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Pathogenic Bacterial Strains from a Pristine Mangrove Ecosystem - Virulence Determination through Biochemical Fingerprinting

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***Abstract*-- Global health scenario is constrained by the emergence of novel, mutated, antibiotic-resistant highly virulent microbes, which impair human health and results in increased mortality rates. Among these pathogens water borne bacteria are the most dominant. Indiscriminate discharge of untreated waste water from households, industries and hospitals, turns natural water resources into reservoirs of potential pathogens. In this study, four pathogenic strains of bacteria were isolated, characterized and identified from the water samples collected from the mangrove ecosystem of Poovar, South Kerala, India. The bacterial colonies were isolated by serial dilution and spread plating. Morphologically distinct bacterial colonies were propagated in pure culture for biochemical and molecular characterization. Two Gram positive and two Gram negative pathogenic bacteria were isolated and subjected to substrate utilization, enzyme secretion, acidification and antibiotic resistance tests as per the "*Bergey's Manual of Determinative Bacteriology*". The metabolic fingerprints of the isolates confirmed their pathogenicity and indicated the biochemical pathways that have evolved in these isolates, for virulence determination and host specific invasion. For precise taxon identification, the 16S rRNA sequences of the isolates were elucidated and aligned for homology with the standard sequences of NCBI GenBank through BLAST analysis. Based on the morphological, biochemical and molecular characteristics, the individual isolates were identified as *Kocuria kristinae, Granulicatella elegans, Acinetobacter lwoffii and Acinetobacter baumannii.* The presence and load of such highly virulent pathogenic strains of bacteria in natural ecosystems like the mangroves are alarming, since mangroves are the repositories that replenish the aqua fauna which serves the health and wealth of global mankind. The ability of the pathogenic microbes to survive in the highly fluctuating mangrove ecosystem, when read in conjunction with the threat of antibiotic resistance, horizontal gene transfer, co-evolution and guided evolution, indicate multiplied risk magnitude in public health and hence, we suggest that the environmental quality of the mangroves must be regularly monitored in order to avoid future health hazards.**

Key words - Nosocomial, antibiotic resistance, meningitis, virulence, pathogenicity

I. INTRODUCTION

Mangroves are unique ecosystems, found in the transition zone between land, sea and rivers in tropical and subtropical regions around the world [1]. Mangroves provide breeding and feeding grounds and refuge for a variety of organisms and replenish aquafaunal diversity, ensure fishery availability and maintain local climate, biogeochemical cycles and nutrient turnovers [2, 3]. Bacterial fauna dominates the structure and function of mangroves and have decisive roles in ecosystem productivity, conservation and recovery [2, 3, 4]. But the health of mangrove ecosystems is deteriorated by anthropogenic pollution and the major threat is the invasion of harmful bacteria through riverine influx which will exert adverse effects on human health. The characterization and identification of bacterial communities in mangroves is a suitable and ideal indicator of environmental quality since, the structure of microbial community determines the quality of their habitat [5].

Mangroves are the first to be impacted with the negative outcomes of urbanization and industrialization [6]. The hazardous effluents from terrestrial sources eventually end up in aquatic habitats by natural water flow, flooding, erosion and sewage. Mangroves being the interface of terrestrial, marine and freshwater habitats, the magnitude of environmental dilapidation will be synergistic. With the elevation in global pollution rates, an alarming increment is seen in the number of mortality and morbidity cases reported as resulting from water borne infections. Presence of pathogenic bacteria that are resistant to a wide spectrum of antibiotics used for human, aquaculture and livestock treatment regimens was reported by Heuer *et al* (2011) and Marisol*et al* (2000) in tropical mangrove ecosystems [7,8]. Mangroves harboring pathogenic bacteria pose a serious threat because the environment favors horizontal gene transfers and hence the indigenous flora can turn as a reservoir for antibiotic resistance and virulence plasmids. Human infection then results from contact and consumption of contaminated water and seafood [9, 10]. Antibiotic resistance genes are quickly transferred from fish pathogens to human pathogens and from there to the normal human gut bacteria. This causes dysbiosis of gut bacteria and will result in a multitude of health issues including allergic reactions, inflammatory bowel syndrome, obesity, diabetes and cancer [11]. Spreading of antibiotic resistant pathogenic bacteria in humans results in treatment failures and epidemics. Colonization of these infectious agents in invasive diagnostic tools leads to secondary nosocomial infections [12].

In this study, the mangrove ecosystem of Poovar, South Kerala, India was screened for the presence of aquatic pathogenic bacteria since, the site is highly depended by local fisherman for livelihood and by tourists for recreation. Assessment of aquatic bacteria is a suitable and important indicator of the quality of available fishery resources and environmental health [13], and hence this study is undertaken. Since bacterial pathogenicity is determined by their metabolic capabilities, the isolates were tested for selected substrate utilization and enzyme secretion, in order to detect the metabolic pathways which are employed by the pathogens as virulence strategy. The growth response of the pathogens towards inhibitory agents was also tested to understand bacterial resistance towards antibiotics. Phylogenetically meaningful taxon identification of bacterial isolates demands molecular characterization and hence 16S rRNA sequencing and analysis were performed. Based on the morphologic, biochemical and molecular characteristics, the bacterial isolates were identified up to their species taxon level. The presence of pathogenic bacteria at the study site indicates the precarious and alarming health status of the mangrove ecosystem and emphasizes the need for conservation.

II. MATERIALS & METHODS

A. Study site and sampling

Poovar mangrove (Fig.1) lies very close to Vizhinjam, a natural harbor in South Kerala, India. The 56 km Neyyar River flows into the Arabian Sea near Poovar. The geographical location is 81^0 09’ 054’’N 77^0 03’44.6’’ E. Water samples were collected from three designated areas of the mangrove forest (site 1: Freshwater mouth, site 2: Near human settlement area, site 3: Near the sea) during January (dry season) and July (rainy) months of the year2018based on standard protocol (IS 1622-1981). Water samples were collected in triplicates from each designated site and transferred to the laboratory within 3 hours of collection for bacterial isolation.

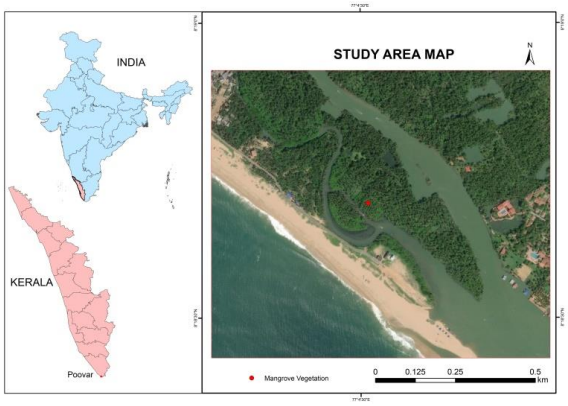

Fig.1: Study site

B. Total Plate Count (TPC)

1ml of the collected water sample was thoroughly mixed with 9ml of sterile distilled water and the resulting solution was serially diluted from 10^{-2} up to 10^{-5} using sterile distilled water. 0.1ml aliquot was taken from each dilution and spread plated in triplicates onto standard agar plates and incubated at 37˚C for 24 – 48 hours to isolate viable colonies within countable range. After incubation, colony counts and morphologies were recorded. Four colonies of suggestive morphology were selected (PMB1-4) and sub-cultured on agar slants for further analysis (Fig.2).


Fig 2: Bacterial isolates on agar slants

C. Gram staining

Isolates PMB1 to PMB4 were Gram stained following the standard protocol and examined microscopically for cell morphology at 40 X magnification.

D. Metabolic Profile analysis for virulence determination

The selected bacterial colonies were subjected to metabolic analyses using Vitek 2 system working on growth based technology. The instrument uses advanced photometric technology to determine individual biochemical reactions contained in a variety of microbe identification cards. After inoculation with a standardized suspension of the unknown organism, each self-contained card is incubated and the test result is read by the instrument's internal optics. Raw data is compared to threshold values to determine the response towards each test.

D.1 Reagent card selection, inoculation and result reading

Each reagent card has 64 wells, each containing an individual test substrate and each substrate measures a specific metabolic activity including reactions for carbon source utilization, enzyme production and growth in the presence of antibiotics. Four reagent cards are available and each card is designed based on the reactions standardized in *Bergey's Manual of Determinative Bacteriology*, to measure the unique identifying metabolic features of specific groups of bacteria. The type of reagent card to be used for biochemical profiling is determined on the basis of the Gram staining response of colony members. Two types of reagent cards were used in this study – GN card and GP Card. For the Gram negative rods 47 reactions were tested using GN Card and for Gram positive isolates 43 reactions were tested using GP card. Inoculation density of reagent cards were adjusted to the required McFarland turbidity range (Table.1) using 0.3l% sterile saline. Results of test reactions were recorded every 15 minutes and compared with threshold values for proximity to the database taxa. More than 99% similarity in biochemical profile was accepted for species level identification.

E. Molecular characterization and identification of isolated bacteria

The 16S rRNA of the four pathogenic bacteria were isolated, sequenced and aligned with standard library sequences for phylogenetic homology and taxon identification.

E.1 16S rRNA isolation

The bacterial suspension was mixed with 10 microliter Tris-EDTA buffer (pH 8.0) and centrifuged at, 5000rpm until a pellet was obtained. Tris-EDTA buffer and lysozyme were added to the bacterial pellet and incubated at 37^0C for 30 minutes. For cell lysis 3microliters of 10% Sodium dodecyl sulfate and 3 microliters of acetyl trimethyl ammonium bromide were added and extracted using chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 rpm. Isopropanol (0.6ml) was added to the supernatant and the mixture was again centrifuged at 10,000 rpm for 5min, after which the supernatant was removed. The ethanol washed, air dried pellets were suspended in Tris - EDTA buffer and stored at 4^oC until used [14].

TABLE 1
Reagent card Specification

Sl. No:	Reagent card Selected	MC Farland turbidity range for card Inoculation
1	GN	0.50-0.63
2	GP	0.50-0.63

E.2 16S rRNA gene amplification and sequencing

16S rRNA genes were amplified by PCR using the Universal primer 27 F (5'-AGAGTTTGATCATGGCTCAG-3') as the forward and 1492R (5'-GGTTACCTTGTTACGACTT-3') as the reverse primers [14]. The PCR conditions were: initial denaturation of 2min at 94^0C, followed by 35 cycles of 1min at 94^0C, 1.5 min at 55^0C and 1min at 72^0C and a final extension at 72^0C for 3 minutes. Sequence analysis was performed in the fully automated Applied Biosystems 3500/3500xL Genetic Analyzer. The sequence analysis reagent used was Big Dye Terminator (BDT) kit and the data collection software used was Microseq^R ID Analysis Software V2.2. The maximum number of contiguous bases in the analyzed sequences was with an average QV $\geq$ 20.

E.3 BLAST Analysis

The raw data of codon sequences obtained were employed as the query sequences for comparison with the genomic database deposited in NCBI Library using Megablast to compare with highly similar sequences. Species level identification of the clone was accepted when the 16S rRNA sequences had a similarity of $\geq$ 98% [14] with that of the prototype sequences available in the Genbank.

III. RESULTS

A. Microbial isolation

a. Colony morphology and TPC

The colonies on the Petriplates differed in their shape, size and colour (Table.2) indicating the presence of different species of bacteria on the agar plate (Fig. 3).

The count of colony forming units (cfu) showed significant variation across the sites and also across the seasons (Table.3). For all the three sites colony counts obtained during the monsoon season (July) were significantly higher than that of the dry season (January) (Fig.4).

Highest counts of colony forming units were observed in the collection from site 2 and lowest from site 3.The colony count ranged between 10 X10-^{3} and 15 X10-^{4}cfu/ml.

TABLE.2
Colony morphology of isolates

Sl No	Selected colonies	Morphology of bacterial colonies on standard agar Petriplates
1	PMB_1	Circular,pulvinate, Shiny white colony with undulate margin
2	PMB_2	Circular, off-white, convex colony with entire margin
3	PMB_3	Circular, convex, mucoid white smooth colony with entire margin
4	PMB_4	Circular, raised, transluscent, smooth colony with lobate margin

TABLE 3
Cfu/isolation

Isolation sites	No: of Colony forming units(cfu/ml)	
	Dry season (M±SE)(10^{-3})	Rainy season (M±SE) (10^{-4})
Site 1	22±0.58	7±1.73
Site 2	10±1.15	15±1.15
Site 3	3.6±0.88	3±1.73

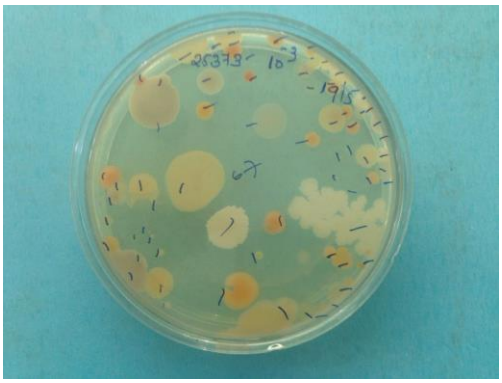

Fig.3: Aquatic bacterial colonies on Agar plate

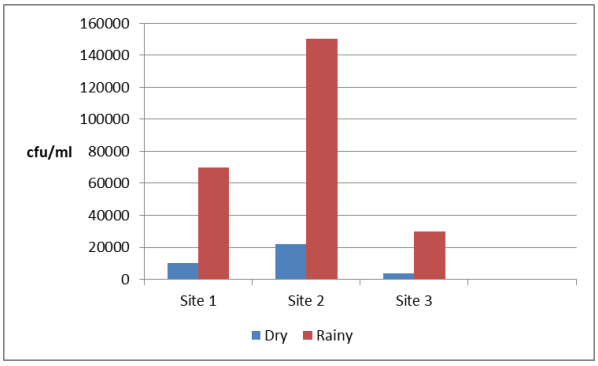

Fig.4: Cfu/ Isolation from the three sites

b. Gram staining

Out of the four bacterial isolates, the colonies marked PMB1 and PMB 2 were Gram positive and PMB3 and PMB4 were Gram negative. Individual colony members varied in their cell morphology (Table. 4).

Table 4:

Gram staining morphology of isolates

Sl. No	Isolate	Gram Staining Result	Cell Morphology
1	PMB_1	Positive	Cubical
2	PMB_2	Positive	Coco-bacilli
3	PMB_3	Negative	Rod
4	PMB_4	Negative	Rod

c **Virulence identification based on biochemical assay**

The biochemical profiles of the bacterial isolates were summarized in Table 5 & 6. The Gram positive isolatePMB1 was positive for the production of aminopeptidases (alanine arylamidase, leucine arylamidase, tyrosine arylamidase and aspartate arylamidase) and urease. The isolate utilized arginine, D- mannose and D-xylose as the carbon substrates and was positive for L-lactate alkalinization. The isolatePMB2 was positive for the production of aminopeptidases (alanine arylamidase, aspartate arylamidase and arginine dihydrolase), urease and D-xylose utilization. PMB 2 was negative for the utilization of arginine, D- Mannose, citrate,malonate, coumarate and succinate.The Gram negative isolate PMB3waspositive for the production of prolinearylamidase, tyrosine arylamidase and urease. The carbon sources utilized were D-glucose, D- mannose, citrate and succinate. The isolate PMB 4 was positive for L-lactate and succinate alkalinization, L-malate assimilation and production of the enzymes tyrosine arylamidase, lipase and urease. The isolate was resistant to the vibrio-static agent 0129 (2, 4-diamino-6, 7-di-isopropylpteridine phosphate). Carbon sources utilized by the isolate were D-glucose, D- mannose, ,citrate, malonate and coumarate

TABLE 5
Biochemical profile of Gram positive isolates.

Isolate ID	PMBI	PMB2
AMY	-	-
APPA	-	-
Leu A	+	-
AlaA	+	+
dRIB	-	-
NOVO	-	-
D RAF	-	-
OPTO	-	-
PIPLC	-	-
CDEX	-	-
ProA	-	-
Tyr A	+	-
ILATK	+	-
NC 6.5	-	-

0129R	-	-
dXYL	+	-
ASPA	+	+
BGURr	-	-
dSOR	-	-
LAC	-	-
D MAN	-	-
SAL	-	-
ADHI	+	+
BGAR	-	-
AGAL	+	-
URE	-	-
NAG	-	+
dMNE	+	-
SAC	-	-
BGAL	-	-
AMAN	-	-
PyrA	-	-
POLYB	-	-
dMAL	-	-
MBdG	-	-
dTRE	-	-
AGLU	-	-
PHOS	-	-
BGUR	-	-
d GAL	-	-
BACI	-	-
ADH2S	-	-
Identification	*Kocuriakristinae*	*Granulicatella elegans*

[AMY: D-amygdalin,APPA:Ala-phe-pro arylamidase, Leu A: Leucine arylamidase, Ala A: alanine arylamidase, dIB-D-ribulose, NOVO: novomycin resistance, D RAF; D-raffinose, OPTO-optochin resistance, PIPLC: phosphatidyl inosityl phospholipase C, CDEX:cyclodextrin, ProA: proline arylamidase, Tyr A: tyrosine arylamidase, ILATK:L-lactate alkalinisation, NC 6.5: growth in 6.5% NaCl, 0129 R: comp.vibrio resistance, dXYL: D-xylose, Asp A: aspartate arylamidase,, BGUrr: β-glucuronidase, dSOR: d-sorbitol, LAC: Lactose, D-Man: D-mannose, Sal: salicin, ADH1: Arginine hihydrolase, BGAR: β-galactopyranosidase, AGAL: α-galavtopyranosidase, URE Urease, NAG: N acetyl glucosaminidase, dMNE: D-mannose, SAC: sucrose, BGAL:β-galactosidase, AMAN:α- mannosidase, Pyr A: pyrolydonyl arylamidase, POLYB:polymixin B resistance, dMAL: D-maltose, MBdG: methyl β-D glucopyranoside, dTRE: D-trehalose, AGLU: α-glucosidase, PHOS:phosphatase, BGUR:β-glucuronidase, dGAL :D-galactose , BACI;Bacitracin resistance, ADH2Saarginine dihydrolase 2]

TABLE.6
Biochemical profile of Gram negative isolates

Reaction Substrate		
Isolate ID	PMB3	PMB4
APPA	-	-
H2S	-	-
BGLU	-	-
ProA	+	-
SAC	-	-
ILATK	-	+
GLYA	-	-
O129R	-	+
ADO	-	-
BNAG	-	-
dMAL	-	-
LIP	-	+
dTAG	-	-
AGLU	-	-
ODC	-	-
GGAA	-	-
PyrA	-	-
AGLTP	-	-
dMAN	-	-
dTRE	-	-
SUCT	+	+
LDC	-	-
IMLTa	-	+
IARL	-	-
dGLU	+	+
DMNE	+	+
TyrA	+	+
CIT	+	+
NAGA	-	-
THISa	-	-
ELLM	-	-
dCEL	-	-
GGT	-	-
BXYL	-	-
URE	+	+
MNT	-	+
AGAL	-	-
CMT	-	+
ILATa	-	-
BGAL	-	-
OFF	-	-
BALap	-	-
dSOR	-	-
5KG	-	-
PHOS	-	-
BGUR	-	-
Identification	*Acinetobacter woffi*	*Acinetobacter baumanii*

d. Molecular characterization and identification of bacterial isolates

The forward FASTA sequences(Table. 7) of the isolates had ≥99 % similarity (Table. 8) with the GenBank sequences. Based on 16S rRNA sequence homology, cellular morphology and biochemical characteristics, the isolate PMB1 was identified as *Kocuria kristinae (99.8%),* PMB2 as *Granulicatella elegans (99.88%),* PMB 3 as *Acinetobacter lwoffii (99.05%) and* PMB 4 as *Acinetobacter baumanii (99.90%).*

Sample code	Forward primer sequence of 16S rRNA of isolated bacteria
PMB 1	CTGGTGCTTGCACCGGGTGGATGAGTGGCGAACGGGTGAGTA ATACGTGAGTGACCTGCCTTTGACTCTGGGATAAGCCTGGGA AACTGGGTCTAATACCGGATGCGACTACTGCCCGCATGGGCT GGTGGTGGAAAGGGTTATGTACTGGTCTTAGATGGGCTCACG GCCTATCAGCTGGTTGGTGGGGTAATGGCCTACCAAGGCGAC GACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGA CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCG TGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGG GAAGAAGCGGAAGTGACGGTACCTGCAGAAGAAGCGCCGGC TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGC GTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT GTCGCGTCTGCTGTGAAAGCCCGGGGCTTAACTCCGGGTGTG CAGTGGGTACGGGCAGGCTAGAGTGCAGTAGGGGTAACTGG AATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAA CACCGATGGCGAAGGCAGGTTACTGGGCTGTTACTGACGCTG AGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTG GTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACA TTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGC CTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGA CGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT GCAACGCGAAGAACCTTACCATGACATACACCGGATCGTTCC AGAGATGGTTCTTCCCTTTTGGGCTGTACAGGTGGTGCATGGT TGTCGTCAGCTCGTGTCGTGAGATGTTGAGGCT

PMB 2	ACGAGAGCGACCGGTGCTTGCACTGGTCAATCTAGTGGCGAA CGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAGGGGGAT AACATTCGGAAACGGATGCTAAAACCGCATAGGTTCTTGAGT CGCATGACTGAAGAAGGAAAAGAGGCTTCGGCTTCTGCTGAT GGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGC TCACCAAGGCCGTGATGCATAGCCGACCTGAGAGGGTGATCG GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG CAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGA GCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACT CTGTTGTTAGAGAAGAACAGCGCATAGAGTAACTGTTATGCG TGTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCC AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT TATTGGGCGTAAAGCGAGCGCAGGCGGTCAATTAAGTCTGAT GTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG GTTGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTA GCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGA AGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGC GTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGAGTGCT AAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGTTAACGC ATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATG TGGTTTCGGTATAATTCGAAGCAACGCGAAGAACCTTACCAA GTCTTGACATCCTTTGACCACTCTAGAGATAGAGGTTTCCCTT CGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC TTATTACTAGTTGCCAGCATTGAGTTGGGCACTCTAGTGAGAC TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAAT CATCATGCCCCTTATGACTTGGGCTACACACGTGCTACAATG GATGGTACAACGAGCAGCGAACTCGCGAGGGTAAGCGAATC TCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCC TACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCC GCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAC ACCACGAGAGTTTGTAACACCCAAAGTCGGTGAGGTAACCTT ATGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGA AGTCG
PMB 3	ATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGG GGAAAKGTAGCTTGCTACMTWACCTAGCGGCGGACGGGTGA GTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTC GAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGC AGGGGACCTTCGGGCCTTGCGCTAATAGATGAGCCTAAGTCG GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT CTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA TTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTG TGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAACCGGCGAGG AGGAGGCTACCGAGATTAATACTCTTGGATAGTGGACGTTAC TCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGG TAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTA AAGCGCGCGTAGGTGGCCAATTAAGTCAAATGTGAAATCCAG CTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGAGTATG GGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT AGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGG CCTAATACTGACACTGAGGTGCGAAAGCATGGGGAGCAAAC AGGATTAGATACCCTGGTA
PMB 4	GTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTG AGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGG GGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA CGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCC AGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTG CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC CCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCGGGTTAA GCCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGA AGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGG GCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACC TCGCGAGAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGG ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAG TAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCC TTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCA GAAGTAGGTAGTCTAACCTTAGGGGGGACGCTTACCACGGTG TGGCAGATGACTGGGGTGATTGGA
	AACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGGG AAGGTAGCTTGCTACCGGACCTAGCGGCGGACGGGTGAGTAA TGCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAA GGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGG GATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCGGATT AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGT AGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGAC ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG GACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGA AGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGG CTACTTTAGTTAATACCTAGAGATAGTGGACGTTACTCGCAG AATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC AGAGGGTGCGAGCGTTAATCGGATTTACTGGTTAAAAGCGTG CGTAGGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTA
	ACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTATGGGA GAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA GATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTA ATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCC ATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGC TTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAG TACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCC GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA AGAACCTTACCTGGCCTTGACATACTAGAAACTTTCCAGAGA TGGATTGGTGCCTTCGGGAATCTAGATACcGGTGCTGCATGGC TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA ACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGG AACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGG GGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACA CACGTGCTACAATGGTCGGTACAAAGGGTTGCTACACAGCGA TGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGA GTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCG CGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC ACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGTA GCTAGCCTAACTGCAAAGAGGGCGGTTACCACGGTGTGGCCG ATGACTGGGGTGAAGT

TABLE 7
Forward FASTA sequence of 16s rRNA of the pathogenic bacterial isolates

Sl No	Sample code	% Identity of 16S rRNA sequence	Identification	GenBank Accession No:
1	PMB 1	99.8	*Kocuriakristinae*	MF193735
2	PMB 2	99.88	*Granulicatellaelegans*	NJN801174
3	PMB 3	99.05	*Acinetobacterlwoffii*	AB626125
4	PMB 4	99.90	*Acinetobacterbaumanii*	NR 026206.1

TABLE 8

BLAST ANALYSIS SUMMARY AND IDENTIFICATION OF THE PATHOGENIC ISOLATES

IV. DISCUSSION

In this study total bacterial count obtained from all the three sites were significantly higher during the rainy season than during the dry season, since mixing up of bacterial communities from water, land and anthropogenic origin is higher during the rainy season as the run-off water is more dispersed and dynamic in terms of bacterial burden[14].The total colony count of bacteria was higher in the samples collected from the second site, which is close to human settlement and was significantly higher than site 1during the entire period of observation. This indicates the effect of domestic effluents in adding up non endogenous micro flora to open water resources. Lowest colony count was observed at site 3, which is closer to the sea where the higher salinity denies bacterial growth. The conditions remain unfavorable for microbial growth except for the recalcitrant bacteria, resulting in the low density of bacterial population at site 3 [15].

Biochemical profiling of isolates enables the identification of virulence factors that the pathogens employ to successfully invade and establish themselves within the host [16]. All the four isolates obtained in this study were positive for lipases production. This enzyme enables the pathogens to invade the

host cell by disrupting the membrane and helps in the establishment of replication niche during the process of infection [17]. Lipases give direct access to host polymeric nutrients and also confer the pathogens ability to manipulate host cell signal transduction pathways [18]. Pathogenic bacteria are restricted by host immune system by curtailing the availability of nutrients and to counteract this, the pathogens have evolved to acquire virulence determinants that aid in nutrient acquisition [18]. The pathogenic bacteria isolated in this study were capable of utilizing a wide variety of carbon sources including D- mannose, adonitol, malonate, D-tagatose, citrate, coumarate, D-xylose and D-glucose. This metabolic plasticity of pathogens is crucial to trigger virulence [19] and have a major impact on host colonization [20]. Studies report a positive correlation between mannose utilization and biofilm formation in bacteria [21]. Specific arylamidases were produced by all the four isolates for the hydrolysis of the aminoacids tyrosine, alanine, proline, arginine etc. and this facilitates adaptation of the bacterial strains to different host environments, by taking advantage of and managing with the available nutrients such as aminoacids and smaller peptides [22]. Proline and arginine are reported to activate secondary metabolic virulence factors and simultaneously serve as an alternative energy source for the metabolic reactions that shift appropriately with changing environments. Proline also confers pathogens the ability to overcome the immune barriers of the hosts [22].The metabolic flexibility of these pathogens enables them to compete advantageously over normal host probiotic microflora promoting their own colonization and enhancing pathogenesis. Both PMB 1 and PMB 2 were positive for succinate alkalinization which has been attributed to their efforts to overcome acid stress [22]. Lactate assimilation in PMB 4 plays a key role in their biofilm forming ability [23].

Phylogenetically meaningful taxon identification in bacteria make use of 16S rRNA sequence homology [14] and the identified isolates *Kocuria kristinae, Granulicatella elegans, Acinetobacter lwoffii* and *Acinetobacter baumanii* maintained above 98% similarity with standard genomic library sequences. *K. kristinae*is an emerging human pathogen[24] and is etiologically associated with catheter-related bacteremia and acute cholecystitis [25]. In addition, reported cases of *K. kristinae* include urinary tract infections, dacryocystitis, canaliculitis, keratitis, valve endocarditis, peritonitis, descending necrotizing mediastinitis, brain abscess and meningitis[26].The majorities of the reported cases are device-related, acquired nosocomially or endogenous. .Different *Kocuria* species appear to share a common etiology of peritonitis. The overall disease burden associated with *Kocuria* appears to be high[27].It is a matter of concern that the guidelines for treatment of diseases associated with *Kocuria* have not yet been clearly defined[28]. Presence of *Kocuria* in mangrove water is posing a serious challenge to human health since water borne microbes can directly reach unsuspecting people through contaminated aquatic products[29] and can initiate a chain of nosocomial infectious outbreaks by colonizing invasive diagnostic devices, when affected individuals seeks treatment.

G. elegans is reported to cause infectious endocarditis [30, 31] and thoracic emphysema [32]. *A. lwoffi*i is associated with gastritis, ventilator- associated pneumonia and blood stream infections [33].*A. lwoffii* is a common skin colonizer of humans and cause opportunistic infections when there are skin burns or deep wounds [34]. *A. baumannii* is increasingly recognized as an important cause of community-acquired pneumonia [35].*A. baumannii* is a member in the infectious ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiellia pneumonia, A.baumanii Pseudomonas aeruginosa and Enterococcus species*) group of bacteria which are the substantial cause of nosocomial infections and they represent the vast majority of isolates with broad resistance to antibiotics [36]. The isolated colony members in this study were resistant to the vibrio-static agent 0129 (2, 4-diamino-6, 7-di-isopropylpteridine phosphate).A dauntingly large number of medical reports were published across the world on scepticemia [37] meningitis, brain abscess, lung abscess [38] infections of eyes, urinary tract, skin and wounds [39,40]caused by *Acinetobacter*.20 -70% rise in mortality rate was reported to be associated with *Acinetobacter* when compared with other pathogens causing nosocomial infections [41].

Opportunistic pathogenic bacteria is a major threat to public health when antibiotic resistant, virulent pathogens reaches unique environments like mangroves by the release of untreated hospital effluents to open water sources or poor sterilization and disinfection practices in hospitals. This can cause epidemic outbreaks, especially during natural calamities. Studies suggest that heavy metal pollutions in such ecosystems trigger co-selection of antibiotic resistance in *in-situ* bacterial fauna [42]. Irrational use of antibiotics in aquaculture and animal husbandry further complicates the scenario. Since spread of antibiotic resistance is not necessarily restricted by phylogenetic, geographic and ecological borders, it spreads from its origin to human niches on a global scale. It is suggested that monitoring of the water quality in mangroves, especially with respect to pathogenic bacteria and human coliforms should be conducted on a regular basis and treatment of waste water should be done at the source itself. Such practices in hospitals, domestic households and house boats will ensure that the quality of such pristine and ecologically important water bodies is not compromised.

V. CONCLUSION

This study concludes the presence of pathogenic bacterial strains in the mangrove water of Poovar and their presences indicate anthropogenic influence. Biochemical profile of the isolates reveals similar metabolic pathways in all the four isolates, which were identified as their virulence determinants. The isolates were identified as K. *kristinae, G. elegans, A. lwoffii* and A. *baumanii*, based on 16S rRNA sequence analysis. The presence of these pathogens warns us of higher chances of water borne diseases in the local community since, open well water is their most dependent domestic water source and are within the range of tidal influence. Conservation strategies need to be planned keeping in mind the effluent pollution of mangroves that pose long lasting health hazards

by guiding evolution of recalcitrant, sturdy and drug resistant bacteria. The potential threat from pathogenic bacteria through food source of consumers is extremely alarming. The result of this study is indicative evidence of untreated hospital effluents being dumped into the water bodies and this calls for stringent enforcement of laws that have been enacted to prevent the abuse of natural resources.

VI. REFERENCES

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