

Presence and Antibiogram of Inhabiting Marine Bacterial Population at the Upper Sub-surface Water of the South East Coast of Bangladesh, Northern Bay of Bengal

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ABSTRACT: The present study was conducted to assess the presence of bacterial populations at the upper sub-surface water from the offshore areas near the South East Coast of Bangladesh, the northern Bay of Bengal and to evaluate their antibiotic resistance pattern. Water samples were collected from 5 stations near the South East Coast of Bangladesh, the northern Bay of Bengal. About 38 marine isolates were primarily identified using conventional cultural methods (Mannitol Salt Agar, Chromocult Coliform Agar and Cetrimide Agar). Among them, two gram negative viz. *Pantoea* spp. and *Pseudomonas aeruginosa* and one gram positive viz. *Micrococcus* spp. were identified and confirmed by biochemical and analytical profiling index techniques. The antibiogram results showed that *Pantoea* spp. and *Micrococcus* spp. were sensitive to ciprofloxacin, tetracyclin, erythromycin and chloramphenicol, whereas *P. aeruginosa* was sensitive to ciprofloxacin, azithromycin and streptomycin. However, *Pantoea* spp. and *Micrococcus* spp. were resistant to at least 3 antibiotics (oxacillin, cefixime and polymyxin B). But *P. aeruginosa* was resistant to a number of antibiotics such as oxacillin, cefixime, ampicillin, novobiocin, cephalixin, tetracycline, amoxicillin and kanamycin. Increasing drug resistance potential of microbial organisms gives us signal to go for immediate necessary action on the maintenance of water quality of estuarine and coastal areas.

Keywords: Marine microorganisms, Antibiogram, Drug resistance, Antibiotics, Bay of Bengal

INTRODUCTION

Ocean is the great sink of biodiversity including microscopic organisms to giant whale. Marine microbes show great potential for various biotechnological applications in the area of health, environment and agriculture (Azam and Worden, 2004). The number of potential antimicrobial compounds isolated from marine organisms have almost soared and this number now exceeds 10,000 with hundreds of new compounds are still being discovered every year (Proksch et al., 2002). More and more research have been conducted on marine organisms as sustainable sources because of low content of known active compounds in marine animals and plants as well as limitation of bioresource supply.

Ocean is a promising untapped source of novel

bioactive compounds. Because of having potential antibacterial and antifungal properties of marine organisms against bacterial pathogens, they have been profoundly used in the pharmaceutical industries. Jones (1959) collected sea water samples from both oceanic and neritic areas of the Pacific Ocean and used five different cultural and two direct microscopic methods to estimate the abundance of microorganisms. Jannasch and Padmavathy et al. (2015) isolated 51 bacterial strains from five different coastal locations in Gulf of Mannar region, Tamilnadu, India and ten bacterial isolates showed antagonistic activity varied from 14 to 20 mm against the entire gram positive, gram negative and fungal test pathogens. Overall, globally numerous studies have been conducted to assess the distributions and abundance of microorganisms in marine waters and sediments and analyzed for their potential antimicrobial compounds (Mudryk and Skórczewski, 1998; Do Thi et al., 2012; Valli et al., 2012; Hassan et al., 2015).

The Bay of Bengal, the largest bay of the world, occupies an area of 2.2 million km² and reaches a

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maximum depth of 5,258 meters. The Bay of Bengal, a northern extended arm of the Indian Ocean, is located between 5° N and 22° N latitudes and 80° E and 100° E longitudes. The Bay of Bengal is full of biological diversity that includes coral reefs, fish and shellfish spawning and nursery areas, feeding grounds and coastal mangroves. However, most part of the Bay of Bengal is untapped and there is great prospect to explore the antimicrobial activities of marine organisms in the Bay. Some studies were conducted in different parts of the Bay of Bengal, for example, Sarma et al. (2000), Ramesh et al. (2006), Ramesh and Mathivanan (2009), Sekhar et al. (2012), Verma et al. (2016), etc. to assess antimicrobial activities of marine organisms, but those studies are scattered. Pathak et al. (2012) isolated 6 *Pseudomonas* and 12 *Actinomyces* from rhizoidal soil of coastal vegetation of Rameshwaram, Pichavaram and Thiruvananthapuram regions of the Bay of Bengal and conducted in vitro antimicrobial test using agars well and disc diffusion methods to test their resistance capacity towards antibiotics. Sekhar et al. (2012) collected 28 marine sediment samples from different locations of Visakhapatnam coast of the Bay of Bengal, India and reported that marine microorganisms could be used as vital sources for bioactive molecules as they showed useful antimicrobial activity. However, in northern part of the Bay of Bengal, there is scarce study on marine microorganism's abundance, distributions and their bioactive compounds. So, the present study was conducted to assess the presence of microbes at the upper sub-surface water from the offshore areas near the South East Coast of Bangladesh, the northern Bay of Bengal and to evaluate their antibiotic resistance pattern.

MATERIALS AND METHODS

Site selection and sample collection

Water samples were collected from a one-month winter cruise of the Department of Oceanography, University of Dhaka using Agro Food-4 Fishing Trawler of Sea Resource Company Ltd. from five (5) stations of the south of south patches off the South East Coast of Bangladesh. The sample water collected from a depth of 5 meter (Table 1 and Figure 1) by Niskin bottles in January, 2016. Special care was taken during sampling and collecting water samples. The samples were preserved in individual clear plastic

bottles in order to prevent cross contamination of bacteria between water samples.

Table 1. Sampling Stations of the South East Coast of Bangladesh, Northern Bay of Bengal

Sample Number	Date	Time	Longitude	Latitude	Tidal Condition
40	28/01/2016	2.05 PM	20°45.577'	91°53.095'	Low tide
54	29/01/2016	12.00 PM	20°53.072'	91°50.238'	High tide
68	30/01/2016	11.35 AM	20°41.939'	91°39.760'	High tide
78	31/01/2016	9.35 AM	20°41.658'	91°52.009'	High tide
83	31/01/2016	12.00 PM	20°34.870'	91°54.70'	High tide



Figure 1. Sampling Stations of the South East Coast of Bangladesh, the Northern Bay of Bengal

Laboratory investigation

Sample analyses were carried out in the Food Microbiology and Research Laboratory of the Center for Advanced Research in Sciences (CARS), University of Dhaka.

Bacteriological methods

Inoculation in Marine Agar

At the beginning, Marine Agar (MA) media (DIFCO Marine Agar 2216) was prepared for inoculation of water sample in anaerobic condition.

Then samples name, date and sample number were labelled with the MA plates carefully. MA plate was stored at 37°C incubator for 24 hours and checked the contamination level. The fresh plates were inoculated with 100 µl of the seawater sample by pipetting 100 µl onto the plate in biosafety cabinet. After spreading, the plates were incubated at 37°C temperature and incubated plates were examined for colony formation after 24-48 and 72 hours.

Isolation of bacterium in selective growth media

Streaked each of the colonies for further analysis on fresh MA plates to isolate single colonies. Colonies were streaked onto CHR (Chromo Cult Coliform Agar), MSA (Mannitol Salt Agar) and CET (Cetrimide Agar) plates for pure culture as well as for confirmation. For growth of isolated colony, the organisms were incubated at 37°C for 24 to 72 hours. After then, isolates were transferred to another 1.5 ml eppendorfs containing 20% glycerol broth and stored at -20°C temperature. Rest of the plates were sealed with parafilm and store at 4°C temperature.

Biochemical test

Presumptive identification and confirmation of specific bacterial population were done by conventional biochemical tests. These biochemical tests included Methyl Red/Voges-Proskauer (MR/VP), Indole, Simmon's Citrate, Catalase, Oxidase and Carbohydrate Fermentation (Sucrose and Dextrose) tests. Tests were performed for the identification of the isolates following the methods of Cappuccino and Sherman (1996).

Identification of isolates using analytical profile index (API) system

Three types of API viz. API 20 E, API 20 NE and API STAPH test were performed to identify the isolates following the methods of Holmes et al. (1978).

KIRBY-BAUER test for antibiotic sensitivity

Kirby-Bauer test (Bauer et al., 1966) was employed to evaluate antibiotic sensitivity for different bacteria.

Statistical analysis

Microsoft Excel (version-13) were employed for data analysis and to prepare the tables and graphs.

RESULTS AND DISCUSSION

Cultural properties of the samples

Colonies of bacterial populations showed different colors in different selective agar media (Table 2 and Figure 2). The presence of bacterial colonies in water samples are agar specific and depending on their morphological characteristics, biochemical and API tests are performed for their confirmation. In this study, around 38 marine isolates were primarily identified using conventional cultural methods (MSA, CHR and CET). Out of 38 marine isolates, two gram negative bacteria viz. *Pantoea* spp. and *Pseudomonas aeruginosa* and one gram positive bacteria viz. *Micrococcus* spp. were identified and characterized (Table 2).

Table 2. Colony Characteristics of Bacterial Population in Different Selective Agar Media

Organisms	Media	Colony size	Colony shape	Colony color	Elevation	Texture
<i>Micrococcus</i> spp.	MSA	Small	Round	Light pink	Raised	Smooth
<i>Pantoea</i> spp.	CHR	Medium	Convex	Off white	Flat	Smooth
<i>Pseudomonas aeruginosa</i>	CET	Medium	Rod	Bright green	Flat	Translucent smooth

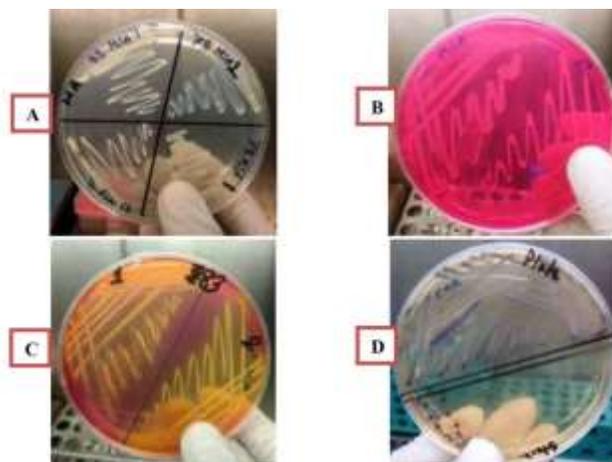


Figure 2. Bacterial Colonies on – A. MA (Marine Agar), B and C. MSA (Mannitol Salt Agar), and D. CHR (Chromocult Coliform Agar)

Biochemical tests

Bacterial colonies from different agar media were used for biochemical tests. *Micrococcus* spp., *Pantoea* spp. and *P. aeruginosa* showed positive results only for VP, Catalase and Oxidase; MR, Catalase and Sucrose; and Catalase, Oxidase and Citrate, respectively (Table 3). None of the bacteria showed

any result for Indole, Lactose and Dextrose tests. All these samples were later used for API test for bacterial confirmation.

Table 3. Biochemical Properties of Marine Bacteria Isolated from Upper Sub-surface Water from the Offshore Areas Near the South East Coast of Bangladesh, the Northern Bay of Bengal

Organisms	Name of biochemical tests								
	MR	VP	Indole	Catalase	Oxidase	Citrate	Lactose	Sucrose	Dextrose
<i>Micrococcus</i> spp.	-	+	-	+	+	-	-	-	-
<i>Pantoea</i> spp.	+	-	-	+	-	-	-	+	-
<i>Pseudomonas aeruginosa</i>	-	-	-	+	+	+	-	-	-

API tests to confirm the identity of the isolates

After cultural identification and biochemical test, API 20 E (bioMerieux, France) tests were performed to confirm the identity of *Pantoea* spp. (Enterobacteriaceae group) from water off the South East Coast of Bangladesh, the northern Bay of Bengal according to the reading table and identification software (Table 4).

In contrast, API 20 NE (bioMerieux, France) tests were performed to confirm the identity of *P. aeruginosa* from water off the South East Coast of Bangladesh, the northern Bay of Bengal according to the reading table and identification software (Table 5). API 20 E (bioMerieux, France) tests did not give any result for *P. aeruginosa* since it showed positive results for Oxidase test. API 20 E is used for gram negative bacteria that are oxidase negative.

Table 4. Interpretation Table of API 20 ETtest (adapted from bioMerieux.com)

Tests	Active ingredients	Qty (mg/cup)	Reactions/Enzymes	Results	
				Negative	Positive
ONPG	2-nitrophenyl β -D-galactopyranoside	0.223	β galactosidase	Colorless	Yellow
<u>ADH</u>	L-arginine	1.9	Arginine Dihydrolase	Yellow	Red/Orange
<u>LDC</u>	L-Lysine	1.9	Lysine Decarboxylase	Yellow	Red/Orange
<u>ODC</u>	L-Ornithine	1.9	Ornithine Decarboxylase	Yellow	Red/Orange
<u>CIT</u>	Tri sodium citrate	0.756	Citrate Utilization	Pale green/ yellow	Blue-green/ blue
<u>H₂S</u>	Sodium Thiosulphate	0.075	H ₂ S production	Colorless/greyish	Black deposit/thin line
<u>URE</u>	Urea	0.76	Urease	Yellow	Red/Orange
TDA	L-tryptophan	0.38	Tryptophan deaminase	TDA /Immediate	
				Yellow	Reddish Brown
IND	L-tryptophan	0.19	Indole Production	James/ Immediate	
				Colorless	Pink
				Pale green/yellow	
VP	Sodium Pyruvate	0.19	Acetoin Production (Voges Proskauer)	VP ₁ +VP ₂ / 10 min	
				Colorless	Pink/red
Gel	Gelatin (Bovine origin)	0.6	Gelatinase	No difusion	Diffusion of black pigment
GLU	D-glucose	1.9	Fermentation/Oxidation (GLU)	Blue/Blue-Green	Yellow/Greyish yellow
MAN	D-Mannitol	1.9	Fermentation/Oxidation(MAN)	Blue/Blue-Green	Yellow
INO	Inositol	1.9	Fermentation/Oxidation (INO)	Blue/Blue-Green	Yellow
SOR	D-Sorbitol	1.9	Fermentation/Oxidation(SOR)	Blue/Blue-Green	Yellow
RHA	L-Rhamnose	1.9	Fermentation/Oxidation(RHA)	Blue/Blue-Green	Yellow
SAC	D-Sucrose	1.9	Fermentation/Oxidation(SAC)	Blue/Blue-Green	Yellow
MEL	D-Mellibiose	1.9	Fermentation/Oxidation(MEL)	Blue/Blue-Green	Yellow
AMY	Amygdaline	0.57	Fermentation/Oxidation(AMY)	Blue/Blue-Green	Yellow

ARA	L-arabinose	1.9	Fermentation/Oxidation(ARA)	Blue/Blue-Green	Yellow
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Table 5. Interpretation Table of API 20 NE Test (adapted from bioMerieux.com)

Tests	Active Ingredients	Qty (mg/cup)	Reactions/Enzymes	Results	
				Negative	Positive
NO3	potassium nitrate	01.36	reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min	
			reduction of nitrates to nitrogen	Colorless	Pink-Red
TRP	L-tryptophane	0.2	indole production (TRyptOPhane)	Zn / 5 min	
				Pink	Colorless
GLU	D-glucose	1.92	fermentation (GLUcose)	Blue to Green	Yellow
ADH	L-arginine	1.92	Arginine DiHydrolase	Yellow	orange / pink /red
URE	Urea	0.76	UREase	Yellow	orange / pink /red
ESC	Esculin ferric citrate	0.56 0.072	hydrolysis (β -glucosidase) (ESCulin)	Yellow	grey / brown /black
GEL	gelatin (bovine origin)	0.6	hydrolysis (protease) (GELatin)	no pigment diffusion	diffusion of black pigment
PNPG	4-nitrophenyl- β Dgalactopyranoside	0.22	β -galactosidase (Para-NitroPhenyl- β DGalactopyranosidase)	Colorless	Yellow
GLU	D-glucose	1.56	assimilation (GLUcose)	transparent	opaque
ARA	L-arabinose	1.4	assimilation (ARABinose)	transparent	opaque
MNE	D-mannose	1.4	assimilation (ManNosE)	transparent	opaque
MAN	D-mannitol	1.36	assimilation (MANnitol)	transparent	opaque
NAG	N-acetyl-glucosamine	1.28	assimilation (N-Acetyl-Glucosamine)	transparent	opaque
MAL	D-maltose	1.4	assimilation (MALtose)	transparent	opaque
GNT	potassium gluconate	1.84	assimilation (potassium GlucoNate)	transparent	opaque
CAP	capric acid	0.78	assimilation (CAPric acid)	transparent	opaque
ADI	adipic acid	1.12	assimilation (ADIPic acid)	transparent	opaque
MLT	malic acid	1.56	assimilation (MaLaTe)	transparent	opaque
CIT	trisodium citrate	2.28	assimilation (trisodium CITrate)	transparent	opaque
PAC	phenylacetic acid	0.8	assimilation (PhenylACetic acid)	transparent	opaque

Table 6. Interpretation Table of API STAPH Test (adapted from bioMerieux.com)

Tests	Active Ingredients	Qty (mg/cup)	Reactions/Enzymes	Results	
				Negative	Positive
0	No Substrate		Negative control	red	-
GLU	D-glucose	1.56	(Positive control) (D-glucose)	red	yellow
FRU	D-Fructose	1.4	acidification (D-Fructose)		
MNE	D-Mannose	1.4	acidification (D-Mannose)		
MAL	D-Maltose	1.4	acidification (Maltose)		
LAC	D-Lactose	1.4	acidification (Lactose)		
TRE	D-Trehalose	1.32	acidification (D-Trehalose)		
MAN	D-Mannitol	1.36	acidification (D-Mannitol)		
XLT	Xylitol	1.4	acidification (Xylitol)		

MEL	D-Melibiose	1.32	acidification (D-Melibiose)		
NIT	Potassium Nitrate	0.08	Reduction of Nitrates to nitrites	NIT 1+NIT 2 / 10 min	
				colorless-light pink	red
PAL	β -naphthyl phosphate	0.0244	Alkaline phosphatase	ZYM A=ZYM B / 10 min	
				yellow	violet
VP	Sodium pyruvate	1.904	Acetyl-methyl-carbinol production (voges proskauer)	VP 1=VP 2 / 10 min	
				colorless-light pink	violet pink
RAF	D-raffinose	1.56	acidification (Raffinose)	red	yellow
XYL	D-xylose	1.4	acidification (Xylose)		
SAC	D-sacchrose (Sucrose)	1.32	acidification (Saccharose)		
MDG	methyl- α D- glucopyranoside	1.28	acidification (Methyl- α D- glucopyranoside)		
NAG	N-acetyl-glucosamine	1.28	acidification (N-acetyl- glucosamine)		
ADH	L-arginine	1.904	Arginine Dihudrolase	yellow	orange-red
URE	Urea	0.76	UREase	yellow	red-violet

In case of *Micrococcus* spp., API STAPH (bioMerieux, France) tests were performed to confirm the identification of *Micrococcus* spp. from water off the South East Coast of Bangladesh, the northern Bay of Bengal (Table 6).

Antibiogram of identified bacterial populations

Biochemically identified bacterial populations were further screened for different antibiotics and some isolates showed positive results. This study showed that *Pantoea* spp. and *Micrococcus* spp. were highly sensitive to ciprofloxacin, tetracyclin, erythromycin and chloramphenicol (Figure 6 and 7), whereas *P. aeruginosa* was sensitive to ciprofloxacin, azithromycin and streptomycin (Figure 8).

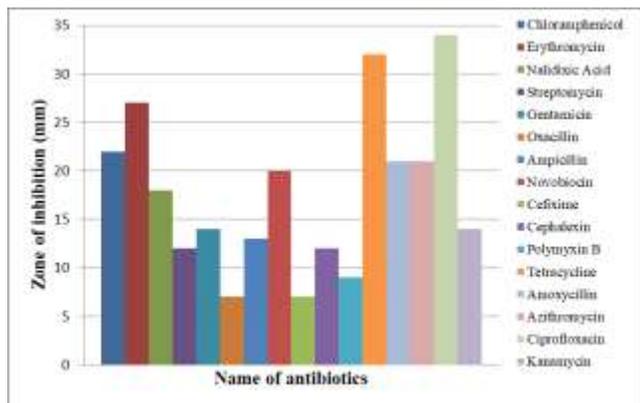


Figure 6. Antibiogram Patterns for *Pantoea* spp.

However, the antibiogram results showed that *Pantoea* and *Micrococcus* spp. were resistant to at least 3 antibiotics (oxacillin, cefixime and polymyxin B) (Figure 6 and 7). But *P. aeruginosa* was resistant to a number of antibiotics such as oxacillin, cefixime,

ampicillin, novobiocin, cephalosin, tetracycline, amoxicillin and kanamycin (Figure 8). Possible resistant pattern might be occurred due to chromosome and plasmid mediated drug resistance of respective bacteria. Some other reasons e.g., drug efflux pump might also be responsible for drug resistance of respective bacteria. The increase of resistance potential micro flora shows the importance of water quality maintenance.

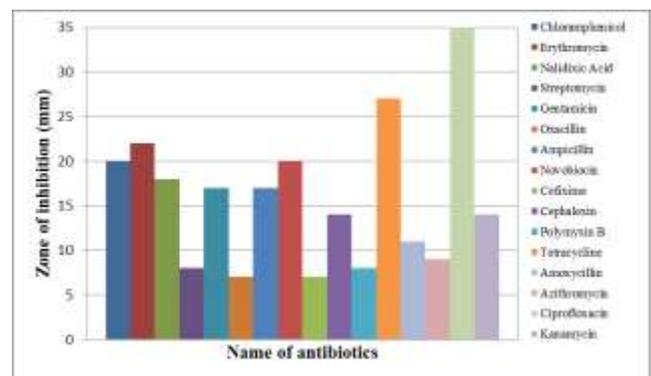


Figure 7. Antibiogram Patterns for *Micrococcus* spp.

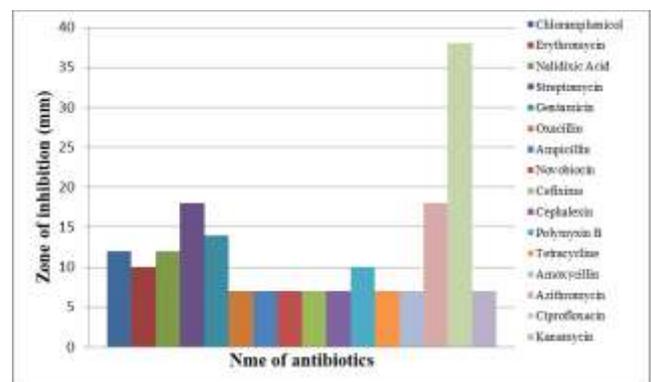


Figure 8. Antibiogram Patterns for *Pseudomonas aeruginosa*

Marine habitats are important sources for supplying living and non-living resources including social and economic goods and services. Among the three major habitats of the biosphere, the marine realm which covers 70% of the earth's surface provides the largest inhabitable space for living organisms, particularly microbes. Marine microbes flourish not only in the surface waters of the sea, but also in the lower and abyssal depths from coastal to the offshore regions, and from the general oceanic to the specialized niches like blue waters of coral reefs to black smokers of hot thermal vents at the sea floor (Das et al., 2006). Assessing and exploring microbial diversity is clearly a topic of considerable importance and interest. Besides, analysis of microbial biodiversity helps in isolating and identifying new and potential microorganisms having high specificity for recalcitrant compounds (Ely et al., 2004; Wietz et al., 2010; Stincone and Brandelli, 2020).

This study identified around 38 isolates from the water off the South East Coast of Bangladesh which might provide many bioactive compounds that have biotechnological prospective as antibiotics, biosurfactants, antifungal, or anticancer agents (Demain and Sanchez, 2009; Hibbing et al., 2010). Among 38 isolates, this study found two gram negative bacteria viz. *Pantoea* spp. and *P. aeruginosa* and one gram positive bacteria named *Micrococcus* spp. from the sub-surface coastal water of the northern Bay of Bengal. The role of *Pantoea* spp. is ambiguous because of its epiphytic and endophytic nature (Dutkiewicz et al., 2015). However, it could produce bioactive compounds effective in dealing with cancer and other diseases of humans, defeats the growth of numerous plant pathogens, stimulates plant development, and appears as a potentially effective biofertilizer and bioremediator (Dutkiewicz et al., 2016). *P. aeruginosa* and *Micrococcus* spp. can also produce bioactive compounds shown by, for example, de Oliveira et al. (2016), Anayo et al. (2019), Karbalaeei-Heidari et al. (2020), etc.

However, marine bacteria are increasingly becoming antibiotic resistant that impose a great risk for environmental safety and human health concern (Korajkic et al., 2020). In this study, it was found that *Pantoea* spp., *Micrococcus* spp. and *P. aeruginosa* were highly sensitive to only a few antibiotics such as

ciprofloxacin. In contrast, they are resistant to a number of antibiotics such as oxacillin, cefixime and polymyxin B. *P. aeruginosa* is a key pathogen accountable for infections in patients who suffer from breathing diseases (Saxena et al., 2014). *P. aeruginosa* can result in hard-to-treat life threatening diseases because of its high resistance to antibiotics and to the ability to form antibiotic tolerant biofilms (Papa et al., 2015). The increasing antibiotic resistant power of marine microbes have threatened human health.

The incidence of high percentages of marine bacteria resistant to antimicrobials indicate that marine environment is contaminated by different sources which make bacteria resistant against antimicrobials. For example, entrance of non-indigenous bacteria in marine ecosystems (de Oliveira et al., 2010), coastal runoff from terrestrial sources, anthropogenic antibiotic runoff which challenges native bacteria to become resistant and antibiotic production in marine environments (Hatosy and Martiny, 2015). Increasing drug resistance potential of microbial organisms gives us signal to take immediate necessary actions and strategies in order to conserve the estuarine and coastal water quality from degradation. This study suggests proper management strategies should be taken to monitor microbial community in marine environment and control antibiotic resistant pathogens.

CONCLUSIONS

In this study, presence of bacterial populations at the upper sub-surface water from the offshore areas near the South East Coast of Bangladesh, the northern Bay of Bengal was assessed and their antibiogram were tested. Around 38 marine isolates were primarily identified using cultural, biochemical and immune assay techniques. Among them, two gram negative and one gram positive bacteria were identified. The antibiogram results revealed that these three bacteria showed antibiotic resistant to a number of antibiotics such as oxacillin and cefixime. Since bacteria can live and reproduce in a variety of environments including animals, humans, water, soils and foods, their drug resistant power could impose a threat for human health. In addition, microorganisms increasing drug resistance potential reflects an alarming signal for water quality maintenance. Unnecessary and excessive use of antibiotics are responsible for occurrence of the drug resistant pathogenic bacteria in

the aquatic environment. More studies should be conducted in the Bay of Bengal for proper understanding about the distribution of microbial populations, their life cycle, antibiogram, and to monitor trends in antibiotic susceptibilities.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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