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Multiple drug resistant *Edwardsiella tarda* associated disease outbreak in *Labeo catla*

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Abstract

The present study documented an *Edwardsiella tarda* associated disease outbreak in a *Labeo catla* commercial fish farm in Tamil Nadu, India. Samples of infected fish (n=10) were collected from the *L.catla* farm in Thanjavur, Tamil Nadu, India that had reported an outbreak of disease. Bacterial isolation was done from kidney, eye, liver, intestine, brain and gills and the isolates were biochemically characterized, confirmed by PCR amplification and nucleotide sequencing as *E.tarda* (E4L/SRLAAH/2020). Antibiogram showed that the isolate was either resistant or intermediately resistant to Chloramphenicol, Ampicillin, Oxytetracycline, Cefotaxime, Vancomycin, Amoxicllin, Erythromycin, Ceftazidime, Kanamycin and Tetracycline and sensitive to five antibiotics viz., Norfloxacin, Ciprofloxacin, Nalidixic acid, Oflaxacin and Co-trimazole.

Keywords: *Edwardsiella tarda*, fimbrial gene, antibiotic resistance.

Introduction

In India, traditional fish culture of Indian major carps viz., *Labeo catla*, *Cirrhinus mrigala* and *Labeo rohita* and their hybrids are widely practiced due to their high economic importance as food fishes (Szekely *et al.*, 2015). Edwardsiellosis is one of the important diseases caused by the bacterial pathogens of *Edwardsiella* genus of the family Enterobacteriaceae. *E.tarda* is a gram-negative, motile, facultative anaerobic, short rod shaped bacterium (Park *et al.,* 2012) that could be isolated from a wide range of water salinity (0-4% NaCl), pH (5-10) and temperatures (14 to 45ºC). The fish species commonly affected by Edwardsiellosis include eels, channel catfish, mullet, tilapia, carps and flounder (Thune *et al.,* 1993). *E.tarda* enter the fish hosts through gills and gastrointestinal tract and multiply inside various internal organs (Ling *et al.,* 2001) causing gross pathological changes of skin and muscle lesions, necrotic liver, intestine and liquefaction of the kidney (Choudhury *et al.,* 2017). Fimbrial adhesin-like protein, are important for attachment and penetration into the epithelial cells of hosts (Srinivasa Rao *et al.,* 2003). The internal clinical signs viz., hemorrhagic ascetics, hypertrophy of the kidney and spleen, reddening of the spleen, inflammation in the adipose tissue, peritoneum and intestine and/or congestive mottle of the liver (Sakai *et al.,* 2007). *E. tarda* also has the ability to multiply in large numbers and upgrade the local infection to a systemic infection (Leung *et al.*, 2012). There are no established successful commercial treatments available for this disease (Mohanty and Sahoo, 2007). However, administration of commercial probiotics containing *Bacillus subtilis, Lactobacillus acidophilus, Clostridium butyricum* and *Saccharomyces cerevisiae* enhanced the non-specific immune system, slightly improved the survival of fishes and reduced mortality due to

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E. tarda infection (Taoka *et al.,* 2006). Hence, the best way to prevent this disease is to maintain proper environmental conditions in ponds and in improving the health and immune status of the fish. As *E.tarda* is considered zoonotic in nature (Leung *et al.,* 2012), the practice of manual handling of fish, consuming partially cooked fish, and unhygienic farming practice would pose a higher risk of contracting the disease. In the present study, we report the isolation of multidrug resistant *E.tarda* associated with disease and mortality of *L.catla* in a commercial fish farm in Thanjavur district, Tamil Nadu, India.

MATERIALS AND METHODS

Sampling and bacteriology

Morbid fishes 25±2 cm, 250±50g were collected from the commercial fish farm undertaking catla culture in Edaiyathi village (10°25'12.4"N 79°10'35.2"E), Thanjavur District, Tamil Nadu, India. Behavioral changes and clinical abnormalities of the diseased fish were recorded on site. The fish samples were thoroughly rinsed with sterile saline and excess was wiped off and the specimens were aseptically dissected. The inocula from the kidney, liver, intestine, and gills from the fish sample were aseptically collected in Hiculture transport swabs (HiMedia, India), inoculated onto *Edwardsiella ictaluri* broth and was incubated overnight at 35 ± 2°C. A loopful of colonies from the broth were streaked onto *Edwardsiella ictaluri* Medium (EIM Agar) (Shotts and Waltman, 1990) and incubated at 37°C for 24h. Distinct green transluscent colonies with black centers grown in EIM Agar were sub-cultured into a fresh EIM agar to obtain pure colonies. The colonies of interest were further subjected to a series of biochemical tests (Wyatt *et al*., 1979; Grimont *et al.,* 1980).

Molecular characterization

Genomic DNA was extracted from the biochemically identified isolate, (E4L/SRLAAH/2020) using QIAamp DNA mini kit (Qiagen, Germany) as per manufacturer's protocol. PCR was carried out in a thermal cycler (Biorad, USA) in a total volume of 25µl following the primers and protocols of Fimbrial genes (eftA, eftB, eftC, eftD) conserved to *Edwardsiella* genus (Sakai *et al.*, 2007) and 16S small subunit ribosomal RNA gene (Weisburg *et al.,* 1991) (Table.1). The PCR products were resolved on a 1.5% agarose gel and documented in a gel documentation unit (Biorad, Germany). Nucleotide sequencing (forward and reverse) was done by commercial sequencing services (Eurofins, India). The forward and reverse sequences were assembled by DNA Baser Sequence Assembler v3.5.3 (2012). The sequence information was subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov) and submitted to GenBank, NCBI to obtain accession number.

Table. 1. Details of primers used in the study

Primer code/ target	Primer Sequence	Length (bp)
16s rDNA gene	5'-AGAGTTTGATCCTGGCTCAG-3' 5'-ACGGCTACCTTGTTACGACTT-3'	1500
etf A (Major fimbrial subunit)	5'-CGGTAAAGTTGAGTTTACGGGTG-3' 5'-TGTAACCGTGTTGGCGTAAG-3'	415
etf B (Fimbrial chaperon protein)	5'-CTATATGGTGCAGACCTG-3' 5'-GCTGAAGGAGACTGTATTG-3'	493
etf C (Fimbrial usher protein)	5'-AACACCGGTATCAGCGGAAC-3' 5'-GTTGGAATCGGTATGGCGTC-3'	357
etf D (Fimbrial subunit)	5'-GGTAACCTGATTTGGCGTTC-3' 5'-GGATCACCTGGATCTTATCC-3'	445

Antibiotic susceptibility test

Sensitivity of the bacterial isolate (E4L/SRLAAH/2020) was tested against 15 commonly used antibiotics, oxytetracyline (30 mcg), norfloxacin (10 mcg), chloramphenicol (30 mcg), ampicillin (10 mcg), cefotaxime (30 mcg), ciprofloxacin (5 mcg), vancomycin (30 mcg), nalidixic acid (30 mcg), amoxycillin (30 mcg), co-trimazole (25 mcg), oflaxacin (5 mcg), kanamycin (30 mcg), erythromycin (15 mcg), tetracycline (30 mcg) and ceftazidime (30 mcg) by agar disc diffusion method (Bauer *et al.* 1966) on Mueller Hinton agar and incubated for 24h at 37ºC and the diameter of the zone of inhibition (mm) was measured and compared with the zone size interpretation chart (CLSI document M100-S22, 2012).

RESULTS AND DISCUSSION

The clinical signs observed in the diseased fishes include discoloration of the skin, lethargy, anorexia, haemorrhage of the internal organs, pale liver and empty intestine. Inocula from the liver and intestine showed green colonies with black centres on EIM agar (Fig.1). The bacterial isolate was Gram-negative short rod, motile, O/F, oxidase, VP, inositol, xylose, rhamnose, salicin, sorbitol, sucrose, lactose, and arabinose negative, positive for catalase, H2S production, citrate, methyl red, indole tests, mannitol, glucose, dextrose and Trehalose utilization. Molecular characterization by PCR using primers for amplification of 16s rRNA gene and *Edwardsiella* genus and the virulent fimbrial genes such as eftA, eftB, eftC and eftD of Type 1 fimbrial gene cluster of *E. tarda* (Fig.2) which will help in the differential identification the pathogenic and non-pathogenic strains of *E.tarda* (Sakai *et al.,* 2007). PCR amplified 16srRNA product (1500bp) confirmed by sequencing as *E tarda* (E4L/SRLAAH/2020) and submitted to the GenBank (NCBI Acc. No MT422189).

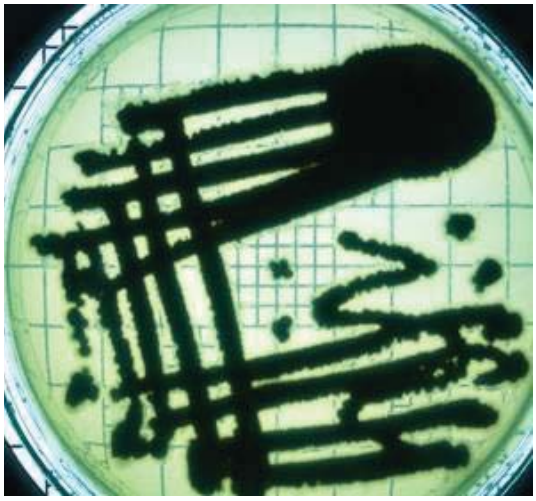

Fig.1. *E.tarda* bacterial culture grown as green colonies in EIM agar.

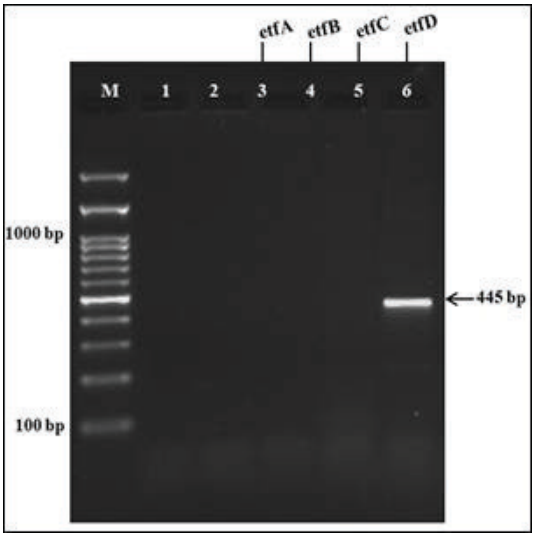

Fig. 2. *E.tarda* fimbrial protein etfD gene profile of the *E.tarda* isolate. Lane 6 showing specific amplified product at 445 bp.

The diseased *L.catla* fishes were found to be asymptomatic without any external signs but with the symptoms such as lethargy, overall paleness, anorexia and external skin lesions as reported earlier (Muratori *et al* 2000). Increased *E.tarda* incidences could be correlated when there is a rise in temperature and higher organic load (Meyer and Bullock, 1973). The observations of our study showed that although there were no external symptoms observed on the fish, but the water quality analysis revealed a high organic load of ammonia (1.5ppm) but the optimal range is below 0.01ppm. Outbreaks of acute *E.tarda* infection are mostly found in culture systems of channel catfish during rise in temperature, overcrowding and high organic load, although mortality often remains low and the infection becomes chronic (Meyer and Bullock, 1973; Noga, 2010). Fimbrial proteins were found to be induced more significantly under higher sodium chloride concentrations (Mahmoud, 2006) and this explains the reason for the amplification of fimbrial gene eftD alone in our isolate.

E.tarda isolate (E4L/SRLAAH/2020) was susceptible to antibiotics viz., norfloxacin, ciprofloxacin, nalidixic acid, oflaxacin and co-trimazole and resistant and/or intermittently resistant to antibiotics viz., chloramphenicol, ampicillin, oxytetracycline, cefotaxime, vancomycin, amoxicllin, erythromycin, ceftazidime, kanamycin and tetracycline. This indicates the multiple drug resistance of the *E.tarda* isolate (E4L/SRLAAH/2020) and this result was consistent with the observations of Stock and Wiedemann (2001).

Antibiogram of the *E.tarda* isolate (E4L/SRLAAH/2020) showed that it is susceptible to all the quinolones group of antibiotics, norfloxacin, ciprofloxacin, nalidixic acid, oflaxacin which is in accordance with other previous report (Reger et al., 1993). In addition to that, the isolate was also susceptible to folate pathway inhibiting drug, co-trimazole as reported earlier (Miniero *et al.,* 2018). However, the isolate exhibited multiple drug resistance either complete or partial to antibiotics such as chloramphenicol, ampicillin, oxytetracycline, cefotaxime, vancomycin, amoxicillin, erythromycin, ceftazidime, kanamycin and tetracyclines reported in earlier studies (Wimalasena, 2018; Ogunleye *et al,* 2020; Algammal, 2022). There is an increasing trend in the documentation in antimicrobial in bacterial pathogens of fish. Enterococcus spp. Strain from diseased zebrafish showed either resistance and/or intermittently resistance to amoxyclav, ampicillin, penicillin G, streptomycin, kanamycin, gentamicin, vancomycin, erythromycin, norfloxacin, ciprofloxacin, sulfisoxazole, nitrofurantoin and oxytetracycline (Uma *et al.,* 2020). Multiple antibiotic resistant *E. faecalis* have been isolated from Tilapia and catfish (Rahman *et al.,* 2017, Uma *et al.,* 2017) and *A. hydrophila* isolated from diseased catfish of *Clarius sp* (Uma *et al.,* 2014). *E.tarda* isolated from diseased Oscar fish (*Astronotus ocellatus*) showed multi-drug-resistance towards antibiotics belonging to aminoglycosides, tetracyclines, and quinolones categories with a MAR index of 0.32 (Vishnupriya *et al.,* 2024). As there is a growing evidence to suggest that lateral gene transfer as one of the major causes of multidrug resistance due to the indiscriminate use of antibiotics (Alanis, 2005), the stringent use of antibiotics and other chemicals need to be followed in the fish farming practices besides adopting the use of probiotics and immunomodulators to improve the host's health (Taoka *et al.,* 2006).

Conclusion

These results of this study suggest that commercially important food fishes cultured in farms may potentially act as carriers of pathogenic bacteria such as multiple antibiotic resistant *E. tarda* and this pathogen also has a risk of causing gastrointestinal problems to

human beings due to its zoonotic nature which may further complicate the treatment for humans due to its antibiotic resistance. Therefore, appropriate good management practices must be practised to prevent such pathogens from affecting commercially important food fish farms.

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