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 for isolation of Edwardesiella spp. From fresh water fish and molecular identification of some virulence genes as 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 Kafr-elsheikh 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 isolates were 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, cdsl was indicated in one out of the three isolates (33.3%), pvsA was in two isolates (66.7%) and edw1 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: Edwardesiellalatardata, 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]. Edwardsiellalatardata was one of the most important bacterial disease occurring in both freshwater and marine fish such as carp, tilapia, eel, catfish, salmon, trout and flounder [2]. Edwardsiellalatardata 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]. Edwardsiellalatardata 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 pili that form different serovars and serotypes in different fish species [5]. Edwardsiellalatardata virulence factors responsible for its pathogenicity includes stable enterotoxin and hemolysins, dermatonecrotictoxin, chondroitinase activity, complement-mediated resistance, hemaggulination 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 Edwardesiella 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 Edwardesiellalatardata [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/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 100 naturally diseased fish (50 Oreochromis niloticus and 50 Clarias gariepinus) were collected from fish farms and fish markets respectively, of different weights at Kafrelsheikh Governorate. Oreochromis niloticus were transferred live in an aerated plastic pags and C. gariepinus to the laboratory of Animal Health Research Institute, Kafrelsheikh branch, Egypt 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 Tryptic 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 subculutred 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- prskauer (VP), Citrate utilization, Urease, Hydrogen sulphide production (H2S), Gelatin hydrolysis, Nitrate reduction, Ornithine decarboxylase (ODC), Detection of L-lysine decarboxylase (LDC), Detection of Ornithine decarboxylase (ODC), Detection of L-sulphide production Voges II B3 (Oxoid, UK.) plates for further identification. [18].

III-Molecular Identification of Edwardsiella 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 25 µl reactioncontaining 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 Edwardsiella virulence associated genes and annealing temperature.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers sequence (5’ → 3’)</th>
<th>Size bp*</th>
<th>°Cb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R: TTGAGAGTGCCTGTCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrB1</td>
<td>F: GCATGGAGACCTTCAGCAAT</td>
<td>415</td>
<td>50</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>R: GCCGAGATTTTGCCTCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cds1</td>
<td>F: TCTCCACCCATAATGCCAAG</td>
<td>435</td>
<td>55</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>R: CAAAACCCGCTGCTGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>edwI</td>
<td>F: ATCCGCGACGAGATGCCG</td>
<td>360</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GAAGGATACAGATGGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pvsA</td>
<td>F: CTGGAGCAGTACGCGAAG</td>
<td>313</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CGATGCTGCGGTAGTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Base Pair of amplicon size, °C Annealing Temperature, fimA; Edwardsiella speciesfimbrial gene, gyrB1; gyrase gene as taxonomic marker for E. tarda, cds1: chondroitinase, edw I: AHL-synthase; pvsA: 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: Haemorrhagic patches all over the body, haemorrhagic protruded anus and exophthalmia.
B: Ulcer on abdomen and haemorrhage 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 haemorrhagic 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, with distended gall bladder; while kidney and spleen were congested and inflamed; and abdominal cavity was filled with ascetic fluid as 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 cocccobacilli 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 for 11 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.

<table>
<thead>
<tr>
<th>Fish samples</th>
<th>No. of examined</th>
<th>No. of isolates</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. niloticus</td>
<td>50</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>C. gariepinus</td>
<td>50</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>

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 Edwardsiella spp. (37.5%) (Isolates No. 2, 6, 7 from O. niloticus) (Figure 3).
Fig 3: A garose gel electrophoresis of PCR of fimA gene (848 bp) for characterization of Edwardsiella 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. tardo gyr 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 fimbriae.

In this study, isolated Edwardsiella 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 ofgyrB1 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].
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]. edw1Gene was negative in all isolates in the current study at the reverse amplified in the strains according to [20] edw1 gene encodedN-acylhomoserinelactones (AHL-synthese) 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 Edwardsiella 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 replication and virulence of bacteria, this result may serve as a very useful tool in the development of new antibacterial agent against E. tarda infections.

References


