Contribution to Vibriosis in Cultured Eels *(Anguilla Anguill)*

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Abstract: One hundred and twenty cultured eels *(Anguilla anguilla L.)* were collected from Behera Governorate were investigated for the isolation of Vibrio species. The isolation of Vibrio spp. was achieved from the ulcers, blood, liver, kidneys and spleen of naturally infected eels (alive and freshly dead). The recovered bacteria were studied for the virulence, pathogenicity and antimicrobial sensitivity. Blood samples were collected for determination of serum AST and ALT, total protein , albumin , globulin , cholesterol , cortisone , Glucose , direct and indirect bilirubin . Forty eight isolates of Vibrio species were identified as *V.anguillarum* (22) ,*V.ordalii* (12) , *V. parahaemolyticus (7), V.vulnificus* (4) and *V.algnolyticus*(3). The results of LD50 in eels *A. anguilla* injected with *V.anguillarum* was 10^{-2} cfu / ml , while the sublethal dose 1/10 X LD_{50} equal 10^{-3} cfu/ml . The experimentally infected eels showed severe hemorrhages over the body and congestion of the head. Internally, enlargement of spleen which became cherry red and loss of its sharp edges as well as severe congestion of kidney.
The histopathological alterations revealed hepatic cell necrosis and hyperactivation of the melanomacrophage centers of kidneys in acute phase while in thrombus formation in the branchial artery of gills and severe glycogen deposition in liver in chronic stage. The five recorded isolates of the Vibrio species were sensitive to Ampicillin, Doxycycline, Colistin sulphate and Amoxicillin, but totally resistant to Oxytetracycline and Nalidixic acid. The Antibody titers in *A. anguilla* injected with booster dose of bacterin of *V.anguillarum* were higher than in group injected by one dose of bacterin. Asignificant increase in enzymatic activity, hypoproteinaemia ,hypoalbuminaenia and hypoglobulinaemia. Also significant increase level of adrenocorticotrophic hormone,glucose and cholesterol in chronic infection(1/10LD_{50})
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Key words: Vibrio species , *V.anguillarum*_ , *A. anguilla* , LD50 , Pathogenicity virulence , chronic infection , sensitivity , histopathology , Antibody titer serum cortisol ,serum glucose ,serum AST, ALT levels serum cholesterol serum protein,albumin ,globulin and serum bilirubin)

1. Introduction

Vibrio is a human and animal pathogen that carries the highest death rate of any food- borne disease agent. It colonizes shellfish and forms biofilms on the surfaces of plankton ,algae and fish to decrease its load in filter feeder and biotic surface and control the occurrence of invasive disease **(Nahamchik et al ., 2008).**

Vibriosis is emerging as the scourge of marine and freshwater fish as well as shellfish. There are seven species of Vibrio, namely *V. alginolyticus* , *V.anguillarum* , *V.carchariae* , *V. cholerae* , *V. damsele* , *V. ordalii* and *V. vulnificus*, have been described as pathogens of fish**(Toranzo and Barja , 1993)**

Eel (*A.anguilla*) are important food fish in many tropical and subtropical countries.More than 20species of the genus *A.anguilla* has been cultivated in devolping countries due to their high tolerance to adverse environmental condition. Thier relatively fast growth,high coast,high content of phospholipid (omega 2 and 3)as well as easy handling for breeding **Guerrero (1982).**

The objects of this study were to survey the isolation of the Vibrio species from *A. anguilla* , determination of pathogenesis of isolated *V. anguillarum* in eels . Also, attempt to develop vaccine that would effectively. protect eels against infection with isolated *V.anguillarum* determine biochemical changes associated with *V.anguillarum* infection and vaccination.

2. Materials and Methods

Fish for primary isolation:

The isolation of Vibrio species was achieved from ulcers, liver, kidneys and spleen of naturally infected 120 eels *(Anguilla anguilla)* with an average body weight of 170- 190 g. Fish were collected from private farms at Kafr AL dawwar, Behera Governorate. The examined fish showed hemorrhagic patches at the trunk and the base of fins as well as superficial hemorrhagic ulcers at the abdominal wall.

Experimental fish:

A total of 120 apparently healthy eels with an average weight of 50 ±15g were obtained from a private fish farm at Kafr EL-Sheikh Governorate. They were kept in glass aquaria provided with aerated dechlorinated tap water, and kept at temperature of 22±1 ^{o} C.

Aquaria:

12 glass aquaria (90 x 50 x 35 cm) were used for holding the experimental fish throughout the period of the present study and supplied with chlorine free tap water according to **Innes, (1966).** Continuous aeration was maintained in each aquarium using an electric air pumping compressors Water temperature was kept at 22 ± 1 C^{o} by using electric heater.

Fish diet:

Fish were fed on commercial fish food containing 35% crude protein. The diet was provided daily at 3 % body weight as described by **Eurell et al. (1978) .**

All fish samples were clinically examined according to the method described by **Amlacher (1970)**. The postmortem examination of all examined fish were done.

Isolation of Vibrio species

The isolation of vibrio spp. was achieved from ulcers, blood, liver, kidneys and spleen of naturally infected eels (alive and freshly dead). Primary isolation was made on tryptcase Soya agar at different concentrations of sodium chloride (1.5-8 %) according to the methods described by **Sherbina (1973) .**

-Identification and biochemical characterization of isolates:-

Identification of the isolates was carried out by determining their morphology, cultural and biochemical characteristics according to the criteria of **Baumann and Baumann (1981)** and the established methodologies of **Davis et al. (1980)**.

Antibiotic susceptibility tests*:*

These tests were done according to the method described by **Bauer et al. (1966)**.**Determination of *Vibrio anguillarum* isolates virulence by calculation of the lethal concentration 50 (LD_{50}).**

A total of 60 apparently healthy eels (*Anguilla anguilla)* were used in this experiment with average body weight of 50-60g . Fish divided into 6 groups (10 fish / group). Five groups were injected intramuscular (i.m) with 0.2 ml from different dilutions of selected *V.anguillarum* isolate (10^{-1} , 10^{-2}, 10^{-3} , 10^{-4} , and 10^{-5} cfu/ml) and the 6th group was injected with 0.2 ml sterile saline (i.m) and served as control . Clinical signs, gross lesions and mortalities were recorded throughout the experiment (7day). Specimens from dorsal musculature, liver, kidney and spleen were taken for histopathological studies. The LD_{50} was determined according to the method described by **Behrens and karber (1953)**

Reisolation and identification of injected bacteria was done from freshly dead fish according to **Sherbina (1973**) and **Davis et al. (1980)**.

Chronic experiment:

A total of 60 apparently healthy eels (50±10g each) were used in this experiment after being checked and proved to be free from examined bacteria. The eels were divided into two groups (30 fish / group). The first group was injected with 0.2 ml from sublethal dose of *V.anguillarum* (10^{-3}cfu/ml) and the second group was injected with 0.2 ml from sterile saline and kept as a control group. All experimental groups were kept under daily observation for 4 weeks. The clinical signs, mortality and postmortem lesions were recorded . Reisolation of injected bacteria was done from dead fish for verification of death.

At the end of chronic experiment , the survival *A. anguilla* from both infected and control(20eels for each group) are injected with 0.2 ml/fish of formalin inactivated and adjuvant bacterial suspension . Control fish were similarly injected (IP) with 0.2 ml/fish sterile saline. All experimental groups were kept under daily observation for 4 weeks. The clinical signs, mortality and postmortem lesions were recorded. Reisolution of injected bacteria was done from dead fish for verification of death.

V. anguillarum virulent isolate was used in the bacterin preparation according to the method described by **Sakai et al. (1984)** .

Safety and sterility tests of the prepared bacterin were carried out according to **Anderson and Conroy (1970).**

Challenge test: (Booster dose of bacterin)

At the end of the previously mentioned experiment both of infected and control groups (10eels for each group and 10 eels control-ve) were injected with 0.2 ml of virulent strain of *V. anguillarum* previously adjusted at Macfarland's No.2 ($6x10^{8}$cells/ml). Clinical signs and mortality were recorded for one week. Blood samples were collected for 4 weeks Specificity of death was determined by reisolation of injected bacteria from dead fish during the period of experiment.

The potency of bacterin was examined by calculating the relative level of protection (RLP)

According to the procedure **of Newmen and Majnarich (1982)**

Blood and serum sampling:

Blood samples were collected from the caudal vein of control & infected eels were collected weekly for 4 weeks of each experiment by using disposable syringe, collected blood was kept overnight in the refrigerator at 2 - 8 ^{o}C. Serum was separated by centrifuging at 6000 rpm for 10 minutes. Aspiration of supernatant serum using sterile pipette was carefully done and stored at -20 ^{o}C until use.

Clinical biochemical analysis:

Serum aspartate aminotransferase (S.AST) and serum alanine aminotrasnferase (S.ALT) were estimated according to **Reitman and Frankel (1957).**

Total protein was determined according to **Doumas et al. (1981)**

Albumin was determined according to **Reinhold (1953)**

Globulin was determined by subtract the total serum albumin from total serum protein according to **Coles (1974)**

Cholesterol was determined according to **Allian et al. (1974)**

Glucose was determined according to **Trinder, (1969)**

Determination of direct and indirect bilirubin according to **Baumgartner and Skalicky (1979).**

Cortisone was determined according to **(Farmer and Pierce, 1974).**

Preparation of antiserum:

The preparation of antiserum was carried out according to the method of **Badran (1990)**

After 28 days post – injection with inactivated bacterin , blood collection was carried out from the caudal vein of inoculated fish . Collected blood was kept overnight in the refrigerator. Serum was separated and stored at – 20^{o}C until use.

Preparation of stained antigen:

To 10 ml of the bacterin suspension (6x 10 ^{8} cells / ml) in sterile saline solution one drop of lofeller's alkaline methylene blue (10%) was added to increase the visibility of the serological reaction (**Collins et al., 1976)**. The prepared antigen was used for serum antibody detection.

Antibody titration against V.anguillarum bacterin :

Detection of immune response to *V.anguillarum* was evaluated by micro agglutination (MA) test according to the method described by **Badran (1990)** . In a standard micro titer plate (U-shaped wells) , serial two fold dilution of serum were made in sterile saline solution , using a 0.025 ml pipette dropper and 0.025 ml microdiluter. *V.anguillarum* stained antigen (0.025ml) was added to the diluted serum. The suspensions were mixed and incubated overnight at room temperature (24^{o}C). A positive serological reaction was indicated by bacterial agglutination. Agglutination titers were expressed as log $_{2}$ of the highest serum dilution still giving a clear agglutination **(Badran, 1990).** The negative controls consisted of:

I. One drop of sterile physiological saline and one drop of tested serum
II. One drop of sterile physiological saline and one drop of stained antigen.

The positive control was carried out using collected positive antisera

Histopathological studies:

Tissue specimens were collected from gills, Liver, spleen, kidney and dorsal musculature of sacrificed fish after determination of the lethal and sublethal dose of *V. anguillarum*, then fixed in 10% formalin saline. Five microns paraffin sections were obtained by using rotatory microtome and stained by hematoxyline and eosin stain **(Carlton, 1967).**

The obtained data were calculated and statistically analysis according to **Snedecor and Cochren(1980).**

3. Results

Clinical examination of infected eels:

The clinical signs in naturally infected eels were hemorrhagic patches on the caudal peduncle area and base of the fins as well as superficial hemorrhagic ulcers at the abdominal wall. The postmortem changes were characterized by deep seated muscle lesions, enlargement and congestion of the spleen which became cherry red in color an losses its sharp edges. Moreover, ascites and corneal opacity were also noticed in some examined fish.

Isolation and identification of Vibrio species:

Attempts to isolate Vibrio spp . from different organs (kidney , ulcers , blood , liver and spleen) of naturally infected eels (*A. anguilla*) gave forty eight isolates that grow on trypticase soya agar and added different concentration from Na Cl (1.5 to 8 %) . The sites of the isolation of each of the isolates are shown in Table (1). They were Gram – negative motile rods and gave a presumptive identification of Vibrio species.

Table (1): Sites of isolation of different Vibrio species from different orangs of naturally infected *Anguilla anguilla* fish .

Name of isolates	Total numbers	Sites for isolation				
		Ulcer	Blood	Spleen	Liver	Kidney
V. anguillarum	22	4	4	2	5	7
V. ordalii	12	1	3	1	2	5
V. alginolyticus	3	-	1	-	1	1
V .parahaemolyticus	7	-	4	-	1	2
V. Vulnificus	4	-	1	-	1	2
Total	48	5	13	3	10	17

Table (2): The cultural and biochemical characters of *V. anguillarum*, isolated from examined *Anguilla anguilla*

Test	*V.anguillarum*
Colonies with green , rounded and transparent , convex in shape , change from green to yellow colour	
Motility	+
Oxidase	+
Catalase	+
Indole	+
Fermentation	
Lactose	-
Glucose	+
Arabinose	+
Sucrose	+
Citrate utilization	-
Arginine dihydrolase	+
Ornithine decaboxylase	-
Novobiocine	Inhebition zoan

Antibiotic susceptibility:

In vitro susceptibility of Vibrio species isolates to variety of antibiotics are shown in Table (3). The data revealed that the isolates were susceptible to Doxycycline (30mg), Colistin sulphate (50mg), Amoxicillin (25mg), Oxytetracycline (30 mg) and Nalidixic acid (30mg).

Table (3): Sensitivity of different Vibrio species to different antibiotics isolated from naturally infected *(Anguilla anguilla)*

Vibrio / strains	**Ampicillin (50IU)**	**Doxycycline (30mg)**	**Colistin sulphate (50mg)**	**Amoxicillin(25mg)**	**Oxytetracycline (30mg)**	Aureomycine**(28mg)**	**Nalidixic acid (30mg)**
V.anguillarum	S+	S+++	S++	S+	S+++	R	S+++
V.ordalii	R	S+++	S+++	S++	S+++	R	S++
V.parahaemolyticus	R	S+++	S++	S++	S+++	S+	R
V.vulnificus	R	S+++	S++	S++	S++	R	S

S++ +: Highly susceptible (Sensitive) S++: Moderately susceptible (Sensitive)
S+: Slightly susceptible (Sensitive) R: Resistant

Experimental studies:

Determination of the LD $_{50}$ of selected *V. anguillarum* in eel:

The results of determination of the virulence of selected *V.anguillarum*_isolate by calculation of the lethal dose 50 (LD_{50}) are summarized in Table (4) . Moreover , the results showed that the LD50 and sub lethal dose *of V. anguillarum* in *A.anguilla*_ was 10^{-3} bacterial cells / ml .

Table (4) : Results of LD_{50} in eels (*A. anguilla*) injected with *Vibrio anguillarum*_

Bacteria dilution inoculated	Number dead	Number alive	Accumulated number		Proportion Dead / total	Percent dead
			Dead	Alive		
10^{-1}	2	3	7	3	7/10	70
*10^{-2}	3	2	5	5	5/10	50
10^{-3}	2	3	2	8	2/10	20
10^{-4}	0	5	0	10	0/10	0
10^{-5}	0	5	0	10	0/10	0

* Sublethal dose 1/10 x LD50 = 1/10 x 10^{-2} = 10^{-3}

Table (5) : Results of vaccination and relative level of protection of *V. anguillarium* in *Anguilla anguilla* :

Treatment	Results*	Survival	Mortality	Relative Protection
		%	%	Level
Booster dose	0/20	100	0	100
One dose	4/20	80	20	32
Control	12/20	40	60	65

number of fish dying while the denominator is the number of fish inoculated

Results of histopathological studies:

Histopathological sections from different organs of examined fish revealed the following results.

A.Liver:

In case of injection of lethal concentration (LD_{50}) there was thrombus formation Also, in case of sub lethal dose injection, there Is hepatocytic cell necrosis in between swollen cells an normal hepatocytes.

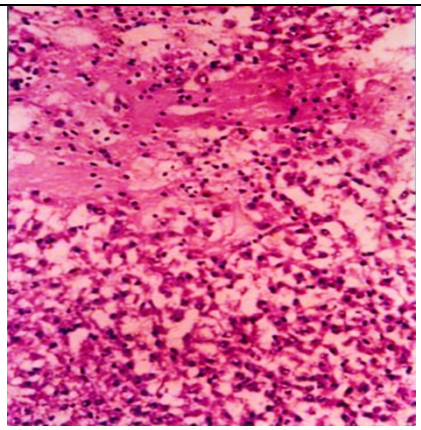
Electron micrograph of the liver in case of (LD_{50}) indicate the presence of vacuolation of hepatocytes with severe glycogen deposition and severe endoplasmic dilatation **Fig(1)**

B. Kidneys:

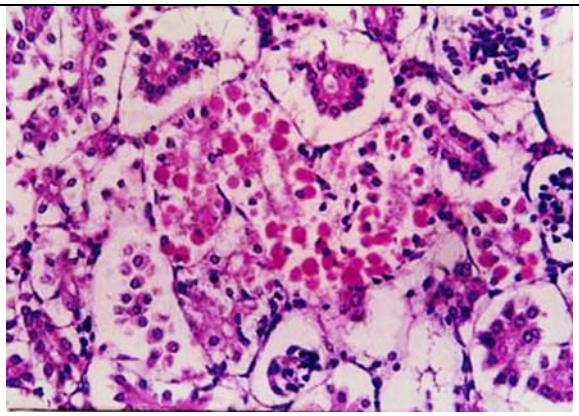
In case of injection of lethal concentration (LD_{50}) , the kidneys showed hyper activation of the melanomacrophage centers . The melanomacrophage centers were seen around and within the tunica media of the long arterioles. **Fig (2)**

D. spleen:

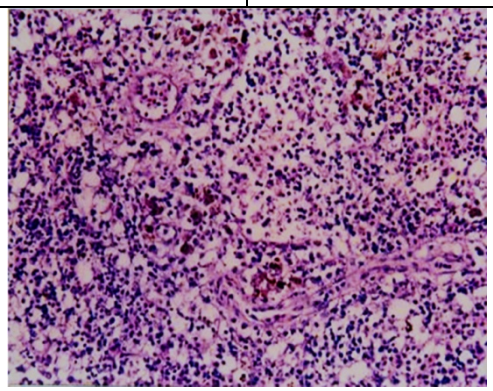
In case of sublethal dose injection , there is hyper activation of melanomacrophage centers (MMCS). **Fig (3)**



Fig(1): liver of *A. anguilla* infected with *v.anguillarum* showing coagulative necrosis of hepatocytes H,E.(x250)



Fig(2): kidney of *A.anguilla* infected with V.angiullarum showing sever hyaline droplet degeneration of some convoluted tubules H,E.(x250)



Fig(3): spleen of *A.anguilla* infected with *V.anguillarum* showing hyper-activation of melano macrophage center centers H,E.(x160)

Table (6): Effect of different treatments on enzyme levels, cholesterol , glucose, direct bilirubin, indirect bilirubin, total protein, albumin, globulin and cortisone levels of eel at different periods of experiment .

Weeks	Group	N	S.AST (IU/L)	S.ALT (IU/L)	Cholesterol (mg/dl)	Glucose (mg/l)	Direct bilirubin (mg/dl)	Indirect bilirubin (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Cortisone (pmol / L)
1st week	One dose of bacterin	10	72.58±1.68Bb	48.80±2.73Bb	207.67±2.03Bb	73.55±0.67Dd	3.22±0.1Cc	0.88±0.02^{Bb}	2.19±0.17^{DC}	1.60±0.11^{DC}	0.59±0.13^{De}	551.33±7.67^{Ee}
	Booster dose of bacterin	10	75.58±1.57 Aa	63.78±2.37Aa	195.00±0.58Cd	75.66±1.15Dd	3.62±0.1Cc	0.75±0.01^{Bc}	4.53±0.285^{Aa}	0.85±0.228^{Ed}	3.68±0.077^{Aa}	635.67±6.88^{Ef}
	Infected (1/10dose of LD_{50})	10	70.7±2.49 Cc	46.30±1.41Bb	273.11±2.33Aa	121.33±1.82Cc	4.15±0.2Bb	1.72±0.04^{Aa}	2.00±0.269^{Dc}	0.147±0.029^{Ed}	1.85±0.249^{Cc}	678.33±10.67^{Dd}
	Control (injected saline)	10	60.50±1.99Ed	30.65±1.41 Cc	208.00±0.58Bb	77.00±1.15Dd	3.48±0.1Cc	1.09±0.02 ^{Ba}	3.53±0.151 ^{Cb}	1.16±0.698^{Dc}	2.36±0.538^{Ba}	568.00±8.55^{Ee}
2nd week	One dose of bacterin	10	73.23±0.05Bb	47.53±1.81Bb	208.67±1.45Bb	71.21±0.82Dd	2.00±0.1Cc	0.85±0.02 ^{Bb}	3.26±0.25^{Cb}	2.04±0.14^{Cb}	1.22±0.28 ^{Cc}	563.67±7.88^{Ee}
	Booster dose of bacterin	10	75.74±2.23Aa	62.57±1.78Aa	208.67±0.88Bb	75.33±0.67Bb	3.24±0.1Cc	0.71 ±0.01 ^{Bc}	5.11±0.10 ^{Aa}	2.50 ±0.14 ^{Ba}	2.60 ±0.08^{Ba}	511.33±6.67 ^{Gg}
	Infected (1/10dose of LD_{50})	10	69.78±2.37Cc	45.23±2.31Bb	289.15±3.44Aa	138.55±1.97Bb	4.52±0.2Bb	1.69±0.02^{Aa}	2.07±0.15^{Dc}	1.40±0.09 ^{Dc}	0.66±0.09 ^{De}	703.67±12.33^{Cc}
	Control (injected saline)	10	61.39±1.60Ed	50.75±3.33Cd	208.00±1.73Bb	79.67±0.88Dd	3.35±0.1Cc	0.95±0.01^{Bb}	3.85±0.053^{Cb}	1.90±0.085^{Cb}	1.94±0.089^{Bb}	554.33±7.83^{Ee}
3rd week	One dose of bacterin	10	73.37±3.11Bb	50.15±2.53Bb	200.00±1.73 Cd	70.67±0.88Dd	2.78±0.1 Dd	0.79±0.01^{Bc}	4.49±0.13^{Ba}	1.98±0.04^{Cb}	2.50±0.09^{Ba}	752.67±9.47^{Ee}
	Booster dose of bacterin	10	75.53±2.14Aa	61.51±2.31Aa	212.67±0.88Bb	71.22±2.31 Dd	2.53±0.1 Dd	0.82±0.02^{Bb}	5.20±0.07^{Aa}	2.90±0.15Aa	2.40±0.13^{Ba}	508.24±6.88^{Gg}
	Infected (1/10dose of LD_{50})	10	71.41±4.89Cc	46.21±1.18 Bb	257.33±2.73 Aa	152.00±2.05 Aa	5.21±0.3 Aa	1.89±0.04^{Aa}	1.65±0.13^{Ed}	1.25±0.08^{Dc}	0.40±0.09^{Ef}	812.75±15.67^{Bb}
	Control (injected saline)	10	60.25±1.05Ed	30.78±1.18Cb	208.67±0.88 Bb	77.67±0.58 Dd	3.67±0.2 Cc	1.13±0.03^{Ba}	3.72±0.10^{Cb}	1.960±0.12^{Cd}	1.85±0.05^{Bb}	572.67±7.42^{Ee}
4th week	One dose of bacterin	10	73.34±1.17Bb	48.31±1.60 Bb	207.67±0.88 Bb	80.67±0.33 Dd	2.41±0.1 Dd	0.86±0.01^{Bb}	4.42±0.11^{Ba}	2.30±0.03^{Ba}	2.19±0.14^{Cd}	568.67±8.33^{Ee}
	Booster dose of bacterin	10	75.14±2.13Aa	62.14±2.31Aa	209.00±1.45 Bb	81.00±1.73 Dd	1.68±0.1 Ee	0.93±0.02^{Ba}	5.72±0.10^{Aa}	2.92±0.12^{Aa}	2.85±0.05^{Ba}	466.67±6.33^{Hh}
	Infected (1/10dose of LD_{50})	10	70.41±6.23Cc	45.22±1.23Bb	203.54±1.58Cd	164.33±2.18	5.92±0.4 Aa	1.95±0.06Aa	1.59±0.24^{ed}	1.37±0.17^{Dc}	0.38±0.12^{Ef}	973.11±17.33^{Aa}
	Control (injected saline)	10	62.21±0.85 Ed	30.17±0.73Cd	209.00±1.15 Bb	80.67±0.33Dd	3.81±0.2 Cc	0.92±0.01^{Ba}	3.69±0.21^{Cb}	2.05±0.14^{Cb}	1.64±0.15^{Cc}	536.33±7.67^{Ff}

Means within the same column of different litters are significantly different at ($P < 0.05$).
N= Number of samples.

4. Discussion

Various kinds of gram –ve rod shaped bacteria were classified as genus Vibrio in the family Spirillaceae . F Vibrio species were described by **Breed, (1957)**.

In 7th Edition of Bergey's Manual of Determinative Bacteriology, 207 species names were listed in Index of Bergey **Buchan et al . (1966)**

The genus Vibrio is common in aquatic habitats, particularly in marine, several species are pathogenic for freshwater especially where organic loads are high **Roberts (1978)** and **Alicia, et al., (2005)** .

Only certain species are pathogenic, and while particular strains within a species may be highly pathogenic, other may be innocuous or act only as secondary invaders. The Vibrios,

(*V*.anguillarum, *V.ordalii, V. parahaemolyticus, V. vulnificus , V. algindyticus)* are fish pathogens . All are associated with acute bacterial septicemia or chronic focal lesions in infected fish. Generally, vibriosis in fish accompanies some other stress or physical trauma but some strain, especially of *V.anguillarum* and V.salmoniscida appear to be highly infections primary pathogens **Roberts et al. (1978)**.

V. anguillarum was the first Vibrio to be isolated, from eels in the Mediterranean, meanwhile, *V.anguillarum* itself, however , and the two species designated from closely related strain **Schieve, et al . (1981)** and **Egidius et al. (1986**). The clinical examination on the examined eels collected from farms cleared the severe hemorrhagic ulcerations and erosions over the dorsal musculature of the caudal peduncle. These results were nearly similar with those of **(Ivanova et al ., 2001**) , where they indicated that , eel affected by vibriosis showed typical signs of haemolysins , protease and cytotoxin produced by Vibrio **(Nabila , 2000 and Alicia et al., 2005)** which produce generalized septicemia . The most important postmortem lesions included enlargement of spleen an severe congestion of the kidney. These findings were parallel with those of **Lunder et al . (2000),** where they observed that, sever ascitis , septicemia and hemorrhages of different internal organs . These clinical signs as post mortem lesions may be attributed to the exotoxin .

Vibrio anguillarum was isolated from ulcer , blood , spleen , liver and kidney of naturally infected eels and that agree with finding of **Stoskopf (1993)** . The observed results may be due to bacteramia. These results agreed with those of **Kumar et al. (2006)** who isolated *V.* anguillarum from skin and kidney.

Concerning the morphological and biochemical characters of different Vibrio isolates, they were Grm – negative aerobic , motile , oxidase positive , highly liquefied gelatin . Table 2 showing

the colony characters of isolates , the results agree with those reported by, **Roberts (1975) , and Alicia et al. (2005).**

The LD_{50} of *Vibrio anguillarum*_was found to be 10^2 microorganisms / ml which was completely different than that those determined by **Reham Abd El-Aziz (2009**) 10^{3-5} microorganisms /ml.

Regarding the pathogencity of the selected of *Vibrio anguillarum* which were injected at a dose of $1/10^{th}$ the dose of LD_{50} (10^3 bacterial cells / ml for 4 weeks) subcutaneously in *Anguilla.* the colony characters of isolates , the results agree with those reported by **Roberts (1978) and Alicia et al . (2005)**

Regarding the pathogencity of the selected of *Vibrio anguillarum*_which were injected at a dose of $1/10^{th}$ the dose of LD_{50} (10^3 bacterial cells / ml for 4 weeks) subcutaneously in *Anguilla anguilla* , the mortality rate were 20 , 40 , 60 and 90% respectively along the course of chronic infection. Similar findings were reported by**, Chen, et al. (1982), Nabila (2000) and Reham Abd El-Aziz (2009**).

Concerning the antibiotic sensitivity of *Vibrio anguillarum* , the isolates were highly sensitive to Doxycycline , Oxytetracycline , Nalidixic acid , slightly sensitive to Colistin Sulphate , Amoxicillin , Ampicillin , while it was resistant to Aureomycin . Similar results were reported by **Nabila (2000)** who mentioned that Vibrio species were sensitive to oxytetracycline . On the other hand, **Lenntte, et al. (1985); Mohney et al. (1992); Murray et al. (1999) ; Austin and Austin (1999) , Volk et al., (1996)** and **Reham Abd El-Aziz (2009**) who found that *Vibrio anguillarum* were fully sensitive to Chloramphenicol , Sulphonamide , Streptomycin , Gentamycine , trimethoprin and Erythromycin respectively .

The present results (Table 6) revealed significant increase in S.AST, S ALT , allover the period of experiment (4weeks) . It has been reported that, the increased serum transaminases (AST and ALT) may reflect the hepatocellular damage and inflammatory reaction leading to extensive Liberation of the enzymes into the blood circulation **(Kachmor , 1970** and **Vermu, et al., 1981)** . Moreover, serum ALT and AST activities are considered as a sensitive indicator to evaluate. hepatocellular and myocardial damage by **Raa (1984)** and **Abo – Hegab et al . (1992).** These results agree with those obtained by **Nabila (2000)** who reported increase in AST and ALT due to inflammatory reaction in the experimentally infected fish with *Vibrio ordalii* **Ma-Qian et al. (2010)** enzymatic activity changes caused by bacterial infection were influeinced by both non-specific immune factors and stress reaction.

The significant increase in cholesterol level at infected (1/10dose of LD_{50}) due to decrease in kidney function **Younis , (2003). Bruno and Munro (1989)** found that rainbow trout experimentally infected with bacterial kidney diseases suffered from a significant increase in serum cholesterol especially at the end of experiment .**park et al. (2005)** blood lipo protein has been shown to be important defense factor against the bacterial infection. also **Kim and Kim (2002)** cited that cholesterol inactivats *Vibrio vulnificus cytolysin* (vvc) moreover , LDL inactivated hemolytic activity of vvc in

adose – dependent manner . The significant decrease in serum cholesterol level at Booster dose of bacterin agree with **Waagbo et al (1988)** who reported significantly reduced in serum triglycerides and total cholesterol in diseased fish .

Total serum protein is useful in diagnosis of fish diseases **Mulcahy (1967).** In the present work , significant decrease in total protein allover the period of chronic infection(4 weeks) by *Vibrio anguillarum* and this agree with**, Waagbo et al. (1988)**. **Khalil , (1998**) and **Nabila , (2000)** Hypoproteinaemia , hypoalbuminaemia and hypoglobulinemia which were observed may be due to hepatocellular damage as well as increase capillary permeability for plasma protein and degradation of protein by proteolytic enzyme released from endothelial cells destroyed by causative agents **Coles,(1986)** and **Stoskoph, (1993).** On the other hand results showed a significant increase in serum globulin fraction in booster dose of Bacterin this suggests that differences exist in antigen presentation and naïve lymphocyte stimulation, a prerequisite for the initiation of adaptive immune responses **Chavespozoe et al. (2005)** this agree with **Esteve-Gassent et al (2003)** who found that the immune response in mucus was faster (peak at 3-4 days) than in serum (peak at 7days significantly elevated for more than 25days).

In addition, the significant increase in direct and indirect bilirubin levels all over the chronic infection was observed this may be due to hepato-renal damage which may lead to major dangerous sequels in body metabolism.

Serum glucose level was significantly increase (hyperglycemia)in chronic infection 1/10 dose of (LD_{50}) could be resulted from stress action of corticosteroids on carbohydrate metabolism that results in the process of glyconeogenesis **Ducan and Prasse (1989). Shieh and Maclean (1976)** cited that the infection of brook trout with *A. salmonicida* lead to increase in serum glucose level **Marco-noales et al. (2001)** demonstrated that ability of the pathogen to colonize both hydrophilic and hydrophobic surfaces was inhibited by glucose. **Ackerman et al. (2006)** observed significant increase in plasma glucose concentration as effect of sub acute level of ammonia on physiological and immunological system of fish.

In the current study chronic infection with *V.anguillarum* increase levels of adrenocorticotrophic hormone (Cortisol) all over the periods of experiments (4 weeks). These increases of this hormone may explain the previous mentioned parameter and increase the susptability to infection . These results agreed with those of **Mangood (2004) and Haggag (2004), Deane and woony (2001),** who reported serum cortisol levels were 14-fold increasd in moribund fish.

On the same manner **Svein, et al., (1993)** monitored the plasma cortisol and glucose levels in large number of hatchery reared of Atlantic salmon and rainbow trout following a standardized confinement stress . They noticed that the cortisol concentration were higher than, the glucose in both species . Also, **Pickering and Pottinger (2005)** recorded that the acute stress of both brown trout , Salmo trutta L.and rainbow trout , Salmo garidenri_ Richardson such as handling or 1h confiment caused a temporary elevation of the plasma cortisol levels (40-200 ng /ml) compared to 10 ng/ ml in control one . **Gregory and Roger (2008)** using plasma cortisol concentration after a 3-h crowding stress in rainbow trout as a measure of stress responsiveness to infection with *Yersinia ruckeri*. They mentioned the strong correlation between level of cortisol and incidence of disease. Where, **Valiente et al. (2008)** suggest that pathology caused by vibriosis in eels is not caused bye massive bacterial growth in the blood and internal organs but, rather by the effect of potent toxic factores. Inconclusion this study proved that *V .anguillarum* induced extensive damage of haemobiotic tissue of eels *(Anguilla Anguilla l.).* vaccination can minimize the impact of vibriosis .

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