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Isolation, Identification and Pathogenicity Characterization of *Edwardsiella tarda* Isolated From *Oreochromis niloticus* Fish Farms in Kafr-Elshiekh, Egypt

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ABSTRACT

Edwardsiella septicemia is a systemic bacterial disease caused by *Edwardsiella tarda* that is considered one of the most important bacterial microorganisms causing severe economic losses due to morbidity and mortality among wide fish species in many countries. In this study *Edwardsiella tarda* (*E. tarda*) was isolated and identified from *Oreochromis niloticus* fish farms using traditional and molecular PCR techniques using *E. tarda* specific primers. Fifty-six isolates of *E. tarda* were isolated from 300 fish samples during spring, summer and autumn. While in winter it couldn't be isolated from 100 samples. The summer season showed the highest prevalence with 28% then the spring season with 18% finally autamn10%. Experimental infection using the isolated *E. tarda* was performed to determine the pathogenicity in O. niloticus through mortality rate, clinical sings, post mortem lesions and histopathological changes that were observed in the liver, spleen, kidney and gills of the infected fish. The result also showed that there is no significant difference between in vivo and in vitro effect of antibiotic on *E. tarda*. Norfloxacin showed the highest protection in vivo with 90% survival while Ampicillin showed no protection. These results indicated that the antibiotic of choice for treatment of *E. tarda* was Norfloxacin.

1. INTRODUCTION

Fish and their products are an important source of easily digested and delicious animal protein with high biological value (Faber *et al*., 2010). They compromise many essential amino acids, vitamins, poly saturated fatty acids, omega-3, essential minerals as well as amounts of trace elements (Abdullahi *et al*., 2001; Surette, 2008). Nile tilapia (Oreochromis niloticus) represent the most important and economic fish species in Egypt representing 71.38 % of total cultured fish in Africa and 1.54 % of total cultured fish all over the world (FAO, 2012).

Egypt is the third largest tilapia producing country after China and Indonesia the most important governorates in Egypt that produce 80 percent of the farmed tilapia in Egypt are Kafr-Elshikh, Behira and Sharkia (EL-sayed, 2013). Disease outbreaks are one of the main and high significant limiting factors affecting fish industry, especially that result from bacterial infections (Jehan, 2001; Roberts, 1978; saad, 2002). *Edwardsiella tarda* is a common fish pathogen that causes one of the most significant septicemic diseases responsible for mass mortality in freshwater fishes and consequently high economic losses in fish farms in many countries as North America, Japan, Taiwan, Thailand and Africa It leads to mass mortality in various populations and age groups of fish species including carp, tilapia, eel, cat fish, mullet, Salmon, trout and flounder (Baya *et al*., 1997; Bragg, 1991; Durborow *et al*., 1991; Galal,2002).

E. tarda also important due its zoonotic aspects infected fish processed for human consumption is a source of gastroenteritis and meningitis mainly among individuals with impaired immune systems, so it is of great health importance (Mikamo *et al*., 2003; Mizunoe *et al*., 2006)

This study was conducted for the isolation and characterization of *E.tarda* from *Oreochromis niloticus* collected from Kafr El-Shiekh fish farms. Furthermore, experimental study was carried out to determine its pathogenicity through mortality rate, clinical sings, P.M lesions and histopathological changes in internal organs. Furthermore, determination of the antibiotic of choice for treatment of experimentally infected fish and the significant deference between in vivo and in vitro reaction of sensitive and resistant drugs.

2. MATERIALS AND METHODS

2.1. Fish Samples:

A total number of 400 *O. niloticus* with average body weight 75± 20g B. Wt. were collected randomly alive through the four seasons (summer, autumn, winter, spring) during 2016 from private farms at Kafr El-Sheikh governorate the samples were transported directly on ice to the laboratory of the Dept. of Microbiology, Faculty of Veterinary Medicine, Damanhur University.

2.2. Clinical and Postmortem examination

Fish were clinically examined to detect edwardsiellosis associated clinical abnormalities as previously described by Kimberley (2004). Fish were also subjected to post-mortem examination according to the method described by Noga (2000).

2.3. Isolation and identification of *E. tarda*

After disinfection of the outer surface of fish skin with70% ethyl alcohol, samples from internal organs (kidney, liver, spleen and muscles) were inoculated into nutrient broth and incubated at 30°C for 24 hrs. Salmonella-Shigella agar media and Brain heart infusion agar media were streaked with a loopful of the cultured broth and incubated at 30°C for 24- 48 hrs. as previously described by Lima *et al*. (2008).

2.4. Phenotypic identification of *E. tarda*

Colony morphology, culture and microscopic characters were identified as previously described by Muratori *et al*. (2001) and Xiao *et al*. (2009) followed by API 20E system, which was carried out according to manufacturer's instructions. The isolates were characterized by biochemical tests as described by Fang *et al*. (2006).

2.5. Molecular identification of *E. tarda* by polymerase chain reaction

PCR was applied for detection of gyrB gene of *E. tarda* isolated from naturally infected Nile tilapia. DNA extraction was performed as previously described by Choresca *et al*. (2011). The concentration was measured by spectrophotometer then preserved at -20°C. DNA was amplified using GoTaq® Hot Stored Green Master Mix (Promega, USA) and oligonucleotide primers target 415 bp fragment of gyrB gene of E. tarda according his manufacturer's instructions. The primers used and PCR reaction were performed as previously described by Lan *et al*. (2008).

2.6. Antimicrobial susceptibility test for *E. tarda*

Disc diffusion method was used for antimicrobial sensitivity test on Muller-Hinton agar plates according the method carried out by Bauer *et al*. (1966), using the following antibiotics Norfloxacin (10µg), Streptomycin (10µg), Gentamicin (10µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Doxycycline (30µg), Ampicillin (30µg), Flumequine (30µg), Neomycin (30µg), Tetracycline (30µg), Sulfamethoxazole (100µg), Cefotaxime (10 µg) diameter of the inhibition zone was measured and interpreted according to clinical and laboratory standards institute **(CLSI,2006)**.

2.7. Inoculum preparation for pathogenicity test

The inoculum was prepared as intraperitoneal injections (I/P) according to Eissa and Yassien (1994). The bacterial isolates were sub-cultured on Salmonella-Shigella agar (S.S agar) plates and incubated for 24 hrs. A typical colony was picked up and inoculated into nutrient broth and incubated at 30ºC for 24- 48hr. The broth culture was centrifuged for 3000 rpm for 4 minutes and the bacterial cells were re-suspended in phosphate buffered saline and standardized for the optical density of McFarland's No. 1 (each ml contained approximately 3×10^8 CFU bacterial cells) according to Cruickshank *et al*. (1982).

2.8. LD_{50} of *E. tarda* in *O. niloticus* calculation

A total of 70 apparently healthy Nile tilapia fish weighting 70 ± 20 grams were selected and left for adaptation in glass tanks for one month and grouped into 7 groups. The first group was inoculated with 0.3 ml sterile phosphate buffered saline (control group). The second group was inoculated with 0.3 ml of 3×10^8

CFU of *E. tarda* /ml (McFarland 1). The other five groups were inoculated with the serially diluted bacterial solutions according to **Mekuchi *et al*. (1995)** to determine the LD_{50} to be used in experimental design.

2.9. Experimental design

Forty apparently healthy Nile tilapia each weighing 70 ± 20 gm were divided into 4 groups each of 10 fish as follows: Group 1 (negative control) was I/P inoculated with 0.3 ml of sterile phosphate buffered saline. Group 2 (positive Control): was I/P inoculated with 0.3 ml of phosphate buffered saline containing 3 × 10^6 CFU of *E. tarda* (LD_{50}). Group 3: was I/P inoculated with 0.3 ml of phosphate buffered saline containing 3 × 10^6 CFU of *E. tarda* plus feeding on ration containing Norfloxacin; 100 mg / kg twice daily for 10 days. Group 4: was I/P inoculated with 0.3 ml of phosphate buffered saline containing 3 × 10^6 CFU of *E. tarda* plus feeding on ration containing Ampicillin; 50 mg/kg twice daily for 10 days. All groups were observed daily for 14 days to record the general health condition, clinical signs and mortalities and re isolation and identification of *E. tarda* from experimentally infected fish. Postmortem examination was performed on morbid and dead fish and specimens for histopathological studies were collected.

2.10. Histopathological examination

Following necropsy, tissue specimens were collected from gills, hepatopancreas, spleen, kidney, intestine and muscles of Nile tilapia experimentally infected with *E. tarda* and examined for histopathological changes. Tissue specimens were fixed in 10% neutral buffered formalin solution for at least 24 hrs. The fixed specimens were processed through the conventional paraffin embedding technique. 5 µ thick sections were obtained from paraffin blocks and were stained with Hematoxylin and Eosin (H&E) according to the method described by Culling (1983).

3. RESULTS

3.1. Seasonal incidence of edwardsiellosis among cultured *O. niloticus* fish farms in Kafr El-Shiekh governorate

A total of 400 randomly collected *O. niloticus* were caught during different seasons in 2016. Incidence of edwardsiellosis was 0%, 18%, 28% and 10% during Winter, Spring, Summer and Autumn, respectively.

3.2. Morphological characters

E. tarda colonies on SS agar varied in size and were small transparent with black centers to predominantly black colonies. Microscopically, *E. tarda* isolates were Gram-negative short bacilli by Gram stain. Biochemical profile by the API 20E biochemical system of all the isolates was able to produce indole and were positive in hydrogen sulphide production test, citrate utilization test, ornithine decarboxylase test and able to ferment glucose. While negative in Voges-Proskauer test, arginine dihydrolase test, gelatin hydrolysis test and non-fermentable to mannitol, sorbitol, rhamnose, saccharose, melibiose, amygdaline and arabinose.

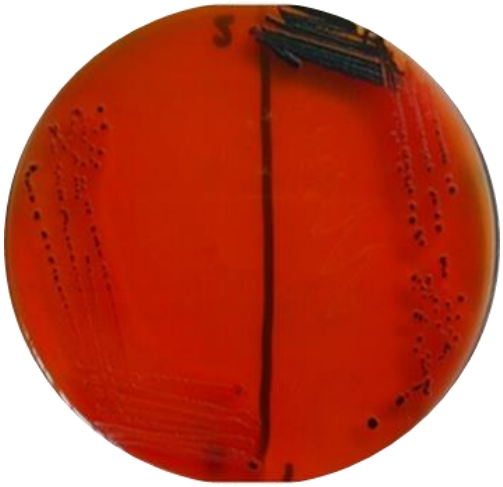

Figure 1*: E.tarda* colonies on SS agar showing varied size. They appeared small transparent with black centers to predominantly black colonies.

3.3. Molecular Identification of *E. tarda* using PCR

By PCR using specific primers targeting 415 bp fragment of gyrB gene of *E. tarda* showed the amplification of the target DNA from the isolates collected during spring, summer and autumn as show in fig.2.

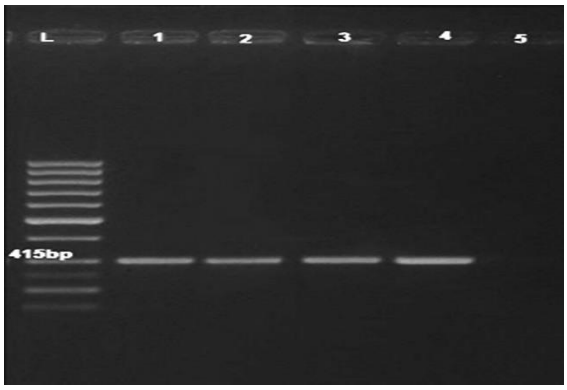

Figure 2: PCR amplification of *E. tarda* gyrB gene. L, 100 bp DNA marker; 1, Summer isolate; 2, Spring isolate; 3, Autumn isolate; 4, control positive *E. tarda*; 5, negative control.

3.4.Antimicrobial susceptibility test for *E. tarda*

Agar disc diffusion test revealed that isolated *E. tarda* were sensitive to Norfloxacin, Streptomycin, Gentamicin, and Chloramphenicol, intermediately sensitive to Ciprofloxacin, Doxycycline and resistant to Ampicillin, Flumequine, sulfamethoxazole, Neomycin, Tetracycline and Cefotaxime indicating multiple antimicrobial resistant (Table1).

Table1: Antimicrobial susceptibility test for *E. tarda*

Antimicrobial	Interpretation
Streptomycin (10 µg)	S
Ciprofloxacin (5 µg)	I
Gentamicin (10 µg)	S
Doxycycline 30µ g	I
Ampicillin (30 µg)	R
Flumequine (30 µg)	R
Cefotaxime (10 µg)	R
Sulfamethoxazole (100 µg)	R
Neomycin (30 µg)	R
Norfloxacin (10 µg)	S
Tetracycline (30 µg)	R
Chloramphenicol (30 µg)	S

S=sensitive R=resistant I= intermediate

3.5. Lethal Dose 50 of *E. tarda* in *O. niloticus*

The results revealed that the concentration of 3×10^6 CFU caused 50% mortalities of the inoculated fish (LD_{50}) and was used in experimental design (Table 2).

Table 2: Lethal Dose 50 of *E. tarda* in *O. niloticus*

Fish groups	Dose / fish (0.3ml of concentration)	Mortality %
1	(3×10^8) CFU	80%
2	(3×10^7) CFU	60%
3	(3×10^6) CFU	50%
4	(3×10^5) CFU	30%
5	(3×10^4) CFU	20%
6	(3×10^3) CFU	0%
Control	Saline	0%

3.6. Experimental infection of *O. niloticus* with natural isolates of *E. tarda*

The experimental infection showed sever clinical signs, clear PM lesions and histopathological changes in internal organs. The results also showed that Norfloxacin; 100 mg / kg is effective for treatment of *E. tarda* infection with no significant difference between in vivo and in vitro effect of the antibiotic. Norfloxacin provided high protection (90%) in contrast to Ampicillin that showed no protection (Fig 3).

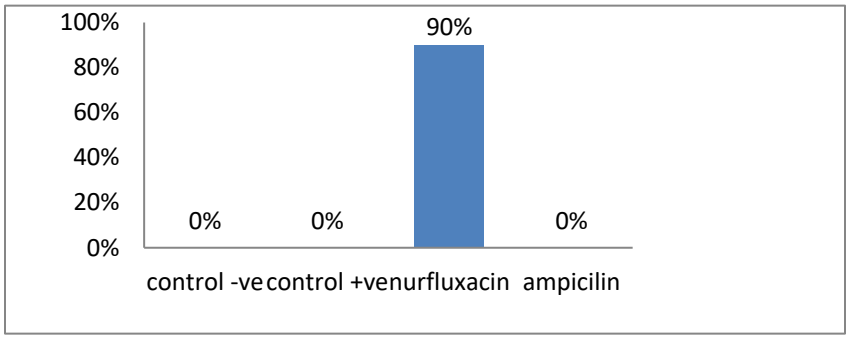

Figure 3: Protection percentage following antimicrobial treatment
Control –ve 0%, control +ve 0%, Ampicillin 0% and Norfloxacin 90%.

3.7. Clinical sings and postmortem lesions of experimentally infected fish

O. niloticus infected with *E. tarda* showed loss of scales from some areas of the body, congestion and hemorrhages all over the fish body especially at the base of the pectoral fin, hemorrhages at anal opening, fin and tail rot (Fig 4A). It also showed exophthalmia and ascites (Fig 4B) as well as protruded congested vent (Fig 4C) ulcer and scale loss (Fig 4D). The post-mortem examination of infected fish revealed a wide variety of lesions in the form of abdominal distention with ascetic fluids, distended gall bladder and congested enlarged Hepatopancreas and kidney (Fig 4E and 4F).

Figure 4: Clinical signs and postmortem lesions of *O. niloticus* experimentally infected with E. tarda. (A) Scales loss from some areas of the body, fin and tail rot, congestion and hemorrhages all over the fish body especially at base of pectoral fin and anal opening. (B) Exophthalmia and ascites. (C) Protruded congested vent and skin darkness (D) Ulcer and scale loss. (E) Abdominal distention with ascetic fluids and kidney congestion. (F) Congested and enlarged gall bladder and Hepatopancreas.

3.8. Histopathological changes in experimentally infected fish

Histopathological changes were detected in tissue specimens collected from gills, hepatopancreas, spleen, kidney, intestine and muscles with apparently gross lesion of Nile tilapia experimentally infected with *E. tarda*. Gills showed congestion in branchial blood vessel, abundant mucous cells in tip of primary gill lamellae, inflammatory cells infiltration at the base of primary gill lamellae, eosinophilic granular cells (EGCs) infiltration and congestion of blood vessels. Gill arch showed congestion of blood vessels inflammatory cells infiltration, EGCs infiltration. Hepatopancreas as well showed congested and distended bloodless hepatic sinusoid, necrotic pancreatic acini with inflammatory cell infiltration. Furthermore, the posterior kidney showed interstitial nephritis with inflammatory cells infiltration in the interstitium. The spleen showed moderate activation of the Melano Macrophage Centers. But intestine showed hyperplasia of the goblet cells. Finally, muscle showed segmental lytic necrosis and congestion of blood vessels (Fig. 5).

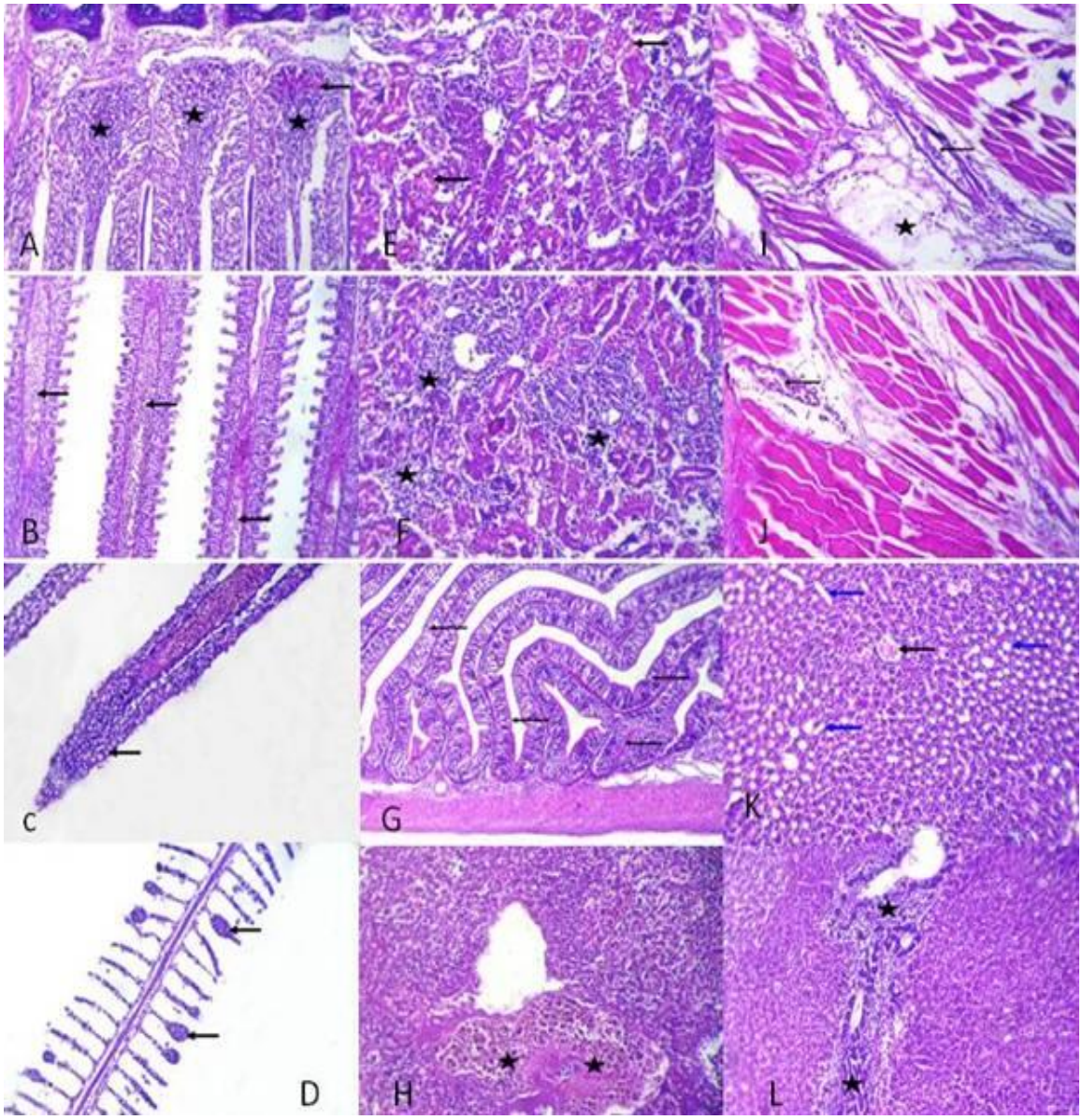

Figure 5: Histopathological changes in O. niloticus experimentally infected with E. tarda. (A) Gill arch showing congestion of blood vessels and inflammatory cell infiltration (stars), (B) Gills showing congestion in bronchial blood vessel (arrows), (C) Abundant mucous cells in tip of primary gill lamellae, (D) Gills showing lamellar telangictasis (arrows) H&E. (X 250). (E)

The posterior kidney showing hyaline droplets formation in tubular epithelium of renal tubules (arrows), (F) Showing interstitial nephritis with inflammatory cell infiltration in the interstitium (stars) H&E. (X 160), (G) Intestine showing hyperplasia of the goblet cells (arrows), (H) Spleen showing activation of Melano Macrophage Centers (MMCs) H&E. (X 250). (I) Muscle showing congestion of blood vessels (arrow) and edema (stars), (J) Muscle showing segmental lytic necrosis and congestion of blood vessels, (K) Hepatopancreas showing congestion in blood vessels (black arrows), and distended bloodless hepatic sinusoid (blue arrows), (L) Hepatopancreas showing necrotic acini with inflammatory cell infiltration (stars). H&E. (X 160).

4. DISCUSSION

Edwardsiella tarda is considered as one of the most important bacterial microorganisms that causes severe economic losses due to morbidity and mortality among various populations and age groups of fish in many countries (Jun and Yin, 2006). There is less information about the incidence of *E. tarda* from different locations in Egypt. Our study proved that the high *E. tarda* incidence was in summer (28%) and in spring (18%) at Kafr El-Shiekh. These results agreed with Korni *et al*. (2012) who recorded the prevalence of edwardsiellosis among the cultured *O. niloticus* in the spring season 13.33 % at Beni-Suef Governorate and Bullock and Roger (1985) who recorded outbreaks of *E. tarda* infections restricted essentially to water temperatures of 24-28 °C in May-June and September. On the other hand, Abd El-Mageed *et al*. (2002) recorded the incidence of *E. tarda* in *O. niloticus* collected from different localities in Egypt to be 0%. This may suggest that the location of the study, water quality parameters and culture density may be the reasons of the difference in prevalence of *E. tarda*.Diagnosis using polymerase chain reaction (PCR) depending on specific primers of gyrB gene proved that morphologically and biochemically identified isolates were positive for *E. tarda* gyrB gene as described by Lan *et al*. (2008); Baird *et al*., (2003) and Lima *et al*. (2008). PCR identification and diagnosis of the causative agent provides rapid treatment and decreases economic loses.

Antimicrobial sensitivity test revealed that isolated *E. tarda* were sensitive to Norfloxacin, Streptomycin, Gentamicin, and Chloramphenicol, while intermediately sensitive to Ciprofloxacin and Doxycycline but resistant to Ampicillin, Flumequine, sulfamethoxazole, Neomycin and Tetracycline. These results indicate multiple antimicrobial resistance of isolated bacteria specially those commonly used in fish farms as Ciprofloxacin and Tetracycline. This result closely similar to that reported by Choresca *et al*. (2011)

The experimental infection showed that natural isolates of *E. tarda* were pathogenic with mortality rate reaching 100% at 6 $X10^8$ CFU and lethal dose 50 (LD_{50}) at 3×10^6 CFU. These results are similar to those reported by Ling *et al*. (2000) who recorded that virulent *E. tarda* strains having LD_{50} values 10^6CFU in blue gourami. Ibrahim *et al*. (2011) recorded that LD_{50} of the *E. tarda* in African sharptooth catfish was 10^4 CFU/ml and caused 60% mortality in Nile tilapia. Mekuchi *et al*. (1995) recorded that the LD_{50} was 7.1x10, $1.7x10^2$, $3.6x10^6$ and $1.3x10^6$ CFU/ml by intra muscular injection, inter peritoneal injection, immersion and oral administration, respectively, in Japanese flounder. The difference in LD_{50} concentration may be due to the difference in species and route of injection. There we used intraperitoneal route of injection method according to Amandi *et al*. (1982) and Eissa and Yassien (1994).Experimental infection and treatment revealed that Norfloxacin; 100 mg / kg was the drug of choice for the disease treatment with no significant difference between in vivo and in vitro effect of the antibiotic. This result is similar to those reported by Sahoo and Mukherjee, (1997); Zhang *et al*., (2005) and Zhu *et al*., (2006) who reported that Norfloxacin, ciprofloxacin, gentamicin, chloramphenicol, cefazolin and aztreonam were effective in controlling *E. tarda* infection. Pathogenicity was determined through mortality rate, LD_{50}, clinical signs, PM lesions and histopathological changes in experimentally infected fish with *E. tarda*. Clinical sings of experimentally infected *O. niloticus* showed scales loss, congestion and hemorrhages, fin and tail rot, exophthalmia, ascites, protruded congested vent and ulcer that are similar to those reported by Lima *et al*. (2008) and Ibrahim *et al*. (2011). The post-mortem examination of infected fish revealed a wide variety of lesions, in the form of abdominal distention with fluids, distended gall bladder and congested enlarged Hepatopancreas and kidney. These results were similar to those reported by El-Deeb *et al*. (2006); lima *et al*. (2008); Ibrahim *et al*. (2011) and Korni *et al*. (2012). Histopathological changes in experimentally infected Nile tilapia in this study showed results that agreed to those reported by Soliman (1991); Galal *et al*. (2002); Galal *et al*. (2005) and Ibrahim *et al*. (2011).

The histopathological changes could be due to nephric and hepatic virulence factors of *E. tarda* (Miwa and Mano, 2000 and Mathew *et al*., 2001).

In this study, our results showed that *E. tarda* has high incidence of *E. tarda* in summer and spring seasons, thus, good hygienic management during these seasons is required to improve water quality and decrease the disease incidence and economic losses. Although, the API 20E system is a good primary method for biochemical differentiation of *E. tarda*, the PCR is a rapid and accurate diagnostic method for differentiation and diagnosis. Furthermore, Norfloxacin proved to be effective and can be used in *E. tarda* elimination providing high level of protection.

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