

## **Toxigenic *Escherichia coli* with high antibiotic resistance index recovered from sands of recreational beaches of Mumbai, India**

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### **Abstract**

Mumbai, India's seven-island city, is known for its sandy beaches as a major tourist attraction, but urbanization and industrialization have weakened the environment. Unregulated sewage disposal and untreated effluents off the coast have made the beach environment vulnerable. Therefore, monitoring water and sand quality at beaches should be mandatory. This study was thus designed to determine the microbiological status of selected sandy beaches, viz. Versova, Juhu, and Girgaon. The study found fecal coliforms in the sand, with *stx1* and *stx2* genes specific for shiga toxin-producing *E. coli* pathotypes in 5.5% of isolates, whereas the presence of *eaeA* gene specific for enteropathogenic *E. coli* pathotype was detected in 12.2% of isolates, and the presence of the *LT* and *ST* genes specific for enterotoxigenic *E. coli* pathotype was detected in 6.6% of isolates. Multiple antibiotic-resistant indices indicated high-risk contamination sources. The study suggests routine monitoring of pollution levels at coastal cities' beaches.

**Keywords:** Anthropogenic pollution, *Escherichia coli*, Sandy beaches, toxin genes

## 1. Introduction

A sandy beach is a habitat that supports many autochthonous and allochthonous microbes, including bacteria, fungi, viruses, and protozoa. Pathogenic and opportunistic microorganisms, together with fecal indicator bacteria, can be found in beach sand and can have a direct impact on the quality of bathing water (Abreu et al., 2016). Studies from around the globe indicate the presence of a variety of pathogens in beach sands, and recent epidemiology studies have found some evidence of health risks associated with sand exposure (Whitman et al., 2014; Robalo et al., 2023). *Campylobacter* (Khan et al., 2013), *Salmonella* (Yamahara et al., 2012), *Vibrio vulnificus* (Abdelzaher et al., 2010; Shah et al., 2011), *Staphylococcus aureus* (Plano et al., 2011), and fecal indicator bacteria (FBI) like enterococci (Zhu et al., 2011) are a few examples of pathogenic bacteria found in beach sand. The origin of fecal indicator bacteria on beaches is unknown; however, exogenous sources (such as sewage, warm-blooded domestic animals, urban and/or agricultural runoff, and bathers) and/or indigenous sources (regrowth in the sediment itself) can be taken into consideration as likely sources (Abreu et al., 2016). Pathogen exposure can occur through a variety of methods, including ingestion, inhalation, contact with the eyes and ears, and skin contact (Solo-Gabriele et al., 2016). Numerous studies have looked into the role of *E. coli* as an indicator of risk to the health of recreational marine waters (Rodrigues et al., 2016). Although *E. coli* is typically not harmful, some strains have the ability to cause a variety of infections in the urinary, gastrointestinal, or central nervous systems due to their virulence. Currently, six categories of *E. coli* that cause diarrhea have been identified based on genes that encode virulence factors: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC or EAaggEC), and diffusely adherent *E. coli* (DAEC) (Maloo et al., 2018).

Infections caused by these pathogenic bacteria are further aggravated by the emergence and dissemination of antimicrobial resistance among these pathogens. Reported outbreaks due to multiple antibiotic-resistant pathogenic bacteria from India further challenge the efficiency of antibiotic treatment in the country (Behera et al., 2023; Abhirosh et al., 2011). *E. coli*, the predominant fecal indicator bacteria. The emergence of *E. coli* isolates with multiple antibiotic-resistant phenotypes, involving co-resistance to four or more unrelated families of antibiotics, is a serious matter of concern (Maynard et al. 2003). Pathogenic strains of *E. coli* in clinical specimens, foods, and environmental samples can be detected.

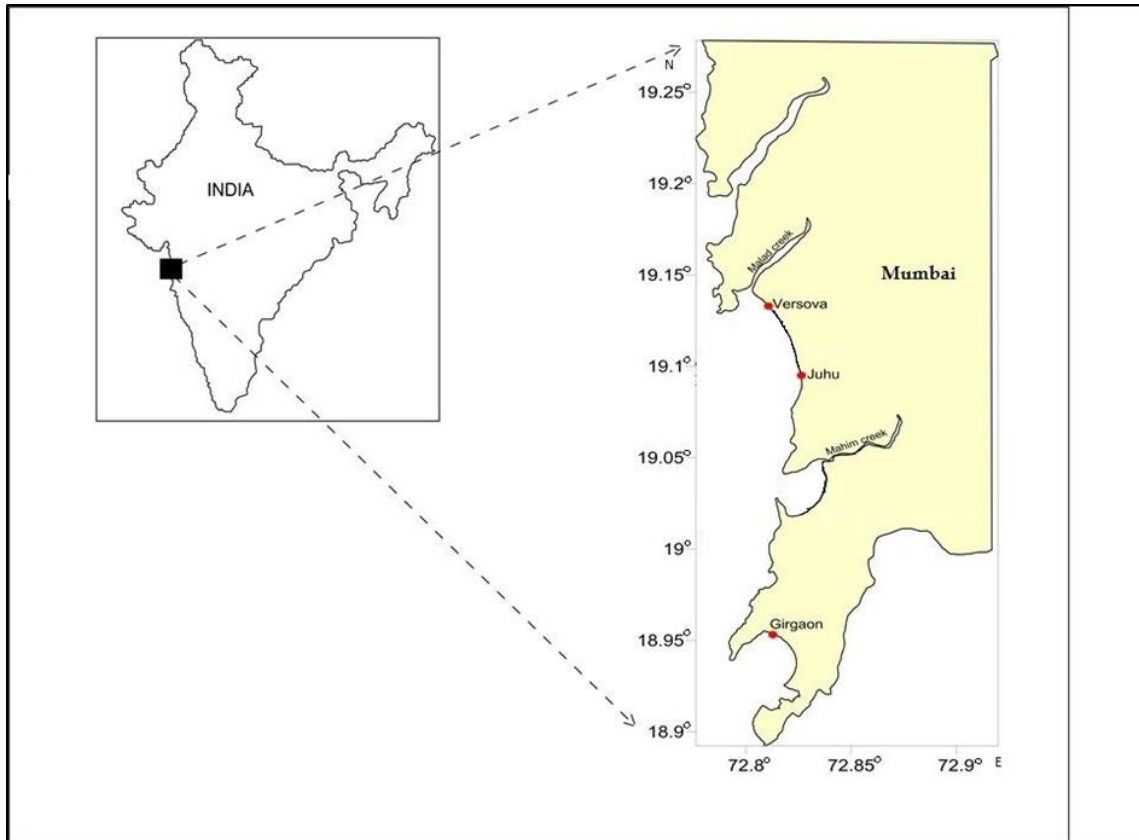
Three major sandy beaches in Mumbai, viz., Girgaon Chowpatty (beach), Juhu Beach, and Versova Beach, were selected for this study. These beaches are extensively used not only for recreational and

holistic purposes but also to support the livelihoods of hundreds of hawkers, slum dwellers, and other vendors (Dutta et al., 2022). Slum clusters have sprouted on these beaches, and slum dwellers utilize the long coast as a dumping ground, as domestic sewage, human waste, and solid waste are all discarded off the coast. This increases the health risk for the public when recreating on the beach sand and water. As multiple non-point sources of contamination exist on these beaches (Dhage et al., 2006). This study was designed to unveil the microbiological status prevailing in Mumbai beach sand, with special emphasis on *E. coli*-bearing toxin genes.

## **2. Materials and methods**

### **2.1 Sample collection and analysis**

Beach sand samples were collected in triplicate in sterile 50-ml Falcon tubes (Tarsons, India) from the swash zone of three beaches in Mumbai, India, viz. Versova, Juhu, and Girgaon, for a period of three seasons between May 2015-December 2015 i.e. pre-monsoon in May 2015, monsoon in September 2015, and post-monsoon in December 2015 in triplicates on the same day from the same location. Sampling locations are illustrated in Figure 1. All samples were collected by taking the precautions required for microbiological analysis, held in an icebox, and processed within 6–8 hours of collection. One gram of sand was transferred to a sterile, 99-mL, 0.8% saline-containing Erlenmeyer flask, swirled vigorously in the flask for approximately 1 minute, and then allowed to settle for a few minutes. Supernatant was used to prepare standard dilutions. The total viable bacterial counts (TVC) from the appropriate dilutions were estimated via the spread plating method on marine agar plates. The enumeration of total coliforms (TC), fecal coliforms (FC), and *Escherichia coli* was carried out using membrane filtration according to the standard method of APHA (1998). In brief, 0.1–1 ml of suitable dilutions of sand samples, depending on the sample turbidity, were filtered through 0.45µm pore-sized cellulose acetate filters (Millipore) and aseptically placed on selective media plates, viz., MacConkey Agar for TC, M-FC Agar for FC, and M<sub>7</sub>HrFC Agar for EC (HiMedia Ltd., Mumbai, India). All of the media plates, except M-FC agar plates, were incubated at 37°C±1°C for 24–48 h, and final counts of colonies were noted. M-FC agar plates were incubated at 44.5°C ± 1°C for 24–48 h. Colonies from M<sub>7</sub>HrFC Agar plates were isolated and grown on Hicrome *E. coli* agar for confirmation and isolation of *E. coli* as per the manufacturer's instructions. Well-isolated blue *E. coli* colonies were selected, biochemically characterized (data not shown), transferred onto nutrient agar slants for instant analysis, and also maintained as glycerol stock for further studies.



**Figure 1:** Map showing studied sandy beaches of Mumbai

## 2.2 Detection of virulence and toxin genes by PCR

Out of 200 randomly selected presumptive *E. coli* colonies, genomic DNA from 90 biochemically confirmed *E. coli* was extracted using the heat lysis method described by Masoji et al. (2017) for different samples. The DNA extracts were used as a template for the detection of five different Virulence Genes (VGs): *stx1*, *stx2*, *eaeA*, *LT1*, and *ST1* in confirmed *E. coli* strains. This was carried out by conventional singleplex PCR using primers previously described by Maloo et al. (2018). The PCR amplification was carried out using an Eppendorf Vapoprotect thermocycler system, set to the following run conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing between 44-63.7°C, extension at 72 °C for 1.30 min, and final extension at 72°C for 8 min. A PCR reaction was set up using optimized reaction conditions such as MgCl<sub>2</sub> being added at 8 mM for *stx1*, *stx2*, and *eaeA* and 25 mM for *LT1* and *ST1*, wherein dNTP mix was considered at 1.2 mM for *stx1*, *stx2*, and *eaeA*, and 10 mM dNTP mix for *LT1* and *ST1* with Taq polymerase 5U/μl and Primers at 10pm. Positive control: Genomic DNA of *Escherichia coli* O157 (EDL 933, Sigma, Germany) for *stx1*, *stx2* and *eaeA*; *Escherichia coli* MTCC 723 (CSIR-IMTECH, India) for *LT1* and *ST1*. The master mix reaction for 25 μl was as follows:

<b>Components</b>	<b>Volume(<math>\mu</math>l)</b>
Buffer10X	2.5
dNTP	1.5
MgCl <sub>2</sub>	2.5
Primer	1.0
Primer	1.0
Taq Polymerase	0.5
DNA template	5.0
Nuclease free water	11.0
<b>Total</b>	<b>25 <math>\mu</math>l</b>

Identification of the band size was done by comparison of the band with 100-1000bp molecular ladder (Thermo Scientific, EU, Lithuania). Samples were considered to be positive for a specific VG if the visible band was of the same size as that of the positive control DNA. Detail of primer sequences are given in the Table 1.

Table1: Primer sequences, annealing temperatures and their expected product sizes

Target gene	Primer sequence (5' → 3')	Annealing temp (°C)	Product size (bp)
1. <i>stx1</i>	stx1 F: CAGTTAATGTGGTGGCGAAGG stx1 R: CACCAGACAATGTAACCGCTG	63.7	348
2. <i>stx2</i>	stx2 F: CTTCTGGTATCCTATTCCTGG stx2 R: GGATGCATCTCTGGTCATTG	55.6	478
3. <i>eaeA</i>	eaeA F: GAAGCCAAAGCGCACAAAGACT eaeA R: CTCCGCGGTTTTAGCAGACAC	44	413
4. <i>LT1</i>	LT1 F: TCTCTATGTGCATACGGAGC LT1 R: CCATACTGATTGCCGCAAT	49	322
5. <i>ST1</i>	ST1 F: CTTTCCCCTCTTTTTAGTCAG ST1 R: TAACATGGAGCACAGGCAGG	49	175

### 2.3 Determination of Susceptibility to Antimicrobial agents

VG-bearing isolates were screened for antimicrobial susceptibility using the disk diffusion method and antimicrobial impregnated rings (Hi-Media Ltd., Mumbai, India) as described by the Clinical and Laboratory Standards Institute (CLSI, 2018). Each test was performed in duplicate for each *E. coli* isolate, and the antibiotics used are given in Table 2. Data for susceptibility to antibiotics tested for each bacterial isolate has been reported as resistant, intermediate (isolates with reduced susceptibility), and sensitive based on Clinical and Laboratory Standards Institute break points (CLSI, 2018). The MAR index was calculated following the prescription of Krumperman (1983) and as previously described by Maloo et al. (2018) as the number of antibiotics to which the isolate is resistant divided by the total number of antibiotics against which the isolate was tested.

Table 2. List of antibiotics used in this study and their concentration.

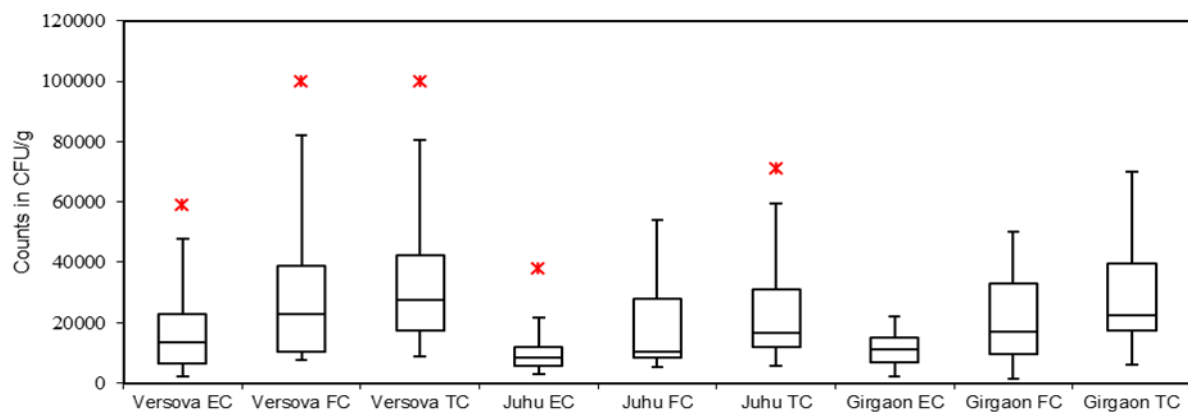
Antibiotic	Concentration
Cefuroxime	30mcg
Ceftazidime	30mcg
Cefepime	30 mcg
Cefotaxime	30 mcg
Ceftriaxone	30 mcg
Cefoxitin	30 mcg
Cefoperazone	75 mcg
Ceftizoxime	30 mcg
Ceftazidime/Clavulanicacid	30/10 mcg
Ciprofloxacin	5 mcg
Ofloxacin	5 mcg
Gatifloxacin	5 mcg
Levofloxacin	5 mcg
Gentamicin	10 mcg
Amikacin	30 mcg
CoTrimoxazole	20 mcg
Aztreonam	75 mcg
Ampicillin/Sulbactam	10/10 mcg
Ticarcillin/Clavulanicacid	75/10 mcg
Augmentin	30 mcg
Piperacillin/Tazobactam	100/10 mcg
Imipenem	10 mcg
Meropenem	10 mcg
Ampicillin	10 mcg
Nalidixic acid	30 mcg

### 3. Results and Discussion

#### 3.1 Indicator bacterial count in beach sand

In this study, the densities of total coliforms (TC), fecal coliforms (FC), and *E. coli* ranged from 5600–100000 CFU/g, 5200–100000 CFU/g, and 2000–59000 CFU/g, respectively, as given in Table 3. The distribution and variability of TC, FC, and EC counts on these beaches are presented in Figure 2, indicating a high coliform and *E. coli* count at Versova Beach compared to the other two beaches.

Numerous studies have shown that beach sand can harbor significant levels of indicator bacteria, mainly *E. coli*, and are often present in higher concentrations than in the water column (Whitman et al., 2003; Alm et al., 2006; Ishi et al., 2007; Wade et al., 2010; Shibata and Solo-Gabriele, 2012; da Costa Andrade et al., 2015; Meyers & McLellan, 2022). Swimming-associated illnesses like diarrhea and infections of the skin, ear, respiratory, eye, gastrointestinal, and wound are contracted by people recreating in polluted waters (Bonilla et al., 2007). Also, recreational activities like playing football, volleyball, and cricket, infants building sand castles, sand arts, and burying themselves under such contaminated sand involve direct exposure, may represent an additional health risk (Solo-Gabriele et al., 2016). Further, DeFlorio-Barker et al. (2018) summarized that the estimated national cost of getting sick from recreating in polluted water is \$2.9 billion nationwide. People involved in various activities on contaminated beaches may serve as vectors for disease-causing germs and can spread infections among others (Nana et al., 2023). Remedial measures like cleanliness drive programs, the removal of plastic trash, garbage, unwanted wreckage, and fishing nets should be undertaken by local authorities and residents to preserve the aesthetic value of the coastline.



**Figure 2** Box and whisker plot of distribution and variability of average total coliforms, fecal coliforms and *Escherichia coli* counts throughout three seasons in sand of Mumbai beaches. Only highest outlier is shown in boxplot. The red x depicts max outlier.

### 3.2 Detection of virulence and toxin genes

Although commensal, few strains of *Escherichia coli* harbor specific virulence attributes that allow them to cause a wide spectrum of life-threatening diseases. Out of 90 (30 *E. coli* isolates from each beach) screened for the presence of virulent genes, 22 isolates showed the presence of at least one of the VGs (*eaeA*, *stx1*, *stx2*, *LT1*, and *ST1* genes). Details of the number of *E. coli*-bearing toxin genes on all three beaches are given in Table 3. Overall, the *eaeA* gene, which codes for intimin protein and is necessary for intimate attachment to host epithelial cells in both the enterohemorrhagic *E. coli*



(EHEC) and enteropathogenic *E. coli* (EPEC) pathotypes, was the most prevalent gene (12.2%) in the *E. coli* isolates from sands. The frequency of occurrence of this gene was significantly higher (ANOVA,  $p < 0.01$ ) than that of *stx2* and *LT1*, which were infrequently detected. The reason for the high frequency of *eaeA* in our study sites is unclear, but similar findings were reported in the sands of Great Lakes recreational beaches (Bauer and Alm, 2012) and in the surface waters of estuaries in Brisbane (Masters et al., 2011), wherein gene *eaeA* dominated the presence of *stx1* and *stx2* genes. The *STI* gene, a heat-stable gene playing an important role in traveler's diarrhea isolates, was detected in 5.5% of the isolates, while the *LT1* heat-labile toxin was detected in 1.1% of the isolates. The *stx1* gene, which codes for shigatoxin 1, was present in 4% of the isolates and was only detected in isolates from wet periods. Also, it was observed that the highest frequency of *E. coli* with VGs was in Girgaon (30%), followed by Versova (23%), and Juhu (20%). On the basis of pathotypes, *E. coli* belonging to EPEC (12.2%) dominated the sand of the beaches studied here, followed by enterotoxigenic *E. coli* (ETEC) (6.6%) and Shiga toxin-producing *E. coli* (STEC) (5.5%). None of the isolates screened showed the presence of *stx1*, *stx2*, and *eaeA* together, a combination required for EHEC pathotypes, which may indicate human fecal contamination is the main source of pollution. According to Sidhu et al. (2013), the lower prevalence of the EHEC pathotypes as compared to other pathotypes suggests that human fecal contamination is the main source of diarrheagenic *E. coli* pathotypes in surface water as opposed to contamination from animals. The prevalence of *stx1* and *stx2* may also be low if they are not required in the extraintestinal environment and are therefore negatively regulated under environmental conditions (House et al., 2009). It is also possible that the *E. coli* strains persisting in the beach sand environment are evolving such that they are losing the *stx1* and *stx2* genes. However, the presence of *stx1* and *stx2*-bearing *E. coli* was also detected in the surface waters of beaches in Mumbai, indicating a health risk to beachgoers (Maloo et al., 2018). Additional research is necessary to identify factors influencing the abundance of *E. coli* possessing *eaeA* in Mumbai beach sands and to determine whether the scarcity of *stx1* and *stx2* is a function of adaptation to the beach sand environment or a result of inadequate sampling and/or analysis methods. Similar studies have been carried out in the fresh waters of India, as demonstrated by Ram et al. (2007, 2008, 2009), and Singh et al. (2010), wherein the virulence gene profiles of *E. coli* are employed as indicators of water quality. Also, it may be noted that the presence of a single or multiple toxin genes in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of toxin genes to cause disease in the host (Stange et al., 2016). Therefore, further screening of the *E. coli* isolates with these unusual VG patterns in tissue culture or animal models would be required to demonstrate their pathogenicity, as also suggested by Titilawo et al. (2015).

Table 3. Microbial counts (CFU/g), number and percentage of virulence genes with respect to each pathotypes, number and percentage of resistant isolates with respect to class of antibiotics and Multiple Antibiotic Resistance (MAR) index of different beach.

Parameters	Name of Sandy beaches			
	Versova	Juhu	Girgaon	
TVCx10 <sup>4</sup>	12-900	9-1000	16-2000	
TC x10 <sup>2</sup>	90-1000	56-710	60-700	
FC x10 <sup>2</sup>	76-1000	52-360	52-500	
ECx10 <sup>2</sup>	20-590	30-380	20-220	
No. of <i>E. coli</i> screened for presence of VGs	30	30	30	
Pathotypes	Genes	No. and percentage of isolates bearing virulence genes		
STEC	<i>stx1</i>	2(6)	1(3)	1(3)
	<i>stx2</i>	0	0	1(3)
EPEC	<i>eaeA</i>	3(10)	4(13)	4(13)
ETEC	<i>LT</i>	1(3)	0	0
	<i>ST</i>	1(3)	1(3)	3(10)
Overall no. and percentage		7(23.3)	6(20)	9(30)
No. of <i>E. coli</i> tested for antibiotic susceptibility		7	6	9
Class of Antibiotics	No. and percentage of resistant isolates (percentage in parenthesis)			
Cephalosporins		5(68)	5(79)	6(61)
Fluoroquinolones		5(71)	4(67)	6(69)
Aminoglycosides		1(14)	1(8)	2(22)
Penicillin combinations		5(75)	5(79)	8(83)
Carbapenems		6(86)	4(67)	4(44)
Cephalosporins combinations		7(100)	6(100)	8(89)
Sulphonamides		4(57)	5(83)	5(56)
Quinolones		7(100)	6(100)	9(100)
Penicillins		6(86)	6(100)	8(89)
Monobactams		6(86)	6(100)	6(67)
<b>MAR index Range</b>		0.4-0.92	0.6-0.8	0.32-0.84

## **Resistance to Multiple Antimicrobial agents**

The increasing resistance of bacteria to antibiotics used for treatment is a major cause of concern worldwide. In this study, the overall resistance of *E. coli* to antibiotics was high, and this result is consistent with our earlier findings (Maloo et al., 2017; Maloo et al., 2018). Chandran et al. (2008), Sukumaran and Hatha (2012) reported the presence of antibiotic-resistant *E. coli* from the Cochin estuary, India. Similarly, Leonard et al. (2015, 2018) and O-Flaherty et al. (2019) estimated the human exposure risk associated with recreational waters contaminated with MAR *E. coli* in the United Kingdom and Italy, respectively. In the study presented here, antibiotics generally used for treating infections caused by the Enterobacteriaceae family were used to determine the antibiotic susceptibility profile and found that all isolates were resistant to almost 32–92% of the antibiotics tested. 100% of the isolates were resistant to quinolones (Nalidixic acid), and only 16% of the isolates were resistant to aminoglycosides (Gentamicin and Amikacin). Resistance to broad-spectrum first-generation Quinolone antibiotics are reported to be slowly emerging in Asian, South American, and African countries (Sreela et al., 2011) and are also evidenced in this study, as 100% of the isolates studied here expressed resistance to Nalidixic acid. High sensitivity to Gentamicin and Amikacin has been recorded from previous studies conducted in India (Bharathi et al., 2002; Nalini et al., 2013). Also, the multiple antibiotic resistance index ranged between 0.32-0.92, indicating a high-risk source of contamination. The problem could become more severe when these organisms acquire resistance to multiple drugs because infections caused by such organisms will be difficult to treat. Thus, it is imperative that the determination of antibiotic susceptibility patterns of isolated microbes be part of the microbial monitoring process in coastal waters.

## **4. Conclusion**

The presence of attachment and virulence/toxin genes associated with *E. coli* pathotypes in selected recreational beach sand indicates a health risk to beachgoers. Beach sand may serve as a reservoir for pathogenic *E. coli* that could contribute to the emergence of novel pathogens. Moreover, growing antibiotic resistance among bacteria in environmental waters is a matter of concern. Further studies are necessary to quantify the presence of these virulence genes as well as antibiotic resistance genes to determine whether their corresponding proteins are being expressed.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethics approval and consent to participate:**

No human or animal subjects have been used in the experimental analysis of this paper.

**Availability of data and material:**

Data will be made available on request.

**Competing interests:**

The authors have no conflicts of interest to declare.

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**Authors' contributions:**

Aayushi Maloo wrote the main manuscript and Abhay B. Fulke and Soniya Sukumaran guided and reviewed the manuscript.

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