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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF EDWARDSIELLA TARDA BACTERIA CAUSING SEVERE MORTALITIES IN CULTURED OREOCHROMIS NILOTICUS FISH WITH TREATMENT TRIALS

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ABSTRACT

The present study was carried out to isolate *Edwardsiella tarda* (E. tarda) from cultured *Oreochromis niloticus* (*O. niloticus*) and that identified by both Biochemical tests (API 20 E) and PCR. A total of 2 E. tarda isolates were isolated from 50 cultured *O. niloticus* fish collected randomly from the ponds of private fish farms at Kafr El Sheikh Governorate, Egypt. The clinical picture of the collected fish exhibited loss of escape reflex; skin darkness; bilateral exophthalmia with corneal opacity and ulcers varied in their degrees, inflammation, congestion, hemorrhage and enlargement of most internal organs were apparent in postmortem examination. The isolated *E. tarda* was screened for presence of 3 virulence genes (esrB, gyrB and gadB genes) using multiplex PCR and the results showed the amplification of the concerned gene at molecular size esrB (311 bp), gadB (583) in one strain and gyrB (415) in the other strain. Histopathological examination for haemobiotic organs liver, spleen and kidney as well as gills revealed necrosis of most internal organs, inflammatory reaction, associated with hemosiderosis. In vitro antibiotic sensitivity pattern of E. tarda isolates was conducted by disc diffusion method for five antibiotic discs where, the isolate was found to be sensitive against florfenicol, Ciprofloxacine and sulphadimethexine and resistant to oxytetracycline and ampicillin. Isolated E. tarda was used for experimental infection of healthy fish; typical symptoms in naturally infected fish appear in experimentally infected fish. Treatment trials for experimental infected fish reveal effectiveness of florfenicol, Ciprofloxacine and sulphadimethexine.

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INTRODUCTION

Egypt occupied the 8th position in the world with 1350535 tons/year (1.54%) of aquaculture world. It imports 316165 tons of fish while, export about 6110 tons (FAO, 2014). In Egypt, aquaculture is concentrated on inland farms, with the main species culture being tilapia and in semi-intensive and poly culture fish farms which well-established systems that proved both productive and economic efficiency (Ibrahem *et al.,* 2011). Bacterial agents are considered highly encountered causes of diseases in environment stressed on cultured fish in warm water (Abd El-Kader, 2015; Katharios Pantelis, 2015). *Edwardsiella tarda* is bacterial pathogen infecting different ages of some fish species in warm freshwater (Bin *et al.,* 2015).

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Itis a Gram negative, motile bacterium oftheEnterobacteriacae family which can be intracellular during infection (Ling *et al.,* 2001; Okuda *et al.,* 2014), making it less sensitive to antibiotic treatment (Xu and Zhang, 2014). Its importance as a fish pathogen has been gaining increasing interest lately as it is associated with heavy losses in freshwater cultured fish (Taguchi *et al.,* 2014; Soto *et al.,* 2012). Also, it is considered a dangerous bacteria and causes septicemic disease with high economic losses in infected fish (Choresca *et al.,* 2011)), the seriousness of *Edwardsiella tarda* infection is expanding in various fish species (Alcaide *et al.,* 2006; Mohanty and Sahoo, 2007). It infects catfish causing small cutaneous lesions on the anterio-lateral part of the body which developed to cause emphysematous putrefactive disease (Darwish *et al.,* 2000). *Edwardsiella tarda* is one of the bacterial fish pathogens which is widely distributed in aquatic organisms in nature and convert live of fish into jeopardy with consequent negative impact on growth, fecundity and productivity (Eissa, and Yassien, 1994).

PCR is considered the rapid and confirm method for diagnosis of *Edwardsiella tarda* in Nile tilapia and by DNA extraction and amplified using GoTaq® Hot Start Green Master Mix and oligonucleotide primer target (Iregui *et al.,* 2012; El Seedy *et al.,* 2015)**.** The histopathological examination of Nile tilapia revealed hydropic degeneration in most of hepatocytes. The kidneys revealed necrobiotic changes in the convoluted tubules. There were increase in melano-macrophages centers and depletions in lymphoid follicles of spleen (Ibrahem *et al.,* 2011)**.** While, in red sea bream were suppurative interstitial nephritis,hepatitis and purulent inflammatory changes in spleen (Park *et al.,* 2012) and severe hemorrhage occurred in almost organs in motile eel in China (Mo *et al.,* 2015). *Edwardsiella tarda* has acquired resistance to almost antimicrobial agents (Xu and Zhang, 2014). This isolate, when causing disease, may be difficult to control. This strain was resistance to tetracyclines (McPhearson *et al.,* 1991). On the opposite side, it was highly susceptible to florfenicol (McGinnis *et al.,* 2003)**.** In contrast, (Ho *et al.,* 2000), isolated *E. tarda*, from aquatic animals in Taiwan was less susceptible to florfenicol. Thus the present study was aimed to investigate pathogenesis, diagnosis and histopatholgical alterations of naturally infected*O. niloticus* with *Edwardsiella tarda* in private fish farms in Kafer El-sheikh governorate with trials for control and treatment of naturally infected fish using florfenicol, Ciprofloxacine and sulphadimethexine.

MATERIALS AND METHODS

Fish: A total of 50 random cultured Oreochromis niloticus fish with average body weight (150±15g) showed apparent clinical signs of disease were collected randomly alive from private fish farms, from Kafr El- Sheikh Governorate were transported in battery aerated tanks to the wet lab. At the Animal Health Research Institute, Kafr El-Sheikh branch, Egypt for examination.

Examination

1. Clinical and post mortem examination: Naturally infected *Oreochromis niloticus* were carefully examined in ponds in the fish farm, swimming, feeding and any abnormal sings on the body. Also, any post mortem lesions were examined and recorded according to (Austin and Austin, 1999)**.**

2. Bacteriological Examination

A. Isolation: Incomplete aseptic conditions bacteriological isolation was carried out from poolded samples from spleen, liver, kidney and skin lesions of infected fish and inoculated into tryptic soya broth at 30 ^{o}C for 24hrs then cultured into *E. tarda* agar media and incubated at 30 ^{o}C for 24-48 hrs according to method described by (Muratori, 2001)**.**

B. Identification: Smears of suspected bacterial colonies of cultured samples were prepared, stained with gram stain and examined microscopically then bio-chemical testes to the suspected purified isolates were carried according to (Austin, and Austin, 1999) by the API -20E rapid identification system test strips (Biomerieux 20 100 Marcy-l' Etiole, France) for bacteriological diagnosis. Furthermore, multiplex PCR technique were applied for detection of 2 types of virulence genes (esrB, gyrB and gadB genes) in fish pathogenic *E. tarda* isolates according to (Wang *et al.,* 2012)**.**

Antibiogram for treatment *E. tarda:* Antibiogram (sensitivity test) was performed for treatment *E. tarda* using many types of antibiotics for detection of largest inhibition zone was taken to be of choice for treatment of *E. tarda*. Antibiotic sensitivity testing: was applied according to guide lines stipulated by the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS, 2002) using Muller Hinton agar. Bacterial isolates were tested for their susceptibility to 5 different antimicrobial discs included CIP: Ciprofloxacin (5µg), SXT: sulphadimethexine (25µg), AM:Ampicillin (20µg),T: Oxytetracycline (30µg), Florafenicol (30µg) (Xian-jieLiua, 2015).

Experimental infection: 60 apparently healthy O. niloticus fish110 - 140 gm body weight were divided into 4 groups, each group 15 fish in glass aquarium supplied with de-chlorinated water and source of aeration. Injection of each fish intra-peritoneal with 0.3 ml of 10^8 CFU/ml *E. tarda* suspensions previously prepared according to (28). Recording for clinical signs and changes and re-isolation of *E. tarda* from these fish was carried. Typical symptoms were appearing on experimentally infected fish.

Treatment trial: Usage of the high sensitive antibiotics ciprofloxacin, florafenicol and sulphadimethexine according to the antibiogram for treatment of experimentally infected fish for 5 days each antibiotic was mixed well to fish ration in concentration of 3gm antibiotic/kg ration and feeding was 3% from fish weight in divided amount per day.

Polymerase Chain Reaction (PCR)

1. Primer sequences of *E. tarda* used for PCR identification system: Application of PCR for esrB (TTSS regulator), gyrB (gyrase B) and gadB (glutamate decarboxylase) genes as virulent factors of E. tarda was performed essentially by using Primers (Pharmacia Biotech) as shown in the following table:

2. DNA Extraction using QIA amp kit (29)**:** Genomic DNA was extracted from every isolate of *E. tarda* using DNA extraction kit (QIAamp). Isolated DNA samples were checked for purity and quantified in ND-1000. The samples were then resolved on agarose gel (0.8%) with 4 µl of template DNA mixed with 1 µl of loading dye (xylene cyanol + bromophenol blue) and electrophoresed at 120 volts for 70 min. DNA samples showing intact bands were used for polymerase chain reaction (PCR) amplifications.

3. DNA amplification of *E.tarda* (25)**:** The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The targeted genes of *E. tarda* isolates were esrB, gyrB and gadB. To amplify the genes, 25 µl of reaction mixture was made containing 20ng of template DNA, 20 pM of primers, 160 µM of dNTP mix, 1.25 U Taq polymerase, 1×Taq buffer, and 0.5 mM $MgCl_2$. Seven genes were amplified individually using the specific primers with 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR amplified products were analyzed by 1.5% of agarose gel (Sigma- USA), stained with ethidium bromide and visualized as well as captured on UV transilluminator, then compared with the marker DNA ladder (100 bp).

Histopathological examination: Fresh tissue specimens from the liver, kidneys, spleen and gills were collected from

naturally infected *O. niloticus* 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 (30)**.**

(Table 1) :

Group No.	No. of fish	Anti-biotic for treatment	No. of dead fish	Survival rate
Group 1	15	Florafenicol	1	93.5%
Group 2	15	Ciprofloxacin	2	87%
Group 3	15	sulphadimethexine	2	87%
Control	15	No treatment	13	13%

(Table 2) :

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
esrB (F)	**5′** TCGTTGAAGATCATGCCTTGC **′3**		
esrB (R)	**5′** TGCTGCGGGCTTTGCTT **′3**	311	
gyrB (F)	**5′** GCATGGAGACCTTCAGCAAT **′3**		
gyrB (R)	**5′** GCGGAGATTTTGCTCTTCTT **′3**	415	[25]
gadB (F)	**5′** ATTTGGATTCCCGCTTTGGT **′3**		
gadB (R)	**5′** GCACGACGCCGATGGTGTTC **′3**	583	

RESULTS

Clinical sings of naturally infected fish: Naturally infected *O. niloticus* showed hang head up in the water and exhibit corkscrew spiral swimming, followed by death, most fish continue to eat and petechial, hemorrhagic patches all over the fish body with exophthalmia. (Fig1). Fish also suffered from putrefied areas on the body especially on caudal peduncle. (Fig, 2).

Postmortem lesions: The post mortem examination of naturally infected *O. niloticus* revealed pale or congested liver with distended gall bladder, abdomen filled with bloody fluid and congestion of the internal organs (Fig, 3, 4).

Isolation and identification of *Edwardsiella tarda:* Colonies on *Edwardsiella* agar media identified by both API20E system and conventional method which include size, motile, negative to cytochrome oxidase test and positive to H_2S production and indole, also they were able to ferment maltose and non-fermented to xylose, sucrose, lactose and mannitol, which revealed the 2 isolates, are *E. tarda*.

Polymerase Chain Reaction (PCR): by the PCR, confirmed the obtained results from previous mentioned identifications through detection of the most common 3 virulence genes of E. tarda.

Antibiogram for treatment *E. tarda*: Florafenicol, Ciprofloxacine and sulphadimethexine were the largest inhibition zone for *E.tarda* thus, they were chosen for treatment of edwardsiellosis in infected *O.niloticus* fish.

Histopathological Results: Histopathological examination of the naturally diseased *O. niloticus* revealed that liver showed congestion of central vein and hepatic cells and hydropic degeneration in which the cells swollen with irregular vacuoles in the cytoplasm (A). Others showed fatty changes in which the cells showed circumscribed vacuoles in the cytoplasm. (B) Some hepatic cells revealed signs of coagulative necrosis. (C) There were multi-focal areas of hemorrhages in-between the hepatic cells. (D) Renal tubules of kidney showed different necrobiotic changes as cloudy swelling, hydropic degeneration and even necrosis. (E)The spleen was studded with large numbers of erythrocytes. There were increase in numbers of

Fig 1: Naturally infected *O. niloticus* showing hemorrhagic spots on the body

Fig 2: Naturally infected *O.niloticus* showing putrefied area on the caudal peduncle

Fig 3: Natural infected *O. niloticus* showing pale liver with distended gall bladder and congested kidney

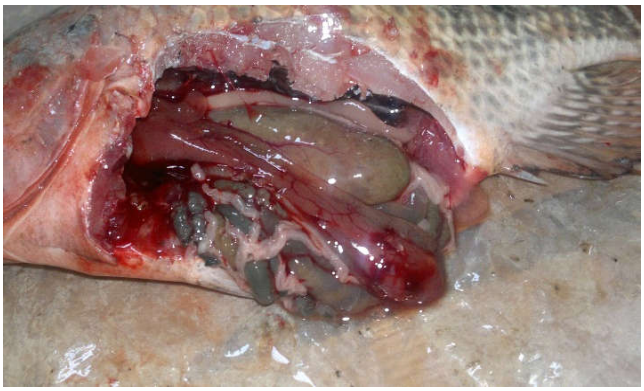
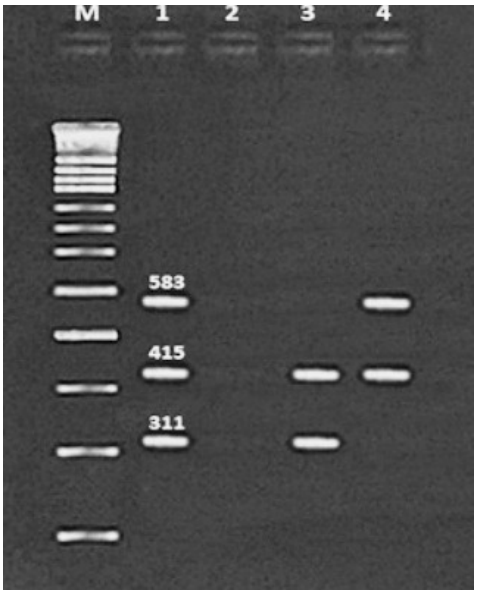


Fig. 4: Natural infected *O. niloticus* showing congestion of stomach, kidneys and liver with bloody fluids in abdominal cavity

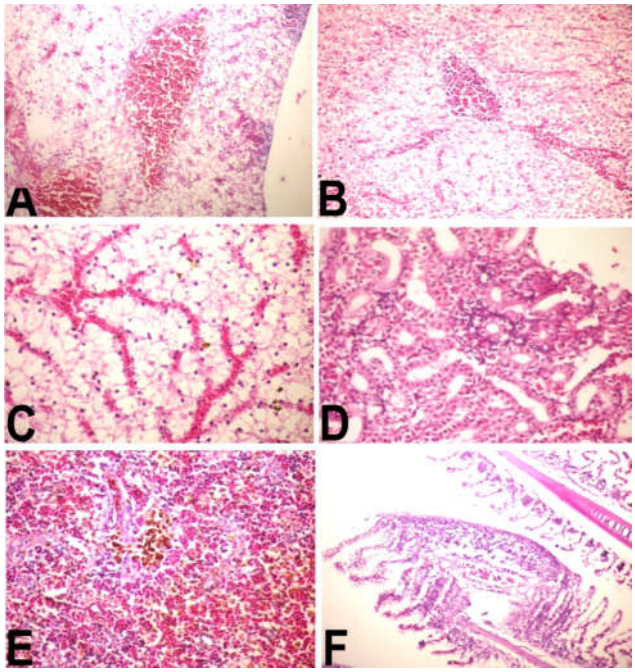
Fig 5: Experimental infected *O. niloticus* fish with E. tarda showing skin ulcer on caudal peduncle with exophthalmia and losing of scales

Fig. 6: Experimental infected*O. niloticus* fish with E. tarda showing exophthalmia with corneal opacity



Photograph (1): Agarose gel electrophoresis of multiplex PCR of esrB(311 bp), gyrB (415) and gadB (583) genes as virulence factors for characterization of Edwardesiella tarda

Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive of *E. tarda*for esrB, gyrB and gadB genes.
Lane 2: Control negative.
Lane 3: Positive *E.tarda*strain for esrB and gyrB genes.
Lane 4: Positive *E.tarda*strain for gyrB and gadB genes.



melano-macrophages centers, with haemociderosis and depletion of lymphocytes. (F) Gills showed hyperplasia of the secondary gill lamellae with odema and infiltration of inflammatory cells. Histopatholgical figures, showed (A) in liver of *O. niloticus* congestion of central vein and hepatic cells degeneration, coagulative necrosis(B), multi-focal areas of hemorrhages in-between the hepatic cells(C), necrobiotic changes in renal tubules of kidney (D). In spleen was studded with large numbers of erythrocytes, increase of melano-macrophages centers, with haemociderosis and depletion of lymphocytes (E).Gills showed hyperplasia of the secondary gill lamellae with odema and infiltration of inflammatory cells (F).

DISCUSSION

Edwardsiella tarda is the etiological agent for *Edwardsiellosis* in many commercially freshwater and marine fish; it is considered a dangerous septicemic disease with high economic losses and affected on public health (Austin and Austin, 1999; Choresca, 2011). Edwardsiellosis is considered one of the most dangerous bacterial diseases causes massive mortalities of cultured freshwater fish caused by a Gram-negative bacterium belonging to Enterobacteriaceae and accompanied with fish handling by nets, overcrowded, bad water quality and high organic matters and water temperature act as predisposing

factors to infections with *E. tarda* (Darwish *et al*., 2000; Hossain *et al*., 2011; Fatma Korui, 2012).

In the present study, the clinical pictures of naturally infected *O. niloticus* showed hang head up in the water and exhibit corkscrew spiral swimming, followed by death, almost fish continue to eat and petechial hemorrhages and hemorrhagic patches on the flank region with swollen protruding anus and corneal opacity these results may be due to toxin produced by E.tarda. These results nearly agree with that recorded by (Ibrahem *et al*., 2011) above plus swollen abdomen with yellowish ascetic fluid and pale coloration. These same authors attributed the obtained signs due to extra cellular bacterial toxin excretion (haemolysine and dermatoxines).

The internal clinical pictures of naturally infected *O. niloticus* showed severe congestion with hemorrhagic spots and patches in pale or congested liver also kidney, spleen and intestine were congested with bloody ascetic fluid surround it. Such results agree with that recorded by (Ibrahem *et al*., 2011; Fatma Korui, 2012) and disagree with that record by (El Seedy *et al*., 2015) who record thatwhite nodules in liver and kidney. This difference in the result may be due to the *E.tarda* isolate was from different kind of fish and different area of study in addition to different environment. The results were agreement with that recorded by (Abdelraheim, 2016; Qin *et al*., 2014; Shetty *et al*., 2014; Michal Ucko *et al*., 2016; Micaela Ferreira Pinto *et al*., 2017; Kumar *et al*., 2009; Hashiem *et al*., 2012; El Seedy *et al*., 2015). This result may be attributed to the extra cellular products of E. tarda particulary haemolysine and dermatoxines.

Regarding the identification of the isolated *E. tarda* strain was differentiated from other colonies on Edwardsiella agar media by naked eye and traditional biochemical tests, the isolated strains were negative to cytochrome oxidase test and positive to H_2S production and indole and able to ferment maltose and non-fermentedto xylose, sucrose, lactose and manitole, these results disagree with that recorded by (Srinivasa *et al*., 2003) who reported that no variation in citrate utilization test among E. tarda and agree with (Micaela Ferreira Pinto *et al*., 2017) who recorded that exhibited variation in citrate utilization in 14 isolates from 16 strains. Identification was confirmed using API -20E rapid identification systemtest.

The only accurate and rapid interference test for identification of *E. tarda* in diseased fish is PCR methods. In this study, *E. tarda* was confirmlly identified by detection of type esrB, gyrBand gadB virulence genes which are specific for identification and pathogenicity of E. tarda isolates (Ibrahem *et al*., 2011; Abayneh *et al*., 2013; Jo *et al*., 2013; Pridgeon *et al*., 2014; Monir and Rahman, 2015). Concerning specific antibacterial therapy is the effective method commonly used to protect fish from E. tarda. In the present study, the sensitivity tests on many of antibacterial drugs were performed. Antibacterial drugs florafenicol, ciprofloxacine and sulphadimethexine were successful drugs in controlling edwardsiellosis, the results were consistent with that recorded by (Castro *et al*., 2016; Shu-Peng *et al*., 2000) and disagree with (Ali Md *et al*., 2014) who reported that *vitro* antibiotic sensitivity pattern of *E. tarda* isolate was (Shu-Peng *et al*., 2000) conducted by disc diffusion method for eight antibiotic discs where, all of the isolates were found to be sensitive against ciprofloxacin, streptomycin, chloramphenicol and gentamycin. (Bullock, 1985) who recorded that the drug of choice for control of edwardsiellosis was feeding terramycin at the rate of 2.5-3.0 g/l00 lb of fish per day for 10 days. These results may be attributed to the antibiotic sensitivity results largely differs between different strains and between different species and localities from which those strains were isolated. These results may be attributed to bacterial species resistance is due to mutations in the gyrase or to poisomerase genes. These results supported by (Sørum, 2006). The results of sensitivity recorded that the high drug sensitive to *E. tarda was* florfenicol. This result similar to that recoded by (Gaunt *et al*., 2003) who reported that florfenicol has instead become available and rapidly became popular in several animal industries, including aquaculture. The results revealed that the effectiveness of florfenicol and sulphadimethexine and Ciprophloxacin while oxytetracycline, ampicillin were non effective (Thangapalam Jawahar Abraham *et al*., 2015; Pankaj Kumar *et al*., 2016; Ahamad *et al*., 2012; Anyanwu *et al*., 2014).

Regarding to the histopathological alterations in naturally infected fish with edwardsiellosis, the present study revealed that, the main lesion in liver of *O. niloticus* was congestion of central vein and hepatic cells showed hydropic degeneration. Others showed fatty changes in which the cells showed circumscribed vacuoles in the cytoplasm. Some hepatic cells revealed signs of coagulative necrosis. There were multi-focal areas of hemorrhages in-between the hepatic cells. These findings agree with that recorded by (Ibrahem *et al*., 2011) and dis agree with (Monir and Rahman, 2015) who found massive necrosis in the hepatic cells revealed signs of coagulative necrosis.

In this study, renal tubules of kidney showed different necrobiosis changes as cloudy swelling, hydropic degeneration and even necrosis. The spleen was studded with large numbers of erythrocytes. There were increase in numbers of melano-macrophages centers, with haemociderosis and depletion of lymphocytes. Gills showed hyperplasia of the secondary gill lamellae with odema and infiltration of inflammatory cells, the obtained results were supported by (Darwish *et al*., 2000; Ibrahem *et al*., 2011). Spleen of naturally infected fish with edwardsiellosis, showed increase in numbers of melano-macrophages centers, with haemociderosis and depletion of lymphocytes. These results go with (Darwish *et al*., 2000; Zhou *et al*., 2014) who found increase in numbers of melano-macrophag centers, depletation of lymphocytes and congestion of blood vessels. Regarding gills of naturally infected *O. niloticus* revealed that hyperplasia of the secondary gill lamellae with odema and infiltration of inflammatory cells. These results nearly agree with that recorded by (Taguchi *et al*., 2014; Huong *et al*., 2014; Eman Moustafa Moustafa *et al*., 2016) and disagree with (Woo and Bruno, 2011) who found granulomas in examined fish. This may be attributed to different site and kind of study and kind of fish and environmental condation.

The present study was concluded that edwardsiellosis infection in *O. niloticus* leads to high morbidity and mortality rate resulting great economic losses. PCR was the most rapid and confirmed diagnosis of *E. tarda*. Florafenicol, Ciprofloxacine and sulphadimesoxine on sensitivity tests are considered promising drugs for control of Edwardsiellosis in *O. niloticus* fish. This study emphasizes the need to be vigilant and control the use of any antimicrobial drugs in the sense that it should not be used indiscriminately.

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