Biofilm forming ability and antibiotic susceptibility of bacterial strains isolated from Kavaratti Island, India

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ABSTRACT

Objective: This study aimed to investigate the prevalence of biofilm-forming bacteria and antibiotic resistance in the Kavaratti Island, an ecological hotspot of Lakshadweep archipelago.

Methods: Fourteen bacterial strains were isolated by routine microbiological procedure and characterized by the 16 sRNA sequencing. The disc diffusion assays, minimum inhibitory concentrations (MICs), biofilm formation and degradation assay were carried out by standard methods.

Results: Antibiotic susceptibility tests revealed the highest incidence of antibiotic resistance against vancomycin (57.2%), followed by methicillin (7.1%) and trimethoprim (7.1%). A total of eight bacterial strains showed biofilm formation. Analysis of biofilm formation in the bacterial isolates indicated their role in exhibiting phenotypic resistance against antibiotics based on varied MIC values. MIC values of vancomycin and trimethoprim were found to be upraised in biofilm-forming and biofilm states as compared to planktonic forms of bacteria. Sub-inhibitory concentrations of vancomycin (2µg/ml) and trimethoprim (4µg/ml) promoted biofilm formation.

Conclusions: This study demonstrated that the coastal realm of Kavaratti Island represented a rich source of bacterial diversity. High incidence of antibiotic resistance among isolated bacterial strains is captivating and might be a consequence of increased number of antibiotic resistance genes in the coastal resistome and their cross-generational transfer.

KEY WORDS: Antibiotic resistance
Biofilms
Coastal regimes
Minimum Inhibitory Concentration

Introduction

Marine environment is a diverse habitat, fostering a wide range of microorganisms. Various bacteria indigenous to the marine environment are potential human pathogens [1].Taking into account, the wide potential of microbes dwelling in the marine environment, it is indeed noteworthy to determine the microbial community structure and its pathogenic potential [1, 2]. The majority of the biomass of microorganisms on the earth exists in biofilms [3]. Most, if not all, bacteria are able to form biofilms, which are communities of cells organized within a permeable extracellular matrix [4]. Zobell illustrated the first ever biofilms in natural aquatic environments (1943). Bacteria fluctuate between two forms: planktonic and cell-forming biofilms in the natural environment as well as in their host [3]. Bacteria living within the biofilm are less susceptible to antibiotics than their planktonic cultures [3, 5]. The contrast between the sessile and planktonic forms of some pathogenic bacteria for antibiotic resistance confirmed biofilm production to be the main reason for the emergence of infections and diseases [6]. These precursive structures are responsible for the majority of chronic bacterial infections in humans [7]. It has been reported that genes conferring antibiotic resistance in human pathogenic bacteria have originated from the natural environment [8]. Environmental bacteria bear genes encrypting antibiotic resistance which might be a consequence of subjection of environmental bacteria to antibiotics as well as other antibiotic resistant bacteria.Another aspect escalating the phenomenon of multidrug resistance is the formation of persister cells that evade antibiotic killing by assuming a physiologically latent state. This phenotypic switch of regularly growing bacteria into persister cells can be induced by the change in environmental cues [9].

Lakshadweep is a tropical archipelago of 36 atolls and coral reefs in the Laccadive Sea, located off the south-western coast of India in the Arabian Sea between latitude 8˚ and 12˚30' N and between longitude 71˚ and 74˚ E [10].

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Kavaratti is the capital of the Union Territory of Lakshadweep and lies 360 km off the coast of the state of Kerala at 10.57°N 72.64° E.

Coastlines are becoming the hotspots of some new emerging diseases [11]. Many opportunistic pathogens, including *Aeromonas*, *Clostridium*, *Klebsiella*, *Legionella*, *Listeria*, *Pseudomonas*, and *Vibrio*, are naturally enterprising in oceans [1]; some can persevere in latent, but viable states. When present in coastal waters, these opportunistic pathogens can persist and infect humans through recreational exposures or consumption of contaminated sea-food [12].

Though there have been reports investigating the distribution of fecal, supplemental-indicator, human pathogenic bacteria in the Kavaratti Island of the Lakshadweep archipelago [10], no studies have been done so far which link biofilm formation and antibiotic resistance to it. In this regard, it would be interesting to isolate biofilm-forming bacteria from natural environments and deduce their antibiotic susceptibility patterns.

Materials and Methods

Sample collection

Sample collection was done from different sites of Kavaratti islands viz; Soil sediment, Ocean Eastern Jetty, Middle well water & Lagoon water. Samples were collected in sterilized containers and preserved at 4˚C.

Isolation of bacteria

Bacterial colonies were isolated from above cited samples by serial dilution and spread plate techniques, making use of different culture media viz; Trypticase Soy Agar, Zobell Marine Agar, Mannitol Salt Agar and Nutrient Agar respectively, following incubation at 37°C for 24-48 hours. Predominant bacterial colonies were sub-cultured on the nutrient agar medium post incubation and purified bacterial isolates were preserved in glycerol stock [34].

Identification of bacteria

For the identification of bacterial isolates, genomic DNA was extracted using QIAGEN QIAamp® genomic DNA extraction kit and 16S rRNA gene was amplified using a set of universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1390R (5'-ACGGCTACCTTGTTACGACTT-3'). PCR product was purified using QIAGEN QIAquick Gel Extraction Kit [5, 34]. Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) was performed using 10-mer random primers Rba11 (5'CCTGGGTGCA3') and Rba13 (5'GTCAGAGTCG3') for the typing of bacterial isolates and analysis of closely related species. Molecular identification by 16S rRNA gene sequencing was carried out using ABI-PRISM 377 automated DNA sequencer, as described in previous reports [5, 11, 34].

Biofilm assay

For the detection of biofilm formation, overnight bacterial cultures were diluted in the ratio of 1:100 in Trypticase Soy Broth/Sautons Fluid Medium (HiMedia) in a 24-well flat bottom polystyrene plate with a total volume of 2mL and incubated at 37˚C for 24-48 h in static conditions. Culture medium containing 0.5% Tween-20 (Sigma-Aldrich) served as negative control where as biofilm forming strain *S.aureus* ATCC 33591 was used as a positive control. Following incubation, plates were observed under the microscope at 8X magnification for biofilm formation.

For the quantification of biofilm formation, overnight cultures of bacterial strains were diluted in a ratio of 1:100 in Trypticase Soy Broth/Sautons Fluid Medium (HiMedia) and incubated statically in a 96-well flat bottom polystyrene plate at 37˚C for 24 hours. Planktonic bacteria were removed and the wells were washed carefully with sterile 1X phosphate buffer saline (PBS) thrice. This was followed by staining with 0.1% crystal violet (HiMedia) for 10 minutes, washing the stained wells with sterile water thrice and air drying. Crystal violet in the wells was solubilized by adding 30% acetic acid (SRL), allowing it to stand for 15 minutes and recording the absorbance at OD_{595}. Biofilm assay was performed for monospecies cultures as well as multispecies combinations [12-14].

Antibiotic susceptibility test

Kirby-Bauer Disc Diffusion test

Antibiotic susceptibility patterns of bacterial isolates were determined against a basic set, each of bacterial cell wall synthesis inhibiting (Methicillin, Vancomycin), bacterial protein synthesis inhibiting (Gentamycin, Erythromycin) and bacterial nucleic acid synthesis inhibiting (Nadifloxacin, Trimethoprim) antibiotics, using Kirby-Bauer disc diffusion method [11]. Following antibiotic-impregnated

discs (6mm diameter) were used for this purpose: Vancomycin (10µg), Methicillin (10µg), Gentamicin (10µg), Erythromycin (15µg), Nadifloxacin (5µg) and Trimethoprim (5µg) [11].

Minimum inhibitory concentration (MIC) determination in the planktonic state

Different concentrations (0.5-512µg/ml) of vancomycin and trimethoprim were loaded into defined rows of 96 well microplates from their stock solutions. The negative control rows for each case consisted of the serial dilutions of vancomycin and trimethoprim (0.5-512 µg/ml) and the culture medium (Mueller-Hinton broth) but no bacterial suspension. The positive control rows consisted of culture medium (Mueller-Hinton broth) and the bacterial suspension but no antibiotic. After inoculation and incubation, the plates were subjected to scanning of absorbance at a wavelength of 600nm. The experiment was done in triplicates [15].

Biofilm formation and degradation assay

It was performed to assess the ability of bacteria to form biofilm in the presence of antibiotics- vancomycin and trimethoprim. Overnight grown cultures of bacterial strains were diluted to OD_{600} of 0.05. The same procedure was followed as used for evaluating the MIC of planktonic states, except that the incubation of plates was done under static conditions. Post incubation, crystal violet staining was done to evaluate the biofilm biomass, absorbance was recorded at OD_{590} and Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradicating Concentration (MBEC) of vancomycin and trimethoprim were reported [16].

Biofilm degradation assay

It was performed to test the ability of antibiotics- vancomycin and trimethoprim to degrade preformed biofilm, evaluating the biofilm biomass and cell viability after treatment. Biofilms were allowed to form in 96-well flat bottom polystyrene plates. Planktonic bacteria were discarded, wells were added with fresh medium containing serial dilutions of vancomycin and trimethoprim respectively and the plates were incubated at 37°C for 24 h without agitation. Evaluation of bacterial viability was done using MTT assay and Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradicating Concentration (MBEC) of vancomycin and trimethoprim were reported [16].

Statistical analysis

Data entry and analysis were performed using MS-Excel 2007. The data were expressed as average values and percentages.

Results

Isolation and identification of bacteria

Different types of bacterial colonies were isolated from sediment and water samples (Figure 1). Based on the colony morphology, 14 predominant bacterial colonies were selected and designated from S1 to S14. Bacterial strains were identified by using PCR amplification of 16S rRNA gene followed by sequencing. Taxonomic identification by 16S rRNA gene sequencing unveiled the presence of 10 distinct genera (Table 1). Partial 16S rRNA gene sequences were submitted to the GenBank database.

Table 1. Bacterial strains isolated and identified from different sampling sites.

Bacterial isolate	Bacterium Identified	CFU/ml	Sampling site
S1	*Bacillus cereus*	$1x10^3$	Kavaratti sediment
S2	*Bacillus anthracis*	$3.2x10^3$	Kavaratti sediment
S3	*Microbacterium paraoxydans*	$4.2x10^3$	Kavaratti sediment
S4	*Acinetobacter sp.*	$2.9x10^3$	Kavaratti sediment
S5	*Bacillus mycoides*	$2.1x10^3$	Kavaratti sediment
S6	*Bacillus sp.*	TNTC	Kavaratti sediment
S7	*Pseudomonas stutzeri*	$1.9x10^2$	Ocean Eastern Jetty
S8	*Marinobacter sp.*	$5x10^2$	Ocean Eastern Jetty
S9	*Enterobacter cloacae*	$0.07x10^2$	Middle well water
S10	*Mesoflavibacter zeaxanthinifaciens*	4.1x102	Lagoon water
S11	*Bacillus endophyticus*	$8x10^2$	Kavaratti sediment
S12	*Exiguobacterium profundum*	$2x10^2$	Ocean Eastern Jetty
S13	*Microbacterium sp.*	$1.2x10^2$	Middle well water
S14	*Mesorhizobium sp.*	$3x10^2$	Lagoon water

Biofilm assay

Out of fourteen, eight bacterial isolates exhibited biofilm formation. Most of them formed moderate to strong biofilms. O_{D595} >0.2 indicated significant biofilm formation where as that in the range of 0.1-0.2 indicated moderate biofilm formation. The results of biofilm formation in monospecies and multispecies cultures were as tabulated (Table 2 (a), (b) respectively). *B. anthracis*, *Bacillus sp*., *E. cloaceae*, *E. profundum*, *Mesorhizobium* and *B.endophyticus* didn't form biofilms in either of the culture medium as evident from microscopic examination as well as crystal violet staining.

Table 2 a. Biofilm assay - Monospecies culture.

Bacterial isolate	Biofilm formation (Monospecies) Medium	
	Trypticase Soy Broth	Sautons Fluid Medium
Bacillus cereus	+	+
Bacillus anthracis	-	-
Microbacterium paraoxydans	+	+
Acinetobacter sp.	+	+
Bacillus mycoides	+	+
Bacillus sp.	-	-
Enterobacter cloacae	-	-
Exiguobacterium profundum	-	-
Microbacteriumsp.	+	+
Mesorhizobium sp.	-	-
Pseudomonas stutzeri	+	+
Marinobacter sp.	+	+
Mesoflavibacter zeaxanthinifaciens	+	+
Bacillus endophyticus	-	-

'+' = Biofilm formed, '-' = Biofilm not formed

Antibiotic susceptibility tests

Kirby-Bauer Disc Diffusion test

Antibiotic susceptibility test by Kirby Bauer Disc Diffusion method employed the use of antibiotics which are frequently used for human administration, against bacterial infections and comprise all major bacterial targets. Results showed that the highest incidence of antibiotic resistance was against vancomycin (57.2%), followed by methicillin (7.1%) and trimethoprim (7.1%). The pattern of resistance against these three antibiotics is as depicted in the Venn diagram (Figure 2(i)). Since methicillin and vancomycin belong to the same class of antibiotics and have similar mechanisms of action, only vancomycin was chosen for MIC determination out of the two.

Table 2 b. Biofilm assay - Multispecies culture.

Bacterial isolate	Biofilm formation (Multispecies) Medium	
	Trypticase Soy Broth	Sautons Fluid Medium
Bacillus cereus+ Acinetobacter	+++	+++
Bacillus cereus+ Bacillus mycoides	++	++
Microbacterium paraoxydans+Bacillus mycoides	+++	+++
Microbacterium paraoxydans+ Microbacterium	+++	+++
Microbacterium paraoxydans+ Pseudomonas stutzeri	+	+
Microbacterium paraoxydans +Marinobacter	+++	+++
Acinetobacter+ Pseudomonas stutzeri	+++	+++
Acinetobacter+ Marinobacter	+++	+++
Bacillus mycoides+ Microbacterium	+++	+++
Bacillus mycoides+ Pseudomonas stutzeri	+++	+++
Bacillus mycoides+ Marinobacter	+	+
Pseudomonas stutzeri+ Marinobacter	+	+

'+++' = strong, '++' = moderate, '+'= weak

MIC determination in planktonic state

The results of broth micro-dilution method with vancomycin and trimethoprim gradient range of 0.5-512 µg/ml revealed high MIC values of trimethoprim for most of the isolates, being 128µg/ml for more than 50%. The lowest vancomycin concentration that resulted in no significant turbidity at OD_{600} was found to be 4µg/ml for most of the isolates. MIC values of different bacterial isolates against vancomycin and trimethoprim were as tabulated (Table 3).

Biofilm formation and degradation assay

The MIC for biofilm-forming state of different bacterial isolates was as tabulated (Table 4). It was observed that sub-inhibitory concentrations of vancomycin and trimethoprim promoted biofilm formation (Figure 2(ii)).

Table 3. MIC determination in planktonic state

Cell state: Planktonic	Minimum Inhibitory Concentration (µg/ml)	
Bacterial isolate	Vancomycin	Trimethoprim
Bacillus cereus	4	64
Bacillus anthracis	4	64
Microbacterium paraoxydans	4	64
Acinetobacter sp.	4	128
Bacillus mycoides	4	128
Bacillus sp.	4	64
Enterobacter cloacae	4	128
Exiguobacterium profundum	8	128
Microbacteriumsp.	8	64
Mesorhizobium sp.	16	64
Pseudomonas stutzeri	8	128
Marinobacter sp.	32	128
Mesoflavibacter zeaxanthinifaciens	8	128
Bacillus endophyticus	64	128

All the experiments were carried out in triplicates and the results expressed as average values of vancomycin and trimethoprim gradients of 0.5 –512 µg/ml respectively

Table 4(a). Biofilm formation and degradation assay in monospecies culture.

Cell state: Biofilm forming	MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)
	Vancomycin		Trimethoprim	
Bacillus cereus	16	64	64	128
Microbacterium paraoxydans	32	>512	128	512
Acinetobacter sp.	32	256	128	512
Bacillus mycoides	256	>512	128	512
Microbacteriumsp.	32	>512	64	512
Pseudomonas stutzeri	32	64	128	>512
Marinobacter sp.	16	64	512	>512
Mesoflavibacter zeaxanthinifaciens	16	256	128	>512

All the experiments were carried out in triplicates and the results expressed as average values of vancomycin and trimethoprim gradients of 0.5 –512 µg/ml respectively

Biofilm degradation assay

The MIC values for biofilms of most of the bacterial isolates was >512 µg/ml, for both vancomycin and trimethoprim. The MIC values were as tabulated (Table 5). The MIC values for both vancomycin and trimethoprim were quite high in biofilm-forming and biofilm states as compared to planktonic bacterial cultures. Bacteria responded distinctively and defensively to sub-inhibitory concentrations of vancomycin and trimethoprim in biofilm-forming states.

Table 4(b). Biofilm formation and degradation assay in multispecies culture.

Cell state:Biofilm forming	MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)
	Vancomycin		Trimethoprim	
Bacillus cereus + *Acinetobacter*	16	64	64	128
Microbacterium paraoxydans+*Bacillus mycoides*	32	>512	128	512
Microbacterium paraoxydans + *Microbacterium*	32	256	128	512
Microbacteriumparaoxydans +*Marinobacter*	256	>512	128	512
Acinetobacter+ *Pseudomonas stutzeri*	32	>512	64	512
Acinetobacter+ *Marinobacter*	32	64	128	>512
Bacillus mycoides+ *Microbacterium*	16	64	512	>512
Bacillus mycoides+ *Pseudomonas stutzeri*	16	256	128	>512

All the experiments were carried out in triplicates and the results expressed as average values of vancomycin and trimethoprim gradients of 0.5 –512 µg/ml respectively

Table 5. Biofilm degradation assay.

Cell state: Biofilm	MBIC (µg/ml)	MBEC (µg/ml)	MBIC (µg/ml)	MBEC (µg/ml)
	Vancomycin		Trimethoprim	
Bacillus cereus	512	>512	>512	>512
Microbacterium paraoxydans	8	>512	128	>512
Acinetobacter sp.	64	>512	256	>512
Bacillus mycoides	128	>512	>512	>512
Microbacterium sp.	64	>512	>512	>512
Pseudomonas stutzeri	16	>512	>512	>512
Marinobacter sp.	256	>512	>512	>512
Mesoflavibacter zeaxanthinifaciens	32	>512	>512	>512

All the experiments were carried out in triplicates and the results expressed as average values of vancomycin and trimethoprim gradients of 0.5 –512 µg/ml respectively

Discussion

A culture approach was used in this study to isolate, identify and screen biofilm-forming bacteria from different sites of Kavaratti Island. Major phylum representative of most of the bacterial isolates was found to be Firmicutes followed by Proteobacteria, Actinobacteria, and Bacteriodetes [11].

Figure 1. Biofilm assay (microscopy): (a) Blank (b) *Bacillus cereus* (c) *S.aureus* ATCC 33591 (d) *B.cereus* and *Acinetobacter* (e) *M. paraoxydans* and *B.mycoides* (f) *B.cereus* and *E.cloacae* (g) *M. paraoxydans* and *E.cloacae* (h) *M. paraoxydans* and *E.profundum* (i) *E.profundum* and *B.endophyticus*.

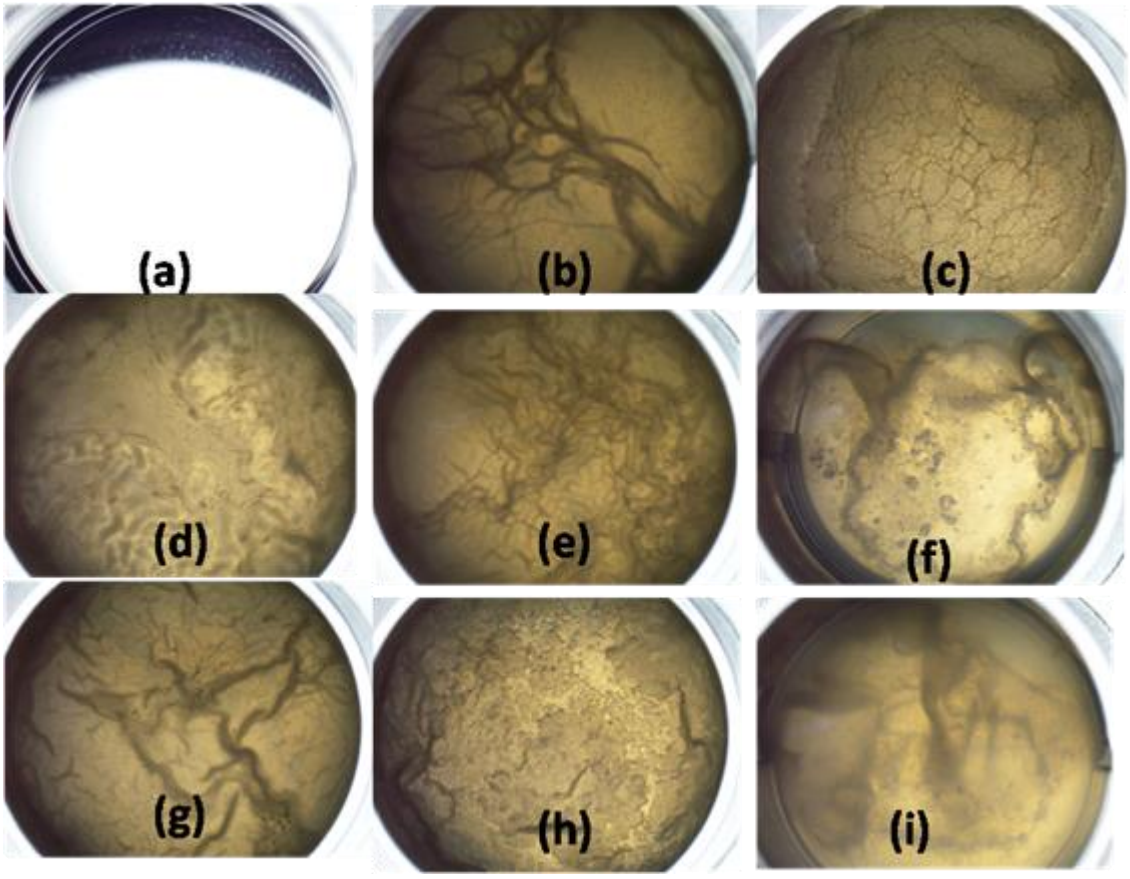
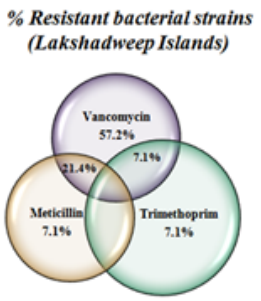
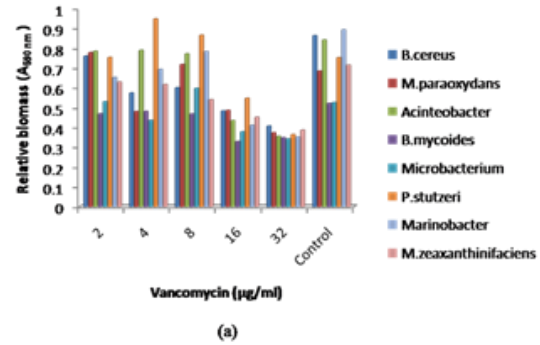


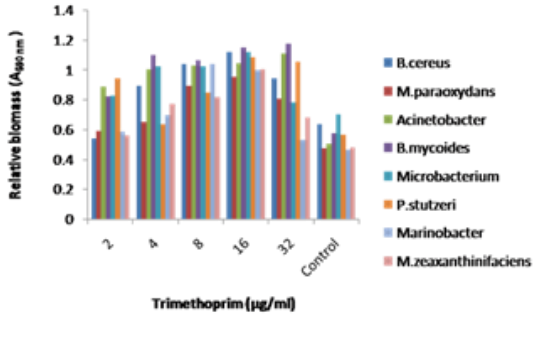
Figure 2. (i). Pattern of resistance against vancomycin, methicillin and trimethoprim
(ii) Biofilm genesis assisted by sub-inhibitory concentrations of (a) Vancomycin (b) Trimethoprim



(i)



(a)



(b)

(ii)

Members of these phyla have been reported to be pervasive in coastal environments [17, 18]. Among different microflora, *Bacillus sp*. was found to be predominant. Most of the bacterial strains isolated in the study viz; *Bacillus cereus*, *Acinetobacter*, *Pseudomonas stutzeri*, *Enterobacter cloacae* etc. are potential human pathogens [1]. Multispecies culture demonstrated very high affinity of biofilm formation between *B.cereus & Acinetobacter*, *M.paraoxydans & Microbacterium*, *M.paraoxydans & P.stutzeri*, *Acinetobacter & P.stutzeri*, *Acinetobacter & Marinobacter*, *B.mycoides & Microbacterium* and *B.mycoides & P.stutzeri* respectively (Figure 1). Most of them are either opportunistic or established human pathogens and possess the ability to cause biofilm-related infections in monospecies state or polymicrobial infections, when present in combinations. It has been reported that multispecies interactions are involved in the persistence of pathogens in the environment [5]**.** Interspecies interactions [33] have been demonstrated to further enhance the tolerance against antibiotics as compared to single-strain biofilms and their native planktonic states [5]. Low concentrations of antibiotics trigger transcription at a low level and favor the augmentation of genotypic and phenotypic resistance to antibiotics in susceptible bacteria [19]. Also, the sub-inhibitory concentrations of antibiotics activate the genes regulating virulence, stress response, biofilm formation and mutation, aiding bacterial survival under adverse conditions [19]. This could be a contributing factor to the diversity and probable outcomes of antibiotic resistant bacterial communities in soil and water [20].

The coastal realm of Kavaratti Island represented a rich source of bacterial diversity. High incidence of antibiotic resistance among isolated bacterial strains is captivating and might be a consequence of increased number of antibiotic resistance genes in the coastal resistome and their cross-generational transfer. Environment may be recognized as a giant repository of resistance agents and the propensity of environmental bacteria to form biofilms may be implicated as a selective advantage to survive in the natural environments and spawn infectious diseases. This study might prove as an early caution system for the early detection and management of potentially pathogenic bacteria in order to prevent biofilm-associated infections [35] in the coastal area of Kavaratti Island and can serve as an elementary data to several future studies aspiring to understand the ecology of marine habitats.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgments

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