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Prevalence of Multiple Drug Resistant *Escherichia coli* Serotypes in a Tropical Estuary, India

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A total of eighty-one *Escherichia coli* isolates belongs to forty-three different serotypes including several pathogenic strains such as enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli* (UPEC) isolated from a tropical estuary were tested against 12 antibiotics to determine the prevalence of multiple antibiotic resistance (MAR), antimicrobial resistance profiles and also to find out high risk source of contamination by MAR indexing. The results revealed that more than 95% of the isolates were multiple antibiotic resistant (resistant to more than three antibiotics). Resistance to vancomycin, novobiocin, kanamycin, oxytetracycline, tetracycline, streptomycin was high (>80%), resistance to other antibiotics was relatively less. The MAR indexing of the isolates showed that all these strains were originated from high risk source of contamination. The incidence of multiple antibiotic resistant *E. coli* especially the pathogenic strains in natural water will pose a serious health risk to the human population and also act as a 'manmade' reservoir of resistance genes for (potentially) pathogenic bacteria. The determination of antibiotic susceptibility/resistance patterns of isolated microbes is a part of the microbial monitoring process of the water which would be important for the meaningful interpretation of sanitary water quality data.

Key words: antibiotic resistance, *Escherichia coli*, MAR index, estuary, India

Antimicrobial resistance has been recognized as an emerging worldwide problem in human and veterinary medicine[1,7] both in developed and developing countries. The effect could be severe in heavily populated developing country such as India where there is no strict monitoring programme regarding the use of antibiotics in animals and humans. Clinical misuse of antibiotics may be more common among private practitioners than among public health personnel. More drugs are available in private clinics and medical shops than in public hospitals, even without prescription. It is also well documented that widespread use of antibiotics in agriculture and medicine is accepted as a major selective force in the high incidence of antibiotic resistance among gram-negative bacteria[20]. These microorganisms may be shed in faeces with subsequent contamination of soil, food, and aquatic environments. The presence and persistence of antibiotic resistant bacteria in surface water[15] municipal drinking water[21], groundwater[20] and sea food[17] is a growing public health concern.

Escherichia coli is the natural intestinal inhabitant of humans and other warm-blooded animals. Its presence in food or water is generally considered to indicate direct or indirect fecal contamination and the possible presence of enteric pathogens[16]. Antimicrobial resistance in *E. coli* is of particular concern because it is the most common Gram-negative pathogen in humans, the most common cause of urinary tract infections, and a common cause of both community and hospital-acquired bacteraemia[28]. Several diarrhegenic serotype of *E. coli* has also been recognized, including enterotoxigenic (*ETEC*), enteroinvasive (*EIEC*), enterohemoragic (*EHEC*) *E. coli,* enteropathogenic (*EPEC*), enteroaggregative (*EAggEC*) and enteroadhesive (*DAEC*) *E. coli*[27] which is the main cause of worldwide morbidity and mortality especially in young children in developing countries[14]. In addition, resistant *E. coli* strains have the ability to transfer antibiotic resistance determinants not only to other strains of *E. coli,* but also to other bacteria within the gastrointestinal tract[4] and to acquire resistance from other organisms[23]. Conjugative and transductional transfer of these factors (R plasmid) among microbial strains in the aquatic environment has already been demonstrated[12]. A number of reports have documented the emerging resistance to multiple antimicrobial agents in verocytotoxin-producing *E. coli* including *E. coli O157:H7*[30].

One of the most serious aspects of drug resistance is that the presence of indigenous bacteria that harbor R plasmids in recreational and drinking water sources could pose a serious health hazard due to their potential resistance to normal antibiotic treatments and ability to transfer resistance to other pathogenic bacteria. This health risk is enhanced when potentially pathogenic bacteria survive for prolonged periods in aquatic environments, as indicated by the high densities of coliform bacteria in tropical waters in the absence of known human fecal sources[29]. As commensal bacteria constitute a reservoir of resistance genes for (potentially) pathogenic bacteria their level of resistance is considered to be a good indicator for selection pressure by antibiotic use and for resistance problems to be expected in pathogens[22]. Hence in the present investigation different pathogenic and non pathogenic serotypes of *E. coli* isolated from estuarine water were tested against commonly used antibiotics to find out the

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prevalence, multiple antibiotic resistance profiles and the high risk source of contamination by MAR indexing.

Materials and methods

Description of the study area

The present study has been carried out in Cochin Estuary, a part of Vembanadu lake, the most important Ramsar site and extensive lake water system in Kerala. It had undergone considerable pollution in the last decade resulting mainly from the development of satellite Township all along the estuary. The pollution of the estuary is mainly of microbial, as the industrial development had declined in this region. The only industry which contributes to microbial pollution of Cochin estuary is seafood industry, as the waste water from many factories, which is rich in organic matter, end up in the estuary. The sampling stations were fixed in an around Cochin city as they were suspected to high level of sewage inputs.

Collection and Transportation of Samples

The water samples were collected monthly from five different stations along the Cochin estuary for a period of one year from November 2001 to October 2002. The stations were selected based on their closeness to satellite townships and waste input. The water samples were collected between 7 am – 9 am in sterile plastic bottle (Tarson, India) one foot below the surface to get a better representation of the sample. Water samples were transported to the laboratory in an icebox and subjected to bacteriological examination within 4 hours of collection. The water samples were processed for microbial parameters such as faecal coliform and *E. coli*.

Isolation of and identification of E. coli

A three tube most probable number (MPN) method was used for the isolation of *E. coli* using EC broth (Hi-Media Laboratories, India) as medium. Ten ml, 1 ml and 0.1 ml of appropriately diluted samples were inoculated into respective dilution tubes containing inverted Durham's tubes. Inoculated tubes were incubated at 44.5°C for 24 hours and observed for growth and gas production. Inocula from tubes showing growth and gas production were streaked on Eosin Methylene Blue (EMB) agar for the isolation of *E. coli* and incubated at 37°C for 24 hours. Typical *E. coli* like colonies were selected, restreaked to ensure purity and confirmed by Indole, Methyl Red, Voges-Proskauer and Citrate (IMViC) tests. The cultures giving ++−− reaction were confirmed as *E. coli*. Confirmed *E. coli* cultures were serotyped at National *Salmonella* and *Escherichia* Centre, Kasauli, Himachal Pradesh, India.

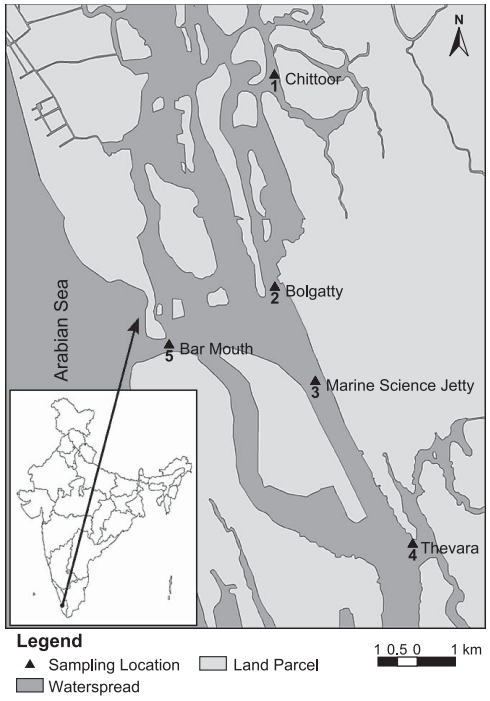

Fig. 1. Cochin estuary map showing sampling locations.

Antibiotic resistance analysis

Antimicrobial susceptibility testing was performed using a disk diffusion method[2]. Disks with the following drugs and concentration were used. Ampicillin (10 mcg); Amikacin (30 mcg); Chloramphenicol (30 mcg); Ciprofloxacin (10 mcg); Gentamycin (10 mcg); Kanamycin (30 mcg); Nalidixic acid (30 mcg); Novobiocin (30 mcg); Oxytetracyline (30 mcg); Streptomycin (30 mcg); Tetracycline (30 mcg) and Vancomycin (30 mcg).

Pure cultures of different serotypes *E. coli* were enriched in nutrient broth at 37°C for 6–8 hrs. The cultures were then streaked over previously prepared sterile Muller Hinton agar plates using a sterile cotton swab. The antibiotic disks were dispensed using a disk dispenser sufficiently separated from each other so as to avoid overlapping of inhibition zones. After 30 min the plates were inverted and incubated at 37°C for 16-18 hrs. Results were recorded by measuring the inhibition zones and compared with the interpretive chart of performance standards for antimicrobial disks susceptibility tests, supplied by the Hi-media laboratories, Bombay and classified as resistant, intermediate and sensitive. All the bacteriological media and antimicrobial disks were purchased from Himedia Laboratories, Bombay, India.

MAR index of the individual isolates and sampling station were done according to Krumperman[16]. MAR index of an individual isolate was calculated by dividing the number of antibiotic to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed. The MAR index greater than 0.2 is considered to originating from high risk source of contamination. The MAR index of a sample site or area is calculated by taking the aggregate antibiotic resistance score of all isolate from the sampling station divided by the number of antibiotics tested multiplied by number of isolates from the sample.

Results and Discussion

A total of 81 *E. coli* strains belonging to 43 different 'O' serotypes isolated from five stations in Cochin estuary was tested against 12 different antibiotics. *E. coli* were isolated consistently from all the stations, though there were differences in their prevalence level (Table 1). The prevalence of the *E. coli* serotypes revealed remarkable diversity of these strains in the system, which includes potential pathogens such as enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli* (UPEC). The EHEC and UPEC level was considerably higher and this is for the first time the isolation of these emerging pathogens is being reported from the Cochin estuary. The interesting observation was that EHEC and UPEC were isolated from the station near to Cochin city, which suggests the possible release of this organisms through hospital waste from many of the hospitals in an around Cochin city. Though prevalence of faecal indicator bacteria from Vembanadu lake has been reported earlier[13], serological characterization and existence of different serotypes were not reported so far.

Overall percentage resistance of *E. coli* against different antibiotics is given in Table 2. Among the antibiotics high resistance was observed against vancomycin (93%) followed by novobiocin (91%) kanamycin (85%) and oxytetracycline

Table 1. Different serotypes of *E. coli* and percentage incidence during the study period

E. coli serotypes	% incidence (n= 81)	*E. coli* serotypes	% incidence (n= 81)
O1[a]	4.9	O63	2.5
O39	2.5	O19	1.2
O173	1.2	O51	2.5
O78[b]	1.2	O139[b]	2.5
O157[c]	3.7	O86[d]	1.2
O8[b]	2.5	O113[c]	1.2
O101	7.4	O150	1.2
O91[d]	1.2	O32	1.2
O165	2.5	O135	1.2
O106	1.2	O102	1.2
O22[a]	4.9	O15[c]	1.2
O104	1.2	O29	1.2
O107	1.2	O9	3.7
O25[b]	8.6	O88	2.5
O14	1.2	O80	1.2
O69	2.5	O131	1.2
O117[c]	1.2	O66	1.2
O105	1.2	O30	1.2
O156	3.7	O20	1.2
O113	1.2	O2[a]	1.2
O60	1.2	O116[b]	1.2
O33	7.4	UT[e]	3.7

[a]Uropathogenic *E. coli* (UPEC), [b]Enterotoxigenic *E. coli* (ETEC), [c]Enterohaemorrhagic *E. coli* (EHEC), [d] Enteropathogenic *E. coli* (EPEC) [e]Uuntypable

Table 2. Percentage antibiotic resistance of *E. coli* strains (n=81) isolated from Cochin Estuary

Name of Antibiotic	Percentage of resistance
Ampicillin (A)	62
Amikacin (Ak)	50
Chloramphenicol (C)	10
Ciprofloxacin(Cf)	30
Gentamycin (G)	14
Kanamycin(K)	85
Nalidixic acid (Na)	36
Novobiocin (Nv)	91
Oxytetracycline (O)	84
Streptomycin (S)	80
Tetracycline (T)	83
Vancomycin(Va)	93

(84%). The least resistance was detected against Chloramphenicol and Gentamycin. Several authors reported varying degrees of resistance of *E. coli* to some of these antibiotics. McKeon *et al*.[20] observed that the resistance against novobiocin, ampicillin and tetracycline are most common among Gram negative bacteria in water. A high ampicillin resistance was noted from rural and urban waters[3] and much lower resistance to ampicillin were reported by Parveen *et al*.[25]. Amundsen *et al*.[1] observed the most common resistance directed towards ampicillin, cephalothin, nitrofurantoin, and tetracycline. Gomathinayagam *et al*.[9] reported that *E. coli* showed predominant resistance to penicillin G, novobiocin and neomycin. Lin *et al*.[18] observed a high resistance of enteric bacteria against novobiocin and ampicillin and also suggests that environmental, industrial and/or human activities impact on the level of antibiotic resistance in the environment.

The results of the antibiotic resistance analysis revealed that more than 95% of *E. coli* was multiple antibiotic resistant. MAR index and resistant pattern of *E. coli* serotypes is represented in Table 3. Among all the serotypes 72 different resistance patterns were observed and the MAR index ranged from 0.25-1. Interestingly all of the emerging pathogenic serotypes such as *EHEC, ETEC, EPEC* and *UPEC* were multiple antibiotic resistant and the number of antibiotics to which these organisms were resistant ranged from 3 to 10. The most common resistance pattern observed among all the serotypes was ampicillin, amikacin, ciprofloxacin, gentamycin, kanamycin, nalidixic acid, novobiocin, oxytetracyline, streptomycin, tetracycline, vancomycin. Cardonha *et al.*[6] reported almost 36% of the *E. coli* strains isolated from water was resistance to more than one antibiotic including enteroinvasive (O143, O112 and O124) and enteropathogeic (O111 and O125) serotypes. The multiple antibiotic resistance among these human pathogens is worrisome because disease caused by these organisms will be very difficult to treat and they are the main cause of child hood diarrhea worldwide with over 2 million deaths occurring each year[14]. It is thus imperative that the determination of antibiotic susceptibility/resistance patterns of isolated microbes is a part of the microbial monitoring process of the water.

The incidence of MAR organisms is higher in the present study (95%) when compared to earlier investigations in India; 45%[20] 32% Gaur *et al*.[8] and Ramteke[26] and 80% by Parveen *et al*.[25] clearly indicating the increasing trend in the spread of drug resistant bacteria. The level of antibiotic resistance observed in this study was also greater than that of previous reports from urban and rural water by Kaspar *et al.*[15]. They found that 90% of all isolates were resistant to one or more antibiotics. In another study, investigators showed that 80% of strains from municipal waste, river and estuarine water displayed antibiotic resistance[31]. Much lower resistance, ranging from 31 to 75%, has been reported for *E. coli* isolates from various aquatic environments[9,10,24]. Recently Kumar *et al*.[17] reported that Seafood from India contains multiple antibiotic resistant strains of *E. coli* which may serve as a reservoir for antibiotic resistance genes in the aquatic environment and pose a greater risk in the form of transfer of resistance to other pathogenic bacteria.

The occurrence of antibiotic resistance among *E. coli* isolates is probably due to widespread use of chemotherapeutic drugs and may reflect the occurrence of plasmid transfer in the alimentary tract of humans and in the microbial milieu of sewerage system[10,19]. Recent studies have shown that antibiotics can accumulate in the environment, and even persist for up to a year[33]. It has been well documented that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in stagnant areas of waste water systems[11]. It has been also suggested that MAR microorganisms are fit than its nonresistant counterpart and is therefore able to survive under harsh conditions[5].

The multiple antibiotic resistance indexing of the isolates

Table 3. MAR index and resistant pattern of *E. coli* serotypes isolated from the estuary

E. coli serotypes	Mar Index	Resistance pattern	*E. coli* serotypes	Mar Index	Resistance pattern
O1[a]	0.58	Cf KNvOSTVa	O33	0.75	AkCKNaNvOSTVa
O1[a]	0.75	AkCfKNaNvOSTVa	O33	0.75	AkCCfKNvOSTVa
O1[a]	0.66	AkCfKNvOSTVa	O33	0.58	AKNvOSTVa
O39	0.41	KNvOSVa	O63	0.83	ACCfGKNaNvOTVa
O39	0.66	CfKNaNvOSTVa	O63	0.58	AAkKNv OTVa
O173	0.66	AAkKNvOSTVa	O156	0.5	KNv OSTVa
O78[b]	0.83	AAkCfGKNvOSTVa	O156	0.75	AAkCfKNaNvOSVa
O157[c]	0.58	AkKNvOSTVa	O156	0.75	AAkCfKNv OSTVa
O157[c]	0.66	AAkKNvOSTVa	O113[c]	0.5	AkKNvOTVa
O8[b]	0.66	AAkKNvOSTVa	O22[a]	0.66	AAkKNvOSTVa
O8[b]	0.83	AAkCCfGKNvOTVa	O22[a]	0.66	AkCKNvOSTVa
O101	0.91	AAkCfGKNaNvOSTVa	O22[a]	0.66	ACKNvOSTVa
O101	0.5	ACfKNaNv ST	O22[a]	0.5	CNvOSTVa
O101	0.5	KNvOSTVa	O102	0.83	AAkCKNaNvOSTVa
O91[d]	0.58	AAkKNvOTVa	O117[c]	0.83	AAkCfKNaNvOSTVa
O165	0.66	A AkKNvOSTVa	O2[a]	0.66	AAkKNvOSTVa
O165	0.58	AKNvOSTVa	O105	0.5	KNvOSTVa
O106	0.66	AAkKNvOSTVa	O60	0.5	KNvOSTVa
O104	0.83	AAkCfKNaNvOSTVa	O116[b]	0.58	ANaNvOSTVa
O107	0.58	AKNvOSTVa	O150	0.75	AAkNvKNvOSTVa
O14	0.41	KNvSTVa	O132	0.75	AAkGKNvOSTVa
O69	0.66	AAkKNvOSTVa	O135	0.5	ANaNvOTVa
O69	0.75	AAkKNaNvOSTVa	O15[c]	0.75	AAkKNvOSTVa
O19	0.58	ANaNvOSTVa	O29	0.75	ACfKNaNvOSTVa
O51	0.66	AKNaNvOSTVa	O9	0.75	ACfKNaNvOSTVa
O25[b]	0.58	AKNv OSTVa	O9	0.75	AkCfKNaNvOSTVa
O25[b]	0.33	NvOSVa	O9	0.5	AKNaNvOVa
O25[b]	0.5	KNvOSTVa	O88	0.5	AKNvOTVa
O25[b]	0.5	KNvOSTVa	O88	0.58	ACfKNvOTVa
O25[b]	0.58	AKNvOSTVa	O80	1.0	AAkCCfGKNaNvOSTVa
O139[b]	0.41	Nv OSTVa	O131	0.75	ACfkNaNvOSTVa
O139[b]	0.25	Nv OVa	O66	0.5	KNvOSTVa
O86[d]	0.58	AKNvOSTVa	O30	0.75	ACCfKNaNvOSVa
O33	0.75	AkCfGKNvOSTVa	O20[d]	0.66	AAkKNvOSTVa
O33	0.58	AkNaNv OSTVa	UT[e]	0.5	KNvOSTVa
O33	0.41	KNv OTVa	UT[e]	0.58	KNaNvOSTVa

[a]Uropathogenic *E. coli* (UPEC), [b]Enterotoxigenic *E. coli* (ETEC), [c]Enterohaemorrhagic *E. coli* (EHEC), [d]Enteropathogenic *E. coli* (EPEC) [e]Uuntypable

A-Ampicillin, Ak-Amikacin, C-Chloramphenicol, Cf-Ciprofloxacin, G-Gentamycin, K-Kanamycin, Na-Nalidixic acid, Nv-novobiocin, O-Oxytetracycline, S-Streptomycin, T-Tetracycline, Va-Vancomycin

showed that more than 95% of the isolates originated from high risk source of contamination. According to Krumperman[16] the choice of MAR index of 0.2 to differentiate between low and high risks contamination is arbitrary. Indices between 0.2 and 0.25 are in a range of ambiguity, and samples in this range require careful scrutiny. The MAR indexing of the isolates in our study ranged from 0.33 to 1 and it is greater than 0.25 and probability originated from high risk source of contamination. We also calculated the FC/FS ratio to find out the source of contamination and it was considered to be human origin. While FC/FS ratio of 4.4 or more indicates pollution from human faecal contamination the values between 0.1 to 0.6 are considered to have originated from non human faecal sources such as poultry, dairy cattle and pig. The MAR index of all the sampling station also exceeded the high risk level (0.25) (Table 4). Here MAR index of the different stations exceeded the arbitrary level which revealed that all stations were highly polluted with feacal bacteria originated from high risk source. A similar observation made by Gomathinayagam *et al.*[9] who reported that the *E. coli* isolates from Bhavani river originated from

Table 4. Multiple antibiotic resistance index of different sampling stations

Station No.	No. of *E. coli* isolates	% of isolates resistant to seven or more antibiotic	MAR index of sampling station
1	31	90.32	0.68
2	10	40.00	0.46
3	12	75.00	0.64
4	8	75.00	0.66
5	20	65.00	0.64

high risk sources such as night soil, commercial poultry farms and the MAR index of the sampling location exceeds the high risk level (0.25). Our results substantiate previous observations[9,15,16] that urban sources harbour MAR *E. coli* as our study area located in a highly urban area.

Percentage of isolates resistant to seven or more antibiotic and the MAR index of sampling station from which the samples were taken are given in the Table 4. In station 1 about 90.32% of the isolates were resistant to more than seven antibiotics and the MAR index is 0.68 followed by station 3, 4 and 5 (75% resistance and MAR index 0.64, 75% resistance and MAR index is 0.66, 65% resistance and MAR index 0.64) respectively. In station 2, 40% of the isolates were resistant to more than 7 antibiotics and the MAR index is 0.466. Generally aquatic environments are exposed to wide range of pollutants from different sources by discharged waste. The high prevalence of multiple antibiotic resistant *E. coli* in all the station studied revealed that frequent discharge of sewage containing antibiotic resistant *E. coli* into the estuary. The unregulated use of antibiotic in agriculture, medicine might have contributes significantly the high antimicrobial resistance in bacteria and also the runoff from different regions also contribute the high prevalence of the resistant strains in the estuarine water. The rise in frequency of drug resistant isolates supports the view that widespread use of antibiotics results in the selection of resistant strains carrying plasmid encoding resistance[19]. These resistant strains may spread into different ecological niches including normal intestinal flora leading to a further increase in the number of drug resistant bacteria. The ability of a strain to tolerate drug concentration depends upon the efficacy of expression of a gene encoding for drug resistance.

Conclusion

The high diversity of MAR *E. coli* serotypes recorded in the present study indicates its high range of contamination and its presence is a potential health hazard to the people who consume this water for different purposes. Such polluted water is directly or indirectly act as a common source of disease in man and animals. The health hazard imposed by the resistant factor (R-factor) is not restricted to drug resistance; they may enhance the infectivity and virulence of some pathogens. The MAR indexing of the isolates revealed that all *E. coli* strains originated from high risk source of contamination and also pointing out that the water body is subjected to severe contamination and sewage input from in and around Cochin city and will also act as an artificial 'manmade' reservoir of multiple antibiotic resistance gene for (potentially) pathogenic bacteria. Poor sanitation facilities and inadequate infrastructure to treat and dispose human waste results in the high levels of faecal contamination together with the high levels of multiple antibiotic resistance amongst the isolated enteric bacteria are a major cause for concern. This is likely to have serious consequences for health care management and prevention within the local communities[32].

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