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SHORT COMMUNICATION

Multiple antibiotic resistance indexing of coliforms to identify high risk contamination sites in aquatic environment

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Abstract Bacteriological analysis of the water samples collected from upstream, midstream and downstream points along the bank of the river revealed high populations of *Escherichia coli*, *Citrobacter freundii*, *Citrobacter diversus*, *Enterobacter aerogens* and *Klebsiella* species. All these isolates were screened against eight antibiotics to determine the prevalence of multiple antibiotic resistance among isolates at different sites of the river. The study revealed that multiple antibiotic resistance was prominently seen in coliforms at downstream sites (Average multiple antibiotic resistance index, MAR Index = 0.43) while it was low in coliforms at upstream sites (MAR Index = 0.15). These differences in MAR indices provide a method for distinguishing high risk contamination sites in aquatic environment.

Keywords Antibiotic resistance · Fecal coliforms · *E. coli* · MAR Index etc.

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Introduction

Coliforms are representative of the important group of indicator bacteria in water, soil and other environments and are often considered as a measure of water quality. They represent one of the major contaminants in surface and ground water in developing countries. In recent decades, the increased usage of antibiotics has led to antibiotic resistance among enteric bacteria. Bacteria with highest level of resistance are isolated from environments such as hospital effluents, sewage and waste water, contaminated with antimicrobial agents [1]. River water is the main receptacle reservoir of antibiotics and antibiotic resistant bacteria in the environment. They are directly introduced into surface water through fisheries, animal farms and agricultural practices [2]. A large volume of sewage and effluent containing antibiotic resistant bacteria is discharged into rivers, streams, lakes and sea water [3]. The antibiotic resistance bacteria in drinking water are a prime concern to public health [4].

Nanded is one of the important tourist (pilgrims visit famous Gurudwara) centers in India. Thousands of pilgrims take bath and perform various religious activities on the bank of the Godavari River. Hence, microbial quality of river water sources at different sites along the bank of river is of utmost importance. Keeping in view, the current scenario of rivers and ground water conditions prevailing in the region, the present work was undertaken to study antibiotic resistance among coliforms isolated from one of the major river, Godavari flowing from the tourist district of Nanded.

The study sites were selected along the bank of river Godavari which flows through the tourist district of Nanded city. Water samples were collected from 10 kms stretch of

the river. Six sites were divided into three parts. Two sites located upstream of the city were Kaleshwar (K) and Vishnupuri (V) site. Osmanshahi mill area (U) was considered as midstream point, while the three sites of Govardhan Ghat (G), Banda Ghat (B) and Nagina Ghat (N) were selected as downstream sites. Upstream sites are selected from the rural area of Vishnupuri village, while urban area starts from midstream points. Five polluting points (Nallahs) collecting city's domestic waste discharge the waste into the river through sewages.

Water samples were collected from all six sites of the river in sterile glass bottles 0.5 m below the surface of river and transported to laboratory on ice and were processed within 6 hrs of collection. The standard procedure prescribed by American Public Health Association was used for sample collection. Three samples were collected from each site [5].

The samples were analyzed for total coliforms (TC) & faecal coliforms (FC) count by multiple tube fermentation method [6]. Appropriate volume of water samples were incubated in series of MacConkey's broth, both single strength and double strength tubes incubated at 37°C for 24 hrs for total count and 44°C for faecal counts. The counts were determined on the basis of results of positive fermentation tubes. A loop from the positive multiple fermentation tube from every sample was streaked on MacConkey's agar plates and incubated at 37°C for 24 hrs. Dark pink colonies developed on the plate were selected and identified on the basis of indole production, methyl red, Voges-Proskauer and citrate utilization (IMViC) test.

Antibiograms of all coliforms were determined on Muller & Hinton Agar (Hi-Media Pvt. Ltd. Mumbai) using Kirby-Bauer disk diffusion method [7]. The antimicrobial agents were chosen on the basis of their importance in treating human or animal infections caused by gram negative bacteria of the family Enterobacteriaceae. The antibiotics selected for the study is comprised of Ampicillin (10 μg), Gentamycin (10 μg), Streptomycin (10 μg), Tetracycline (30 μg), Colistin (10 μg), Nitrofurantoin (300 μg), Ciprofloxacin (10 μg), and Co-Trimoxazole (25 μg).

Multiple Antibiotic Resistance (MAR Index) Index of the samples was calculated by the formula [8].

$$\text{MAR Index} = y/nx.$$

Where, y= Total number of resistance scored; n = number of isolates; x = Total number of antibiotics tested.

The population of indicator organisms in the river at six isolated sites is depicted in Table 1. It was observed from the data that there was a gradual increase in TC & FC counts from upstream sites, Kaleshwar (K), and Vishnupuri (V). Increase in populations of TC & FC was observed at downstream sites with maximum concentrations at Nagina Ghat site (N). Total sixty coliform isolates were identified from six sites by taking ten isolates from each site. Among coliforms the number of *Escherichia coli* was maximum (27) followed by *Citrobacter freundi* (16), *Citrobacter diversus* (6) *Enterobacter aerogens* (7) and *Klebsiella species* (4) (Fig. 1).

Table 2 depicts the changes in antibiogram of coliforms at different sites of the river. A marked resistance was shown by coliforms towards the antibiotic ampicillin in the range of 40 to 100% with maximum (i.e. 100%) at downstream sites. Second most commonly observed antibiotic resistance was nitrofurantoin resistance followed by tetracycline and streptomycin. The lowest resistance was observed for Gentamycin. Ciprofloxacin and Co-Trimoxazole resistance was observed only in downstream samples, while it was totally absent in upstream and midstream samples. A large variation was seen in colistin resistance pattern.

The overall resistance of isolates towards eight antibiotics is represented as multiple antibiotic resistance index (MAR Index). The average MAR index value in upstream samples was 0.15 and it increased from upstream to downstream (average 0.43). The midstream samples showed moderate MAR index value i.e. 0.28 (Fig. 2). Changes in antibiotic resistance among coliform species revealed that *E. coli* isolates showed greater resistance towards all the tested antibiotics than *C. freundi* and other coliforms (Table 3). Resistance to more than three antibiotics was also common in *E. coli* (40.44%) than *C. freundi* (6.25%) (Table 4).

In this study, bacteriological analysis and antibiotic susceptibility profiles of water sources at different sites were examined in order to establish the bacteriological safety of water sources and to provide updated data on antibiograms, which helps in identifying the high risk contamination sites in the aquatic environment. The coliforms are indicative of general hygienic quality of the water and potential risk of infectious diseases from water [9]. In the present investigation, the quantitative assessment of water samples revealed increase in TC and FC counts from upstream to midstream and then downstream. Both TC and FC were found alarm-

Table 1 Population of Indicator Organisms per 100 ml

Sites	Total Coliforms (TC)	Fecal Coliforms (FC)
Kaleshwar (K)	920	540
Vishnupuri (V)	16×10^2	920
Usmanshahi (U)	24×10^2	16×10^2
Govardhan Ghat (G)	16×10^3	9.2×10^3
Banda Ghat (B)	24×10^3	16×10^3
Nagina Ghat (N)	16×10^4	92×10^3

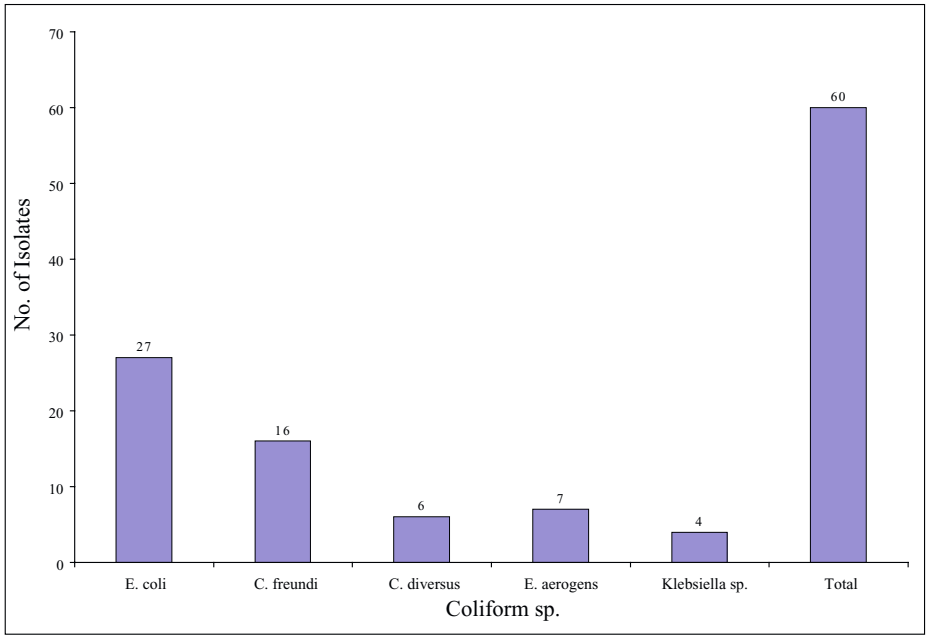

Fig. 1 Distribution of different Coliform sp. in samples collected from river water

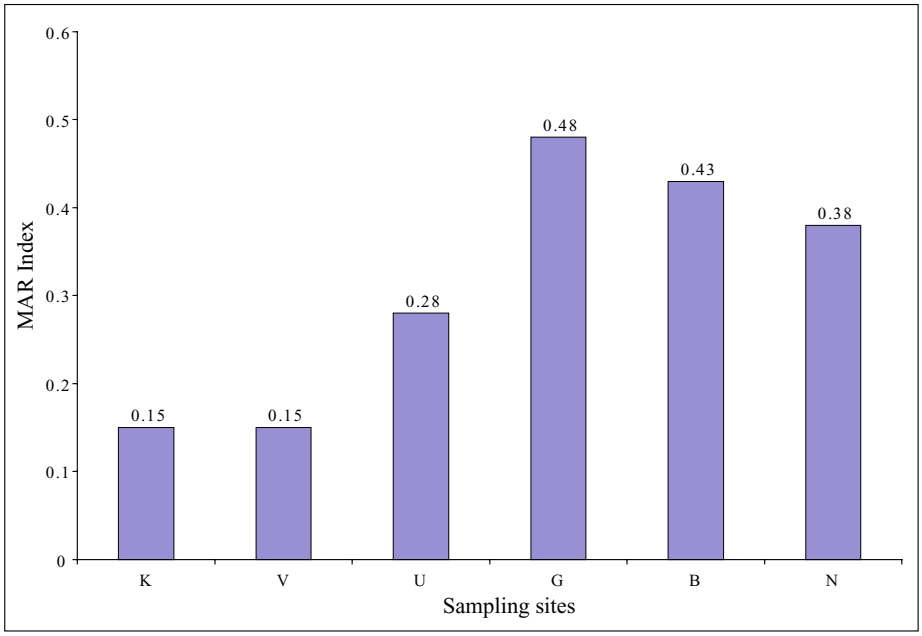

Fig. 2 Multiple antibiotic resistance index (MAR) at different sites

ingly high at all the downstream sites examined. High count of indicator organisms at downstream sites indicates the influence of domestic waste and urban runoff on quality of water.

The results of antibiogram also showed 94.34% of the isolates studied were found to be resistant to one or more antibiotics used and only 6.66% were sensitive to all antibiotics. The sensitive isolates were mainly recovered from upstream sites. The levels of antibiotic resistance at different sites of the river revealed that antibiotic resistance was low in upstream samples and then gradually increased at midstream and reached maximum in downstream sample. The trend of resistance from upstream to downstream clearly indicated the influence of domestic waste and urban runoff on the antibiotic resistance levels, as more number of drains discharging domestic waste and hospital waste of the city enter into river from midstream to downstream sites. The MAR indices of upstream sites were found in low

Table 2 Antibiotic resistance at different sampling sites (% resistance)

Antimicrobial Agent	K	V	U	G	B	N	Overall n= 60
Ampicillin (A)	40	50	90	90	100	90	76.66
Ciprofloxacin (Cf)	0	0	0	20	10	30	10.00
Colistin (Cl)	20	0	10	40	50	10	21.66
Co-trimoxazole (Co)	0	0	0	40	20	30	15.00
Gentamycin (G)	0	0	10	10	10	0	05.00
Nitrofurantoin (Nf)	60	60	70	90	70	50	66.66
Streptomycin (S)	0	10	20	30	30	40	21.66
Tetracycline (T)	0	0	20	60	50	50	30.00

Table 3 Changes in antibiotic resistance among coliforms (% resistance)

Coliform Species	A	Cf	Cl	Co	G	Nf	S	T
E. coli (n = 27)	81.48	14.81	18.51	18.51	7.40	85.18	33.33	40.74
C. freundii (n = 16)	68.75	6.25	6.25	18.75	6.25	62.50	12.50	25.00
Other Coliforms (n=17)	76.47	5.88	29.41	0	0	52.94	17.64	17.64

Table 4 Multiple antibiotic resistance among coliforms (% resistance)

Coliform Species	No Antibiotic	One Antibiotic	Two Antibiotic	Three Antibiotic	>Three Antibiotic
E.coli	3.70	14.81	22.22	14.81	40.44
C. freundi	12.5	25.00	31.25	25.00	6.25
Other coliforms	5.88	27.52	35.29	17.64	11.76

range (0.15) indicating low risk of contamination at sites. While the index was moderate at midstream site (0.28) and higher values of MAR indices were observed at three downstream sites of the river (site G, B and N) with MAR indices of 0.48, 0.43 and 0.38 respectively. The upstream sites were located in rural region while the urban area starts from midstream point. The MAR indexing determined for upstream sites were below arbitrary value of risk contamination of 0.2 indicating low risk contamination sites [10]. The downstream samples showed MAR indexing above 0.25 indicating high risk contamination sites of the river. The difference in MAR indexing from upstream to downstream indicated impact of urbanization on antibiotic resistance levels.

The study also revealed the quantitative changes in antibiotic resistance at different sites of the river. The highest level of resistance was observed towards ampicillin. The trend of resistance among coliforms for eight antibiotics was found to be A> NF>T>S>Cl>CO>CF>G. A high incidence of ampicillin, nitrofurantoin, and tetracycline resistance in aquatic environments were reported [11–13]. The frequency of ampicillin resistance was more in human isolates as compared to tetracycline and nitrofurantoin resistance observed in the isolates obtained from animal sources. The emerging ciprofloxacin resistance from downstream sites is of serious concern, as this is the drug of choice, for many gram negative bacteria. Low resistance to gentamycin and colistin in the present study might be due to the less use of these antibiotics in clinical practices and veterinary medicine.

All the isolates of coliforms and fecal coliforms showed rising trend of resistance from upstream to downstream. The differences in resistance profiles in this ecological study clearly reflect the differences in selection pressure in the investigated locations. The data correlates with higher TC and FC counts at downstream sites with higher MAR indices. The higher level of resistance to antibiotics among coliforms of midstream and downstream sites of Godavari River is alarming because most of the pilgrims take bath in rivers at midstream and downstream sites whereas some residents and non-residents (pilgrims) use the holy water for drinking purpose.

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