



Multiple drug resistant *Enterococcus* spp. causes disease and mortality in Zebra fish (*Danio rerio*)

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ABSTRACT

The present report describes the isolation and identification of a multiple drug resistant *Enterococcus* spp. from diseased zebrafish from a commercial rearing facility in Chennai, Tamil Nadu, India. Zebrafish (*Danio rerio*) has recently gained great significance as a vertebrate animal model, as its immune system is remarkably similar with that of the humans. However, zebrafish are still susceptible to microbial infection. Gram positive diplococci isolated from kidney was identified as *Enterococcus* spp. using 16S rRNA gene sequencing analysis. The *Enterococcus* spp. isolate was either resistant and or intermediately resistant to 14 antibiotics assessed by agar disc diffusion method. This communication is the first report on isolation and confirmation of *Enterococcus* spp. associated with disease and mortality in zebrafish.

Key words: Antibiotics, 16S rRNA, Drug resistance, Model fish, Pathogens

Zebrafish, *Danio rerio* possesses both innate and adaptive immune system similar to humans, making it a good animal model for research on infectious diseases. The small size of adult zebrafish (3–5 cm), the high population density (5 fish/liter) at which they can be maintained, large clutches of eggs and ease of spawning had made the zebrafish the vertebrate model of choice for forward genetic screens (Meijer and Spink 2011). Bacterial pathogens, viz. *Aeromonas* sp., *Pseudomonas fluorescens*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus* sp. and *Enterococcus* spp. are reported to be commonly present in the fish rearing facilities (Osman *et al.* 2017) and their infection are often sub-clinical and without apparent signs. *Enterococcus* sp., an anaerobic Gram-positive commensal bacteria has emerged as one of the important fish pathogens in aquaculture worldwide (Martins *et al.* 2008, Rahman *et al.* 2017). Large scale production and economic losses due to *Enterococcus* spp. infection have been reported in cultured yellowtail, *Seriola quinqueradiata* (Kusuda *et al.* 1991); turbot, *Scophthalmus maximus* (Nieto *et al.* 1995) and Tilapia, *Oreochromis* sp (Uma *et al.* 2017). Report on *Enterococcus* spp. infection causing mortality in fishes from India is scanty (Uma *et al.* 2017). In the present study, we report the isolation of *Enterococcus* spp. associated with disease and mortality of

zebrafish for the first time at a rearing facility in Tamil Nadu, India.

MATERIALS AND METHODS

Sampling and bacteriology: Diseased zebrafish (≈ 211 mg; size 2.3–3.5 cm) collected from a fish rearing facility in Kolathur, Chennai, Tamil Nadu, India, that reported a disease outbreak during October 2016 were examined. At site, behavioral abnormalities, gross and clinical signs of diseased zebrafish were noted and dissected aseptically (Heil 2009). Inoculum from the kidney of morbid zebrafish were streaked on to brain heart infusion agar (BHIA), *Aeromonas* isolation agar (AIA) supplemented with ampicillin (10 µg/ml), *Streptococcus* isolation agar (SIA) and Bile Esculin Azide agar plates (HiMedia, India), and incubated at $30 \pm 2^\circ\text{C}$ for 24–48 h. Based on dominance and definite colony morphology, one representative white colony from SIA, was picked, purified on BHIA and maintained on BHIA slants at room temperature ($28 \pm 2^\circ\text{C}$) and also as glycerol stock at -20°C for further characterization. Identification of the bacterial strain up to genus level was done following a series of biochemical tests (Collins *et al.* 2004, Austin and Austin 2012). A Gram-positive and catalase negative diplococcal isolate (Z2S) was isolated and characterized biochemically using Rapid HiStrep™ biochemical test kit (HiMedia, India).

Molecular characterization: For molecular characterization of the bacterial strain, The genomic DNA was extracted from the isolate (Z2S) using QIAamp DNA mini kit (Qiagen, Germany) as per manufacturer's protocol. Presumptive characterization of the bacterial isolate, Z2S

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was done by amplifying a fragment of *tuf* gene conserved to *Streptococcus* genus and some Enterococcal species (Picard *et al.* 2004) in a T-100™ thermal cycler (Bio-Rad, USA). Confirmatory identification of the isolate (Z2S) was done by amplifying the 16S small subunit ribosomal RNA gene (16S rRNA) (Weisburg *et al.* 1991). The PCR products were resolved by gel electrophoresis on a 1.5% agarose gel and documented. 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) to form consensus sequence. Phylogenetic analysis was performed on a selection of nineteen 16S rRNA gene sequences comprising the new consensus sequence of bacterial isolate Z2S and closely related sequences of same genus and other representatives of lactic acid bacteria which was determined by Basic Local Alignment Search Tool (BLAST) of NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov>). *Edwardsiella tarda* (Acc. No. KP898211), a member of Enterobacteriaceae family, was used as an out-group. Sequence alignment was performed by Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (Edgar 2004) and phylogenetic tree was constructed using MEGA6 software (Tamura *et al.* 2013). Evolutionary distance was evaluated by neighbor-joining method using Kimura 2-parameter model (Kimura 1980) with 1000 bootstrap replicates.

Antibiogram: Sensitivity of the bacterial isolate, Z2S from diseased zebrafish was tested against 15 antibiotics, viz. amoxyclav (30 µg), ampicillin (10 µg), penicillin-G (10 units), streptomycin (10 µg), kanamycin (30 µg), vancomycin (30 µg), erythromycin (15 µg), clindamycin (2 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), gentamicin (10 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg) and sulphafurazole (300 µg) by agar disc diffusion technique (Bauer *et al.* 1966) on Mueller Hinton agar. The agar plates were incubated for 24 h at 35±2°C and the diameter of the zone of inhibition (mm) was measured. Interpretation of sensitivity was done based on the zone size interpretation chart (CLSI document M100-S22 2012).

RESULTS AND DISCUSSION

The present study demonstrated the role of *Enterococcus* spp. in diseased zebrafish. The gross and clinical signs observed in diseased zebrafish were lethargy, poor escape response, erratic movement, exophthalmia, focal cutaneous haemorrhages on abdomen and hyperaemia around the base of the pectoral fins, abdominal ascites, organs discoloration, necrosis of the spleen and haemorrhages in kidney which were in accordance with the previous reports (Nieto *et al.* 1995, Uma *et al.* 2017).

Inocula from kidney yielded predominantly round, white, glossy semi-convex colonies on SIA plate within 48 h. The bacterial isolate from kidney on SIA was Gram positive, diplococci, oxidase, catalase, ONPG, arabinose and sorbitol negative, positive for Vogues Proskaur test, hydrolysed

esculin, PYR, arginine, glucose, lactose and sucrose. As the isolate, Z2S never matched with any of the *Streptococcus* sp. described in Rapid HiStrep™ biochemical test kit, the bacterial isolate Z2S was presumptively considered as *Enterococcus* spp. Thus, the Rapid HiStrep™ biochemical test kit data was used only to characterize the diplococcal isolates phenotypically and molecular characterization for confirmative identification isolate by PCR amplification of *tuf* gene resulted in a band at 197 bp. Enterococcal species carries two divergent *tuf* genes, among which one gene shares a common ancestor with the *tuf* gene of streptococci (Picard *et al.* 2004). Phylogenetic analysis based on 16S rRNA gene sequencing and analysis of gene sequence similarity showed 88–92% homogeneity with other such species of the genus *Enterococcus* (Z2S) (GenBank Accession No: MH229461) which clustered with *E. durans* in the NCBI GenBank database and formed a monophyletic group with high nodal support (Fig. 1). The *E. faecalis* clustered together forming a separate clade. The lactic acid bacteria of the genus *Lactococcus*, *Streptococcus* and *Vagococcus* branched out separately with high node support (Fig. 1). The 16S rRNA gene sequence of this isolate was closely related to *E. durans*. The gene sequence of *Enterococcus* spp. (Z2S) isolate of the present study was closely related with 92% similarity to *E. durans* isolated from fish viscera with accession numbers, viz. KC213474, KC213475, KC213476, KC213477, KC213478, KC213479 and KC213480. The 16S rRNA gene sequencing confirmed that the novel sequence belonged to the family Enterococcaceae, phylum Firmicutes, and fell within the evolutionary radiation of the genus *Enterococcus*.

Antibiotic sensitivity test showed that the *Enterococcus* spp. isolate Z2S was either resistant and/or intermittently resistant to more than 9 classes of antibiotic groups, viz. penicillin and β-lactamase inhibitors (amoxyclav, ampicillin, penicillin G), aminoglycosides (streptomycin, kanamycin, gentamicin), glycopeptides (vancomycin), macrolide (erythromycin), first generation synthetic fluoroquinolone (norfloxacin), second-generation fluoroquinolone (ciprofloxacin), sulfonamides (Co-trimoxazole, sulfisoxazole), Imidazolidinedione antibacterial agent (nitrofurantoin), broad-spectrum tetracycline group (oxytetracycline) and another broad-spectrum antibiotic (chloramphenicol). The isolate was observed to be susceptible only to chloramphenicol. Multiple antibiotic resistant fish pathogenic *E. faecalis* have been isolated from Tilapia and catfish (Rahman *et al.* 2017, Uma *et al.* 2017) which corroborates with the present study. The high resistant rate to various antibiotics could be due to the frequent and imprudent use of antibiotics (Abuseliene and Daud 2011).

In the present study, the entry of *Enterococcus* sp. into the culture ponds is still unknown. The fecal-oral route, body injuries and contaminated diets are believed to be responsible for the outbreak of *Enterococcus* spp. infection. High counts of *Enterococcus* spp. in intestinal samples from marine fish, suggests that Enterococci may be a member of

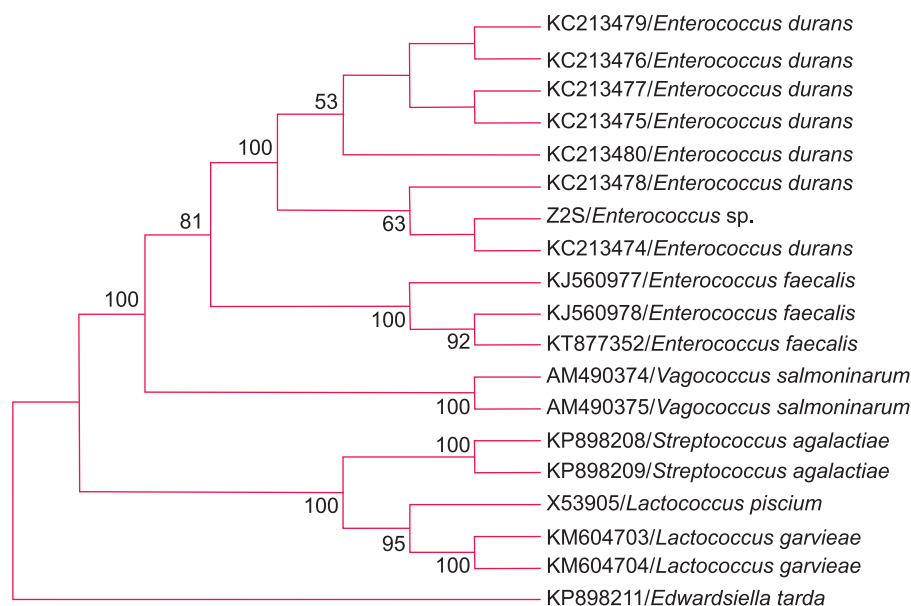


Fig. 1. Phylogenetic tree of *Enterococcus* strains isolated from diseased zebrafish, *Danio rerio* generated by neighbor-joining method with 1,000 bootstrap replicates. The GenBank accession number is provided for each species.

the normal intestinal flora of fish (Kanoë and Abe 1988). This suggests that *Enterococcus* spp. may be an opportunistic pathogen found in the normal intestinal flora of fish.

This report confirms the identification of multiple drug resistant *Enterococcus* spp. from diseased zebrafish, in Tamil Nadu, India. Use of specific pathogen-free stock, better quarantine measures for new arrival of fish stock, avoidance of overcrowding and overfeeding, keeping separate water supplies for culture systems, minimizing unnecessary handling or transportation, removing dying and dead fish frequently, feeding pathogen free ration, and keeping excellent sanitary conditions will help in reducing the risks of bacterial infection. Further research is warranted for continuous surveillance and to understand the pathology to determine whether *Enterococcus* spp. is an opportunistic pathogen found in the normal intestinal flora of fish and finally evolve the disease management strategies that do not use antibiotics to prevent the outbreak of *Enterococcus* spp. infection.

The study was conducted to identify the bacterial isolate associated with the disease and mortality of zebrafish at a rearing facility in Tamil Nadu, India. A multiple drug resistant *Enterococcus* spp. isolate (Z2S) has been identified and confirmed to be the causative of the diseased zebrafish. This appears to be the first report on the outbreak of disease caused by *Enterococcus* spp. in zebrafish in Tamil Nadu. Stringent bio-security, quarantine measures and better management practices should be followed while rearing any fish species to prevent the spread of this emerging disease, as it can infect other species, which share the common aquatic environments.

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