histopathological alterations may be attributed to the virulence factors of ***T. maritimum*** such as the synergistic interaction of the toxins and enzymes present in the extra cellular products (ECP) which have high proteolytic activity with an ability for degradation of gelatin, amylase, casein and nucleases as well as a positive cytotoxic activity [23]; this factor gave the ability to *T. maritimum* to cause hemorrhagic skin ulcerations as found in infected *M. capito*. Similarly, Adhesion is important also to increase the infectivity of the bacterium [24]; hemagglutinating activity [25]; the lipopolysaccharides (LPS) of cell wall is considered as endotoxin [26]; Capsular structure [27] and iron uptake mechanisms [28]. All these previously mentioned factors contribute together to produce the clinicopathological picture of infected *M. capito*. Finally, Results obtained concluded that the marine bacterium, *T. maritimum* can produce a serious clinical disease in cultured *M. capito* in Egypt.

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