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Biofilm of *Edwardsiella tarda* isolated from fresh water fishes and its role in the bacterial virulence

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ABSTRACT

The Pathogenic *Edwardsiella tarda* is the causative agent of edwardsiellosis which infected marine and freshwater fishes and has many virulence factors which enhance pathogenesis of bacteria in fishes and cause severe losses in aquaculture . In this study *Edwardsiella tarda* was isolated from 150 diseased fish samples (60 Nile tilapia (*Oreochromis niloticus*), 60 African catfish (*Claris gariepinus*), 30 (Mullet (*Mugil cephalus*) (bayad). from internal organs after clinical and postmortem examinations The results revealed that, about Twenty-four isolates of *Edwardsiella tarda* 12 (20%); 7 (11.67%) and 5 (16.66%) were isolated from *C. gariepinus* ,*O. niloticus*, and *M. cephalus* fishes respectively. Eight strains formed strong formation biofilm (black colonies), eleven moderate and Six negative on Congo red agar. Eight out Twenty-four showed resistance to Five Antimicrobial agents, (oxsacillin, ampicillin, sulfamethoprim, gentamicin and norfloxacin. The pathogenicity of the *Edwardsiella tarda* in the 8 resistance isolates are positive for production of chondroitinase enzyme *cds*1 gene, Nacylhomoserine lactones *edwI* gene, vibrioferringene *pvsA* gene and sensor protein implicated gene *qseC* . The sequences obtained for *qseC* and *edwI* genes had accession number MW362141 and MW362142at GenBankand were identical to the corresponding GenBank sequences.

1. INTRODUCTION

Edwardsiella tarda (E. tarda) is member of the Enterobacteriaceae which produce H2S and indole, found in Water, mud and reptilian intestines, eels, catfish and marine mammals. *E. tarda* infection in fish is characterized by surfacing with a corkscrew swimming movement, postmortem examinations of infected fishes showed loss of pigmentation, opacity of the eyes, swelling of the abdominal surface, petechial hemorrhage in fin and skin, ulceration, enteritis while in case of chronic infection showed red swelling on the head, swollen anus due to the accumulation of fluid, abscesses in muscle, liver and kidneys and hemorrhagic septicemia (Plumb 1999; Abbott and Janda 2006; Park et al. 2012; Markey et.al. 2013). *E. tarda* has several virulence factors which enable it to survival and pathogenesis in fishes. Siderophores is an iron acquisition system and one of the virulence factors of *E. tarda* which are necessary for pathogenicity and provide *E. tarda* with iron from host to survive and replicate in the host environments. Vibrioferrin siderophore one type of siderophores was encoded with many genes as *pvsA* , *pvsD, pvsE* and *pvuA* genes in the genome of *E. tarda* and found in many gram-negative bacteria as *Edwardsiella species* and *Vibrio species* (Yamamoto et al. 1994). Another virulence factors in *E. tarda* was chondroitinase Enzyme which is hydrolytic enzyme in case of chronic infection of *E. tarda* lead to degradation of cartilage of fishes ('hole-in-the-head) lesion (WaltmanShotts and Hsu 1986; Cooper et.al.1996.; Schaechter et al. 1998).

The ability of *E. tarda* to form biofilm is consider one of the important virulence factor of bacteria as bacteria lives in biofilms are tough to remove from surfaces, and has the ability to resist to antimicrobial agents and resist the immune system of host, easy to adhere to host tissues lead to relapses of the infection, outbreaks of serious diseases and virulence factors production (Oana and Tim 2011). For formation of biofilm bacteria must reach quorum sensing (a self-regulation behavior) which is a method of communication between bacteria that enables bacteria to reach certain density form biofim matrix (Brownand Smith, 2003). Many genes in *E. tarda* help it to reach to quorum sensing for formation of biofilm by transcriptional activator and an autoinducer which is quorum sensing signaling molecules act to genes expression upon the basis of cell density to form complex cell–cell communication systems to reach to certain density for formation biofilm mass such as switching between the flagella gene and the gene for pili for the development of a biofilm and reach to Biofilm maturation, regulates social behaviours and secretion of virulence

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(March and Bentley2004, Williams et al. 2007; Romero et al. 2014). Two genes in *E. tarda* act as an autoinducers which help in the production of virulence factors as N Acylhomoserine lactones and sensor protein implicated in quorum sensing BC system (Ma et al. 2018). N Acylhomoserine lactones (AHLs) are a class of signaling molecules involved in bacterial quorum sensing. Self-regulation behavior (autoinducer) for reach to quorum sensing and control the expression of virulence factors secretion, production of exoenzyme and biofilm formation in gram-negative fishes bacteria (Morohoshi et al. 2004; Defoird et al. 2005). Another autoinducer genes help in forming biofilm in *E. tarda* is *qseC* which is sensor protein in quorum sensing consider as global regulator of many phenotypes as virulence genes, biofilm formation and regulate the expression of genes of flagella and motility and secretion system enhancing the pathogenicity of *E.tarda* (Xin et.al.2011; Weigel and Demuth 2015).
The current study aimed to detected biofilm formation of E. tarda.

2. MATERIAL AND METHODS

2.1. Samples collections
The samples were taken from 150 diseased fish (spleen, livers, gilles , muscles and kidney) represented by 60 *Oreochromis niloticus* ,60 *Claris gariepinus*, and 30 *Mugil cephalus* were examined from different fish markets at Qalyubia Governorate for bacteriological examination. The examined fish were of different ages and both sexes and subjected to clinical and postmortem examination. Samples were taken from internal organs.

2.2. Bacteriological examination
The samples from internal organs (kidney, liver, spleen, gilles and muscles) were inoculated in the Tryptic Soya broth (Oxoid) and then incubated at 30°C for 24 h. Loopful from Tryptic Soya broth was inoculated on Macconkey agar (LABM045) , XLD (HAMEDIA) and. S.S. (HAMEDIA) agar media at 30°C for 24 h. target colonies (black colonies without fermentation of lactose in SS agar and XLD while pale on Macconkey agar) were picked up and was inoculated on Tryptic Soya agar (LAB011) then incubated at 37°C for 24 h. for biochemical identification according to (Lima *et al*. 2008; Markey et.al., 2013).

2.3. Biofilm of Edwardsiella tarda isolates was conducted following to (Pramodhini et al., 2012)
The detection of biofilm production by using Congo Red Agar (CRA) medium.(37g\L of Brain Heart Infusion broth (HIMEDIA), 10 g/L agar No. 1 (HAMEDIA) , 50 g/L Sucrose (ADWIC) and Congo Red indicator (alpha chemika) 8 g/L) Congo Red stain was prepared as a concentrated aqueous solution separately and all medium autoclaved (121oC for 15 minutes) as it qualitative method. Then inoculated plates incubated at 37ºC for 24 h aerobically. Black colonies with a dry crystalline consistency indicated strong biofilm production and Weak biofilm producers remained pink.The experiment was performed in triplicate and repeated three times.

2.4. Antimicrobial sensitivity test of Edwardsiella tarda isolates were done according to (Markey et.al.,(2013)
By using the following antibiotic discs: ampicillin (AMP10mcg), trimethoprim + sulfamethoxazole (COT23.75\1.25mcg), oxacilline (oxiod OX1mcg) , gentamicin (GEN10mcg) and norofloxacin (NX10mcg) All antibiotic discs were obtained from HIMEDIA INDIA except oxacilline from OXIOD.

2.5. Detection of some virulence genes of biofilm producing Edwardsiella tarda strains by PCR
The Four specific primers, Metabion (Germany) in Table (1) were used to detection of some virulence factors and biofilm formation *cds1), edwI (, qseC* and *pvsA* , Genomic DNA Extraction from *E. tarda* strains were done by using Patho Gene-SpinTM DNA/RNA Extraction kit iNtRON cat. No. 17154 Korea, Preparation of PCR Master Mix , temperature and time conditions of the primers during PCR according to Emerald Amp GT PCR mastermix (Takara)Code No.RR310Akit as shown in table (1): negative control (sterile distilled water), positive control (strain of Edwardsiella local isolates(AHRI) and 100- to 3000-bp ladder (BIO-HELIX Co.,LTD were loaded to 1% (w/v) agarose gel electrophoresis for (30-45 minutes at room temperature)at 1-5 volt/cm in TAE (Tris-acetate-EDTA). The gel was transferred to UV cabinet and photographed by a gel documentation system and the data was analyzed through computer software according to Sambrook *et al.,(*1989)

2.6 The traditional Sanger technology with the new 454 technology method Sanger et al., (1977)
The genomes be sequenced and analyzed **.** Purification PCR product was done using Thermo Scientific GeneJET PCR Purification kit Amino acid Sequences were done using the BioEdit sequence alignment editor ,CLUSTALX software for multiple sequence alignment and were compared with other strains published on GenBank using BLASTsearch programs, (National Center for Bio-technology Information NCBI "http://www.ncbi.nlm. nih.gov/), The phylogenetic trees were constructed using MegAlign (DNASTAR, Lasergene®,Version 7.1.0. USA) for tree reconstruction of sequences by Neighbor-joining method based on ClustalW. Bootstrapping values

Table 1 Oligonucleotide primers sequences , and cycling conditions of the primers during PCR

Target gene	Primers sequences 5`-3	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			One cycle	Reference
cds1	TCTCCACCCATAATGCCACG	435 bp		2nd denaturation	Annealing	Extension	Final Extension	Castro *et al*., 2016
	CAAACGGCGTCGTGTAGTCG		94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
edwI	ATCCGCAGCATCGAATGGCT	360 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
	GAAGGATAACGATGTGGTGT							
qseC	CAGCAGTAGCAGGATCACCA	260 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
	ATGGACGTATGCTGCTCAAC							
pvsA	CTGGAGCAGTACCTCGACGG	313 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
	CGATGCTGCGGTAGTTGATC							

3. RESULTS

3.1 Postmortem examination-

Most fishes showed severe degeneration in the liver, and in the kidney, epithelial hyperplasia, edema and ulcer in gills and abdominal cavity was filled with ascetic fluid

3.2 Cultural and biochemical Characters of isolate

The recovered isolates in the present study are grow well on MacConkey agar giving non-lactose fermented colonies, Pale black colonies on SS Agar and Reddish/black centre (alk) in XLD agar Gram –negative, straight rods bacilli or cocccibacilli, oxidase, Citrate, Gelatin liquefaction, ONPG (beta-galactosidase), Voges–Proskauer, urease are negative while catalase, Lysine decarboxylase, Motility, H_2S, indol and Methyl red are positive. The numbers and level of positive samples are in Tilapia was 8/60(13.3%), in Catfish was 13/60 (21.7%) and in Mullet was 3/30. (10%)

3.3 Biofilm results of Edwardsiella tarda isolates

Twenty-four strains of *E. tarda* were applied to biofilm formation on Congo red agar. Five isolates gave pale or weak biofilm formation as in figure 1A. Twelve isolates gave moderate biofilm formation characterized by red colonies (Figure 1B), and Eight isolates gives strong biofilm formation (metallic black colonies) figure 1C.

3.4 Results of in vitro antimicrobial sensitivity tests (Antibiogram)

The sensitivity to different therapeutic agents was applied on the isolates showed variable degree of resistance to different used antibacterial Most isolate were resistance to oxacillin and ampicillin and sulphamethoprim while sensitive to gentamicin and norfloxacin as in table (2)

3.5 The results of detection some virulence genes of Edwardsiella tarda:-

As in table (3) and figures 4,5,6,7 four genes detected in the isolates E. tarda all isolates give positive to cds1, edwI, qseC, five isolates positive to pvsA as sample number 1 to 6 belonged to Catfish and Seven and Eight belonged to Nile tilapia.

3.6 sequence of edwI (AHL-synthase), qseC (sensor protein implicated in quorum sensing), of E. tarda

The sequence were submitted to Gene Bank and have accession numbers (MW362142 for *edwI* and MW362141 for *qseC*). The sequences obtained were identical to the corresponding GenBank sequences which isolated from different sources and different countries as shown in fig(8 and 9) in phylogenetic tree

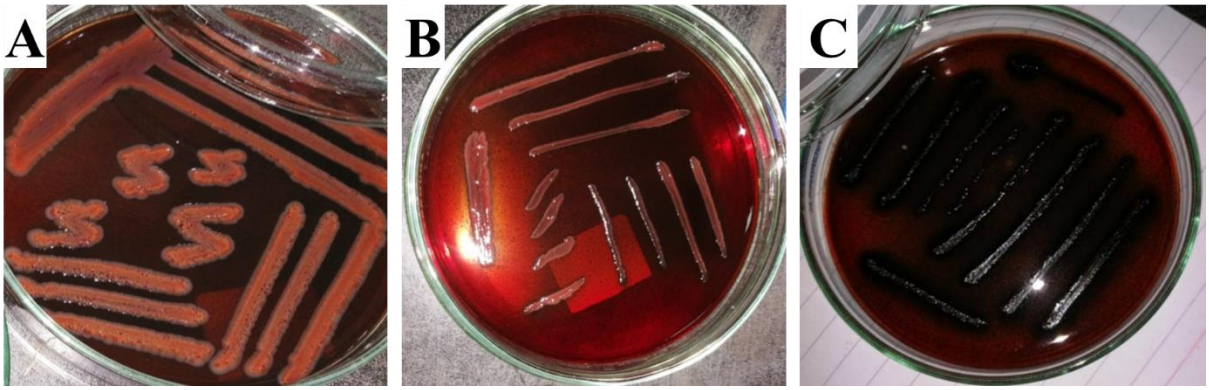

Fig. (1) of *E. tarda* biofilm formation on Congo red agar. Note: Strong biofilm formation (metallic black colonies) in the pic.(C)

Table 2 In-Vitro anti-microbial Sensitivity test for the isolates according to the reference for interpretation

Type of fishes And NO.	Sxt		gentamicin			ampicillin		oxacillin		Norfloxacin	
Total number of isolates	R	S	R	m	S	R	S	R	S	R	S
Catfish(13)	6	7	6		7	9	4	9	4	6	7
Nile tilapia (8)	2	6	2		6	4	4	4	4	2	6
Mullet*(3)*	0	3	0		3	1	2	1	2	0	3

R = Resistant S = Sensitive

Table 3 Results of four detected virulence genes in *E. tarda*

Samples	*cds1*	*edw1*	*qseC,*	*pvsA*
1	+	+	+	_
2	+	+	+	+
3	+	+	+	_
4	+	+	+	+
5	+	+	+	+
6	+	+	+	_
7	+	+	+	+
8	+	+	+	+

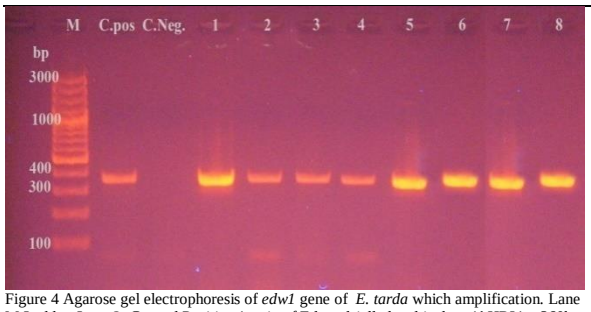

Figure 4 Agarose gel electrophoresis of *edw1* gene of *E. tarda* which amplification. Lane M:Ladder, Lane 2: Control Positive (strain of Edwardsiella local isolates(AHRI)at 360bp Lane 3: Control Negative, Lane 1-8: samples are positive at 360bp

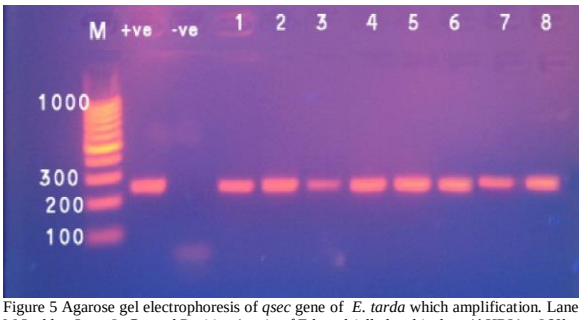

Figure 5 Agarose gel electrophoresis of *qsec* gene of *E. tarda* which amplification. Lane M:Ladder, Lane 2: Control Positive (strain of Edwardsiella local isolates(AHRI)at 260bp Lane 3: Control Negative, Lane 1-8: samples are positive at 260bp

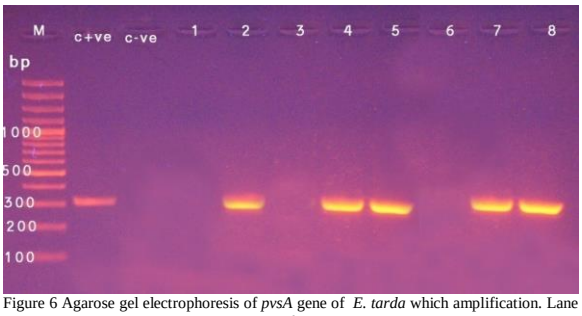

Figure 6 Agarose gel electrophoresis of *pvsA* gene of *E. tarda* which amplification. Lane M:Ladder, Lane 2: Control Positive (strain of Edwardsiella local isolates(AHRI) at 313bp Lane 3: Control Negative, Lane 2, 4, 5, 7, and 8: samples are positive at 313bp Lane 1, 3, and 6: samples are Negative

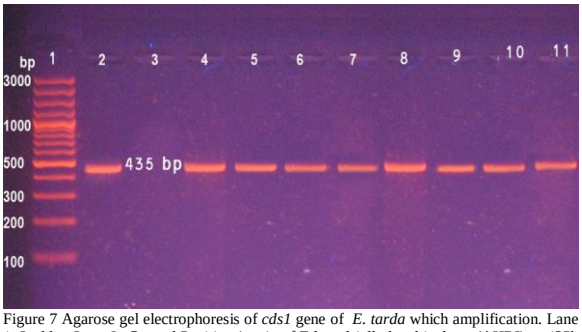

Figure 7 Agarose gel electrophoresis of *cds1* gene of *E. tarda* which amplification. Lane 1: Ladder, Lane 2: Control Positive (strain of Edwardsiella local isolates (AHRI) at 435b Lane 3: Control Negative, Lane 1-8: samples are positive at 435bp

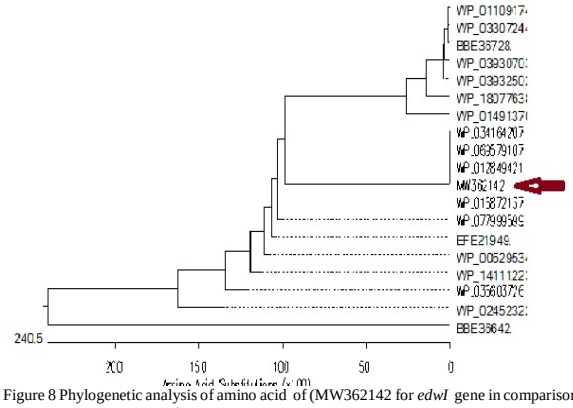

Figure 8 Phylogenetic analysis of amino acid of (MW362142 for *edwI* gene in comparison with other selected strains form gene bank

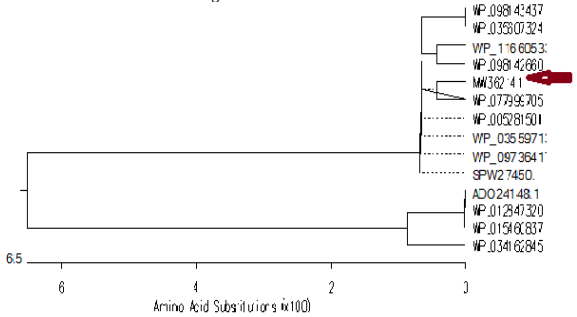

Figure 9 Phylogenetic analysis of amino acid of (MW362141 for (***qseC***) gene in comparison with other selected strains form gene bank

4. DISCUSSION

E. tarda is a member of the Enterobacteriaceae. Which capable of producing H2S and indole , found in Water, mud and reptilian intestines, infecting fresh water and marine Fishes and mammals . in Egypt .in our study isolated 24\150 (8%) strains from,(60 Nile tilapia (*Oreochromis niloticus*), 60 Catfish (*Claris gariepinus*), 30 (Mullet (*Mugil cephalus*). Many studies detected E. *tarda* nearly similar to this incidence as Eissa et al. (2016) detected the incidence of E. tarda isolates (9.6%) among all examined marine fishes, while Abd El-tawab et.al. (2020) detected the incidence of *E. tarda* isolates 21% from *O.niloticus* and *C. gariepinus*. In addition, Adanech and Kassa (2018) isolated *E. tarda* from 12 *Clarias gariepenus* and 88 *Oreochromis niloticus*. The ability of *E. tarda* to form biofilm for resisting the undesired environmental changes, and for intracellular living. ,adhere , invade and replicate in host cells. In the present study 8 out of 24 isolates give strong biofilm formation and 11 isolate give moderate biofilm. This results agreed with Michael et.al. (1991) who recorded that most isolates of *E. tarda* positive on congo red medium. *edwI,* and *qseC* genes which help *E. tarda* to reach to Quorum sensing and formation biofilm, in this study the two genes detected in all isolated *E. tarda* which they positive on congo red agar and were Sequencing, phylogenetic analyses and has accession numbers MW362142 and MW362141 were showed similarity to those in gene bank which isolated from fishes in different countries. The results had confirmed the virulence of the obtained isolate and agree with Castro et al. (2016) and Sherif et al (2020) studies on *E. tarda* have identified many virulence factors associated with its pathogenicity as chondroitinase enzymes which effect on fish cartilage lead to destroyed it in chronic infection (Shotts and Cooper 1992; Xu et al. 2013) In this study all isolates detected for chondroitinase enzyme gene *(cds1*) were positive which agree with Castroet al. (2016) who recorded

that gene encoding a chondroitinase was present in all the European turbot isolates of *E. tarda*, which similar to the gene present in the EIB202 strain with Asian origin., *E. tarda* vibrioferrin is a type of the siderophores that provide *E. tarda* to iron which essential to growth in host and expressed to its virulence factors that helps in the survival and replication of *E. tarda* Kokubo et al. (1990) in this study one type of vibrioferrin (*pvsA*) gene was detected in the half of detected isolate many authors detected vibrioferrin in *E. tarda as* Castro et al. (2016) who detected four types of vibrioferrin in *E. tarda*.

5. CONCULOSIONS

There is a link between the presence of virulence genes of the *E. tarda* infected fishes and its responsible for biofilm formation. Virulence-related genes typically involved in bacterial pathogenesis as the production of vibrioferrin siderophore (*pvsA*) gene and chondroitinase as chondroitinase enzyme gene (*cds1*). Detected of virulence factors of *E. tarda* may help in new treatment of *E. tarda* and new ways to control it.

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