Fishery Technology 60 (2023) : 196 - 205

Efflux Pump and Biofilm Forming Capabilities of Antibiotic Resistant, *Serratia marcescens* from Freshwater Fish Farms in Andhra Pradesh, India

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Abstract

Serratia marcescens gains much attention due to its ubiquitous distribution in soil, water and plant, on the surface of animals, as well as in the intestinal tract of animals, posing problems for both public and animal health. There is a scarcity of information on its distribution in aquaculture settings. Hence, the present study was taken to identify *S. marcescens*, its antibiotic-resistant pattern, and underlying mechanisms in freshwater fish farms in Andhra Pradesh, India. Sampling was done in 54 freshwater fish farms from August 2021 to April 2022, and 31 isolates were confirmed as *S. marcescens* by standard morphological and biochemical methods. The majority of strains were isolated from water (n=13) followed by gill swabs (n=9), skin swabs (n=5) and sediment (n=4). Antibiotic susceptibility testing revealed that 71 %, 48 %, 48 % and 9.7 % of isolates were resistant to furazolidone, oxytetracycline, doxycycline hydrochloride and co-trimoxazole, respectively, whereas 100 % of isolates were sensitive to ciprofloxacin and enrofloxacin. The minimum inhibitory concentrations for furazolidone, oxytetracycline, and doxycycline were found to be 256 μg/ml, 192 μg/ml and 64 μg/ml, respectively. Only three isolates were found to be multi-drug resistant, having resistance to furazolidone, oxytetracycline, and co-trimoxazole. All the isolates had the ability to form biofilms, and 48.4 %, 32.2 % and 19.4 % of the strains were strong, moderate, and weak biofilm formers, respectively. Spearman rank correlation revealed a strong correlation between biofilm formation and antibiotic resistance. Twenty-five resistant isolates possessed efflux pump activity, as evident in 0.5 to 2.5 mg/L concentrations of ethidium bromide containing agar plates. Biofilm formation and efflux pump activities of *S. marcescens* contribute to enhanced antimicrobial resistance to different antibiotics in the environment and could cause serious public health implications. To the best of our knowledge, this is the first report of *S. marcescens* isolated from freshwater finfish farms in India.

Keywords: Antibiotic resistance, *Serratia marcescens,* fish farms, biofilm, efflux pump activity

Introduction

Antimicrobial use (AMU) is the driving force behind antimicrobial resistance (AMR) in humans and animals (Dadgostar, 2019; Schar et al., 2020). Unfortunately, the overuse and indiscriminate use of antibiotics in the globally interconnected ecosystem has resulted in a massive increase in AMR, which eventually leads to morbidity and mortality (Lu et al., 2014; Kotwani et al., 2021). In aquaculture, production intensification and an increase in the prevalence of aquatic animal pathogens are driving AMU and AMR in a variety of farmed species (Cabello, 2006; Cabello et al., 2013 & 2016; Miller & Harbottle, 2018; Reverter et al., 2020). Unregulated antibiotic use in humans and animals, the use of antibiotic-fed animal waste, as well as pharmaceutical industry effluents, are all sources of antibiotic metabolites, antibiotic-resistant bacteria (ARBs) and antibiotic-resistant genes (ARGs) in soil and water bodies (Meersche et al., 2020).

Serratia marcescens a Gram-negative bacterium that infects plants, insects and animals, including

Received 28 January 2023; Revised 08 April 2023; Accepted 20 July 2023

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humans, often exhibits a high level of resistance to a wide range of antimicrobials used in humans and persists for long periods in the hospital environment (Khanna et al., 2013; Gadhiya et al., 2021). Gram-negative bacteria have prominent survival mechanisms confer antibiotic resistance, contributing to pathogenicity and virulence (Ray et al., 2017; Alav et al., 2018). *S. marcescens* can form biofilms, which protect it from antibiotics and other toxic compounds in the environment (Mehlen, 2011). Bacterial biofilms are extracellular matrices composed of polysaccharides, proteins and extracellular DNA, which contribute to increased antimicrobial tolerance (Flemming et al., 2016; Satpathy et al., 2016). High population densities and cell proximity in biofilms also increase the likelihood of genetic exchange among bacteria, transforming biofilms into antibiotic-resistance hotspots (Balcazar et al., 2015). Efflux pumps are membrane proteins found in all bacteria that are responsible for transporting a substance out of the cell (Poole, 2005). It has been reported that biofilms can interact synergistically with efflux pumps resulting in an overall effect of increased resistance by the quorum-sensing communication system (Jonas et al., 2007; Alav et al., 2018). Although *S. marcescens* has been isolated from fish farms, it is not a fish-associated bacterium and may play an important role in antimicrobial resistance dissemination through horizontal gene transfer among bacteria in the environment (Sandner-Miranda et al., 2018). It has a tremendous ability to survive in the environment through hospital effluents and sewage and may contaminate adjacent aquatic environments and remain viable at sub-optimal concentrations of antibiotics and disinfectants used in aquaculture practices (Gadhiya et al., 2021). Although we did not find S. *marcescens* associated with diseases in fishes, the incidence and frequent occurrence (3 %) of *S. marcescens* in aquaculture farms observed during the preliminary survey and their relatively high resistance to antibiotics is a matter of concern. The present study was undertaken to identify the distribution of antibiotic-resistant *S. marcescens* in freshwater fish farms. The findings highlight the importance of monitoring *S. marcescens* and its spread in the natural environment, as well as the need for continuous surveillance to support effective control measures.

Materials and Methods

All the chemicals including tryptone soya agar (TSA) and broth, Luria-Bertani (LB) broth, Muller-Hinton agar, glacial acetic acid, ethanol, crystal violet, antibiotic discs and MIC E-strips were purchased from HiMedia Laboratories Pvt. Ltd, India.

The current study was conducted in freshwater fish farms in Krishna and West Godavari of Andhra Pradesh, India. This region is known for the cultivation of Indian major carp, exotic carp and other commercially important species. The aquaculture farms selected for this study were large-scale fish farms (> 3-10 Ha), practicing polyculture (Indian major carp, exotic carps and cat fishes) and following partial harvesting. Sampling was conducted at randomly selected fish farms from August 2021 to April 2022. Data regarding farming activities, disease incidence, chemical usage, preventive strategies and other relevant information have been collected using a questionnaire.

Sediment (n=48), water (n=50) and fish samples (n=110) were collected from the 54 selected fish farms following Austin & Austin (2007) procedures. Approximately 100-200 g of sediment was taken from different stretches of each farm and pooled together. 10 ml and 10 g of water and sediment was added to 90 ml of tryptone soya broth (TSB) and incubated at 37 °C for 24 h. From each farm, an average of 2–3 fishes were collected and transported to the laboratory within 2–3 h in sterile sealed bags under iced conditions for further analysis. For animal samples, gills and skin swabs were collected and directly transferred into TSB and incubated at 37 °C for 24 h. Following incubation, a loopful of enriched samples were streaked on to TSA plate and incubated at 37 °C for 24 h. The red pigment colonies were selected and used for further identification.

Further, the suspected *S. marcescens* was identified based on its morphological and biochemical characteristics. The isolates producing round and red pigment colonies were subjected to further biochemical tests such as Gram staining, catalase, oxidase, citrate, lysine, ornithine decarboxylase, hydrogen sulfide, DNase and gelatinase activities, urease, arabinose and lactose fermentation, which were performed following the guidelines of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The sample type, number of samples examined, and number of *S. marcescens* isolated from the study are presented in Table 1.

Antimicrobial susceptibility testing was performed on Muller-Hinton agar plates by the Bauer et al. (1966) disc diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI, 2021). Overnight-grown bacterial cultures were adjusted to 0.5 McFarland standards and a 100 µL of the sample was plated on Mueller-Hinton agar plates and incubated at 28 °C for 24 h. The following panel of antibiotic discs were used: furazolidone (100 µg), oxytetracycline (30 µg), doxycycline hydrochloride (30 µg), co-trimoxazole (25 µg), enrofloxacin (10 µg) and ciprofloxacin (5 µg). The zones of inhibition were recorded and the isolates were categorized as susceptible, intermediate and resistant according to the zone diameter interpretation standards described in the CLSI (2021). Isolates that exhibited resistance to three or more classes of antibiotics were categorized as multidrug-resistant (MDR) as per Magiorakos et al. (2012). The MIC was determined for selected resistant isolates using MIC E-strips embedded with antibiotics ranging (from 0.016-256 µg/ml).

The biofilm formation assay was performed using the 96-well tissue culture plate (TCP) method described by Mathur et al. (2006). Individual colonies of *S. marcescens* isolates were inoculated into Brain Heart Infusion (BHI); 200 µL of bacterial suspension was loaded into the 96-well microtiter plate and incubated at 37 °C for 24 h. 200 µL of sterile BHI broth poured into wells served as negative control. After overnight incubation, the wells were washed thrice with sterile deionized water and air-dried for 45 min, following which 200 µL of 0.1 % (v/v) crystal violet solution (HiMedia, India) was added and incubated for 45 min at room temperature. The wells were then washed four times with sterile deionized water. The dye was solubilized by adding 200 µL of 33 % glacial acetic acid, and optical density (OD) was measured at 650 nm using the iMark™ Microplate reader (Biorad, USA). The assay was carried out in triplicates, and the biofilm-forming potential was recorded as mean OD values, which Hassan et al. (2011) defined as strong (>0.108), moderate (0.108–0.083) and weak (<0.083).

The efflux pump activity of resistant isolates was determined using the Ethidium bromide (EtBr)-agar Cartwheel (EtBrCW) method described by Martins et al. (2011). This method provides information on the ability of each isolate to extrude EtBr from the cells by efflux on the basis of fluorescence emitted from isolates swabbed in EtBr-containing agar plates. The technique employed was to use different sets of freshly prepared plates of TSA with concentrations of EtBr ranging from 0.2 to 2.5 mg/L were kept protected from light until use. Overnight-grown cultures of the bacterial isolates were prepared in LB broth and adjusted to 0.5 McFarland standards. Then, from the center of the plate to the margin, the bacterial cultures were swabbed on EtBr-TSA plates and incubated at 37 °C for 24 h. After incubation, the plates were examined using a gel imaging system (Biorad, USA). The higher the concentration of EtBr required for producing fluorescence of the bacterial mass, the greater the efflux capacity of the bacterial cells (Girijan et al., 2020).

The Spearman rank correlation was used to determine the relationship between biofilm formations and antibiotic susceptibility. Correlation coefficient (ñ) values of +1 and -1 were considered to be perfectly positive and negative correlation respectively.

Results and Discussion

In the present study, 54 freshwater finfish farms in Krishna and West-Godavari districts of Andhra Pradesh, India, were investigated to better understand management practices adopted for aquaculture production. The selected fish farms adopted intensive and semi-intensive culture methods for rearing Indian major carp, exotic carp and other commercially important freshwater fishes. The majority of farms were found to have high stocking densities with ineffective farm management. All the farms had been in operation for more than three years at the time of sampling. Bacterial and parasitic diseases are the major threats to fish culture in this area, causing significant economic losses.

This study found a significant distribution of S. marcescens with human pathogenic potential in aquaculture facilities. The suspected isolates are red, smooth, convex, entire and round colonies on TSA plates. Furthermore, the Gram staining showed that they were Gram-negative bacilli which were catalase positive and oxidase negative. The isolates were motile and they showed DNAse and gelatinase production. The remaining biochemical tests, including those for urease, hydrogen sulfide, arabinose, and lactose fermentation, were all negative; however, the tests for citrate, lysine, and ornithine decarboxylase were all positive. Table 1 shows a

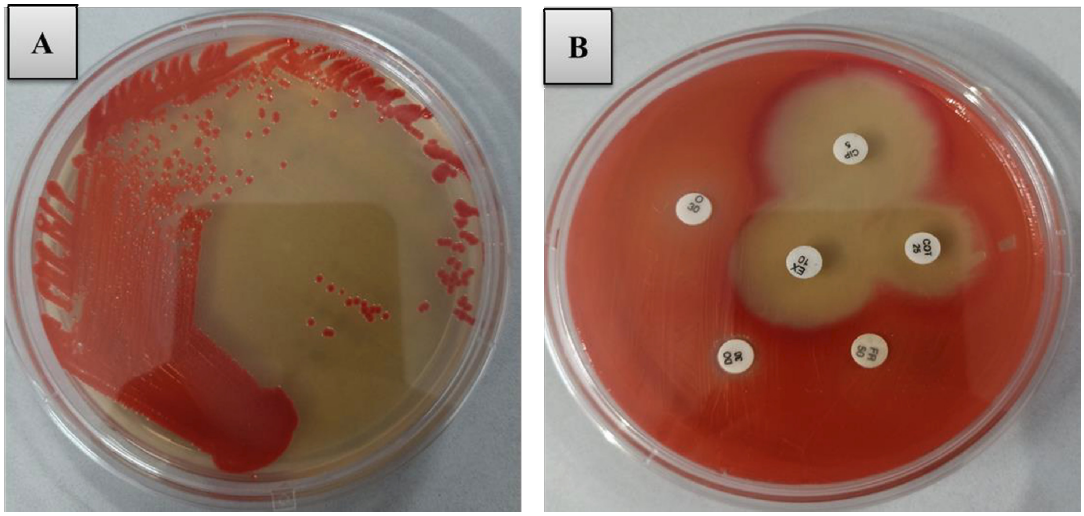

Fig. 1. A. *S. marcescens* grown on TSA agar, B. Antimicrobial susceptibility pattern to different antibiotic discs

total of 31 isolates from various samples that were positively identified as *S. marcescens.* The majority of strains were isolated from water (n=13) followed by gill swabs (n=9), skin swabs (n=5) and sediment (n=4). Concerning the species-wise isolation, a total of 20 isolates originated from Pangasius farms and the remaining 11 isolates from Indian major carp *(Catla catla* and *Labeo rohita)* farms. All 31 isolates were further examined for antibiotic susceptibility, biofilm formation and efflux pump activity.

Thirty-one isolates characterized as *S. marcescens* demonstrated varied resistance patterns to the antibiotics tested. Antimicrobial susceptibility testing revealed that 71 %, 48 %, 48 % and 9.7 % of isolates were completely resistant to furazolidone, oxytetracycline and doxycycline hydrochloride, co-trimoxazole, respectively, while 100 % of isolates were completely susceptible to both ciprofloxacin and enrofloxacin (Fig. 1 and Fig. 2). The average minimum inhibitory concentrations for furazolidone, oxytetracycline and doxycycline were found to be 256 µg/ml, 192 µg/ml and 64 µg/ml, respectively. Only three isolates were altered as multi-drug resistant (MDR) having resistance to furazolidone, oxytetracycline and co-trimoxazole. The AMR pattern of representative isolates and the presence or absence of biofilm formation and efflux pump activity is shown in Table 2.

S. marcescens is known for its biofilm formation that could contribute to antimicrobial resistance and pathogenicity. In the present study, all isolates had the ability to form biofilms and 15 (48.4 %), 10 (32.2 %) and 6 (19.4 %) were categorized as strong, moderate and weak biofilm formers, respectively. The Spearman rank correlation revealed that there is strong correlation between antibiotic resistance and biofilm formation in *S. marcescens*. The correlation coefficient (ρ) values for different antibiotics are depicted in Table 3.

The efflux pump activity of resistant isolates from different freshwater fish farms was assessed. Following incubation, variation in fluorescence of bacterial mass were noticed, depending on their

Table 1. Number of *Serratia marcescens* isolates from different samples from fish farms

S. No	Sample type	Number of samples	Number of *S. marcescens*
1	Gills swab	110	9
2	Skin swab	110	5
3	Water	50	13
4	Soil	48	4

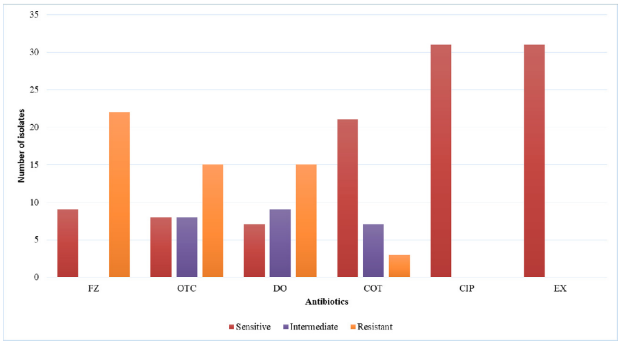

Fig. 2. Antibiotic susceptibility profiles of *S. marcescens*. FZ: Furazolidone; OTC: Oxytetracycline; DO: Doxycycline; COT: Co-trimoxazole; CIP: Ciprofloxacin; EX: Enrofloxacin

ability to efflux EtBr. In the present study, 15 resistant isolates showed efflux activity on TSA plates containing 2.5 mg/L EtBr concentrations considered as active efflux pump isolates and 10 resistant isolates that fluoresced between 0.5 and 1.0 mg/L of EtBr concentration were considered intermediate efflux active isolates, and those with no fluorescence below 0.2 mg/L of EtBr concentration were considered EtBrCW-negative isolates (Fig. 3). It was observed that an active efflux pump was present in 60 % of strong biofilm formers. However, there was no active efflux in moderate or weak biofilm formers.

S. marcescens is a Gram-negative *Enterobacteriaceae* species, initially considered non-pathogenic due to low virulence in healthy populations (Baden & Eisenstein, 2000). Over the last three decades, this bacterium has emerged as an important opportunistic pathogen responsible for diseases in humans and animals (Rice et al., 2005). This bacterium has been shown to cause a wide range of infectious diseases, including urinary, respiratory and biliary tract infections, peritonitis, wound infections and intravenous catheter-related infections, which can also lead to fatal bacteremia (Henjyoji et al., 1971). The current study highlighted the autochthonous nature of *S. marcescens* in aquaculture facilities, as this bacterium does not induce disease in fish. Yet, there is a chance that it will become resistant to antimicrobials used in aquaculture. Furthermore, it is widely distributed in soil, water, plant, animals, as well as in the intestinal tract of animals represents a problem for public and animal health (Chen et al., 2020; Cristina et al., 2019; Ferreira et al., 2020; Pei et al., 2015). In the present study, *S. marcescens* (n=31) was isolated from different samples from freshwater fish farms, indicating that the bacterium is opportunistically distributed in the aquatic environment. The majority of strains were isolated from water (n=13) followed by gill swabs (n=9), skin swabs (n=5) and sediment (n=4). Concerning the farm-wise isolation, a total of 20 isolates originated from

Table 2. Representative *S. marcescens* isolates showing different AMR patterns, biofilm and efflux pump activity

Isolate ID	AMR pattern	Biofilm formation	Efflux pump activity
SM0121	R (FZ+OTC+DO+ COT)	P	P
SM0221	R (OTC+DO+COT)	P	P
SM0321	R (OTC+DO+COT)	A	P
SM0421	R (OTC+DO)	P	P
SM0521	R (OTC+COT); I (DO)	P	P
SM0621	R (FZ+OTC+COT)	A	A
SM0721	R (FZ+DO+COT)	P	A
SM0821	R (FZ+OTC)	A	A
SM0921	R (OTC+COT)	P	P
SM1121	I (OTC+DO+COT)	P	P
SM1221	R (FZ)	P	P

FZ = furazolidone; OTC = oxytetracycline; DO = doxycycline hydrochloride; COT = co-trimoxazole; R = resistant; I = intermediate; P = present; A = absent

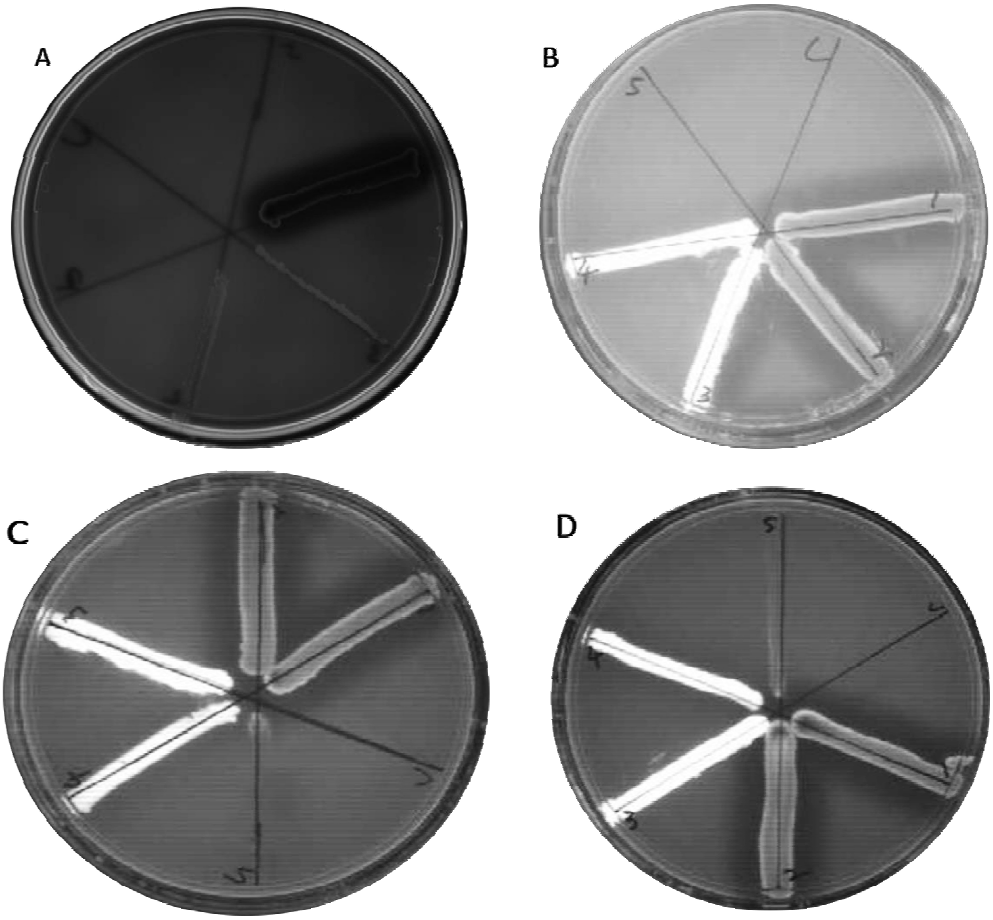

Fig. 3. EtBr-agar cartwheel method applied to different resistant isolates. A. 0.2 mg/L; B. 0.5 mg/L; C. 1.5mg/L; D. 2.5mg/L

Pangasius farms and the remaining 11 isolates are from Indian major carp *(C. catla* and *L. rohita).* Biosecurity and good aquaculture practices are essential for disease prevention and control. However, in the present study, the majority of farms were practicing continuous cultures, partial harvesting, high stocking densities and animal-originated manures without proper farm management. This might be the reason for the entry and distribution of *S. marcescens* in aquaculture facilities. Several previous studies have reported that zoonotic bacterial pathogens reach aquaculture systems through sewage and the use of untreated waters from other farming sectors and poultry manures (Mishra et al., 2017; Reverter et al., 2020; Schar et al., 2020).

Recent epidemiological analyses have revealed an increase in the prevalence of antimicrobial resistance and multidrug-resistant strains among *S. marcescens* isolates (Luzzaro et al., 1998; Ivanova et al., 2008; Gadhiya et al., 2021). The antibiotic resistance in *S. marcescens* is mediated by either the presence of resistance genes on the chromosomes or by the acquisition of such genes via horizontal transfer (Sandner-Miranda et al., 2018). Treatment of infections caused by *S. marcescens* has become challenging since it exhibits resistance to different classes of antibiotics (Biscoe & Hakeem, 2016; Fournier et al., 2016; Ray et al., 2017; Alav et al., 2018). According to Stock et al. (2003) and Sandner-Miranda et al. (2018), *S. marcescens* possesses resistance to penicillins and cephalosporins, nitrofurans, tetracyclines, macrolides, fluoroquinolones and colistin. Similarly, in our study, 71 %, 48 %, 48 %, and 9.7 % of the isolates were resistant to furazolidone, oxytetracycline and doxycycline hydrochloride, co-trimoxazole respectively. This may be due to the frequent use of such antibiotics in aquaculture to prevent and control bacterial diseases. Furthermore, bacterial susceptibilities to antibiotics may differ depending

Table 3. Correlation of AMR and biofilm forming abilities in *S. marcescens* isolated from fish farms

Antibiotics	Resistance			ρ
	SBF (%)	MBF (%)	WBF (%)	
Furazolidone	100	91.6	20	0.705
Oxytetracycline	66.6	75	0	0.488
Doxycycline hydrochloride	55.5	58.3	30	0.262
Co-trimoxazole	11.1	8.33	10	-0.129
Enrofloxacin	0	0	0	a
Ciprofloxacin	0	0	0	a

SBF: Strong biofilm formers, MBF: Moderate biofilm formers, WBF: Weak biofilm formers, ρ: Spearman rank correlation coefficient, a: ρ was not determined

on the antibiotics previously used in a specific environment (Lefort et al., 2005). Mishra et al. (2017) have also reported that disinfectants, antibiotics, pesticides and other growth-promoting agents are very frequent applications for aquatic animal health management. Interestingly, 100 % of isolates were completely susceptible to both ciprofloxacin and enrofloxacin. However, it is important to consider that *S. marcescens* is highly adaptable, so rates of resistance to fluoroquinolones and other antibiotic classes can diverge considerably (Sader et al., 2014). The formation of biofilms is critical for the establishment of pathogenicity and resistance in bacteria to environmental and chemical stressors (Donlan, 2002; Hoiby et al., 2010). All the isolates in the current study were able to form biofilm and showed a varied degree of resistance to all antibiotics tested. 48.4 %, 32.2 % and 19.4 % of isolates were strong, moderate and weak biofilm formers respectively. Ugwuanyi et al. (2021) conducted a similar study in *Pseudomonas aeruginosa* and found a strong correlation between biofilms, efflux pumps and antibiotic resistance. In this study, Spearman rank correlation revealed positive correlation between biofilm formation and antibiotic resistance among *S. marcescens* strains (Table 3). In addition to biofilm formation, active efflux pumps are prominent mechanisms that contribute to multidrug resistance in bacteria (Martins et al., 2011; Balcazar et al., 2015). Three resistance-nodulation-division (RND type) efflux pumps have been identified in *S. marcescens,* namely SdeAB, SdeCDE, and SdeXY (Chen et al., 2003; Kumar & Woroboc, 2005). In the present study, 15 resistant isolates showed strong efflux pump activity on agar plates containing 2.5 mg/L EtBr concentrations, whereas 10 resistant isolates that fluoresced between 0.5 and 1.0 mg/L of EtBr concentration were considered intermediate efflux pump active isolates, respectively. It was also observed that 60 % of the strong biofilm formers possessed active efflux pump activity by EtBrCW. The present study findings are similar to that of Alav et al. (2018), who reviewed and proposed that efflux pumps play multiple roles in biofilm formation.

In interconnected ecosystems, the interaction between humans, animals and environments is crucial for the development and spread of AMR (Schar et al., 2020). One of the most important factors is the discharge of antibiotics from hospitals and pharmaceutical manufacturing plants, which propagates AMR and pollutes the environment (Kotwani et al., 2021). Fouz et al. (2020) reported that wastewater is the main contributor to the transmission of antimicrobial resistance in the environment. All of the isolates in this study were collected from fish farms, suggesting that effluents from hospitals, municipal solid waste and animal husbandry could transport antibiotic resistant bacteria (ARBs) and antibiotic resistant genes (ARGs) into fish rearing facilities if discharged without further disinfection or treatment. Previous studies demonstrate the presence of ARBs and ARGs in the environment (Girijan et al., 2020; Kotwani et al., 2021).

In summary, our findings clearly demonstrate that *S. marcescens* is distributed in aquaculture settings and may pose a threat to aquaculture development and sustainability if proper management measures are not taken. Although it is not a fish pathogen, it may play an important role in the development and dissemination of antibiotic resistance through hori-

zontal gene transfer among the bacteria in the environment. All the resistance isolates were capable of producing biofilms and efflux pump activities that contribute to enhanced resistance, leading to the development of superbugs. Like other zoonotic pathogens (*Klbesiella pneumoniae*, *Edwardsiella tarda*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*), *S. marcescens* also may pose a threat to aquatic animals in the future. Furthermore, the suboptimal or imprudent use of antibiotics in fish farms is a matter of concern since *S. marcescens* is highly adaptable to a wide variety of antibiotics. Hence, there is a need for prudent use of antibiotics across the sectors and meticulous hygiene practices to prevent the entry or transmission of human and animal-borne pathogens into aquaculture. In addition to this, a collaborative approach from all policymakers, regulators, manufacturers, researchers, civil society and the general public is needed to reduce the risk of AMR through safe and effective antibiotic use in humans and animals in order to maintain environmental sustainability.

Acknowledgement

We are thankful to Kerala University of Fisheries and Ocean Studies, Kochi, Kerala, India for supporting this work.

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