

# The prevalence of antibiotic resistance among different species of bacteria isolated from an estuary in Kerala, South India.

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**ABSTRACT:** The current investigation was carried out to determine the antibiotic-resistance status of bacteria isolated from Ponnani estuary. Antibiotic sensitivity was determined by the disc diffusion method. A total of 39 samples were collected from three stations of the estuary. Of the fifty-one bacteria isolated, 56.8% were gram-negative, and 43.1% were gram-positive. *Vibrio* sp. 13(25.49%) *Pseudomonas* sp. 1(1.9%) *Flavobacterium* sp. 3(5.9%) *E. coli* 6 (11.7%) *Alcaligenes* 6 (11.7%) *Staphylococcus* sp. 21 (41.7%) and *Micrococcus* sp. 1 (1.9%) were isolated from various part of the estuary. Members of *Vibrio* sp. are found to be resistant to 8 of the 10 antibiotics employed whereas *Flavobacterium* sp. and *Alcaligenes* sp. showed resistance to 7 antibiotics used. All members of *Vibrio* sp. showed resistance to cefoxitin, and cefuroxime while only 76.9% showed resistance to nalidixic Acid. All the members of *E. coli* were resistant to ampicillin and tetracycline. All the members of the gram-positive bacteria were resistant to methicillin while 95% of them were resistant to nalidixic acid followed by penicillin (86%) and erythromycin (68%). The antibiotics vancomycin and chloramphenicol seems to be highly sensitive to gram positive isolates. In the current investigation, we found an MAR index of 0.1 to 0.7 among gram-negative bacteria and an index of 0.2 to 0.5 among gram-positive bacteria.

Key word: antibiotics, antibiotic-resistance, MAR index, Ponnani

## 1. Introduction

### 1.1. Incidence of bacteria in estuaries

Estuaries are water bodies where saltwater from sea mixes with fresh water from rivers. Estuarine water contains a large number of bacteria of different species, and it is estimated that there are about  $10^6$  to  $10^7$  bacteria per milliliter of water and  $10^8$  to  $10^9$  per dry weight of the sediments. Within the water column, high densities may be found in the surface layer. Aerobic and facultative anaerobic bacteria are the most abundant group bacteria found in the estuaries. *Pseudomonas* and *Vibrio* are the most isolated bacteria from the inlets (Day *et al.*, 1989). The physicochemical parameters and various fauna and flora of the estuary influence the microbial diversity of the bay. The climatic and geographical factors have a significant impact on physicochemical parameters, and thus tropical estuaries are more complex than temperate one. As a result of the combined effect of all these factors, there is both seasonal and spatial variation in the distribution of various species of bacteria in estuaries.

Coliforms are a group of bacteria originating from the gut of warm-blooded animal. The incidence of coliforms indicates recent entry of faecal matter into the estuary. Along with faecal matter pathogenic bacteria like *Salmonella*, *Shigella*, *Vibrio* and enteric viruses enters the inlet and may survive for an extended period. Thus coliforms are excellent indicators of faecal contamination in estuaries (Chandran *et al.*, 2008)

### 1.2. The emergence of antibiotic-resistant strains of bacteria

An antibiotic is a chemotherapeutic agent that inhibits the growth of microorganism such as bacteria, fungi, or protozoa (Kummerer, 2009). Antibiotic sensitivity is the susceptibility of bacteria to the antibiotics. Antibiotic sensitivity studies are usually carried out to determine the efficiency of antibiotics in killing a

bacterial population in vivo. In recent years many medicines have become ineffective in treating bacterial infection due to the emergence of antibiotic-resistant strains. Antibiotic-resistance bacteria in estuaries originate a result of the careless discharge of urban and animal waste (Goniurriza *et al.*, 2000). The unregulated use of antibiotics in human and animals for treatment leads to release of antibiotics and antibiotic-resistant strain into the environment (Ghafur *et al.*, 2010). Another adverse effect of the improper use of antibiotics is the origin of multidrug resistant bacteria. Multidrug resistant bacteria are the bacteria that show resistance to three or more class of antibiotics. The main reason for multidrug resistance in bacteria is the overuse of antibiotics in agriculture, and aquaculture (Mcmanus and Stockwell, 2001). In countries like in India, there is no regulation or monitoring in the application of antibiotics in any of the fields mentioned above.

### 1.3. Ponnani estuary

Ponnani estuary is situated at the mouth of Bharathapuzha and it is bounded by the Arabian Sea on the West. The tidal mouth of Bharathapuzha is Ponnani harbour where Bharathapuzha and Tirur River join together into the Arabian Sea. Municipal sewage and hospital waste carried by the river Bharathapuzha ultimately reaches Ponnani estuary where it combines with the tidal influx from the sea influencing the microbial flora of the bay.

## 2. Materials and Methods

### 2.1. Sampling

Water samples from estuary were collected monthly for six months. All samples were collected aseptically in sterile containers from three sampling sites and brought to the laboratory for analysis.

### 2.2. Isolation and identification of bacteria

Bacteria were isolated by standard procedure (Downs and Ito, 2001). The isolates thus separated were identified by the schemes and methods of Buchanan and Gibbons (1974).

For isolation of *E. coli*, water samples were enriched in lauryl tryptose broth. From positive lauryl tryptose broth, one loopful was transferred to tubes with EC broth for the confirmation of the presence of faecal coliforms in the water samples. From EC broth one loopful was transferred to Eosine Methylene Blue Agar and incubated at 37°C for 24 hours. Colonies with green metallic sheen were presumptively identified as *E. coli* and later confirmed by IMViC tests (Downs and Ito, 2001).

### 2.3. Antibiotic sensitivity studies

We employed disc diffusion method to determine the Antibiotic resistance of bacteria (Baur *et al.*, 1966). The isolates were challenged with various antibiotics. For gram-negative bacteria following antibiotics were used: ampicillin (10µg), tetracycline (10µg), nitrofurantoin (300µg), amikacin (10µg), cefuroxime (10µg), nalidixic acid (30µg), gentamicin (30µg), chloramphenicol (30µg) ciprofloxacin (5µg) cefoxitin (30µg). Another set of antibiotics were employed against gram-positive bacteria and included antibiotics like nitrofurantoin (300µg), amikacin (10µg), nalidixic acid (30µg), gentamicin (30µg), chloramphenicol (30µg) ciprofloxacin (5µg) cefoxitin (30µg) methicilin (5µg), penicillin(10µg) and vancomycin (30µg).

A bacterial suspension of the overnight culture was prepared. A sterile cotton swab was used to inoculate the bacterial suspension on the surface of the Mueller- Hinton Agar plate. Antibiotic- impregnated disc (Hi-Media, Mumbai) were dispensed on the surface of the inoculated agar plate and incubated at overnight at 37°C. The isolates were scored as sensitive and resistant according to the inhibition zone around the disc.

### 2.4. Multiple Antibiotic resistances Index

The Multiple Antibiotic resistance Index (MAR index) was measured by the formula (MAR index= Number of antibiotics to which an isolate is resistant/ Total number of antibiotics employed).

### 3. Results and discussion

#### 3.1. The incidence of different species of bacteria

Bacteriological analysis of water samples from Ponnani estuary shows the presence of bacteria belonging to different species (Fig-1). Of the 51 isolates, 21 isolates (41.17%) were *Staphylococcus sp*, 13 isolates were *Vibrio sp*, and 6 (11.7%) isolates were confirmed as *E. coli*. The remaining isolates were identified as *Alcaligenes* (5.8%), *Flavobacterium* (11.7%), *Micrococcus* (1.78%) and *Pseudomonas* (1.9%). Thus a total of 29 gram-negative isolates and 22 gram-positive isolates were obtained in the current investigation. Almost identical results were obtained in another investigation in Meenachil River in Kottayam district. Investigators isolated bacterial species like *Salmonella sp*, *Escherichia coli*, *Vibrio sp*, *Staphylococcus sp*, and *Pseudomonas sp* (Vincy *et al.*, 2015). However similar studies in Krishna Godavari basin showed an altogether different species of bacteria comprising *Bacillus sp*, *Actinobacter sp*, *Klebsiella*, *Enterobacter sp*, *Yersinia sp* and *Proteus sp* (Ruban and Gunaseelan, 2011). The survival of bacteria in the water bodies are influenced by geographical and physicochemical factors and bacterial species composition also varies accordingly.

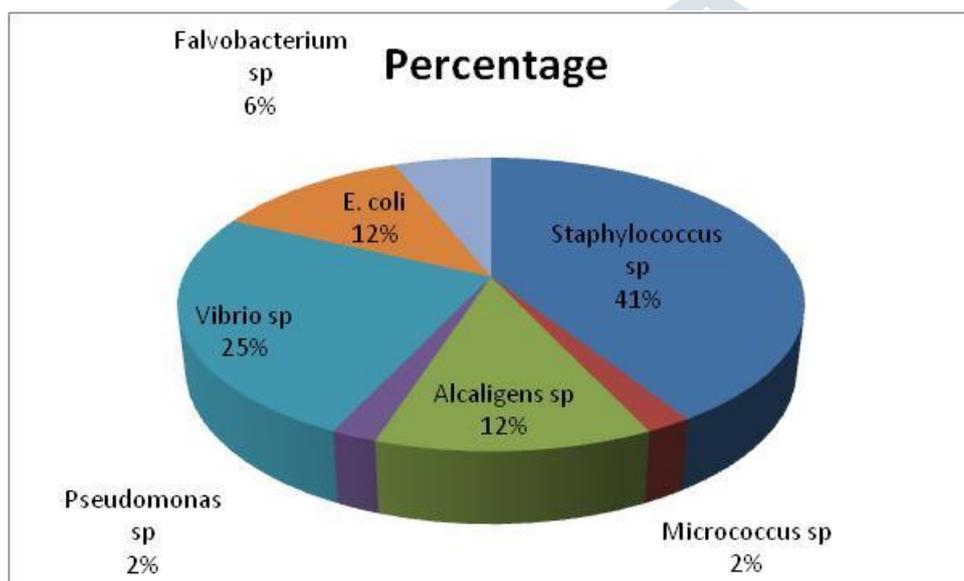


Fig-1 Different species of bacteria isolated from Ponnani estuary

#### 3.2. Antibiotic sensitivity studies

The combination of antibiotics employed against the gram-negative and gram-positive bacteria were slightly different. Some antibiotics were employed against both gram-negative and gram-positive bacteria. Antibiotics like methicillin and vancomycin were used only against gram-positive strains.

The current investigation revealed the incidence of antibiotic-resistant bacteria in Ponnani estuary. Among the twenty-nine gram-negative isolates 58.6% were resistant to cefuroxime and 55.1% to cefoxitin. More than fifty percent of isolates (51.7%) showed resistance to nitrofurantoin and ampicillin. All the isolates were sensitive to gentamycin and chloramphenicol. Members of the *Vibrio sp* were resistant to eight of the ten antibiotics employed whereas *E. coli* and *Alcaligenes sp*. showed resistance to seven antibiotics used. All members of *Vibrio sp* species showed resistance to cefoxitin and cefuroxime. All members of *E. coli* were resistant to ampicillin and tetracycline. (Table-1) In another study, various species of bacteria isolated from Ganga River showed an almost similar pattern of resistance against different antibiotics employed. (Manisha *et al.*, 2011).

All the 22 gram-positive isolates were resistant to methicillin indicating entry of hospital strains into estuaries. Twenty-one of these isolates were *Staphylococcus* and the remaining one isolate was *micrococcus*. The clinicians indiscriminately prescribe this drug for infections by gram-positive bacteria as penicillin had lost its antibacterial effect. As a result, even methicillin has lost the therapeutic effect it enjoyed in the past. However, in the current investigation only 86% of isolates showed resistance to

penicillin. At the same time 95% of isolates were resistant to nalidixic acid (Table-2). The emergence of methicillin-resistant *Staphylococcus* has become a major challenge for medical practitioners since a suitable drug with the same effect has not yet introduced.

**Table-1 Antibiotic resistance status of gram-negative bacteria isolated from Ponnani estuary**

Species	No of isolates	Percentage of isolates showing resistance to different antibiotics									
		GEN	NIT	C	NA	CIP	CB	TE	AK	CX	AM
<i>Vibrio sp.</i>	13	0	61.5	0	76.9	38.4	100	7.6	30.7	100	53.8
<i>Pseudomonas sp.</i>	1	0	100	0	0	0	0	100	0	0	100
<i>Alcaligenes sp.</i>	6	0	50.0	0	50.0	0	33.3	16.6	16.6	16.6	33.3
<i>Escherichia coli</i>	6	0	33.3	0	0	16.0	16.0	100	16.6	16.6	100
<i>Flavobacter sp.</i>	3	0	33.3	0	0	33.3	33.3	0	66.6	33.3	0
<b>Total</b>	<b>29</b>	<b>0</b>	<b>51.7</b>	<b>0</b>	<b>44.8</b>	<b>24.1</b>	<b>58.6</b>	<b>31.0</b>	<b>27.5</b>	<b>55.1</b>	<b>51.7</b>

GEN- Gentamycin, NIT-Nitrofurantion. C -Cholramphenicol, NA –Nalidixic acid. CIP- Ciprofloxacin, CB-Cefuroxime TE-Tetracycline, AK-Amikacin, CX-Cefoxitin, AM- Ampicillin.

**Table-2 Antibiotic-resistance status of gram-positive bacteria isolated from Ponnani estuary**

Species	No of isolates	Percentage of isolates showing resistance to different antibiotics									
		GEN	AK	C	CIP	MET	VA	NA	ER	CX	P
<i>Micrococcus</i>	1	0	0	0	100	100	0	100	100	0	0
<i>Staphylococcus</i>	21	19.0	9.5	0	4.7	100	0	95.0	66.6	4.7	90
<b>Total</b>	<b>22</b>	<b>18.0</b>	<b>9.0</b>	<b>0</b>	<b>9.0</b>	<b>100</b>	<b>0</b>	<b>95.0</b>	<b>68.0</b>	<b>4.5</b>	<b>86</b>

GEN- Gentamycin, AK-Amikacin, C -Cholramphenicol, CIP- Ciprofloxacin, MET- Methicillin, , VA-Vancomycin NA –Nalidixic acid, ER- Erythromycin, CX-Cefoxitin, AM- Ampicillin

### 3.3. Multiple Antibiotic Resistance

Multiple antibiotic resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.*, 2011). In the current investigation, some strains were multiple antibiotic-resistant, and few isolates showed resistance to even seven antibiotics. In the present study, 68% of gram-negative bacteria and 95% gram-positive bacteria were multiple antibiotic resistant. In Hooghly estuary, 71% of bacterial isolates were multiple antibiotics resistant (Mohanta and Goel, 2014). Twenty-five different antibiotic resistant patterns (Table-4) were observed among gram-negative bacteria while only 10 antibiotic resistant patterns (Table-3) were found among gram-positive bacteria. High variability of resistance pattern among the isolates indicates the different sources of origin of these isolates. Similar to the results of the present study, investigators observed fifty different patterns of antibiotic resistance in bacteria isolated from Periyar River (Krishna *et al.*, 2014).

We found high variation in MAR index in bacteria isolated from Ponnani estuary. Similarly, investigators have observed MAR index of 0.1 to 0.7 among bacterial isolated from bottled drinking water collected from different cities in India (Jeena *et al.*, 2005). In the current investigation, we found an MAR index of 0.1 to 0.7 among gram-negative bacteria and an index of 0.2 to 0.5 among gram-positive bacteria.

Table-3 MAR pattern of Gram-positive bacteria isolated from Ponnani estuary

Name of bacteria	Antibiotic resistance pattern	MAR index	Percentage
<i>Micrococcus sp.</i>	1MET, CIP, NA, ER	0.4	100
<i>Staphylococcus sp.</i>	1,P, AK, MET,NA	0.4	4.7
	2,P, MET, CIP,NA,ER	0.5	23.0
	3, P, MET,NA,ER	0.4	4.7
	4, P,MET,AK,NA,ER	0.5	19.0
	5,P, MET,NA	0.3	28.0
	6,PMET,CX,NA,ER	0.5	4.7
	7,P, MET,GEN,NA ER	0.5	4.7
	8,MET, ER	0.2	4.7
	9.MET,NA, ER	0.3	4.7

GEN- Gentamycin, AK-Amikacin, C -Cholramphenicol, CIP- Ciprofloxacin, Vancomycin, NA –Nalidixic acid, MET- Methicillin, VA- acid, ER- Erythromycin, CX-Cefoxitin, AM- Ampicillin

Table-4 MAR pattern of Gram-negative bacteria isolated from Ponnani estuary

Name of bacteria	Antibiotic resistance pattern	MAR index	Percentage
<i>Vibrio sp.</i>	1,NIT,NA,AK,CIP,CB,AM,CX	0.7	7.0
	2,NIT,NA,CIP,CB,CX	0.5	7.0
	3,NA, CIP,CB,AM,CX	0.5	7.0
	4,CB,CX,	0.2	15.0
	5, NIT,NA,CB,CX	0.4	7.0
	6,NA,CB,CX,AM	0.4	15
	7, C NIT,AK,CIP,CB,AM,CX	0.7	7.0
	8,NIT,NA,AK,CB,AM,CX	0.6	7.0
	9, NIT,NA, CB,AM,CX	0.6	7.0
	10,NIT, NA,CB,AM,CX	0.5	7.0
	11,NA,AK,CB,CX	0.5	7.0
<i>E. coli</i>	1,NIT,TE,AM	0.3	16.0
	2,TE,CB,AM	0.3	16.0
	3, 1E, AM	0.2	33.0
	4,NIT,TE,CIP,AM	0.4	16.0
	5,CX,TE,AM	0.3	16.0
<i>Flavobacterium</i>	1,AK,CB CX	0.3	33.0
	2,AK	0.1	33.0
	3, NIT,AK,CIP	0.3	33.0
<i>Alcaligenes</i>	1,NIT,NA, CB	0.3	33.0
	2,NIT,AM,CX	0.3	16.0
	3,NA,AK	0.2	16.0
	4, AM	0.1	16.0
	5,TE	0.1	16.0
<i>Pseudomonas</i>	NIT,TE,AM	0.3	100

GEN- Gentamycin, NIT-Nitrofurantion. C -Cholramphenicol, NA –Nalidixic acid. CIP- Ciprofloxacin, CB-Cefuroxime, TE-Tetracycline, AK-Amikacin, CX-Cefoxitin, AM- Ampicillin.

#### 4. Conclusion

The presence of antibiotic-resistant forms in estuaries reveals the unregulated use of antibiotics for various purposes other than therapeutic function. Poultry and cattle based industries regularly employs antibiotics as growth promoters. In aquaculture farmers regularly apply antibiotics as a therapeutic and prophylactic agent without any regulation for an extended period. It is sold without any prescription in developing countries like India. The present investigation demonstrates that, apart from clinical strains environmental strains of bacteria also have become resistant to various antibiotics and brings forth need for stringent regulation in the procurement and application of antibiotics.

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