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Edwardesiellosis in fresh water fish with special reference for detection of some virulence genes by PCR

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Abstract
This study was performed forisolation of *Edwardesiella* spp. From fresh water fish and molecular identification of some virulence genesas the etiological agent of edwardsiellosis in many fish species. 50 *Oreochromis niloticus* and 50 *Clarias gariepinus*, were collected live from fish farms and markets respectively, in Kafrelsheikh Governorate, Egypt. Isolates obtained from the internal organs of examined fish subjected to bacteriological and biochemical identification. Some suspected isolates were confirmed by conventional PCR and three virulence genes highly associated with the pathogenicity of the isolates were identified. A total 21 isolateswere identified phenotypically as *E. tarda* from both fish species with the higher isolation rate from *O.niloticus* than *C. gariepinus.* Eight isolates were identified genotypically by *fimA* gene and were detected as three *Edwardesiella* species (37.5%) and these three isolates were confirmed as *E. tarda* by using *gyrB1* gene (100%). Regarding virulence genes, *cds1* was indicated in one out of the three isolates (33,3%), *pvsA* was in two isolates (66,7%) and *edwI* was absent (zero %) in the three isolates. In conclusion, Presence of these genes render *E. tarda* highly pathogenic so rapid control was required and fish handlers should take care to avoid infection as it has public health hazard.

Keywords: *Edwardesiellatarda*, Fresh water fish, PCR, Virulence genes

Introduction
The infectious diseases constituted a major constrain to aquaculture production with a consequent effect on the economic development, the bacterial agents were among the highly encountered causes of aquaculture diseases [1]. Edwardsiellosis was one of the most important bacterial disease occuring in both freshwater and marine fish such as carp, tilapia, eel, catfish, salmon, trout and flounder [2]. *Edwardsiellatarda* was the cause of septicemic disease with high economic losses in infected fish. Furthermore, it has public health hazards in humans including gastroenteritis, liver abscesses, meningitis, skin abscesses and valvular endocarditis [3, 4]. *Edwardsiellatarda* is a member of the *enterobacteriaceae* that infects fresh and marine water fish species. The outer surface of this bacterium have different antigenic components such as fimbriae and pilli that form different serovars and serotypes in different fish species [5]. *Edwardsiellatarda* virulence factors responsible for its pathogenicity includes stable enterotoxin and hemolysins, dermatonecrotictoxin, chondroitinase activity, complement-mediated resistance, hemagglutination mediated by nonfimbrialadhesins, and siderophore production, invasive ability and a type III (T3SS) and type VI (T6SS) secretion of virulence factors [6]. There were great differences in *E. tarda* biochemical characters specially Indole and H2S tests [7]. In addition to, several biochemical variations among *Edwardsiella* spp. as ornithine decarboxylase, citrate utilization, indole and hydrogen sulfide production, and fermentation of mannitol and arabinose [8, 9]. So, PCR was the rapid and confirmative method for diagnosis of *Edwardsiellatarda* [10]. Using the *ED* primer set targeted to the upstream region of fimbrial geneconsiders rapid technique for the detection of fish infected with *E. ictaluri* or *E. tarda* by PCR [11]. Moreover, the *gyrB1* gene was a suitable phylogenetic marker for the identification and classification of *E. tarda*in diseased fish [12, 13].
The present work was carried out to determine the incidence of *Edwardesiella* spp. in some fresh water fish species by bacteriological methods. In addition to molecular characterization (PCR) of some virulence genes as a marker of pathogenicity.

Materials and Methods

Fish Sampling

A total number of 100naturally diseased fish (50 *Oreochchromisniloticus* and 50 *Clarias gariepinsus*) were collected from fish farms and fish markets respectively, of different weights at Kafrelsheikh Governorate. *Oreochchromis niloticus* were transferred live in an areated plastic pags and *C. gariepinsus* to the laboratory of Animal Health Research Institute, Kafrelsheikh branch, Eygpt immediately and subjected to clinical examination according to [14], post-mortem examination according to [15].

Bacteriological Examination

A-Isolation: Aseptically, samples from internal organs (kidney, liver, spleen, intestine and gills) were inoculated in Trypticase soya broth (TSB) (Oxoid, UK) and incubated at 30°C for 24 hrs. followed by inoculation on Xylose Lysine Deoxycholate (XLD) agar (Lab M Limited, UK) according to [16] at 30°C for 24 hours. The suspected colonies were carefully selected and subcultured on MacCkonkey agar (Lab M Limited, UK) plates. All lactose non fermenting colonies (pale colonies) were subcultured on Tryptic Soy Agar (TSA) (Oxoid, UK.) plates for further identification.

B- Identification

I-Gram's staining forfilms of 24hrs cultures and motility were performed [17].

II-Biochemical: Catalase, Oxidase, Indole, Methyl red (MR), Voges- praskauer (VP), Citrate utilization, Urease, Hydrogen sulphide production (H2S), Gelatin hydrolysis, Nitrate reduction, Ornithine decarboxylase (ODC), Detection of L-lysine decarboxylase (LDC), Detection of Argininedihydrolase (ADH), Detection of β- galactosidase (ONPG) and Fermentation of sugars were carried out according to [18].

III-Molecular Identification of *Edwardesiella* SPP. And Detection of Virulence Genes by PCR

The DNA from random phenotypically identified isolates was extracted using QIA amp DNA mini Kit (Qiagen, Catalogue no. 51304). Species specific primer (*fimA* and *gyrB1*) were used. Primers were supplied from (Metabion, Germany). In addition, three selected virulence genes; *cds1, edwI, pvsA* genes, were investigated by conventional PCR. The primers used for amplification of different genes and sizes of PCR amplicons in the current study were listedin (Table1). *Edwardsiellatarda* (ATCC® 15947™) was used as a quality control strain, for other virulence genes Positive and negative controls were represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference laboratory for veterinary quality control on poultry production, Animal health research institute, Egypt. For PCR amplification; primerswere utilizedin a25 µl reactionscontaining 12.5µl of Emerald Amp GT PCR master mix (Takara, Japan), 1 µl of each primer (forward and reverse) of 20 pmol concentration, 4.5µl of PCR grade water and 6µl of template DNA then the reaction was performed in a T3 thermal cycler (Biometra) with primary denaturation at 94°C for 5 min. followed by 35 cycles each consisted of denaturation at 94°C for 30 sec., annealing according to each primer pairs for 40 sec. (Table 1), extention at 72°C for 45 sec. except *pvs* A and *edwI* were for 40 sec. and final extention at 72°C for 10min. The products of PCR were analyzed by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) and gels were photographed by Gel documentation system (Alpha Innotech, Biometra).

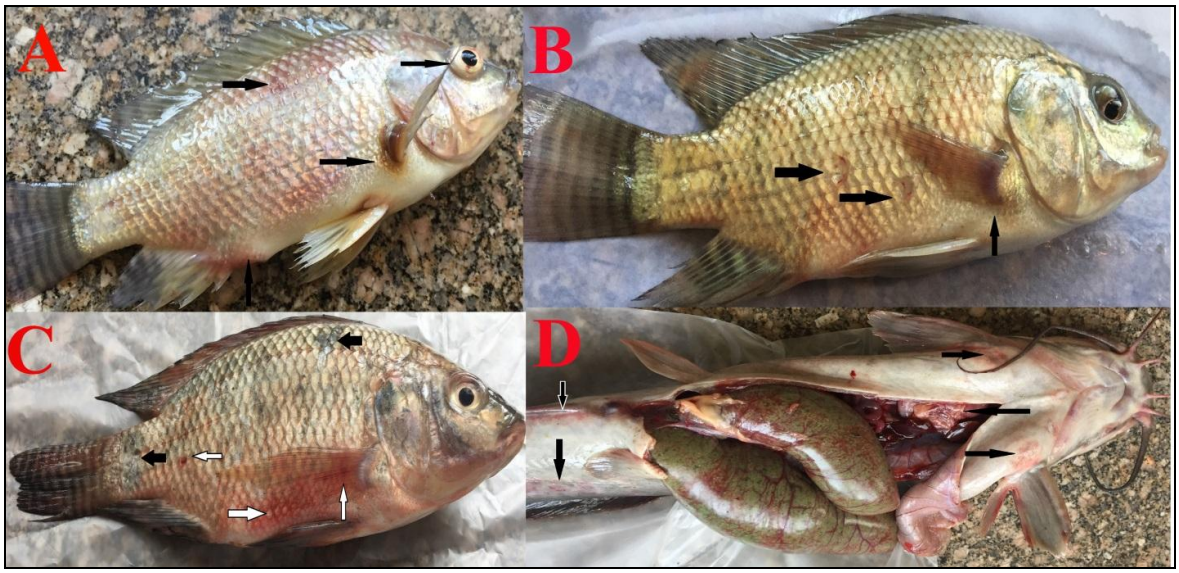
Table 1: primers used for PCR amplification of genes of *Edwardesiella* virulence associated genes and annealing temperature.

Target genes	Primers sequence (5` →3`)	Size bp[a]	°C[b]	Reference
fimA	F: ACAGCCTGGAAGAGTCCTAC	848	55	[11]
	R: TTGAGAGTCGCTGCTTAC			
gyrB1	F: GCATGGAGACCTTCAGCAAT	415	50	[19]
	R: GCGGAGATTTTGCTCTTCTT			
cds1	F: TCTCCACCCATAATGCCACG	435	55	[20]
	R: CAAACGGCGTCGTGTAGTCG			
edwI	F:ATCCGCAGCATCGAATGGCT	360	55	
	R:GAAGGATAACGATGTGGTGT			
pvsA	F:CTGGAGCAGTACCTCGACGG	313	55	
	R:CGATGCTGCGGTAGTTGATC			

[a]Base Pair of amplicon size, [b] Annealing Temperature, *fim*A: *Edwardsiella* speciesfimbrial gene, *gyr* B1: gyrase gene as taxonomic marker for *E. tarda*, *cds* 1: chondroitinase, *edw* I: AHL-synthase; *pvs*A: vibrioferrin synthesis.

Results

Clinical observation of naturally infected *O. niloticus* showed scales detachment, cutaneous haemorrhagic lesions, ulcers, gills erosion, exophthalmia and protruded hemorrhagic anus. While as *C. gariepinus* showed cutaneous lesions including skin discoloration, excessive mucous, hemorrhage and ulcer (Figure1).



A: Haemorragic patches all over the body, haemorragic protruded anus and exophthalmia.
B: Ulcer on abdomen and haemorrage at base of pectoral fin and skin.
C: Scale detachment and ulcer at the dorsal surface and caudal peduncle, haemorrhage on skin and fin.
D: *C. gariepinus* infected with *E. tarda* showing haemorragic patches, ulcers on ventral surface of abdomen; congestion of the pectoral fin and all internal organs.

Fig 1: *O. niloticus* naturally infected with *E. tarda* showing:

Post mortem (p.m)finding:liver showed enlargement, congested or pale coloration, withdistended gall bladder; while kidney and spleen were congested and inflamed; andabdomenal cavity wasfilled with ascetic fluidas well as enlarged emphysematous intestine free from food, (Figure 2).

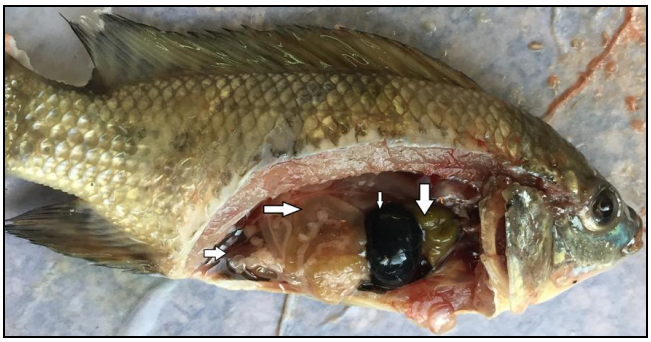


Fig 2: O. niloticus naturally infected with *E. tarda*was showing yellowish discoloration of the liver, distended gall bladder, distended intestine empty from food, congested enlarged spleen with ascetic fluid in the abdomen.

The morphological characters of colonies on MacConkey agar were pale colonies (non lactose fermenter) and on XLD agar were small circular colonies ranging from 1mm to 3mm in diameter with black centers, some without.
The isolates were motile and by Gram staining, Gram negative rods to coccobacilli shaped bacteria appeared.
Biochemical results: Catalase, Indole, MR, ODC, Nitrate reduction and LDC were positive but Oxidase, VP, Citrate utilization, Urease, Gelatin hydrolysis, ADH, ONPG and sugar fermentation were negative except (sucrose and arabinose were variable). H2S was positive for11 isolates however, it was negative in 10 isolates.
Based on phenotypic characters, out of 100 samples 21 *E. tarda* isolates were preliminary identified with prevalence 21% (12 (24%) isolates from *O. niloticus* and 9 (18%) from *C. gariepinus*) (Table 2).

Table 2: Showing prevalence of *E. tarda* isolation from *O. niloticus* and *C. gariepinus.*

Fish samples	No. of examined	No. of isolates	Prevalence (%)
O. niloticus	50	12	24
C. gariepinus	50	9	18
Total	100	21	21

The molecular characterization of random eight isolates, four isolates from *O. niloticus* and another four from *C. gariepinus* identified by *fimA* gene, resulted in three from the selected eight isolates were positive *Edwardesiella* spp. (37.5%) (Isolates No. 2, 6, 7from *O. niloticus*) (Figure3).

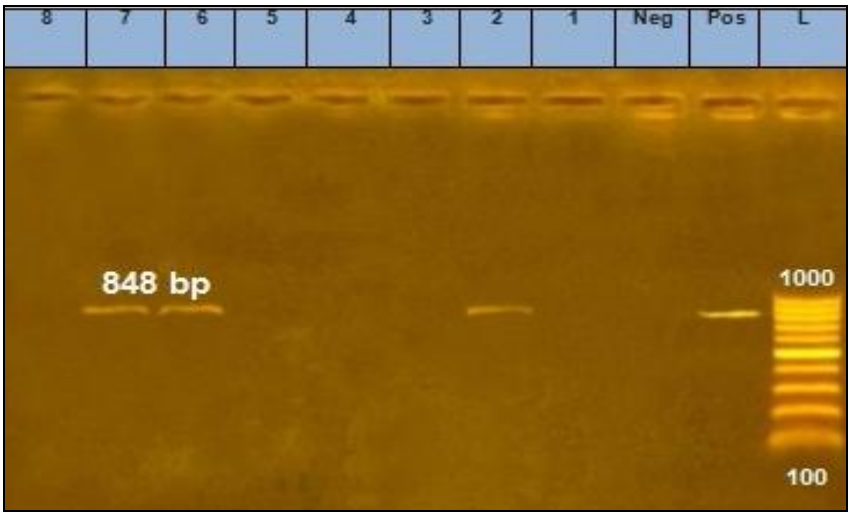


Fig 3: A garose gel electrophoresis of PCR of *fimA* gene (848 bp) for characterization of *Edwardesiella* species. L: 100 bp ladder as molecular size DNA marker. Pos and Neg: positive and negative control. Lanes 2, 6 and 7: Positive isolates for *fimA* gene. Lanes 1, 3, 4, 5 and 8: negative isolates for *fimA* gene.

By using *gyrB1* as a taxonomic marker for *E. tarda*, further identification to the three positive isolates for *fimA* at the species level were confirmed as *E. tarda* by (100%) (Figure4).

Regarding to the virulence genes: *cds1* gene was amplified in isolate number 7 only (33,3%), *pvsA* gene was in isolates No. 2 and 7(66.7%) while *edwI* gene was negative in all (zero%) (Figure4).

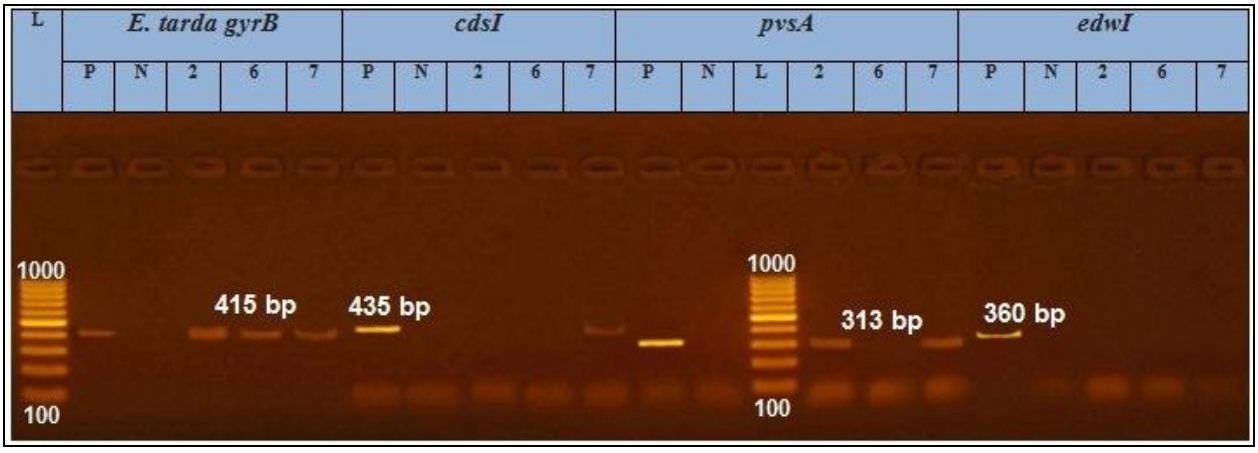


Fig 1: Agarose gel electrophoresis for virulence genes amplification in *E. tarda* isolates No. (2, 6 and 7). L: 100 bp ladder as molecular size DNA marker; P and N: positive and negative control; *E. tardagyr B*: 2, 6 and 7 positive isolates at 415bp.; *cds1*: negative in 2 and 6 isolates, isolate No.7 was positive to *cds1* at 435bp. ; *pvsA*: positive for isolates No. 2 and 7 at 313bp, negative for isolate 6; *edwI* gene: negative for 2, 6 and 7 isolates.

Discussion

Edwardsiella species was the cause of mass mortality in a considerable number of commercially important fish populations worldwide that associated with major economic losses [21]. *Edwardsiellatarda* was a heterogeneous species [22].

The clinical signs and p.m of examined fish were similar to that recorded with [21, 23, 24] recorded similar clinical and p.m signs in *E. tarda* infected cat fish that may be attributed to its virulence factors including extracellular products particularly haemolysine and adherence ability to the host surface by fimberiae.

In this study, isolated *Edwardesiella* spp. were Gram negative rods or coccobacilli, motile which grew well on XLD and MacConkey agar. These results go hand with that recorded by [25]. The conventional biochemical results were similar to [7] specially in H2S variable results [21]. reported variable H2S and sugar fermentation results. The high prevalence rate of *E.tarda* in this study, clearly its dangerous role in fish diseases in Egypt. The result was higher than [26] which the prevalence was (3.5%); [27] was (10.42%) but, the current prevalence rate was lower than observed by [28] who reported incidences of *E. tarda* in African cat fish and Nile tilapia at 50% and 34%, respectively [29]. Isolated similar colonies on XLD and by conventional biochemical methods found 8 isolates of *E. tarda;* 4 from cat fish and 4 from tilapia with prevalence rate 7.2% (8/111).

For the *Edwardsiella* species confirmation, *fimA* gene amplification was performed and was positive for DNA fragment at 848bp as obtained by [11]. The 3 isolates were from *O. niloticus* but no amplification occurred with isolates from *C. gariepinus.* Results of*gyrB1* gene at 415bp agree with [12, 30] but [31] employed strains did not generate any bands during amplification.

The conventional biochemical tests yielded more isolates of *E. tarda* than in molecular techniques that may propose the high degree of variable phenotypes within bacterial species which diminish the accuracy of biochemical identification [32].

Regarding the selected virulence genes in the genome of *E.*

tarda strains isolated from *O. niloticus*, *cds1* gene was positive in isolate number 7 only but the results of [20] were positive in all strains. *cds1* gene encodes chondroitinase activity that was the cause of hole in the head lesion in fish by cartilage degradation during invasion [33], *pvsA* gene was positive for isolate number 2 and 7. *pvsA* gene (vibrioferrin synthesis) representing presence of siderophore-mediated ferric uptake systems, supply *E. tarda* with replication and survival advantages in the marine environment as well as in iron-restricted host environments [20]. *edwI*gene was negative in all isolates in the current study at the reverse amplified in the strains according to [20], *edwI* gene encodedN-acylhomoserinelactones (AHL-synthase) which were indicative for quorum sensing(QS) systems, especially in Gram-negative bacteria [34]. The more virulent isolate was number 7that was positive for *pvsA* and *cds1* followed by isolate number 2 which contained *pvsA* only. At the reverse, the three selected virulence genes were absent in isolate number 6, this mean it may be less virulent or involve other virulence genes. Our results were suggesting a high degree of genetic heterogeneity among *O. niloticus* isolates. Thus, further studies on virulence and pathogenicity are required.

Conclusion

There were many virulent strains of *Edwardesiella* spp. causing sever diseases that greatly affecting on the industry of fish production due to fish mortality. Hence, rapid intervention is required depending on rapid accurate diagnosis by PCR to control spread of infection and avoid public health hazard. The isolate No.7 was the most virulent one. The siderophores related gene (*pvsA*) is important for both multiplication and virulence of bacteria, this result may serve as a very useful tool in the development of new antibacterial agent against *E. tarda* infections.

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