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Isolation, Molecular Identification and Antibiotic Resistance of *Enterococcus faecalis* from Diseased Tilapia

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A B S T R A C T

Aquaculture is one of the fastest growing food production sectors globally. Tilapia is the second widely farmed fish species in the global fish production. Enterococcus sp. is one of the leading causes of nosocomial infections in urinary tract, surgical wound and endocarditis in humans. These infections can be hard to treat because of the rising incidence of multiple antibiotic-resistances. The spread of antibiotic resistance has become a major concern in both human and veterinary medicine. In this study, we isolated and characterized an *Enterococcus faecalis* isolate from a diseased cultured Tilapia. Initial isolation of putative *E. faecalis* was carried out on streptococcus enrichment broth for 36 h. Characteristic, gram-positive, black color colony was selectively sub-cultured and subsequently identified by 16 rRNA sequencing analysis as *Enterococcus faecalis* (Genbank Acc no. KT877352). Furthermore, when the isolate was subjected to profiling against 16 antibiotics, it was found to be highly resistant to amoxyclave, ampicillin, erythromycin, gentamicin, kanamycin, nitrofurantoin, penicillin G, streptomycin, sulphafurazole, and vancomycin. The findings of present study showed that E. faecalis infects the cultured Tilapia species and the isolate (SRLFDA/TIL-1/15) possess multiple antibiotic resistance, which emphasizes the urgent need for targeted alternate bio-control strategies for control of emerging diseases like *Enterococcus* sp. infection in Tilapia culture.

Introduction

Aquaculture has emerged as one of the important food production sector over recent decades (FAO, 2000). Food fish supply has been reported to increase at the rate of 8.3 % annually (FAO, 2014). Tilapia is the second most farmed fish species in the world with the estimated global production of around 5.5 million metric tons (mmt) in 2016, but is predicted to increase by 4.5 % in 2017 reaching 5.8 mmt (RGCA, 2016). Tilapia may play an important role in the growth of aquaculture and continue to contribute in the future food demand in developing and developed countries.

As in any other fish culture operations, disease is the major factor that adversely affects the production of farmed Tilapia. Although, Tilapia is considered hardy with high disease resistance, bacterial diseases caused due to *Streptococcus* sp (Atyah *et al.*, 2010; Chen *et al.*, 2015; Li *et al.*, 2015; Shen *et al.*, 2016) and *Enterococcus* sp (Martins *et al.*, 2009) have been reported. *Enterococcus*

sp. is a commensal gram-positive, diplococcal bacterium which are dominant in fish, shellfish, and other aquatic animals (Wilson and McAfee, 2002; Sonsa Ard *et al.,* 2015; Chajecka-Wierzchowsk *et al.,* 2016; Paganelli *et al.,* 2017). Nonetheless, *Enterococcus* sp. in contaminated food fishes can lead to life-threatening illness in human such as endocarditis (Dahl and Bruun, 2013), bacteremia (Stuart *et al.,* 2006), urinary tract infection and meningitis (Tebruegge *et al.,* 2011) and its resistance to antibiotics is emerging as a major problem in treating these infections (Koch *et al.,* 2004). In addition, the ubiquity of *Enterococcus* sp in food has been reported to be mainly a result of their resistance to unfavorable environmental conditions during production technology with food fish storage conditions and their adaptability (Sarra *et al.,* 2013). Because of their relative abundance and their resistance to environmental factors, *Enterococcus* sp have been proposed as an indicator bacteria for hygiene quality, as well as for antimicrobial resistance in food and water (Boehm and Sassoubre, 2014). *Enterococcus* sp. has emerged as important healthcare associated pathogen (Arias and Murray, 2012; Khan *et al.,* 2015), as they are intrinsically resistant and tolerant to numerous commercial antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposing acquisition containing genetic sequences that confer resistance in other bacteria (Eaton and Gasson, 2001; Ben Belgacem *et al.,* 2010; Hammerum Lester and Heuer, 2010). In the last decade, several virulence factors have been described in *Enterococci* including cytolysins (Vankerckhoven *et al.,* 2004), gelatinase (Mannu *et al.,* 2003), serine protease (Mohamed et al. 2004), hyaluronidase, aggregation substance (Muscholl-silberhorn *et al.,* 2000) and extracellular surface protein (Shankar *et al.,* 1999). The cell wall adhesion and biofilm formation properties of *Enterococcus* sp. have also been described (Barbosa, Gibbs and Teixeira, 2010).

The presence of commensal microbiota in environmental ecosystems (Salyers and Shoemakers, 2006), human ecosystems and in food suggest that microorganisms can play a essential role in transfer of antibiotic resistant genes and the food chain may play a key role in the transmission of resistance between the environment and human (Marshall and Levy, 2009). Although, the detection of virulence factors may indicate a virulence potential in food isolates, food-borne Enterococcal infection have never been reported (Giraffa, 2002; Foulquie-Moreno *et al.,* 2006; Valenzuela *et al.,* 2010). However, the consumption of food carrying antibiotic-resistant bacterial populations is considered a possible transfer route (Kruse and Sorum, 1994). In recent years, growing interest in the consumption of fish foods, which are considered balanced healthy foods have been observed. In the present study *E. faecalis* was isolated from a diseased Tilapia, identified by 16S rRNA amplification and sequencing and its antibiotics resistance was studied.

Materials and Methods

Samples

Diseased Tilapia sample (Average weight 57 g, average 18 cm) was collected from a fresh water aquaculture farm in Chennai, Tamilnadu, India. At site, behavioral abnormalities, gross and clinical sign of the diseased Tilapia were recorded (Heil, 2009). Morbid tilapia fish with typical disease symptom was first rinsed in sterile saline and dissected aseptically. Inoculum from the kidney of tilapia was collected aseptically and was spread plated onto brain heart infusion agar (BHIA) and *Streptococcus* selective isolation broth (SIB) supplemented with 6.5% NaCl. Presumptive single (black; 1mm dia)

colony on bile-esculin agar (Himedia, India) were streaked on to *Streptococcus* selective isolation agar (SIA) (Himedia, India) plates for further purification at 30±2°C for 24-36 h and maintained on BHIA slants at room temperature (28±2°C).

Phenotypic characterization

A series of biochemical tests were performed to identify the isolate up to genus level (Svec and Devriese, 2015). Biochemical characterization like, gram strain, catalase test, and growth at 6.5% NaCl/ 45°C, was done using Rapid HiStrepTM biochemical test kit specific for *Streptococcus* sp. (HiMedia, India). The phenotypic characteristics documented in earlier reports (Murray, 1990; Teixeira *et al.*, 2011) were compared for presumptive identification of the isolate.

Antibiotics susceptibility test

The antimicrobial susceptibility of the isolate was determined by disc diffusion technique using Muller-Hinton's agar (Bauer *et al.*, 1966). The isolate was tested against 16 antibiotics (Himedia, India) viz., amoxyclave (AMC), ampicillin (AMP), chloramphenicol (C), ciprofloxacin (CIP), clindamycin (CO), co-trimoxazole (COT), erythromycin (E), gentamicin (GEN), kanamycin (K), nitrofurantoid (NIT), norfloxacin (NX), oxytetracycline (O), penicillin-G (P), streptomycin (S), sulphafurazole (SF), and vancomycin (VA). The isolate (Himedia, India) was grown overnight (OD 2 at 600nm) in tryptic soy agar and spreaded on Muller-Hinton's agar. After 30 min, four dissimilar antibiotics discs were positioned on the plates and incubated for 10-24 h at 37°C. After incubation, the zone of inhibition (by mean of diameter in mm) was measured around the discs and compared with the interpretive chart (Clinical and Laboratory Standards Institute, CLSI guidelines, 2012).

Molecular identification

DNA extraction

The genomic DNA was extracted from the *Enterococcus* sp isolate (SRLFDA/TIL-1/15) using QIAamp genomic DNA kit (Qiagen, Germany) as per manufacturer′s protocol.

16S rRNA gene amplification and sequence analysis

The 16S ribosomal RNA gene (16S rRNA) of the isolate was amplified using a set of universal prokaryotic primers 8F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTTACGACTT-3' (Eden *et al.*, 1991). The PCR amplification was performed in a 50 µl reaction volume with 25 µl of PCR master mix (Ampliqon, Denmark), 2.0 µl each of forward and reverse primers, and 2.0 µl (100 ng) of genomic DNA template and (19 µl) nuclease-free water. The PCR reaction was carried out in T-100 TM thermal cycler (Bio-Rad, USA). Amplification was done by initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s with a final extension at 72 °C for 5 min. The PCR product was resolved on a 1.5% agarose gel containing 0.5µg/mL ethidium bromide in 1X Tris-Borate-EDTA (TBE) buffer and electrophoresed at 100 V.

Sequencing analysis

The amplified 16S rRNA gene PCR product was purified using HiyieldTM Gel/PCR DNA mini kit (real genomic, Taiwan) as per the manufacturer's instructions. Nucleotide sequencing (forward and reverse) was done with a commercial sequencing service (Eurofins, India). The forward and reverse

sequences were assembled by DNA baser sequence assembler v3.5.3 (2012) to form consensus sequence and identified by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search algorithm.

PCR detection of tetracycline resistance genes

The isolate SRLFDA/TIL-1/15 was examined for the presence of the tetracycline resistance encoding genes viz., tet (K) tet (L), following the primers and protocols of the previous researchers (Aarestrup *et al.,* 2000; Garofalo *et al.,* 2007; Ullah *et al.,* 2012).

Results and Discussion

Isolation and identification of *E. faecalis*, SRLFDA/TIL-1/15

The clinical symptoms recorded in the Tilapia sample were lethargy, abdominal ascites, organ discoloration, necrosis of the spleen and haemorrhages in kidney. The isolate recovered from kidney samples yielded a predominant black colony on bile-esculin agar (BEA) and *Streptococcus* selective isolation (SIA) agar. Microscopic observation of the stained smear revealed Gram-positive cocci arranged in diplococci or short chain and displayed oxidase and catalase negative activity. The isolate could be grown at above 45 °C on BHI medium containing 6.5 % NaCl, at pH 7.5. Biochemical characterization of the isolate (MLTEC) as assessed by Rapid HiStrepTM biochemical test is presented in table 1.

Enterococci are one of the most common group of bacteria present in foods (Paganelli *et al.,* 2017), mainly due to their resistance to adverse environmental conditions during production technology, as well as food storage conditions and their high adaptability (Boehm and Sassoubre, 2014). *Enterococci* bacterial contamination in seafood products have been documented (Wilson and McAfee, 2002; Sonsa-Ard *et al.,* 2015).

Molecular confirmation by 16S rRNA

PCR amplification of the 16S rRNA of the isolate (SRLFDA/TIL-1/15) resulted in an amplified product of 1450 bp size (Fig. 1). On the basis of gene sequence similarity carried out by BLAST NCBI, the isolate was identified as *E. faecalis* (Genbank Acc. No. KT877352) with 99-100 % homology with other *E. faecalis* strains in the GenBank database (NCBI).

16S ribosomal RNA present in bacteria plays a major role in gene coding due to the highly conserved region. It is considered as a standard marker for bacterial phylogenetic analysis to differentiate the species (Nagpal *et al.,* 1998). Recent studies demonstrated that the different *Enterococcus* strains isolated from diverse sea water environment elucidated unique nucleotide position and evolution of *Enterococcus* and its related species Chajęcka -Wierzchowska *et al.,* 2016; Prichula et al. 2016). Moreover, many recent reports have been published on the 16S rRNA sequences of *Enterococcus* sp and the phylogenetic relationship deduced from analysis of these sequences (Deasy *et al.,* 2000; Mannu *et al.,* 2003; Ben Belgacem *et al.,* 2010; Galimand *et al.,* 2011; Galloway-Pena *et al.,* 2012).

Antibiotic susceptibility profile

Antibiotic sensitivity test showed that the *E. faecalis* (SRLFDA/TIL-1/15) was either resistant and/or intermediately resistant to more than nine classes of antibiotic groups (Table 2). The isolate showed resistance to amoxyclave (AMC), ampicillin (AMP), erythromycin (E), gentamicin (GEN), kanamycin (K), nitrofurantoin (NIT),

oxytetracycline (O), penicillin-G (P), streptomycin (S), and sulphafurazole (SF) and was intermediately resistant to chloramphenicol (C), ciprofloxacin (CIP), clindamycin (CO), norfloxacin (NX), and vancomycin (VA). The isolate was observed to be susceptible only to co-trimoxazole (COT), (Table 2). PCR amplification of the tet (tet K and tet L) genes showed that the *E. faecalis* isolate (SRLFDA/TIL-1/15) from Tilapia carry tet K (360 bp) and tet L (1077 bp) genes (Fig. 2).

Table.1 Biochemical characterization of *Enterococcus faecalis* strain (SRLFDA/TIL-1/15) isolated from diseased Tilapia

Biochemical tests	***Enterococcus faecalis* SRLFDA/TIL-1/15**
Gram reaction	+
Voges–Proskauer's	+
Bile-Esculin agar (black)	+
Esculin hydrolysis	+
PYR	+
ONPG	–
Arginine utilization	nd
Glucose	+
Lactose	+
Arabinose	–
Sorbitol	+
Sucrose	+
Mannitol	+
Raffinose	–
Salt tolerance (6.5 % NaCl)	+

Fig.1 PCR amplification of 16S rRNA of *E. faecalis* isolate from Tilapia

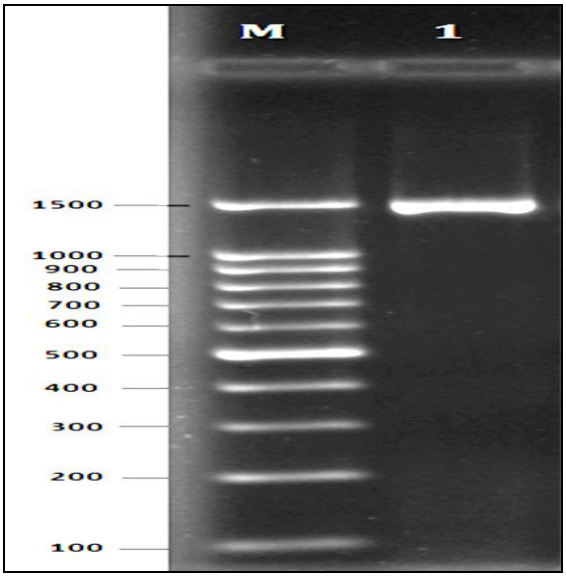
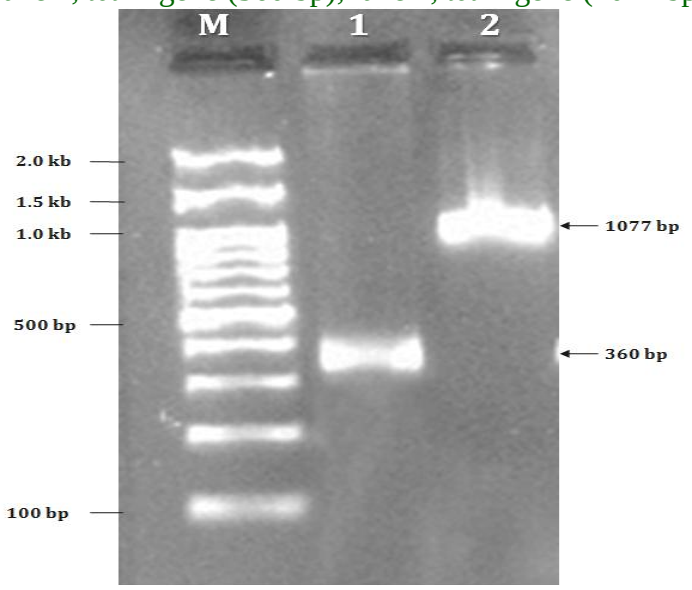


Table.2 Antibiotic resistance profile of *Enterococcus faecalis strain* (SRLFDA/TIL-1/15) isolated from diseased Tilapia

S. No	Antibiotics	TE
1	Amoxyclave (AMC)	0(R)
2	Ampicillin (AMP)	7(R)
3	Chloramphenicol (C)	17(1)
4	Ciprofloxacin (CIP)	18(1)
5	Co-Clindamycin (CO)	12(1)
6	Co-Trimoxazole (COT)	20(S)
7	Erythromycin (E)	9(R)
8	Gentamicin (GEN)	7(R)
9	Kanamycin (K)	0(R)
13	Nitrofurantoin (NIT)	12(R)
10	Norfloxacin (NX)	16(1)
11	Oxy*tet*racycline (O)	0(R)
12	Penicillin-G (P)	17(R)
14	Streptomycin (S)	0(R)
15	Sulphafurazole (SF)	11(R)
16	Vancomycin (VA)	16(1)
	Ratio S: I: R	**1: 5: 10**

Zone of inhibition measured (mm) S=sensitive, I=intermediate, R=resistant, 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).

Fig.2 PCR amplified fragments of the tetracycline resistance genes in *E. faecalis* isolated from diseased Tilapia. Lane M- 100 bp DNA ladder; Lane 1, *tet* K gene (360 bp); lane 2, *tet* L gene (1077 bp)



E. faecalis isolate (SRLFDA/TIL-1/15) was resistant to drugs frequently used to treat bacterial infections in humans and veterinary medicine, including erythromycin, ciprofloxacin, norfloxacin and vancomycin (Bates *et al.*, 1994; Prichula *et al.*, 2016).

Antibiotics represent one of the most prominent aquatic pollutants (Tello *et al.*, 2012). The presence of antibiotics in water can cause serious environmental issues, such as the emergence of resistance due to selective pressure (Baquero *et al.,* 2008). Recently, several studies have reported the development of multiple antibiotic resistance in the microbes of the aquaculture systems (Stalin and Srinivasan, 2016; Prichula *et al.*, 2016; Uma and Ronald, 2016). Although, most of the studies on antibiotic resistance and virulence of *Enterococci* sp have been carried out in strain isolated from clinical samples, recent reports have suggested that environment and food could play a significant role in the transmission of resistance to humans (Gomes *et al.,* 2008; Koluman, 2009; Barbosa *et al.,* 2010; Chajęcka - Wierzchowska *et al.,* 2016). The *E. faecalis* isolate (SRLFDA/TIL-1/15) was found to be resistant to 62% of the antibiotics tested in this study with multiple resistances to ten different antibiotics. Resistances to upto eight antibiotics have been reported in isolates from other aquaculture sources (Akinbowale *et al.*, 2006). The amplification of (tet K and tet L) resistant genes and the tetracycline resistance of the *E. faecalis* isolate (SRLFDA/TIL-1/15) in the antibiotics sensitivity test showed that the resistance shown against tetracycline could be due to the expression of these genes. tet genes are reported to be widely disseminated in the environment (Pallecchi et al., 2008; Di Cesare et al., 2012). The identification of tetracycline resistance determinants may be used as additional genotypic markers for the purpose of outbreak investigation and evolution of gene exchange (Koike *et al.*, 2007; Ng *et al.,* 2001; Ullah *et al.,* 2012; Rico *et al.,* 2014; Prichula *et al.,* 2016).

In conclusion, this is the first report from India on the isolation and confirmation of *E. faecalis* from diseased Tilapia. The presence of antibiotic resistant genes, tet K and tet L shows that there is a need for judicious use of antibiotics in aquaculture and to adopt alternate and safe measures for the management of disease in aquaculture.

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