Assessment of the susceptibility of polyculture reared African Catfish and Nile tilapia to *Edwardsiella tarda*

M. D. Ibrahem[1], Iman, B. Shaheed [2*], H. Abo El-Yazeed[3], and H. Korani[3]

[1]Department of Fish Diseases and Management. [2] Department of Pathology, [3] Department of Microbiology, Faculty of Vet. Med.,Cairo University. [*]imanshaheed@yahoo.com

Abstract: The study aimed to clarify the relative susceptibility of polyculture rearing African sharptooth catfish (*Clarias gariepnus*) and Nile tilapia (*Oreochromis niloticus*) , the two main reared species in Egypt to Edwardsiella tarda. Experimental infection of catfish and Nile tilapia with *E. tarda* was carried out after determination of the Mean lethal dose in African catfish (LC50). Infectivity pattern and pathology of *E. tarda* in catfish and Nile tilapia were tested via intra-peritoneal inoculation of 0.2ml of 10^4 CFU/ml of the bacteria at 25 °C. The mortality rates were 70% and 60% in catfish and Nile tilapia respectively. Congestion and hemorrhages in fish body were detected in both species. African catfish showed abdominal distention together with ulcers and excessive mucus in the skin. Internally; pinpoint white nodules in the liver was the main lesions observed. Histopathological examination of organs of both species revealed presence of myositis, and degenerative changes in liver and kidneys. Establishment of infection was confirmed with the laboratory diagnosis; culture characters; biochemical reactions; API -20E test kits in addition to molecular studies based on detection of the 1106-bp PCR product in tissue samples from experimentally infected fishes at 24 hr post experimental infection. In conclusion: *E. tarda* can express a potential role in polyculture fish farming. The African catfish exhibited severe pathological lesion and histopathological changes in comparison to Nile tilapia which show moderate to mild lesions. Direct probing for the presence of *E. tarda* in infected fish by PCR is reliable and helpful in diagnosis, anticipating and rapid interference.

[M. D. Ibrahem, Iman, B. Shaheed, H. Abo El-Yazeed, and H. Korani**. Assessment of the Susceptibility of polyculture reared African Catfish and Nile tilapia to *Edwardsiella tarda*].** Journal of American Science 2011;7(3):779-786]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: African sharptooth catfish, *Edwardsiella tarda*, experimental infection, histopathology, Nile tilapia, PCR

1. Introduction:

Intensification of fish culture has made the producers think wisely of developing suitable husbandry strategies, based on novel nutritional and management practices that enhance performance without significant increase in the cost of production (Lovell, 1989). The majority of fish farms in Egypt can be classified as semi-intensive water pond farms. Current developments in fish culture production are centered on the changes in the structure of the fish farming community (GAFRD, 2006). The polyculture fish farms are well established systems that proved both productive and economical efficiency. Nile tilapia (*Oreochromis niloticus*) and African Catfish (*Clarias gariepinus)* are two major species that are mixed reared in the aquaculture. Bacterial agents are among the highly encountered causes of diseases in stressed warm water aquaculture (Pavanelli etal., 1998. and Noga, 2000) *Edwardsiella tarda* infection is considered a dangerous septicaemic disease with high economic losses (Meyer and Bullock, 1973), the seriousness of *Edwardsiella tarda* infection is its expanding fish host range (Alcaide et al.,2006 and Mohanty and Sahoo, 2007). It infect catfish causing emphysematous putrefactive disease (EPD) [Darwish et al., 2000] Despite the fact that, *E. tarda* is a bacterium of fish, it infects humans posing a public health threats; causing gastroenteritis, meningitis, liver and skin abscesses and valvular endocarditis in patient with acquired immune deficiency syndrome (AIDS) (Mikamo et al., 2003 and Mizunoe et al., 2006). With respect to the most recent diagnostic methods for Edwardsiellosis, polymerase chain reaction (PCR) represents a widely-used alternative to traditional identification methods. Although pathogenic and virulent genes have been used as target regions; it is now accepted as a tool for identification purposes (Chen and Lai 1998). The Goal of this study was to determine the relative susceptibility of African catfish *(Clarias gariepinus)* and Nile tilapia *(Oreochromis niloticus)* two fish species that are commonly cultured together in Egyptian aquaculture to E. tarda . Furthermore we assessed pathological and histopathological features of *E.tarda*. Tentative characterization and confirmation of the *Edwardsiella tarda* isolate using molecular identification (PCR), conventional biochemical assays and the API -20 E system.

2. Materials and Methods:

Fish:

The fishes used for the experimental infectivity were obtained from semi-intensive farms, Egypt. Two hundred and twenty African catfish *(Clarias gariepinus)* with average body weight of 10 g ± 1.0 g and 120 Nile tilapia *(Oreochromis niloticus)* with average body weight of 20 g ± 1.0 g were used in the pathogenicity assays and the experimental infection. Ten fish were submitted for bacteriological examination to verify the absence of E. tarda. The fishes acclimatized in 16 separate glass aquaria (30x 40x 80cm) contain chlorine-free tap water for 14 days before the onset of the experiment according to Best et al., (2002). Temperature was thermostatically controlled to 24^0C ± 2^0C simulating the water temperature of the fish pond when the E. tarda strain was isolated. Aquaria were supplied with oxygen through electrical air pumps (Sicc- Alis and Pieters, Italy). Fish were fed to satiation twice daily (09:00 and 15:00) with 35% protein commercial fish pellets.

Bacterial isolate:

Edwardsiella tarda strain was isolated in the summer of 2008 from diseased African catfish farm in Fayoum governorate; Egypt. Confirmation was achieved using the conventional biochemical assays and the API 20 E system. Five typical colonies of the MacConky's agar cultured isolate were picked up; suspended in Mueller Hinton broth (Oxoid). The broth was incubated at 27°C for 24 hours until its turbidity exceeds that of the standard McFarland tube number 0.5. The turbidity was adjusted to match the McFarland tub number 0.5 (1×10^4 bacteria/ml) by adding sterile saline according to Sahoo et al., (2000).

Pathogenicity assays:

The pathogencity of *Edwardsiella tarda* was determined following the methodology described by **Nieto et al., (1984)**. Groups of 5 catfish (10±1 g body weight) were inoculated intraperitoneally with the bacterial isolate using 0.1 ml of serial bacterial dilutions containing 10^3-10^8 CFU. A control was provided using same number of fish inoculated with 0.1 ml PBS. Inoculated fish were observed daily for 7 days, all mortalities were recorded. Freshly dead fish were submitted for bacterial isolation and re-identification of E. tarda to verify specificity of mortality.

Experimental infection:

The experiment was carried on 3 replicate. Three groups of catfish; 40 fish/group; with a total of 120 catfish. Another 3 groups of Nile tilapia*; 40* fish/group; with a total of 120 were used. The assigned groups were subjected to intraperitoneal injection using a dose of 0.2 ml of 10^4 bacteria ml^{-1} at 25 ± 1 °C, after Darwish et al., (2000). A group of 40 fish/ group from each species were reared under the same experimental conditions, injected I/P with 0.1 ml PBS and served as controls.

Clinical and post mortem examination:

The experimentally infected catfish and Nile tilapia were grossly examined to determine any abnormalities. The fish were opened under aseptic condition; interior of the body was exposed and examined for changes according to the Noga, (2000) and Kimberley (2004).

Sampling:

Fish samples from the experimentally infected catfish and Nile tilapia*;* were collected from each group daily, starting on day one until day 7 post infection. Liver, spleen, kidney and muscle tissues were tested for bacteria using the method provided by Buller, (2004). Parts of the collected samples were collected in sterile cry- tubes and stored at -80 C for PCR test.

Isolation and Identification:

For bacterial re-isolation, all samples were streaked on MacConky's agar, and the selective medium, EIM (*Edwardsiella ictaluri medium*), according to Shotts and Waltman, (1990). The identification were performed by the biochemical tests and the API -20E rapid identification system test strips (Biomerieux 20 100 Marcy-l' Etiole, France) bacteriological diagnosis (Austin and Austin, 1999).

PCR detection of the hemolysin gene fragment in visceral organs of fish:

PCR was applied for detection of the hemolysin gene fragment in visceral organs (liver, spleen & kidneys) of experimentally infected catfish and Nile tilapia 24 hour post experimental infection. Chromosomal DNA was extracted from 100 µl of tissue samples (tissue sample suspended in 100 µl of sterile saline) using DNeasy tissue extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The PCR targeted the hemolysin gene using the method reported by **Chen and Lai,** (1998). The forward primers — hem – F (5'CCTTATAAATTACTCGCT3) and the reverse primers hem — R –5'TTTGTGGAGTAACAGTTT3 were used. Buffers and reagents used prepared according to (Paton and Paton, 1998) .

Histopathological examination:

Fresh tissue specimens from the liver, kidneys, spleen, skin and muscle were collected from morbid experimentally infected fishes; Specimens were fixed in 10 % neutral buffer formalin ,processed by

conventional method , embedded in paraffin, sectioned and stained with Hematoxyline and Eosin stain according to Bancroft and Gamble (2008).

3. Results:

The Pathogenicity assay

The mean lethal dose 50% (LC_{50}) of the *Edwardsiella tarda* in African sharptooth catfish was 10^4 CFU/ml.

Experimental infectivity of catfish and Nile tilapia *with E. tarda*:

Clinical abnormalities were clear in experimentally infected African catfish and Nile tilapia with *E. tarda*, from day one tell the end of 7 days post infection at 25 ± 1 °C. Regarding catfish infected groups; mortality rate was 70%. Grossly, there were petechial hemorrhage and cutaneous ulcers (Figs, 1 and 2); raised edematous areas with liquefaction of underlying tissues in the caudal peduncle and the site of injection. Congestion of the fins and petechial hemorrhages allover the body surface was also observed.

The experimental infection of Nile tilapia resulted in 60% mortality and range of abnormalities as sluggish movement and loss of escape and defense reflexes. Scale detachment and pale coloration (Fig, 3), severe edematous swelling at the site of injection, swollen abdomen with yellowish ascetic fluid, protruded hemorrhagic anus with opaqueness eyes were constant findings. Internally, both catfish and Nile tilapia showed severe hemorrhagic enteritis with adhesion between organs, the body cavity was filled with abundant yellowish mucoid fluid; in catfish; the liver showed a multiple tiny white foci (Fig, 4) while the intestine contained thick white opaque mucus.

Histopathological Examination.

The main lesion in liver of African catfish was congestion of central veins. The hepatocytes showed disorganization and disorientation of hepatic plates. Some hepatic cells showed hydropic degeneration in which the cells swollen with irregular vacuoles in the cytoplasm. Others showed fatty changes in which the cells showed circumscribed vacuoles in the cytoplasm (Fig 5). Some hepatic cells revealed signs of coagulative necrosis. There was increase in the melano-macrophages cells. The kidneys showed congestion of renal blood vessels with multi-focal areas of hemorrhages. The renal tubules showed different necrobiotic changes as cloudy swelling, hydropic degeneration and even necrosis (Fig 6).The spleen was studded with large numbers of erythrocytes. There were increase in numbers of melano-macrophages centers, depletion of lymphocytes and congestion of blood vessels (Fig 7).

The skin showed focal areas of necrosis in epidermal layer forming erosions or even the necrosis extended to the basement membrane leaving an ulcer (Fig 8). The dermis showed signs of dermatitis in which there were congested blood vessels with large areas of hemorrhages and inflammatory cells mainly macrophages and lymphocytes. Some areas of skin showed hyperplastic activity of epidermal cells including the mucus cells (Fig 9). The muscles showed Zenker's necrosis of muscle bundles in which the sarcoplasm stained deeply eosinophilic with losing of striation. There was inflammatory cells infiltration mainly macrophages and lymphocytes in--between the muscle bundles (Fig 10). The histopathological examination of Nile tilapia revealed hydropic degeneration in most of hepatocytes (Fig11). The kidneys revealed necrobiotic changes in the convoluted tubules. There were increase in melano-macrophages centers and depletions in lymphoid follicles of spleen.

Bacteria recovered from mortalities and euthanized fish of both species had phenotypic characteristic consistent with E. tarda. Genotypic confirmation of E. tarda was performed and amplifying an 1106 bp portion of hemolysin gene was detected (Fig 12).

4. Discussion:

Edwardsiella septicemia is an economically important bacterial disease affects mainly catfish, the disease incidence increases with the rise in water temperature as environmental stress (Palacois and Ghittino, 1987; Noga, 2000 and Roberts, 2001). As a result of the aggressive behavior of the catfish; injuries can be awaited from Catfish toward tilapia. Skin injuries are a potential route of pathogen entry and disease occurrence (Noga, 2000). In order to clarify the possible negative impact of the polyculture rearing of catfish and Nile tilapia, experimental infectivity of the two species *with E. tarda* isolated from African catfish using the intra-peritoneal route was carried out. The route of infection was in accordance to Amandi et al., (1982) and Eissa and Yassien, (1994) who recorded that the pathogenicity of *E. tarda* was demonstrated experimentally in salmon by I/P injection.

The LC50 of Edwardsiella was found to be 10^4 CFU/mL. Mekuchi et al., (1995) recorded following LC50 7.1X10, $1.7X10^2$, $3.6X10^6$ and $1.3X10^6$ CFU/ml by intramuscular injection, interpertioneal injection, immersion and oral administration respectively in Japanese flounder. The difference in calculated dose may by due to differences in types of fish and conditions of experiment.

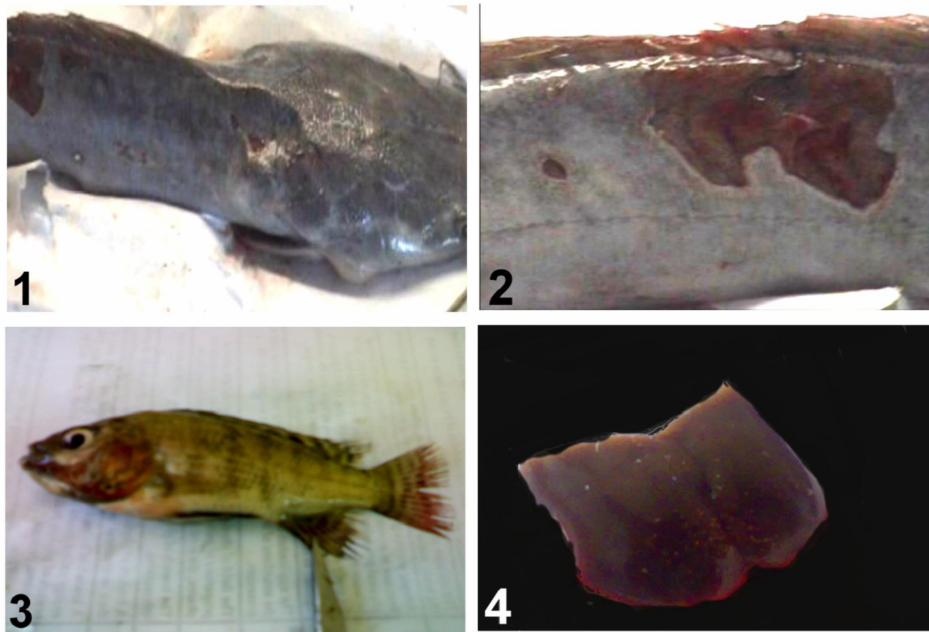

Fig 1: catfish experimentally infected by E tarda showing external skin hemorrhages and ulcers and fin rot
Fig 2: catfish experimentally infected by E tarda showing deep ulceration reached the dorsal muscles
Fig 3: Nile tilapia experimentally infected by E tarda showing signs of septicemia
Fig 4: liver of catfish showing a multiple tiny white foci and congestion

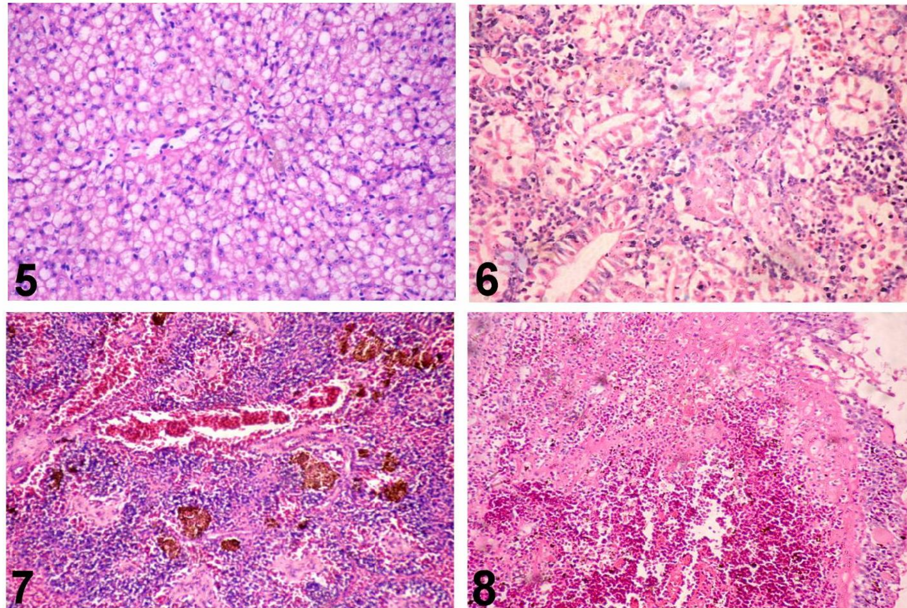

Fig 5: Liver of African catfish experimentally infected I/p by E. tarda showing fatty changes in most of hepatocytes. H&E X200.
Fig 6: Kidney of African catfish experimentally infected I/p by E. tarda showing necrobiotic changes in renal tubules. H&E X400.
Fig 7: Spleen of African catfish experimentally infected I/p by E. tarda showing congestion of blood vessels and increase in melano-macrophage center. H&E X200.
Fig 8: Skin of African catfish experimentally infected I/p by E. tarda showing necrosis of epidermal layer with hemorrhage and congestion of dermal layer. H&E X200.

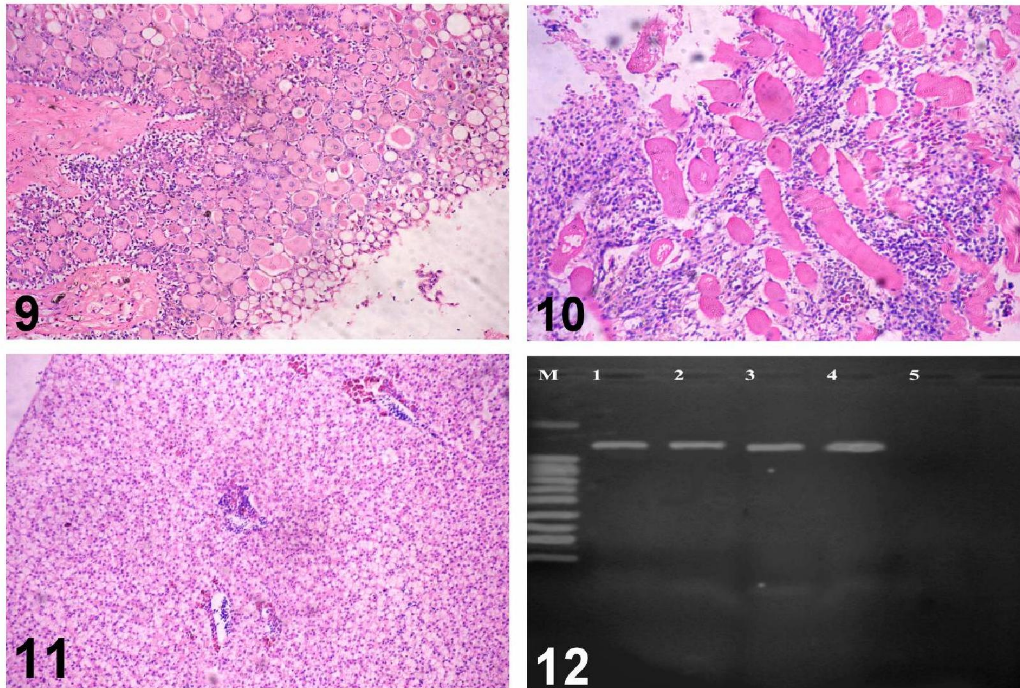

Fig 9: Skin of African catfish experimentally infected I/p by E. tarda showing hyperplasia of epidermal layer especially mucus secreting cells with increase of its activity. H&E X200.

Fig 10: Muscle of African catfish experimentally infected I/p by E. tarda of showing severe infiltration of mononuclear cells in-between the muscle bundles with necrosis of some bundles. H&E X200.

Fig 11: Liver of Nile tilapia experimentally infected I/P by E. tarda showing hydropic degeneration in most of hepatocytes. H&E X100.

Fig 12: Agarose gel electrophoresis: lane 1: positive Control (DNA from ATCC E. tarda); lane 2: liver sample; lane 3: spleen sample; lane 4: kidney sample showing characteristic 1106-bp PCR product; lane 5: control negative. Marker: 100 increment DNA ladder.

There is no available literature dealing with *E. tarda* in African cat fish but the clinical signs and gross lesion observed in the experimentally infected African catfish were in agreement with Meyer and Bullock (1973) who reported that with mild infections of channel catfish,the external signs of disease were 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 experiments were also similar to those reported in largemouth bass ,*micropterus salmoides*, infected with *E. tarda* (Francis-Flody et al., 1993). Soliman et al., (1991) reported that Catfish injected with *E. tarda* showed hemorrhagic raised edematous areas with liquefaction of underlying tissues, congestion of the fins and petechial hemorrhages allover the body surface. Much evidence from the present study, *E. tarda* is a fish pathogen that causes systemic infections in fish. The results were in accordance with Stoskopf, (1993) and Ling et al., (2000) who stated that infected fish showed hemorrhages allover the fish body, pale skin areas with detached scales, hemorrhagic protruded vent, and abdominal dropsy.

The experimental infection of Nile tilapia resulted in 60% mortalities, sluggish movement and loss of escape and defense reflexes and scale detachment, severe edematous swelling at the site of injection, the presence of swollen abdomen filled with yellowish ascetic fluid, protruded hemorrhaged anus and opaqueness to the eyes the findings are much likely reported by Kubota et al.,(1981). Internally, both Catfish and Nile tilapia, showed severe hemorrhagic enteritis with adhesion between organs, the body cavity was filled with abundant yellowish or sanguineous mucoid fluid; in catfish; the liver showed multiple tiny white foci; while the intestine contained thick white opaque mucus. The findings were in agreement with Baya et al., (1997)

and Noga, (2000). The pathogenicity mechanisms were investigated by Ullah and Aria (1983a, b) and Suprapto, et al., (1995), they reported that *E. tarda* secrete hemolysins and dermatotoxins, (exoenzymes) which may confer pathogenicity on *E. tarda* as it produce both an exotoxine extracellular products (ECP) and endotoxin intracellular components (ICC) that are lethal to eels and flounders and play an important role in the pathogenicity of this organism. Hemolysin, which is identified as an endotoxin because it is not secreted outside of the cell (Watson and White 1979, Ullah and Arai 1983a, b; Janda et al.,. 1991 and Janda and Abbott 1993 a, b).

Recently, Singh and Singh (1997) revealed that all the pathogenic *E. tarda* had either type 1 fimbriae or colonization factor. On iron deficient medium *E. tarda* produced siderophore, which permit the pathogen to scavenge for iron in the blood of the host (Park et al., 1983; Kokubo et al., 1990 and Ouyang et al., 1998). Certainly the ability of *E. tarda* to acquire iron or produce toxin is an important part of the infection process. The clinical and P.M lesions may be attributed to the action of bacterial toxins. This explanation supported by the statement of Braude (1964) and Nowotny (1979). They reported that the nature of pathogenesis caused by all gram-negative bacteria was almost similar and the disease process was caused by endotoxins and exotoxins. Meanwhile, Ullah and Arai (1983 b) reported that, E. tarda did not produce endotoxins as other Gram-negative bacteria did, but it produced two extoxins 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 (Eissa and Yassien (1994), Baya et al., (1997) and Miwa and Mana, (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 microorganism (Hirono et al., 1997 and 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 Mana, 2000 and Mathew et al., 2001).

The re-isolation, phenotypic and biochemical characterization of E. tarda from fishes were similar to those reported by other investigators (Ling et al., 2001 and Roberts, 2001 and Quinn et al., 2002).

The 1106-bp PCR product was detected in tissue samples obtained from the experimentally infected catfish and Nile tilapia (liver, spleen and kidneys) .The PCR gave positive results as early as 24 h post infection, by which the same samples gave negative for the bacteriological detection of the bacteria from experimentally infected catfish and Nile tilapia. This result suggests that live bacteria residing in the visceral organs of infected fish can be rapidly and easily detected by using the oligomers of the hemolysin gene as primers for PCR assay. Direct PCR detection of the hemolysin gene of live bacteria is potentially simple, accurate, rapid and helpful in anticipating and preventing epidemics which otherwise occur so frequently on fish farms.

The investigation of tissues alterations different predilection body organs and tissues could be an important aid for diagnosis of infectious diseases.

regarding the histopathological studies, the lesions in internal organs observed in the present work could be attributed to potential virulence factors of E. tarda such as siderophores, cell adhesion factors and cell invasion activity and two types of hemolysin, dermotoxin, cytotoxin , entertoxin which induced the necrosis and the degenerative changes in most organs especially liver , kidney , skin and muscle (Chen et al., 1996 and Mathew et al., 2001). Also the capability of a pathogen to infect its host is partly due to its ability to detoxify various toxic forms of oxygen (Free radicals) present in the host body by producing enzymes such as catalase, peroxidase, and superoxide dismutase (Yamada and Wakabayashi 1999).Lymphoid depletion observed in our study may be due to extensive apoptosis in lymphocytes induced by E. tarda. Also it may be due to systemic immunosuppression result in this lesion (Pirarat et al., 2007).

The histological alterations in infected Nile tilapia in this study consistent with those reported by Areechan and Plump (1983) and Soliman et al., (1991).

5. Conclusion:

Based on the present study, *E. tarda* may evoke serious ecological and economical problems. Polyculture rearing of catfish and Nile tilapia may lead to disease outbreaks. The experimental infection cleared that E, tarda in African Catfish induce clinical signs and pathology were more or less the same as others species of catfish. Oreochromis niloticus can get infected by *E. tarda* isolated from catfish and expressed clear septicemia and mortalities. The African catfish exhibited severe pathological lesion and histopathological changes in comparison to Nile tilapia which show moderate to mild lesions. Early detection of *E. tarda* using PCR technique is recommended in fish farms in the warm areas and/or under stress; as early diagnosis of the disease before the onset of the clinical picture evokes a better prognosis and allows the rapid interference and treatment.

Corresponding author
Prof.Dr. Iman, B. Shaheed,
Department of Pathology, Faulty of Veterinary Medicine, Cairo University, Egypt.
imanshaheed@yahoo.com

6. References:

1. Alcaide, E., Herraiz S., and Esteve, C. (2006): Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain. *Disease of Aquatic Org*anism 173,77-81
2. Amandi, A., Hiu, S. F., Rohovec J. S. and Fryer J. L. (1982): Isolation and characterization of *Edwardsiella tarda* from fall Chinook salmon (*Oncorhynchus tshawytscha). Applied Environmental .Microbiology* 43, 1380 -1384
3. Areechon, N. and Plumb, J.A. (1983): Pathogenesis of *Edwardsiella icteluri* in Channel cat fish *(Ictalurus punctatus).Journal of the World Maricultural Society* 14, 249 - 260.
4. Austin, B. and Austin , D. A. (1999) : Bacterial fish pathogens. Diseases in farmed and wild fish. 3rd Ed. Ellis Harwood Limited. New York, London.
5. Bancroft, J.D and Gamble, M. (2008): Theory and Practice of Histological Techniques, 6th ed. Elsevier Health Sciences. China.
6. Braude , A.(1964) : bacterial endotoxins. Sci. Amer., 210, 36.
7. Baya, A. M., Romalde, J.L., green, D. E., Navarro, R.B., Evans, J., May, E. B., and Toranzo, A. E. (1997): Edwardsiellosis in wild striped bass from the Chesapeake Bay. *Journal Of Wildlife Disease*, 33, 517-525.
8. Best, J.H., Pflugmacher, S.,.Wiegand, C., Eddy, F.B., Metcalf, J.S., Codd, G.A. (2002): Effects of enteric bacterial and cyanobacterial lipopolysaccharides, and of microcystin–LR, on glutathione S-transferase activities in zebra fish (Danio rerio). *Aquatic Toxicology* 60, 223–231.
9. Buller, B.N. (2004): Bacteria from fish and other aquatic animals: A practical identification manual Page 83-116/CABI publication Oxford UK.
10. Chen, J. D., Lai, S. Y. and Huang, S. L. (1996): Molecular cloning, characterization, and sequencing of the hemolysin gene from Edwardsiella tarda. *Archives Of Microbiology* 165,9-17.
11. Chen, J. D.and Lai, S. Y. (1998): PCR for direct detection of Edwardsiella tarda from infected fish and environmental water by application of the hemolysin gene. *Zoological Science.* 37,169-176.
12. 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
13. Eissa, I. A. M. and Yassien, M.A. (1994) : Some studies on EPD among catfish Clarias lazera in Lake manzala ISSN110-2047 Alex. *Journal of veterinary Science* 10, 41-48.
14. Francis-Floyd, R., Reed P., Bolon B., Estes J. and Mckinney S. (1993): An epizootic of *Edwardsiella tarda* in large mouth bass (*Micropterus salmoides*). *Journal of Wildlife Diseases* 29, 334-336.
15. GAFRD, (2006): General Authority for Fish Resources Development. Fishery Annual Report, Cairo. Egypt.
16. Hirono, I., Tange, N. and Aoki, T.(1997) : Iron-regulated hemolysin gene from *Edwardsiella tarda* . *Molecular .Microbiology* 24, 851 –856
17. Janda, J.M. and Abbott, S. L. (1993a): Expression of an iron-regulated hemolysin by *Edwardsiella tarda* . *FEMS Microbiology Letters* 111, 275 .280
18. Janda, J.M. and Abbott, S.L. (1993b) : Infections associated with the genus *Edwardsiella* :the role of *Edwardsiella tarda* in human disease. *Clinical Infectious Diseases* 17, 742 .748
19. 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*ournal Of Clinical Microbiology* 29 1997 .2001
20. Kimberley, A. W. (2004): Finfish and shellfish Bacteriology manual techniques and procedures. 15-28 / Blackwell publishing company USA.
21. Kokubo, T., Iida, T. and Wakabayashi, H. (1990): Production of siderophore by *Edwardsiella tarda*. *Fish Pathology* 17, 243 -256
22. Kubota, S.S., kaige, N., Miyazaki, T. and Miyashita, T. (1981): Histopathological studies on edwardsiellosis of tilapia. Bulletin of the Japanese Society of Scientific Fisheries 9,155-165.
23. Ling, S. H. M., Wang, X. H. , Xie, L. ,Lim, T. M and Leung, K. Y. (2000) : Use of green fluorescent protein (GFP) to track the invasive pathways of *Edwardsiella tarda* in the *in vivo* and *in vitro* fish models. *Microbiology* 146, 7-19
24. Ling, S. H. M., Wang X. H., Lim T. M.& Leung K. Y. (2001): Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. *FEMS Microbiology Letters* 194, 239-243
25. Lovell R.T. (1989): Nutrition and feeding of fish. Auburn University Van.Nostrand Reinhold, New York.
26. 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.
27. Mekuchi, T., Kiyokawa, T., Honda, K., Nakai, T., and Muroga, K. (1995): Vaccination trials in the Japanese flounder against edwardsiellosis. Fish Pathology 30: 251-256.
28. Meyer, F. P. and Bullock, G. L. (1973) : *Edwardsiella tarda, a* new pathogen of channel catfish (*Ictalurus punctatus*).*Applied Microbiology* 25, 155-156

29. Mikamo, H., Ninomiya, M., Sawamura ,H. and Tamaya, T. (2003): Puerperal intrauterine infection caused by *Edwardsiella tarda*. *Journal of Infectious Chemotherapy* 9, 341 .343.
30. Miwa, S. and Mana, N., (2000): Infection with Edwardsiella tarda causes hypertrophy of liver cell in the Japanese flounder Paralicthys Olivaceus. Dis. Aquat. Organ. 28, 42(3): 227-231.
31. Mizunoe, S., Yamasaki, T., Tokimatsu, I. , Matsunaga, N. , Kushima, H. , Hashinaga, K. and Kadota, J. (2006): A case of empyema caused by *Edwardsiella tarda .Journal of Infection* 53, 255-258
32. Mohanty, B .R and Sahoo, P. K. (2007): Edwardsiellosis in fish: a brief review. Journal of Bioscience 32, 7:1-14.
33. Nieto, T.P., Toranzo, A.E. and Barja, J.L. (1984): Comparison between the bacterial microflora associated with fingerlings of rainbow trout cultured in two different hatcheries in North west of Spain. Aquaculture 42, 193-206.
34. Noga. E. J. (2000): *Fish disease diagnosis and treatment* Iowa: Iowa State University Press.
35. Nowotny, A. 1979: Basic exercises in immunochemistry, 2nd ed. Springer Verlag, New York.
36. Ouyang, Z., Chen, H. and Lu, C.P. (1998): Pathogenic properties of Edwardsiella tarda. Acta Hydrobiolgica Sinica 22 , 11-15
37. Palacois, F.M. and Ghittino, P. (1987): Primer case de edwardsiellosis enla anguillicultura italiana. Riv. Ital. Pisci. Lttiopatiol 22, 105-110.
38. Park, S., Wakabayashi, H. and Watanabe, Y. (1983): Serotype and virulence of *Edwardsiella tarda* isolated from eel and their environment. *Fish Pathology*18, 85-89.
39. Paton , A. W. and Paton, C.J. (1998) : Detection and characterization of shiga toxigenic Escherichia coli by using Multiplex PCR assays for stx1, stx2, eaeA, Enterohemorrhagic hlyA, rfb O111 and rfbO157.Journal of Clinical Microbiology 36 ,598-602.
40. Pavanelli, G. C., Eiras, J. C. and Takemoto, R. M. (1998): Doen as de peixes: profilaxia, diagnosticos e tratamentos Eduem (Ed.) pp. 125-166. Nupleia, Maringa, Brazil.
41. Pirarat, N., Maita M., Endo, M. and Katagiri, T. (2007): Lymphoid apoptosis in Edwardsiella tarda septicemia in tilapia *Oreochromis niloticus*. Fish and Shellfish Immunology 22, 608 –616
42. Quinn, P. J., Markey B. K., Carter M. E., Donnelly W. J. and Leonard F. C. (2002): Veterinary Microbiology and Microbial Disease. First Published Blackwell Science Company, Iowa, State University Press.
43. Roberts, R. J. (2001): Fish Pathology, 3rd ed. Bailliere Tindal, London, England.
44. Sahoo, P.K, Swain, S.K. S., Mukherjee, S.C. and Sahu, A.K. (2000): Pathology caused by the bacterium E.tarda in Anabas testudineus. *Asian Fisheries science* 13,357-362.
45. Shotts, E.B. and Waltman, W.D. (1990): A medium for the selective isolation of *Edwardsiella ictaluri*. *Journal of Wildlife Diseases* 26, 214-218.
46. Singh, B.R. and Singh, K.P. (1997): Virulence factors of *Edwardsiella tarda* isolated from fish. *Journal of Food Science andTechnology* 34, 450-453.
47. Soliman, M.K. and Kitao. T., branson, E. and yoshida, T. (1991): Pathogenesis of *Edwardsiella ictaluri* in African labyr in the cat fish (*Clarias lezera*). *Alex. Journal of Veterinary Science* 6&7,143-153.
48. Suprapto, H., Nakai, T.and Muroga, K. (1995): Toxicity of extracellular products and intracellular components of *Edwardsiella tarda* in the Japanese eel and flounder. *Journal of Aquatic Animal Health* 7, 292-297.
49. Stoskopf, K. M. (1993): Fish medicine. W. B. Saunders Company, Harcoort Bruce Javanrich. Incist Ed.
50. Ullah, M. A. and Arai, T. (1983 a): Pathological activities of the naturally occurring strains of *Edwardsiella tarda*. *Fish Pathology* 18, 65-70
51. Ullah, M. A. and Arai, T. (1983 b): Exotoxic substances produced by *Edwardsiella tarda*. *Fish Patholology* 18, 71 - 75
52. Watson, J. and White, F. H. (1979): Hemolysins of *Edwardsiella tarda*. *Canadian Journal of Comparative Medicine* 43, 78–83.
53. Yamada, Y. and Wakabayashi, H. (1999): Identification of fish pathogenic strains belonging to genus Edwardsiella by sequence analysis of sod B. *Fish Pathology* 34 , 145 –150.

3/3/2011