International Journal of Hygiene and Environmental Health 222 (2019) 235–248

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

# Screening of tropical estuarine water in south-west coast of India reveals emergence of ARGs-harboring hypervirulent *Escherichia coli* of global significance

Sukumaran P. Divya*, A.A. Mohamed Hatha

*Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Lakeside Campus, Cochin, 682 016, Kerala, India*

ARTICLE INFO

*Keywords:*
*Escherichia coli*
Estuarine water
Antibiotic resistance
ARGs
ExPEC
STEC

ABSTRACT

The goal of this study was to investigate the involvement of a tropical Indian estuary in the emergence of antibiotic resistance genes (ARGs)-harboring hypervirulent *E. coli* of global significance. A total of 300 *E. coli* isolates was tested for antibiotic susceptibility to β-lactams, aminoglycosides, chloramphenicol, quinolones, sulphonamides, tetracyclines, and trimethoprim. The *E. coli* isolates were screened for the presence of antibiotic resistance genes ($bla_{TEM}$, $bla_{CTX-M}$, *tetA*, *tetB*, *sul1*, *sul2*, *strA*, *aphA2*, *catI, dhfr1*, and *dhfr7*), integrase (*int1*, *int2*, and *int3*), Shiga toxin genes (*stx1* and *stx2*) and extraintestinal virulence genes (*papAH*, *papC*, *sfa/focDE*, *kpsMT II*, and *iutA*). The highest prevalence of antibiotic resistance was observed for ampicillin, followed by tetracycline, and nalidixic acid. Among *E. coli* isolates, 64% were resistant to at least one of the 15 antibiotics tested, and approximately 40% were multiple antibiotic-resistant (MAR). More than 40% (n = 122) of *E. coli* isolates had ARGs. Integrase 1 (*int1*) was found in 7.6% of *E. coli* isolates. Among *E. coli* isolates, 16.3% (n = 49) were extraintestinal pathogenic *E. coli* (ExPEC), and approximately 34.6% (n = 17) of ExPEC had ARGs. A hypervirulent ARGs-harboring STEC was isolated. The prevalence of Shiga toxin-producing *E. coli* (STEC) was low (n = 1). The prevalence of ARGs-harboring pathogenic *E. coli* isolates was higher in stations close to the City (urban area), than that of other stations. ERIC-PCR (enterobacterial repetitive intergenic consensus sequence polymerase chain reaction) analysis revealed a high degree of genetic diversity among the ARGs-harboring *E. coli* isolates. The results demonstrate a high prevalence of ARGs-harboring *E. coli* in estuarine water and confirm the need for a better wastewater treatment facility and proper control measures to reduce the discharge of sewage and wastewater into the aquatic environments.

## 1. Introduction

Antibiotic resistance in pathogenic bacteria from the environment is a global problem in public health (Berendonk et al., 2015). The misuse and overuse of antibiotics have resulted in the global increase in antibiotic resistance (Neill, 2016). Moreover, antibiotic resistance genes (ARGs) are a form of pollution, which are also entering into the aquatic environment by the release of fecal bacteria that already exposed to high levels of antibiotics in the human or animal digestive system (Alonso et al., 2001; Martinez, 2009). Pathogenic *E. coli* strains are good candidates to carry ARGs from the environment back to the human gut because they adapted to grow in the gut environment and to grow outside the host (Winfield and Groisman, 2003).

Russo and Johnson (2000) classified *E. coli* strains into three categories: (1) commensal strains, (2) intestinal pathogenic strains (diarrhoeagenic *E. coli*), and (3) extraintestinal pathogenic *E. coli* (ExPEC). STEC (Shiga toxin-producing *E. coli*) are important intestinal pathogens cause hemorrhagic colitis and hemolytic uremic syndrome in humans. STEC are characterized by their ability to release Shiga-toxin, which kills host cells in the intestine and can enter the bloodstream to affect other organs such as the kidneys and brain (Kintz et al., 2017). The ExPEC strains cause human and animal infections that occur outside of the intestinal tract such as urinary tract and bloodstream infections. These strains contain a broad range of virulence genes (VGs) such as adhesins, iron acquisition systems, polysaccharide coatings, and toxins (Johnson and Russo, 2002). The presence of multiple ARGs in pathogenic *E. coli* complicates the therapeutic management of infections (Mellmann et al., 2012).

Recently, there are increasing studies about the spread of antibiotic-resistant bacteria (ARB) and ARGs in environmental settings. ARGs-

* Corresponding author.
*E-mail address:* divyapsbio@gmail.com (S.P. Divya).

https://doi.org/10.1016/j.ijheh.2018.11.002
Received 11 May 2018; Received in revised form 8 November 2018; Accepted 14 November 2018

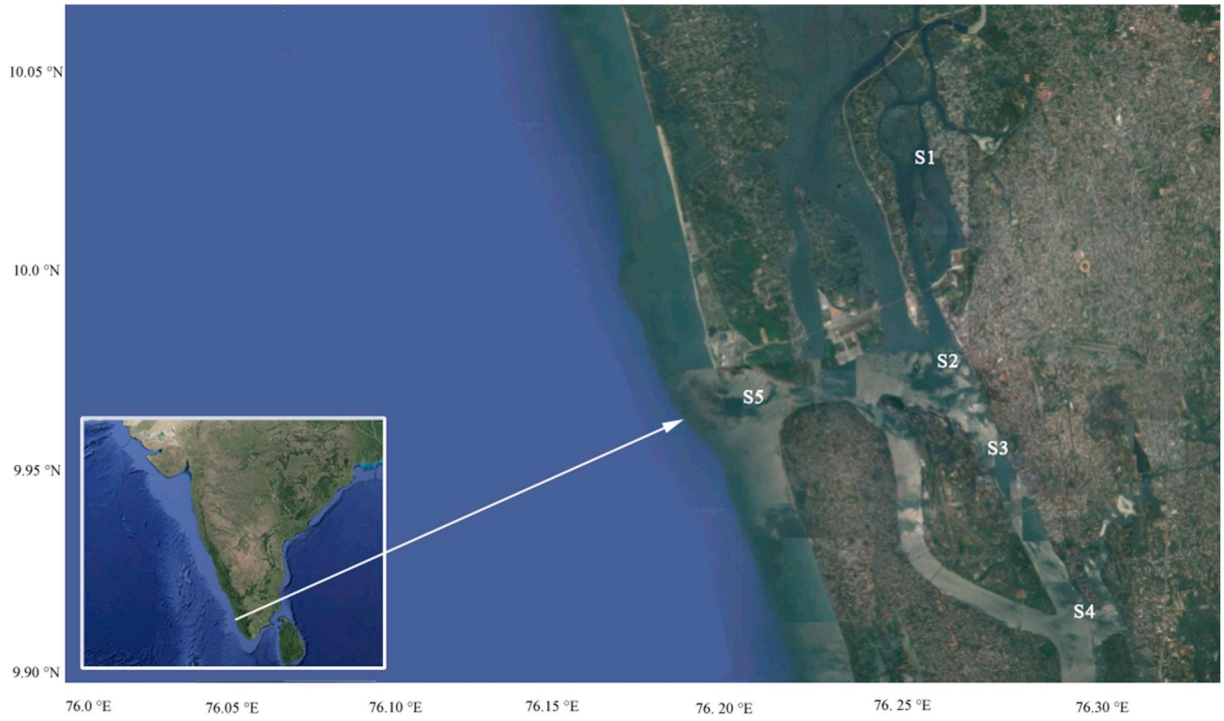


**Fig. 1.** Cochin estuary map showing sampling locations: S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth.

harboring *E. coli* isolates have been detected from wastewater (Bibbal et al., 2018; Mao et al., 2015; Rodriguez-mozaz et al., 2015; Um et al., 2015), aquatic environments (Leonard et al., 2018; Sabri et al., 2018; Stange et al., 2016; Tacao et al., 2014) and other sources (Beukes et al., 2017; Reinthaler et al., 2010). A few studies have focused on estuaries (Chen et al., 2013; Guo et al., 2017; Pereira et al., 2013). However, no studies focused on the presence of ARGs-harboring ExPEC or STEC from estuaries.

Estuarine environments lie at the boundary between terrestrial/freshwater and marine ecosystems and are a hotspot for anthropogenic impacts (Lotze et al., 2006). Estuaries are the major receptacle for pollution from both point sources (sewage outfalls, hospital, agricultural, and industrial discharges) and non-point sources (diffused sewage discharges, stormwater runoff, ballast water, and animal fecal inputs) (Garcia-Armisen et al., 2005; Guo et al., 2017; Pereira et al., 2013). Estuaries are a key area for understanding ARB fate after releasing into the aquatic environment (Na et al., 2018). Estuary may provide an ideal setting for the exchange of genetic material and the spread of antibiotic resistance (Na et al., 2018). The spread of antibiotic resistance genes present in gene-transfer units and dissemination of ARGs-harboring bacteria in nature may have consequences for human health and the evolution of hypervirulent bacteria (Martinez, 2009). The emergence of the hypervirulent *E. coli* strains are a threat to public health in the modern world (Buchholz et al., 2011). The presence of *E. coli* in estuarine water is a growing public health concern (Laroche et al., 2009; Pereira et al., 2013). The lack of high-level sewage treatment and direct discharge of untreated sewage are the reasons for the degradation of estuarine water quality in India (CPCB, 2011).

Cochin estuary is a part of Vembanad Lake and a Ramsar site in India. It is the largest estuary along the south-west coast of India (Menon et al., 2000). A full range of recreational opportunities offers boating, swimming, sports fishing, and relaxing, which attracts a large number of domestic and international tourists to Cochin City (TOI, 2018). Backwaters and coastal areas of Cochin are witnessing increasing ship traffic (TNIE, 2017). Because of the increasing sea commerce, there is an emerging threat of transportation of bacteria via ballast water from one region to another. The movements of ballast waters from one continent to another via ships cause a global distribution mechanism for ARGs-harboring pathogenic bacteria (Ruiz et al., 2000). The water quality of Cochin estuary is degraded by industrialization and urbanization in Cochin City and adjacent area (Babu et al., 2006).

The objectives of the present study were to: (i) evaluate the prevalence of antibiotic resistance; (ii) investigate the prevalence of ARGs and integrase; (iii) determine the prevalence of ExPEC and associated VGs; (iv) investigate the prevalence of STEC; and (v) investigate the genetic diversity of ARGs-harboring *E. coli* isolates from five different stations in the Cochin estuary. A total of 300 *E. coli* was isolated and tested for antibiotic susceptibility to β-lactams, aminoglycosides, chloramphenicol, quinolones, sulphonamides, tetracyclines, and trimethoprim. The *E. coli* isolates were screened for the presence of antibiotic resistance genes ($bla_{TEM}$, $bla_{CTX-M}$, *tetA*, *tetB*, *sul1*, *sul2*, *strA*, *aphA2*, *cat1*, *dhfr1*, and *dhfr7*), integrase (*int1*, *int2*, and *int3*), Shiga toxin genes (*stx1* and *stx2*) and extraintestinal virulence genes (*papAH*, *papC*, *sfa/focDE*, *kpsMT II*, and *iutA*). Furthermore, ERIC-PCR was used to evaluate the genetic diversity of ARGs-harboring *E. coli* isolates from the Cochin estuary.

## 2. Materials and methods

### 2.1. Study area

Cochin estuary is a shallow brackish water body situated on the south-west coast of India. Its length varies from 60 to 80 km, width from 500 to 4000 m and depth range of 2–7 m. The estuary is opening to the Arabian Sea by Cochin Barmouth (450 m width) at Cochin.

We selected two upstream stations (Chitoor [S1], Thevara [S4]), two in the central part of the estuary (Bolgatty [S2], Off Marine Science Jetty [S3]), and one at Barmouth [S5] (Fig. 1) (Suppl. Fig. 1A-E). Station 1 is located in a rural area, whereas S2 and S3 are situated near Cochin City. It is a major port City on the south-west coast of India. The City has a population of 612,343 and a metropolitan population of over 2.1 million, making it the largest urban region in Kerala. Cochin City is classified as a Tier-II City by the Government of India. Station 4 is a

**Table 1**
Antibiotic resistance of *E. coli* isolates from five different stations in Cochin estuary. S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth. Statistically significant differences ($p < 0.05$) in the prevalence of antibiotic resistance between stations are indicated by superscript letters. [a] Between S1 and S2, [b] between S1 and S3, [c] between S1 and S4, [d] between S1 and S5, [e] between S2 and S3, [f] between S2 and S4, [g] between S2 and S5, [h] between S3 and S4, [i] between S3 and S5, [j] between S4 and S5.

| Antibiotics | Percentage (no. of isolate) | | | | | Total (n = 300) |
|---|---|---|---|---|---|---|
| | S1 (n = 47) | S2 (n = 75) | S3 (n = 69) | S4 (n = 49) | S5 (n = 60) | |
| Ampicillin [a, b, c, d] | 27.6 (13) | 50.6 (38) | 43.5 (30) | 51 (25) | 43 (26) | 44 (132) |
| Cefotaxime | 6.3 (3) | 9.3 (7) | 8.6 (6) | 8.1 (4) | 5 (3) | 7.6 (23) |
| Cefoxitin | 19.1 (9) | 18.6 (14) | 23.1 (16) | 18.3 (9) | 23.3 (14) | 20.6 (62) |
| Cefpodoxime | 17 (8) | 13.3 (10) | 15.9 (11) | 14.2 (7) | 15 (9) | 15 (45) |
| Ceftazidime | 0 | 1.3 (1) | 1.4 (1) | 0 | 3.3 (2) | 1.3 (4) |
| Ceftriaxone [j] | 2.1 (1) | 1.3 (1) | 2.8 (2) | 0 | 5 (3) | 2.3 (7) |
| Cefuroxime [b, c,] | 0 | 2.6 (2) | 5.7 (4) | 8.1 (4) | 3.3 (2) | 4 (12) |
| Chloramphenicol [e, f] | 4.2 (2) | 8 (6) | 1.4 (1) | 0 | 3.3 (2) | 3.6 (11) |
| Ciprofloxacin [a, b, c, d, e, f, g] | 0 | 18.6 (14) | 7.4 (5) | 8.1 (4) | 5 (3) | 8.6 (26) |
| Co-trimoxazole [a, c, d, h, i] | 6.3 (3) | 29.3 (22) | 10.1 (9) | 22.4 (11) | 26.6 (16) | 20.3 (61) |
| Gentamicin [f, g] | 4.2 (2) | 12 (9) | 7.2 (5) | 2 (1) | 0 | 5.6 (17) |
| Nalidixic acid [a,b, c, d, e] | 10.6 (5) | 37.3 (28) | 23.1 (16) | 26.5 (13) | 33.3 (20) | 27.3 (82) |
| Streptomycin [b, e, h, i] | 10.6 (5) | 14.6 (11) | 27.5 (19) | 14.2 (7) | 10 (6) | 16 (48) |
| Tetracycline [a, b, c, d] | 10.6 (5) | 33.3 (25) | 34.7 (24) | 34.6 (17) | 38.3 (23) | 31.3 (94) |
| Trimethoprim [a,b, c, d] | 6.3 (3) | 24 (18) | 17.3 (12) | 22.4 (11) | 26.6 (16) | 20 (60) |

semi-urban area. Station 5 is the meeting place where freshwater from Cochin estuary enters into the sea through a single narrow outlet (Vishnu et al., 2018).

Water samples (0.3 m depth, 2 m apart from the estuary bank) were collected in sterile 1L plastic bottles during low tide from five stations in the Cochin estuary. The water samples were transported on ice to the laboratory for processing. A total of 225 water samples was tested during the study period, which extended from June 2009 to February 2013. The more details of the sampling stations and water collection are given in Suppl. Table 1.

### 2.2. Water analysis

Physical factors such as temperature, salinity, and pH were measured in situ using a water quality analyzer (Systronics, India). Nitrate ($NO_3^-$), nitrite ($NO_2^-$), ammonium ($NH_4^+$), and phosphate ($PO_4^{3-}$) were analyzed by the methods described by Grassholf et al. (1983). Dissolved oxygen (DO) was determined using Winkler titrations (Grassholf et al., 1983).

### 2.3. Isolation and identification of E. coli from water samples

Water samples were analyzed for fecal coliforms (FC) by most probable number method (MPN) using lactose broth (APHA, 1998). Ten mL, 1 mL, and 0.1 mL of water samples were inoculated into respective dilution tubes containing inverted Durham's tubes. Presumptive positive tubes were streaked onto eosin methylene blue agar (EMB) (Himedia, India) and incubated at 37 °C for 24 h. Colonies with green metallic sheen were considered as *E. coli* and isolated for biochemical analyses (Barrow and Feltham, 1993). DNA from the bacterial genome was extracted as per standard Proteinase-K digestion method (Sambrook et al., 1989). The identification of *E. coli* isolates were performed by the amplification of the *uidA* gene (Bej et al., 1991). The primers are shown in Suppl. Table 2. PCR (polymerase chain reaction) reactions were performed with a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). PCR mixes of 25 µL final volume were prepared with 1 µL of total DNA, 0.2 µM of each primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). The cycling conditions were as follows: 1 cycle of initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 1.5 min), extension (72 °C, 2 min) and final extension (72 °C, 5 min). PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by gel documentation system (BioRad Gel Doc™ EZ Imager, United States). *E. coli* isolates (two *E. coli* colonies from each positive water sample) were serotyped at the National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli, Himachal Pradesh, India. If *E. coli* isolates showed the same serotype, we considered the strains as the same. Duplicates were avoided for further study.

### 2.4. Detection of Shiga-toxin producing genes

*E. coli* isolates (n = 300) were screened for the presence of Shiga-toxin genes (*stx1* and *stx2*) (Blanco et al., 2003). PCR reactions were performed with a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). PCR mixes of 25 µL final volume were prepared with 1 µL of total DNA, 0.2 µM of each primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). The cycling conditions were as follows: 1 cycle of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 1 min) and final extension (72 °C, 5 min). PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, United States). The primers are shown in Suppl. Table 2.

### 2.5. Detection of extraintestinal virulence genes

*E. coli* isolates (n = 300) were screened by PCR for five key virulence genes (*papAH*, *papC*, *sfa/focDE*, *iutA*, and *kpsMT II*) of extraintestinal pathogenic *E. coli* (Johnson and Stell, 2000). Isolates were classified as ExPEC if they were found to be positive for two or more virulence genes such as *papAH*, *papC* (P fimbrial assembly), *sfa/focDE* (S and F1C fimbriae), *iutA* (aerobactin receptor), and *kpsMT II* (group II capsule). PCR mixes of 25 µL final volume were prepared with 1 µL of total DNA, 0.2 µM of each primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). PCR conditions were used in the following way: 1 cycle of initial denaturation (94 °C for 5 min), followed by 30 cycles of denaturation (94 °C for 30 s), annealing (64 °C, 30 s), extension (68 °C, 3 min) and final extension (72 °C, 10 min) on a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). The primers are shown in Suppl. Table 2. PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, United States).

**Table 2**
The characteristics of the ARGs-harboring *E. coli* isolates from Cochin estuary. R, Rough; UT, Untypable; S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth; Amp, Amipicillin; Ctx, Cefotaxime; Cx, Cefoxitin; Cpd, Cefpodoxime; Caz, Ceftazidime; Ctr, Ceftriaxone; Cxm, Cefuroxime; C, Chloramphenicol; Ctr, Ceftriaxone; Cip, Ciprofloxacin; Cot, Co-Trimoxazole; Gen, Gentamicin; Na, Nalidixic acid; S, Streptomycin; Te, Tetracycline; Tr, Trimethoprim.

| Strain No | Phylogenetic group | Serotype | Station No | Resistance patterns | MAR index | ARGs | integrase | Virulence genes |
|---|---|---|---|---|---|---|---|---|
| EA3 | F | R | S3 | Gen | 0.06 | *aphA2* | | |
| F4 | D | O13 | S5 | Amp Ctx Cx Cpd Caz Ctr Cxm C Cot S Tr | 0.73 | $bla_{CTX-M}$, *sul1, sul2, tetA, tetB, strA, aphA2, cat1, dhfr1* | *int1* | *iutA, kpsMT II* |
| ES69 | A | O59 | S2 | Amp Cx C Gen | 0.26 | $bla_{TEM}$ | | |
| A84 | A | R | S3 | Amp Na Te | 0.2 | $bla_{TEM}$ | | |
| ES43 | A | O64 | S5 | Amp Cx Na | 0.2 | $bla_{TEM}$ | | |
| A24 | A | O37 | S3 | Amp Na | 0.13 | $bla_{TEM}$ | | |
| EC95 | A | O11 | S4 | Amp | 0.06 | $bla_{TEM}$ | *int 1* | |
| EC9 | B1 | R | S4 | Amp Cx Cpd Cxm Cip Na Te | 0.46 | $bla_{TEM}$ | | |
| EC10 | B1 | O9 | S4 | Amp Cx Cpd | 0.2 | $bla_{TEM}$ | | |
| ES1 | B1 | O60 | S1 | Amp Cpd | 0.13 | $bla_{TEM}$ | | |
| ES72 | B1 | O21 | S1 | Amp Na | 0.13 | $bla_{TEM}$ | | |
| ES51 | B1 | O41 | S2 | Amp | 0.06 | $bla_{TEM}$ | | |
| A20 | B1 | O149 | S3 | Amp Na | 0.13 | $bla_{TEM}$ | | *iutA, kpsMT II, papC* |
| A21 | B1 | O170 | S3 | Amp Na | 0.13 | $bla_{TEM}$ | | |
| EC36 | B2 | O25 | S5 | Amp Cx Cpd | 0.2 | $bla_{TEM}$ | | |
| A32 | B2 | O75 | S2 | Amp | 0.06 | $bla_{TEM}$ | | *iutA, kpsMT II* |
| EC43 | B2 | O25 | S5 | Amp | 0.06 | $bla_{TEM}$ | | |
| EC55 | B2 | O166 | S4 | Amp Cx Cpd Na | 0.26 | $bla_{TEM}$ | | *iutA, kpsMT II* |
| ES74 | D | O91 | S1 | Amp Cx Cpd Na | 0.26 | $bla_{TEM}$ | | |
| ES38 | D | R | S5 | Amp Cot Te Tr | 0.26 | $bla_{TEM}$ | | |
| E17 | D | O102 | S5 | Amp Cx Cpd | 0.2 | $bla_{TEM}$ | | |
| EC53 | F | UT | S5 | Amp Cx Cpd Ctr Na | 0.33 | $bla_{TEM}$ | | *iutA, kpsMT II* |
| ES10 | B2 | UT | S3 | Amp Ctx S | 0.2 | $bla_{TEM}$, $bla_{CTX-M}$, *strA* | *int1* | *iutA, kpsMT II* |
| EC96 | B2 | UT | S4 | Amp S | 0.13 | $bla_{TEM}$, *strA* | | |
| A29 | B1 | O75 | S2 | Amp Te | 0.13 | $bla_{TEM}$, *tetA* | | |
| MS8 | A | O135 | S3 | Amp Cx Cpd Te | 0.26 | $bla_{TEM}$, $bla_{CTX-M}$ | *int 1* | |
| N10 | A | O20 | S3 | Amp Co S Te Tr | 0.33 | $bla_{TEM}$, $bla_{CTX-M}$ | | |
| MS6 | B1 | O135 | S3 | Amp Cx Te | 0.2 | $bla_{TEM}$, $bla_{CTX-M}$ | *int1* | |
| EC41 | D | O28 | S4 | Amp Cx Cpd | 0.2 | $bla_{TEM}$, $bla_{CTX-M}$ | | |
| ES63 | E | R | S5 | Amp Ctx Cx Ctr Cpd Cxm Caz | 0.46 | $bla_{TEM}$, $bla_{CTX-M}$ | | |
| EC32 | F | UT | S2 | Amp Cx Cpd Cip Cot Gen Na Te | 0.53 | $bla_{TEM}$, $bla_{CTX-M}$, *aphA2* | *int1* | |
| EC85 | D | O163 | S1 | Amp Cx Cpd Ctr C Cot Gen Na S Te | 0.66 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1, sul2, strA, aphA2, cat1* | *int1* | |
| N13 | D | UT | S3 | Amp Ctx Cx Cpd Caz Ctr Cxm S Te | 0.6 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1, sul2, tetA, strA* | *int1* | *kpsMT II, papC* |
| A77 | A | O59 | S5 | Amp Cx Cpd C Cot Na Te Tr | 0.53 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1, sul2, tetA, tetB, cat1* | *int1* | |
| EC6 | C | R | S2 | Amp Cip Cot Na S Te Tr | 0.46 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1, sul2, tetA, tetB, strA, aphA2* | | |
| N16 | E | UT | S3 | Amp S Te | 0.2 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1, tetA, tetB, strA* | | |
| ES 11 (CUSMBES11) | B2 | UT | S2 | Amp Cx Ctx Caz Cpd Ctr Cxm Cip C Cot Gen Na S Te Tr | 1 | $bla_{TEM}$, $bla_{CTX-M}$, *sul1,sul2, tetA, tetB, strA, aphA2, cat1, dhfr1, dhfr7* | *int1, int3* | *iutA, kpsMT II, papC, papAH, sfa/focDE, stx1* |
| N7 | D | UT | S3 | Amp Cx Cpd Ctr Cip C Cot Gen Na S Te Tr | 0.8 | $bla_{TEM}$, $bla_{CTX-M}$, *tetA, dhfr1* | | |
| A58 | B2 | UT | S3 | Amp Cx Cpd Cxm Na S Te Tr | 0.53 | $bla_{TEM}$, *strA* | | |
| ES77 | A | O59 | S2 | Amp Cot Tr | 0.2 | $bla_{TEM}$, *sul1* | | |
| ES33 | A | O20 | S2 | Amp Cot Tr | 0.2 | $bla_{TEM}$, *sul1* | | |
| ES45 | A | O4 | S2 | Amp Cx Cpd Cot Tr Na | 0.4 | $bla_{TEM}$, *sul1* | | |
| ES67 | A | R | S3 | Amp Cx Cpd Cxm Cot Tr | 0.4 | $bla_{TEM}$, *sul1* | | |
| ES47 | B1 | O4 | S2 | Amp Cip Cot Tr | 0.26 | $bla_{TEM}$, *sul1* | | |
| A78 | B1 | R | S5 | Amp Cot Na Te Tr | 0.33 | $bla_{TEM}$, *sul1* | | |
| ES48 | B1 | O4 | S2 | Amp Cip Cot Tr | 0.26 | $bla_{TEM}$, *sul1, sul2* | | |
| ES55 | B1 | O106 | S2 | Amp Cx Cpd Co Na Tr | 0.4 | $bla_{TEM}$, *sul1, sul2, tetA, tetB* | | |
| ES94 | A | UT | S2 | Amp Ctx Cx Cpd Cxm C Cip Cot Na S Te Tr | 0.8 | $bla_{TEM}$, *sul1, sul2, tetA, tetB, strA* | | |
| ES36 | A | UT | S1 | Amp Cot Na Te Tr | 0.33 | $bla_{TEM}$, *sul1, tetA* | | |
| EC29 | B2 | UT | S5 | Amp Cx Cip Cot Na Te Tr | 0.46 | $bla_{TEM}$, *sul1, tetA* | | |
| ES31 | A | UT | S2 | Amp C Cip Cot Gen Na S Te Tr | 0.6 | $bla_{TEM}$, *sul1, tetA, strA, aphA2* | | |
| A79 | B1 | UT | S5 | Amp Cot Na Te Tr | 0.33 | $bla_{TEM}$, *sul1, tetA, tetB* | | |
| E138 | A | O69 | S5 | Amp Cip Cot Na Te Tr | 0.4 | $bla_{TEM}$, *sul1,sul2, tetA* | | |
| EC37 | B1 | UT | S4 | Amp Cx Cpd Cxm Cip Cot Na Te Tr | 0.6 | $bla_{TEM}$, *sul1,sul2, tetA, tetB, strA, dhfr1, dhfr7* | *int1* | |
| ES7 | A | UT | S3 | Amp Cx Cpd Na Te | 0.33 | $bla_{TEM}$, *tetA* | *int1* | |
| A82 | A | O21 | S3 | Amp Na Te | 0.2 | $bla_{TEM}$, *tetA* | | |

(*continued on next page*)

**Table 2** (*continued*)

| Strain No | Phylogenetic group | Serotype | Station No | Resistance patterns | MAR index | ARGs | integrase | Virulence genes |
|---|---|---|---|---|---|---|---|---|
| EC50 | D | O5 | S2 | Amp Cx Cpd Cip Na Te | 0.4 | *$bla_{TEM}$, tetA* | *int1* | |
| ES96 | B1 | O135 | S2 | Amp Cx Cpd Na S Te | 0.4 | *$bla_{TEM}$, tetA, strA* | | |
| EC5 | B2 | R | S2 | Amp Cx cpd Cip Na S Te | 0.46 | *$bla_{TEM}$, tetA, strA* | | *iutA, kpsMT II* |
| ES46 | A | O4 | S2 | Amp Cip Cot Te Tr | 0.33 | *$bla_{TEM}$, tetA, tetB* | | |
| ES97 | A | O25 | S2 | Amp Na S Te | 0.26 | *$bla_{TEM}$, tetA, tetB* | | |
| ES37 | D | O60 | S5 | Amp Cot Te Tr | 0.26 | *$bla_{TEM}$, tetA, tetB* | | |
| ES5 | E | O1 | S5 | Amp Cot Te Tr | 0.26 | *$bla_{TEM}$, tetA, tetB* | | |
| ES6 | A | UT | S3 | Amp Na Te | 0.2 | *$bla_{TEM}$, tetA, tetB* | | |
| A35 | A | O75 | S1 | Amp Ctx Cx Cpd Gen S Te Tr | 0.53 | *$bla_{TEM}$, tetA, tetB, strA, aphA2* | | *iutA, papC* |
| MS2 | B2 | O106 | S3 | Amp Ctx Cx Cpd Cip Cot Na S Te Tr | 0.66 | *$bla_{TEM}$, tetA, tetB, strA, aphA2* | *int1* | *iutA, papC* |
| EC58 | B1 | O112 | S1 | Amp Cx Cpd | 0.2 | *$bla_{TEM}$, $bla_{CTX-M}$* | | |
| EC59 | B1 | O58 | S1 | Amp Cx Cpd | 0.2 | *$bla_{TEM}$, $bla_{CTX-M}$* | | |
| EC81 | B2 | O1 | S2 | Amp Cx Cpd C Cot Gen Na S Te Tr | 0.66 | *$bla_{TEM}$, $bla_{CTX-M}$, sul1, tetA, tetB, strA, aphA2, cat1, dhfr1* | *int1, int3* | *iutA, papC* |
| A74 | B1 | O29 | S5 | Amp Cx Cpd Cot Na TeTr | 0.46 | *$bla_{TEM}$, $bla_{CTX-M}$, sul1, sul2, tetA* | | |
| A38 | A | O18 | S1 | S | 0.06 | *strA* | | |
| A46 | A | UT | S1 | S | 0.06 | *strA* | | |
| A61 | A | O52 | S3 | S | 0.06 | *strA* | | |
| A64 | A | O149 | S3 | S | 0.06 | *strA* | | |
| EC97 | B2 | UT | S4 | S | 0.06 | *strA* | | |
| EC78 | B2 | UT | S2 | Amp C Cot Gen Na S Te | 0.46 | *strA, aphA2* | | *kpsMT II, papC* |
| EC3 | A | R | S2 | Amp Cip Cot Na Te Tr | 0.4 | *sul1, tetA, tet B* | | |
| ES56 | A | O60 | S4 | Amp Cot Na Tr | 0.26 | *sul1* | | |
| EC94 | A | UT | S4 | Cot | 0.06 | *sul1* | | |
| ES62 | B1 | O147 | S3 | Amp Cx Cot Tr | 0.26 | *sul1* | | |
| ES76 | E | O37 | S2 | Amp Cx Cpd Cot Tr | 0.33 | *sul1* | | |
| ES40 | B1 | O60 | S3 | Amp Cip Cot Na S Tr | 0.4 | *sul1, strA* | | |
| EA69 | B1 | O29 | S2 | Cot Te Tr | 0.2 | *sul1, sul2* | *int1* | |
| EC98 | A | UT | S4 | Amp Ctx Cx Cot Te Tr | 0.4 | *sul1, sul2, tetA* | | |
| ES49 | A | O60 | S4 | Amp Cot Tr | 0.2 | *sul1, sul2, tetA* | | |
| A9 | A | O149 | S5 | Cot Te Tr | 0.2 | *sul1, sul2, tetA* | *int1* | |
| ES12 | B2 | O1 | S1 | Amp Cx Cpd Cot Te Tr | 0.4 | *sul1, sul2, tetA, strA, aphA2* | | |
| EC2 | B1 | R | S4 | Amp Cip Cot Na S Te Tr | 0.46 | *sul1, sul2, tetA, strA, aphA2, dhfr1* | *int1* | |
| E135 | B1 | O103 | S5 | Amp Cot Cip Na Te Tr | 0.4 | *sul1, sul2, tetA, tetB* | | |
| A16 | A | O170 | S4 | Cot Te Tr | 0.2 | *sul1, sul2, tetA, tetB, aphA2* | *int1* | |
| A1 | C | R | S5 | Cot Te Tr | 0.2 | *sul1, sul2, tetA, tetB, aphA2* | | |
| EC1 | E | R | S4 | Amp Cip Cot Na Te Tr | 0.4 | *sul1, sul2, tetA, tetB, dhfr1* | *int1* | |
| A63 | C | O149 | S3 | Cot S Te Tr | 0.26 | *sul1, sul2, tetA, tetB, strA* | | *iutA, papC* |
| A14 | A | UT | S5 | Cot Te Tr | 0.2 | *sul1, tetA* | *int1* | |
| A10 | B1 | O149 | S5 | Cot Te Tr | 0.2 | *sul1, tetA, tetB* | | |
| ES127 | B2 | R | S4 | Cot Na Te Tr | 0.26 | *sul1, tetA, tetB* | | |
| ES134 | B2 | O8 | S4 | Amp Cxm Cot S Te Tr | 0.4 | *sul1, tetB* | | |
| A5 | A | R | S2 | Cot Te Tr | 0.2 | *sul2, tetA, tetB, strA, aphA2* | | |
| A62 | A | O9 | S3 | Cot S Gen | 0.2 | *sul2, strA* | | *iutA, papC* |
| N8 | B2 | O4 | S3 | Amp Cot Gen Te Tr | 0.33 | *sul2, tetA* | | |
| ES3 | B2 | O157 | S2 | Amp Cip Cot Na Te Tr | 0.4 | *sul2, tetA, tetB* | | *iutA, kpsMT II* |
| A80 | A | O44 | S3 | S Te | 0.13 | *tetA* | | |
| A59 | B1 | O149 | S3 | Na Te Tr | 0.2 | *tetA* | | |
| A69 | B1 | O29 | S2 | Cot Te Tr | 0.2 | *tetA* | *int 1* | |
| A4 | B1 | R | S5 | Amp Te | 0.13 | *tetA* | | |
| EC24 | B2 | O52 | S2 | Amp Na Te | 0.2 | *tetA* | | |
| A50 | A | O147 | S4 | Na Te | 0.13 | *tetA* | | |
| ES126 | B2 | UT | S4 | S | 0.06 | *tetA, strA* | | |
| EC100 | A | O90 | S4 | S | 0.06 | *tetA, tetB* | | |
| A66 | B2 | O75 | S3 | S Te | 0.13 | *tetA, tetB, strA* | | *iutA, kpsMT II* |
| EA67 | B2 | O29 | S3 | Amp Cpd S Te | 0.26 | *tetA, tetB, strA* | | |
| EA75 | B2 | O37 | S5 | Na S Te | 0.2 | *tetA* | *int1* | |
| ES34 | A | O20 | S1 | C S Te | 0.2 | *tetA, strA* | | |
| A7 | A | R | S5 | Na S Te | 0.2 | *tetA, strA* | | |
| A72 | B1 | UT | S5 | Na S Te | 0.2 | *tetA, strA* | | |
| A51 | C | O75 | S4 | Na Gen Te | 0.2 | *tetA, strA* | | |
| EC68 | A | UT | S4 | Amp Cx Cpd Te | 0.26 | *tetA, tetB* | | |
| ES75 | E | O87 | S2 | Amp Cx Cot Tr | 0.26 | *tetA, tetB* | | |
| EC42 | F | O25 | S2 | Amp Cip Cot Gen Na Te | 0.4 | *tetA, tetB* | | |
| EC8 | A | UT | S3 | Amp Cx Cpd S Te | 0.33 | *tetA, tetB, strA* | | |
| A23 | A | O148 | S3 | S Te Tr | 0.2 | *tetA, tetB, strA* | | |
| A6 | A | R | S5 | Na S Te | 0.2 | *tetA, tetB, strA* | | |

**Table 3**
Distribution of antibiotic resistance genes among ARGs-harboring *E. coli* isolates from five different stations in Cochin estuary. S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth. Statistically significant differences ($p < 0.05$) in the prevalence of ARGs between stations are indicated by superscript letters. [a] Between S1 and S2, [b] between S1 and S3, [c] between S1 and S4, [d] between S1 and S5, [e] between S2 and S3, [f] between S2 and S4, [g] between S2 and S5, [h] between S3 and S4, [i] between S3 and S5, [j] between S4 and S5.

| ARGs | Percentage (no. of isolate) | | | | | Total (n = 122) |
|---|---|---|---|---|---|---|
| | S1 (n = 12) | S2 (n = 31) | S3 (n = 31) | S4 (n = 22) | S5 (n = 26) | |
| $bla_{TEM}$ [c, f, h, j] | 66.6 (8) | 67.7 (21) | 54.8 (17) | 31.8 (7) | 57.6 (15) | 55.7 (68) |
| $bla_{CTX-M}$ [a, c, e, f, h, j] | 25 (3) | 12.9 (4) | 22.5 (7) | 4.4 (1) | 15.3 (4) | 15.5 (19) |
| *sul1* [a, c, d, e, h, i] | 25 (3) | 45.1 (14) | 19.3 (6) | 45.4 (10) | 46.1 (12) | 36.8 (45) |
| *sul2* [e, h, i] | 16.6 (2) | 25.8 (8) | 12.9 (4) | 27.2 (6) | 26.9 (7) | 22.1 (27) |
| *tetA* [a, b, c, d, i] | 33.3 (4) | 58 (18) | 48.3 (15) | 54.5 (12) | 69.2 (18) | 55.7 (68) |
| *tetB* [a, b, c, d, e] | 8.3 (1) | 38 (12) | 25.8 (8) | 31.8 (7) | 34.6 (9) | 30.3 (37) |
| *strA* [a, c, d, e, g, h, i, j] | 50 (6) | 29 (9) | 45.1 (14) | 27.2 (6) | 15.3 (4) | 31.9 (39) |
| *aphA2* [b, c, d, e, f, g] | 25 (3) | 22.5 (7) | 6.4 (2) | 9.0 (2) | 7.6 (2) | 13.1 (16) |
| *cat1* [b, c, e, f, i, j] | 8.3 (1) | 6.4 (2) | 0 (0) | 0 (0) | 7.6 (2) | 4 (5) |
| *dhfr1* [a, c, h, j] | 0 (0) | 6.4 (2) | 3.2 (1) | 13.6 (3) | 3.8 (1) | 5.7 (7) |
| *dhfr7* [c, h, j] | 0 (0) | 3.2 (1) | 0 (0) | 4.5 (1) | 0 (0) | 1.6 (2) |

### 2.6. Antibiotic susceptibility test

Antibiotic resistance patterns of *E. coli* isolates (n = 300) were determined by the disc diffusion method (Bauer et al., 1966) on Mueller-Hinton agar (Hi-Media, India). The antibiotics and concentrations are listed in Suppl. Table 3A. One of the MAR (multiple antibiotic-resistant) isolates (CUSMBES11) was further analyzed using additional antibiotics disks (Suppl. Table 3B). The isolates were classified as sensitive, intermediate, or resistant according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2012), after 24 h of incubation at 37 °C. MAR index was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics used (Krumperman, 1983).

### 2.7. Detection of antibiotic resistance genes and integrase genes

Eleven primer pairs were used for target genes encoding resistance to: β-lactams [$bla_{TEM}$ (Marynard et al., 2003), $bla_{CTX-M}$ (Batchelor et al., 2005)], tetracycline [*tetA* and *tetB* (Marynard et al., 2003)], gentamicin [*aphA2* (Marynard et al., 2003)], streptomycin [*strA* (Rosengren et al., 2009)], sulphonamide [*sul1* and *sul2* (Marynard et al., 2003)], trimethoprim [*dhfr1* and *dhfr7* (Navia et al., 2003)], and chloramphenicol [*catI* (Marynard et al., 2003)]. The presence of integrons was analyzed through PCR amplification of *int1* (Kraft et al., 1986), *int2,* and *int3* integrase genes (Goldstein et al., 2001). PCR mixes of 25 µL final volume were prepared with 1 µL of total DNA, 0.2 µM of each primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). The PCR reactions were carried out using ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States) under conditions as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing (varied temperature, given in Suppl. Table 2) for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C, 5 min. The primers are shown in Suppl. Table 2. PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by gel documentation system (BioRad Gel Doc™ EZ Imager, United States).

### 2.8. Phylogenetic analysis

*E. coli* isolates positive for at least one virulence gene or ARG was analyzed for phylogenetic groups. Phylogenetic grouping of the isolates was determined by using the recently-developed quadruplex PCR protocol by Clermont et al. (2013). This method led to the assignment of an *E. coli* isolate to one of the seven phylogenetic groups (A, B1, B2, C, D, E, and F) and *Escherichia* cryptic clades I to V. All PCR reactions were carried out in a final volume of 25 µL containing 1 µL of total DNA, 0.2 µM of each primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). PCR reactions were performed under the following conditions: denaturation 4 min at 94 °C, 30 cycles of 5 s at 94 °C and 20 s at 57 °C (group E) or 59 °C (quadruplex and group C), and a final extension step of 5 min at 72 °C, on a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). The primers are shown in Suppl. Table 2. PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, United States).

### 2.9. ERIC fingerprinting

ARGs-harboring *E. coli* isolates were analyzed using the enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) with primer ERIC-2 (Meacham et al., 2003). PCR mixes of 25 µL final volume were prepared with 1 µL of total DNA, 0.2 µM of primer, and 12.5 µL of EmeraldAmp® GT PCR Master Mix (Takara, Japan). The cycling conditions were as follows: 1 cycle of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 1 min), extension (72 °C, 1 min) and final extension (72 °C, 5 min) on a ProFlex™ 3x32-Well PCR system (Applied Biosystems, United States). The primer is shown in Suppl. Table 2. PCR products were analyzed by electrophoresis on a 2% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by gel documentation system (BioRad Gel Doc™ EZ Imager, United States). Fingerprint data analysis Gelcompare II version 6.0 software (Applied Maths, Texas) was used to analyze the ERIC-PCR fingerprints of the *E. coli* isolates. Dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA).

### 2.10. Statistical analysis

Pearson's Chi-squared test was used to test whether antibiotic resistance, ARGs-harboring *E. coli* isolates, ExPEC and VGs were evenly distributed among sampling stations. It was also used to compare the prevalence of ARGs between *int1*-positive and *int1*-negative multiple antibiotic-resistant *E. coli* isolates. One-way Analysis of Variance (ANOVA, Games-Howell) was applied to test whether MPN load was evenly distributed among sampling stations. It was also applied to test the frequency of distribution of different ARGs in *E. coli* isolates from Cochin estuary. Pearson's correlation test was used to investigate the positive and negative relationships of the physico-chemical parameters with the occurrence of ARGs-harboring *E. coli*. Statistical analysis of the results was carried out using IBMSPSS version 22 (IBM Corporation, United States). Statistical significance was set at a *p*-value of $< 0.05$.

**Table 4**
Prevalence of virulence genes in ARGs-harboring *E. coli* isolates and Non ARGs-harboring *E. coli* isolates. *Significant *p* value; ($p < 0.05$).

| Virulence gene | $bla_{TEM}$ | | $bla_{CTX-M}$ | | *sul1* | | *sul2* | | *tetA* | | *tetB* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Present (n = 68) | Absent (n = 232) | Present (n = 19) | Absent (n = 281) | Present (n = 45) | Absent (n = 255) | Present (n = 27) | Absent (n = 273) | Present (n = 68) | Absent (n = 232) | Present (n = 37) | |
| *iutA* (n = 39) | 14.7% (10) | 12.5% (29) | 21.0% (4) | 12.4% (35) | 8.8% (4) | 13.7% (35) | 18.5% (5) | 12.4% (34) | 13.2% (9) | 12.9% (30) | 21.6%* (8) | |
| *kpsMT II* (n = 35) | 11.7% (8) | 11.6% (27) | 21.0%* (4) | 11% (31) | 6.6% (3) | 12.5% (32) | 14.8% (4) | 11.3% (31) | 8.8% (6) | 12.5% (29) | 10.8% (4) | |
| papC (n = 28) | 8.8% (6) | 9.4% (22) | 15.7% (3) | 8.8% (25) | 8.8% (4) | 9.4% (24) | 14.8% (4) | 10.9% (30) | 8.8% (6) | 9.4% (22) | 13.5% (5) | |
| *papAH* (n = 1) | 1.4% (1) | 0 | 5.2%* (1) | 0 | 2.2% (1) | 0 | 3.7% (1) | 0 | 1.4% (1) | 0 | 2.7% (1) | |
| *sfa/focDE* (n = 1) | 1.4% (1) | 0 | 5.2%* (1) | 0 | 2.2% (1) | 0 | 3.7% (1) | 0 | 1.4% (1) | 0 | 2.7% (1) | |
| *stx1* (n = 1) | 1.4% (1) | 0 | 5.2%* (1) | 0 | 2.2% (1) | 0 | 3.7% (1) | 0 | 1.4% (1) | 0 | 2.7% (1) | |

| Virulence gene | *tetB* | *strA* | | *aphA2* | | *cat1* | | *dhfr1* | | *dhfr7* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Absent (n = 263) | Present (n = 39) | Absent (n = 261) | Present (n = 16) | Absent (n = 284) | Present (n = 5) | Absent (n = 295) | Present (n = 7) | Absent (n = 293) | Present (n = 2) | Absent (n = 298) |
| *iutA* (n = 39) | 11.7% (31) | 15.3% (6) | 11.8% (31) | 31.2%* (5) | 11.9% (34) | 40%* (2) | 12.5% (37) | 42.8%* (3) | 12.2% (36) | 50%* (1) | 12.7% (38) |
| *kpsMT II* (n = 35) | 11.7% (31) | 17.9 (7) | 10.7% (28) | 18.7% (3) | 11.2% (32) | 40%* (2) | 11.1% (33) | 28.5%* (2) | 11.2% (33) | 50%* (1) | 11.4% (34) |
| papC (n = 28) | 8.7% (23) | 20.5%* (8) | 7.6% (20) | 37.5%* (6) | 7.7% (22) | 40%* (2) | 8.8% (26) | 28.5%* (2) | 8.8% (26) | 50%* (1) | 9% (27) |
| *papAH* (n = 1) | 0 | 12.5% (1) | 0 | 6.2%* (1) | 0 | 20%* (1) | 0 | 14.2%* (1) | 0 | 50%* (1) | 0 |
| *sfa/focDE* (n = 1) | 0 | 12.5% (1) | 0 | 6.2%* (1) | 0 | 20%* (1) | 0 | 14.2%* (1) | 0 | 50%* (1) | 0 |
| *stx1* (n = 1) | 0 | 2.5% (1) | 0 | 6.2%* (1) | 0 | 20%* (1) | 0 | 14.2%* (1) | 0 | 50%* (1) | 0 |

## 3. Results

### 3.1. Water quality

The physico-chemical parameters of the water from the different sampling stations are shown in Suppl. Table 4A. Significant differences in physico-chemical parameters except temperature pH, and nitrite were evident among the different stations in Cochin estuary (Suppl. Table 4B–C). The surface water temperature varied from 26 to 33.5 °C. The pH of the water varied from 7.2 to 8.6. Salinity varied from 0 to 34 ppt. The water was consistently aerobic with the dissolved oxygen concentration of above 2.5 mg/L in all five stations. Station 5 had significantly higher salinity and DO than that of other stations ($p < 0.05$). Station 4 had significantly lower nitrate concentration (mean, 1.3 mg/L) than that of S1, S2, and S5 ($p < 0.05$). The nitrite concentration ranged from 0.5 mg/L-0.7 mg/L. Station 2 had significantly high ammonium concentration (mean, 64.2 mg/L) than that of S1, S3, and S4 ($p < 0.05$). Station 3 had statistically lower phosphate concentration (mean, 0.9 mg/L) than that of S1, S2, and S3 ($p < 0.05$).

The physico-chemical parameters of the water in different sampling seasons are shown in Suppl. Table 4D. Significant differences in the physico-chemical parameters were evident in Cochin estuary during different seasons (Suppl. Table 4E–F). Pre-monsoon (PRM) had the significantly high temperature, pH, salinity, DO, nitrate, and nitrite compared to monsoon period (MON), whereas post-monsoon (PSM) had significantly high ammonium concentration compared to pre-monsoon period ($p < 0.05$). Post-monsoon had significantly high phosphate concentration compared to pre-monsoon. Water temperature showed a variation, with a mean of 27.8 °C in monsoon, and a mean of 31 °C in pre- and post-monsoon period. The pH values of water samples ranged as follows: 7.5 mg/L-8.5 mg/L (PRM), 7.2 mg/L-8.3 mg/L (MON), and 7.2 mg/L-8.6 mg/L (PSM). Salinity difference was observed in different seasons, with values in the range of 14-35 ppt (PRM), 0-3 ppt (MON), and 0-33 ppt (PSM). The concentration of DO significantly varied with the seasonal variation, with values ranged from 4.5 to 5.7 mg/L, 3-5.3 mg/L, 2.5-5.7 mg/L in the pre-monsoon, monsoon, and post-monsoon, respectively. The nitrate concentration ranged from 1.3 to 27.2 mg/L, 4.2-15.4 mg/L, and 8.5-18.7 mg/L in the pre-monsoon, monsoon, and post-monsoon, respectively. The nitrite concentration ranged as follows: 0.4-1.4 mg/L (PRM), 0.2-0.9 mg/L (M), and 0.2-8.8 mg/L (PSM). The phosphate concentration significantly varied with the seasonal variation, with values ranged from 1 to 1.7 mg/L, 0.1-2.9 mg/L, and 1.3-3.4 mg/L in the pre-monsoon, monsoon, and post-monsoon, respectively. The results of correlation analyses among the different water quality parameters are presented in Suppl. Table 4G-N. During the monsoon period, nitrite concentration showed a positive correlation with pH, nitrate, and phosphate. Additionally, the ammonium and nitrate concentration showed a positive correlation with phosphate concentration. Temperature showed a negative correlation with salinity during pre- and post-monsoon period. Furthermore, temperature showed a negative correlation with ammonium concentration during pre-monsoon and monsoon period. DO showed a negative correlation with ammonium concentration during pre- and post-monsoon period.

### 3.2. E. coli enumeration and isolation

The results showed that the surface water was contaminated with FC in all the five stations in Cochin estuary (Fig. 2A-B). The MPN load of FC ($Log_{10}$MPN/100 ml) ranged as follows: 1.1-4.0 (S1), 1.3-5.0 (S2), 1.4-5.0 (S3), 1.3-3.9 (S4), and 1.3-4.0 (S5). Significant differences in MPN values were found among different stations (ANOVA, $p < 0.05$). Station 2 had significantly higher FC load than that of other stations ($p < 0.05$) during the monsoon period. Furthermore, S2 and S3 had significantly higher FC load than those of S1, S4, and S5 ($p < 0.05$) during pre- and post-monsoon period (Suppl. Table 5Aa-Cc).

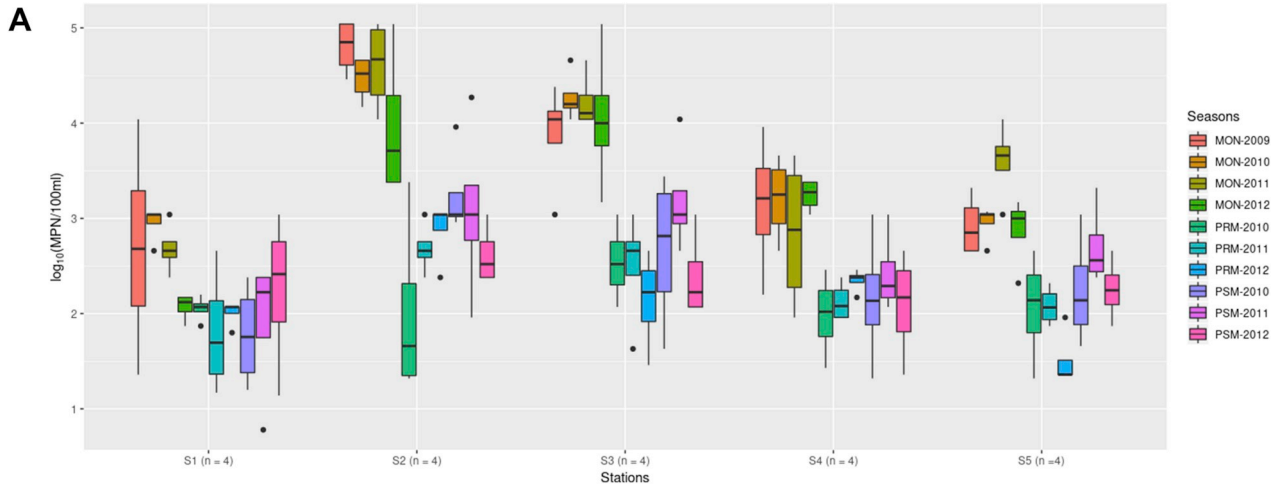


**Fig. 2A.** Fecal coliform concentration during monsoon, pre- and post-monsoon during the study period (2009-2012) at different stations in Cochin estuary. X-axis labels indicate different sampling stations, n = no. of sample, Y-axis label indicates $\log_{10}$ (MPN/100 ml). S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth. The Box plot representing (from top to bottom), the maximum, upper-quartile, median, lower-quartile and minimum values of the data. Pre-monsoon (PRM [n = 4], February to May); Monsoon (MON [n = 4], June-September); Post-monsoon (PSM [n = 4], October-January). Data of the sampling period 2013 (January [n = 1, PSM] - February [n = 1, PRM]) are not shown in Fig. 2A. Data of the sampling period PSM-2009 are not included in Fig. 2A, because sample number is low, n = 3 (October, November and December).

### 3.3. Antibiotic resistance

Among *E. coli* isolates, 64% were resistant to at least one of the 15 antibiotics tested. The highest prevalence of antibiotic resistance was observed for ampicillin (44%), followed by tetracycline (31%), and nalidixic acid (27%). Furthermore, between 20% and 10% of *E. coli* isolates were resistant to cefoxitin (20%), co-trimoxazole (20%), trimethoprim (20%), streptomycin (16%), and cefpodoxime (15%). The prevalence of ciprofloxacin, gentamicin, cefuroxime, chloramphenicol, cefotaxime, ceftriaxone, and ceftazidime resistance was low, with 8.6%, 5%, 4%, 3.6%, 3.3%, 2.3%, and 1.3%, respectively. Significant differences were observed in the prevalence of antibiotic resistance except cefotaxime, cefoxitin, cefpodoxime, and ceftazidime among the different stations ($p < 0.05$) (Table 1). Station 2 had significantly highest prevalence of ciprofloxacin, co-trimoxazole, chloramphenicol, gentamicin, and nalidixic acid resistant isolates ($p < 0.05$). Whereas, S3 had the highest prevalence of streptomycin resistant isolates ($p < 0.05$). Furthermore, the prevalence of cefuroxime resistant isolates was significantly high in S4 and S3 than those S1 ($p < 0.05$).

Approximately, 37.6% of *E. coli* isolates were multiple antibiotic-resistant. The MAR index ranged as follows: 0.2-0.6 (S1 and S4), 0.2-1 (S2), 0.2-0.8 (S3), and 0.2-0.7 (S5).

### 3.4. Prevalence of antibiotic resistance genes

Among the 300 tested isolates, 40.6% (n = 122) of *E. coli* had antibiotic resistance genes (ARGs). More than 69% of ARGs-harboring isolates had two or more ARGs. The isolate (CUSMBES11) had multiple ARGs (all eleven ARGs tested). ARGs-harboring *E. coli* isolates mainly belonged to phylogenetic group A (36.8%), B1 (23.7%), B2 (19.6%) and to a lesser extent, to phylogenetic group D (8.9%), E (4.9%), C, and F (3.2%) (Fig. 3). The characteristics of the ARG-harboring *E. coli* isolates are shown in Table 2.

Significant differences were found in the frequency of different ARGs from Cochin estuary (ANOVA, $p < 0.05$) (Suppl. Table. 6A-C). The β-lactamase gene, $bla_{TEM}$ was detected more frequently among *E. coli* isolates. Games-Howell analysis showed that the percentage of distribution of $bla_{TEM}$ and *tet*A genes was significantly higher than to

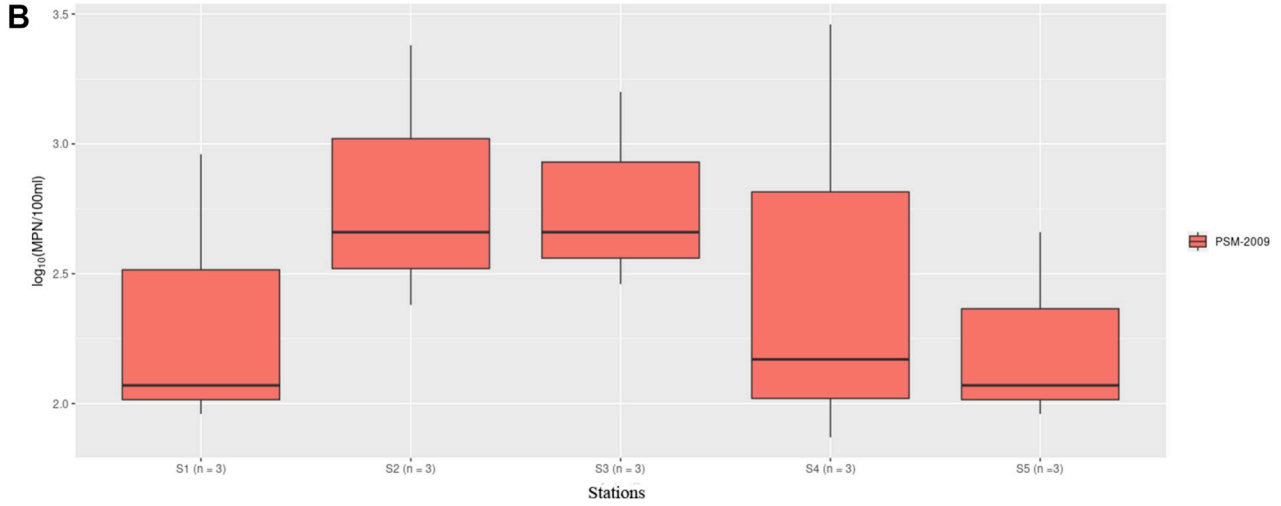


**Fig. 2B.** Fecal coliform concentration at different stations in Cochin estuary during 2009 post-monsoon (PSM) period.

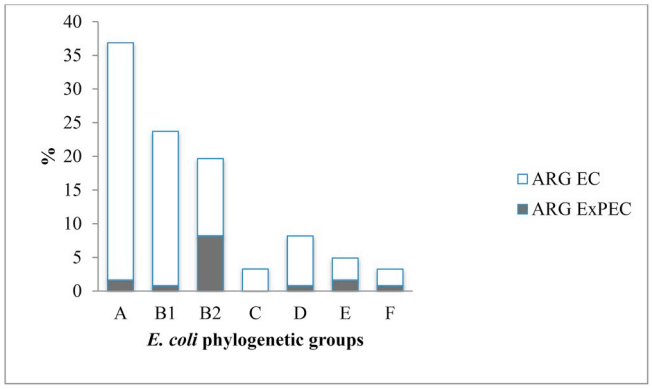


**Fig. 3.** Phylogenetic group distribution of ARGs-harboring *E. coli* isolates. ARG EC indicates ARGs-harboring *E. coli* isolates. Filled bars (ARG ExPEC) indicate the percentages of ARGs-harboring ExPEC isolates.

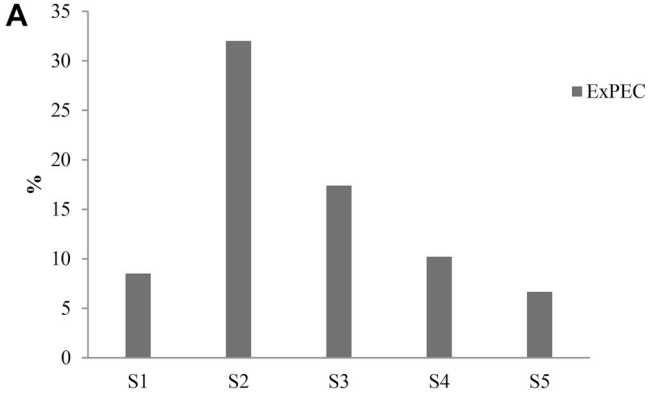


**Fig. 4A.** Prevalence of ExPEC among five different stations in Cochin estuary. S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth.

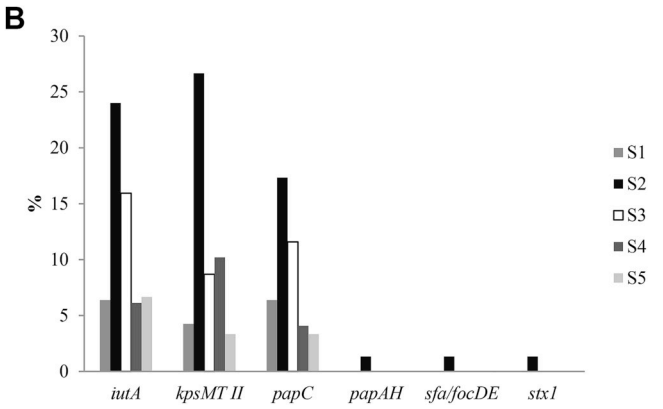


**Fig. 4B.** Prevalence of virulence genes among five different stations in Cochin estuary. S1, Chitoor; S2, Bolgatty; S3, Off Marine Science Jetty; S4, Thevara; S5, Barmouth.

$bla_{CTX\text{-}M}$, *aphA2*, *catI*, *dhfr1*, and *dhfr7* ($p < 0.05$). Among tetracycline-resistant strains (n = 94), 72.3% and, 39.3% had *tetA,* and *tetB,* respectively (Suppl. Table 7). The $bla_{CTX\text{-}M}$ harboring *E. coli* isolates were dominated in phylogenetic group D. (Suppl. Fig. 2). The dominant gene encoding sulphonamide resistance was *sul1*. Among trimethoprim-resistant isolates (n = 60), 11.6%, and 3.3% had *dhfr1*, and *dhfr7*, respectively. Among streptomycin-resistant isolates (n = 47), 81.2% had *strA* gene. Among gentamicin-resistant isolates (n = 17), 94.1% had *aphA2* gene. The *sul1*, *sul2*, and *strA* were absent in phylogenetic group F. Among chloramphenicol-resistant isolates (n = 11), 45.4% had *cat1* gene. Significant differences were observed in the prevalence of ARGs among five different stations ($p < 0.05$) (Table 3). Moreover, the prevalence of *sul1*, *tetA*, *tetB,* and *dhfr1* was significantly higher in S2 compared to S1 ($p < 0.05$). Conversely, the prevalence of $bla_{CTX\text{-}M}$ and *strA* was significantly higher in S1 compared to S2 ($p < 0.05$). The prevalence of *aphA2* harboring *E. coli* isolates was significantly higher in S1 and S2 than that of other stations ($p < 0.05$). The prevalence of $bla_{TEM}$ and $bla_{CTX\text{-}M}$ harboring *E. coli* isolates was significantly lower in S4 ($p < 0.05$) than that of other stations. The prevalence of *sul1* and *sul2* harboring *E. coli* isolates was significantly lower in S3 than that of S2, S4, and S5 ($p < 0.05$).

Pearson's correlation results showed that the physico-chemical parameters had no effects on the year-wise occurrence of ARGs-harboring *E. coli*. Conversely, a significant negative correlation with nitrite concentration and the occurrence of ARGs-harboring *E. coli* was evident during the monsoon period.

### 3.5. Prevalence of integrase

The prevalence of *int1*-harboring *E. coli* isolates was 7.6% (23/300). Moreover, two *E. coli* isolates had both *int1* and *int3* genes. No *int2* gene was detected. Among *int1*-positive isolates, 94% (20/23) were multiple antibiotic-resistant. A significant positive association was observed between the occurrence of ARGs ($bla_{CTX\text{-}M}$, *sul1*, *sul2*, *aphA2*, *cat1*, *dhfr1*, and *dhfr7*) and the occurrence of *int1* among multiple antibiotic-resistant *E. coli* isolates (Suppl. Table. 8A). Station 1 had a significantly lower prevalence of *int1* than that of other stations ($p < 0.05$) (Suppl. Table. 8B).

### 3.6. Prevalence of ExPEC and STEC

Extraintestinal virulence genes (*papAH*, *papC*, *sfa/focDE*, *kpsMT II*, and *iutA*) were screened in 300 *E. coli* isolates. Isolates were classified as ExPEC if they were found to be positive for two or more VGs such as *papAH*, *papC* (P fimbrial assembly), *sfa/focDE* (S and F1C fimbriae), *iutA* (aerobactin receptor) and *kpsMT II* (group II capsule). Extraintestinal virulence genes were detected in 52 *E. coli* isolates and 16.3% (49/300) were ExPEC. ExPEC isolates mainly belonged to phylogenetic group B2 (57.1%) followed by D (20.4%), B1 (8.1%), A (6.1%), E (4.1%), F, (2.1%), and E (2.1%). The prevalence of ExPEC and virulence genes among different stations is shown in Fig. 4A. The prevalence of ExPEC isolates was significantly higher in S2 than that of other stations ($p < 0.05$) (Suppl. Table 9A). The *iutA* (aerobactin acquisition), *papC* (P fimbriae) and *kpsMT II* (group 2 capsule synthesis) genes were frequent among ExPEC isolates (Fig. 4B). The prevalence of virulence genes except for *stx1*, *papAH*, and *sfa/focDE* was varied between 9% and 13% (Suppl. Table. 9B). The *iutA* and *kpsMT II* was the most frequent combination of virulence genes detected. Twenty-five strains showed this combination. Significant differences were observed in the prevalence of *papC*, *kpsMT II*, and *iutA* among five different stations ($p < 0.05$). S2 had the highest prevalence of virulence genes such as *kpsMT II*, *iutA*, and *papC* ($p < 0.05$). The prevalence of *stx1* was low (0.3%). Only one isolate had *stx1* gene. This STEC isolate (CUSMBES11) also had extraintestinal virulence genes.

### 3.7. Co-occurrence of antibiotic resistant genes and virulence genes

Fifty-five percent (27/49) of the ExPEC isolates were resistant to at least one antibiotic, and approximately 26.5% (13/49) were multiple antibiotic-resistant. Resistance to ampicillin, nalidixic acid, sulphonamides, and cefoxitin was relatively frequent among ExPEC. More than, 34% (17/49) of ExPEC had ARGs. Furthermore, between 14 and 27% of ExPEC had *strA*, $bla_{TEM}$, *tetA*, *sul2*, *tetB*, and *aphA2*. A statistically significant association was observed between six virulence genes (*papAH*,

*papC*, *sfa/focDE*, *kpsMT II*, *iutA*, and *stx1*) and ARGs (*cat1*, *dhfr*, and *dhfr7*) ($p < 0.05$) (Table 4). All the virulences genes except *kpsMT II* showed a statistically significant association with *aphA2* ($p < 0.05$). ARGs-harboring pathogenic *E. coli* isolates were significantly high in stations (S2 and S3) close to Cochin City ($p < 0.05$) (Suppl. Table. 9C). The *kpsMT II*, *papAH*, *sfa/focDE*, and *stx1* showed a significant positive association with the $bla_{CTX\text{-}M}$ gene ($p < 0.05$). Moreover, *iutA* showed a positive association with *tetB*, whereas *papC* associated with *strA* ($p < 0.05$).

### 3.8. ERIC fingerprints

ARGs-harboring *E. coli* isolates were characterized by ERIC PCR to determine the genetic diversity among the strains. All the isolates were typeable and produced amplicon size ranging from 150 to 1400 bp. *E. coli* isolates were grouped in eleven different clusters (Fig. 5). In ERIC-PCR, ES11 yielded amplicon sizes of 150, 275, 350, 500, 700, 800, and 1400 bp and clustered separately (Suppl. Fig. 3).

### 3.9. Detection of a hypervirulent E. coli

Isolate CUSMBES11 had the highest number of resistance to tested antibiotics (n = 35, antibiotic classes: penicillins, cephalosporins, vancomycin, aminoglycosides, tetracyclines, macrolides, chloramphenicol, quinolones, rifampin, sulfonamides, and trimethoprim). Furthermore, the isolate had multiple ARGs ($bla_{TEM}$, $bla_{CTX\text{-}M}$, *tetA*, *tetB*, *sul1*, *sul2*, *strA*, *aphA2*, *catI*, *dhfr1*, and *dhfr7*), integrase (*int1*and *int3*), Shiga toxin gene (*stx1*), and extraintestinal virulence genes (*papAH*, *papC*, *sfa/focDE*, *kpsMT II*, and *iutA*). The isolate belonged to the B2 phylogenetic group. Moreover, this most potentially pathogenic strain was isolated from S2. The GenBank accession number of the strain is JX183942.1.

## 4. Discussion

We sampled estuarine water to investigate the involvement of estuary in the emergence of ARGs-harboring hypervirulent *E. coli*. *E. coli* are one of the possible indicators to assess the antibiotic resistance status in environmental settings, as these are the most frequent carriers of acquired ARGs (Berendonk et al., 2015). In India, only 12.45% waste is scientifically processed and remaining sewage flows into the water bodies or the ground without any treatment (CPCB, 2013). Only two sewage treatment plants are operational (STPs) in Cochin City and rely upon activated sludge tanks to reduce the organic load of the primary effluents (CPCB, 2015b). Municipal sewage treatment plants are considered as “hotspots” for horizontal gene transfer and the release of ARB and ARGs into the aquatic environment (Bibbal et al., 2018; Reinthaler et al., 2010; Um et al., 2015). Because of the inadequate sewage treatment facility, Cochin estuary receives $1.04 \times 10^5\ m^3$ of untreated industrial effluents and $260\ m^3$ of domestic or sewage waste (Gupta et al., 2016). The major polluting industries in the region include Hindustan Insecticides Ltd., Fertilizers and Chemicals Travancore Ltd., Mercham Ltd., Indian Rare Earth Ltd., and Binamizinc. The products include dichlorodiphenyltrichloroethane (DDT), endosulfan, dicofol, thiazoles, sulphamides, and zinc ingots (CPCB, 2015a).

The remarkably low salinity during the monsoon period indicated the freshwater influx in the study area (Jyothibabu et al., 2006). The high amount of organic material is responsible for the decrease of dissolved oxygen in the backwaters (Babu et al., 2006). Industrial and domestic sewage that reach directly into Cochin estuary may contribute to the prevailing high nutrient concentration in the study area (CPCB, 2015a; Jyothibabu et al., 2006). The present study found that the MPN load of FC exceeded by far the maximum limits recommended by standards (USEPA, 2003). Station 2 and 3, close to Cochin City had high MPN load. The fecal contamination is predominantly due to the lack of modern and efficient sewage treatment facilities in the Cochin City (CPCB, 2015b). Biomedical waste treatment facilities are not available in Cochin City (CPCB, 2016). Fish markets in Cochin City and adjacent area are dumping 27.5% of seafood solid waste into canals and backwaters (Sasidaran and Saleena, 2011). Seafood processing units in Cochin City and adjacent area are equipped with effluent treatment plants for the discharge of liquid waste effluents into the backwaters (Sasidaran and Saleena, 2011).

*E. coli* isolates were showed resistance against critically important and highly important class of antibiotics listed by the World Health Organization (WHO, 2017). WHO has categorized antibiotics into 3 categories (critically important, highly important, and important) based on their importance to human medicine. According to the latest revised edition of the document (WHO, 2017), cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, and ampicillin are ranked as critically important, while cefuroxime, cefoxitin, chloramphenicol, co-trimoxazole, tetracycline, and trimethoprim, feature among the highest important, highlighting the relevance of the antibiotic resistance detected in the current study. The antibiotic resistance of *E. coli* isolates was higher than those previously reported from the Seine estuary in France (Laroche et al., 2009). The prevalence of antibiotic resistance against tetracycline, cefotaxime, and ciprofloxacin was higher than those previously reported from Tagus estuary in Portugal (Pereira et al., 2013). Station 2 had the highest prevalence of antibiotic resistance. This report supports the finding of the previous study in which isolates from wastewater receiving sites showed higher antibiotic resistance (Korzeniewska et al., 2013).

The prevalence of ARGs-harboring *E. coli* isolates was higher than those previously reported from the Tagus estuary in Portugal (Pereira et al., 2013). In their study, the prevalence of $bla_{TEM}$, *tetA*, *tetB*, and *sul1* was 45.5%, 46.1%, 40%, and 30%, respectively. The β-lactamase gene, $bla_{TEM}$ was predominant in the present study. The findings presented here were consistent with previously reported results from the hospital and municipal sewage in Poland (Korzeniewska et al., 2013), municipal wastewater treatment plant in France (Diallo et al., 2013), and sewage sludge in Austria (Reinthaler et al., 2010). The extended spectrum β-lactamase gene, $bla_{CTX\text{-}M}$ was detected more frequently among cefotaxime-resistant *E. coli* isolates. This is in agreement with other studies showing that $bla_{CTX\text{-}M}$ gene was predominant in *E. coli* from the aquatic environment (Leonard et al., 2018; Tacao et al., 2012).

Our data showed that the *tetA* gene was more frequently detected than *tetB* among tetracycline-resistant isolates. This is consistent with previously reported results from aquatic environments in Portugal (Tacao et al., 2014), and Japan (Hu et al., 2008). In sulfonamide-resistant *E. coli*, *sul1* was predominant. Sulfonamides resistance genes could be transferred from commensal bacteria, via integrons, transposons or plasmids, into more virulent bacteria in the human intestine (Guerra et al., 2003). *Sul1* gene is one of the most commonly detected sulphonamide-resistant genes in the environment (Pei et al., 2006). The prevalence of *strA*, and *aphA2* was high among streptomycin-resistant and gentamicin-resistant isolates, respectively. Diallo et al. (2013) reported the presence of *strA* gene from City wastewater in France. The *cat1* gene showed a moderate prevalence among chloramphenicol-resistant isolates. The prevalence of *aphA2* and *cat1* was higher than those previously reported from Lake Ontario in Canada (Hamelin et al., 2006). Trimethoprim resistance genes, *dhfr1* and *dhfr7* were highly detected from S2 and S4. This is consistent with previously reported results from the United States in which isolates from effluents receiving sites had *dhfr* genes (Suhartono et al., 2016). The ARGs ($bla_{TEM}$, $bla_{CTX\text{-}M}$, *sul1*, *sul2*, *aphA2*, and *int1*) monitored in the current study have been listed as possible candidate genes, frequently occurring in environmental settings (Berendonk et al., 2015). Significant differences were found in the distribution of different ARGs among different stations. This result is in agreement with other studies showing that the release of wastewater into the aquatic environment has resulted in a high prevalence of ARGs-harboring *E. coli* in surface water (Czekalski et al., 2014; Rodriguez-mozaz et al., 2015).

No significant correlation was found between different physico-

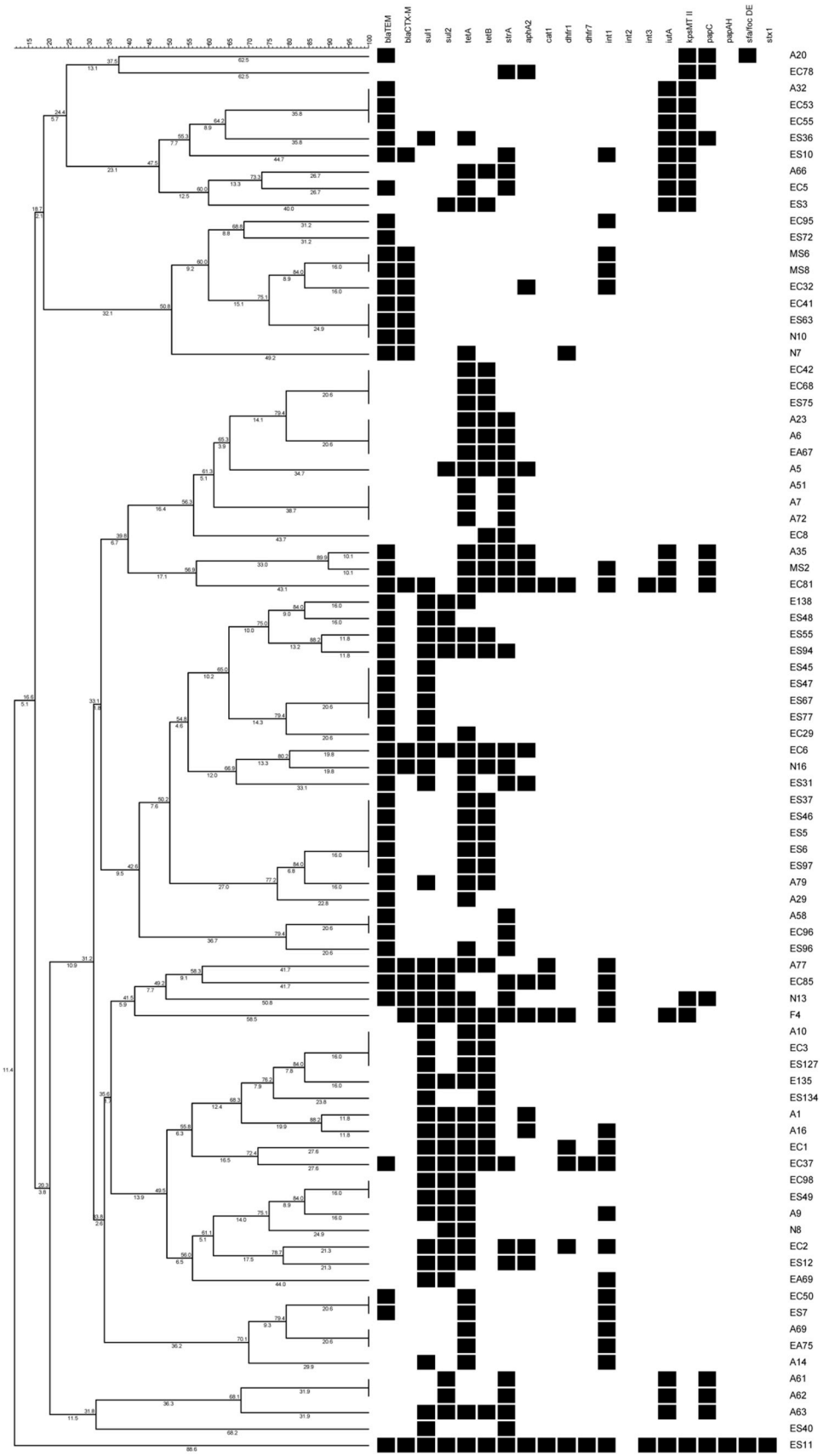


**Fig. 5.** Genetic heterogeneity of ERIC-PCR profiles of ARGs-harboring *E. coli* isolates from Cochin estuary. Antibiotic resistance genes ($bla_{TEM}$, $bla_{CTX-M}$, *sul1*, *sul2*, *tetA*, *tetB*, *strA*, *aphA2*, *catI*, *dhfr1*and *dhfr7*); integrase (*int*, *int2*, and *int3*); extraintestinal virulence genes (*papAH*, *papC*, *sfa/focDE*, *kpsMT II* and *iutA*); Shiga toxin gene (*stx1*).

chemical parameters and year-wise occurrence of ARGs-harboring *E. coli*. However, a significant negative correlation was found between nitrite concentration and occurrence of ARGs-harboring *E. coli* during the monsoon period. Although physico-chemical parameters have the potential to enhance the effect of selective pressures and promote bacterial evolution towards antibiotic resistance, there is still a poor understanding of the environmental factors that may alleviate the spread of antibiotic resistance (Berendonk et al., 2015). Na et al. (2018) reported that antibiotic-resistant strains were less successful with increases of salinity and nutrient availability. Sabri et al. (2018) reported a positive correlation with ammonium concentration and ARGs and a negative correlation with DO and ARGs in a wastewater effluent-receiving river in the Netherlands.

The prevalence of *int1* positive *E. coli* (7.6%) was low in Cochin estuary, which was similar to previous observations (Laroche et al., 2009; Pereira et al., 2013). Our results showed a strong correlation between *int1* positive isolates and ARGs in agreement with previously reported results from various environments in the People's Republic of China (Ma et al., 2017) and wastewater treatment plants in Tunisia (Rafraf et al., 2016). The gene cassettes of class 1 integrons could carry diverse antibiotic resistance genes (ARGs) and conduct horizontal gene transfer among microorganisms (Ma et al., 2017). Class 1 integrase (*int1*) has considered as a good proxy for anthropogenic pollution (Berendonk et al., 2015; Ma et al., 2017).

The prevalence of ExPEC isolates was 16.3%. The presence of ExPEC in Cochin estuary is a matter of concern, because it is known to be detected from clinical settings (Chakraborty et al., 2016). The prevalence of *papAH* and *sfa/focDE* was low (0.3%, 1/300). This result is in agreement with other studies showing that *papAH* and *sfa/focDE* positive isolates have been isolated from extraintestinal infections (Chakraborty et al., 2016; Martinez-Medina et al., 2009). Similar to our findings, several authors reported the occurrence of extraintestinal virulence genes in *E. coli* isolated from wastewater (Biswal et al., 2014; Diallo et al., 2013). The increasing ARG-harboring *E. coli* would make the future management of extraintestinal infections more challenging (Chakraborty et al., 2016).

The prevalence of *stx1* gene was very low. The isolate CUSMBES11 had the *stx1* gene. Our results are in agreement with other studies showing a lower prevalence of *stx* genes from City wastewater and slaughterhouse wastewater in France (Bibbal et al., 2018; Diallo et al., 2013). Moreover, the STEC was multiple antibiotic-resistant and had multiple ARGs and extraintestinal virulence genes. This is, to our knowledge, for the first time a hypervirulent strain detected from the estuarine water. This pathogenic strain might have acquired antibiotic resistance genes either horizontal gene transfer or continuous exposure to antibiotic agents (Kristiansson et al., 2011; Li et al., 2009). This is of particular concern because certain strains of *E. coli* are emerging as a pathogen of global significance with an increased number of disease outbreaks and consequent deaths. The causative agent of one of the world's largest outbreak was a hypervirulent strain harboring ESBL genes $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ (Beutin and Martin, 2012; Grad et al., 2013; Mellmann et al., 2012; Muniesa et al., 2012).

ERIC-PCR analysis revealed high genetic diversity among the ARGs-harboring *E. coli* isolates. The high genetic diversity in *E. coli* isolates might be due to a different source of pollution or difference in the rate of horizontal gene transfer (Luna et al., 2010). ERIC-PCR can be extremely useful for understanding the genetic diversity and improve our knowledge of potentially pathogenic strains. The prevalence of antibiotic resistance, ARGs, ExPEC and VGs was high in S2 and S3, stations close to Cochin City. The present study highlighted the fact that stations close to the urban region contribute to the dissemination of antibiotic-resistant and pathogenic *E. coli* in the aquatic environment. The Cochin estuary is used for different recreational and sporting activities and also for swimming. Direct contact with the polluted estuarine water can lead to colonization or infection (Leonard et al., 2018). Thus, using the Cochin estuary for recreational purposes might result in potential health hazards.

The present study forms one of the most important records from this region depicting antibiotic resistance. It is very important to take necessary steps towards the control of antibiotic resistance in environmental settings. International organizations such as World Health Organization (WHO, 2015), European Union (EU, 2017), and United States Center for Disease Prevention and Control (US-CDC, 2015) have proposed several guidelines and recommendations to curb antibiotic resistance. These recommendations and action plans can aid in systematizing ARB and ARGs containment in India and make it comparable to global efforts. Given the public health threat posed by antibiotic resistance, the national and international policy and management options should be prioritized to tackle antibiotic resistance in the environment.

## 5. Conclusion

- This is the first report of an environmental multiple antibiotic-resistant STEC harboring multiple ARGs and extraintestinal virulence genes.
- Prevalence of ARG-harboring *E. coli* in estuarine water could be a cause for concern because *E. coli* has high potential to disseminate ARGs.
- Co-occurrence of ARGs and VGs could lead to a serious problem in the treatment of human ExPEC infections.
- Stations close to the City (S2 and S3) had the high prevalence of antibiotic-resistant *E. coli*, ARGs, and VGs.
- Anthropogenic interactions play a major role in the emergence of new resistant hypervirulent bacterial strains.
- The findings confirm the need for a better wastewater treatment facility and proper control measures to reduce the discharge of sewage and wastewater into the aquatic environments.
- Continuous monitoring of the estuarine environment is necessary for future studies.

## Declarations of interest

None.

## Acknowledgments

Dr. Divya would like to thank Council of Scientific and Industrial Research (CSIR), Govt of India for the research fellowship to carry out the work. The authors are also thankful to the Head, Dept. of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology for providing the facilities.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2018.11.002.

## References

Alonso, A., Sanchez, P., Martinez, J.L., 2001. Environmental selection of antibiotic resistance genes. Environ. Microbiol. 3 (1), 1–9. http://doi.org/10.1046/j.1462-2920.2001.00161.x.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. American Public Health Association, Washington, DC.

Babu, M.T., Das, V.K., Vethamony, P., 2006. BOD-DO modeling and water quality analysis of a waste water outfall off Kochi, west coast of India. Environ. Int. 32, 165–173.

Barrow, G.I., Feltham, R.K.A., 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria. Cambridge University Press, Cambridge, pp. 317.

Batchelor, M., Hopkins, K., Threlfall, E.J., Clifton-Hadley, F.A., Stallwood, A.D., Davies, R.H., Liebana, E., 2005. *bla*CTX-M genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrob. Agents Chemother. 49 (4), 1319–1322. http://doi.org/10.1128/AAC.49.4.1319-1322.2005.

Bauer, A.W., Kirby, W.M.M., Skerris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single diffusion method. Am. J. Clin. Pathol. 45 (4),

493–496.
Bej, A.K., DiCesare, J., Haff, L., Atlas, R.M., 1991. Detection of *Escherichia coli* and *Shigella* sp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. 57 (4), 1013–1017.
Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, Sorum, H., Norstrom, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.S., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. http://doi.org/10.1038/nrmicro3439.
Beukes, L.S., King, T.L.B., Schmidt, S., 2017. Assessment of pit latrines in a peri-urban community in KwaZulu-Natal (South Africa) as a source of antibiotic resistant *E. coli* strains. Int. J. Hyg Environ. Health 220 (8), 1279–1284. http://doi.org/10.1016/j.ijheh.2017.08.002.
Beutin, L., Martin, A., 2012. Outbreak of shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J. Food Prot. 75 (2), 408–418. http://doi.org/10.4315/0362-028X.JFP-11-452.
Bibbal, D., Um, M.M., Diallo, A.A., Kerouredan, M., Dupouy, V., Toutain, P.L., Bousquet-Melou, A., Oswald, E., Brugere, H., 2018. Mixing of Shiga toxin-producing and enteropathogenic *Escherichia coli* in a wastewater treatment plant receiving City and slaughterhouse wastewater. Int. J. Hyg Environ. Health 221 (2), 355–363. http://doi.org/10.1016/j.ijheh.2017.12.009.
Biswal, B.K., Mazza, A., Masson, L., Gehr, R., Frigon, D., 2014. Impact of wastewater treatment processes on antimicrobial resistance genes and their co-occurrence with virulence genes in *Escherichia coli*. Water Res. 50, 245–253. http://doi.org/10.1016/j.watres.2013.11.047.
Blanco, M., Blanco, J.E., Mora, A., Rey, J., Alonso, J.M., Hermoso, M., Hermoso, J., Alonso, M.P., Dahbi, G., Gonzalez, E.A., Bernardez, M.I., Blanco, J., 2003. Serotypes, virulence genes, and intimin types of shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. J. Clin. Microbiol. 41 (4), 1351–1365. http://doi.org/10.1128/JCM.41.4.1351-1356.2003.
Buchholz, U., Bernard, H., Werber, D., Bohmer, M.M., Remschmidt, C., Wilking, H., Delere, Y., Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Hohle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Stark, K., Kuhne, M., 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. N. Engl. J. Med. 365 (19), 1763–1770. http://doi.org/10.1056/NEJMoa1106482.
Chakraborty, A., Adhikari, P., Shenoy, S., Rao, S., Dhanashree, B., Saralaya, V., 2016. Molecular characterization and clinical significance of extraintestinal pathogenic *Escherichia coli* recovered from a south Indian tertiary care hospital. Microb. Pathog. 95, 43–48. http://doi.org/10.1016/j.micpath.2016.03.001.
Chen, B., Yang, Y., Liang, X., Yu, K., Zhang, T., Li, X., 2013. Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. Environ. Sci. Technol. 47 (22) 12753 − 1276. http://doi.org/10.1021/es403818e.
Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylotyping method revisited : improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5 (1), 58–65. http://doi.org/10.1111/1758-2229.12019.
CLSI, 2012. Performance Standard for Antimicrobial Susceptibility Testing. Twenty-second Informational Supplement. CLSI Document M100-s22. Clinical and Laboratory Standards Institute, Wayne, PA, USA.
CPCB, 2011. Annual Report 2010-2011. Central Pollution Control Board. accessed on 22.09.2018. http://cpcb.nic.in/annual-report.php.
CPCB, 2013. Performance Evaluation of Sewage Treatment Plants under NRCD. Central Pollution Control Board. accessed on 22.09.2018. http://cpcb.nic.in/openpdffile.php.
CPCB, 2015a. Annual Report 2013-2014. Central Pollution Control Board. accessed on 22.09.2018. http://cpcb.nic.in/annual-report.php.
CPCB, 2015b. Inventorization of Sewage Treatment Plants. Central Pollution Control Board. accessed on 22.09.2018. https://nrcd.nic.in/.../NewItem_210_Inventorization_of_Sewage-Treatment_Plant.pdf.
CPCB, 2016. Annual Report 2014-2015. Central Pollution Control Board. accessed on 19.10.2018. http://cpcb.nic.in/annual-report.php.
Czekalski, N., Diez, E.G., Burgmann, H., 2014. Wastewater as a point source of antibiotic resistance genes in the sediment of a freshwater lake. ISME J. (8), 1381–1390. http://doi.org/10.1038/ismej.2014.8.
Diallo, A.A., Bruge're, H., Ke'roure'dan, M., Dupouy, V., Toutain, P., Bousquet-Me'lou, A., Oswald, E., Bibbal, D., 2013. Persistence and prevalence of pathogenic and extended-spectrum beta-lactamase-producing *Escherichia coli* in municipal wastewater treatment plant receiving slaughterhouse wastewater. Water Res. 47 (13), 4719–4729. http://doi.org/10.1016/j.watres.2013.04.047.
EU, 2017. The New EU One Health Action Plan against Antimicrobial Resistance. Europian union accessed 17.09.2018. https://ec.europa.eu/health/amr/sites/amr/files/amr_summary_action_plan_2017_en. pdf.
Garcia-Armisen, T., Touron, A., Petit, F., Servais, P., 2005. Sources of faecal contamination in the Seine estuary (France). Estuar. Coast 28 (4), 627–633. http://doi.org/10.1007/BF02696073.
Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G., Maurer, J.J., 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. 45 (3), 723–726. http://doi.org/10.1128/AAC.45.3.723-726.2001.
Grad, Y.H., Godfrey, P., Cerquiera, G.C., Mariani-Kurkdjian, P., Gouali, M., Bingen, E., Shea, T.P., Haas, B.J., Griggs, A., Young, S., Zeng, Q., Lipsitch, M., Waldor, M.K., Weill, F.X., Wortman, J.R., Hanage, W.P., 2013. Comparative genomics of recent shiga toxin-producing *Escherichia coli* O104:H4: short-term evolution of an emerging pathogen. Ambio 4, 00452. http://doi.org/10.1128/mBio.00452-12.
Grassholf, K., Ehrhardt, M., Kremling, K., 1983. Methods of seawater analysis. In: Grassholf, K., Ehrhardt, M., Kremling, K. (Eds.), Verlag Chemie, Weinheim, pp. 89–224.
Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S., Helmuth, R., 2003. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. J. Antimicrob. Chemother. 52, 489–492. http://doi.org/10.1093/jac/dkg362.
Guo, X., Yang, Y., Lu, D., Niu, Z., Feng, J., Chen, Y., Tou, F., Garner, E., Xu, J., Liu, M., Hochella, M.F., 2017. Biofilms as a sink for antibiotic resistance genes (ARGs) in the Yangtze Estuary. Water Res. 129, 277–286. https://doi.org/10.1016/j.watres.2017.11.029.
Gupta, G.V.M., Sudheesh, V., Sudharma, K.V., Saravanane, N., Dhanya, V., Dhanya, K.R., Lakshmi, G., Sudhakar, M., Naqvi, S.W.A., 2016. Evolution to decay of upwelling and associated biogeochemistry over the south eastern Arabian Sea shelf. J. Geophys. Res. 121, 159–175. http://doi.org/10.1002/2015JG003163.
Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.A., Bekal, S., Fairbrother, J.M., Harel, J., Marynard, C., Masson, L., 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational waters. Appl. Environ. Microbiol. 72 (6), 4200–4206. http://doi.org/10.1128/AEM.00137-06.
Hu, J.Y., Shi, J.C., Chang, H., Li, D., Yang, M., Kamagata, Y., 2008. Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. Environ. Sci. Technol. 42 (9), 3415–3420.
Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181 (6), 261–272. http://doi.org/10.1086/315217.
Johnson, J.R., Russo, T.A., 2002. Extraintestinal pathogenic *Escherichia coli* (ExPEC): the 'other bad *E. coli*'. J. Lab. Clin. Med. 139, 155–162. http://doi.org/10.1067/mlc.2002.121550.
Jyothibabu, R.E., Madhu, N.V., Jayalakshmi, K.V., Balachandran, K.K., Shiyas, C.A., Martin, G.D., Nair, K.K.C., 2006. Impact of freshwater influx on microzooplankton mediated food web in a tropical estuary (Cochin backwaters - India). Estuar. Coast. Shelf. 69, 505–518. http://doi.org/10.1016/j.ecss.2006.05.013.
Kintz, E., Brainard, J., Hooper, L., Hunter, P., 2017. Transmission pathways for sporadic Shiga-toxin producing *E. coli* infections: a systematic review and meta-analysis. Int. J. Hyg Environ. Health 220 (1), 57–67. http://doi.org/10.1016/j.ijheh.2016.10.011.
Korzeniewska, E., Korzeniewska, A., Harnisz, M., 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. Ecotoxicol. Environ. Saf. 91, 96–102. http://doi.org/10.1016/j.ecoenv.2013.01.014.
Kraft, C.A., Timbury, M.C., Platt, D.J., 1986. Distribution and genetic location of Tn*7* in trimethoprim-resistant *Escherichia coli*. J. Med. Microbiol. 22 (2), 125–131. http://doi.org/10.1099/00222615-22-2-125.
Kristiansson, E., Fick, J., Janson, A., Grabic, R., Rutgersson, C., Weijdegard, B., Soderstrom, H., Larsson, D.G., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PloS One 6 (2), e17038. http://doi.org/10.1371/journal.pone.0017038.
Krumperman, P.H., 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk source of fecal contamination of foods. Appl. Environ. Microbiol. 46 (1), 165–170.
Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., Peti, F., 2009. Occurrence of antibiotic resistance and class1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). FEMS Microbiol. Ecol. 68, 118–130. http://doi.org/10.1111/j.1574-6941.2009.00655.x.
Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant *E. coli* in UK coastal water users: environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. http://doi.org/10.1016/j.envint.2017.11.003.
Li, D., Yang, M., Zhang, J., Liu, R., Gu, X., Zhang, Y., Wang, Z., 2009. Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. Environ. Microbiol. 11 (6), 1506–1517. http://doi.org/10.1111/j.1462-2920.2009.01878.x.
Lotze, H.K., Lenihan, H.S., Bourque, B.J., Brabury, R.H., Cooke, R.G., Kay, M.C., Kidwell, S.M., Kirby, M.X., Peterson, C.H., Jackson, J.B.C., 2006. Depletion, degradation, and recovery potential of estuaries and coastal seas. Science 5781 (312), 1806–1809. http://doi.org/10.1126/science.1128035.
Luna, G.M., Vignaroli, C., Rinaldi, C., Pusceddu, A., Nicoletti, L., Gabellini, M., Danovaro, R., Biavasco, F., 2010. Extraintestinal *Escherichia coli* carrying virulence genes in coastal marine sediments. Appl. Environ. Microbiol. 76 (17), 5659–5668. http://doi.org/10.1128/AEM.03138-09.
Ma, L., Li, A.-D., Yin, X.-L., Zhang, T., 2017. The prevalence of integrons as the Carrier of antibiotic resistance genes in natural and man-made environments. Environ. Sci. Technol. 51, 5721–5728. http://doi.org/10.1021/acs.est.6b05887.
Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., Alvarez, P.J.J., 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. Water Res. 85, 458–466. http://doi.org/10.1016/j.watres.2015.09.010.
Martinez, J.L., 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc. Biol. Sc. 276 (1667), 2521–2530. http://doi.org/10.1098/rspb.2009.0320.
Martinez-Medina, M., Mora, A., Blanco, M., Lopez, C., Alonso, M.P., Bonacorsi, S., Nicolas-Chanoine, M.H., Darfeuille-Michaud, A., Garcia-Gil, J., Blanco, J., 2009. Similarity and divergence among adherent-invasive *Escherichia coli* and extra-intestinal pathogenic *E. coli* strains. J. Clin. Microbiol. 47 (12), 3968–3979. http://

doi.org/10.1128/JCM.01484-09.
Marynard, C., Fairbrother, J.M., Bekal, S., Sanschagrin, F., Levesque, R.C., Brousseau, R., Masson, L., Lariviere, S., Harel, J., 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. J. Antimicrob. Chemother. 47 (10), 3214–3221.
Meacham, K.J., Zhang, L., Foxman, B., Bauer, R.J., Marrs, C.F., 2003. Evaluation of genotyping large numbers of *Escherichia coli* isolates by enterobacterial repetitive intergenic consensus-PCR. J. Microbiol. 41 (11), 5224–5226. http://doi.org/10.1128/JCM.41.11.5224-5226.2003.
Mellmann, A., Harmsen, D., Cummings, C.A., Zentz, E.B., Leopold, S.R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S.F., Henkhaus, J.K., Leopold, B., 2012. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6 (7), 22751. https://doi.org/10.1371/journal.pone.0022751.
Menon, N.N., Balchand, A.N., Menon, N.R., 2000. Hydrobiology of the Cochin estuary system a review. Hydrobiologia 43, 149–183.
Muniesa, M., Hammerl, J.A., Hertwig, S., Appel, B., Brussow, H., 2012. Shiga toxin producing *Escherichia coli* O104:H4: a new challenge for microbiology. Appl. Environ. Microbiol. 78 (12), 4065–4073. http://doi.org/10.1128/AEM.00217-12.
Na, G., Lu, Z., Gao, H., Zhang, L., Li, Q., Li, R., Yang, F., Huo, C., Yao, Z., 2018. The effect of environmental factors and migration dynamics on the prevalence of antibiotic-resistant *Escherichia coli* in estuary environments. Sci. Rep. 8, 1663. http://doi.org/10.1038/s41598-018-20077-x.
Navia, M.M., Ruiz, J., Sanchez-Cespedes, Vila, J., 2003. Detection of dihydrofolate reductase genes by PCR and RFLP. Diagn. Microbiol. Infect. Dis. 46 (4), 295–298.
Neill, J.O., 2016. Tackling drug-resistant infections globally: final report and recommendations," in "Review on antimicrobial resistance. accessed 12.10.2018. https://amr-review.org/sites/default/files/160518.
Pei, R., Kim, S.C., Carlson, K.H., Pruden, A., 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 40 (12), 2427–2435. http://doi.org/10.1016/j.watres.2006.04.017.
Pereira, A., Santos, A., Tacao, M., Alves, A., Henriques, I., Correia, A., 2013. Genetic diversity and antimicrobial resistance of *Escherichia coli* from Tagus estuary (Portugal). Sci. Total Environ. 461–462, 65–71. http://doi.org/10.1016/j.scitotenv.2013.04.067.
Rafraf, I.D., Lekunberri, I., Anchez-Melsi, A.S., Aouni, M., Borrego, C.M., Balcazar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. https://doi.org/10.1016/j.envpol.2016.10.062.
Reinthaler, F.F., Feierl, G., Galler, H., Haas, D., Leitner, E., Mascher, F., Melkes, A., Posch, J., Winter, I., Zarfel, G., Marth, E., 2010. ESBL-producing *E. coli* in Austrian sewage sludge. Water Res. 44 (6), 1981–1985. http://doi.org/10.1016/j.watres.2009.11.052.
Rodriguez-mozaz, S., Chamorro, S., Marti, E., Luis, J., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. http://doi.org/10.1016/j.watres.2014.11.021.
Rosengren, L.B., Waldner, C.L., Reid-Smith, R.J., 2009. Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and virulence genes of fecal *Escherichia coli* isolates from healthy grow-finish pigs. Appl. Environ. Microbiol. 75 (5), 1373–1380. http://doi.org/10.1128/AEM.01253-08.
Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A., Colwell, R.R., 2000. Global spread of microorganisms by ships. Nature 408, 49–50. http://doi.org/10.1038/35040695.
Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extra-intestinal pathogenic isolates of *Escherichia coli*: ExPEC. J. Infect. Dis. 181 (15), 1753–1754. http://doi.org/10.1086/315418.
Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H.M., Langenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in The Netherlands. J. Environ. Chem. Eng. http://doi.org/10.1016/j.jece.2018.03.004.
Sambrook, J., Fritsch, E., Maniatis, V., 1989. Molecular Cloning: a Laboratory Manual. Cold spring Harbor Laboratory Press, New York.
Sasidaran, A., Saleena, M., 2011. Current status of fish waste management in Kerala, India. Waste Manag. 31, 1897–1900.
Stange, C., Sidhu, J.P.S., Tiehm, A., Toze, S., 2016. Antibiotic resistance and virulence genes in coliform water isolates. Int. J. Hyg Environ. Health 219 (8), 823–831. http://doi.org/10.1016/j.ijheh.2016.07.015.
Suhartono, S., Savin, M., Gbur, E.E., 2016. Genetic redundancy and persistence of plasmid-mediated trimethoprim/sulfamethoxazole resistant effluent and stream water *Escherichia coli*. Water Res. 103, 197–204. http://doi.org/10.1016/j.watres.2016.07.035.
Tacao, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *blaCTX-M*-like genes. Appl. Environ. Microbiol. 78 (12), 4134–4140. http://doi.org/10.1128/AEM.00359-12.
Tacao, M., Moura, A., Correia, A., Henriques, I., 2014. Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water Res. 48, 100–107. http://doi.org/10.1016/j.watres.2013.09.021.
TNIE, 2017. Cochin Port registers 17.9 per cent growth, handles 18.9 million tonnes of cargo in 2017-18. The New Indian Express. accessed 10.10.2018. http://www.newindianexpress.com/business/2017/dec/24/cochin-port-registers-179-per-cent-growth-handles-189mt-cargo-in-2017-18-1735531.html.
TOI, 2018. Tourism in Cochin. Tourism of India. accessed on 10.10.2018. https://www.tourism-of-india.com/cochin/.
Um, M.M., Barraud, O., Kerouredan, M., Gaschet, M., Stalder, T., Oswald, E., Dagot, C., Ploy, M.C., Brugere, H., Bibbal, D., 2015. Comparison of the incidence of pathogenic and antibiotic-resistant *Escherichia coli* strains in adult cattle and veal calf slaughterhouse effluents highlighted different risks or public health. Water Res. 88, 30–38. http://doi.org/10.1016/j.watres.2015.09.029.
US-CDC, 2015. U.S. National action plan for combating antibiotic-resistant bacteria (National Action Plan). The centers for Disease Control and Prevention, Atlanta, United States accessed 17.09.2018. https://www.cdc.gov/drugresistance/us-activities/national-action-plan.html.
USEPA, 2003. Bacterial Water Quality Standards for Recreational Waters. US environmental protection agency, Washington, D.C Report No. EPA/821/R-97/004.
Vishnu, P.S., Shaju, S.S., Tiwari, S.P., Menon, N., Nashad, M., Joseph, C.A., Raman, M., Hatha, M., Prabhakaran, M.P., Mohandas, A., 2018. Seasonal variability in bio-optical properties along the coastal waters off Cochin. Int. J. Appl. Earth. Obs. Geoinformation. 66, 184–195. https://doi.org/10.1016/j.jag.2017.12.002.
WHO, 2017. Critically Important Antimicrobials for Human Medicine 5th Revision. World Health Organization, Geneva, Switzerland accessed 17.09.2018. http://who.int/foodsafety/publications/antimicrobials-fifth/en/.
WHO, 2015. Global Action Plan on Antimicrobial Resistance. World Health Organization, Geneva, Switzerland accessed 17.09.2018. http://www.who.int/antimicrobial-resistance/global-action-plan/en/.
Winfield, M.D., Groisman, E.A., 2003. Role of non host environments in the lifestyles of *Salmonella* and *Escherichia coli*. Appl. Environ. Microbiol. 69 (7), 3687–3694.