Characterization of *Edwardsiella* Species Isolated from Fish by Using Genomic DNA Fingerprinting Technique

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Abstract:

Nowadays, *Edwardsiella* is considered the most dangerous bacterial problem in cultured fish farms. In this study, 100 diseased Nile tilapia (*Oreochromis niloticus*) and 100 diseased sea bass (*Dicentrarchus labrax*) were collected randomly from kafer El-sheikh cultured fish farms and also from Damietta Government during the period from May 2016 till May 2017. All diseased fishes showed hemorrhages, all over the body surface accompanied by swelling of abdomen were observed clinically and post-mortem. Liver, kidney and spleen samples were subjected to bacteriological examinations, for isolation of *E. tarda*. The suspected isolates were characterized morphologically and biochemically including microbact 24. PCR technique was applied for detection of some virulence genes (*etf*A, *etf*D, *gyer*B genes) in *E. tarda* isolates. Total prevalence of well identified *E. tarda* isolates was 12% in *Oreochromis niloticus* while 7% in *Dicentrarchus labrax*. Most *E. tarda* isolates were sensitive to ciprofloxacin, streptomycin chloramphenicol and gentamycin, conversely, most of isolates were resistant to tetracyclin, nalidixic acid, ampicillin, and sulphamethoxazole. PCR results recorded that all recovered *E. tarda* isolates contain *gyer*B at 415bp and *etf*D at 445 bp, while not all *E. tarda posses etf*A at 415bp.

Keywords: *E. tarda, Oreochromis niloticus, Sea bass, PCR*

Introduction:

In Egypt, majority of fish farms can be classified as semi-intensive earthen pond farms, Nile tilapia (*O.niloticus*) in fresh water fish and sea bass (*Dicentrarchus labrax*) in marine fish are the two main species reared in these farms ***(Younes et al., 2015)***

Bacterial agents are considered highly encountered causes of diseases in environment stressed on cultured fish in warm water ***(Castro et al 2016).*** *E. trada* causes Edwardsiellosis in freshwater and

marine fishes of both farmed and wild population all over the world ***(El-Jakee et al., 2008).*** This pathogen is intimidate the aquaculture industry worldwide, due to devastating economic losses incurred ***(Loh et al ., 2014).***

E. tarda has been shown that different strains, serotypes, genotypes and biotypes of bacterial pathogens vary in their ability to cause disease within aquaculture ***(Das et al., 2014)***, so accurate identification and characterization of a pathogen was important for both control and epidemiological investigations**.**

Detection of type I fimbrial gene of *E. tarda* by PCR is important factor to confirm identification and pathogenicity of *E. tarda* isolates. *E. tarda* has acquired resistance to most antimicrobial agents when causing disease, may be difficult to control that it due to intracellular infection ***(Okuda et al., 2014).*** This strain was resistance to tetracyclines. On the opposite side, it was highly susceptible to cholomphenicol ***(Noor El Deen et al., 2017)***.

Material and Methods

Samples:

Fishes showed any clinical abnormalities on the external body surface such as scale detachment, skin ulceration, hemorrhages and swollen abdomen with yellowish ascetic fluid were collected aseptically from fresh and marine farms, each sample was marked and placed in an ice box and transferred to the laboratory for bacteriological examination according to ***(Austin and Austin, 2007).***

Isolation and identification of *E. tarda:*

Samples of examined fresh and marine fish were gathered aseptically from internal organs as (kidneys, liver, spleen) and homogenized separately in sterile stomacher bags, then inoculated in Brain Heart Infusion (BHI) broth and incubated under aerobic condition at 35 c° for 24 hrs. Then cultivated on S.S agar for 24 hrs. ***(Wei and Musa, 2008)*** small clear transparent colonies with black center and pink peripheral ring with 1-2 mm in diameter were picked up and streaked on TSA slope containing 0.5 % NaCl at 37 c° for 24 hrs for further identification. Biochemical confirmation was carried by rapid identification system test strips (Microbact 24) according to***(Tinsley et al. 2010).***

Antibiotic Sensitivity of isolated *E. tarda*

According to ***(Xian-jieLiua, 2015)*** all recovered isolates were tested against 8 different antimicrobial discs included; CIP: Ciprofloxacin (5µg), SXT: sulfadimethoxine (25µg), AM: Ampicillin (20µg), T: Tetracycline (30µg), C: chloramphenicol (30µg**),** K: kanamycin (30 µg**),** GN**:** Gentamycin (10 µg**),** NA: Nalidixic acid 30(µg**).**

PCR detection of *E. tarda* virulence genes

Detection of *etf*A, *etf*D, *gyr*B genes as virulent factors of *E. tarda* by using Primers as in table (1). Chromosomal DNA was extracted employing Insta-Gene Matrix (Bio-Rad) according to ***(Lan et al., 2008)***.

Three species-specific primer pairs described by ***(Sakai et al., 2007)*** were synthesized by Sigma-Genosys and employed in this work for the identification of *E. tarda*.

Table (1) *Oligonucleotide primers sequences for the detection of E. tarda*

Name of product	Sequence (5' to 3')	Target gene	ProductSize (bp)	Source
etfA	5'-CGG TAA AGT TGA GTT TAC GGG TG-3'	etfA (major fimbrial subunit)	415	*Sakai et al.* (2007)
	5'-TGT AAC CGT GTT GGC GTA AG-3'			
etfD	5'-GGT AAC CTG ATT TGG CGT TC-3'	etfD(fimbrial subunit)	445	*Sakai et al.* (2007)
	5'-GGA TCA CCT GGA TCT TAT CC-3'			
gyrBF1/gyrBR1	5'-GCA TGG AGA CCT TCA GCA AT-3'	gyrB (gyrase)	415	*Lan et al.* (2008)
	5'-GCG GAG ATT TTG CTC TTC TT-3'			

Results

Clinical and post-mortem examinations of *Oreochromis niloticus*

The most clinical signs noticed on the examined tilapia fish were congestion with increased mucous secretion, abdominal distention and full with offensive odor mucous exudate, hemorrhage, eroded fins and wounds at the base of the dorsal and caudal fins as in photos 1, 2

Clinical and post-mortem examination of *Dicentrarchus labrax*

The most clinical signs showed abdominal swelling, abdomen filled with exudate in addition to offensive odor beside hemorrhage on fins and tail. Gills showed nearly brown coloration with sloughed parts in some cases, eye swelling and exophthalmia, scale detachment, enlargement of liver, kidney, and hemorrhage in the intestine as well as skin erosion and ulcer as in photos 3,4

The isolates appeared as G –ve short rod and appeared as small punctuate grayish white small, circular, raised, with black center on S.S agar. All isolated strains were oxidase negative. All isolates were positive for indol, Methyl Red, Catalase, H_2S production, and glucose fermentation. They were negative for Lactose fermentation, sucrose, Urease, Voges – Proskauer. Out of 19 strains, 5 strains showed citrate – ve about 26%

Prevalence of *E. tarda* in tilapia and sea bass

The *E. tarda* isolates recovered from internal organs of tilapia fish were 4.1% in liver, 3.3% in kidneys and 2.5% in spleen as in table (2), while in sea bass were 4.2 in liver and 2.8% of both kidney and spleen, as in table (3).

Antibiotic sensitivity.

Results showed that most of isolates were sensitive to ciprofloxacin, streptomycin, chloramphenicol, and gentamycin, conversely, most of isolates were resistant to tetracycline, nalidixic acid, ampicillin and sulphamethoxazole

Molecular identification of *E. tarda* and it is virulence genes

In this study, 5 DNA extracted from *E. tarda* isolates contain Major fimbrial subunit gene *etfA*, visible bands appeared at 415bp as shown in photo 5. While, 8 DNA extracted from *E. tarda* isolates contain Major fimbrial subunit gene *etfD*, visible bands appeared at 445bp as shown in photo 6

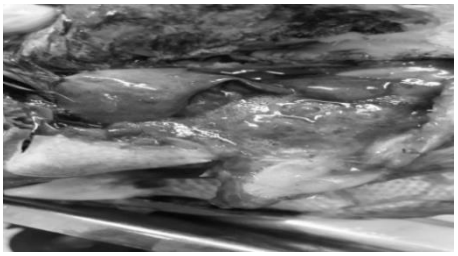

Photo1. Congestion and skin erosion

Photo 2. Abdomen with offensive exudates

Photo 3. Exophthalmia, corneal opacity **Photo 4. Congested internal organs**

Phenotypic identification:

Table (2) *Distribution of E. tarda in internal organs of tilapia:*

	Samples	Positive *E. tarda*		Liver		Kidney		Spleen	
Nile tilapia	No.	No.	%	No.	%	No.	%	No.	%
	100	12	12%	5	4.1	4	3.3	3	2.5

Table (3) *Distribution of E. tarda in internal organs of sea bass:*

	Samples	Positive *E. tarda*		Liver		Kidney		Spleen	
Sea bass	No.	No.	%	No.	%	No.	%	No.	%
	100	7	7%	3	4.2	2	2.8	2	2.8

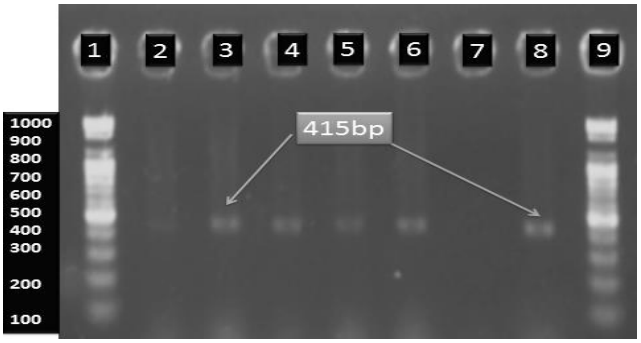

Photo (5)Agarose gel electrophoresis of PCR products using specific primers targeting Major fimbrial subunit gene (*etfA*) in *E. tarda* isolates. Lanes1 and 9 M: DNA ladder (100 bp; lane 8: control positive; lanes 3, 4, 5 and 6 DNA extracted from *E. tarda* isolates showing bands at 415 bp; lane 2 extracted DNA negative and 7: negative control (sterile saline).

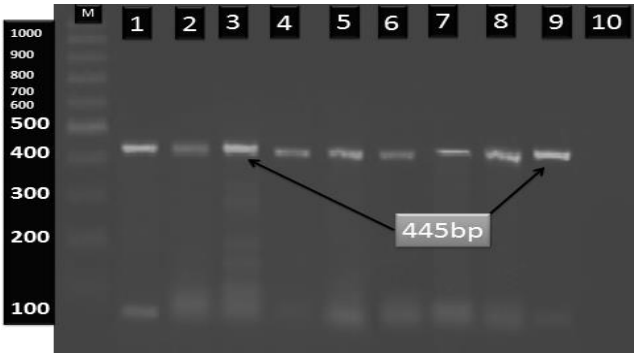

Photo (6)Agarose gel electrophoresis of PCR products using specific primers targeting Major fimbrial subunit gene (*etfD*) in *E. tarda* isolates. Lane M: molecular weight marker 100 base pair; lane 9: control positive; lanes 1-8: DNA extracted from *E. tarda* isolates showing bands at 445 bp and lane 10: negative control (sterile saline).

Discussion

E tarda infection is considered a dangerous septicaemic pathogen with high economic losses and of highly encountered causes of diseases in stressed warm water aquaculture ***(Ibrahem et al., 2011).*** The clinical examination of examined tilapia fish revealed number of clinical signs as abdominal distension, hemorrhages, congestion of fins with ulcers and erosion in addition to presence of ascitic fluid. Such results were agreed with those reported by ***Noor El Deen (2017).*** On the other hand, the clinical examination on sea bass revealed erosion and skin ulcers, eye swelling, hemorrhage on fins in addition to congestion and

enlargement of internal organs and intestinal hemorrhage with accumulation of exudate in the abdomen ***(Ibrahem et al., 2011 and Abdelrahem, 2016).***

The results revealed prevalence of *E. tarda* among the examined tilapia was 12%, this result nearly agreed with ***Korni et al. (2012)*** who recorded prevalence of *E. tarda* in tilapia by 13.13 %. Our results also higher than ***Sayed et al (2015)*** who isolated *Edwardsiella* by 3.7 %. On the other side ***El – Seedy et al. (2015)*** isolated *Edwardsiella* by 4.3 %. It was found that *Edwardsiella* isolated from sea bass as7 %. This result is lower than that recorded by ***Abdelraheim (2016***) who recorded isolation of *E. tarda* by 11.1 among examined marine fish.

The difference in the prevalence of *E. tarda* either in tilapia or sea bass may be attributed to the difference in water temperature, seasonal variation and location of study, the nutritional status of the fish ***Nuru (2007)***.

E. tarda affect intestine, liver, and kidney of sea bass and tilapia, the highest percentage of the pathogen was isolated from liver. This could be due to the metabolic activities of the organs The higher percent of *E. tarda* in tilapia fish (fresh water fish) than Sea bass (Marine fish) may refer to other factors which act as stress predisposing factors as recorded by ***Korni et al. (2012)***

The results of *E. tarda* sensitivity agree with ***Noor El Deen et al. (2017)*** and disagree with ***Eissa et al. (1994)*** who recorded that *Edwardsiella* isolates sensitive to oxytetracycline ,also disagree with ***Lee et al. (2011)*** that recorded that most of *Edwardsiella* isolates sensitive to Ampicillin and Nalidixic acid, intermediate to tetracyclin and resistant to sulphamethoxazole, disagree with ***Ogbonne et al. (2018)*** who recorded that all isolates were resistant to Chloramphenicol , streptomycin and also our results disagree also with ***Wimalasena et al. (2018)*** who mentioned that all or most of isolates were susceptible to tetracyclin, Nalidixic acid. This could be attributed to the difference in location, change of environmental factors and also it could be due to the variation of genotype and phenotype of fishes. The difference in sensitivity and resistance of *Edwardsiella* isolates may be refer to the intra cellular infection of *Edwardsiella* making it less sensitive to antibiotics ***Okuda et al. (2014)***

In this study, DNA extracted from *E. tarda* isolates contain Major fimbrial subunit gene *etfA and eftD* genes visible band appeared at 415bp and 455 bp as shown in photos (5, 6) our results agree with ***El-Seedy et al. (2015)*** who used major fimbrial subunit gene (*etfA*) and *(gyrB*) gene for PCR while disagree with ***Noor El Deen et al. (2017)*** who used (*esrB*), and(*gadB*) genes as virulent factors of *E. tarda*. PCR method considered as a rapid and accurate method for diagnosis of *E. tarda* and has high sensitivity and specificity and can

improve the level of detection within few hours. The difference in the result may be due to the *E.tarda* isolate was from different types of fish and different areas of study in addition to different environment

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الملخص العربى

يعتبرميكروب الايدواردسيلا من الميكروبات التى تتواجد بشكل طبيعى الا انه قد يسبب مشاكل مرضية اذاتعرضت الاسماك لعوامل الاجهاد ولذلك فقد تناولت هذه الدراسة تصنيف وعزل ميكروب الايدواردسيلا على عدد 200 سمكه من المياه العذبة والمالحة ممثله فى (100 سمكة بلطى نيلى و 100 سمكة قاروص) والتى جمعت عشوائيا من مزارع خاصة فى محافظتي كفرالشيخ ودمياط فى الفترة من مايو 2016 الى مايو 2017 وقد تم اجراء الفحوصات الظاهرية والبكتيريولوجية والبيوكيميائية وقد تم اخذ العينات من الطحال والكبد والكلى وقد تبين من الدراسة مايلى :أعلى نسبه لبكتيريا الايدواردسيلا فى الكبد بنسبه 4.1% يلي الكلى بنسبة 3.3% ثم الطحال بنسبة 2.5% وكانت فى اسماك القاروص نسبه الايدواردسيلا فى الكبد 4.2% والكلى والطحال نفس النسبة2.8%. وتم عمل الفحص البيكتيريولوجى والاختبارات االبيوكيميائية وقد اسفرت النتائج عن نسبة الاصابة بالايدواردسيلا فى سمك البلطى(مياه عذبة) 12% اعلى من الاصابه بسمك القاروص(مياه مالحة) 7%. تم عمل اختبارالحساسية للمضادات الحيوية المختلفة كانت النتائج ان معظم العترات حساسة للكلورمفينيكول ,الاستربتومايسين ,السيبروفلوكساسين والجنتاميسين هذا وقد تم اختبارعدد من المعزولات التى تم عزلها اللتاكد انها تنتمى لجنس الايدواردسيلا بإختبارالبلمرةالجزيئية وأنها تحمل بعض جينات الضراوه