Prevalence and Molecular Detection of Edwardsiella tarda in Cultured Tilapia Species of Fish Farms of Punjab in Pakistan and their Postmortem Examination

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ABSTRACT
Edwardsielllosis caused by an emerging fish pathogen Edwardsiella tarda, is one of the major problems in aquaculture, associated to massive economic losses due to high mortality of a wide variety of fish species worldwide. In the current study, we isolated, identified, detected E. tarda and performed phylogenetic tree analysis of its 16S rRNA gene. Postmortem examination of infected fish revealed skin degeneration, exophthalmia, swollen abdomen, enlarged liver, white bacteria filled nodules in liver, kidney and intestine. Biochemical identification of E. tarda showed negative results in citrate, lactose, amylase and arginine tests while methyl red, H₂S, catalase, indole and glucose tests showed positive results. Amplification of esrB, gadB, gyrB, blaTEM, qnrA, and sul3 gene by PCR revealed bands at 312, 585, 414, 801, 654, and 444bp respectively. We recorded maximum 18% prevalence in intestine with respect to organs, 32.3% in male with respect to fish sex, 38.8% in O. niloticus with respect to fish species, 37.8% at fish farm FMG-10 of Muzaffargarh with respect to sampling site and 38.9% in summer at 37°C with respect to season. Overall 27.2% prevalence of E. tarda was recorded at all selected fish farms resulting 4.7% mortality. Chi-square test of independence showed significant difference (P<0.05) with respect to sampling sites. Our all isolated E. tarda strains showed 100% similarity with E. tarda strain isolated in USA. We concluded that virulence genes of E. tarda and high temperature in association with high stocking density and pollutant water also increase prevalence of E. tarda and cause mortality in fish.


INTRODUCTION
The world’s rapidly increasing population has been facing major challenge of food security and fish is considered a sustainable diet in future (Froehlich et al., 2018). Fish and its products are major contributors to animal protein, fatty acids and minerals, important for health status of humans worldwide (FAO, 2016). Oreochromis niloticus is a cichlid fish native to Africa and considered commercially and economically important fish species of freshwater due to its efficient FCR, fast growth performance, high resistance against disease, high consumption rate and easy breeding nature (Sousa et al., 2013). Nile tilapia contributed 8.3% (4525.4 thousand tonnes) to inland fisheries worldwide in 2018 (FAO, 2020).

Edwardsiella species are one of the top causative pathogens which cause severe infections and mortality in wide variety of fish species worldwide (Oh et al., 2020). Edwardsiella tarda is an emerging fish pathogen (Algammal et al., 2022) and a serious threat for fish which has affected worldwide aquaculture badly (Preena et al., 2022). E. tarda spreads Edwardsielllosis, important bacterial infectious disease, causes high prevalence (Eissa et al., 2016; Rodrigues et al., 2019; Butar-Butaret al., 2020) and massive mortality in wide variety of wild and cultured fish species of freshwater and marine worldwide (Kumar et al., 2016; Charles et al., 2020) such as Seriola quinquedem, Anguilla japonica, Pagrus major, O.
nioticus, Cyprinus carpio, Labeo rohita, Clarias gariepinus etc. (Kumar et al., 2016; Butar-Butaret et al., 2020). E. tarda causes huge economic loss in commercially important fish species due to its high resistance against multiple antibiotics (Nagy et al., 2018; Nantongo et al., 2019) and transmission of the resistance from antibiotic resistant E. tarda strains to non-resistant E. tarda strains (Kumar et al., 2016; Niu et al., 2019). These antibiotic-resistant strains have antimicrobial resistance genes and virulence genes which cause pathogenicity (Nantongo et al., 2019; Preena et al., 2022) and severe outbreak at fish farms of tilapia and catfish (Wimalasena et al., 2018; Algammal et al., 2022). E. tarda causes serious infections such as lesions, ascites, hemorrhages and exophthalmia in infected fish (Rodrigues et al., 2019).

E. tarda is an opportunistic pathogen and infects fish under environmental stress, low water quality, high temperature, organic content and stocking density (Park et al., 2012; Nagy et al., 2018). Prevalence of E. tarda is increased by multiple factors such as inadequate environmental conditions, virulence and antimicrobial resistance genes (Wimalasena et al., 2018). Increase in temperature causes high prevalence of E. tarda in kidney, fish muscles, intestine and liver (Kumar et al., 2016), in all seasons but the highest in summer (Butar-Butaret al., 2020) followed by spring (Eissa et al., 2016), but intermediate in autumn (Rodrigues et al., 2019) and minimum in winter (Eissa et al., 2016; Nagy et al., 2018). Male fish is observed as more infected than female fish with less significant difference (Kebede and Habtamu, 2016) of mud pond, wild fish and cultured fish (Butar-Butaret al., 2020).

The current study was performed to identify and detect E. tarda by PCR in infected fish sampled from selected fish farms of three districts of Punjab, Pakistan. Prevalence of E. tarda was recorded with respect to fish species, sex, organs, season and site of sampling. Phylogenetic tree was constructed to compare its phylogenetic relationship with other Edwardsiella species.

MATERIALS AND METHODS

Sample collection: Total 540 samples of tilapia fish species O. niloticus, O. mossambicus and O. aureus, were collected randomly from selected fish ponds of district Muzaffargarh, Mandi Bahauddin and Kasur of Punjab in Pakistan. Ice-treated fish samples were transported to the laboratory of department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences Lahore, Pakistan. GIS map of sampling sites is shown in Fig. 1.

Clinical and postmortem examination: Suspected fish samples were examined for external and internal abnormalities during clinical and postmortem examination (Noga, 2010).

Isolation of E. tarda: Suspected fish samples were disinfected with 70% ethanol and swabs from isolated organs (kidney, gills, liver, spleen, stomach, intestine, heart and tail fins), were collected and inoculated onto trypticase soy agar (TSA, Oxoid, England) media plates and incubated at 37°C overnight. A single colony from this culture was inoculated onto brain heart infusion agar (BHIA, LAB, England) media plates to get pure culture of E. tarda and incubated at 37°C for 24 hours (Muratoriet al., 2001). Pure culture of E. tarda isolates, was stored at -20°C.

Phenotypic characterization and biochemical identification of E. tarda: A single E. tarda colony from freshly isolated pure culture, was subjected to phenotypic characterization and biochemical identification (Austin and Austin, 2016).

DNA Isolation: DNA was extracted using Salting out method (Miller et al., 1988) and Gentra Puregene Kit (Qiagen, Valencia, CA) following the manufacturer’s suggested protocol for Gram-negative bacteria and quantified spectrophotometrically (Nanodrop, USA). Extracted DNA was stored at -20°C.

Molecular detection of E. tarda by PCR: E. tarda was detected by amplification of esrB, gadB, gyrB, blaTEM, qnrA, sul3 and 16SrRNA gene of E. tarda isolates by PCR (Bio-Rad, USA) using species specific primers (Macrogen, Seoul Korea). Detection of E. tarda was confirmed by Gel-electrophoresis and bands were visualized in Gel-documentation apparatus (Bio-Rad). Conditions for PCR are shared in Table 1.

Sequencing and Phylogenetic tree analysis of 16SrRNA gene of E. tarda: PCR products revealing the thickest bands, were sequenced by Sanger’s method at BGI Hong Kong Company Limited, China. The obtained sequences were analyzed and compared for taxonomic identification using NCBI- Nucleotide BLAST and submitted on the GenBank sequence database. Phylogenetic relationship of E. tarda was checked by phylogenetic tree analysis of 16S rRNA gene of E. tarda by bootstrap method using MEGA 11.0 (Molecular Evolutionary Genetic Analysis) with 1000 bootstrap replications (Shah et al., 2009).

Statistical analysis: Descriptive statistics such as proportions and percentage (%) were applied to summarize the data of prevalence. Chi-square test of independence was applied to compare the prevalence of E. tarda with respect to fish species, organs of isolation, seasons, sampling sites and fish sex using IBM SPSS Statistics V21.0 (IBM, USA).

RESULTS

Clinical and post-mortem examination: Infected fish showed variety of external and internal abnormalities such as dark spots, hemorrhages, and skin lesions. Protruded and haemorrhaged anus, congested gills, vent and fins, hernia, and swollen abdomen filled with ascitic fluid (ascites) were also observed in infected tilapia. Liver, spleen and kidney were enlarged, and white, bacteria-filled nodules were observed in liver, intestine, gills, spleen and kidney. Clinical and post-mortem abnormalities in infected tilapia fish are shown in Fig. 2.
Phenotypic and biochemical characterization of *E. tarda*: Results of phenotypic characterization revealed round and circular with grayish white coloured colonies on TSA media plates while opaque, translucent, large irregular, and whitish color colonies on BHIA media plates. Microscopic examination of *E. tarda* colonies revealed *E. tarda* as motile and rod-shaped. All the isolates showed positive results (100%) in motility, H₂S and indole production, methyl red, catalase, ornithine decarboxylase, and glucose fermentation tests while 100% negative results in Gram-staining, citrate utilization, cytochrome oxidase, lactose fermentation, amylase, arginine dihydrolase, urease, and Voges-Proskauer tests.

Molecular detection of *E. tarda*: Amplification of esrB, gadB, gyrB, blaTEM, qnrA, sul3, and 16SrRNA gene of *E. tarda* by PCR revealed bands at 312, 585, 414, 801, 654, 444 and 1503bp, respectively. Accession numbers allotted by NCBI GenBank against esrB, gadB, gyrB, blaTEM, qnrA, sul3, and 16SrRNA gene of *E. tarda*, are OP879871, OP879869, OP879867, OP919346, OP901503, OP919350 and ON524406, respectively.
Phylogenetic tree analysis: Phylogenetic tree revealed that our isolated *E. tarda* strains, KSHF741 (ON524406), KSHF148 (ON524380) and KSHF23 (ON415284) shared 100% similarity among them, and with *E. tarda* strain UCD-69E (MN199659) previously isolated in USA, TL2 (MN493069, India) and *E. ictaluri* strain EIV (OP847078, China) while 99% with *E. tarda* strain VMCU06 (KU860461, Thailand). Phylogenetic tree analysis of 16SrRNA gene of *E. tarda* is shown in Fig. 3.

Prevalence of *E. tarda*: Overall 27.2% prevalence was recorded in fish samples of selected fish farms. We recorded maximum 18% prevalence in intestine with respect to fish species, 37.8% mortality while sampling site and 38.9% in summer at 37°C with respect to season. *E. tarda* caused overall 7.69% mortality while 7.7% at fish farms of Muzaffargarh.
followed by Mandi Bahauddin (4.07%) and Kasur (1.7%). Maximum 32.06% was recorded in July. Prevalence of *E. tarda* is showed in Table 2-6. Results of statistical analysis are shown in Table 7.

## DISCUSSION

Virulence and antibiotic resistance genes of pathogenic bacteria enable them to colonize their hosts ultimately resulting serious infections in variety of hosts. In the current study, we isolated 16SrRNA gene, three virulence genes (esrB, gadB and gyrB) and three antibiotic resistance genes (blaTEM, qnrA and sul3) of pathogenic *E. tarda*. In a previous study, esrB gene (Preena et al., 2022), gadB and gyrB were also detected in *E. tarda* by Wanget al. (2012) with almost similar conditions of amplification. In prior studies, blaTEM, qnrA and sul3 (Ge et al., 2015; Niu et al., 2019), were detected by Wimalasena et al. (2018) and Sedek et al. (2020) but their conditions for amplification and size of amplified fragment (bp) were different. These variations in conditions of amplification were due to variation in frequency, quantity and application of antibiotics. In current study, *E. tarda* isolates showed positive results in motility, methyl red, H₂S and indole production tests while negative in Gram-staining, citrate, lactose, amylase and urease tests. Similar results of biochemical identification of *E. tarda*, were reported by El-Seedy et al. (2015) and Moustafa et al. (2016).

Virulence genes of pathogenic bacteria cause serious infections in organs of their hosts. In current study, we recorded 18.15% prevalence of *E. tarda* in intestine, liver 15.93%, stomach 12.96%, kidney 11.5%, spleen 9.64%, gills 7.8%, heart 6.78% and 5.2% in tail fin. There was insignificant (P>0.05) difference with respect to organs. However, Algammal et al. (2022) reported 54.4%, 36.4% and 9.1% in liver, kidney and spleen respectively. Moreover, Kebede and Habtamu, (2016) also reported 6.5% in liver, 2.4% in intestine and 0.8% in kidney. Similarly, Kumar et al. (2016) reported 6.6% in gills, 12.98% in intestine, 2.38% in skin and 0% in kidney. In comparison to our findings, Nemo et al. (2017) also isolated 1% *E. tarda* from kidney, liver 1.9% and 4.8% from intestine of Nile tilapia of Lake Hawassa and Bishofu lakes. However, Charles et al. (2020) concluded 87.5% in gills and 62.5% in intestine.

*E. tarda* causes mass mortality in commercially and economically important fish species and it reduces the market value of infected fish (Park et al., 2012). Prior studies reveal that *E. tarda* has caused disease outbreaks at fish farms of Nile tilapia which caused massive mortality ultimately huge economic loss for fish farmers.

In current study, we recorded 38.79% prevalence of *E. tarda* in *O. niloticus* followed by *O. mossambicus* 15.94% and *O. aureus* 8.74% which was due to high stocking density in polyculture system. Likewise, Rodrigues et al. (2019) observed 10 to 36.67% in *O. niloticus* which was due to fish stress and poor water quality. Similarly, Algammal et al. (2022) concluded 14% in *Clarias gariepinus* and *O. niloticus* 10%. Nantongo et al. (2019) and Nemo et al. (2017) observed 7.2% and 7.6%. But Muratori et al. (2001) reported 30.5% to 60% in *O. niloticus* of Sao Paulo state which might be due to stress caused by high stocking density of fish and low water quality. However, Korni et al. (2012) reported 13.3% in *O. niloticus* of Beni-Suef Governorate, Egypt. El-Seedy et al. (2015) reported 4.3% in *O. niloticus*. Issa et al. (2016) and Wamala et al. (2018) also reported 9.6 and 8.3%, respectively. Similarly, Kumar et al. (2016) reported 14.41% whereas Charles et al. (2020) reported 62.5% in *O. niloticus* of fish farms in Ibadan, Nigeria. There was non-significance (P>0.05) difference with respect to fish species. Kebede and Habtamu, (2016) who also reported insignificant results of prevalence with respect to fish species.

*E. tarda* infects its hosts and causes serious infections mostly in summer and autumn. In our current study, we recorded 38.97% prevalence in summer followed by spring 22.22%, 28% in autumn and 8.57% in winter. There was insignificant (P>0.05) difference with respect to season. Unlike these results Muratori et al. (2001) reported 52% in summer but 60% autumn in *O. niloticus* of fish farms of Minas Gerais state in Brazil which was due to low water quality and highly fish stocking stress. However, Korni et al. (2012) recorded 13.33% in Nile tilapia in spring in Beni-Suef Governorate. Similarly, Issa et al. (2016) recorded 15% in spring, 5% in fall and 3.3% in winter. Nagy et al. (2018) recorded 28% in summer, spring 18 and 10% in autumn in fish farms of *O. niloticus* in Kafr-Elshiekh, of Egypt. In this study, there is no significant difference in prevalence of *E. tarda* with respect to fish sex, organs and fish species. Similar to the results as reported in our study, Kebede and Habtamu (2016) also reported non-significant results of prevalence with respect to fish sex.

Prevalence of *E. tarda* varies under different environmental conditions and stocking density. In this study, we recorded maximum 37.77% prevalence at fish farm FMG-10 of Muzaffargarh while minimum 20% in fish farms of FKS-1 and FKS-4 of Kasur. Main reasons behind this difference of prevalence among fish farms of Muzaffargarh and Kasur, were high fish stocking density, water temperature, organic content and salinity. In contrast to our results, Charles et al. (2020) reported 50% at site B and D, 66.6% at C and 100% at site A of their sampling sites. Unlike our results, Nantongo et al. (2019) observed 8.3% at fish farm. Our study recorded

### Table 6: Prevalence (%) of *E. tarda* with respect to water temperature and pH

<table>
<thead>
<tr>
<th>Month</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Prevalence of <em>E. tarda</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Apr-20</td>
<td>25.38</td>
<td>7.92</td>
<td>5%</td>
</tr>
<tr>
<td>May-20</td>
<td>26.5</td>
<td>7.68</td>
<td>15%</td>
</tr>
<tr>
<td>Jun-20</td>
<td>28.91</td>
<td>7.42</td>
<td>45%</td>
</tr>
<tr>
<td>Jul-20</td>
<td>37</td>
<td>6.67</td>
<td>58.6%</td>
</tr>
<tr>
<td>Aug-20</td>
<td>36.17</td>
<td>6.8</td>
<td>54.3%</td>
</tr>
<tr>
<td>Sep-20</td>
<td>28.75</td>
<td>7.87</td>
<td>33.3%</td>
</tr>
<tr>
<td>Oct-20</td>
<td>26.83</td>
<td>7.95</td>
<td>23.3%</td>
</tr>
<tr>
<td>Nov-20</td>
<td>24.75</td>
<td>8.27</td>
<td>22%</td>
</tr>
<tr>
<td>Dec-20</td>
<td>19.92</td>
<td>8.4</td>
<td>20%</td>
</tr>
</tbody>
</table>

### Table 7: Results of statistical analysis; chi-square test of independence showing X²-value and P-value with respect to selected parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>X²-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.644</td>
<td>0.656**</td>
</tr>
<tr>
<td>Season</td>
<td>0.233</td>
<td>1.00***</td>
</tr>
<tr>
<td>Organs</td>
<td>3.262</td>
<td>0.999**</td>
</tr>
<tr>
<td>Fish Farms</td>
<td>7.364</td>
<td>0.998**</td>
</tr>
<tr>
<td>Sampling site</td>
<td>294</td>
<td>0.00***</td>
</tr>
</tbody>
</table>

*** = Shows significant result, ns = non-significant result
significant difference in prevalence of *E. tarda* with respect to sampling sites. Similarly, Kebede and Habtamu (2016) also concluded significant results with respect to site.

Our study recorded overall 7.69% mortality and 5.35% in *O. niloticus* followed by *O. mossambicus* (4.52%) and *O. aureus* (3.47%), which is less than mortality reported by Algmanal et al. (2022) who recorded 40% in *O. niloticus* fish farms of Dakahlia Governorate, Egypt. Preena et al. (2022) concluded 60% mortality in infected fish of farms of Ermakulam, Kerala. Similarly, Muratori et al. (2001) reported maximum 40% mortality in autumn, 63.6% in winter and 69.9% in spring due to temperature variation and different geographical locality.

**Conclusions:** *E. tarda* is an opportunistic bacterium, which causes multiple abnormalities and mass mortality in infected fish under high temperature and low pH in association with high stocking density, polluted water and high organic content. There is no significant difference in prevalence of *E. tarda* with respect to fish sex, organs and fish species. But its prevalence varies with respect to sampling sites due to different environmental conditions and farm management.

**Declaration of competing interest:** The authors declare that they have no known financial interests or personal relationship that could have appeared to influence the work reported in this paper.

**Authors contribution:** KM and FR conceived and designed the study. KM executed the experiment and finalized the data. NK and KM helped in sampling. SP assisted in experimentation. KM wrote the manuscript. KM and FR contributed equally to this work. All authors assisted in experimentation. KM wrote the manuscript. FR and KM finalized the data. NK and KM helped in sampling. All authors read and approved the final manuscript.

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