Studies on Vibrio Infection in Cultured Freshwater Fish

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Abstract: During the course of this study 10 isolates were isolated from *M.capito* collected from several farms in Behera province .The morphological and biochemical characters of isolated bacteria were proved to be belong to *V.anguillarum* (2 isolates),*V.ordalii* (6 isolates) and *V. parahaemolyticus* (2 isolatea).The isolation of the 3 Vibrio sp. from internal organs of naturally infected *M.capito* indicated that isolates are able to induce infection in *M.capito* .The examined *M.capito* showed signs of septicemia in the form of hemorrhagic patches on the caudal peduncle area and base of the fins , superficial ulcers, ascites and congestion of internal organs .Up on injection of *V.ordalii* in eels both the clinical signs and postmortem lesions were more severe than that observed in naturally infected *M.capito* .The histopathological changes were severe hyperplasia of secondary gill lamellae, hepatocyts necrosis, activation of melanomacrophage centers and bacterial colonization in the ellipsoid of the spleen .The vaccinated eels respond positively to the injected *V.ordalii* bacterin with relative level of protection of 100% .To the best knowledge of the authors it is the first time to isolate *V.ordalii* from *M.capito* in Egypt .Moreover, isolation of *V. parahaemolyticus* is an alarm not only as fish pathogen but also as human hazard .
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1. Introduction

One of the main factors affecting fish production and efficiency is the fish diseases and especially that resulted from bacterial diseases which are responsible for heavy mortality among wild and cultured fish **(Saad., 2002)**

Vibrio species are gram negative bacteria affected all type of fish of either marine or freshwater fish allover the world in the different areas of Asia, America ,Australia ,Africa and Europe **.(Toranzo and Barja 1993 ,.and Austin and Austin., 1999).**

Genus **Vibrio** comprises more than 45 species, most of which are which are widely distributed in the marine environment. Bacteria of this genus constitute the dominant intestinal microbiota of a wide range of marine fish **) Sakata et al., 1980 ;Onarheim and Raa. (1990).**

Fish affected by **Vibriosis** suffered from severe congestion at the base of the fins, erosion of the fins, excessive mucoid secretion on gills, severe congestion of gills, hemorrhagic ulcerations, linear hemorrhages over different parts of the body and severe congestion or hemorrhagic protrusion of the anal opening **(Toranzo et al., 2005)** . Morovere ,the most important postmortem lesions were congestion of internal organ and distention of gall bladder **) Xio et al., 2005 and Reader et al., 2007(**

In Egypt ,production of fish has significantly increased during the last ten years, due to the improvement in culture techniques specially in *Oreochromis niloticus* farming . However, disease outbreaks have bean reported with economic losses: The outbreaks of Vibriosis were common problems among cultured marine and freshwater fish which have occurred at various stages of cultured and caused serious economic losses (**Rasheed.,1989 a.)**

Vibrio spp **.**has been isolated from freshwater environment and isolation rates increased with increase environmental temperature and organic pollution) Rhades et al 1986 ,.and Reham,Ali.(2009) The aim of this study was isolation and identification of *V .ordalii* that affect cultured *M.capito* and clarify the pathogenicity in cultured eel (*Anguilla Anguilla)***.**

2. Materials & Methods

Naturally infected fish

A total number of 80 *Mugil capito* (50± 5 mg)were collected moribund and alive from a private fish farm in Behera Province. Fish were subjected to clinical and microbiological examinations according to **Austin and Austin.,(1987)** and **Schaperculaus et al ., (1992)** .Isolation of Vibrio spp .was achieved from ulcers , liver , kidneys and spleen of naturally infected *M. capito* alive and freshly dead.

Experimental fish

A total of 130 apparently healthy eel *(Anguilla anguilla)* with an average weight of 40± 5 gm were obtained from natural sources in Behera province .

They were Kept in glass aquaria provided with aerated dechlorinated tap water and kept at temperature of 22± 1 C . with continuous aeration according to **Innes (1966)** . The fish were fed on commercial diet containing 40% crude protein at the level of 5% of body weight according to **Eurell et al ., (1978).** They were used for evaluation of the pathogenicity of isolated Vibrio Spp . Ten eels were random collected and submitted for bacteriological examination to verify the absence of *Vibrio ordalii*.

Primary isolation was don from internal organ of examined *M.capeto* according **to Eleonor., et al (1997**) ,on Tryptcase soya agar (TSA) .

Isolation and identification of the isolated bacteria.

Primary isolation was done on trypticase soya agar (TSA) supplied with different concentrations of sodium chloride (1.5-8%) according to **Eleonor et al (1997),** incubated for 24 hours at 30C .The recovered suspected colonies were picked up and purified for further identification according to culture, morphological and biochemical characterization

Morphological characters, colonial and growth feature on TSA as well as biochemical were used for identification of isolated bacteria according to **Berge's(1982) and Whitman. (2004).**

Moreover the API.20E system (Analytab products, Plainview New York) was also used for biochemical characteristics of all suspicious isolates.

Detection of the pathogen city of isolated bacteria in eel *(Anguilla anguilla).*

Medial lethal dose 50 (LD_{50})

Medial lethal dose 50 (LD_{50}) for the isolate (N 10) was estimated in *A. anguilla* according to **Reed and Muench-(1938**). Graded doses ranged from 10^{-1} to 10^{-7} CFU /ml was used. A total number of 80 apparently healthy eels (40+.5 gm) were grouped into 8 groups (10 eels / group). The first seven groups were injected intra peritoneal (I.P.) with one ml of specified bacterial concentrations. The eight groups was injected I.P with one ml of sterile saline and served as control. Mortalities were recorded for 7 days post injection. Freshly dead fish were submitted for bacterial isolation and re-isolation and identification of tested bacteria was done to verify specificity of mortality.

Experimental infection:

A total of 40 eels (40 + 5 g) was allotted to four equal groups . Fish of the first three groups were injected I. m with 0.2 ml of 0.5 dose of LD_{50} according to **Shehate et al., (1988)** . The fourth group was injected with 0.2 ml of sterile saline and served as control. Infected and control groups were kept under daily observation for two weeks. Both clinical sings and mortalities were recorded.

All freshly dead eels were submitted to bacterial isolation and *V. ordalii* isolated was re-identified to verify the specificity of mortality.

Histopathological and ultra changes were carried out from organs of experimentally infected eels according to **Culling, (1983).**

Evaluation of potency of prepared vaccine against *V.ordalli* were done according to the method described by **Sakai et al., (1984)** and **Badran and Eissa, (1991)**. The formalin inactivated bacterin were mixed with an equal volume of 0.85% sterile saline and adjusted to Macfarland's No.5 (approximately $6x10^8$ cells/ml) .

Twenty eels were injected with 0.2 ml bacterin / fish (IP) . Twenty eels was also injected with 0.2 ml (IP) sterile saline control. After 2 weeks the injected eels received booster dose from bacterin (Same dose) and control group injected with 0.2 ml sterile saline.

Blood collection was carried out after 28 days post injection for serum collection ,The antibody titer was evaluated by microagglutination test according to **Badran and Eissa (1991)** .

After 28 days both infected and control groups were injected with 0.2 ml of virulent isolate of *V. ordalii* previously adjusted to $6X10^8$ cfu/ ml .

Clinical signs and mortality were observed for one week. The potency of bacterin was examined by calculating the relative level of protection (RLP) by the following formula:

RLP = % 1- mortality of vaccinated eels x100 / % mortality of control

According to **Newman and Majnarich, (1982)**.

All groups of eels in this study were anesthetized with a solution containing 1 gm of benzocaine (ethyl aminobenzoate) in 10 ml ethanol prior to injection.

3. Results

Results of clinical examination of naturally infected fish:

The clinical signs in *Mugil capito* , were hemorrhagic patches on the caudal peduncle area and base of fins as well as superficial hemorrhagic ulcers at the abdominal wall (Fig.1). The postmortem changes in *Mugil capito* were characterized by deep seated muscle lesions, enlargement and congestion of the spleen which became cherry red in colour and losses its sharp edges .Moreover, ascites and corneal opacity were also noticed in some examined fish (Fig. 2)

Isolation and identification of Vibrio species :

Attempts to isolate Vibrio spp. from different organs (kidneys, liver and spleen) of

naturally infected *M. capito* gave ten isolates that grow on trypticase soya agar with different concentration from NaCl (1.5% to 8%). The colonies appeared after 24 hrs post – incubated at 30C.*Colonies* were of medium size (2-3 mm in diameter) and creamy in color . They proved to be Gram – negative, motile rods and gave presumptive identification of Vibrio species .

Biochemical characterization of isolates :

The biochemical and growth characteristics of the isolates using traditional tests indicated that all the isolates were positive for oxidase and motility tests. Variable results were obtained in case of lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase .Moreover, all isolates gave positive results in case of sucrose fermentation except isolate No.9 and 10 . The other tested sugars gave variable results . Further biochemical characterization of 10 isolates, was carried out by using the API 20E system . All tested isolates were positive for sodium pyruvaate (VP) (except N9 and N10), gelatin liquefaction and tryptophane (except N9 and N10) Negative results were obtained for tryptophane (IND) (except N9 and N10), sodium thiosulphate (H2S) and orthonitrophenyl galactoside (except isolates N1 and N2). Variable results were observed for arginine |(ADH), lysine (LDC), ornithine (ODC), sodium citrate (Cit) and urea (URE).

Concerning the results of sugar fermentation by using API20E system, the tested isolates gave positive results for glucose, mannitol, sucrose, sorbitol and rhamnose except (N2).Variable results were noticed in case of other sugars

According to both morphological and biochemical characters of Vibrio species, the tested isolates were identified as *V.anguillarum* (2 isolates), *V.ordalii* (6 isolates) and *V.parahaemolyticus* (2 isolates) .

Pathogenicity assay

Due to the unique isolation *of V.ordalii* for the first time in Egypt according to the best knowledge of the authors , the most virulent one which proved by developing rapid and severe clinical and MP lesions was used for further experimental studies in *Anguilla Anguilla* .

The results of determination of the virulence of selected *V. ordalii* isolate by calculation of the lethal concentration 50 (LD50) showed that the LD50 of *V. ordalii* was $10^{2.4}$ bacterial cells / ml.

Experimental infection with *V.ordalii*:

This experiment was done to determine the nature of experimental infection of selected *V.ordalii* isolate in eel (*A. Anguilla*).

After 2 day post injection, the eels became sluggish and the escape reflex was too weak . The clinical signs were characterization by server hemorrhages over the body, in some cases these hemorrhages covered the whole body surface and congestion of the head region (Figs.3+4). Haemorrhagic ulcers were recognized at the body surface of infected eel.

The postmortem lesions were in the form of server congestion of the liver which some times became edematous. Enlargement of spleen which became cherry red and loss its sharp edges in addition to severe congestion of the kidneys and inflammation of the intestine (Fig 5). Fifteen eels were died during the course of experiment.

Evaluation of antibody titers of *Vibrio ordalii* in eel :

The antibody response in *A anguilla* infected with *V.ordalii* was detected 28 days post-injection of bacterin. The detected antibody titer was 2^5.

The injected *A.anguilla* were firstly examined to verify their freedom from Vibrio species infection and proved to be free. The results revealed that fish vaccinated with prepared bacterin gave complete protection when challenged with 10^4 cells /ml of live bacteria, were the relative protection level reached 100% .

The re-isolation of injected bacteria was positive in case of freshly dead infected eel.

Hstopathological changes :

Histopathological sections from different organs of injected eels with ***V. ordalii*** revealed the following results.

Gills:

The major histopathological changes were characterized by severe hyperplasia and filamental thickening adhesion with mononuclear cell infiltration (Figs. 6). There were also thrombus formation in the bronchial artery. There was also slight oedema and diffuse lymphocytic infiltration in the gill arch and epithelial hyperplasia at the base of the secondary lamellae .

Liver:

The lesion in the liver were characterized by extensive areas of oedematous, vacuolated and atrophied hepatocytes. There were hepatocytic cell necrosis in between swollen cells and normal hepatocytes (Fig.7), as well as thrombus formation.

Kidneys :

The kidneys showed hyper activation of the melanomacrophage centers. The melanomacrophge centers were seen around and within the tunica media of the long arterioles . The interstitial tissues of the kidney showed depletion and cell necrosis (Fig.8).

Skin and underlying musculature :

The skin showed excessive melanosis in the dermis with multifocal slight dermal oedema (Figs .9). Leucocytic infiltration of muscle was observed .Oedema and extensive haemorrhages as leucocyte

infiltration were noted between muscle fibers as well as muscle necrosis .

Spleen:

In most samples, the spleen was extensively colonized by *V. ordalii* Bacteria accumulated mainly in the ellipsoide .The spleen was hyperaemic and the number of macrophages and neutrophils increased considerably in the periellipsoidal area and red pulp. These macrophages were hypertrophied and contained phagocytosed erythrocytes, bacteria, melanin granules and cell debris. .

Ultra changes :

The ultra changes in liver of eel previously injected with *V.ordalii* were pronounced and clear. Electron micrograph of liver in case of infected eel revealed vacuolation of hepatocyte with server glycogen deposition (Fig.10) . Moreover, server endoplasmic dilatation and server glycogen deposition were observed .

4. Discussion

The Vibrio, *V. alginolyticus, V. anguillarum, V. ordalii* are fish pathogens. All are associated with acute bacterial septicemia or chronic focal lesions in infected fish. Generally, Vibriosis in fish accompanies with some other stress or physical trauma but some strains, especially of *V.anguillarum, V.ordalii* and *V.* salmonicida appear to be highly infectious primary pathogens (Robert et al., 1975).

During the course of this study 10 isolates from several outbreaks of Vibriosis among cultured *Mugil capito* were isolated. The isolates were submitted to complete morphological, cultural and biochemical examination by using both tube biochemical method and AP120E system.

The morphological and biochemical characters of isolated bacteria were proved to belong to *V.anguillarum* (2 isolates), *V.ordalii* (6 isolates) and *V. Parahaemolyticus* (2 isolates) .The identification of the previously mentioned Vibrio species were based on the data published by **Grisez et al. (1991**) and the criteria of the manufacturer of AP 120E system . Moreover, the negative results of arginine dihydrolase, reduction of NO_2, Voges-Proskauer production and utilization of both arabinose and sorbitol distinguished *V. ordalii* from *V. anguillarum*.

Also, the positive results of lysine decarboxylase, ornithine decarboxylase, citrate simmons and indole and negative results of Vp and utilization of sucrose and melibiose distinguished the *V. parahaemolyticus* from *V.ordalii* and *V. anguillarum* **(Austin and Austin, 1987, Grisez et al. 1991 and Saeed, 1995).**

The isolation of both *V. anguillarum* and *V.ordalii* from internal organs of naturally infected *Mugil capito* (namely spleen , kidneys and liver) indicated that both Vibrio species are pathogen and able to induce infection in *Mugil capito* . Moreover, the isolation of Vibrio species from internal organs may be attributed to the ability of bacteria to produce septicemia as well as bacteriaemia.

Chart and trust (1984) and Davease et at. (1985) reported that *V. anguillarum* and *V. ordalii* were well known to be the primary pathogens to fish. While**, Austin and Austin (1987)** pointed out that seven Vibrio fish pathogens as follows: *V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae, V. damsela and V. ordalii.* While, **, Noel et al. (1996), Benediktsdottir et al. (1998) and Akhlaghi (1999)** had reported isolation of different types of Vibrio (namely , *V.anguillarum , V. alginolyticus , V . carchariae , V. cholera , V . damsela , V . ordalii , V salmonicida , V. parahaemolyticum* and *V. vulnificys* biotype 2) from different internal organs such as liver , spleen kidneys , muscular lesions of different infected fish species- . Moreover, **Ransom et al. (1984)** reported that *V. ordalii* induced pathogenesis in fish not particularly different from that of *V. anguillarum* but generally less server .They also added that the infection by *V. ordalii* may be occurred via ascending infection from the posterior gut or through the skin .

The isolation of the two isolates which were identified as *V. parahaemolyticus* is very interesting since the *V. parahaemolyticus* has been reported to be implicated in fish disease and human infection as fish food poisoning. Moreover, *V. parahaemolyticus* has been reported to be isolated from human disease situation **(Austin and Austin, 1987).**

Daniels et al. (2000) reported an outbreak of *V. parahaemolyticus* serotype $O_{3:}K_6$ infection in the United States .The same authors added that, the consumers should understand that raw or undercooked fish infected with *V. parahaemolyticus* can cause illness in from of gastroenteritis.

It is worthy to mention that, the less information about the susceptibility of eel to *V. ordalii* directed us to study the pathogenicity of this species in eels.

The result of LD_{50} proved that *V ordalii* (No. 10) used in the present study was highly virulent for eel. LD_{50} being estimated to be $10^{2.4}$ CFU/ fish.

Nordmo et al., (1997) recorded that LD_{50} of *V. ordalii* was 10^6 CFU/L fish in Atlantic salmon. The difference in the LD_{50} value in this study may be attributed to the difference in fish species , bacterial strain and environmental conditions .

The extensively colonization of *V.ordalii* in spleen ellipsoide directed us to believe that the spleen is the predilection sit for *V.ordalii* and organ of choice for isolation of the bacteria.

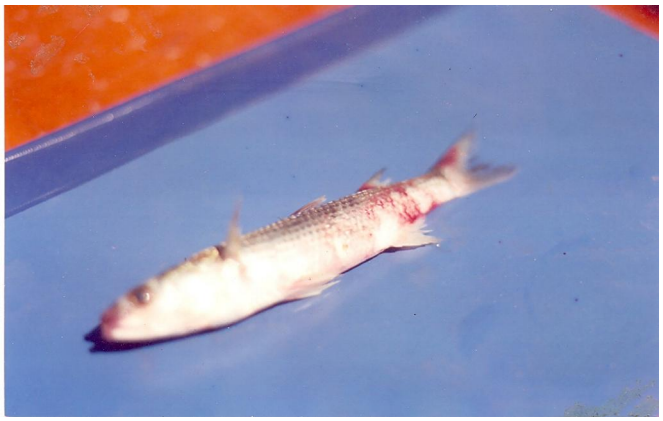

Fig.1 : *Mugil capito* naturally infected with *Vibrio ordalii* showing hemorrhagic patches on the caudal peduncle and base of fins

Fig.2 : *Mugil capito* naturally infected with *Vibrio ordalii* showing congestion and enlargement of spleen .

Fig.3: Eel (*Anguilla Anguilla*) experimentally infected with *Vibrio.ordalii* showing severe hemorrhages over the body .

Fig .4: Eel (*Anguilla Anguilla*) experimentally infected with *Vibrio.ordalii* showing deep hemorrhagic ulcer .

Fig.5: Eel *(Anguilla Anguilla*) experimentally infected with *Vibrio.ordalii* showing congestion of internal organs .

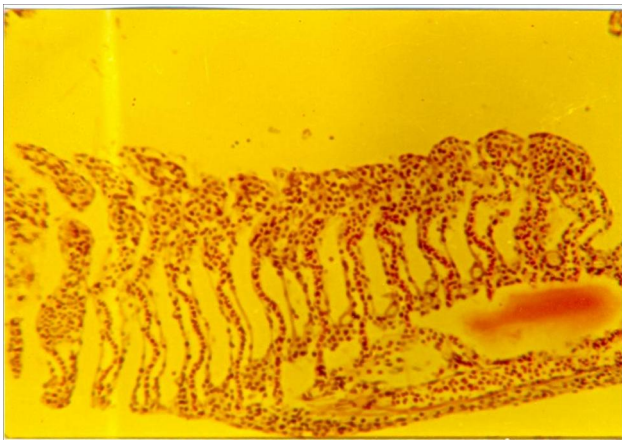

Fig6:Gills of eel experimentally infected with *Vibrio .ordalii* showing severe lamellar hyperplasia with filamental adhesion .(H&E.X160).

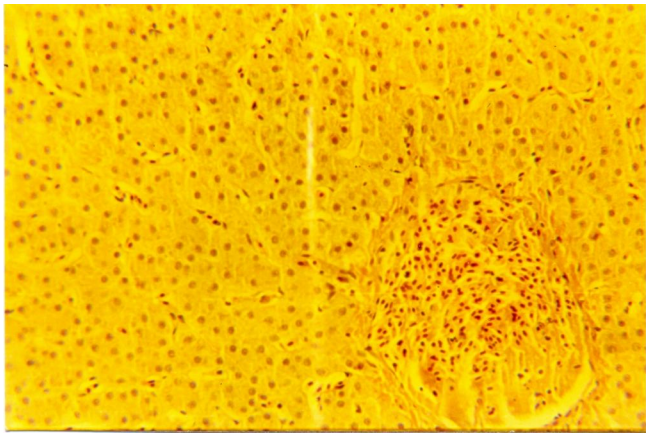

Fig.7: Liver of eel experimentally infected with *Vibrio.ordalii* showing thrombus formation .(H&E.X160).

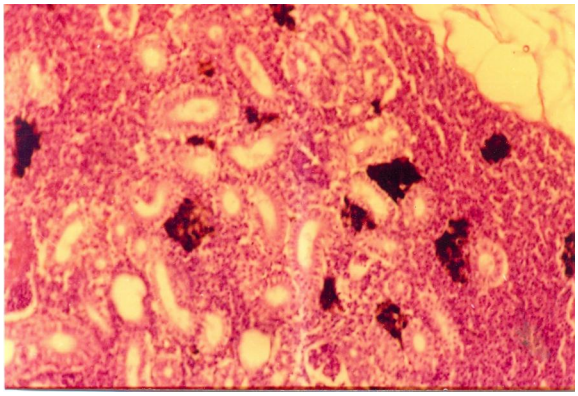

Fig.8:Kidneys of eel experimentally infected with *Vibrio.ordalii* showing hyper activation of the melanomacrophage centres .(H&E.X160).

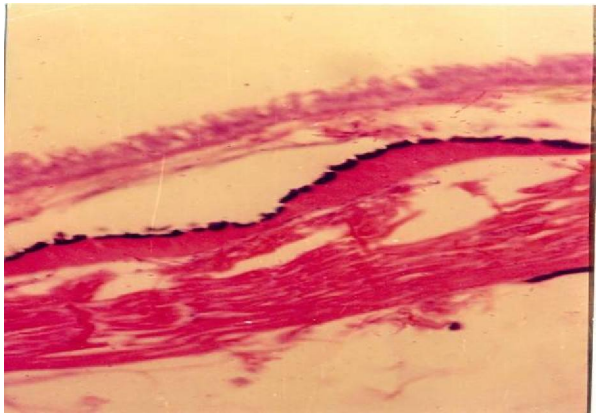

Fig.9: Skin of eel experimentally infected with *Vibrio ordalii* showing excessive melanosis in the dermis with multifocal slight dermal oedema.(H&E.X250).

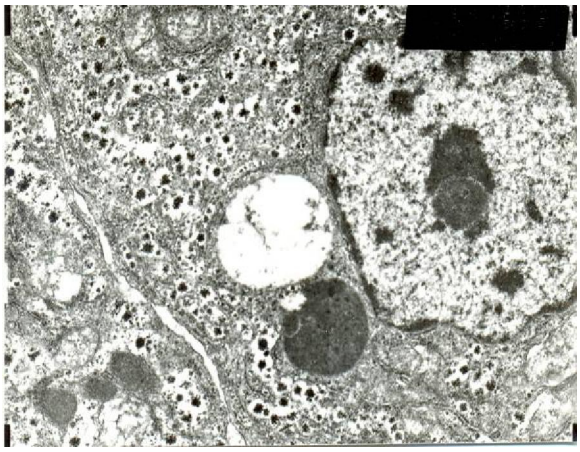

Fig .10: Electron micrograph of liver of eel experimentally infected with *Vibrio.ordalii* showing valuation of hepatocytes with severe glycogen deposition. (x10000).

The histopathological changes due to infection of *V.ordalii* varied and recognized in different organs . These changes may be attributed to the extensive bacterial multiplication and the secretion of cytotoxin, haemolysin and protease by *V.ordalii* **(Santos, et al 1991, kumar et al,. 2006 and Reham 2009)** Ultra structurally, the lesions in liver were not specific and frequently in the liver of fish exposed to toxic agents (**Ghadially, 1988**), The same conclusion was reported by **Lamas et al ., (1994)** in case of Rainbow trout experimentally infected with *V .anguillarum* .

The Relative level of protection (RLP) of vaccinated eel was 100%.This result proved that eel was respond positively to the formalized killed bacterin which prepared from *V. ordalii* isolated from *M. capito* in Egypt. *V. ordalii* isolated from *M.capito* succeeded to produce system infection in eel upon experimental infection .

The experimentally injected eels showed sings of septicemia in the form of sever hemorrhage of the body, hemorrhagic ulcers and congestion of internal organs . The recorded signs may by due to toxins produced by injected bacteria **Nordmo et al., (1997) and Reham(2009) .**

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