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Molecular characterization and antibiotic susceptibility pattern of isolated pathogenic bacterial strains from Sangam water in Allahabad region

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Abstract
This study was carried out to isolate and characterize two pathogenic bacterial strains from Sangam water in Allahabad region. Two pathogens i.e. *Staphylococcus species* and *Vibrio species* were isolated and subjected to morphological, biochemical and molecular characterization, thereafter the species was identified as *Staphylococcus epidermidis* and *Vibrio parahaemolyticus.* The isolates were screened for antibiotics susceptibility pattern and found that *Staphylococcus epidermidis* was resistant for amoxicillin, kanamycin, streptomycin therefore *Staphylococcus epidermidis* was found to be multi-drug resistance. *Vibrio parahaemolyticus* was resistant for streptomycin, erythromycin, gentamycin, tetracycline therefore *Vibrio parahaemolyticus* was also found to be multi-drug resistance. *Staphylococcus epidermidis* is the causative agent of serious recurrent infections like urinary tract infection (UTI), epididymitis, urethritis while *Vibrio parahaemolyticus* can cause diarrhea, gastrointestinal hemorrhage, colitis. Thus, the water of these rivers Ganga and Yamuna was found to be unfit for human consumption without treatment. Better management of resources and research in the area of water remediation, could minimize the elevated levels of water pollution.

Keywords: *Staphylococcus epidermidis, Vibrio parahaemolyticus,* antibiotic susceptibility pattern, 16S rRNA gene, water born microbes

Introduction
Biofilm-associated microorganisms have been shown to be related to more than 65% of all medical infections, including endocarditis, otitis, prostatitis, periodontitis, conjunctivitis, vaginitis, and infections related to cystic fibrosis. In addition, biofilms are important colonizers of a wide variety of medical devices, such as catheters and prostheses. The costs linked to vascular catheter-related blood-stream infections caused by *S. epidermidis* amount to an estimated US$2 billion annually in the United States. In a biofilm, the bacterium is protected against attacks from, the immune system and antibacterial treatment, making *S. epidermidis* infections difficult to eradicate. The inhibition of virulence targets could bring new antibacterial molecules with radically new mechanism of action and represent an innovative therapeutic concept (Trentin *et al*. 2011) [8].

The importance of *Staphylococcus epidermidis*, a gram-positive bacterium with low virulence and weak pathogenicity has been seen as a causative agent of various community acquired diseases and infections is being increasingly recognized. These species can form biofilms, bacterial clusters that attach to materials such as plastics which constitute the major portion of waste found at the site of confluence. Furthermore, biofilm production has been associated with an increased resistance to antibiotics (Meléndez *et al*. 2016) [5].

Vibrio parahaemolyticus is a halophilic gram-negative bacterium widely distributed in marine environments and is recognized as the world's leading cause of gastroenteritis in humans due to raw or undercooked seafood. However, it is ubiquitous in many brackish coastal environments of most continents, and infections associated with seafood contaminated by this pathogen have occurred throughout the world. At present, outbreaks of *V. parahaemolyticus* infections are a significant public health concern in many countries. Filter-feeding bivalve, such as mussels, can concentrate pathogenic strains of *V. parahaemolyticus* resulting in bacterial loads capable of producing infection to the people that ingest them. Consequently, virulent *V. parahaemolyticus* strains are clearly a concern for seafood safety (Yáñez *et al*. 2015; Xu *et al*. 2015) [11, 10].

The heavy use of antibiotics for disease treatment in aquatic animals has resulted in the emergence of antibiotic-resistant pathogens in aquaculture environments making the antibiotic

treatment ineffective and this type of incident has been reported from all areas of aquaculture. Moreover, there is a growing concern over the risks of the transfer of resistance determinants to bacteria of land animals and to human pathogens and the presence of antibiotic residues in aquaculture products that constitutes threats to public health (Kongchum *et al*. 2016) [2].

For the last few decades, multidrug resistance has become an increasing concern for both Gram-positive and Gram-negative bacteria. The number of new molecules has dramatically decreased and antibiotic resistance is now a priority in the international community. Facing this new threat, a large number of new as well as "old" solutions are now being discussed in the medical community to propose an alternative to antibiotic treatments. A first option is to potentiate the effect of existing molecules through combinations to circumvent the individual molecule resistance. The second option is to neutralize either the infectious agent itself or its by-products using specific antibodies. A third option is to use the pathogen signaling mechanism and inhibit the production of virulence factor through quorum sensing inhibition. A fourth pathway would be to interact with the patient's microbiota using either probiotics or faecal transplantation to modulate the innate immune response and improve response to the infectious challenge, but also to act directly against colonization by resistant bacteria by replacing the flora with susceptible strains. The last option is to target the bacteria using phage therapy. Phages are natural viruses that specifically infect target bacteria independently of any antibiotic-susceptibility profile. In the majority of cases, these treatments represent an interesting approach but not the ultimate solution to multi-resistance. Well-performed clinical trials are still missing and the major priority remains to promote good use and appropriate stewardship of antibiotics to decrease resistance. (Brunel and Benoit 2017) [1].

Thus, in view of above, present study has been designed by keeping focus on antibiotic susceptibility pattern of disease causing microbes isolated from Samgan region where several pilgrims take holy dip at Allahabad.

Methodology

Collection of soil samples

Microbial samples were isolated from Sangam water at Allahabad region during the period of famous Magh Mela in the month of February when millions of pilgrims gather to take holy dip and stored in sterilized bottles to avoid any cross contamination.

Isolation of pathogenic strains

The isolates were maintained by sub culturing in fresh media of Nutrient Agar. Isolated organisms were sub-cultured in the Nutrient agar media. The cultures were incubated at 30°C for 24 hrs in a bacteriological incubator and after that the culture were stored in refrigerator at 4 °C. Cultures were revived at 15 days duration in the same media.

The colonies were selected and plated on selected media. Mannitol sail agar (MSA) for isolation of *Staphylococcus* species and Tryptone soya agar (TSA) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS) for *Vibrio* species. Then the soil selected colonies were serially diluted and appropriate dilutions were inoculated on Luria Broth and streaked on media paltes. The plates were incubated at 37 °C in incubator for 24 hrs to 72 hrs.

Characterization of isolates

The plates were incubated at 35±2 °C and identical colonies were selected for further morphological and biochemical studies. Several biochemical tests *viz.* IMVIC, Catalase test, Coagulase test, Hydrogen sulphide production, Nitrate reduction, Urease test and Carbohydrate fermentation tests were performed for the biochemical characterization of selected rhizobacterial isolates using standard procedures according to Bergey's Manual of Determinative Bacteriology.

For Molecular characterization, bacterial genomic DNA was isolated and quantified at 260/280nm. The 16SrRNA gene for both samples was amplified by PCR using forward 5'-CCGAATTCGTCGACAACAGA GTTTGATCCTGGCTCAG-3' and reverse primer 5'-CCCGGGATCCAAGCTTACGGC TACCTTGTTACGACTT-3 primers under standard conditions (initial denaturation 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30s, extension at 72 °C for 60s, and final extension at 72 °C for 7 min) according to Maxton *et al.* (2017) [4]. Amplified product was purified and sequenced (Applied Biosystems, New Delhi) and submitted to Gen Bank of NCBI.

Antibiotic susceptibility test

Antibiotic Susceptibility test of the antibiotics (Gentamycin, Erythromycin, Ciprofloxacin, Amoxicillin, Ampicillin, Streptomycin, Tetracyclin, Vancomycin, Kanamycin) were performed against the isolated bacterial strains. The antibiotic test was done by Agar well diffusion method. In brief, the pure culture of organism was sub-cultured in broth. For bacterial growth, a lawn of culture was prepared by spreading the 200µl fresh culture from broth of each test organism on agar plates with the help of a sterile glass rod spreader. Plates were left standing for 10 minutes to let the culture get absorbed. Then 6 mm wells were punched into agar plates for testing antibacterial activity. Using the micro-pipette, 20µl of antibiotic was poured onto each of wells on the plates. After overnight incubation at 37 °C, the different levels of zone of inhibition were measured (Wanger *et al.* 2017) [9].

Results and Discussions

Isolation and biochemical characterization

The gram staining procedure for the first pathogenic isolate retained the crystal violet and bunch of coccus colonies were observed which was later identified as *Staphylococcus epidermidis* whereas the second pathogenic isolate resulted in pink coloured, comma shaped colonies which was later identified as *Vibrio parahaemolyticus.*

The characterization of the both isolate was done by biochemical analysis as per Bergy's manual of systematic bacteriology. The *S. epidermidis* showed positive reactions for Urease (Table 1), Nitrate reduction (Table 1), Citrate utilization (Table 1) while negative reaction (s) for Indole (Table 1), Methyl Red (Table 1), Voges Proskaure (Table 1), Coagulase (Table 1) and Hydrogen sulphide reduction (Table 1), were observed. These isolates were found to be not able to utilize some carbohydrates Xylose, Starch, Sallicin, Rhamnose, Raffinose, Mannitol, Arabinose, Cellobiose and able to utilized Sucrose, Maltose, Lactose, Glucose whereas *V. parahaemolyticus* showed positive reactions for Methyl Red (Table 1), Nitrate reduction (Table 1), negative reactions for Indole (Table 1)**,** Urease, Voges Proskauer (Table 1)**,** Coagulase (Table 1), Citrate utilization (Table 1) and Hydrogen sulphite reduction (Table 1) were observed. This isolate was found to be not able to utilize some carbohydrates

Xylose, Sucrose, Sallicin, Rhamnose, Raffinose, Lactose, Glucose Arbinose and able to utilized Starch, Mannitol, Maltose, Cellobiose.

Molecular characterization (16s rRNA gene amplification)

The two selected isolates were after morphological and biochemical screening was processed for molecular identification. After confirming morphological and biochemical characterization, total genomic DNA was extracted and run on agarose gel with 1kb ladder. Bacterial culture showed genomic DNA size greater than 10kb size Total genomic DNA was isolated and screened for quantification using UV spectrophotometer at OD 260 and 280 nm to calculate total DNA conc. (µg/ml). After successful qualification, genomic DNA was run in 1.2% agarose gel for 23 min at 75 V. Product of genomic DNA was amplified using forward and reverse primers to screen 16s rRNA gene of bacterial species with 1 kb ladder (Fig. 1).

The identification of species was further confirmed by computational analysis. Gene sequence of 16s RNA of different strains were compared with nucleotide database of NCBI webserver through BLAST tool. Results showed that query sequences were best pairwise aligned with 16S rRNA sequences with sequence similarity and identity of 99%. The query sequences of isolated strains were identified as *Vibrio parahaemolyticus* 180236 under NCBI accession number LC375757.1 and *Staphylococcus epidermidis* 180237 under NCBI accession number LC375757.1.

Phylogenetic relationship of identified strains.

An analysis of 16S rRNA gene of the first isolate of the present study showed that the strain was closely related phylogenetically to members of the genus *Staphylococcus* rRNA group**.** Whereas the second isolate showed that the strain was closely related phylogenetically to members of the genus *Vibrio* rRNA group. For better understanding of the species identification, nucleotide conservation was evaluated by comprising 16S rRNA sequences of the isolated strains through multiple sequence alignment analysis.

The evolutionary history for *Staphylococcus epidermidis* was interred using the neighbour joining method (Saltou and Nei 1987)[6]. The optimum tree with the sum of branch length = 0.00136536 is shown. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetics tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding all positions containing gaps and missing data were eliminated. There were a total of 1467 positions in the final dataset Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016) [3].

The evolutionary history for *Vibrio Parahaemolyticus* was interred using the Neighbour Joining method (Saltou and Nei, 1987) [6]. The optimum tree with sum of branch length = 0.00343603 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) [7] and are in the units of base substitutions per site. The analysis involved 11 nucleotide sequences codon positions included were 1st+2nd+3rd+Noncoding All positions containing gaps and missing data were eliminated. There were a total of 1457 positions in the final dataset Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016) [2].

Antibiotic susceptibility test

Antibiotic Susceptibility test of the antibiotics were performed against the two isolated bacterial strains. The antibiotic test was done by Agar well diffusion method. Then 6 mm wells were punched into agar plates for testing antibacterial activity. Using the micro-pipette, 20 µl of antibiotic was poured onto each of wells on the plates. After overnight incubation at 37 °C, it was observed that the first isolate *i.e. S. epidermidis* showed maximum resistance against Amoxicillin (0mm), Streptomycin (0mm) and Kanamycin (0mm) followed by Ciprofloxacin (15mm), Vancomycin (14mm), Erythromycin (13mm), Ampicillin (11mm), Tetracycline (10mm), whereas it showed sensitivity effects against Gentamycin (12mm). (Fig. 2).

For the second isolate *i.e. V. parahaemolyticus*, it was observed that it showed maximum resistance against Gentamycin (0mm), Erythromycin (0mm), Streptomycin (0mm) and Tetracycline (0mm), followed by Vancomycin (13mm). It showed intermediate effect against inhibitory effects against. Ciprofloxacin (0.8mm) and Kanamycin (16mm), whereas it showed sensitivity effects against Ampicillin (15mm) and Amoxicillin (13mm) (Fig. 3).

Table 1: Cultural, Morphological and Biochemical identification of pathogenic isolates from Sangam water

Colony Morphology		***S. epidermidis***	***V. parahaemolyticus***
Elevation		Convex	Raised
Margin		Entire	Entire
Pigmentation		White	White
Opacity		Opaque	Opaque
Morphological Characteristics	Gram stain reaction	+	_
	Shape	Spherical	Curved Rod
Biochemical Characteristics	Indole Test	_	+
	MR Test	_	+
	VP Test	_	_
	Citrate Utilization Test	+	_
	Nitrate Reduction Test	+	+
	Urease Production Test	+	_
	Catalase Test	+	+
	H_2S Production	_	_
	Coagulase Test	_	_
Carbohydrate Fermentation Test	Arabinose	A^-G^-	A^-G^-
	Cellobiose	A^-G^-	A^+G^-
	Glucose	A^+G^-	A^-G^-

	Lactose	A^+G^-	A^-G^-
	Maltose	A^+G^-	A^+G^-
	Mannitol	A^-G^-	A^+G^-
	Raffinose	A^-G^-	A^-G^-
	Rhamnose	A^-G^-	A^-G^-
	Salicin	A^-G^-	A^-G^-
	Starch	A^-G^-	A^+G^-
	Sucrose	A^+G^-	A^-G^-
	Xylose	A^-G^-	A^-G^-

A^+= Acid Production; A^-= No Acid Production; G^+= Gas Production; G^-=No Gas Production

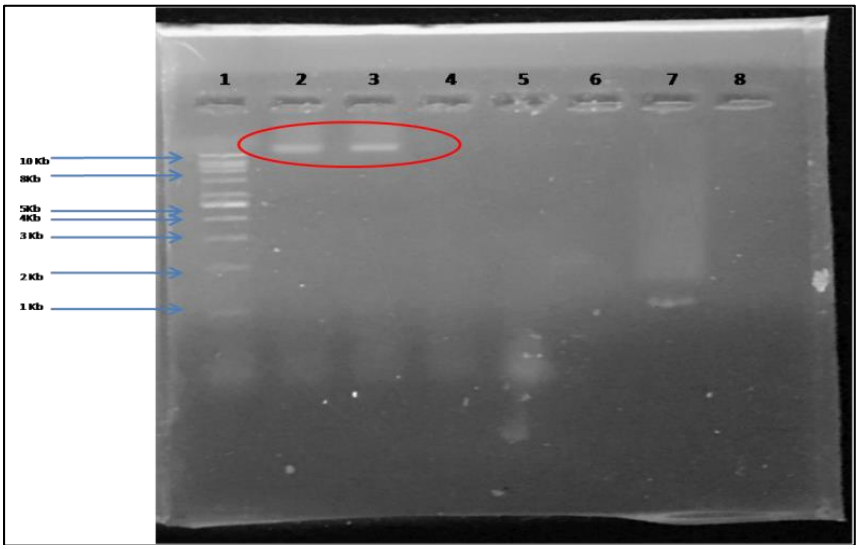

Fig 1: 16s rRNA gene amplification for isolates (Lane 1: 1kb Ladder, Lane 2: *Staphylococcus spp*, Lane 3: *Vibrio spp*.)

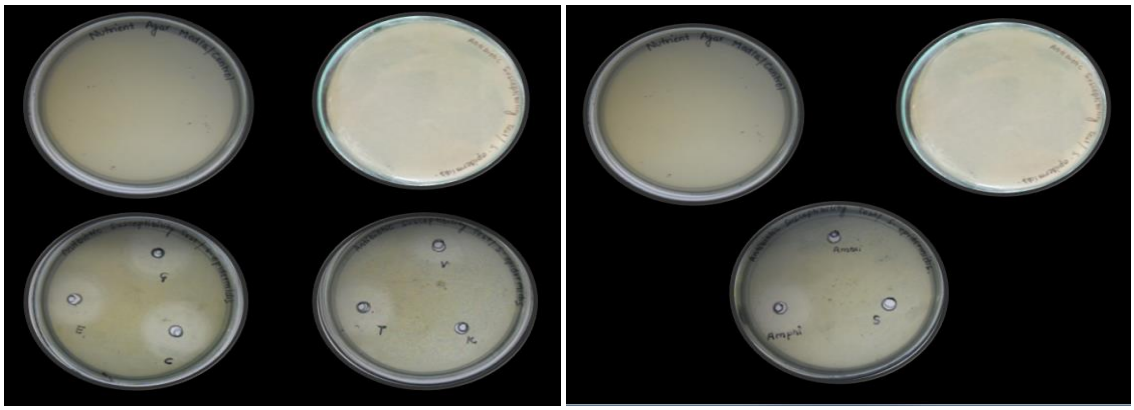

Fig 2: Antibiotic susceptibility test of *S. epidermidis*

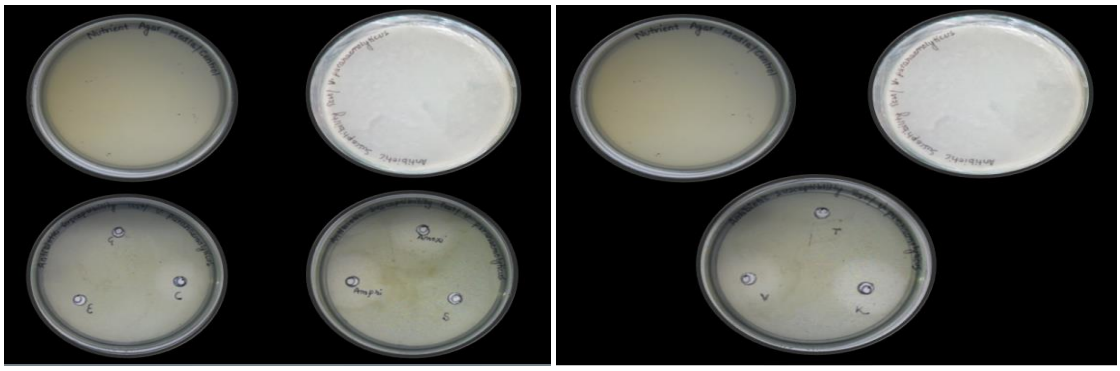

Fig 3: Antibiotic susceptibility test of *V. parahaemolyticus*

Conclusion

Antibiotic Susceptibility test showed that the *S. epidermidis* stain present in the river water has developed resistance towards eight antibiotics out of the nine that it was tested against. Whereas *V. parahaemolyticus* strain present in the river water showed resistance against five antibiotics and intermediate effects against two out of the nine that it was tested against. This could be because of the presence of antibiotic materials in the waste being dumped in the river

water, which has led to the mutating of these strains to develop resistance against them.

It can be said that the water quality at Sangam was very poor, and it can only be attributed towards the ill practices of the visitors taking dip and local community dumping the wastes. People staying in that region mostly defecated in open, washed their clothes in the river, take their cattle for bathing, all these factors contributed towards the river pollution. Better management of resources and provision of basic facilities although, could minimize the elevated levels of water pollution. Mass gatherings and dips poses a complex public health challenge. The difference in healthcare seeking attitude and religious beliefs combined with high crowd mobility make it particularly difficult to measure the disease burden accurately. However, the focus should not be only on precisely measuring the disease burden but also on mitigating the possible risk factors. Disease diagnosis, treatment and prevention will become more targeted if doctors and public health authorities are aware of the common health risks involved during the pilgrimage.

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