Animal Health Research Journal Vol. 7, No. 3, September 2019 pp. 65-77

ISSN: 2356-7767

Molecular characterization of *Edwardsiella tarda* in farmed *Oreochromis niloticus*

Eman, Khalifa[*]; Rasmia, H.M. Abu Leila[**]; Manal, E. Hefny[***] and Mervat, A. Ayoub[****]

*Department of Microbiology, Faculty of Veterinary Medicine, Matrouh University;
**Dept. of Fish Diseases, Dokki; ***Fish Diseases Unit;
****Pathology Unit, Zagazig Branch
Animal Health Research Institute (AHRI),
Agriculture Research Center (ARC), Egypt.

Received in 1/8/2019
Accepted in 1/9/2019

Abstract

Edwardsiella tarda were isolated from 100 naturally infected *Oreochromis niloticus* which suffered from excessive mucus all over the body surfaces with petechial haemorrhages over the dorsal musculature.

The post mortem lesions were in the form of sever congestion in the internal organs. The results of heavy metal concentrations in the collected water sample from pond of naturally infected fish revealed that the iron (Fe) levels were higher than the permissible limits in water sample. PCR was applied for detection of isolated *E. tarda* using specific primers. The isolated *E. tarda* strains were sensitive to Ciprofloxacin; Gentamycin and flumiquin. The results showed that the LD_{50} of *E. tarda* were $10^{-3.4}$ CFU/ml. Trials of vaccination by formalin killed vaccine from the isolated *E. tarda,* which the relative level of protection (RLP) was 90% in case of using bacterin while the (RLP) was 20% of unvaccinated group. Histopathological changes in experimentally infected *O. niloticus* with *E. tarda* were fatty degeneration of the hepatocytes and focal necrotic foci; gill showed separation of surface epithelium of secondary lamellae from the capillary beds. In conclusion, *Edwardsiellosis* is one of the most important bacterial diseases among cultured *O. niloticus* under Egyptian conditions. PCR can be used with great success for definitive identification. Formalin killed bacterin can play an important role for control of *Edward siellosis*.

Keywords: *O. niloticus, E. tarda, PCR, bacterin, histopathology, RLP.*

Introduction

The majority of fish farms in Egypt can be classified as semi-intensive water pond farms. Bacterial agents are among the highly encountered causes of diseases in stressed warm water aquaculture (**Pavanelli *et al.*, 1998** and **Noga, 2000**). *Edwardsiella tarda* infection is considered a dangerous septicaemic disease with high economic losses (**Meyer and Bullock, 1973)**, the seriousness of *E. tarda* infection is its expanding fish host range (**Mohanty and Sahoo, 2007)**. It infects *clarias gariepinus* causing emphysematous putrefactive disease (EPD) (**Darwish *et al.*, 2000**), cutaneous ulcerations, ascites and hemorrhages on the body and fins (**Abdellatief *et al.* 2017**). The pathogenicity of this bacterium is multifactorial and many potential virulence factors have been suggested, namely dermatotoxins (**Ullah and Arni, 1983***),* phagocytic killing (**Ainsworth and Chen, 1990***)*, hemolysins **(Hirono *et al.*, 1997),** serum resistance, and ability to invade epithelial cells **(Janda *et al.*, 1991** and **Ling *et al.*, 2000).** Edwardsiellosis has been known as a disease of primary importance and required, a great attention for successful aquafarming. This bacterium has been described as biochemically homogeneous (**Castro *et al.*, 2011** and

Castro *et al.,* 2012). In the temperature range from 0°C to 25°C, microbiological activity is of great importance. Many bacteria are unable to grow at temperatures below 10°C and even psychrotrophic organisms grow very slowly and sometimes with extended lag phases. When temperatures approach 0° C the growth rate is less than one tenth of the rate at the optimum growth temperature *(***FAO, 1995).** In this concern **Noga (1996)** stated that *E. tarda* was related to freshwater environments, moreover it can infect the skin or cause gastroenteritis or systemic infections to clinicians during a clinical work-up. Temperatures during the outbreak, rearing density, and mortalities were also similar in both cases. Microbiological studies confirmed that in both cases *E. tarda* was the bacterium responsible for the outbreaks *(***Kumar *et al.,* 2007). Salah (2016)** documented that the *E. tarda* are responsible for heavy mortalities in farmed fish. *Edwardsiella* bacterium is one of these diseases and application of the polymerase chain reaction in diagnosis and identification of the fish pathogen considered as a successful tool in diagnosis and identification of such diseases **(Saad *et al.*, 2015)**. In Egypt, little is known about *Edwardsiella* infection in fish and its diagnosis, therefore, this work was planned to fulfill the following objectives; isolation and identification of *E. tarda* from naturally infected *O. niloticus,* application of PCR for definitive diagnosis; determination of LD_{50}, clinical signs, postmortem and histopathological finding of experimentally infected fish, determination of antimicrobial sensitivity and evaluation of autogenous bacterin as protective tool against edwardsiellosis.

Materials and Methods

1-Naturally infected fishes:- In our investigation, a total number of 100 naturally infected *Oreochromis niloticus* (Nile tilapia) with body weight range (120 to 170 gm) were collected from private fish farms at Edku region, Behera governorate, Egypt suffering from high morbidity and mortality. The freshly dead fish specimens were subjected to full clinical, postmortem (PM) lesions, parasitic and bacteriological examinations, according to the methods described by (**Conrory and Herman, 1981)** and **(Austin and Austin, 2012)**.

2. Bacteriology

Bacterial isolation:

For bacterial isolation, samples were aseptically collected from gills, ulcer lesion of skin, kidney, liver, spleen and heart then directly streaked onto MacConkey's agar and Tryptic Soya broth (TSB) (Difco, Detroit, MI, USA) which was used for the growth of some suspected isolates prior to plating. Edwardsiella tarda (ET) medium (*Oxoid Manual, 1982*), the media was supplied originally by (Pioneer Company, Laboratory Service Company, and Egypt) was used for isolation of different strains of *E. tarda* bacterial isolates., and also Blood agar media using 5% sheep RBCs was used. The inoculated plates were incubated at 25 °C for 24 – 48 hrs.

3. Biochemical identification

The isolates were identified using the biochemical characteristics of *E. tarda* according to (**Wyatt *et al*., 1979; Grimont *et al.*, 1980; Fang *et al*., 2006)**.

4. Molecular confirmation of *E. tarda* by PCR.

4.a. Extraction of genomic DNA. (**Chen and Lai, 1998** and **Sakai *et al.,* 2007**):- Strains were cultured on TSA and incubated at 25°C, for 24 hr.

4.b. Oligonucleotide primers:-

These primers were synthesized and supplied originally by Pioneer Co. by Lab Service Co. Egypt. Primers were dissolved in nuclease-free water to obtain 50 – 1000 pmol concentration (**Sambrook *et al.,* 1989)**

Table (1). Primer sets employed in the present study for the detection of *Edwardsiella tarda*:-

Product name	Sequence (5' to 3')	Product size (bp)	Reference
etfD (fimbrial subunit) **Forward primer** **Reverse primer**	 5'-GGT AAC CTG ATT TGG CGT TC-3' 5'-GGA TCA CCT GGA TCT TAT CC-3'	445	**Sakai *et al.*, (2007)**

4.c. Table (2). Programming of thermal cycler

No. of cycles	Temperature (0C)	Time	Target
1 Cycle	94	5 min	Initial denaturation
30 cycles	a) 94	1 min	Denaturation
	b) 65	1 min	Annealing
	c) 72	1.5 min	Extension
1 cycle	72	10 min	Final extension
1 cycle	- 20	Until used	Preservation

4.d. Agarose gel preparation (Pharmacia Biotec Co.) according to **(Sambrook *et al.*,1989**).

5-Sensitivity test of the bacteria:

The antibiotic susceptibility testing was performed by a disc diffusion method according to **Quinn *et al.* (2002)** and interpreted according to **NCCLS, (1994).** The antimicrobial discs (Oxoid) were used: Ciprofloxacin (CIP 5 µg); Gentamycin (GN 10 µg); Flumequine (UB 30 µg); Enrofloxacin (ENR 5 µg); Doxycycline (DO 30 µg); Sulphamethoxazole-Trimethoprim (SXT 25 µg); Oxytetracycline (OT 30 µg); Amoxicillin (AML 10 µg); Erythromycin (E 15 µg).

6-Experimental fish: - A total number of 155 apparently healthy *O. niloticus* with an average body weight (100 ± 5 grams) were collected from private fish farm and transported alive to Fish Diseases Dept., AHRI. Fish were acclimated for two weeks according to **Innes, (1966)** with fully prepared aerated glass aquaria supplied with dechlorinated tap water. Water temperature were maintained at 24±2º C. Random samples (5fish) were taken to be examined to indicate that fish were free from *E. tarda.*

Fish were fed on a commercial fish diet containing 25% crude protein at ratio of 3% of body weight, as described by **Eurell *et al.* (1978).**

6-a-Lethal dose fifty (LD_{50}) determinations:- (Preparation of the inoculum): *E. tarda* isolate was used for determination of LD_{50} according to **Reed and Munch (1938).** The isolate was streaked on TSA plates and incubated at 25ºC for 24hr. A typical *E. tarda* colony was picked up and inoculated into TSB and incubated at 25ºC for 24hr. The culture was centrifuged and supernatant decanted out. The sediment were resuspended in sterile saline and standardized for different optical densities contain bacterial cells ranged from 10^{-1} to 10^{-6} CFU/ml.

6-b-Experimental design of LD_{50}: The fish were divided into seven groups of Nile tilapia and distributed in seven aquariums each contains ten fish. The first six groups were inoculated (i. p.) with 0.1 ml. of different dilutions of bacteria. The last groups were injected with 0.1 ml. of phosphate buffer saline (PBS) and act as a control (table 3).

Table (3). Experimental design of LD_{50}:-

Group	*E. tarda* dilution	Number of *O. niloticus*
1	10^{-1}	10
2	10^{-2}	10
3	10^{-3}	10
4	10^{-4}	10
5	10^{-5}	10
6	10^{-6}	10
7	PBS	10

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

Proportionate Distance (PD) =

$$\frac{\text{(\% mortality at dilution next above 50\%) - 50\%)}}{\text{Mortalities above 50\% - Mortalities below 50\%}}$$

LD50 =
(negative logarithm of the next dilution above 50% mortality + PD)

The clinical signs and PM lesions were recorded daily for about one week. The injected *E. tarda* was reisolated from freshly dead fish for verification of deaths.

Pathogenicity of *E. tarda* : A total number 20 apparently healthy *O. niloticus* were divided into 2 groups (each group10 fish). First group was inoculated (i.p) with 0.1 ml of LD_{50} of tested bacteria. The second group was inoculated (i.p) with 0.1 ml / fish of PBS and served as a control group. All infected and control fish were observed daily to record their general health condition, clinical signs and mortalities. Experimental period was 15 days. Postmortem examination was performed on dead fish **(Badran and Eissa, 1991).**

Preparation of bacterin: *E. tarda* strain was grown for 24-48 hr. in brain-heart infusion (BHI) (Difco). *E. tarda* were inoculated into broth and incubated at 26ºC for 24 hr. Formalin was added to the culture at a final concentration of 2% (v/v) formalin (40%) to the culture. After 24 hr incubation at 26ºC, bacteria were washed three times with PBS and resuspended in PBS. The bacteria were resuspended in sterile saline and standardized for the optical densities of MacFerland No.2 contain approximately bacterial cells 1.5x 10^9 CFU/ml (**Badran, 1990).** A viability test of 1% of the bacterin was conducted on solid medium to determin viability of culture. Prepared bacterin tested for sterility and safety. Formalin killed vaccine preparation was frozen at −20 ^{0}C until use.

Challenge and Immunization: A total number 60 *O.niloticus* was divided into three treatment groups, each group with twenty fishes. First group of fish was non-vaccinated group; the second group was injected (i.p) with 0.2 ml of formalin killed *E. tarda* using tuberculin syringe. Fish in the control group were injected intraperitoneally (i.p) with 0.2 ml of PBS **(Badran, 1990).** The design of experimental for evaluation of immunization of *O. niloticus* against *E. tarda* was summarized in (table, 4).

Table (4). The design of experiment for evaluation of immunization of *O. niloticus* against *E. tarda*:-

Groups	Number of *O. niloticus*
Non Vaccinated	20
Vaccinated of *E. tarda* (Bacterin)	20
Control –ve (0.2 ml PBS)	20

All groups were challenged with 0.1 ml LD_{50} of *E. tarda* after 28 days of vaccination and recorded mortality for 7 days. The relative level of protection (RLP) was calculated using the following formula (**Amend, 1981**):

$$RLP = 1 - \frac{\text{\% mortality of vaccinated fish}}{\text{\% mortality of control}}$$

Histopathological examination: Specimens for histopathological techniques were freshly taken from infected organs and tissues of the experimentally infected *O. niloticus*. and were fixed in 10% neutral buffer formalin solution, dehydrated and embedded in paraffin wax, then sectioned to 4-5 micron thickness and were stained by hematoxylin and eosin for histopathological examination according to (**Roberts, 2001**).

Determination of heavy metals levels: The method for analysis of the heavy metals in the collected water sample from pond of naturally infected fish was carried out according to **APHA (1995).** The heavy metals level was carried out using Atomic Absorption Spectrophotometry (Model Thermo Electron Corporation, S. Series AA Spectrometer with Gravities furnace, UK,). The concentrations of heavy metals were expressed as mg/L for water.

Results

Results of clinical signs and postmortem lesions in naturally infected *O. niloticus* with *E. tarda* Fish showed lethargic, "hang" at the surface, and swim in a spiral or erratic pattern. Loss of scales from some areas of the skin, excessive mucus all over the body surfaces with petechial haemorrhages over the dorsal musculature. The disease can occur as small cutaneous lesions that progress into large abscesses within the muscle. These abscesses become filled with malodorous gas. In some cases, loss of pigmentation, abdominal destension, and different degree of corneal opacity (Figuer 1, A, B, C, D). While, the post mortem lesions of naturally infected *O. niloticus* showed generalized septicemia in most internal organs especially liver, intestinal tract and spleen, and severe distension of gall bladder with bile secretion, congested gills. Gills and liver showed small white nodules. Necrotic foci scattered all over the internal organs especially liver and gaseous formation in the gastrointestinal tract. Sever distension of gall bladder by bloody fluid were observed (Figure 2).

Figure (1). (A) shows petechial haemorrhages over the dorsal musculature; (Fig. B and C) depigmented cutaneous lesions that progress into large abscesses within the muscle and abdominal distension (Fig. D).

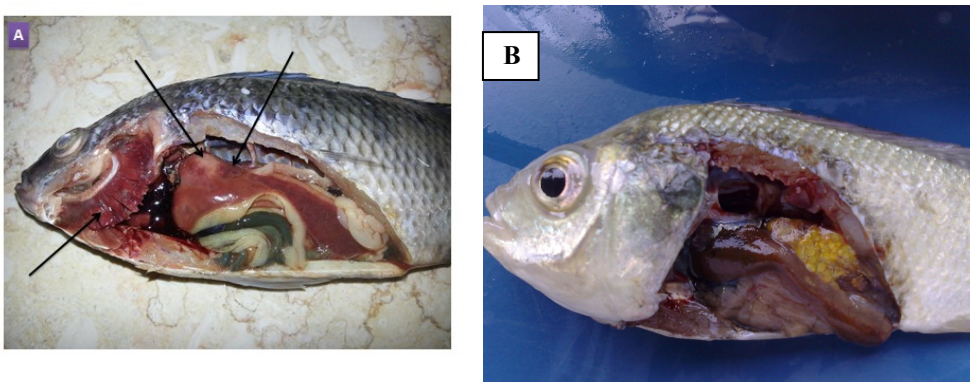

Figure (2). (A) congestion and necrotic foci scattered all over the liver, sever congestion in gills.
(B) Sever congestion in liver and kidney

Bacteriological examinations:-

Different morphological characteristics of different *Edwardsiella tarda*

Examination of 100 naturally infected *O. niloticus* reveled isolation of 5 isolates of *E. tarda* which characterized by: On TSA at 25°C for 24 –48 hrs, circular, smooth, moist, convex, glistening and 1-2 mm in diameter colonies which spread with the increase of the incubation time. The *E. tarda* were Gram negative motile short bacilli, positive for catalase, citrate and H_2S production, and negative for oxidase, indole and urease test. *E. tarda* ferment glucose, lactose and sucrose with acid production.

Molecular characterization:

Molecular characterization of *E. tarda* using specific primers are illustrated in Fig (3) which 5 bacterial isolates gave positive results with molecular weight 445bp

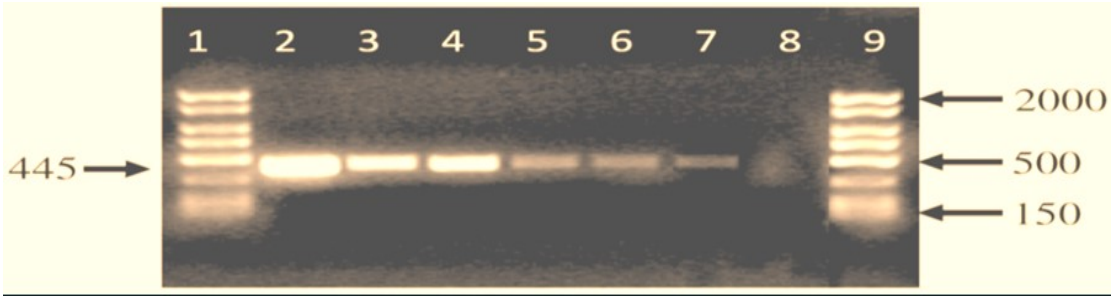

Figure (3). Results of agarose gel electrophoresis of PCR product of E.tarda, Lane (1 and 9): Molecular weight marker; Lane (2): is positive control for *E. tarda.*Lane (3, 4, 5, 6, and 7) *E. tarda* positive samples, Lane (8): negative control.

Antimicrobial sensitivity test

The antimicrobial susceptibility tests against *E. tarda* were illustrated in table (5). It was found sensitive to CIP; GN; UB; ENR; DO; OT; AML; E; and moderately sensitive to SXT.

Table (5). Results of The antimicrobial susceptibility tests against *E. tarda*:-

Antimicrobial disc	Indication of Sensitivity
CIP	++++
CN	+++
UB	+++
ENR	++++
DO	+++
SXT	++
OT	+++
AML	++++
E	++++

- = Resistance; + = weakly sensitive; ++ = moderately sensitive; +++ = quite sensitive; ++++ = highly sensitive.

Determination of LD_{50}:

The obtained results showed that the LD_{50} of *E. tarda* were $10^{-3.4}$ in case of *O. niloticus*.

Table (6). LD_{50} of *E. tarda* in *O. niloticus*

Bacterial dilution			ACCUMULATED VALUES			
				MORTALITY		
	DIED	SURVIED	DIED	SURVIVED	RATIO	RATIO (%)
10^{-1}	7	3	29	3	29/32	0.90
10^{-2}	6	4	22	7	22/29	0.76
10^{-3}	5	5	16	12	16/28	0.57
10^{-4}	4	6	11	18	11/29	0.38
10^{-5}	4	6	7	24	7/31	0.23
10^{-6}	3	7	3	31	3/34	0.08
Control	0	0	0	0	0	0

LD_{50} in *O.* niloticus: proportionate distance (P.D.) = 57-50 / 57-38 = 0.36=0.4
LD_{50} =- 3 + 0.4 = 3.4 = $10^{-3.4}$

Challenge and relative level of protection (RLP) of autogenous bacterin of *E. tarda* in *O.niloticus* revealed that RLP was 90% in case of bacterin; while the RLP% was 20% of non-vaccinated.

Table (7). The RLP% of *O. niloticus* against *E. tarda*:-

Groups	Number of Mortality of *O. niloticus*	RLP%
Non Vaccinated	16	20
Vaccinated with *E. tarda* bacterin	2	90
Control –ve (PBS)	0	0.0

Reisolation of *E. tarda* from dead fish was found positive.

Histopathology:-

The main histopathological changes in experimentally infected *O. niloticus* with virulent strain *E. tarda* were fatty degeneration of the hepatocytes and focal necrotic foci (Fig.4 A). Gills showed congestion of branchial blood vessels with separation of surface epithelium of secondary lamellae from the capillary beds (Fig. 4 B). The posterior kidney showed cloudy swelling of some renal tubules (Fig. 4 C). Spleen showed hyperactivation of Melanomacrophage centers (MMCs) and melanophores overladen with melanin pigment were noticed (Fig. 4 D).

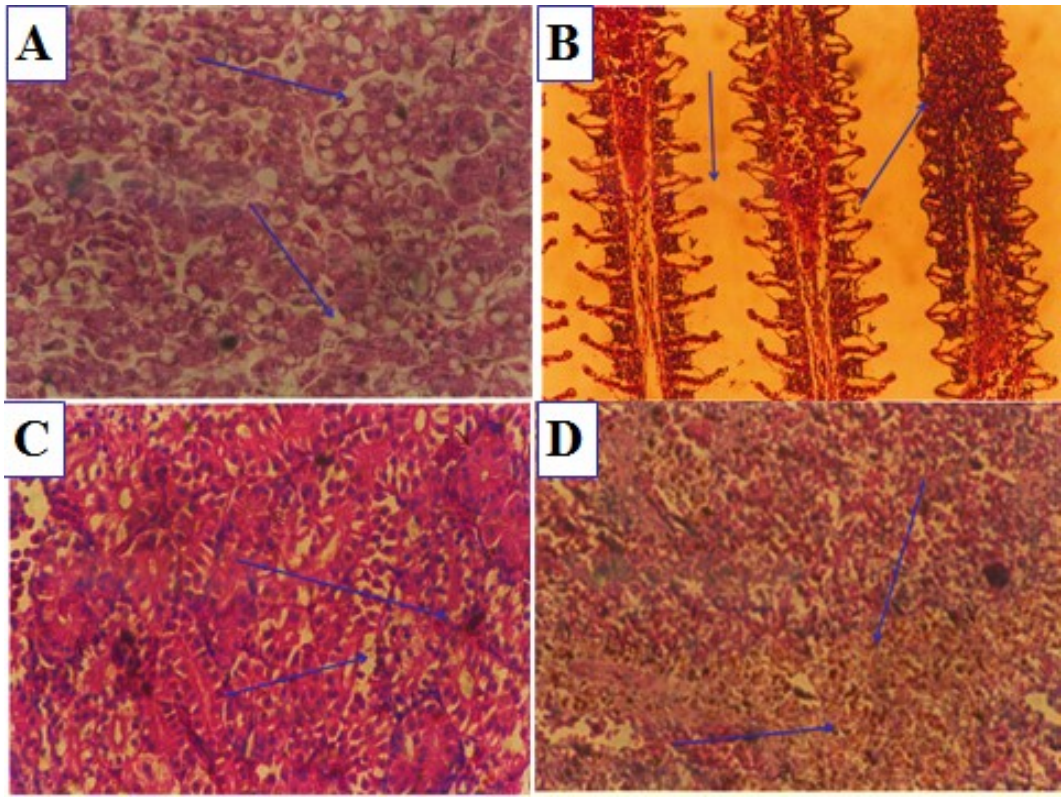

Fig. (4): A Liver of *O.niloticus* infected with *E. tarda* showing fatty degeneration of the hepatocytes and focal necrotic foci (arrows). H & E (X 400). (B) Gills of *O. niloticus* infected with *E. tarda* showing congestion of branchial blood vessels (arrow) with separation of surface epithelium of secondary lamellae from the capillary beds (arrow). H & E (X 400). (C) Posterior kidney of *O. niloticus* infected with *E. tarda* showing cloudy swelling of some renal tubules (arrows) H & E (X 400). (D) Spleen of *O. niloticus* infected with *E. tarda* showing hyperactivation of MMCs where the melanophores over laden with melanin pigment (arrows). H & E (X 400).

Heavy metal concentrations in water: The Cadmium (Cd); Copper (Cu); Zinc (Zn); Mercury (Hg) and Lead (Pb) levels were lower than the permissible limits in water sample (table 8), while the iron (Fe) levels were higher than the permissible limits in water sample.

Table (8). Results of heavy metal concentrations in water sample:-

Parameter (mg /L)	Result	Permissible limits*
Cd	0.001	0.005 mg /L
Cu	0.007	0.005- 0.01 mg /L
Fe	1.321	0.05- 0.5 mg /L
Mn	0.03	0.05 – 0.2 mg /L
Pb	0.002	0.003 mg /L
Zn	0.03	0.01-0.05mg /L

*According to **APHA (1995)**

Discussion

Edwardsiella tarda is mainly responsible for causing a significant disease edwardsiellosis or emphysematous putrefactive disease (EPD) that causes mass mortality in freshwater fishes (**Keskin *et al.*, 2004** and **Galal *et al*., 2005).**

The clinical signs were darkening of the skin, and hemorrhagic skin and fins. Superficial ulceration of the epidermis and musculature, exophthalmia, different degree of corneal opacity and ascites are commonly observed. Internally, the liver was dark red, inflamed with enlarged gall bladder, enlarged kidney, and congested and enlarged spleen. These septicemic lesions of *E. tarda* infected *O. niloticus* may be attributed to its virulence factors including extracellular products particularly haemolysin **(Mathew *et al*., 2001),** and to its adherence ability to the host surface by fimbriae which is controlled by the *E. tarda* specific type 1 fimbrial gene *(***Sakai *et al.*, 2007)**.These results nearly agree with that obtained by **Robert and Moeller (2012)** who reported that infections of catfish with *E. tarda* revealed small cutaneous lesions. With progression of the disease, abscesses develop in the muscle of the body and tail, these abscesses may enlarge and develop into gas-filled hollow areas. The ulcers seen in our study were also similar to those reported in *O. niloticus* (**El-Jakee *et al.* 2008)** and largemouth bass, *Micropterus salmonides*, infected with *E. tarda* **(Francis-Floyd *et al*., 1993).**The results were in accordance with **Han *et al*., 2006** who stated that infected fish with *E. tarda* showed hemorrhages all over the fish body, pale skin areas with detached scales, hemorrhagic protruded vent, and abdominal dropsy. Meanwhile, **Ullah and Arai (1983)** reported that, *E. tarda* produced two exotoxins which could be responsible for the lesions. It was noticed also that *E. tarda* infection spreads from lesions of visceral organs into the musculature and then to the skin, where large lesions develop in the musculature and dermis. These observations agreed with those reported by **Miwa and Mano, (2000)**.

Postmortem findings may be due to septicemia induced by two exotoxins that cause diseases and the most important one of them haemolysin secreted by *E. tarda* **(Mathew *et al*., 2001).** Liver and kidneys of the most examined fish showed congestion and this may be due to the nephric and hepatic virulence factors of *E. tarda (***Miwa and Mano, 2000** and **Mathew *et al*., 2001***)*.

Five isolates of *E. tarda* was isolated from the different external and internal lesions of the *O. niloticus* by conventional methods (morphological and biochemical characterizations) **(Fang *et al*., 2006)** and (**Acharya *et al*., 2007).** The presumptive identification of bacteria has been carried out from their colony morphology over ET plate, which acts as a selective medium for *E. tarda (***Acharya *et al.*, 2007)**. Biochemical characterizations have proved to be a valuable method for typing and differentiation of bacterial fish pathogen **(Austin *et al*., 1997).** In the present study, 30 different conventional biochemical tests have been carried out for proper identification and strain differentiation

of *E. tarda* isolates. Most of the phenotypic characteristic of the isolates were similar as claimed in Bergey s manual of determinative bacteriology

The above finding exhibit similarity with the findings obtained by **Wei and Musa (2008)** and **Kumari (2011).**

The PCR was used to confirm of *E. tarda* isolates and or results indicated that all biochemically identified *E. tarda* gave a positive result using PCR. This result indicated that PCR could confirm the biochemical identification of isolated *E. tarda*. Our results matched with those described by **Chen and Lai (1998)** who reported that PCR could be used for direct detection of *E. tarda* from infected fish and environmental water. Moreover, **Baird *et al.* (2003)** stated that PCR is one of the most important methods used for identification of *Edwardsiella.* We are predicting that the generated 445-bp PCR product described above will very likely be expressed by *E. tarda* at initial stages of diagnosis **(Sakai *et al.*, 2007)**.

The RLP in *O. niloticus* was higher in case of autogenous vaccinated fish than non-vaccinated fish. These results attributed to the action of bacterin on haemopiotic organs ***(*Galal *et al.*, 2005*)***. The RLP was 90% in vaccinated *O.niloticus,* these results agreed with that obtained in a study carried out by **(El-Jakee *et al.* 2008)** who stated that The RLP was 96% in *Clarias gariepinus* after vaccination with formalin killed cells of *E. tarda.* Vaccination with *E. tarda* formalin killed bacterin delayed mortality following experimental *E. tarda* infections **(Song *et al.*, 1982** and **Salati and Kusuda, 1985)** which vaccination with formalin killed bacterin was found to enhance phagocytic activity of Japanese eel leucocytes in vitro.

The histopahological changes due to *E. tarda* infection in the hepatocytes started with dilated blood vessels and ended by hydropic degeneration, while the haemopoietic elements of spleen, anterior and posterior kidney were necrotized and severe degeneration of the renal tubules with detachment of the epithelium from its basal membranes. The possible explanation of these results could be referred to the action of the powerful α- or B-haemolysin of Edwardsiella species and other toxins (**Aukilla and Saad, 2006).**

The results of heavy metal concentrations in water sample revealed that the Cadmium (Cd); Copper (Cu); Zinc (Zn);_mercury (Hg) and Lead (Pb) levels were lower than the permissible limits in water sample. On the other hand, the iron (Fe) levels were higher than the permissible limits in water sample during the present work. The pollution of the aquatic environment with heavy metals becomes a serious health concern during recent years. Lead, mercury and cadmium are the most dangerous metals causing serious health hazards in humans **(Abd El-Hady, 2007).** In regards to Iron (Fe) levels; iron was the most abundant metal in the water samples. These results agreed with that obtained in a study carried out by **(Türkmen *et al*., 2010)** who stated that the average levels of Iron in Gilthead Seabream and Seabass were higher in gill tissue and reported as 38.9 and 28.9 mg kg-1, respectively. The concentration of Iron in the water sample was higher than the permissible level recommended by the (**Egyptian Organization for Standardization, 1993)** which could be attributed to Iron liberation from Sediments as sulphides (**Abo El-Ella *et al.*, 2005**). Generally, Heavy metals are chemical stressors and the development of disease will reflect interactions between the host, the disease causing situation and stressors (**Austin and Austin, 1993**). This may be attributed to suppression of immune system and immune response which provide opportunities for entering of many pathogens. Concerning the relationship between Iron toxicity and the incidence of fish bacterial diseases; our results illustrated that in excess iron concentration in water than the permissible levels can accelerate of bacterial growth as *E. tarda* and this is considered as an excellent bacterial growth stimulant and this may be attributed to many factors as following; its properties as an excellent oxygen transporter, iron tends to stimulate the growth of common bacteria (**Kutsky, 1982**). Also, different pathogens adopt numerous strategies to overcome iron restriction (**Weinberg, 1995**) such as siderophores which are excreted, which chelate iron and return to bacteria via specially induced cell -surface protein receptors, after internalization, the siderophores give up their iron under the

influence of reductases (**Halle and Meyer 1992**).

In conclusion, *Edwardsiellosis* is one of the most important bacterial diseases among cultured Nile tilapia under Egyptian conditions. This study indicated that the PCR can be used successfully as an ideal method for identification and characterization of the *Edwardsiella* from infected fish. Autogenous bacterin can play an important role for control of the *E.tarda* infection.

References

Abd-El-Hady, B.A. (2007). Compare the Effect of Polluted and River Nile Irrigation Water on Contents of Heavy Metals of Some Soils and Plants. Research Journal of Agriculture and Biological Sciences, 3(4): 287 - 294.

Abdellatief, J.I.; Abu Leila, R.H. and Elias, N.S. (2017). Biological control and immunological effect of *Saccharomyces cerevisiae* on Edwardsiellosis infection in *Clarias gariepinus.* Animal Health Research Journal, 5(4), 418-427.

Abo El-Ella, S.M.; Hosny, M.M. and Bakry, M.F. (2005). Utilizing fish and aquatic weeds infestation as bioindicators for water pollution in Lake Nubia, Sudan. Egyptian Journal of Aquatic Biology and Fisheries, (9): 63 - 84.

Acharya, M.; Maiti, N.K.; Mohanty, S.; Mishra, P. and Samanta, M. (2007). Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system. Comp Immunol Microbiol. Infect Dis. 30(1): 33- 40.

Ainsworth, A.J. and Chen, D.X. (1990). Differences in the phagocytosis of four bacteria by channel catfish neutrophils; Dev. Comp. Immunol. 14, 201–209.

Amend, D.F. (1981). Potency testing of fish vaccines. International Symponsium on Fish Biologics: Serodiagnostic and Vaccines. Dev. Bio. Stand., 49: 447-454.

APHA (American Public Health Association) (1995). Standard Methods for the Examination of Water and Wastewater. 19th Eds., Washington DC.

Aukilla, M.A. and Saad, T.T. (2006). Relationship of enterobacteriaceae from microbiological point of view . Assuit 12th conference 273-286.

Austin, B. and Austin, D.A. (1993). Bacterial fish pathogens. Disease in farmed and wild fish. Second edition, Ellis Horwood Limited.

Austin, B. and Austin, A.D. (2012). Bacterial fish pathogens: diseases of farmed and wild-fish. 5th ed. Chichester, UK: Springer/Prazis Publishing.

Austin, B.; Alsina, M.; Austin, D.S.; Blanch, A.R.; Grimont, F. and Grimont, P.A.D. (1997)*:* A comparison of methods for the typing of fish pathogenic Vibrio spp. Syst Appl Microbiol. 20 (1): 89 101.

Badran, A.F. (1990). The role of adjuvants in the immune response of the fish. Zag. Vet. Med. J. 18: 126 – 136.

Badran, A.F. and Eissa, I.A.M. (1991). Studies on bacterial diseases among cultured freshwater fish (Oreochromis niloticus) in relation to the incidence of bacterial pathogens at Ismailia governorate. J. Egypt. Vet. Med. Ass., 51 (4): 837 – 847.

Baird, K.D.; Chikarmane, H.M.; Smolowitz, R. and Uhlinger, K.R. (2003). Detection of *Edwardsiella* infections in Opsanus tau by polymerase chain reaction. Biol. Bull. 205 (2): 235 – 6.

Castro, N.A.; E. Toranzo; S. Devesa; A. Gonzlez; S. NuÇez and B. Magariños (2012). First description of *Edwardsiella tarda* in Senegalese sole, Solea senegalensis (Kaup). Journal of Fish Diseases 2012, 35, 79 –82.

Castro, N.A.; E. Toranzo; A. Bastardo; J.L. Barja and B. Magariños (2011). Intraspecific genetic variability of *Edwardsiella tarda* strains from cultured turbot. Dis Aquat Org Vol. 95: 253–258.

Chen, J.D. and Lai, A.A. (1998). PCR for direct detection of *Edwardsiella tarda* from infected fish and environmental water by application of the hemolysin gene. Zoological Studies. 37 (3): 169 – 176.

Conroy, D.A. and Herman, L.R. (1981). Text book of fish diseases. T.F.H. publ., West Sylvania.

Darwish, A.; Plumb, J.A. and Newton, J.C. (2000). Histopathology and pathogenesis of experimental infection with *Edwardsiella tarda* in channel catfish; J. Aquatic Animal Health 12, 255 -266.

Egyptian Organization for Standardization (1993). Egyptian standard, maximum levels for heavy metal concentrations in food. ES 2360-1993, UDC: 546.19: 815, Egypt.

El-Jakee, J.K.1.; M.S. Marzouk; N.A. Mahmoud and M.A. El-Hady (2008). Trials to create Edwardsiellosis native vaccines for freshwater fish in Egypt. 8 th International Symposium on Tilapia in Aquaculture 2008.

Eurell, T.E.; Lewis, S.D.H. and Grumbles, E.C. (1978). Comparison of selected diagnostic tests for detection of motile Aeromonas septicaemia in fish. Am. J. Vet. Res., 39 (8): 1384-1386.

Fang, H.; Zhang, X.; Chen, C.; Jin, X. and Wang, X. (2006). Studies on the edwardsiellosis and characterization of pathogenic bacteria from diseased flounder (*Paralichthys olivaceus* L.) and turbot (*Scophthalmus maximus* L.); Acta Oceanol. Sin. 25 138–147.

FAO (1995). Water Quality for Agriculture. Irrigation and Drainage Paper No. 29, Rev. 1. Food and Agriculture Organization of the United Nations, Rome.

Francis-Floyd, R.; Reed, P.; Bolon, B.; Estes, J. and Mckinney, S. (1993). An epizootic of *Edwardsiella tarda* in largemouth bass (*Micropterus salmonides*). J Wild Dis. 29 (2): 334 – 6.

Galal, N.F.; S.G.T. Ismail; R.H. Khalil and M.K. Soliman, (2005). Studies on Edwardsiella infection in *Oreochromis niloticus*. Egyptian Journal of Aquatic Research, 31 (1): 460-467.

Grimont, P.A.D.; Grimont, F.; Richard, C. and Sakazaki, R. (1980). *Edwardsiella hoshinae*, a new species of Enterobacteriaceae; Curr. Microbiol. 4: 347–351.

Han, H.J.; Kim, D.H.; Lee, D.C. and Park, S.I. (2006). Pathogenicity of *Edwardsiella tarda* to olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). Journal of Fish Diseases, 29, 601–609.

Halle, F. and Meyer, J.M. (1992). Ferri siderophore reductases of Pseudomonas: purification, properties and cellular location of the *Pseudomonas aeruginosa* ferripyoverdine reductase. Eur. J. Biochem., (209): 613 – 620.

Hirono, I.; Tange, N. and Aoki, T. (1997). Iron-regulated haemolysin gene from *Edwardsiella tarda*. Mol Microbiol 24, 851-856.

Innes, W.T. (1966). Exotoxic aquarium fishes, 19th Aquarium Incorporated, Neogersi, USA.

Janda, J.M.; Abbott, S.L.; Kroske-Bystrom, S.; Cheung, W.K.; Powers, C.; Kokka, R.P. and Tamura, K. (1991). Pathogenic properties of *Edwardsiella* species; J. Clin. Microbiol. 29 1997–2001.

Keskin, O.; Secer, S.; Izgur, M.; Turkyilmaz, S. and Mkakosya, R.S. (2004). *Edwardsiella ictaluri* infection in rainbow trout (*Oncorhynchus mykiss*). Turk. J. Vet. Anim. Sci. 28(4): 649-653.

Kumar, G.; Rathore, G.; Sengupta, U.; Singh, V.; Kapoor, D., Lakra, W. S., (2007). Isolation and characterization of outer membrane proteins of *Edwardsiella tarda* and its application in immunoassays. Aquaculture. 2007, 272: 98-104.

Kumari, S. (2011). Strain Differentiation and Virulence Study of *Edwardsiella tarda*. M.Sc. Thesis. Sikha O Anusandhan University, Bhubaneswar.

Kutsky, R. (1982). Handbook of Vitamins, Minerals and Hormones, 2nd ed., Van Nostr and Reinhold.

Ling, S.H.; Wang, X.H.; Xie, L.; Lim, T.M. and Leung, K.Y. (2000). Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in in vivo and in vitro fish models. Microbiology 2000, 146: 7–19.

Mathew, J.A.; Tan, Y.P.; Srinivasa Rao, P.S.; Lim, T.M. and Leung, K.Y. (2001). *Edwardsiella tarda* mutants defective in siderophore production, motility, serum resistance and catalase activity. Microbiology, 147: 449–457.

Meyer, F.P. and Bullock, G.L. (1973). *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). Appl. Microbiol 25, 155-156.

Miwa, S. and Mano, N. (2000). Infection with *Edwardsiella tarda* causes hypertrophy of liver cells in the Japanese flounder Paralichthys olivaceus. Dis. Aquat. Organ. 42 (3): 227 – 31.

Mohanty, B.R. and Sahoo, P.K. (2007). Immune responses and expression profiles of some immune-related genes in

Indian major carp, *Labeo rohita* to *Edwardsiella tarda* infection. Fish Shellfish Immunol 2010, 28: 613-621.

National Committee for Clinical Laboratory Standered (NCCLS), (1994). performance standered for antimicrobial susciptability testing, fifth international supplement, Document M100-S5. National Committee for Clinical Laboratory Standered, Villanova. Pa.

Noga, E.J. (1996). Fish Disease: Diagnosis and Treatment. S.T. Louis (Ed.) Pp. 139-162. North Carolina State University, Mosby, Missouri.

Noga, E.J. (2000). Fish Disease; Diagnosis and Treatment. Iowa State University Press. 2121 South State Avenue, Ames, Iowa 50014. page number 154.

Oxoid Manual (1982). Oxoid manual 5th Ed. Published by Oxoid Limited Hampshire, England.

Pavanelli, G.C.; Eiras, J.C. and Takemoto, R.M. (1998). Doenòas de peixes: profilaxia, diagnosticos e tratamentos Eduem (Ed.) pp. 125-166. Nupleia, Maringa, Brazil.

Quinn, P.T.; Markey, B.K.; Carter, M.E.; Donnelly, W.J. and Leonard, F.C. (2002). Veterinary Microbiology and Microbial disease. First Published Blackwell Science Company, Lowa, State University Press.

Reed, L.J. and Muench, H. (1938). A simple method of estimating fifty percent end points. Am. J. Hyg. 27: 493-497.

Roberts, R.J. (2001). Fish Pathology 3rd ed., WB Saunders. pp. 305–307.

Robert, B. and Moeller, J.R. (2012). Bacterial Diseases of Fish, Cichlid- forum. com.

Saad, T.T.; El Banna, S.A.; Attalla, A.M.; and Fayza, A.I. El- Tedawy (2015). Studies on some Gram- negative bacterial diseases affecting cultured Mugil cephalus fish and its relation to its economic losses. Journal of Arabian Aquaculture Conference, 10 (2): 89-99.

Sakai, T.; Iida, T.; Osatomi, K. and Kanai, K. (2007). Detection of Type 1 Fimbrial Genes in Fish Pathogenic and Non-pathogenic *Edwardsiella tarda* Strains by PCR. Fish Pathology, 42: 115-117.

Salah, M.A. (2016). A Review of Fish Diseases in the Egyptian Aquaculture Sector. Working Report.

Salati, F. and R. Kusuda (1985). Vaccine preparation used for immunization of eel, *Anguilla japonica*, against *Edwardsiella tarda* infection. Bull. Jap. Soc. Scientific Fisheries, 51 (8): 1233-1237.

Sambrook, J.; Sakozoki, K.P. and Russell, D. (1989). Molecular Cloning: A Laboratory Manual, 3rd ed . Cold Spring Harbor Laboratory Press, New York.

Song, Y.L.; G.H. Kou and K.Y. Chen, (1982). Vaccination conditions for the eel, *Anguilla japonica*, with *Edwardsiella anguillimortifera* bacterins. J. Fisheries Soc. Taiwan, 4 (8): 8-25.

Türkmen, A.; Türkmen, M.; Tepe, Y. and Çekiç, M. (2010). Metals in tissues of fish from Yelkoma Lagoon, northeastern Mediterranean. Environmental Monitoring and Assessment, (168): 223 - 230.

Ullah, M.A. and Arai, T. (1983). Pathological activities of the naturally occurring strains of *Edwardsiella tarda*. Fish Pathol 18, 65-70.

Weinberg, E.D. (1995). Acquisition of iron and other nutrients in vivo. In: Roth JA, Bolin CA, Brogdon KA, Wannemuehler MJ, editors. Virulence mechanisms of bacterial pathogens. Washington: American Society for Microbiology, p. 79 - 94.

Wei, L.S. and Musa, N. (2008). Inhibition of *Edwardsiella tarda* and Other Fish Pathogens by *Allium sativum* L. (Alliaceae) Extract. American-Eurasian J. Agric. & Environ. Sci., 3 (5): 692-696.

Wyatt, L.E.; Nickelson, R. and Vanderzant, C. (1979). *Edwardsiella tarda* in freshwater catfish and their environment; Appl. Environ. Microbiol. 38 710–714.