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“Antibiotic Resistance in the *Vibrio* species isolated from the estuarine sediments of Uttara Kannada Karnataka”

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Abstract

Vibrio species are the marine pathogenic bacterium which causes the health issues in the infected organisms. Antibiotic resistance in the *Vibrio species* is the threat to the human health by the transfer of the resistant gene in the human from the food chain. The present study was carried out to isolate the *Vibrio* species and to study antibiotic resistance from the sediments of two estuaries Kali and Aghanashini from Uttara Kannada, Karnataka. The sampling was done during low tide for the period of ten months from September 2021 to June 2022. The total vibrio count was observed in range between 3.86 to 4.83 Log X10^1 CFU/g. *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio navarrensis* and *Vibrio vulnificus* were isolated and identified from the sediments. The identified *Vibrio* species were resistant to ampicillin and sensitive to chloramphenicol and tetracycline. Due the over use of antibiotics in the aquaculture and mariculture activities there is a possibility of organism to show multiple resistance towards the antibiotics.

Keywords: Sediment, Resistance, Sensitive, Antibiotics, *Vibrio* species

Introduction

Vibrio species are commonly found in coastal environment. These pathogenic organisms causes the gastrointestinal disorders in humans and also causes septicemia. *Vibrio parahaemolyticus* and *Vibrio alginolyticus* are the pathogens for the shellfishes. These species are mainly found in estuarine and marine environment, in free swimming and its motility is confined with a single polar flagellum, which can inert and intimate in the surfaces of zooplanktons, fish, shellfishes and mainly in bivalves which are suspended in the sediments (Gode-Potratz et al., 2011 and V.letchumanan et al.,2014). These causes the gastrointestinal disorders mainly transmitted due to the consumption of raw or uncooked food (Newton et al., 2012), ear, wound infections and septicaemia which are life threatening to the patients with illness (L zhang et al.,2013). Shell fishes are considered as poor mans rich protein food of majority of inhabitants of coastal area. Bivalves are usually consumed by local public and even it is transported to different places.

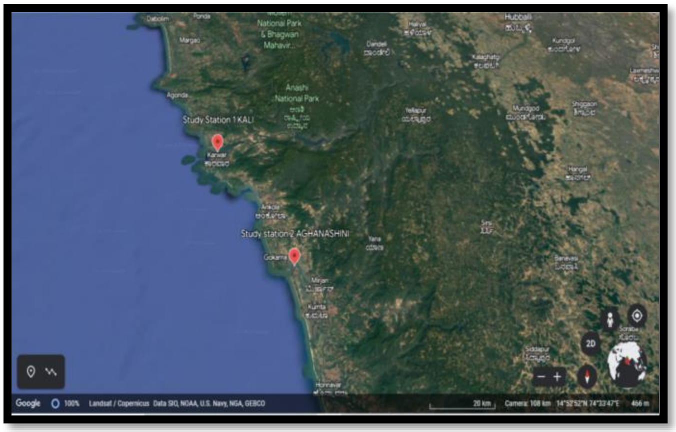
Vibrio bacteria is commonly found in aquatic environment, most bacteria have been observed in fin fishes and shell fishes, most abundantly in shellfishes such as oysters, clams, etc. These bacteria are thus considered to be the natural microflora of the surrounding. Thus, the pathogenic strains arise due the environmental concerns such as increase in the temperature or pollution in the surrounding due to human intervention. It has been reported in studies that during extreme weather conditions, summer, *Vibrio* have been even found in the sediment surrounding the organism as it contains significant amount of organic matter. *Vibrio* infections are usually caused due to improper eating practice of sea foods as most are foodborne pathogens. It is also been observed in reports that these pathogenic strains of *Vibrio* are able to grow at an exponential rate due to the presence of Sodium Chloride (NaCl), so its notable presence in the brackish or estuarine water has been seen. They are able to withstand high pH and salinity. Most *Vibrio* species are able to oxidize and ferment glucose without production of gas (Baumann, et. al., 1984).

In the present study an attempt is made to isolate, identify the pathogenic *Vibrio* species and to study the resistance against the different antibiotics from sediments of Kali and Aghanashini estuary of Uttara Kannada, Karnataka.

Materials and Methods

Study Location: The location of the study was two estuaries Kali and Aghanashini from Uttara Kannada district, Karnataka. The Kali estuary (14^{0}80’58”N & 74^{0}14’26.2”E) located in Karwar taluka. Kali river originates from the place called Diggi in Joida Taluka. It flows to south west into Kadra Reservoir, and is joined by the Thana Halla just below the dam at Kadra. From Kadra the river flows west through marshland to join the Arabian sea in Karwar.

Figure-1 Map showing Study Sites



The Aghanashini estuary or Tadadi River (14^{0}52'25.6"N& 74^{0}36'67.5"E) originates in the Sirsi taluk of Uttara Kannada district in the central Western Ghats of Karnataka State. The river meets the tides of the Arabian Sea and forms a large estuarine expanse in the coastal taluk of Kumta. The estuary has its outlet into the sea in between the villages of Aghanashini in the south and Tadadi in the North.

Sample Collection: The sampling was done on monthly bases for a period of ten months from September 2021 to June 2022. Sediment samples from these estuaries were collected during low tide in the aseptic condition using sterile hand core and immediately transported to laboratory in sterile polythene bags in aseptic condition.

Sample preparation and analysis: Sediment samples were suspended in distilled water to obtain 1:10 (w/v) dilution, vortexed to mix well; the supernatant fluid was used for analysis. 0.5 ml of each required dilution were spread plated on sterilized TCBS Agar plates in duplicate. The inoculated plates were incubated in 37^{0}C for 24 hrs. the green and yellow colonies were isolated and purified for then biochemical test (Catalase test, Oxidase test, Salt tolerance test(0-10%), Indole test, Citrate Utility test, VP-MR test, Nitrate test, VP-MR test, Nitrate test, Urease test, Carbohydrate fermentation test) identified using Noguerola and Blanch 2008 identification keys and molecular identification using 16S rDNA sequencing was done. The bacterial DNA was isolated and evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database.

Antibiotic Sensitivity Test: The *Vibrio spp.* were tested for antibiotic sensitivity against different antibiotics. The previously identified *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio navarrensis and Vibrio vulnificus* were tested on Muller Hilton Agar using eight different antibiotics (ampicillin, cefepime, cefotaxime, ceftazidime, gentamicin, tetracycline, ciprofloxacin and chloramphenicol) by Disc Diffusion method. The test plates were incubated at 37^{0}C for 24hrs and the plates were observed for the zone of inhibition surrounding the antibiotics. The diameter of the zone of inhibition was measured using ruler, by placing the pertiplate over the dark background. The diameters of zone of inhibition was noted for each antibiotics. The results were interpreted with the guidelines of the Clinical and Laboratory Standards Institute M45-A2 (CLSI, 2010). Based on the observed results bacteria was classified into resistant, sensitive and intermediate to antibiotics

Results

In the present study isolation of specific pathogenic bacteria *Vibrio species* from the sediments of two estuaries, the Total *Vibrio* Count in sediments of Kali and Aghanashini estuary was estimated monthly from September 2021 to June 2022 in sediments. In Kali estuary it was ranged between 3.86 to 4.57 Log CFU/g whereas in Aghanashini estuary it was ranged between 3.86 to 4.83 Log CFU/g was observed. In the sediments of Kali estuary, the maximum of 4.57 Log CFU/g in March 2022, followed by 4.34 Log CFU/g in February 2022 and least was 3.86 Log CFU/g in January 2022 was observed. Where as in Aghanashini estuary the maximum was 4.83 Log CFU/g in February 2022, followed by 4.74 Log CFU/g in March 2022 and least of 3.86 Log CFU/g was observed in September 2021. Similar kind of observation was made in *Meretrix meretrix* of Kali and Aghanashini estuary by Revankar,S K et.al.,(2023).

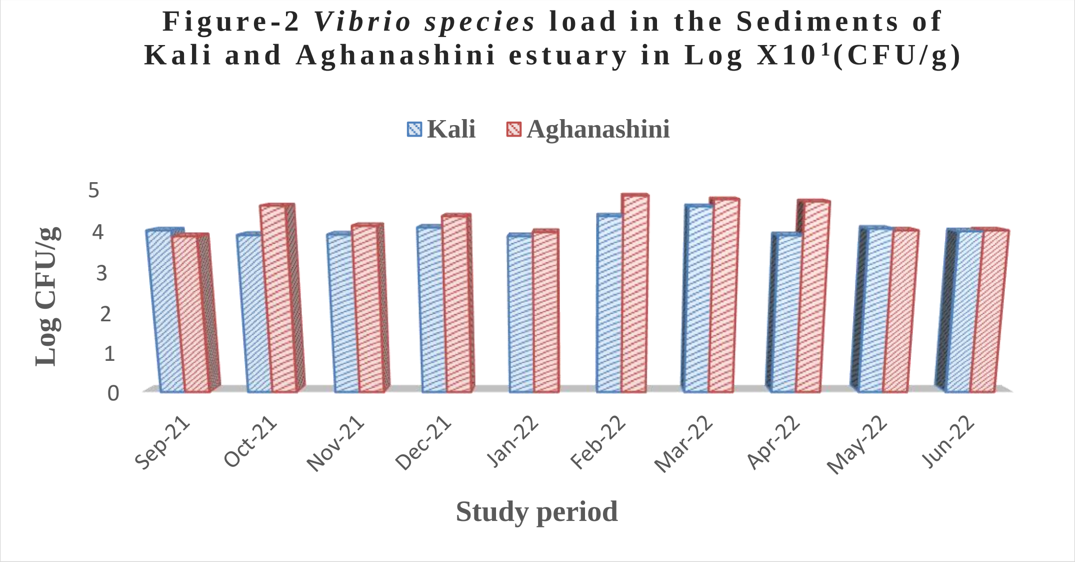
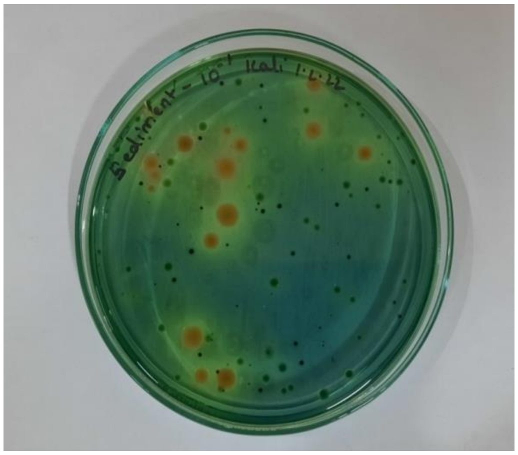


Figure -3 *Vibrio species* on TCBS Agar plate



Biochemical Studies and Molecular identification: Total of 75 isolates showing different colony characters on TCBS plates green, yellow, small yellow and translucent yellow colonies were biochemically tested for different tests and it represented that the bacterial samples were *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio navarrensis and Vibrio vulnificus* with reference to Noguerola and Blanch 2008 identification keys. The green and bluish green isolates showed positive to catalase, oxidase, indole and citrate but showed negative to MR and VP test. These green isolates were able to ferment glucose, arabinose, mannose and mannitol without gas formation and tolerate up to 8% salt (NaCl). Some of the green isolates were partially positive to urease test. Large yellow isolates on TCBS medium showed positive to catalase, oxidase, indole, citrate and VP test. Some of these isolates showed partially positive to urease test. These yellow isolates were able to ferment glucose, sucrose and maltose without gas formation and were able to tolerate up to 10% Nacl. Small yellow isolates were positive to catalase, oxidase and indole but negative to urease, VP and MR. They would ferment some of carbohydrate glucose, sucrose, arabinose and mannitol. They were able to grow in 10% NaCl. Yellowish isolates were positive to catalase, oxidase, indole and were able to tolerate upto 8% NaCl and were able to ferment glucose, sucrose maltose and mannitol without gas formation (Table1). The 16S rDNA sequence which was BLAST with the data base of NCBI gene data base, the result for these samples showed similarities (99.55%) with *V.parahaemolyticus*, (99.40%) *V.alginolyticus*, (99.20%) *V.navarrensis* and (99.35 %) *V.vulnificus* based on nucleotide homology and phylogenetic analysis.

TABLE 1 – BIOCHEMICAL **TESTS**

TEST PARAMETERS	**IDENTIFIED ISOLATES WITH BIOCHEMICAL BEHIVAOUR**			
	V-1	***V-2***	***V-3***	***V-4***
Colour of Colony	Green	Yellow	Small yellow	Translucent yellow
Catalyse	+	+	+	+
Oxidase	+	+	+	+
Methyl Red test	-	+	-	-
Voges – Proskauer test	-	+	-	-
Indole test	+	+	+	+
Citrate test	+	+	-	#
Urease test	#	+	-	+
37^0 C	+	+	+	+
42^0 C	+	+	#	-
0% NaCl	-	+	+	+
2% NaCl	+	+	+	+
6% NaCl	+	+	+	+
8% NaCl	+	+	+	+
10% NaCl	+	+	+	-
Glucose	+	+	+	+
Sucrose	-	+	+	+
Lactose	-	-	-	-
Maltose	-	+	-	+

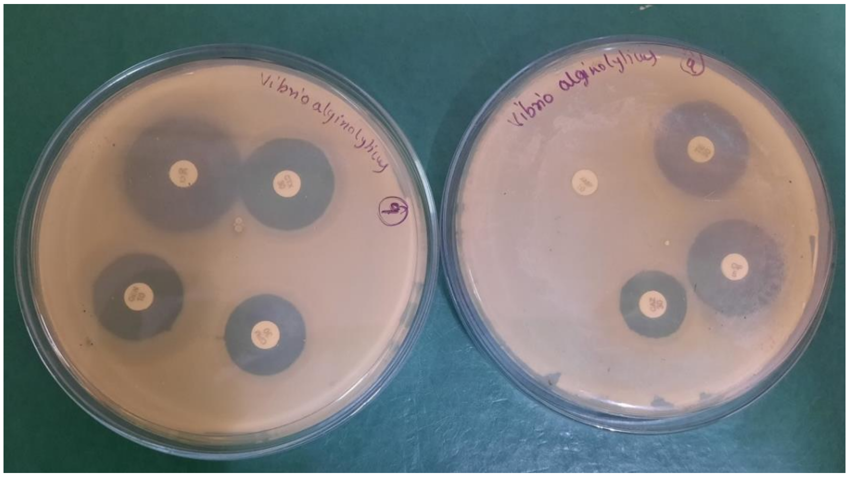
Mannitol	+	-	+	+
Mannose	+	-	-	-
Arabinose	+	-	+	-

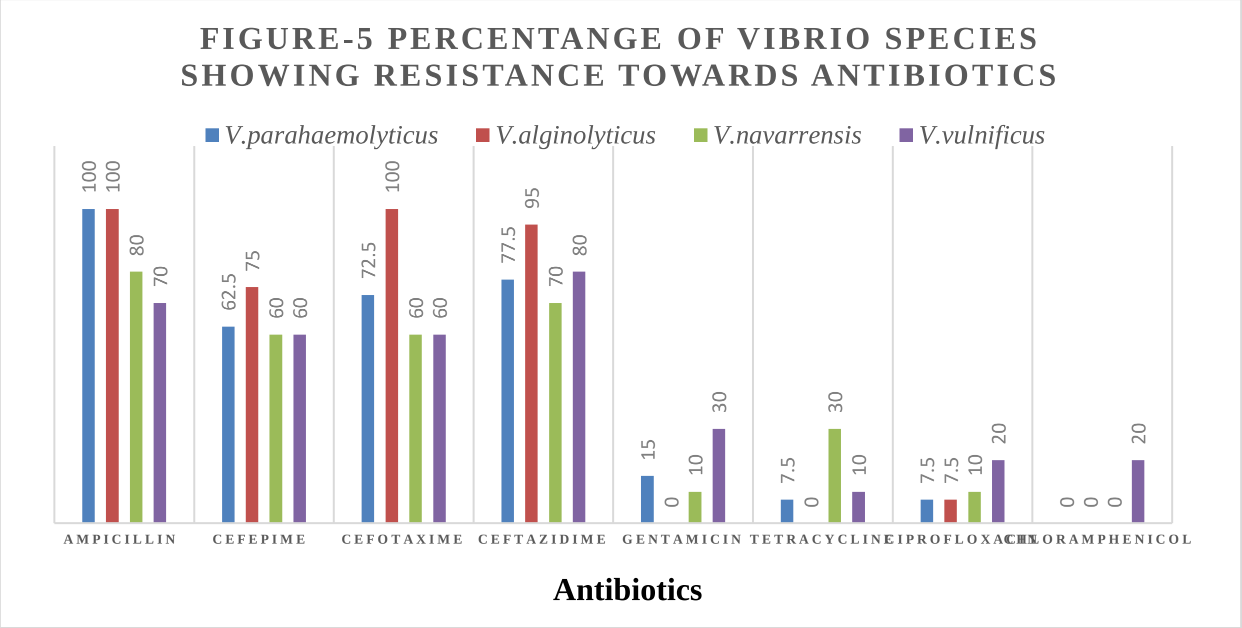
+ Positive; -Negative; # Partially positive

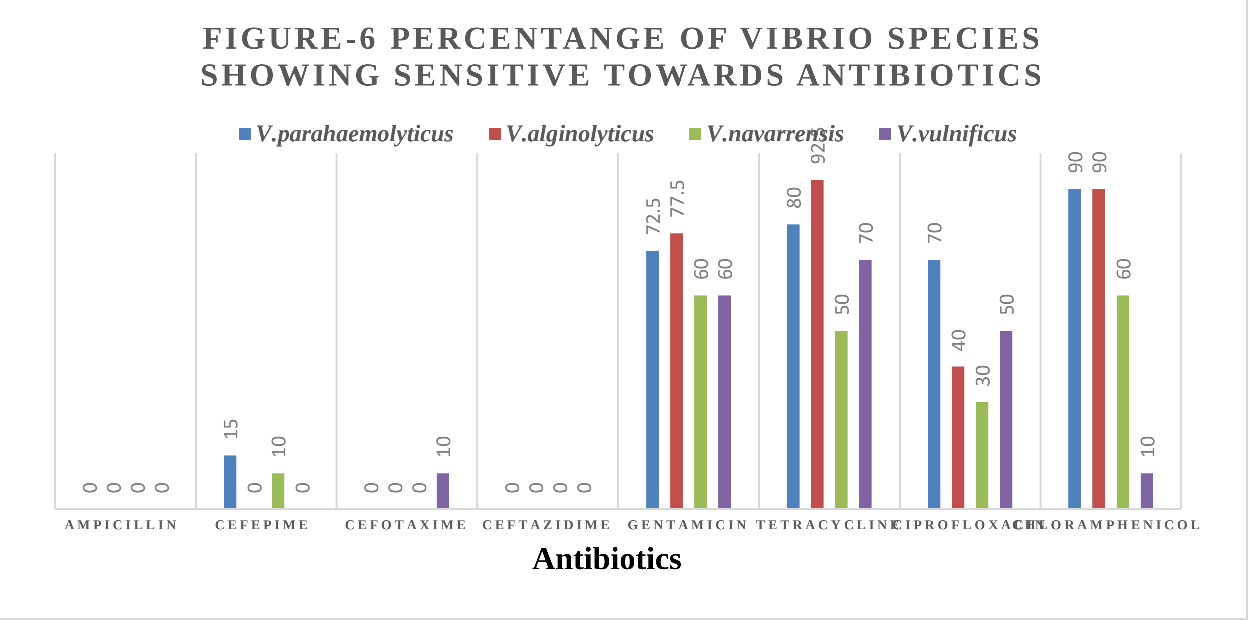
Antibiotic Sensitivity Test

Vibrio species were tested against eight different antibiotics. *Vibrio parahaemolyticus* showed highest resistance to Ampicillin, Ceftazidime, Cefotaxime and Cefepime antibiotics, where as it was sensitive to Chloramphenicol, Tetracycline, Gentamicin and Ciprofloxacin. *Vibrio alginolyticus* showed highest resistance to ampicillin and cefotaxime, followed by ceftazidime and cefepime. Whereas, it was sensitive against tetracycline, chloramphenicol and gentamicin. *Vibrio navarrensis* was resistant and intermediate resistant to ampicillin, ceftazidime, cefotaxime and cefepime antibiotics. Whereas it was sensitive against chloramphenicol, gentamicin and tetracycline. *Vibrio vulnificus* were sensitive against tetracycline, gentamicin and ciprofloxacin. But it was resistant to ceftazidime, ampicillin, cefepime and ceftaxime. But it showed intermediate resistance to all the eight tested antibiotics. In the present investigation the *V. parahaemolyticus, V.alginolyticus, V.navarrensis and V.vulnificus* were tested for the antibiotic sensitivity test using eight different antibiotics, all most all isolates very resistant to ampicillin, ceftazidime, cefotaxime and cefepime antibiotics, but they were sensitive to chloramphenicol, tetracycline and ciprofloxin which is similar to the observation made by Muraleedharan H et.al.,(2009). The assess use of antibiotics in aquaculture farms which was washed off in the estuaries caused antibiotic resistant to the organsisms.

Figure- 4 Image showing Zone of Inhibition







Conclusion

In the present study an attempt was made to isolate *Vibrio species* from the Sediments of Kali and Aghanashini estuary of Karnataka. Estuaries are the considered as the hotspots of the different bacterial flora, due to the anthropogenic activities. The estuarine sediments have 10^8 to 10^{10} bacterial load when compared to that of water samples (Shah et al., 2021), where as in present study the bacterial (*Vibrio*) load was 10^3 to 10^4 which was less compared to study of Shah et al., (2021). *Vibrio parahaemolyticus, Vibrio alginolyticus*, *Vibrio navarrensis and Vibrio vulnificus* were identified from the sediments, *V.parahaemolyticus* and *V.alginolyticus* were dominant species in the estuaries. These *Vibrio species* were resistant to Ampicillin, Ceftazidime, Cefotaxime and Cefepime antibiotic where as they were sensitive to Gentamicin, Tetracycline, Chloramphenicol and Ciprofloxin. The over use of the antibiotics in the aquaculture farms, they are washed off in the estuaries and they antibiotics present in the environment affects the organisms and these organisms can tolerate the antibiotics. Antibiotic resistant *Vibrio species* present in the sediments causes pathological changes in the benthic organisms like bivalves by the filter feeding habit. The study by Revankar,S K et.al.,(2023) identified *Vibrio parahaemolyticus and Vibrio alginolyticus* which caused necrosis and tissue disorganisation in *Meretrix meretrix* from Kali and Aghanashini estuary.

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