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RESEARCH ARTICLE

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

Edwardsiellosis 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 16SrRNA gene. Postmortem examination of infected fish revealed skin depigmentation, 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, H_2S, 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.

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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 overcome food security and considered as major source of 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 Edwardsiellosis, important bacterial infectious disease, causes high prevalence (Eissa *et al.*, 2016; Rodrigues *et al.*, 2019; Butar-Butar*et 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 quinqueradiata*, *Anguilla japonica*, *Pagrus major*, *O.*

niloticus, *Cyprinus carpio*, *Labeo rohita*, *Clarias gariepinus* etc. (Kumar *et al*., 2016; Butar-Butar*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. tard*a 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-Butar*et 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-Butar*et 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 37ºC 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 37ºC for 24 hours (Muratoriet al., 2001). Pure culture of *E. tarda* isolates, was stored at -20ºC.

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 -20ºC.

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 16SrRNA 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.

Fig. 1: GIS map of sampling sites (fish farms) of three selected districts of Punjab

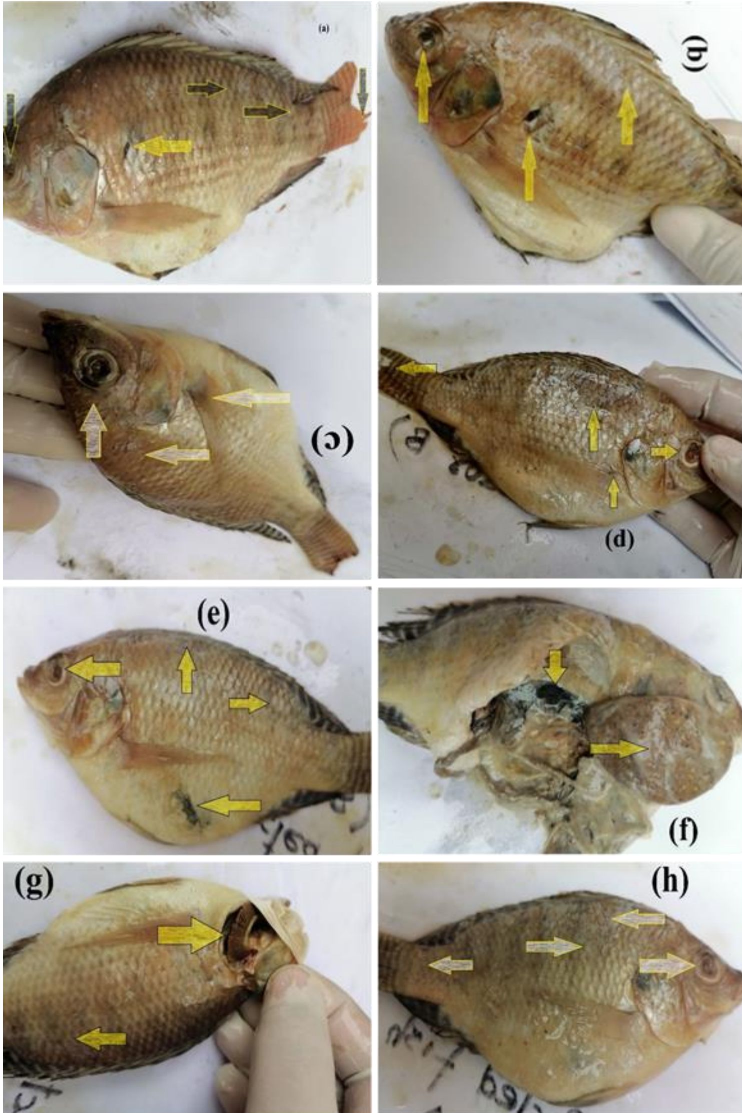

Fig. 2: Clinical and Postmortem abnormalities: (a) Fin rot and scale loss, (b) skin depigmentation, skin lesions and hemorrhages, (c) exophthalmia, swollen abdomen and skin lesions, (d) dark pigmentation, fin rot and corneal opacity (e) severe lesions and hemorrhages on head, vent and tail fin, (f) enlarged liver and spleen with black dots and white bacteria filled nodules, (g) gills rot and congestion (h) swollen abdomen (ascites with ascitic fluid) and protruded and haemorrhaged anus

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, H_2S 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.

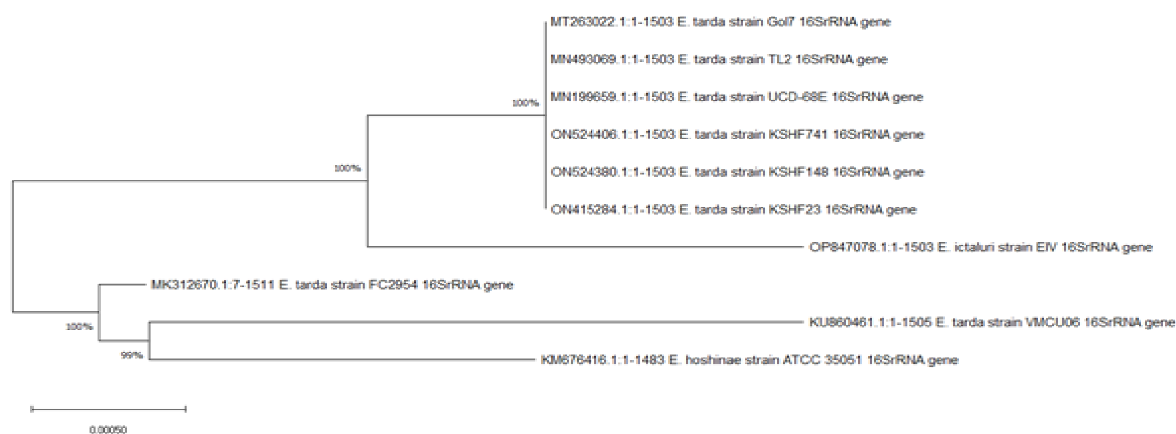

Fig. 3: Phylogenetic tree: Phylogenetic tree of 16S rRNA gene of *E.tarda* by using the Neighbour-joining method with 1000 bootstrap replicates.

Table 1: Primers sequence and conditions for amplification of selected genes of *E. tarda* by PCR

Target Gene	Primer Sequence (5`-3`)	Amplified segment (bp)	Annealing
esrB	F-TCGTTGAAGATCATGCCTTGC	312	55 °C for 1min
	R-TGCTGCGGGCTTTGCTT		32 cycles
gadB	F-ATTTGGATTCCCGCTTTGGT	585	55 °C for 1min
	R-GCACGACGCCGATGGTGTTC		32 cycles
gyrB	FGCATGGAGACCTTCAGCAAT	414	55 °C for 1min
	R-GCGGAGATTTTGCTCTTCTT		32 cycles
qnrA	F-ATTTCTCACGCCAGGATTTG	654	58.5 °C for 1min
	R-GATCGGCAAAGGTTAGGTCA		35 cycles
sul3	F-AGATGTGATTGATTTGGGAGC	444	54.2 °Cfor 30s
	R-TAGTTGTTTCTGGATTAGAGCCT		35 cycles
blaTEM	F-CATTTCCGTGTCGCCCTTATTC	801	52 °C for 30s
	R-CGTTCATCCATAGTTGCCTGAC		35 cycles
16S rRNA	F-AGAGTTTGATCCTGGCTCAG	1503	52 °C for 1min
	R-GGTTACCTTGTTACGACTT		30 cycles

Table 2: Prevalence of *E. tarda* with respect to fish organs

District	Kidney	Gills	Liver	Spleen	Stomach	Intestine	Heart	Tail fins
Kasur	6 (8.8%)	5 (7.3%)	12 (17.6%)	5 (7.3%)	10 (14.7%)	13 (19.1%)	5 (7.3%)	3 (4.4%)
Mandi Bahauddin	11 (16.2%)	7 (10.3%)	15 (22.0%)	10 (14.7%)	12 (17.6%)	16 (23.5%)	8 (11.7%)	4 (5.9%)
Muzaffargarh	14 (20.6%)	9 (13.2%)	16 (23.5%)	12 (17.6%)	13 (19.1%)	20 (29.4%)	6 (8.8%)	7 (10.3%)
Overall	11.5%	7.8%	17.9%	10%	12.96%	18.15%	7%	5.2%

Table 3: Prevalence of *E. tarda* with respect to fish species

Fish species	Mandi Bahauddin	Muzaffargarh	Kasur	Total
Oreochromisniloticus	37 (40.6%)	47 (44.3%)	32 (31.4%)	116
O. Mossambicus	9 (17.3%)	8 (18.6%)	5 (11.6%)	22
O. aureus	4 (10.8%)	3 (9.7%)	2 (5.7%)	9
Total	50 (34.0%)	58 (39.4%)	39 (26.5%)	147

Table 4: Prevalence of *E. tarda* with respect to season

Season	Mandi Bahauddin	Muzaffargarh	Kasur	Total
Summer	26 (40%)	29(44.6%)	21 (32.3%)	76
Spring	7 (23.3%)	8 (26.7%)	5 (16.7%)	20
Autumn	14 (28%)	17 (34%)	11 (22%)	42
Winter	3 (8.6%)	4 (11.4%)	2 (5.7%)	9

Table 5: Prevalence of *E. tarda* with respect to fish farms and fish sex

District	Kasur				Mandi Bahauddin				Muzaffargarh			
Fish Farm	FKS-1	FKS-2	FKS-3	FKS-4	FMB-5	FMB-6	FMB-7	FMB-8	FMG-9	FMG-10	FMG-11	FMG-12
Male	5(18.5%)	7(28%)	6(26.1%)	4(22.2%)	4(25%)	6(25%)	8(42.1%)	10(40%)	10(35.7%)	11(52.4%)	7(36.8%)	9(37.5%)
Female	4(22.2%)	5(25%)	3(13.6%)	5(18.5%)	6(20.7%)	5(23.8%)	7(26.9%)	4(20%)	5(29.4%)	6(25%)	4(15.4%)	6(28.6%)
Overall	9(20%)	12(26.6%)	9(20%)	9(20%)	10(22.2%)	11(24.4%)	15(33.3%)	14(31.1%)	15(33.3%)	17(37.7%)	11(24.4%)	15(33.3%)

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 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 37ºC and 6.67 pH with respect to season. *E. tarda* caused overall 7.69% mortality while 7.7% at fish farms of Muzaffargarh

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

Month	Temperature (°C)	pH	Prevalence of *E. tarda*
Apr-20	25.58	7.92	5%
May-20	26.5	7.68	15%
Jun-20	28.91	7.42	45%
Jul-20	37	6.67	58.6%
Aug-20	36.17	6.8	54.3%
Sep-20	28.75	7.87	33.3%
Oct-20	26.83	7.95	23.3%
Nov-20	24.75	8.27	22%
Dec-20	19.92	8.4	20%

Table 7: Results of statistical analysis; chi-square test of independence showing X^2-value and P-value with respect to selected parameters.

Parameters	X^2-value	P-value
Sex	0.844	0.656^{ns}
Season	0.233	1.00^{ns}
Organs	3.262	0.999^{ns}
Fish Farms	7.364	0.998^{ns}
Sampling site	294	0.00^{***}

*** = Shows significant result, ns = non-significant result

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 Wang*et 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, H_2S 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 Bishoftu 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 Sau 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 13.33% in *O. niloticus* of Beni-Suef Governorate of Beni-Suef Governorate, Egypt. El-Seedy *et al*. (2015) reported 4.3% in *O. niloticus*. Eissa *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, Eissa *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

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 Algammal *et al.* (2022) who recorded 40% in *O. niloticus*of fish farms of Dakahlia Governorate, Egypt. Preena *et al.* (2022) concluded 60% mortality in infected fish of farms of Ernakulam, 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 read and approved the final manuscript.

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