Distribution of antibiotic resistance and virulence factors among the bacteria isolated from diseased *Etroplus suratensis*

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Abstract

Considering the emerging concern with the antimicrobial resistance (AMR) evolution, the study has been designed to identify the antibiotic resistance and virulence properties of culturable bacteria isolated from the diseased fish *Etroplus suratensis*. This has resulted in the purification of 18 morphologically distinct bacterial isolates which were identified by both biochemical and molecular methods. Antibiotic resistance analysis showed the resistance of these isolates to multiple antibiotics and remarkable evolution of AMR. Further screening for virulence factors confirmed five isolates to be positive for haemolytic activity, eight with caseinase, four with DNase, one with gelatinase and three with biofilm-forming properties. In addition to these, the isolates were subjected to PCR-based screening to detect the presence of genes coding for aerolysin and haemolysin. Results showed the presence aerolysin gene in the isolates ESS3.2, ESS3.8, ESI3.3 and ESS3.6, while haemolysin gene was observed to be present in ESG3.1 and ESI3.2. The observed results hence indicate the need for frequent monitoring of these properties among bacterial isolates from diverse environment especially those associated with edible fish.

Keywords *Etroplus suratensis* · Aquaculture · Antimicrobial resistance · Virulence mechanisms

Introduction

Aquaculture is a key sector for the large-scale production of protein-rich food for human consumption (Troell et al. 2014). Hence, the presence of healthy water microbiome is greatly preferred for the generation of microbially safe food from water sources. As the water bodies are continuously subjected to microbial introduction from diverse sources, it naturally favours the rapid exchange of genetic materials

among microorganisms. The highly dynamic nature of aquatic system also supports the exchange of virulence factors and antibiotic resistance which ultimately enhance the microbial threat to aquaculture and humans (Smith 2006). Hence, these processes can accelerate the conversion of more microorganisms into opportunistic pathogens. Indiscriminate use of chemotherapeutics and antibiotics in aquaculture has already been reported to increase the prevalence of drug resistance among fish-associated bacteria (Rose et al. 2013). The unnoticed accumulation of residual antibiotics in water bodies has been reported to trigger the emergence of microbial multidrug resistance (Cabello 2006; Hoque 2014; Chen et al. 2010; Zhou et al. 2010). Management of such resistant bacteria by use of bacteriophages, short-chain fatty acids and polyhydroxyalkanoates has shown to have limited success (Defoirdt et al. 2010). Hence, the determination of virulence factors and antimicrobial resistance among the microorganisms of water bodies are essential to predict the emerging concern on same. Therefore, the present study mainly focused on the analysis of distribution of virulence factors and antibiotic resistance among microorganisms associated with the diseased edible fish *Etroplus suratensis*.

Aswani Ravi and Soumya Das contributed equally to this work.

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The pathogenicity of microorganisms in the aquatic environment is linked to the presence of virulence factors including its ability to form biofilm (Odeyemi 2013). Protease production has been suggested to enable the pathogens to invade and penetrate the fish tissues (Croxatto et al. 2007). DNase production has been considered to favour the release of pathogenic bacteria from infected cells by digesting the host DNA. The exotoxins and haemolysins of microbial origin further cause lysis of erythrocytes and result in hemorrhagic septicemia (Zhang et al. 2001). Biofilm formation by fish pathogenic bacteria has also been considered to favour the disease progression (O'Toole et al. 2000). Since the bacteria enclosed within the extracellular polymeric substances of biofilm are highly protected, it favours the genetic exchange mechanisms to result in emergence of bacteria with enhanced virulence potential.

Etroplus suratensis (pearl spot or Karimeen), the State fish of Kerala is the largest Indian cichlids endemic to peninsular India and Sri Lanka (Padmakumar et al. 2012). Due to the fluctuations in environmental parameters, pearl spot is prone to various diseases. Hence, the present study is focused on the isolation of pathogenic bacteria from different parts of diseased *E. suratensis* and screening of its antibiotic resistance and production of extracellular virulence factors such as proteases, haemolysin, DNase and gelatinase. The study also involved analysis on biofilm formation by obtained isolates. The results of the study showed the presence of multiple virulence factors, multiple antibiotic resistance and biofilm formation among the selected bacterial isolates. These features of the bacterial isolates obtained from the edible fish *E. suratensis* indicate the emerging challenges with bacteria associated with fish.

Materials and methods

Sample collection

Live diseased *Etroplus suratensis* with ulcerative syndrome disease was collected during the period of September 2015 from a local farm of Kottayam, Kerala. This was transported to laboratory in a polybag filled with 1–2 L of water and was anaesthetized using ethyl 3-aminobenzoate methane-sulfonate and immediately used for the isolation of bacteria.

Isolation of bacteria

Bacterial isolation was carried out from the surface, gill and intestine of diseased fish by crushing approximately 1 g of each sample in a sterile mortar and pestle followed by serial dilution (Suganya et al. 2014). From this, 0.1 mL of all the dilutions was spread-plated onto different media including

starch ampicillin agar (HiMedia Laboratories) and incubated at 28 °C for 24–48 h.

Identification of bacterial isolates

After the purification of obtained bacterial isolates, morphologically different isolates were subjected to identification. For this, Gram's staining and motility analysis were initially carried out. Biochemical tests used for identification were catalase, oxidation fermentation, indole production, methyl red, Voges–Proskauer, urease, hydrolysis of esculin and sodium chloride tolerance (Loong et al. 2016). For the molecular identification, genomic DNA was extracted using the bacterial genomic DNA extraction kit (RKN15) from Chromous Biotech Pvt. Ltd, Karnataka, India, and was used as the template for PCR. The amplification was performed using the 16S rDNA-specific primers 16SF (5'-GAG TTT GAT CCT GGC TCA G-3') and 16SR (5'-GAT ATT ACC GCG GCG CCT G-3'). The 50 µL PCR mix used contained 5 µL (50 ng) of genomic DNA, 2 µL of both forward and reverse primers and 25 µL of PCR master mix. PCR was carried out in a Mycycler™ (BIO-RAD, USA) with the initial denaturation at 94 °C for 3 min followed by 35 cycles of the denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min. The amplified product was checked by agarose gel electrophoresis and was subjected to sequencing. The sequence data were further analyzed by BLAST (Basic Local Alignment Search Tool) analysis (Zhang et al. 2002) and the phylogenetic relationship was analyzed using neighbour-joining method of MEGA5 with 1000 boot strap replicates (Tamura et al. 2011; Jasim et al. 2016).

In vitro screening for extracellular virulence factors

The bacterial isolates were then subjected to screening of virulence factors. Gelatinase activity was analyzed by inoculating the isolates on to gelatin agar plates (gelatin 30 g; casein enzymic hydrolysate 10 g; NaCl 10 g; agar 18 g; pH 7.2; distilled water 1 L) followed by incubation at 28 °C for 24–48 h. The protease activity was screened by spot inoculation of isolates on to skim milk agar plates (skim milk powder 28 g; casein enzymic hydrolysate 5 g; yeast extract 2.5 g; dextrose 1 g; agar 18 g; pH 7; distilled water 1 L), followed by incubation at 28 °C for 24–48 h. For DNase activity screening, the isolates were inoculated on to DNase agar plates (casein enzymic hydrolysate 15 g; papaic digest of soyabean meal 5 g; DNA 2 g; NaCl 5 g; agar 18 g; pH 7.3; distilled water 1 L) with incubation at 28 °C for 24–48 h. To investigate the haemolytic activity, the isolates were spot inoculated onto blood agar plates (peptone 5 g; NaCl 5 g; beef extract 3 g; agar 18 g; pH 7.3; human blood 50 mL; distilled water 1 L) and were observed for the zone of clearance

around the colonies after incubation at 28 °C for 24 h (John and Abdulla 2013).

PCR screening for virulence genes

For the genetic analysis for virulence factors, the bacterial isolates were further subjected to PCR-based detection of genes coding for haemolysin and aerolysin. The primers used for haemolysin gene amplification were HF (5'-ATG AGTTTTGCCGATAGTTTATTTTCCTGA-3') and HR (5'-TACGATTCCTGAGCGGGCTTGTGCGCCGCGTG-3') (Erova et al. 2007) and the primers used for the aerolysin gene were AF (5'-CCTATGGCCTGAGCGAGAAG-3') and AR (5'-CCAGTTCCAGTCCCACCACT-3') (Soler et al. 2002). The PCR was carried out using Mycycler™ (BIO-RAD, USA) in a 50 µL reaction volume. The conditions used were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. After the final extension at 72 °C for 5 min, the amplified products were analyzed by agarose gel electrophoresis. The positive samples were further subjected to sequencing and the sequence data were analyzed by BLASTX.

Determination of antibiotic susceptibility

Susceptibility of the isolates to antimicrobial agents was analyzed by the disc diffusion test (Bauer et al. 1996). For this, dilution of broth culture of all the isolated bacteria was made equivalent to 0.5 McFarland standard and was lawn-cultured on the surface of Mueller Hinton Agar (HiMedia Laboratories) plate. Then selected antibiotic discs purchased from HiMedia Laboratories were placed over this. The antibiotics tested were ampicillin (10 µg), methicillin (10 µg), cefoxitin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline hydrochloride (30 µg), gentamycin (10 µg), linezolid (30 µg), nalidixic acid (30 µg), novobiocin (5 µg), oxacillin (5 µg), penicillin G (10 µg), rifampicin (5 µg), tigecycline (15 µg) and levofloxacin (5 µg). After overnight incubation, the degree of sensitivity was determined by measuring the zone of inhibition around the discs using biogram interpretative chart (Bauer et al. 1996; Standards and Testing 2018).

Quantitative assay for biofilm formation

All the obtained isolates were quantitatively screened for its ability to form biofilm by tissue culture plate (TCP) assay. For this, the isolates were inoculated into nutrient broth and incubated at 28 °C for 18 h. After this, 0.5 mL of the culture was transferred into fresh 10 mL trypticase soy broth (TSB) (tryptone 17 g, soya peptone 3 g, sodium chloride 5 g, dextrose 2.5 g, dipotassium hydrogen phosphate 2.5 g,

distilled water 1 L, pH 7.3) and incubated overnight. The cultures were then diluted to 1:10 with fresh TSB and finally 0.2 mL aliquots of each bacterial suspension was added to 96-well flat bottom tissue culture polystyrene plates (HiMedia, Mumbai) in triplicates along with sterile media as negative control. The tissue culture plates were then incubated at 28 °C for 24 h. After this, the content of each well was removed and washed with 0.2 mL of phosphate-buffered saline (PBS, pH 7.2) (HiMedia, Mumbai) for three times. Then, the plates were stained with 0.2 mL (w/v) of 0.1% crystal violet stain and incubated at 28 °C for 15 min. Each well was washed repeatedly with PBS to remove excess stain. Plates were then dried and 200 µL of 90% ethanol was added to each well and after shaking for 5–10 min, the OD values were measured at 570 nm using ELISA plate reader (Bio Rad imark). Isolates with OD value > 1.0 were considered as strong biofilm producers (Yazdani et al. 2006; Farran et al. 2013).

Results

Isolation and identification of bacteria from diseased *E. suratensis*

Among the various bacterial isolates purified from diseased *E. suratensis*, 18 isolates were found to be morphologically distinct and were selected for further studies. The obtained isolates were designated as ESS3.1, ESS3.2, ESS3.3, ESS3.4, ESS3.5, ESS3.6, ESS3.7, ESG3.1, ESG3.2, ESG3.3, ESI3.1, ESI3.2, ESI3.3, ESW3.1, ESW3.2, ESW3.3, ESW3.4 and ESW3.5. Here, ESS represented the isolates from fish surface, ESG from the gill, ESI from the intestine and ESW from the water of storage in which *E. suratensis* was maintained. The isolates were initially identified by Gram staining, motility and biochemical tests (Table S1). PCR amplification of the 16S rDNA of selected isolates was confirmed by the formation of product of 1500 bp size (Fig. 1). Further BLAST analysis has identified the obtained bacteria as those which belong to the genera *Pseudomonas*, *Aeromonas*, *Klebsiella*, *Chromobacterium*, *Enterobacter*, *Pantoea* and *Burkholderia* (Table 1). The phylogenetic analysis of 16S rDNA sequence of obtained bacteria showed distinct clustering (Fig. 2).

Screening for extracellular virulence factors

Among the 18 isolates screened for gelatinase activity, *Aeromonas hydrophila* (ESS3.4) was found to be positive due to the formation of zone of clearance around the bacterial growth. The zone of clearance around the bacterial growth on skim milk agar for the isolates *Aeromonas jandaiei* ESS3.7, *Aeromonas jandaiei* ESS3.2, *Chromobacterium*

Fig. 1 PCR amplification of 16S rDNA from selected bacterial isolates. Lanes 1–9 represent 1500 bp product from the isolates ESS3.1, ESS3.2, ESS3.3, ESS3.4, ESS3.5, ESS3.6, ESS3.7, ESG3.1, and

ESG3.2. Lane M represents 100 bp ladder and Lane 10–18 represent 1500 bp product from ESG3.3, ESI3.1, ESI3.2 and ESI3.3, ESW3.1, ESW3.2, ESW3.3, ESW3.4 and ESW3.5

Table 1 16S rDNA sequence analysis of bacterial isolates from different parts of *E. suratensis*. Here, ESS, ESG, ESI and ESW indicated the surface, gill, intestine and water in which *E. suratensis* was maintained, respectively

Name of isolates	Source of isolation	Closest NCBI match with accession number	% of identity (%)
ESG 3.1	<i>E. suratensis</i> —gill	<i>Klebsiella pneumoniae</i> —KX170832.1	99
ESG 3.2	<i>E. suratensis</i> —gill	<i>Pseudomonas</i> sp.—KX822862.1	99
ESG 3.3	<i>E. suratensis</i> —gill	<i>Burkholderia</i> sp.—KU375115.1	99
ESI 3.1	<i>E. suratensis</i> —intestine	<i>Aeromonas jandaei</i> —KT998868.1	99
ESI 3.2	<i>E. suratensis</i> —intestine	<i>Klebsiella pneumoniae</i> —KX170832.1	99
ESI 3.3	<i>E. suratensis</i> —intestine	<i>Aeromonas jandaei</i> —KT998868.1	100
ESS 3.1	<i>E. suratensis</i> —surface	<i>Chromobacterium violaceum</i> —KL716449.1	99
ESS 3.2	<i>E. suratensis</i> —surface	<i>Aeromonas jandaei</i> —KT998868.1	99
ESS 3.3	<i>E. suratensis</i> —surface	<i>Pantoea pleuroti</i> —KX090187.1	100
ESS 3.4	<i>E. suratensis</i> —surface	<i>Aeromonas hydrophila</i> —KT998822.1	99
ESS 3.5	<i>E. suratensis</i> —surface	<i>Aeromonas veronii</i> —KX828324.1	100
ESS 3.6	<i>E. suratensis</i> —surface	<i>Klebsiella pneumoniae</i> —KT998846.1	99
ESS 3.7	<i>E. suratensis</i> —surface	<i>Aeromonas jandaei</i> —KT998868.1	100
ESW3.1	<i>E. suratensis</i> —water	<i>Pseudomonas</i> sp.—KF870423.1	100
ESW 3.2	<i>E. suratensis</i> —water	<i>Enterobacter asburiae</i> —KX246804.1	98
ESW 3.3	<i>E. suratensis</i> —water	<i>Pseudomonas</i> sp.—KX 350139.1	99
ESW 3.4	<i>E. suratensis</i> —water	<i>Burkholderia</i> sp.—KU375115.1	99
ESW 3.5	<i>E. suratensis</i> —water	<i>Chromobacterium violaceum</i> —KL716449.1	99

violaceum ESS3.1, *Aeromonas veronii* ESS3.5, *Chromobacterium violaceum* ESW3.5, *Burkholderia* sp. ESW3.4, *Aeromonas jandaei* ESI3.1, and *Burkholderia* sp. ESG3.3 indicated its protease activity. The isolates positive for the DNase activity were *Aeromonas jandaei* ESS3.2, *Aeromonas jandaei* ESS3.7, *Aeromonas hydrophila* ESS3.4, and *Aeromonas veronii* ESS3.5 as confirmed by the formation of zone

of clearance after flooding with 1 M HCl. At the same time, the isolates *Aeromonas jandaei* ESI3.3, *Aeromonas jandaei* ESS3.7, *Aeromonas jandaei* ESS3.2, *Klebsiella pneumoniae* ESS3.6 and *Aeromonas hydrophila* ESS3.4 were found to be positive for haemolytic properties (Table 2). PCR-based screening for the haemolysin gene has resulted in the formation of product size of 1320 bp (Fig. 3) for *Klebsiella*

Fig. 2 The phylogenetic relationship of the bacterial isolates obtained from *E. suratensis*

spp. (ESG3.1 and ESI3.2). The formation of product with 431 bp size (Fig. 4) for the isolates ESS3.2, ESS3.8, and ESI3.3 (*Aeromonas jandaei*) and ESS3.6 (*Aeromonas veronii*) indicated the presence aerolysin gene in them. Both the PCR products were further confirmed by sequencing and sequence analysis.

Antibiotic susceptibility test

Among all the isolates, 100% were resistant to methicillin, oxacillin and penicillin G, 88% (16 isolates) showed resistance to linezolid and 83% (15 isolates) were resistant to novobiocin. On the other hand, 100% of the isolates were susceptible to ciprofloxacin and levofloxacin followed by 88% (16 isolates) to chloramphenicol and 72% (13 isolates) to nalidixic acid. Sixty-one percentage (11 isolates) and 50% (9 isolates) of the isolates exhibited intermediate resistance to gentamycin and doxycycline hydrochloride, respectively. Resistance and susceptibility rates of rest of the tested antibiotics varied among the isolates (Fig. 5, Table S2). Among the 15 antibiotics screened, *Pseudomonas* spp. (ESW3.1 and ESW3.3) showed resistance to ten antibiotics. *Enterobacter* sp. (ESW3.2) and *Pantoea* sp. (ESS3.3) were resistant to

nine antibiotics and *Klebsiella* sp. (ESS3.6) showed resistance to eight antibiotics. *Aeromonas* sp. (ESS3.2), *Klebsiella* sp. (ESI3.2), *Pseudomonas* sp. (ESG3.2) and *Burkholderia* sp. (ESG3.3) were resistant to seven antibiotics. *Aeromonas* spp. (ESS3.5, ESS3.7, ESI3.1 and ESI3.3), *Klebsiella* sp. (ESG3.1), *Burkholderia* sp. (ESW3.4) and *Chromobacterium* sp. (ESW3.5) were resistant to six antibiotics where as *Aeromonas* sp. (ESS3.4) and *Chromobacterium* sp. (ESS3.1) were resistant to five antibiotics (Fig. S1). Generally, most of the isolates showed multiple antibiotic resistances which indicate the remarkable evolution of AMR among the bacterial isolates purified from *E. suratensis*.

Quantitative analysis on biofilm formation

Among the 18 isolates screened for biofilm formation, ESG3.1, ESI3.2 (*Klebsiella pneumoniae*), ESW3.3 (*Pseudomonas* sp.), and ESS3.7 (*Aeromonas jandaei*) showed an OD value above 1 at 570 nm and were categorized as strong biofilm producers. Rest of the isolates such as *Pseudomonas* sp. ESG3.2, *Burkholderia* sp. ESG3.3, *Aeromonas jandaei* ESI3.1, *Aeromonas jandaei* ESI3.3, *Chromobacterium violaceum* ESS3.1, *Aeromonas jandaei*

Table 2 Extracellular enzyme production by selected bacterial isolates

Isolates	Protease activity	Gelatinase production	Hemolytic activity	DNase activity
ESS 3.1	+	—	—	—
ESS 3.2	+	—	+	+
ESS 3.3	—	—	—	—
ESS 3.4	—	+	+	+
ESS 3.5	+	—	—	+
ESS 3.6	+	—	+	—
ESS 3.7	+	—	+	+
ESG 3.1	—	—	—	—
ESG 3.2	—	—	—	—
ESG 3.3	+	—	—	—
ESI 3.1	+	—	—	—
ESI 3.2	—	—	—	—
ESI 3.3	—	—	+	—
ESW 3.1	—	—	—	—
ESW 3.2	—	—	—	—
ESW 3.3	—	—	—	—
ESW 3.4	+	—	—	—
ESW 3.5	+	—	—	—

Here, ESS, ESG, ESI and ESW indicated the surface, gill, intestine and water in which *E. suratensis* maintained, respectively

Fig. 3 PCR gel image of haemolysin gene with a product size of 1320 bp. Lane 1—ESG3.1; Lane 2—ESI3.2; Lane M—Marker

ESS3.2, *Pantoea pleuroti* ESS3.3, *Aeromonas hydrophila* ESS3.4, *Aeromonas veronii* ESS3.5, *Klebsiella pneumoniae* ESS3.6, *Pseudomonas* sp. ESW3.1, *Enterobacter asburiae* ESW3.2, *Burkholderia* sp. ESW3.4 and *Chromobacterium violaceum* ESW3.5 were non-biofilm producers (Fig. 6).

Fig. 4 PCR gel image of aerolysin gene with a product size of 431 bp; Lane M—Marker; Lane 1—ESS3.2; Lane 2—ESS3.8; Lane 3—ESI3.3; and Lane 4—ESS3.6

Discussion

Aquaculture contributes significantly to meet the increasing demand for food in many regions of the world. However, various diseases and the environmental factors limit the production of good quality products (Munoz-atienza et al. 2013). The presence of diverse virulence factors and antibiotic resistance among the microflora of aquatic environment can directly favour the disease progression in fish. These can have indirect threat to humans due to the likely transfer of these virulence properties to human-associated microorganisms or pathogens. *E. suratensis* or pearl spot is a high-valued fish and its survival is highly affected by bacterial diseases. Here, most of the infections are caused by multiple organisms with varying virulence properties (Musa et al. 2008; Fish 2012). The emergence of antimicrobial resistant bacteria makes the health of fish more challenging. Hence, the uncontrolled use of antibiotics and chemotherapeutic agents to manage bacterial infections in fish can ultimately result in evolution of highly resistant pathogens (Inglis et al. 1993; Akinbowale et al. 2006). Therefore, an understanding on emergence of virulence properties and antibiotic resistance among bacteria associated with edible fishes like *E. suratensis* is very important as it is subjected to human handling also at various stages.

In the study, bacterial isolates obtained from various parts of diseased fish *E. suratensis* were identified as members of the genera *Aeromonas*, *Pseudomonas*, *Klebsiella*, *Chromobacterium*, *Burkholderia*, *Enterobacter* and *Pantoea*. *Aeromonas* spp. have previously been reported as the most

Fig. 5 Antibiotic resistance, sensitivity and intermediate resistance of the obtained isolates

Fig. 6 Graphical representation of biofilm formation in bacterial isolates from diseased *E. suratensis*

common bacterial genera associated with fish diseases (Igbinosa et al. 2012). At the same time, the features of aquatic environment can favour the fish disease to be progressed by

multiple microbial agents. Hence, the different organisms identified in the study can be the reflection of polymicrobial basis of disease occurred in the selected *E. suratensis*. The

results obtained in the study are in accordance with previous reports on isolation of bacteria from *Tilapia rendalli*, *Oreochromis mossambicus* and from *Scomber scombrus* which support the bacterial role in fish diseases (Sichewo et al. 2014; Eze et al. 2011).

Detection of virulence factor is a key step to identify the potential pathogenicity of the obtained isolates. Fish surface and tissue invasion by the bacterial pathogen is considered to be facilitated by the functioning of virulence factors (Keya Sen and Lye 2007). Chopra et al. (1999) have also reported the role of microbial virulence factors to act multi-mechanistically to result in fish diseases. Haemolysin exert lytic effect on red blood cell (Odeyemi et al. 2013) and its importance in *Aeromonas hydrophila* infection has already been well demonstrated (Samal et al. 2014). Likewise other virulence factors such as DNase, protease and gelatinase activities can also consider to provide effective means to establish disease by microbial agents. Hence, identification of virulence factors is very important with respect to the pathogens of economically important fish like *E. suratensis*. Therefore, all the bacterial isolates obtained in the study were screened for extracellular virulence properties by phenotypic and genotypic methods. Among the 18 isolates studied, 11 isolates were found to have at least one of the screened properties. This is also supportive to consider the observed disease of *E. suratensis* as due to the action of polymicrobial agents. For PCR-based screening of virulence factors, primers specific to haemolysin and aerolysin were used. Among all the 18 samples screened for haemolysin gene, *Klebsiella* spp. (ESG3.1 and ESI3.2) were found to be positive. At the same time, aerolysin gene was identified to be present in *Aeromonas jandaei* (ESS3.2, ESS3.8, and ESI3.3) and *Aeromonas veronii* (ESS3.6). In both cases, the specificity of PCR product formed was confirmed by sequencing and sequence analysis. This is also supportive to the virulence properties of selected bacterial isolates as per previous reports (Wang et al. 2003; Tomas et al. 2012; Pereira and Vanetti 2015). Hence, the results of both phenotypic and genotypic analysis suggested the remarkable virulence features of bacterial isolates purified from the diseased *E. suratensis*.

Distribution of antibiotic resistance observed for the bacterial isolates was comparable to the already reported features of *Aeromonas* sp. from fish samples (Stratev et al. 2016). Similar studies on market fish also described the isolation of *Aeromonas hydrophila* with 100% resistance to methicillin (Vivekanandhan et al. 2002). Chung et al. (2017) have also reported the high prevalence of antibiotic resistance for bacteria isolated from imported pet fish. Higher level of antibiotic resistance has also been reported previously for *Klebsiella* sp., and *Pseudomonas* sp. of fish origin and also for *Chromobacterium violaceum* (Yasemin et al. 2016; Cristiana et al. 2009). The result obtained

in the study hence is an indication of remarkable AMR evolution in the aquatic system. The antibiotic resistance observed among the organisms studied might have either originated due to their direct interaction with residual antibiotics in the environment or they might have acquired this from other organisms by genetic exchange mechanisms. Even though many bacterial strains have been reported to have antibiotic resistance properties, the occurrence of same in edible fish like *E. suratensis* indicates its likely chance to get transferred to human microbiome which can have significant health impact. Hence, these results on AMR of fish-associated bacteria indicate the aquatic environment to be a potential reservoir for emergence of antimicrobial resistance.

Among the 18 isolates screened for biofilm formation, 4 isolates (*Klebsiella pneumoniae* ESI3.2, *Pseudomonas* sp. ESW3.3, *Klebsiella pneumoniae* ESG3.1 and *Aeromonas jandaei* ESS3.7) were identified as strong biofilm producers. Fish pathogens such as *Flavobacterium*, *Vibrio*, *Yersinia* and *Aeromonas* spp. have previously been reported to form biofilm structures to facilitate their survival (Basson et al. 2008). Many *Aeromonas* spp. have also been reported to have the pathogenic nature due to biofilm formation (Tomas 2012; Beaz-Hidalgo and Figueras 2013). However, *Aeromonas* isolated from fresh water and marine sources have been reported to form species-specific biofilm as per their motility, cell surface characteristics and extracellular virulence factors (Chenia and Duma 2017). Hence, the bacterial isolates obtained from the diseased *E. suratensis* are remarkable due to their diverse virulence factors, multidrug resistance and biofilm formation. This indicates the biological potential of these organisms to either cause threat to aquatic life or to act as a source to transfer these to other microbial populations (Morikawa 2006). In the era of antimicrobial resistance emergence, evolution of resistance from diverse sources has to be studied to predict the challenge with currently used antibiotics. In this context, aquatic microbiome is an important reservoir for antimicrobial resistance and current study highlights its significance.

Conclusion

The present study reported the polymicrobial basis of disease in the edible fish *E. suratensis*. The antibiotic resistance and virulence factor analysis of bacteria isolated from *E. suratensis* suggested its association with fish disease. Further, biofilm formation observed for some of the selected isolates indicated its colonization potential on fish surface. Current study thus demonstrates pathogenic properties of bacteria isolated from the edible fish *E. suratensis* and its environmental significance.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

- Akinbowale OL, Peng H, Barton MD (2007) Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Microbiol* 103:2016–2025. <https://doi.org/10.1111/j.1365-2672.2007.03445.x>
- Basson A, Flemming LA, Chenia HY (2008) Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacterium johnsoniae*-like isolates. *Microb Ecol* 55:1–14. <https://doi.org/10.1007/s00248-007-9245-y>
- Bauer AW, Kirby WM, Sherris JC, Turck M (1996) Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 45(4):493–496
- Beaz-Hidalgo R, Figueras MJ (2013) *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease. *J Fish Dis* 36:371–388. <https://doi.org/10.1111/jfd.12025>
- Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol* 8:1137–1144. <https://doi.org/10.1111/j.1462-2920.2006.01054.x>
- Chen R, Zhou Z, Cao Y et al (2010) High yield expression of an AHL-lactonase from *Bacillus* sp. B546 in *Pichia pastoris* and its application to reduce *Aeromonas hydrophila* mortality in aquaculture. *Microb Cell Fact* 9:1–10. <https://doi.org/10.1186/1475-2859-9-39>
- Chenia HY, Duma S (2017) Characterization of virulence, cell surface characteristics and biofilm-forming ability of *Aeromonas* spp. isolates from fish and sea water. *J Fish Dis* 40:339–350. <https://doi.org/10.1111/jfd.12516>
- Chopra AK, Houston CW (1999) Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes Infect* 1:1129–1137. [https://doi.org/10.1016/S1286-4579\(99\)00202-6](https://doi.org/10.1016/S1286-4579(99)00202-6)
- Chung T-H, Yi S-W, Shin G-W (2017) Antibiotic resistance and repetitive-element PCR fingerprinting in *Aeromonas veronii* isolates. *J Fish Dis* 40:821–829. <https://doi.org/10.1111/jfd.12564>
- Cristiana GDO, Regina VA, Luismar MP (2009) *Chromobacterium violaceum* ATCC12472: multi-drug and ethidium bromide resistant. *Nat Proc* 1–12
- Croxatto A, Lauritz J, Chen C, Milton DL (2007) *Vibrio anguillarum* colonization of rainbow trout integument requires a DNA locus involved in exopolysaccharide transport and biosynthesis. *Environ Microbiol* 9:370–382. <https://doi.org/10.1111/j.1462-2920.2006.01147.x>
- Defoirdt T, Darshanee RHA, Karunasagar I et al (2010) Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. *Environ Microbiol Rep* 2:44–49. <https://doi.org/10.1111/j.1758-2229.2009.00043.x>
- Erova TE, Sha J, Horneman AJ et al (2007) Identification of a new hemolysin from diarrheal isolate SSU of *Aeromonas hydrophila*. *FEMS Microbiol Lett* 275:301–311. <https://doi.org/10.1111/j.1574-6968.2007.00895.x>
- Eze EI, Echezona BC, Uzodinma EC (2011) Isolation and identification of pathogenic bacteria associated with frozen mackerel fish (*Scomber scombrus*) in a humid tropical environment. *Afri J Agric Res* 6:1947–1951.
- Farran CAEL, Sekar A, Balakrishnan A, Shanmugam S, Arumugam P, Gopalswamy J (2013) Prevalence of biofilm-producing *Staphylococcus epidermidis* in the healthy skin of individuals in Tamil Nadu, India. *Indian J Med Microbiol* 31(1):19–23
- Fish S (2012) *Etioporus suratensis* (Bloch). The State Fish of Kerala. 37:925–931. <https://doi.org/10.1007/s12038-012-9271-x>
- Hoque F (2014) Biocontrol of β -haemolytic *Aeromonas hydrophila* infection in *Labeo rohita* using antagonistic bacterium *Pseudomonas aeruginosa* FARP72. *Int J Pharm Sci Res* 5(2):490–501. <https://doi.org/10.13040/IJPSR.0975-8232.5490-01>
- Igbinsola IH, Igumbor EU, Aghdasi F, Tom M, Okoh AI (2012) Emerging *Aeromonas* species infections and their significance in public health. *Scientific World Journal*, 625023
- Inglis V, Millar SD, Richards RH (1993) Resistance of *Aeromonas salmonicida* to amoxicillin. *J Fish Dis* 16:389–395. <https://doi.org/10.1111/j.1365-2761.1993.tb00872.x>
- Jasim B, Sreelakshmi S, Mathew J, Radhakrishnan EK (2016) Identification of endophytic *Bacillus mojavensis* with highly specialized broad spectrum antibacterial activity. *3 Biotech* 6:1–10. <https://doi.org/10.1007/s13205-016-0508-5>
- John N, Abdulla MH (2013) Distribution, extracellular virulence factors and drug resistance of motile aeromonads in fresh water ornamental fishes and associated carriage water. *Int J Aquac* 3
- Loong SK, Khor CS, Jafar FL, AbuBakar S (2016) Utility of 16S rDNA Sequencing for Identification of Rare Pathogenic Bacteria. *J Clin Lab Anal* 30:1056–1060. <https://doi.org/10.1002/jcla.21980>
- Morikawa M (2006) Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. *J Biosci Bioeng* 101:1–8. <https://doi.org/10.1263/jbb.101.1>
- Muñoz-Atienza E, Gómez-Sala B, Araújo C et al (2013) Antimicrobial activity, antibiotic susceptibility and virulence factors of Lactic Acid Bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC Microbiol*. <https://doi.org/10.1186/1471-2180-13-15>
- Musa N, Wei SL, Shaharom F, Wee W (2008) Surveillance of bacteria species in diseased freshwater ornamental fish from aquarium shop. *World Appl Sci* 3:903–905
- O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49–79. <https://doi.org/10.1146/annurev.micro.54.1.49>
- Odeyemi OA (2013) Virulence factors and biofilm formation in *Aeromonas* and *Vibrio* species from coastal sources. *Ecology Environ Conservation* 19(4):1271–1278
- Padmakumar K, Bindu L, Manu P (2012) *Etioporus suratensis* (Bloch), the State Fish of Kerala. *J Biosci* 37:925–931. <https://doi.org/10.1007/s12038-012-9271-x>
- Pereira SC, Vanetti MC (2015) Potential virulence of *Klebsiella* sp. isolates from enteral diets. *Braz J Med Biol Res* 48(9):782–789. <https://doi.org/10.1590/1414-431X20154316>
- Rose S, Hill R, Bermudez LE, Miller-Morgan T (2013) Imported ornamental fish are colonized with antibiotic-resistant bacteria. *J Fish Dis* 36:533–542. <https://doi.org/10.1111/jfd.12044>
- Samal SK, Das BK, Pal BB (2014) In vitro and In vivo virulence study of *Aeromonas hydrophila* isolated from fresh water fish. *Int J Curr Res Acad Rev* 2:117–125
- Sen K, Lye D (2007) Importance of flagella and enterotoxins for *Aeromonas* virulence in a mouse model. *Can J Microb* 53(2):261–269. <https://doi.org/10.1139/W06-095>
- Sichewo PR, Gono RK, Muzondiwa J, Mungwadzi W (2014) Isolation and identification of pathogenic bacteria in edible fish: a case study of rural aquaculture projects feeding livestock manure to fish in Zimbabwe. *Int J Curr Microbiol* 3:897–904
- Smith P (2006) Breakpoints for disc diffusion susceptibility testing of bacteria associated with fish diseases: a review of current practice. *Aquaculture* 261:1113–1121. <https://doi.org/10.1016/j.aquaculture.2006.05.027>
- Soler L, Figueras MJ, Chacón MR, Vila J, Marco F, Martínez-Murcia AJ, Guarro J (2002) Potential virulence and antimicrobial susceptibility of *Aeromonas popoffii* recovered from freshwater and seawater. *FEMS Immunol Med Microbiol* 32(3):243–247

- Standards P, Testing (2018) AS M100 performance standards for antimicrobial. 28 th edition
- Stratev D, Odeyemi OA (2016) Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: A mini-review. J Infect Public Health 9:535–544. <https://doi.org/10.1016/j.jiph.2015.10.006>
- Suganya DS, Rajan MRR MR, P.Sivakumar PS (2014) Isolation, Identification, Enzymatic and Molecular Characterization of Intestinal Bacteria of Goldfish (*Carassius Auratus*) and its Role on Growth. Indian J Appl Res 4:9–11. <https://doi.org/10.15373/2249555x/july2014/3>
- Tamura K, Peterson D, Peterson N et al (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. <https://doi.org/10.1093/molbev/msr121>
- Tomás JM (2012) The main *Aeromonas* pathogenic factors. ISRN Microbiol 4:256261. <https://doi.org/10.5402/2012/256261>
- Troell M, Naylor RL, Metian M et al (2014) Does aquaculture add resilience to the global food system? Proc Natl Acad Sci 111:LP-13257–LP-13263. <https://doi.org/10.1073/pnas.1404067111>
- Vivekanandhan G, Savithamani K, Hatha AAM, Lakshmanaperumalsamy P (2002) Antibiotic resistance of *Aeromonas hydrophila* isolated from marketed fish and prawn of South India. Int J Food Microbiol 76:165–168. [https://doi.org/10.1016/S0168-1605\(02\)00009-0](https://doi.org/10.1016/S0168-1605(02)00009-0)
- Wang G, Clark CG, Liu C et al (2003) Detection and characterization of the hemolysin genes in. J Clin Microbiol 41:1048–1054. <https://doi.org/10.1128/JCM.41.3.1048>
- Yasemin BY, Hediye TV (2016) Antibiotic Resistance Profiles Bacteria Isolated Finfish Species Iskenderun Bay Northeastern Mediterranean Sea Turkey. Pak J Zool 49(4):1353–1358
- Yazdani R, Oshaghi M, Havayi A et al (2006) Detection of icaAD gene and biofilm formation in *Staphylococcus aureus* isolates from wound infections. Iran J Public Health 35(2):25–28
- Zhang X, Meaden PG, Austin B (2001) Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi*. 67:316–317. <https://doi.org/10.1128/AEM.67.7.3161>
- Zhang Z, Schwartz S, Wagner L, Miller W (2002) A Greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214. <https://doi.org/10.1089/10665270050081478>
- Zhou X, Wang Y, Yao J, Li W (2010) Inhibition ability of probiotic, *Lactococcus lactis*, against *A. hydrophila* and study of its immunostimulatory effect in tilapia (*Oreochromis niloticus*). Int J Eng Sci Technol 2:73–80