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Antibiotic susceptibility pattern of fish pathogens: A new approach of emerging the bacterial resistance through biofilm formation in *in-vitro* condition

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ABSTRACT

Background: The ability of many bacteria to adhere on the host surfaces and forming biofilms has major implications in a wide variety of industries including the food industry, where biofilms may create a persistent source of contamination. In the same environmental condition, the multiple bacterial species can closely interact with each other and may easily enhance their drug resistance capability, which finally increases the multi-drug resistant (MDR) attribute of the species.
Objective: The present study examined whether the mixed-species biofilm possesses any impact on the enhancement of the antibiotic resistance of the planktonic or single-cell bacterial isolates present in the fish samples.
Methods: In this regard, *Cyprinus rubrofuscus* (Koi), *Heteropneustes fossilis* (Shing) *and Mystus vittatus* (Tengra) fishes were collected and subjected to form an *in vitro* biofilm by shaking condition into the wise bath. The drug-resistant pattern was determined by the Kirby Bauer technique.
Results: All the samples exhibited a huge array (up to 10^7 cfu/ml or g) of bacteria such as *E. coli*, *Klebsiella* spp., *Vibrio* spp., *Salmonella* spp., *Proteus* spp. and *Staphylococcus* spp. The isolates from both the bulk samples and their corresponding biofilms were subjected to antibiogram assay using antibiotics such as Ampicillin (10 μg), Erythromycin (15 μg), Streptomycin (STP 10 μg), Oxacillin (10 μg), Nalidixic acid (30 μg). Before biofilm formation, few of the isolates were found to be sensitive and few were resistant against the antibiotics. But when the species were isolated from the biofilm the sensitive one acquired drug resistance and resistant strain unveiled more resistance towards the same antibiotics. The present study revealed extensive bacterial contamination in fish samples among those some were resistant against the supplied drugs.
Conclusion: After the formation of multi-species biofilm, the isolates became more resistant against the same drugs that is alarming for consumers and major obstacles to maintain sustainable health.

1. Introduction

Food-borne pathogens causing food borne diseases are predominant worldwide. The development of multiple species associated biofilms and further association with the indigenous microbiota in food ecosystems are the main reasons for their adaptation and long-term survival in food (Davey and O'toole, 2000; Donlan, 2002). Thus, the biofilm formation by different species in food plays a harmful role in causing food-borne diseases. Additionally, Biofilm causes substantial implications in fields ranging from

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industrial processes like oil drilling, paper production and food processing, to health-related fields like medicine and dentistry (Dunne, 2002). Therefore, understanding the cellular mechanisms underlying microbial biofilm formation as well as their communicative microbial behavior is now a major concern to researchers. Multiple novel specific intervention strategies are now being used to control problems caused by biofilm formation in the different research fields, particularly for the food-processing environments (Stoodley et al., 2002). Recently, columnaris disease in freshwater fishes like catfish, tilapia, and trout aquaculture causes major economic losses worldwide (Groff and LaPatra, 2000; Figueiredo et al., 2005; Pulkkinen et al., 2010).

These foodborne pathogens are not only able to form biofilms on food like fish, meat, fast foods, vegetables, raw foods and food contact surfaces but also can be the carrier of antibiotic-resistant genes and that is why they have become a big concern to food safety (Chmielewski and Frank, 2003; Brooks and Flint, 2008; Shi and Zhu, 2009; Simões et al., 2010; Jahid and Ha, 2012; Srey et al., 2013).

Several studies in Bangladesh found extended numbers of drug-resistant isolates including the multi-drug resistant (MDR) ones in different food and water sample, fish, or natural environments (Munshi et al., 2012; Noor et al., 2013; Acharjee et al., 2014a,b; Marjan et al., 2014; Ahmed et al., 2014). Unethical usage of non-prescribed antibiotics and clinically unsafe practice of self-medication led to the development of unsolicited resistance by the infection-causing bacteria (Mathew et al., 2007; Dutta et al., 2013). Thus, Antibiotic resistance has become a global interference in disease medication principally shaded by the pathogenic microorganisms, especially in developing countries with a high density of population under the line of poverty as in Bangladesh (Tenover, 2006; Molton et al., 2013; Khan et al., 2013). Many studies have proved that Biofilm formed by monospecies or multiple species and the quorum sensing communication among them, help them to transfer the resistant genomic materials from one another (Stoodley et al., 2002). In this manner, the foodborne pathogens can increase the antibiotic resistance ability by forming biofilm in specific food items (Chavatte et al., 2014). Although many researchers have focused on monospecies, pure cultures or natural environmental biofilms consist of multiple bacterial species as well as fungi, algae, and protozoa (Percival et al., 2000; Manuzon et al., 2007; Chavatte et al., 2014). Recently, multiple-species biofilms have gained importance in food microbiology and food safety since they are more resistant to disinfectants and sanitizers compared to monospecies/pure species biofilms. The multi-species biofilms may indeed offer heightened opportunities for interactions such as horizontal gene transfer and co-metabolism in natural environments, which may take part in the elevated drug resistance.

Most of the studies have focused on drug resistance of the bacteria isolated from the raw samples of different foods including salad vegetables (Munshi et al., 2012; Acharjee et al., 2014a,b; Marjan et al., 2014; Ahmed et al., 2014). However, our previous studies did not focus on the role of biofilm formation for bacterial survival in an adverse environment. Considering all these facts, the present study endeavored to estimate the initial bacterial load from the fish samples and evaluate the ability of the isolated bacteria to form biofilm.

2. Materials and methods

2.1. Study period and sampling

The study was carried out at the Microbiology Laboratory, Stamford University Bangladesh from January 2018 to April 2018. A total of 15 samples (n = 5) of 3 categories of fish samples such as *Cyprinus rubrofuscus* (Koi), *Heteropneustes fossilis* (Shing) *and Mystus vittatus* (Tengra) were randomly collected from different local and supermarkets in Dhaka city and transported to the laboratory at the earliest convenient. For the isolation and enumeration of pathogenic bacteria, 10 g of each sample was homogenized in 90 ml normal saline and diluted up to 10^{-5} according to the standard guidelines (Cappuccino and Sherman 1996; Rahman and Noor 2012).

2.2. Isolation and identification of bacteria

2.2.1. Escherichia coli, Klebsiella spp. and Staphylococcus spp.

From the dilutions, 10^{-3}, 0.1 ml of each sample was spread onto the MacConkey agar and mannitol salt agar (MSA) (Oxoid LTD., Basingstoke, Hampshire, England) for the isolation of coliforms (*Escherichia coli* and *Klebsiella* spp.) and *Staphylococcus* spp. respectively. All the plates were then incubated at 37 °C for 24 h (Rahman and Noor 2012; Acharjee et al., 2014a,b).

2.2.2. Salmonella ***spp.***, Proteus spp. and Vibrio spp.

By considering the possible occurrence of viable but non-culturable (VBNC) cells (Colwell, 2000; Oliver, 2005; Rahman and Noor 2012) 1 ml of each sample from the previous stock was transferred into 9 ml of selenite cystine broth (SCB) and alkaline peptone water (APW) for the enrichment of *Salmonella* spp., *Shigella* spp., and *Vibrio* spp., respectively and incubated at 37 °C for 6 h. After incubation, the samples were diluted up to 10^{-4} and then 0.1 ml of samples from each of the 10^{-2} dilutions were spread onto XLD agar and thiosulfate citrate bile salt sucrose (TCBS) agar (Oxoid LTD., Basingstoke, Hampshire, England) for the isolation of *Salmonella* spp. *Proteus* spp. & *Shigella* spp., and *Vibrio* spp., consecutively. Plates were incubated at 37 °C for 48 h for the detection of typical colonies (Acharjee et al., 2014a,b).

2.2.3. Biochemical tests for the confirmative identification

Finally, the standard biochemical tests were performed for the identification of all the pathogenic isolates found in the samples. For this specific purpose, Triple Sugar Iron media (TSI) was prepared to determine the capability of bacteria for the utilization of different carbohydrates. Methyl Red (MR) test was performed to ensure the ability of organisms to produce stable acids as end products. Voges Proskauer (VP) test was executed to detect the ability of organisms to produce acetyl methyl carbinol and the Simmons' Citrate agar media was used to perceive the capability of bacteria to utilize citrate as a sole source of carbon. The Oxidase test was also performed to evaluate the ability of bacteria to produce cytochrome *c* oxidase and finally, Motility Indole

Urease test was executed to determine the bacterial motility (Table 2) (Cappuccino and Sherman, 1996).

2.2.4. Statistical analysis

All the experiments were performed triplicates and each of the data represents the mean ± SD count. All data were statistically analyzed by determining p-value through *t*-test and the significant level was considered as < 0.1 (Acharjee et al., 2014a,b). The GraphPad Prism (San Diego, CA) was customized to calculate the one-way analysis of variance (one-way ANOVA)

2.2.5. Antibiotic susceptibility test of the isolates

The standard agar-disc-diffusion method (Kirby Bauer technique) was used to examine the antibiotic susceptibility of the pathogenic bacteria on Mueller-Hinton agar (Difco, Detroit, MI) isolated directly from the fish samples. Very commonly available antibiotics were used such as Ampicillin (10 μg), Erythromycin (15 μg), Streptomycin (STP 10 μg), Oxacillin (10 μg), Nalidixic acid (30 μg) (Ahmed et al., 2014; Bauer et al., 1968; Ferraro et al., 2001;

Munshi et al., 2012; Tabassum et al., 2020). The plates were then inverted and incubated at 37° C for 24 h. After incubation, the plates were examined, and the zone of inhibition was measured in mm (Ferraro et al., 2001).

2.2.6. In vitro biofilm formation and recovery of the planktonic cells

For the *in-vitro* biofilm formation, 10 g of each fish sample were added into 90 ml of nutrient broth (NB) and placed in shaking condition into the wise bath at 37° C at 120 rpm for 5–7 days. Free-living bacteria residing in each of the samples were allowed to grow in the broth medium until nutrient was declined which was the selective pressure for biofilm formation. The thin opaque layer suspended surrounding the surface of the medium anchoring flask was the indicative of biofilm formation ([1]Munshi et al., 2018).

The thin layers of free-living bacteria were further revived on to the selective though strick plate methods. MacConkey agar and MSA agar were used for the isolation of coliforms (*Escherichia coli* and *Klebsiella* spp.) and *Staphylococcus* spp. while the isolation of *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. was carried out following the same procedure as described above.

2.2.7. Antibiotic susceptibility pattern of the isolates after biofilm formation

According to the same procedure the antibiotic susceptibility pattern of the bacteria from biofilm matrix were introduced on Mueller-Hinton agar (Difco, Detroit, MI) against same set of antibiotics to examine the bacterial resistance (Bauer et al., 1968; Ferraro et al., 2001; Munshi et al., 2012; Ahmed et al., 2014;). The plates were then incubated at 37 °C for 24 h. After incubation, the plates were examined and the zone of inhibition was measured in mm (Ferraro et al., 2001).

3. Results

3.1. Microbiological quality of the tested fresh fish samples

All the samples were found to contain a huge load of pathogenic bacteria in a range of 7.4 ± 0.055 $\log_{10}$ cfu/ml or g to 5.4 ± 0.477 $\log_{10}$ cfu/ml or g (Table 1). The present study estimated 6 isolates (*E. Coli Klebshilla* spp., *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp., and *Vibrio* spp.) from 3 categories of 15 fish (*Cyprinus rubrofuscus, Heteropneustes fossilis and Mystus vittatus)* samples. Among the 6 isolates, *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp. and *Vibrio* spp were found in every category of samples while *E. Coli and Klebshilla* spp. were found 5.6 ± 0.268 log10 cfu/ml or g and 4.8 ± 0.319 cfu/ml or g in only *Heteropneustes fossilis* (Shing) fish (Table 1). In the case of *Cyprinus rubrofuscus and Mystus vittatus* the contamination of *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp. and *Vibrio* spp. were remarkably significant. All 6 pathogens were expressively found in *Heteropneustes fossilis* fish (Table 1). All the isolated bacteria were further confirmed by the prescribed biochemical tests (Table 2).

Interestingly, pathogenic bacteria that were isolated from the tested samples were not 100% recovered from the biofilms of the samples. In the case of *Cyprinus rubrofuscus* and *Mystus vittatus* biofilm only *Vibrio* spp., *Staphylococcus* spp. and *Proteus* spp. were indentified whereas *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. were found in *Heteropneustes fossilis* biofilm. Several reasons are responsible behind the missing of growth of some species after biofilm formation: 1) during 7 days of incubation the supplied nutrients were gradually declined and the condition enforced some bacteria go forward to the death phase or 2) in environment one species always try to inhibit the growth of another species by producing some toxin or antibiotics for their nutrients and survive.

3.2. Enhanced antibiotic resistance of the isolates recovered from mixed species biofilms

The result of the antibiogram assay for all the samples showed that those bacterial isolates which were found to be sensitive against any of the antibiotics used, exhibited resistance against that antibiotic in every case when they were isolated from biofilms (Tables 3, 4 & 5). All the isolates from both bulk and biofilms showed resistance against Ampicillin (Tables 3, 4 & 5).

If the samples were more specifically assessed, a few of the isolates were found to persist their sensitivity against some antibiotics. The isolates from *Cyprinus rubrofuscus* sample; *Salmonella* spp. *Proteus* spp. and *Vibrio* spp. exhibited sensitivity against Streptomycin (STP 10 μg), Oxacillin (10 μg) and resistance against Ampicillin (10 μg), Erythromycin (15 μg), Nalidixic acid (30 μg) but gained resistance against all the drugs when they were grown in biofilm (Table 3).

In case of *Heteropneustes fossilis* samples, biofilm isolates of *E. coli* gained resistance against all the antibiotics except Streptomycin (STP 10 μg) (Table 4). Isolates *Vibrio* spp. from balk samples were resistant against Erythromycin, *Klebsiella* spp. were resistant against Streptomycin and *Proteus* spp. was resistant against Oxacillin rest of the isolates showed sensitivity. Isolates *E. coli*, *Salmonella* spp. and *Staphylococcus* spp. from biofilm acquired their resistance against all the antibiotics (Table 4).

In case of *Mystus vittatus* samples, *Salmonella* spp. from the bulk samples was found to be resistant against all the antibiotics such as Ampicillin (10 μg), Erythromycin (15 μg), Streptomycin (10 μg), Oxacillin (10 μg), Nalidixic acid (30 μg) and *Proteus* spp. exhibited resistance against Oxacillin (10 μg) but *Proteus* spp., *Vibrio* spp. and *Staphylococcus* spp. were found to be sensitive against all the drugs. The same species (*Proteus* spp., *Vibrio* spp. and *Staphylococcus* spp.) from the biofilm of the same samples turned into resistance against all the antibiotics. Although, *Proteus* spp. from biofilm retained their sensitivity against Streptomycin (10 μg) (Table 5).

4. Discussion

Researchers are now focusing on reducing the rapid expansion of bacterial resistance through the formation of mono or multi-species biofilms in the foods or environment to save the populations who are suffering the drug incompetence and unavailability during medication (Percival et al., 2000; Manuzon et al., 2007). As described in several reports that the bacterial species may have the ability to exchange their genetic materials as well as their co-metabolic features in the natural habitat which directly can escalate the survivability of many sensitive bacteria against the different hostile means (Chavatte et al., 2014). The most serious concern is that such highly persistent bacterial strains could enter the human body via the food chain, weakening the immune system of the host.

Several studies have been conducted previously regarding the bacterial resistance and dissemination of resistant bacteria into the consumer's body through contaminated foods (Acharjee et al., 2019a; Acharjee et al., 2019b; Islam et al., 2020; Akter et al., 2021). So far, many researchers tried to sort out general knowledge about the drug-resistant bacteria and their dissemination in the environment based on the analysis of bulk water, fish, fruit juices, milk, and different street foods samples and patients (Munshi et al., 2012; Dutta et al., 2013; Noor et al., 2013a; Khan et al., 2013; Acharjee et al., 2014a,b; Marjan et al., 2014; Ahmed

[1] "Chowdhury et al., 2016" this reference will be included. the full refernce will be as Chowdhury, F.F.K., Acharjee, M., Noor, R, 2016. Maintenance of Environmental Sustainability Through Microbiological Study of Pharmaceutical Solid Wastes. Clean Soil Air Water. 2016, 44 (3), 309–316. https://doi.org/10.1002/clen.201400777

Table 1
Isolation and quantification of pathogenic bacteria from different fish samples.

Sample (n = 5)	Microbial load (log_{10} cfu/g ± SD)											
	E. coli		*Klebshilla* spp.		*Salmonella* spp.		*Proteus* spp.		*Staphylococcus* spp.		*Vibrio* spp.	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cyprinus rubrofuscus (Koi)	NG	NG	NG	NG	4.1	±0.089	4.7	±0.070	7.1	±0.260	3.1	±0.109
Heteropneustes fossilis (Shing)	5.6	±0.268	4.8	±0.319	4.2	±0.164	5.1	±0.173	7.4	±0.055	2.3	±0.089
Mystus vittatus (Tengra)	NG	NG	NG	NG	5.4	±0.477	5.8	±0.248	6.2	±0.303	2.2	±0.438

SD Standard Deviation.
NG No growth.
The experiments were in triplicates. Average count (cfu/g) from all samples have been shown here. Each of the data has been represented as mean ± SD.
*Bacterial load after enrichment (Prior to enrichment, the recovery was nil).
Shigella spp. was absent in all samples.

Table 2
Biochemical identification of the pathogenic isolates.

Assumed Pathogenic microorganisms	TSI				Motility	Indole Production	MR	VP	Citrate utilization	Catalase	Oxidase
	Slant	Butt	Gas	H_2S							
E.coli	Y	Y	+	–	+	+	+	–	–	+	–
Klebshella spp.											
Salmonella spp.	R	Y	–	+	+	–	+	–	–	+	–
Vibrio spp.	R	Y	–	–	+	–	+	–	–	+	+
Staphylococcus spp.	Y	Y	–	–	+	–	+	–	–	+	–
Proteus spp.	R	Y	–	+	+	–	+	–	+	+	–

TSI triple sugar iron.
Y yellow (acid).
R red (alkaline).

Table 3
Antibiotics susceptibility pattern of the isolates from *Cyprinus rubrofuscus* fish.

Isolates		Antibiotics				
		AMP	ERY	STP	OXA	NAL
Bulk sample	*Salmonella* spp.	R	R	S	S	R
	Proteus spp.	R	S	S	S	R
	Vibrio spp.	R	S	S	S	R
	Staphylococcus spp.	R	R	S	S	R
Biofilm	*Proteus* spp.	R	R	R	R	R
	Vibrio spp.	R	R	R	R	R
	Staphylococcus spp.	R	R	R	R	R

R: Resistant; S: Sensitive.
AMP = Ampicillin (10 μg); ERY = Erythromycin (15 μg), STP = Streptomycin (10 μg), OXA = Oxacillin (10 μg), NAL = Nalidixic acid (30 μg).

Table 4
Antibiotics susceptibility pattern of the isolates from *Heteropneustes fossilis* fish.

Isolates		Antibiotics				
		AMP	ERY	STP	OXA	NAL
Bulk sample	*E. coli*	R	S	S	S	R
	Klebshilla spp.	R	S	R	S	R
	Salmonella spp.	R	S	S	S	R
	Proteus spp.	R	S	S	R	R
	Vibrio spp.	R	R	S	S	R
	Staphylococcus spp.	R	S	S	S	R
Biofilm	*E. coli*	R	R	S	R	R
	Salmonella spp.	R	R	R	R	R
	Staphylococcus spp.	R	R	R	R	R

R: Resistant; S: Sensitive.
AMP = Ampicillin (10 μg); ERY = Erythromycin (15 μg), STP = Streptomycin (10 μg), OXA = Oxacillin (10 μg), NAL = Nalidixic acid (30 μg).

Table 5
Antibiotics susceptibility pattern of the isolates from *Mystus vittatus* fish.

Isolates		Antibiotics				
		AMP	ERY	STP	OXA	NAL
Bulk sample	*Salmonella* spp.	R	R	R	R	R
	Proteus spp.	R	S	S	R	R
	Vibrio spp.	R	S	S	S	R
	Staphylococcus spp.	R	S	S	S	R
Biofilm	*Proteus* spp.	R	R	S	R	R
	Vibrio spp.	R	R	R	R	R
	Staphylococcus spp.	R	R	R	R	R

R: Resistant; S: Sensitive.
AMP = Ampicillin (10 μg); ERY = Erythromycin (15 μg), STP = Streptomycin (10 μg), OXA = Oxacillin (10 μg), NAL = Nalidixic acid (30 μg).

et al., 2014). Recently, scientists are trying to synthesis a new dimension of research on multi-species biofilms which are commonly formed in the environment by the close interaction of bacteria.

As quantified in early findings, common fish, as well as sea fish, can be contaminated with several pathogenic bacteria (*E. Coli*, *Klebsiella* spp., *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp. *Shigella* spp., *Pseudomonas* spp., *Listeria* spp. and *Vibrio* spp. including fungi (Acharjee et al., 2014a,b), which is quite similar to our current findings.

In terms of clinical implications, the existence of such pathogenic bacteria has a significant potential to prolong consumer enteric diseases. Similar results were also reported by Cai and Arias (2017); Matin et al. (2011); King et al. (2004).

Besides the previous findings present study also revealed that the fish, water, vegetables are the good reservoirs of many pathogenic bacteria that are resistant to multiple drugs (Acharjee et al. 2011; Noor et al., 2013; Ahmed et al. 2014; Acharjee et al., 2014a,b).

The data of the present study suggested that multi-species biofilm might actively take part in the frequent spreading of antibiotic resistance genes in the natural environment that will result in extended frequency of MDR cases and hence expand the public health risk (Yamada et al., 2005; Jahid and Ha, 2012; Munshi et al., 2018). Furthermore, while many studies have been conducted previously on the microbiological quality of various fish and fish products, as well as the drug-susceptible properties of isolated bacteria in fish nevertheless few reports have been published on the role of bacterial biofilm in fish samples in enhancing bacterial resistance to commercially available drugs. In our study, we tried to demonstrate how the fish cultivation microbiota can possess biofilm to excel in their sustainable adaptation.

Author contributions

M-A. Designed the study, experimental work, wrote the manuscript, revised and approved the final manuscript, R-H. Conducted the experiment and analyzed the data, S-S-S. Conducted the experiment and analyzed the data, N-T. Draft the manuscript initially, M-R-A, M-R A-A collected the samples and helped during experiment and data analysis. M-A and M-D-R and M-R-M. Critically revised the manuscript and approved.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Compliance with ethical standards

Present study followed proper guidelines in order to use animal species provided by the Government of the People's Republic of Bangladesh, Legislative and parliamentary Affairs Division. The Act NO. VIII of 1973.

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