Insight into the Virulence-Related Genes of *Edwardsiella Tarda* Isolated from Cultured Freshwater Fish in Egypt

Eman Moustafa Moustafa[1]*, Amira Alaa El-Dein Omar[1] and Waleid Sobhy Abdo[2]

[1] *Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Kafr El-Sheikh Governorate, Postal code: 33516, Egypt*
[2] *Department of Animal Pahology, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Kafr El-Sheikh governorate, Postal code: 33516, Egypt*
*Corresponding author`s Email: emantarek2002@yahoo.com

ABSTRACT

One of the most serious fish pathogens infecting both cultured and wild fish species was found to be *Edwardsiella tarda* which contains important virulence factors that enhance bacterial survival and pathogenesis in hosts. The present study aims to isolate, identify and characterize *E. tarda* in freshwater fishes in Kafr El-Sheikh governorate, Egypt using selective differential cultural medium (Rimler Shotts agar), morphological and biochemical tests (oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatine hydrolysis, H_2S production, oxidation-fermentation, nitrate reduction and sugar utilization tests). Pathogenesis of *E. tarda* was checked by experimental infection to *Oreochromis niloticus* fish together with screening of the highly virulent three virulence genes (esrB, mukF and gadB). The obtained results revealed the presence of the three virulence genes in the selected strain of *E. tarda* which gave severe lesions in the experimentally infected *Oreochromis niloticus*. *E. tarda* strain having more than one virulence gene results in more severe lesions than strains having one or even no virulence genes.
Key words: *Edwardsiella tarda*, Virulence genes, Freshwater fish

INTRODUCTION

Edwardsiella tarda (E. tarda) is a predominantly enteric pathogen of both fresh water and brackish water fishes (Plumb, 1994). It is mainly responsible for causing a serious systemic bacterial disease, the edwardsiellosis, having a worldwide distribution among variety of fish taxa (Austin and Austin 1999; Maiti et al., 2009). The bacterium is also a pathogen for wide range of hosts rather than fish namely, reptiles, birds and mammals including humans (Plumb, 1993 and Park and Oh, 2008). *E. tarda* is a Gram-negative bacterium that causes a systemic suppurative disease in fish and humans. Recently, the complete genome sequence of a highly virulent and multidrug resistant *E. tarda* strain isolated in China indicated that this microorganism harbor a number of virulence and toxin secretion system related genes that explain in some extent it capacity to survive within phagocytic cells and to infect a variety of hosts (Verjan et al., 2013).

E. tarda is widely distributed in aquatic environments (Pitlik et *al.,* 1987 and Wyatt et al., 1979). It is one of the serious fish pathogens, infecting both cultured and wild fish species. Research on edwardsiellosis has revealed that *E. tarda* has been considered as a common guest of a high variety of animals including fish, reptiles, amphibians, chickens and other warm-blooded animals as humans (Castro et al., 2016), and contains important virulence factors that enhance bacterial survival and pathogenesis in hosts (Park et al., 2012). *E. tarda* is an intracellular, rod-shaped, Gram negative, non-capsulated, motile, facultative anaerobic bacterium that was first isolated from a pond-cultured eel (Hoshino, 1962).

The basis of pathogenicity depends on the number of virulence factors in *E. tarda*, including the ability to invade epithelial cells (Janda et al., 1991 and Ling et al., 2000), resist serum and phagocyte-mediated killing (Ainsworth and Chen, 1990 and Srinivasa et al., 2001), and production of toxins, such as, haemolysins and dermatotoxins for disseminating infection (Ullah and Arai, 1983 and Hirono et al*.,* 1997). *E. tarda* virulence is believed to be a multifactorial process not yet completely understood. Several potential pathogenic properties have been suggested to contribute to the infection process of *E. tarda*, including secretion of degradative enzymes, adhesions, type III secretion system, type VI secretion system and the ability to survive and replicate in phagocytes. It has been established that the

pathogenic strains of *E. tarda* have virulence genes which might be absent in nonpathogenic strains (Rao et al., 2003 and Castro et al., 2016). Now, many virulence-related genes in *E. tarda* have been cloned or reported, which included catalase (katB), TTSS regulator (esrB), putative killing factor (mukF), Fimbrial operon (fimA), glutamate decarboxylase (gadB), Citrate lyaseligase (citC), pstS, pstC, type III secretion system (ssrB), astA, isor, ompS₂, Hemolysine A (hlyA) and ATPase domain of DNA Gyrase (gyrB) (Rao et al., 2003; Rao et al., 2003; Rao et al., 2004; Tan et al., 2005; Choresca et al., 2011 and Abd El-Kader, 2015). Abd El-Kader, 2015 studied that the Nile Tilapia (*Oreochromis niloticus*) could be used as the animal model for studying the pathogenicity of *E. tarda*.

This study was carried out to isolate, identify and characterize *E. tarda* from freshwater fish in Kafr El-Sheikh governorate, Egypt, and using various culture media, morphological and biochemical tests. In the present study we screened the presence of three virulence-associated genes of *E. tarda* isolated from diseased cultured fishes.

MATERIALS AND METHODS

Ethical approval
Animal ethics committee, faculty of veterinary medicine, Kafr El-Sheikh University, Egypt, approved the protocol and conducting of the study.

Isolation and identification of *E. tarda*
A total number of 120 *Oreochromuis niloticus* fish were collected from Kafr El-Sheikh governorate farms over the seasons of the year 2015. The collected fish showed hemorrhagic patches on different parts of the body and eye exophthalmia externally. Internally, abundant ascetic fluid was found in the abdominal cavity and small white nodules were observed in liver, kidney, spleen and gills.

For bacteriological examination, swabs were collected from the infected parts of the fishes. The collected swabs were firstly pre-enriched on Tryptic Soy Broth (TSB, Oxoid). After which (pre-enrichment on TSB) they were streaked on Rimler Shotts agar (RS) for presumptive identification and incubated at 28 °C for 24 hours. Well-differentiated single bacterial colonies were further streaked onto Tryptic Soy Agar (TSA, Oxoid) for obtaining pure culture.

Biochemical analysis
For phenotypic identification, pure cultures were subjected to Gram staining and viewed microscopically. Further biochemical tests like motility, oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatine hydrolysis, H₂S production, Oxidation-fermentation, nitrate reduction and sugar utilization tests, were performed for the identification as well as strain differentiation of bacteria and results were compared with the reference strain of *E. tarda* (obtained from the food analysis center, faculty of veterinary center, Banha university, Egypt).

Molecular identification by PCR

DNA Extraction using QIA amp kit: Genomic DNA was extracted from every isolate of *E. tarda* using DNA extraction kit (QIAamp: Qiagen inc., USA). 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 (Shah et al., 2009).

Primer sequences of E. tarda used for PCR identification system: Detection of the virulence factors of *E. tarda* represented by esrB (TTSS regulator), mukF (putative killing factor) and gadB (glutamate decarboxylase) was carried out using the peimers which is introduced in Table 1.

DNA amplification of E. tarda: The amplification was performed on a thermal cycler (master cycler, eppendorf, Hamburg, Germany). The targeted genes of *E. tarda* isolates were esrB, mukF 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 MgCl2. The three genes were amplified 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) (Wang et al., 2012).

Table 1. The primers used for the amplification of different virulence associated genes

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
esrB (F)	5′ TCGTTGAAGATCATGCCTTGC ′3	311	Wang et al. (2012)
esrB (R)	5′ TGCTGCGGGCTTTGCTT ′3		
mukF (F)	5′ TTGCTGGCTATCGCTACCCT ′3	357	
mukF (R)	5′ AACTCATCGCCGCCCTCTTC ′3		
gadB (F)	5′ ATTTGGATTCCCGCTTTGGT ′3	583	
gadB (R)	5′ GCACGACGCCGATGGTGTTC ′3		

Experimental infection

Total Bacterial count: Using the drop plate method for estimation of *E. tarda* strain per 1 ml, was used in demonstration of the inoculum dose for the experimental studies according to Cruickshank et al. (1975).

Lethal Dose 50: A total number of 70 apparently healthy *Oreochromis niloticus* fish weighting (50±0.5 gm), were divided into 7 groups, 10 fishes per each group and the seventh group was kept as a control group. All fishes were kept for 15 days under observation prior to injection for accommodation and to confirm that they are free from diseases.

24 hours colony culture of *E. tarda* strain on TSA was used, the colonies were picked up and suspended in sterile saline in a ten fold serial dilution with subsequent incubation at 28 °C for 24 hours for plate counts on TSA. Only the dilutions (10^2-10^7 cfu) were used. Each group was intraperitoneally injected with 0.5 ml/fish of each bacterial dilution. The fishes in the control group were injected with 0.5 ml PBS/fish (Phosphate Buffer Saline). All the injected fish were kept for 1 week post-inoculation for observation. The mortalities were recorded twice/ day according to Ibrahim et al. (2011). The freshly dead fishes were moved for further pm examination. The LD_{50} (the dose which kill 50% of the injected fish) was calculated according to Reed and Muench (1938).

Pathogenicity test: Experimental infection was carried out to determine the pathogenicity of *E. tarda* strain using intra-peritoneal route injection according to Ibrahim et al. (2011). A total number of 60 apparently healthy *O. niloticus* fishes weighting (50±0.5 gm), were divided into 3 groups; 20 fishes per each. Each fish in the first group was intraperitoneally injected with 0.2 ml/fish of LD_{50} dose *E. tarda* strain which was determined previously (1.8 x 10^5 cfu). Each fish in the second group (control negative group), was intraperitoneally injected with 0.2 ml/fish of PBS (Phosphate Buffer Saline). Each fish in the third group (Control positive group), was intraperitoneally injected with 0.2 ml/fish of obtained reference *E. tarda* strain. All injected fishes were observed for a period of 28 days post-inoculation. Mortalities were recorded daily and freshly dead fishes were moved for further pm examination and histopathological studies.

Histopathological examination

Specimens from liver, kidney and spleen of experimentally infected fishes were taken. Specimens were fixed immediately in 10% neutral buffered formalin, dehydrated and embedded in paraffin blocks. Paraffin blocks were sectioned at 4-5 µm thickness and stained with Hematoxilin & Eosin (H&E) and examined under light microscope (Leica) using ×200 and ×400 magnification power according to Bancroft and Gamble (2007).

Table 2. Comparison of the phenotypic and biochemical characteristics between the isolated *E. tarda* strain and the reference strain

Biochemical tests	Isolated *E. tarda* strain	Reference strain
Motility test	+	+
Oxidase test	-	-
Catalase test	+	+
Gram staining	-	-
Indole test	+	+
Methyl red test	+	+
Voges proskaeur test	-	-
Citrate utilization test	-	+
Arginine dehydrolization test	-	-
Acid production from glucose	+	+
Acid production from fructose	+	+
Acid production from maltose	+	+
Acid production from lactose	-	-
Acid production from rhaminose	-	-
Acid production from sucrose	-	-
Acid production from mannitol	-	-
Lysine decarboxylase test	+	+
Ornithine decarboxylase test	+	+
H_2S production	+	+
Lecithinase test	-	-

+ = positive reaction, - = negative reaction

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RESULTS AND DISCUSSION

E. tarda, the causative agent of Edwardsiellosis, is a serious systemic disease of both cultured and wild fishes, has been recognized as one of the most dangerous pathogens causing high economical losses among aquaculture industries worldwide with expanded host range (Hou et al., 2009 and Park et al., 2012) including humans (Xie et al., 2014).

External examination of naturally infected *Oreochromis niloticus* revealed hemorrhagic patches on different parts on the body, eye exophthalmia (Figure 1 and Figure 2) and cutaneous ulcers (Figure 3). The gross lesions displayed in the current study are similar to those reported by several authors (Han et al., 2006; Padros et al., 2006; Shabaan, 2007 and Ibrahim et al., 2011).

However, the postmortem findings exhibited the presence of abundant ascetic fluid in the abdominal cavity together with the enlarged congested gall bladder, liver, kidney and spleen (Figure 4). The postmortem findings displayed in the present study are similar to those reported by Shabaan (2007) and Carlos et al. (2012). The current postmortem findings may be due to the action of bacterial toxins. *E. tarda* secretes Haemolysin and Dermotoxins, Exotoxins Extracellular Products (ECP) and Endotoxin Intracellular Components (ICC) (Suprapto et al., 1995; Hirono et al., 1997 and Mathew et al., 2001). However, liver and kidney congestion might be due to hepatic and nephric virulence factors of *E. tarda* (Miwa and Mana, 2000 and Mathew et al., 2001).

Figure 1. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *E. tarda* showing hemorrhagic patches (arrow) on different parts of the body

Figure 2. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *E. tarda* showing exophthalmia (arrow)
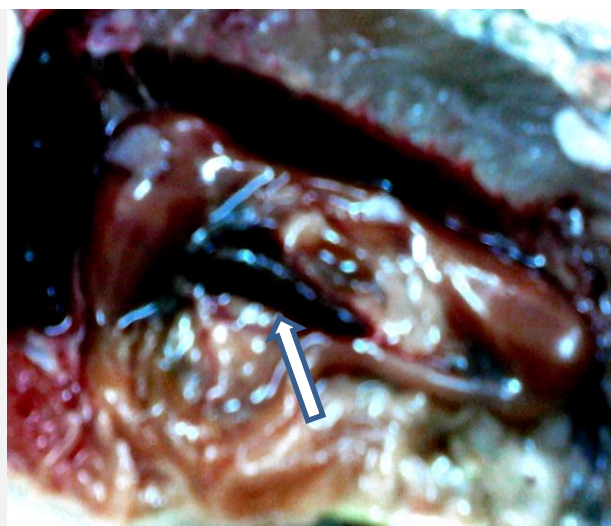

Figure 3. *Oreochromis niloticus,* collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *E. tarda* showing cutaneous ulcers on the body (arrow)

Figure 4. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *E. tarda* showing enlarged and congested spleen (arrow)

The presumptive identification of the bacteria in the recent study was carried out from colony morphology over Rimler Shotts (RS) medium which acts as a selective medium for *E. tarda* (Buller, 2004; Das et al., 2014 and Achrya et al., 2007). They showed typical greenish colonies with black center over RS plates. Microscopically, the bacteria were observed as Gram negative short rod shaped organism. Biochemical characterizations have proven to be a valuable method for typing and differentiation of bacterial fish pathogens (Tison et al., 1982 and Austin et al., 1997). In the present study, based on 20 biochemical tests, a total number of thirty isolates were positively identified as *E. tarda*. All strains were positive for motility, catalase, indole, methyl red, production of gas and acids from glucose, fructose, maltose, H_2S production on TSI agar and lysine and ornithine decarboxylation. But, showed negative results for oxidase, voges proskauer, arginine dehydroxylation production of acid from lactose, rhamnose, sucrose and mannitol. All the obtained biochemical results were compared with the reference strain (Table 1). Most of the phenotypic characteristics of the isolates were similar as reported in Bergey's manual of determinative bacteriology (Holt et al., 1994). Based on the biochemical tests results, all obtained isolates were similar to *E. tarda* reference strain. However, a low degree of heterogeneity was observed in citrate utilization test. All biochemical obtained results were similar to findings of Amandi et al., (1982); Wei and Musa (2008) and Kumari (2011). However, negative results to citrate utilization test was similar to Coles et al. 1978) and Baya et al. (1997).

Pathogenic bacteria may have virulence genes that are absent in non-pathogenic bacteria, making them virulent. Virulence genes may also be present in both pathogenic and non-pathogenic bacteria but may be functional only in pathogenic ones (Rao et al., 2003; Wang et al., 2012 and Castro et al., 2016). One of *E. tarda* positive strains was screened for the presence of three virulence genes (TTSS regulator (esrB), putative killing factor (mukF) and glutamate decarboxylase (gadB) by PCR technique. The results revealed that the selected strain had the three screened virulence genes (esrB, mukF and gadB) (Figure 5). It has been reported that the three virulence genes (esrB, mukF and gadB) obtained in the current study were specific to pathogenic *E. tarda* (Rao et al., 2003; Choresca et al., 2011 and Wang et al., 2012). These genes can therefore be used as biomarkers to perform diagnosis of pathogenic *E. tarda*.

The median lethal dose fifty (LD_{50}) experiments in the present study revealed that the concentration 10^5 cfu was the most potent dilution causing 50% mortalities within (24-48 hr). This result is similar to Shaaban (2007) but, disagrees with Ibrahim et al. (2011) who obtained LD_{50} at concentration of 10^4 cfu. The differences in LD_{50} between different authors may be due to the number of the virulent genes in *E. tarda* strain, pathogen properties as well as size, weight of fish species and temperature difference (Amandi, 1982 and Ibrahim et al., 2011).

The (gadB) recorded in the current study, provides resistance to bacteria towards phagocytes-mediated killing inside the host. The gad system neutralizes acidity and enhances the survival under extreme acid conditions, the glutamate-dependant acid resistance system requires the glutamate decarboxylase gene for protection under acidic condition. Mutation in gadB gene of *E. tarda* resulted in attenuation of the mutant in vivo and acid sensitivity in vitro indicating that the mutant was unable to survive and cause infection inside the host (Srinivasa Rao et al., 2003). Bacteria fight against serum and phagocyte-mediated killing by gadB.
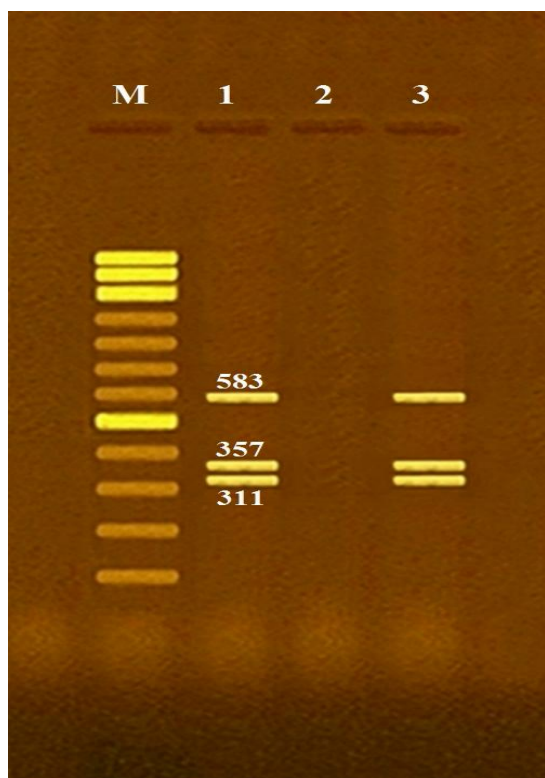

Figure 5. Agarose gel electrophoresis of multiplex PCR of esrB (311 bp), mukF (357 bp) and gadB (583 bp).virulence genes for characterization of *Edwardsiella tarda*

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

The clinical signs of experimentally infected *O. niloticus* with current *E. tarda* strain revealed that fishes were lethargy showing abnormal swimming behavior, loss of escape and defensive reflexes, floating near water surface with gasping atmospheric air followed by fish death with opened mouth. This might be due to stress response and/or development of anaemia leading to insufficient oxygen supplements inside tissues and compensatory increased respiration as a result (Meyer and Bullock, 1973; Walters and Plumb, 1980; Miyazaki and Kaige, 1985; Mohanty and Sahoo, 2007 and Vasquez-Pineros et al., 2010). The Pathogenesis of edwardsiellosis is complex and associated with multiple virulence factors that might be encoded in the chromosome or in conjugative plasmids (Wang *et al.,* 2009). The recorded mortalities of the experimentally infected fishes may be attributed to the presence of more than one virulent gene in the same strain as well as the synergistic effects conferred by combination of several virulence genes including gadB (Shen and Chen, 2005). Besides, the *esrB* and mukF play an important role in virulence of *E. tarda* (Rao et al., 2003; Rao et al., 2004 and Zheng et al., 2005), that appear to confer survival and replication within macrophages (Okuda et al., 2009) or causes pore formation in the host cell membrane (Tan et al., 2005).
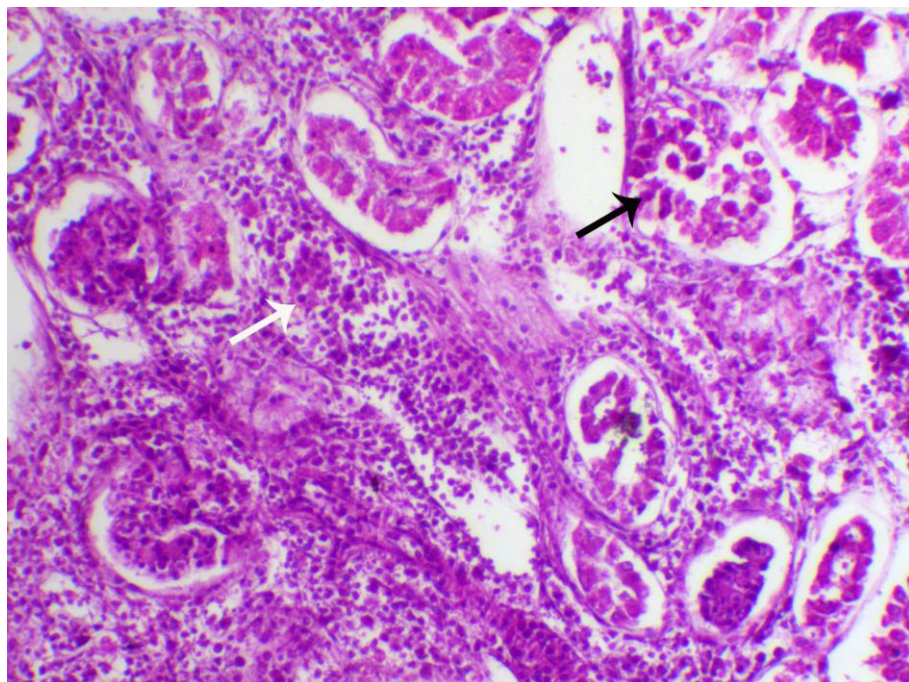

Figure 6. Posterior Kidney of *Oreochromis niloticus* infected with *Edwardsiella tarda* showing interstitial nephritis represented by marked leukocytic infiltration (White arrow) and degenerative tubules (Black arrow), H&E, bar=100µm, ×200
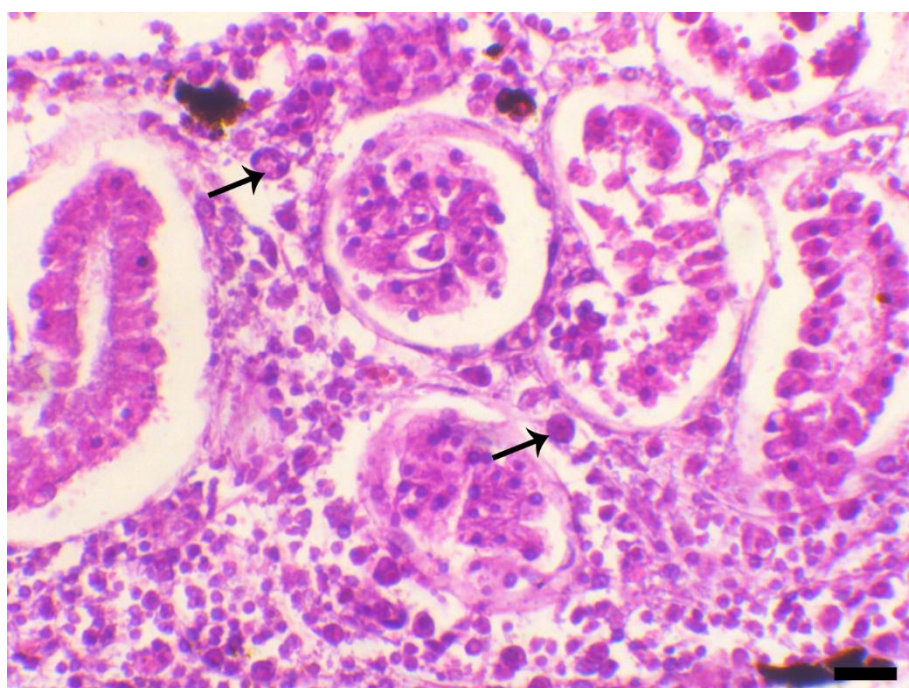

Figure 7. Posterior Kidney of *Oreochromis niloticus* infected with *Edwardsiella tarda* showing leukocytic infiltration consistent mainly from macrophages-laden bacteria (Black arrow), H&E, bar=50µm, ×400

The histopathological findings in the experimentally infected *Oreochromis niloticus* in the present study were obviously seen in the kidney. The kidney showed multifocal interstitial nephritis accompanied with infiltration of mononuclear inflammatory cells and macrophages-laden bacteria (Figure 6 and figure 7). While the liver showed also multifocal necrotic areas associated with inflammatory cells infiltration. These findings were nearly similar to those reported by Darwish et al.*,* 2000; Miwa and Mana, 2000; Shabaan, 2007 and Ibrahim et al.*,* 2011. The findings of histopathological changes could be attributed to the potential virulence factors of *E. tarda* as cell adhesion factors, cell invasion activity, hemolysin, cytotoxin and enterotoxin which might induce the necrosis and degenerative changes in most organs as well as the hepatic and nephric virulence factors (Chen et al.*,* 1996; Miwa and Mana, 2000 and Mathew et al.*,* 2001).

CONCLUSION

E. tarda is a gram negative, rod-shaped, motile bacterium that exhibits a broad geographical distribution and host range, causing significant economic losses to the aquaculture industry. The results of the present work, the three tested virulence genes were correlated with *E. tarda* pathogenicity. The more the number of virulence genes the more the pathogenecity of *E. tarda*. This could be of importance in formulating novel therapeutics and in vaccine development to protect fish against systemic edwardsiella infection.

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Competing interests
Authors have declared that there is no competing interest.

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