Predominance of Genetically Diverse Esbl Escherichia Coli Identified in Resistance Mapping of Largest Fresh Cum Brackish Water of Vembanad Lake, India

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Research Article

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2   Predominance of genetically diverse ESBL *Escherichia coli* identified in resistance mapping of Largest fresh cum

3   brackish water of Vembanad Lake, India

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16   **Running title:** Genetically distinct AMR in *E. coli* of Vembanad Lake, India.

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26 **Abstract**

27   Antimicrobial resistance (AMR) burden in *Escherichia coli* along the 90 km stretch of Vembanad Lake,

28 Kerala, India was assessed. Seventy-seven percent of water samples drawn from 35 different stations of the Lake

29 harbored *E. coli*. Antibiotic susceptibility test performed on 116 *E. coli* isolates revealed 81% were resistant to ≥

30 one antibiotic with 39 AMR profiles, 30% multidrug resistant, 32% extended spectrum β lactamase (ESBL)

31 producers as per CLSI.The probability of isolating cefotaxime resistant *E. coli* was the highest 0.7 (P ≤ 0.05) in the

32 Lake. Genetically diverse ESBL types $bla_{TEM-116}$,$bla_{CTX-M -152}$, $bla_{CTX-M -27}$,$bla_{CTX-M -55}$, $bla_{CTX-M-205,}$ and bla_{SHV-27} were

33 identified. Molecular typing (ERIC PCR, MLST and PBRT) confirmed the diversity among *E. coli* between and

34 within the stations. ST11439 and Single and Double Loci Variants of ST443, ST4533 were identified in Multi locus

35 sequence typing (MLST) analysis. Inc plasmids (B/O, F, W, I1, FIIA, HI1, P-1α, K/B and N) identified in the Lake

36 evidences the transmission potential. Low multiple antibiotic resistance index (average < 0.2) indicating lower risk

37 to the human population albeit, an emerging concern of ESBL resistance in the Lake. The occurrence of genetically

38 variant ESBL *E. coli* in Vembanad Lake signals health hazards and necessitates pragmatizing strategic control

39 measures.

40 **Keywords**:Vembanad Lake; AntimicrobialResistance; Extended Spectrum Beta Lactamase*Escherichia coli*; Water;

41 Molculartyping

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Introduction

The human and animal health care systems, off late have been witnessing the menace of Antimicrobial Resistance (AMR) (Hawken and Snitkin 2019). The antimicrobials used in human therapeutics and animal agriculture finally enter the aquatic environment, thereby potentiating the emergence of AMR in bacteria in the environment (Riedel et al. 2019). The high genetic plasticity of bacterial communities in the aquatic ecosystem makes it a hot reservoir and carrier of AMR genes (Michael et al. 2013; Watts et al. 2017). It is important to map all the aquatic resources for the microbial safety and also in the AMR point of view remains an absolute necessity for appraising the present condition and to devise appropriate mitigation strategies.

Escherichia coli (*E. coli*) is generally a harmless symbiont in the lower intestinal tract of humans and animals, either occurs as commensal or as pathogen in intestinal / extra-intestinal locations of the body (Schroede*r et al*, 2002). It is considered a dominant fecal indicator bacteria for food and water quality testing (Hassuna et al. 2020). AMR in *E. coli* is in an escalating trend worldwide and is a growing concern for both developed and developing countries as they are frequently associated with treatment failures, especially in urinary tract infections (Patterson 2000; Queenan and Bush 2007). Extended Spectrum Beta-Lactamases (ESBL) and carbapenemases are β-lactamase enzymes that are capable of hydrolyzing oximino-cephalosporins, penicillins, cephalosporins, monobactams; and carbapenems respectively which confer resistance and are very important in the realm of AMR. Moreover, ESBLs are often encoded on plasmids that also carry additional genes of resistance for aminoglycosides, chloramphenicol, sulphonamides, trimethoprim, and tetracyclines, thereby extending the resistance profile through cross resistance (Zhang et al. 2016).

A systematic and scientific understanding of the prevalence of AMR bacteria is pivotal to minimize their spread and hence, surveillance becomes an integral part of control strategies (Kronvall 2003; Liu et al. 2016). To evaluate the public health risk in water bodies, it is crucial to understand the development of AMR in this indicator organism and their genetic relatedness (Versalovic et al. 1994). Several molecular tools commonly used in fingerprinting studies of *E. coli* for identifying the source of the contamination include Enterobacterial repetitive intergenic consensus sequences (ERIC-PCR), Repetitive extragenic palindromic-PCR (rep-PCR), BOX sequences (BOX-PCR), Pulse field gel electrophoresis (PFGE), Density gradient gel electrophoresis (DGGE), Amplified fragment length polymorphism (AFLP), Random amplification of polymorphic DNA (RAPD), Multi Locus

Sequence Typing (MLST), Plasmid Based Replicon Typing (PBRT) and phenotypic methods like antimicrobial resistance profiling, carbon utilization, *etc* (Nemoy et al. 2005; Mohapatra et al. 2007). Of all these, PBRT and ERIC PCR are very powerful and cost-effective tools next to MLST, and PFGE for the discrimination of *E. coli* based on the genetic relatedness (Harwood et al. 2014; Kim et al. 2017). Implementation of these phenotypic and genotyping tools in fecal indicator bacteria from water bodies remains an indispensable marker tool for microbial source tracking using bacteria (Kronvall et al. 2003).

The use of clinical breakpoints in determining AMR in health care may not be suitable for assessing the environmental and food associated strains of pathogens. Hence, the use of Epidemiological Cut-off values (designated as Ecoff by EUCAST, EcoV by CLSI) is encouraged (Krumperman 1983; Aarestrup et al. 2007) as it differentiates Wild Type (WT; without resistance) population from Non-Wild Type (NWT; with acquired resistance) population of particular bacterial strains to a specific antibiotic. Multiple Antibiotic Resistance (MAR) index estimates the risk associated to a population with the exposure of *E. coli* isolated from food or water, by distinguishing the origin of the isolate from high or low-risk environments.

Vembanad Lake which spreads over three districts (Alappuzha, Kottayam, and Ernakulam) of Kerala, India is considered to be the longest (96 km) in India (09˚00' -10˚40'N and 76˚00'-77˚30'E). It has an inflow of water from six major rivers and is a complex wetland system (Haldar et al. 2019). Freshwater dominant southern zone and a brackish water dominant northern zone separated by brackish water regulating barrage (bund) are the salient features of the Lake. Livelihood activities in the Lake are agriculture, fishing, tourism, inland navigation, coir retting, and lime shell collection. This biologically diverse Lake is facing threats due to industrialization and urbanization (Selvam et al. 2012). Tourism, the major activity in this lake, is concentrated in the southern zone.

The information on AMR in Vembanad Lake and their genetic characteristics is scant. The present study planned to understand 1. The prevalence of *E. coli* in the Vembanad Lake at different stations with CLSI breakpoints; 2. Determine the prevalent AMR patterns and multidrug resistance (MDR); 3. Estimate the risk associated by multiple antibiotic resistance index (MAR) and 4. To link the genetic diversity of ESBL genes with the ERIC PCR tool as a pilot study for microbial source tracking.

Materials and methods

Study area

Water samples were collected during December 2018 from 35 different stations (A to AJ) of Vembanad Lake, Kerala, India which has Ernakulam, Alappuzha, and Kottayam regions (Fig. 2a and 2b). Lake spreads from the northern estuary region in Ernakulam at Azhikode/Munambam and extends to the south in Kottayam and ends at the Alapuzha district of Kerala. The selection of locations was based on the fisheries activities, tourism, human habitation, inflow mouth of the tributaries, the northern and southern part of the Vembanad Lake, saltwater regulator (Thaneermukham Barrage) and covering all the three regions (Haldar et al. 2019). Surface water (500mL) samples were collected in sterile screw-capped bottles from the boat and brought to the laboratory in chilled condition for further use (Morgan et al. 1976; Baird et al. 2017).

Isolation and identification of *E. coli*

The collected water samples were enriched in 3x sterile Presence-Absence (P-A) broth (1: 3 ratio) and after overnight incubation at 35 ±1°C, streaked on pre-set Eosin Methylene Blue (EMB) agar for primary screening from which 2-10 characteristic colonies were picked and secondary confirmation was carried out on Mac Conkey and HiCrome ECC agars (Baird et al. 2017). Gram-negative rods with catalase production, oxidase non-production, and IMVC test (++--) characteristics were subjected for molecular confirmation. DNA template was prepared with the washed cell suspension from 1mL overnight culture in 1x TE Buffer pH 8.0 by heat shock method and stored at -80°C until further use. PCR reaction was carried out in thermocycler (Veriti, Applied BioSystem) using *uidA* primers (Godambe et al. 2017), and *E. coli* that did not produce amplicons specific for *uidA* were further tested for *phoA* gene-specific primers (Murugadas et al. 2016) and PCR products were analyzed in 2% agarose gel in 1x TAE buffer at 80V for 1h in horizontal gel electrophoresis system and visualized in the gel documentation system.

In vitro antimicrobial susceptibility testing

Phenotypicantimicrobial susceptibility testing (AST) was carried out by Disc Diffusion Assay (DDA) with15 antibiotics belonging to nine different classes (Table. 1) and the plates were incubated at 35°C for 16-20 h. The turbidity of overnight grown cultures was adjusted to 0.5 McFarland standard and swabbed onto Mueller Hinton

128 Agar (MHA) (BD Difco). The AMR pattern was determined in accordance with CLSI (CLSI 2019) and WHONET

129 software version 5.6 (Stelling and O'Brien 1997).

Phenotypic confirmation of ESBL, CRE in *E. coli*

131 Isolates which produced zone diameter of ≤17mm, ≤22mm, ≤27mm, ≤25mm, and ≤27mm against

132 cefpodoxime (10μg), ceftazidime (30μg), aztreonam (30μg), and cefotaxime (30μg), respectively were considered

133 as presumptive ESBL producers (CLSI 2019). These presumptive ESBL isolates (n=83) were further tested in

134 combined disk diffusion (CDD) assay, a confirmatory test with clavulanic acid (10 μg) in MHA agar, and incubated

135 at 35°C for 16 to 18h. Isolates showing ≥5mm zone diameter when clavulanate was combined with cefotaxime or

136 ceftazidime were confirmed as ESBL producers. Those isolates without a significant effect of clavulanic acid and

137 resistant to cefoxitin (zone diameter ≤14 mm) were considered as AmpC-producers (Jacoby 2009). MIC was

138 performed with E-test strips for *E. coli* (n=14) showing reduced susceptibility to imipenem (Himedia, India).

Molecular characterization of ESBL and other Antibiotic Resistance genes

140 *E. coli* isolates (n=94) that were phenotypically resistant to various antibiotics were further screened by

141 PCR for very abundant and important AMR (AR) genes. Presumptive ESBL *E. coli* (n=83) which showed reduced

142 susceptibility to cefpodoxime, ceftazidime, aztreonam, and cefotaxime were tested for $bla_{CTX-M\ group\ 1}$, $bla_{CTX-M group\ 2}$,

143 $bla_{CTX-M\ group\ 9}$, $bla_{CTX-M\ group\ 8/25}$, bla_{SHV}, and $bla_{OXA-1-like}$ genes specificfor broad-spectrum β-lactamases and ESBL

144 detection (Dallenneet al. 2010). *E. coli* (n=14) which showed reduced susceptibility to imipenem were tested for

145 bla_{IMP}, bla_{KPC}, bla_{VIM}, and bla_{NDM} genes specific for carbapenem resistance (Bush and Jacoby, 2010; Sahni et al.

146 2018). *E. coli* (n=23) showing phenotypic tetracycline resistance were tested for *tetA*and *tetB* genes for tetracycline

147 resistance; isolates (n=6) with phenotypic chloramphenicol resistance was tested for *cat A* and *cat B* genes (Kim et

148 al. 2013); *E. coli* (n=14) were tested for *sul1, sul2* and*dfrA* genes for folate pathway inhibitors resistance (Momtaz et

149 al. 2012).

ESBL allele identification by sequencing analysis

151 *E. coli* which produced amplicons of a partial length corresponding to (bla_{TEM}, bla_{SHV}, bla_{CTX-M-}

152 $_{group1}$, $bla_{CTX-M-\ group\ 25}$, $bla_{CTX-M-\ group\ 2}$, $bla_{CTX-M-\ group\ 9}$) gene sequences were amplified as mentioned above (Dallenne

153 et al. 2010) and products were purified by gel elution (Thermofisher Scientific) and outsourced for sequencing in an

154   automated sequencer (ABI 1377) at AgrigenomePvt Lab (Kochi, India). Quality checks and sequence similarity was

155   verified in NCBI https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/.

156   **MLST and eBurst analysis**

157   MLST analysis was carried out for selected eight ESBL *E. coli* with differences in ESBL genes viz.,

158      $bla_{TEM-116}$, $bla_{CTX-M-27}$, $bla_{CTX-M-55}$, $bla_{CTX-M-152}$, $bla_{CTX-M-205}$, and bla_{SHV-27} PCR amplification was carried out with10X

159   Extaq master mix (DSS Takara Bio India). The reactions for all the housekeeping genes viz., *adk, fumC, gyrB, icd,*

160   *mdh, purA, and recA* were amplified as per Enterobase protocol (Wirth et al. 2006). The agarose gel extracted

161   amplicons of various fragments were outsourced for Sanger sequencing at AgrigenomePvt Lab (Kochi, India). After

162   the quality check, the allele numbers for the gene fragments and sequence type for the ESBL *E. coli* were deduced

163   from the public domain PubMLST (https://pubmlst.org/bigsdb?db=pubmlst_escherichia_seqdef). eBurst analysis

164   for the identified STs was carried out in Phylovizsoftware (https://online.phyloviz.net/) taking into account single

165   and double locus variants of the identified clones.

166   **Plasmid Based Replicon Typing**

167   Plasmid characterization of the ESBL *E. coli* isolated from the water of Vembanad Lake was carried out by

168   plasmid-based replicon typing (PBRT) (Carattoli et al. 2005; Johnson and Nolan, 2009). Three multiplex PCR

169   reactions were performed for each ESBL *E. coli* and identified the replicon plasmid present in the ninety-four *E. coli*

170   isolates. Modification in the use of 2X Phusion U Green multiplex PCR master mix (ThermoScientific) and the type

171   of Inc Plasmid identified in the study was deduced (Johnson & Nolan, 2009).

172   **ERIC PCR fingerprinting and Cluster analysis**

173   ERIC – PCR reactions were performed in duplicate for each isolate in 25µl volume containing3µl of *E .*

174   *coli* genomic DNA, 2.5mM $MgCl_2$,1U Taq polymerase, 0.2mM dNTPS, 1X PCR buffer, 1 µM of each primers

175   (ERIC 1 and ERIC 2) and final volume adjusted with nuclease-free water.The reaction was carried out in 0.2 ml

176   PCR tubes always in same thermal cycler (Nemoy et al. 2005; Mohapatra et al. 2007)**.** Ninety four*E. coli* isolates

177   that were resistant at least to one antibiotic were subjected to ERIC PCR analysis. The PCR product was visualized

178   after electrophoresized in 3% agarose gel in maxi preparation with 120V for 3h and gel images were captured in the

179   gel documentation system (Syngene). Phylogenetic tree was constructed in GelJ software after visually comparing

180 the banding pattern for 94 *E. coli* isolates.DNA ladder (100-bp) was used for the normalization. The phylogenetic

181 tree was constructed based on the similarity calculated by Pearson correlation between the fingerprints with the

182 tolerance of 1% and grouping of the fingerprints was carried out with the help of the algorithm unweighted-pair

183 group method using arithmetic averages (UPGMA) (Rasschaert et al. 2005).

Estimation of MDR, MAR index and Epidemiological cut-off

185 MDR was defined as non-susceptibility to at least one agent in three or more antimicrobial categories and

186 the MDR was determined (Magiorakos et al. 2012) and the Multiple Antibiotic Resistance (MAR) index was

187 estimated (Krumperman 1983). The Ecoff for the environment associated *E. coli* was determined as per the

188 normalizedresistance interpretation method of Kronvall (2003) using http:/www.bioscand.se/nri/. Isolates were

189 categorized as either Wild type (WT) or Acquired Resistant type or non-wild type (NWT) based on the Ecoff value

190 determined for each antibiotic.

Statistics and cluster analyses

192 Chi-square statistical analysis was carried out with SAS 9.3 for finding the association between cefoxitin,

193 cefotaxime, cefpodoxime, and ceftazidime to the other antibiotics tested. The binomial logistic regression model was

194 used to predict the probability for the isolate resistant to particular antibiotics under the study in Vembanad Lake

195 water and the binomial logistic regression is given below

196 $\text{logit}\left(\frac{P}{1\text{-}P}\right) = \beta_0,$

197 where P is the probability of the isolate resistant to different antibiotics and β_0 is intercept. The parameter β_0 was

198 estimated by maximum likelihood method. The predicted probability value for the isolate to resistant to different

199 antibiotics is obtained from the formula given below

200 $\hat{P} = \dfrac{\exp(\hat{\beta}_0)}{(1 + \exp\exp(\hat{\beta}_0))}$

201 Cluster analysis was carried out based on hierarchical cluster method for the parameters AMP10, CPM30,

202 CTX30, CX30, CAZ30, MRP10, GEN10, TE30, CIP5, COT25, C30 based on the AMR profiles. The values of

203 the antimicrobial resistance were plotted as 0 or 1 corresponding to the absence or presence of resistance

204   respectively. Binary Squared Euclidean Distance matrix was generated using AMR data between two cultures. The

205   dendrogram was generated based on the similarity matrix in SPSS software version 16.

Results and discussion

Prevalence of *E. coli* in the lake

208   The study is the first of its kind that established the AMR pattern in 35 stations of the Vembanad Lake,

209   Kerala, India. *E. coli* was detected in 77% (27/35) of the sampled stations. After initial enrichment, primary and

210   secondary screening, a total of 116 *E.coli* were detected from 27 different points in the Lake. All 116 isolates

211   yielded specific amplicon in PCR targeted *uid* A (168bp) or *phoA* (999bp) genes and all of them belonged to biotype

212   1 (IMVC result: ++- -). The Alapuzha region of the Vembanad Lake water was comparatively safe in harboring *E.*

213   *coli* (59%) compared to Kottayam (90%) and Ernakulam (100%).

214   Antimicrobial resistance (AMR) is a growing threat to the human population as it significantly curtails

215   treatment options. Surface waters in aquatic bodies, owing to their microbial diversity and moving nature,play a

216   considerable role in the emergence and transmission of AMR (Kittinger et al. 2016). Aquatic reservoirs have been

217   described as hotspots for AMR emergence across the globe (Watts et al. 2017). Monitoring aquatic reservoirs for

218   microbial quality and AMR is an indispensable tool for devising control strategies to protect human health. In this

219   context, Vembanad the largest lake of India was assessed for AMR burden on the environmental. This is important

220   to mitigate the AMR sourge. The present study corroborates with the earlier findings on the incidence of *E. coli* in

221   selected areas of Vembanad Lake as 85.6–86.7% and 100% which attributed to the anthropogenic activity and

222   seafood processing industries (Hatha et al. 2004; Chandran et al. 2008). Variations in the occurrence of *E. coli* in

223   Lake water were observed elsewhere and in coastal water in Kuwait (Al-Mossawi et al. 1982; Riedel et al. 2019).

AMR profiles identified in the Lake

225   Antibiotic resistance profiling revealed that all the 116 *E. coli* isolates were susceptible toGentamicin,

226   however, 81% of the *E. coli* isolates were resistant to a minimum of one and a maximum of nine antibiotics. A total

227   of 94 AMR *E.coli*were isolated from the Lake. AMR was observed in 86% (43/50), 79% (34/43), and 74% (17/23)

228   of the *E. coli* isolated from Kottayam, Alapuzha and Ernakulam regions of Vembanad Lake, respectively (Table S1).

229   High levels of MDR was detected in Kottayam (34%), followed by Alapuzha (30%), Ernakulam (22%). Frequencies

of resistance were estimated by WHONET software version 5.6 with CLSI interpretations (Fig.1, Table 1). A total

of 39 AMR patterns were observed among 94 isolates from 27 positive sampling stations indicating extensive AMR

diversity in the *E. coli*; bothbetween and within the sampling stations of the Lake. CTX pattern alone contributed

39% while CTX-TCY and AMP-CTX-CAZ-CRO-ATM patterns contributed 7.4% each and others contributed 1

to 3% (Table. 2).

E. coli isolated from Cochin estuary were resistant to ampicillin (65.33%) followed by nalidixic acid

(37.33%), tetracycline (33.33%), and others < 17% (Sukumaran et al. 2012). The observations are similar to findings

in the prevalence of lowest resistance to aminoglycosides and chloramphenicol, however, tetracycline resistance was

20% (Sukumaran et al. 2012). Also, MDR was higher (53.33%) compared to the present study (30.17%)(Sukumaran

et al. 2012). The ncreased MDR in the previous study can be attributed to the limited number of sampling stations

(n=5) and proximity to urban habitation and seafood processing factories. Amoxicillin-clavulanate resistance

showed the highest frequency (71.1 %), followed by ampicillin (63.9 %), cefuroxime (21.1 %), ciprofloxacin (17.5

%), cefotaxime (15.7 %), ceftriaxone (10.8 %), and gentamicin (6.6 %) in Somesul Mic River of Romania (Farkas et

al. 2016). However, no study described ESBL producing *E. coli* at different locations representing the entire lake.

Prevalence of ESBL producers

Combined disk diffusion (CDD) assay performed on 83 presumptive ESBL *E. coli* isolates withreduced

susceptibility to CTX, CAZ, CPD, and ATM, revealed that 37 were phenotypically confirmed as Class A ESBL

producing *E. coli* (Kittingeret al. 2016) and were designated as CDD^{+ve}while the remaining 46 isolates that did not

show increased zone size ≥5mm in CDD assay were designated as CDD^{-ve}. ESBL genes screening for CDD^{+ve} and

CDD^{-ve}of *E. coli* isolates revealed that 85.5%, 21.7%, 10.8%, 1.2%, 2.4%, and 2.4% of the isolates harbored

bla$_{TEM}$,*bla*$_{CTX-M group 9}$, *bla*$_{CTX-M group 1}$, *bla*$_{CTX-M group 8/25}$, *bla*$_{CTX-M group 2}$, and*bla*$_{SHV}$genes, respectively (Table 3). Two

isolates from Ernakulam location co-harbored *bla*$_{TEM}^{+}$, *bla*$_{SHV}^{+}$, *bla*$_{CTX-M group 8/25}^{+}$ and *bla*$_{CTX-M group 9}^{+}$,*bla*$_{TEM}$

$^{+}$,*bla*$_{CTX-M group 1}^{+}$ genotypes. Out of 23 phenotypically tetracycline-resistant *E. coli*, only 35% harbored *tetA* gene

but none of the isolates carried *tetB* gene. It is important to note that *bla*$_{OXA-1-like}$gene for ESBL, *sul1*, *sul2*, and *dfrA*

genes for the folate pathway inhibitors resistance, *catA*, *catB* for chloramphenicol resistance, and *bla*$_{NDM}$, *bla*$_{KPC}$,

bla$_{VIM}$, *bla*$_{IMP}$ for carbapenem resistance were not detected in the *E.coli* isolates of Vembanad Lake. The relationship

between phenotypic resistance and its association with the corresponding antibiotic resistant genes remains

257 intriguing. MIC level of phenotypic imipenem resistant isolates in disk diffusion assay (DDA) ranged between 0.19

258 and 0.25μg/ml; the MIC observed was lower than the clinical resistance criterion of CLSI.

259   Tetracycline resistance mediated by *tetA* was very less in the present study compared to the aquaculture

260 setting (Shivakumaraswamy et al. 2019). *E. coli* with phenotypic cefoxitin resistance in DDA showed an increase in

261 zone diameter with clavulanic acid and hence, may not be ampChyper producer(Jacoby, 2009). The identification of

262 *bla*$_{TEM}$ positive non-ESBL producers phenotypically in the present study wasreported earlier in the hospital patients

263 (Bajpai et al. 2017), possibly due to the nonexpression of ESBL genes without the antibiotic pressure or due to the

264 presence of TEM-1, TEM-2, and TEM-13 which are not ESBLs (Paterson&Bonomo, 2005). In CDD assay only 37

265 of the 83 isolates were phenotypically identified as ESBL producers but the genetic determination of antibiotic

266 resistance genes revealed that the majority of the isolates harbored at least one of the ESBL variants. This may be

267 due to several reasons such as the probability of possessing another variant of ESBL genes generally cannot be

268 disregarded or could be the masking of additional enzymes such as AmpC β-lactamases or carbapenamases (Poulou

269 et al. 2014) To confirm that sequencing analysis was carried out for the β-lactamase genes.

270 **Molecular Typing of ESBL *E. coli***

271 **ESBL allele identification by sequencing analysis**

272   Sequencing analysis of partial genes of *bla*$_{TEM}$,*bla*$_{CTX-M\ group\ 9}$, *bla*$_{CTX-M\ group\ 1}$, *bla*$_{CTX-M\ group\ 8/25}$, *bla*$_{CTX-Mgroup}$

273 $_2$, and*bla*$_{SHV}$genes amplicon revealed that *bla*$_{TEM}$genes belonged to*bla*$_{TEM-1,}$ and*bla*$_{TEM-116}$; *bla*$_{CTX-M\ group\ 9}$ belonged to

274 *bla*$_{CTX-M-27}$; *bla*$_{CTX-M\ group\ 1}$ gene belonged to *bla*$_{CTX-M\ -55}$; *bla*$_{CTX-M\ group\ 8/25}$belonged to *bla*$_{CTX-M\ -152.}$; *bla*$_{CTX-M-group\ 2}$

275 belonged to *bla*$_{CTX-M-205}$ and *bla*$_{SHV-27}$ belonged to *bla*$_{SHV-27}$ and the results were summarized (Table3).Resistance

276 mapping for the Vembanad Lake concerning the sampled locations and ESBL subtypes indicates that the majority of

277 ESBL *E. coli* were nearer to the Kottayam region of the Lake and south of the Alappuzha part of the Lake had no

278 CTX mediated ESBL producers (Fig. 2b).

279   Gene sequencing analysis revealed that*bla*$_{TEM}$genes belonged to*bla*$_{TEM-1,}$ and*bla*$_{TEM-116}$ which were

280 identified earlier in the urban aquatic environments of India; 15 of the *bla*$_{TEM}$ were *bla*$_{TEM-1}$indicating it as the broad

281 spectrum β-lactamase producer (non-ESBL). However, *bla*$_{TEM-1}$ gene was carried in the majority of the *bla*$_{CTX-M}$

282 producing isolates of *E. coli* and hence, the isolates were ESBL producers (Paterson and Bonomo 2005; Singh et al.

283   2018). $bla_{CTX-M\ group\ 9}$ belonged to $bla_{CTX-M-27}$ a single nucleotide variant of $bla_{CTX-M-14}$ were identified in Germany,

284   Netherlands and Japan (Matsumura et al. 2015; Franzet al. 2015; Ghosh et al. 2017) and in rivers and lakes of

285   Northwest China (Liu et al. 2018); $bla_{CTX-M\ group\ 1}$ gene belonged to $bla_{CTX-M\ -55}$ identified in Japan, Netherlands

286   (Matsumura et al. 2015; Franz et al. 2015); $bla_{CTX-M\ group\ 8/25}$ belonged to $bla_{CTX-M\ -152,}$ a novel variant form of

287   the$bla_{CTX-M\ group-25}$identified in the water of river Yamuna of India (Azam et al. 2016); $bla_{CTX-M-group\ 2}$ belonged to

288    $bla_{CTX-M-205}$ the particular ESBL type in India is not available in the public domain and probably this is the first

289   report in India for the presence of $bla_{CTX-M-205}$ in the Lake; bla_{SHV}belonged to bla_{SHV-27}has been detected in the

290   Urban riverine environment in India and in the community set up of Morocco (Barguigua et al. 2013; Mondal et al.

291   2019). There is no such study conducted in different geographical stations of the Vembanad Lake. In the context of

292   the epidemiology, $bla_{CTX-M-55}$ is the second most common ESBL-encoding gene in Asian countries, and in the

293   global epidemiology, the genotype $bla_{CTX-M-27}$ a single loci variant of $bla_{CTX-M-14}$ has slowly replaced other CTX-M

294   genotypes although $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ are leading clonesand $bla_{CTX-M-27}$ is now considered as a stable

295   reservoir for the food animals in China (Bevan et al. 2017). In 27 occasions in the Lake, the ESBL *E. coli* co-existed

296   with either $bla_{TEM-116}$,$bla_{CTX-M-27}$, $bla_{CTX-M\ -55}$, or bla_{SHV-27} .Co-existence of CTX-M types, TEM, and SHV were

297   reported in India, Saudi Arabia,and Japan indicating the increased risk of treating these infections and co-evolving

298   of two or three types of ESBL genes within an *E. coli*(Harada et al. 2013; Sharma et al. 2013; Hassan and Baha

299   2014). Resistance mapping of the ESBL types in relation to the sampling points has clearly identified that the

300   Kottayam region only harboured $bla_{CTX-M-205}$.

301   In the Indian health care system, the major variants of ESBL producers harboredbla_{TEM} followed by bla_{CTX-}

302    $_{M\ -1,}$ bla_{OXA}, bla_{SHV} and $bla_{CTX-M-\ group-2}$(Gautamet al.2019); $bla_{CTX-M\ group-1}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-24}$,

303    $bla_{CTX-M-27,}$ bla_{SHV-1} in Lake Zürich and Lake Thun, Switzerland (Abgottspon et al. 2014), and $bla_{CTX-M\ group\ 1}$,

304    $bla_{CTX-M-3}$, $bla_{CTX-M-15}$, $bla_{CTX-M-55}$, $bla_{CTX-M-79}$, $bla_{CTX-M-14}$, and $bla_{CTX-M-27}$in the water samples of Lake in

305   Switzerland (Zurfluh et al. 2013). However, in the present study, $bla_{OXA-1-like}$ was not detected, whereas, bla_{TEM}, and

306    $bla_{CTX-M\ group\ 9}$ were dominant at 85.5% and 21.68%, respectively.

Genotyping of *E. coli* by ERIC- PCR

308   ERIC-PCR image analysis in Gel J software delineated the94 isolates of *E. coli* into five major clusters.

309   Out of five clusters (EC1-EC5), the cluster EC4 contained the maximum number of isolates (n=33) belonging to

310 Kottayam, Alappuzha, and Ernakulam region isolates; Cluster EC1 carried the majority of the Alapuzha region

311 *E.coli*; Cluster EC2 carried Kottayam and Alappuzha region isolates only and cluster EC5 contained isolates

312 belonging to Ernakulam and Kottayam regions. There existed diversity in the *E. coli* isolated from various sites in

313 the Vembanad Lake but similarities also existed between and within the isolates from different geographical

314 locations of the Lake. Within the clusters, several clades were formed that indicated very closely related *E*

315 *.coli*isolates from different sites of the Lake (Fig. 3).

316   ERIC PCR analysis and clustering of the phenotypic AMR profile data revealed that the multidrug-resistant

317 isolates from stations O and P were clustered along with the AC, stations I, and AD2 was grouped in the single

318 cluster indicating the mixing of water of Kottayam and Alappuzha stations near the barrage. Among the 35 stations,

319 16 stations harbored bla_{CTX-M}types of ESBL and higher number of ESBL were detected near the barrage region.

320 **Multi Locus Sequence Typing**

321   MLST analysis of the selected ESBL *E. coli* revealed that $bla_{TEM-116}$,$bla_{CTX-M-55}$,and bla_{SHV-27}belonged to

322 new STs as a single locus variant of previously existing STs, and $bla_{CTX-M-27}$ belonged to ST11439. ESBL *E. coli*

323 belonging to $bla_{CTX-M-152}$ and $bla_{CTX-M-205}$had entirely different allelic profiles with the later ST matched to only one

324 *fumc* locus. eBurst analysis revealed that clones AD1 and X2 had the same profile for 6 loci, J2 and S1 had the same

325 profile for 6 loci, others were distantly grouped in 5 different clonal complexes (Fig 5). X2 and AD1 were double

326 locus variants (DLV) of ST 1049; F6 was a single locus variant (SLV) of ST3188; J5 and S1/J2 were SLV of

327 ST4533 and ST3600 clones, respectively.

328   The ESBL *E. coli*clone ST 11439, SLV of ST4533, and ST10987 identified in the study do not have any

329 clinical implications in human and animal sectors. However, the ESBL *E. coli*clone X2 and AD1 were Double locus

330 variant (DLV) of ST 1049, the descendant of the Clonal Complex (CC) ST155 which has zoonotic potential were

331 reported in sewage and drinking water of Kerala and Maharastra in India (Salim et al. 2019; Rayasam et al. 2019).

332 This clone ST155 has been recognized as the most important strain that has an intrinsic ability to acquire colistin

333 resistance (Matamoros et al.2017). Likewise,the SLV clone of ST443 belonging to clonal complex ST205 ESBL *E.*

334 *coli*was identified in wild birds in Pakistan, Chile, Portugal, Sweden, and Switzerland (Guenther et al. 2011;

335 Hernandez et al. 2013; Zurfluh et al. 2013; Mohsin et al. 2017; Atterby et al. 2017). However, these DLV of ST155

336 and ST205 were isolated as ESBL *E. coli* in the present study from the non-clinical environment which has

337 anthropogenic activity as well as wild bird populations. In the present study, ESBL *E. coli*belonging to *bla*CTX-M-

338 27was not linked to the ST131 clone of Germany and Japan (Matsumura et al. 2015; Ghosh et al. 2017).

Plasmid Based Replicon Typing

340   PBRT analysis revealed that thirty-three of the identified *E. coli /* ESBL *E. coli* carried different types of

341 Inc Plasmids *viz.*, B/O, F, W, I1, FIIA, HI1, P-1α, K/B, and N. Twenty patterns were observed in carrying these Inc

342 plasmids. Eighteen of the *E. coli* carried B/O plasmid, followed by F plasmid. *E. coli* from eighteen stations

343 harbored Inc plasmids. Eight stations in Kottayam harbored Inc plasmids in contrast to Ernakulam and Alappuzha,

344 which harbored in 5 and 6 of their stations, respectively. IncB/O plasmid containing *E. coli* was present in 10

345 stations in the Lake (Table. S1).

346   In the present investigation, the very important mobile genetic element (MGE) *i.e.* plasmid was identified

347 in 33 *E. coli*in the Lake water. ESBL *E. coli* isolates from the Kottayam stations carried multi-replicon plasmid

348 (Table S1) as identified in surface water from watersheds in Northeast Georgia, USA, and elsewhere in the other

349 clinical infectious conditions (Carattoli et al. 2008; Cho et al. 2019). Even though resistance plasmids of the same

350 genetic types were observed in human and animal infections as well as in other environments and food, there exists

351 a heterogeneous genotype when one or two molecular tools/methods were used together for subtyping (Lazarus et al.

352 2015). The same heterogeneity was identified in the present study as evidenced by different ESBL *E. coli* harboring

353 various Inc Plasmids and when compared in MLST they were either SLV or DLV or an entirely new allelic profile

354 (Carattoli 2009; Rozwandowicz et al. 2018).

Epidemiological cut off and MAR index

356   Mean MAR index for environmental *E. coli* was 0.14 (ranged from 0.0 to 0.6) and region-wise MAR index

357 for Alapuzha, Ernakulam, and Kottayam regions werealso below the risk criteriaof 0.2 (Table S1). The majority of

358 the sampling points in the Lake had a MAR index of less than 0.2 and only 25 isolates from 12 stations exceeded the

359 MAR index of 0.2. The geographical points AB and AD of the Lake harboured more AMRisolates with >0.2 MAR

360 index and AC point harbored maximum MAR index of 0.6 which belonged to Kottayam region of the Lake. It is

inferred that the Kottayam region of the Vembanad Lake carried diverse and high-risk AMR isolates which is the major tourist point of the state. High-risk areas identified in the study are marked in the resistance map (Fig. 2b).

The stations of Alapuzha (I, J, O & P) had MAR index greater than 0.2 and harbored more MDR isolates, among the sites O and P had more proximity to fish processing activities while the stations I and J were close to industries. The sites (AB, AC and AD) nearer to the mouth of the rivers and barrage harbored more MDR *E. coli* and crossed the MAR risk index limit. Rivers and lakes are important reservoirs of drug-resistant bacteria which collect effluents from various sources such as wastewater treatment plants, the water of urban or industrial effluents, agricultural runoff, or rain (Lupo et al. 2012; Michael et al. 2013) and the possible reasons for more AMR strains of *E. coli* in the southern part of the Lake could be dense of tourism-related activities, highest population density and ceasing of the tidal flushing action and growth of weeds due to the closure of the barrage (Menonet al. 2000; Michael et al. 2013). The Ernakulam region of the lake had MAR index of < 0.2 indicating low-risk sources of *E.coli*. The present study showed the occurrence of AMR *E. coli,* both in saline and freshwater environments of the Lake which indicates that the microorganism survives and possibly transfers the antibiotic resistance to another species. Hence, these points in the Vembanad Lake need to be targeted for further monitoring and for the development of strategic control measures as described in Fig.2b. The Alapuzha region of the Vembanad Lake water was less in hazard in view of harbouring fewer *E. coli* (59%) compared to Kottayam (90%) and Ernakulam (100%).

Ecoff value determined for the *E. coli* isolates of the present study revealed that the majority of these isolates were wild type in nature for the tested antibiotics; however, the presence of antibiotic resistance genes cannot be disregarded. When applying Ecoff values, the decreased susceptibility to amoxicillin/clavulanic acid (45% of isolates), ceftazidime (39% of isolates), ceftriaxone (48% of isolates), ciprofloxacin (29% of isolates), and imipenem (2% of isolates) was observed. The over and frequent misuse of antibiotics in various sectors resulted in changing antibiotic resistance profiles of microorganisms amongst bacterial populations (Byarugaba 2004). The cephalosporin misuse in the hinterland regions are brought to the Vembanad Lake by different waterways (Chandy et al. 2013)and this selective pressure possibly had resulted in the increased detection of cephalosporin-resistant *E. coli* in this study. Extensive use of third-generation cephalosporins for humans and veterinary purposes has led to an increased incidence and distribution of ESBLs and *AmpC* in bacteria.The MAR index is a good risk assessment indicator tool and the threshold value of >0.2 MAR index has been applied to differentiate low and high-risk

388 regions where antibiotics were inappropriately used (Riaz et al. 2011). Such an analysis exudes an idea of the

389 number of bacteria showing antibiotic resistance in the risk zones of the study. Based on our findings certain

390 locations in the Vembanad Lake, especially the Kottayam region, had MAR indices of >0.2 with more diverse ESBL

391 genotypes were identified confirming that there was selective pressure in this part of the Lake, and the region is a

392 tourist spot too (Davies and Brown 2016). However, the average of the region-wise MAR and overall MAR of

393 Vembanad Lake did not exceed the threshold value of 0.2. The region in this study requires special attention for

394 devising the control strategy.

395 **Statistical and cluster analyses**

396 The Chi-square analysis revealed that AMP resistance was significantly($P<0.01$) associated with CTX,

397 CPD, CAZ resistance; AMC with CPD resistance; CRO with CAZ; ATM withCTX, CAZ; IPM with CTX CPD;

398 NAL with CPD; CIP with CPD; SXT, and CPD. However, TET and CHL resistance were not associated with FOX,

399 CTX, CPD, and CAZ ($P>0.05$). Interpretation based on the logistic regression analysis revealed that the highest

400 probability of *E. coli* in Vembanad Lake being resistant was towardscefotaxime (0.7) followed by ampicillin (0.3)

401 as depicted in Table 1. Clustering of phenotypic AMR profile data produced 3 clusters with all the susceptible

402 isolates grouped to a single cluster EC1along with other fewer resistant isolates; EC2 cluster contained 15 isolates

403 with multidrug resistance from all the three regions, EC3 cluster containing 4 isolates belonged to the Kottayam

404 region of the Vembanad Lake and adjacent of the Alapuzha region with pattern matching to 7

405 antibiotics.Dendrogram with the isolate identity and station number is depicted in Fig. 4.

406 The present study has identified ESBL *E. coli* with genetic makeup *viz.*, ST11439-$bla_{CTX-M-27}$ - IncF

407 plasmid; STnew (SLV ST443) - bla_{SHV-27}; STnew (SLV ST443) - $bla_{CTX-M-55}$; STnew (SLV ST1049) - $bla_{CTX-M-55}$ -

408 Inc (B/O, HI1, I1, F); STnew (SLV ST1049) - $bla_{CTX-M-27}$;STnew (SLV 4533) - $bla_{TEM-116}$.

409 Healthy human and food-producing animals carried ESBL-producing strains of *E. coli*(Huijbers, P.M. et

410 al. 2013). These resistant populations enter the aquatic environment and to the human chain increasing the risk of

411 ESBL resistance transfer to the gut pathogens. Even though, Ecoff and MAR index determination provided the

412 evidence for the presence of more WT strains rather than acquired resistance strains posing "low risk" to the

413 population in the vicinity of the lake, the dissemination of ESBL-producing *E. coli* outside the health care setting

414 through water and food generally cannot be disregarded (Dhanji, H. et al. 2011). Surveillance of antibiotic

415 resistance in water will be a valuable tool for the screening of resistance trends in the human population (Kwaket al.

416 2015) and the results of the present study indicate large diversity between the AMR profiles in *E. coli* isolated from

417 various points of the Lake.

418 With the total population of 35 million, density of more than 859 km^{-2} in the Lake vicinity that is three

419 times as densely to rest of India, and the concomitant pressure on the natural resources, along with the additional

420 pressure from floating population or tourism posesa serious threat to the public health. The lake Vembanad is

421 microbially contaminated as evidenced by this study especially in the Kottayam regions and needs special attention

422 for mitigation. The increasing anthropogenic pressure, chemical and microbiological pollution, the uncontrolled use

423 of antimicrobial agents, and increased antibiotic consumption as well as the free movement of population and goods

424 are the main factors facilitating the global invasion of bacteria with extremely high resistance to antibiotics.The

425 presence of ESBL-producing *E. coli*with different subtypes in the water bodies (Vembanad Lake) meant for various

426 activities viz., fisheries, agriculture including animal agriculture and tourism activities increase the risk factor of

427 exposure and transmission to the human through water or food chain.

428 **Conclusions**

429 The quality assessment of the water in Vembanad Lake at thirty-five locations along the inland and coastal

430 areasthat encompass both freshwater and brackish waterparts of the whole lake revealed the prevalence of *E. coli* in

431 77% of the stations and the distribution of variant AMR and ESBL *E. coli* in the entire stretch of the Lake. The

432 study also revealed the presence of MDR *E. coli* in different locations. The study also identified 39 different AMR

433 patterns among the *E. coli* and 31.8% were extended-spectrum β-lactamase (ESBL) producers; with intra and inter-

434 sample variations in AMR profiles.Despite different ESBL *E. coli*were identified, the MLST has shown that these

435 ESBL *E. coli* are not related to epidemic clones of clinical infections. Epidemiological cut-off (Ecoff) and Multiple

436 Antibiotic Resistance (MAR) index evidenced the predominance of wild type (WT) isolates than *E.coli* with

437 acquired resistance in the Lake Vembanad indicating "low risk" to the population residing in the vicinities of the

438 Lake. However, considering the continuous inflow of floating population of intracountry and international in the

439 form of tourism, the high population density of the region exerting tremendous anthrapogenic pressure on natural

440 resources and so are the microbial pollutants with AMR can pose a serious threat not only human health but also, the

441 animals and the environment. This suggests for identification point and non-point sources of the fecal indicators (*E.*

442 *coli)* by microbial and molecular source tracking tools.

443 **Declarations**

444 **Ethics approval and consent to participate**

445 Not Applicable

446 **Consent for publication**

447 Not Applicable

448 **Availability of data and materials**

449   Data generated from the research work viz., antimicrobial resistance, resistance gene, multi-locus

450 sequencing, plasmid based replicon typing, ERIC PCR clusters are available in the repository of ICAR-CIFT,

451 Microbiology Fermentation and Biotechnology Division.

452 **Competing interests**

453   The authors declare that they have no conflicts of interest in publishing this research work.

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457 **Authors' contributions**

458   Murugadas Vaiyapuri, Madhusudana RaoBadireddy, Visnuvinayagam Sivam, Ravishankar Chandragiri

459 Nagarajarao and Mukteswar Prasad Mothadaka substantially contributed to conceptualization, designing of the

460 work, analysis of data, drafting, and revision of the manuscript. Murugadas Vaiyapuri, Anna SherinPulithara

461 Sebastian, Sandhya SoolamkandathVariem., ShaheerPeeralil., performed the experiments *viz*., isolation,

462 identification of *E. coli*, determination of AMR and PCR analysis; Murugadas Vaiyapuri, Iris George, and Devi

463 Sanjeev performed PBRT and MLST and eBurst analysis; Murugadas Vaiyapuri and ShaheerPeeralil has revised the

18

464 Figure and constructed the phylogenetic tree; Muthulakshmi Thandapani performed PCR for resistance genes;

465 Radhakrishnan NairVasudevan, JoshyChalil George.,Murugadas Vaiyapuri., Visnuvinayagam S., and Sheela Albert

466 Mosesmade the sampling strategy and performedthe statistical analysis.

Appendix A. Supplementary data

472 The base data generated in this study is attached as a supplementary file as Table S1.

References

474 Aarestrup FM, McDermott PF, Kahlmeter G (2007) Antimicrobial susceptibility testing–clinical break points and

475 epidemiological cut-off values. The Community Reference Laboratory for Antimicrobial Resistance.

476 Abgottspon H, Nüesch-Inderbinen MT, Zurfluh K, Althaus D, Hächler H, Stephan R (2014) Enterobacteriaceae with

477 extended-spectrum-and pAmpC-type β-lactamase-encoding genes isolated from freshwater fish from two

478 lakes in Switzerland. Antimicrobial agents and chemotherapy 58(4):2482-4.

479 Al-Mossawi MAJ, Kadri M, Salem A, and Salama M (1982) Incidence of antibiotic resistant fecal coliforms in the

480 coastal waters of Kuwait. Water, Air, and Soil Pollution 17, 141-149.

481 Atterby C, Börjesson S, Ny S, Järhult JD, Byfors S, Bonnedahl J (2017) ESBL-producing Escherichia coli in

482 Swedish gulls—a case of environmental pollution from humans?. PloS one 12(12):e0190380.

483 Azam M et al. (2016) bla CTX-M-152, a Novel Variant of CTX-M-group-25, Identified in a Study Performed on

484 the Prevalence of Multidrug Resistance among Natural Inhabitants of River Yamuna, India. Frontiers in

485 microbiology **7,** 176, doi:10.3389/fmicb.2016.00176.

486 Baird RB, Eaton AD, Rice EW, and Bridgewater L. (Eds.). (2017) *Standard methods for the examination of water*

487 *and wastewater*. Washington, DC: American Public Health Association.

488 Bajpai T, Pandey M, Varma M, Bhatambare GS (2017) Prevalence of TEM, SHV, and CTX-M Beta-Lactamase

489 genes in the urinary isolates of a tertiary care hospital. Avicenna journal of medicine 7(1):12.

490   Barguigua A, El Otmani F, Talmi M, Zerouali K, Timinouni M (2013) Prevalence and types of extended spectrum

491   β-lactamases among urinary Escherichia coli isolates in Moroccan community. Microbial pathogenesis

492   61:16-22.

493   Bevan ER, Jones AM, Hawkey PM (2017) Global epidemiology of CTX-M β-lactamases: temporal and

494   geographical shifts in genotype. Journal of antimicrobial chemotherapy 72(8):2145-55.

495   Bush K, Jacoby GA (2010) Updated functional classification of β-lactamases. Antimicrobial agents and

496   chemotherapy 54(3):969-76.

497   Byarugaba DK (2004) Antimicrobial resistance in developing countries and responsible risk factors. International

498   journal of antimicrobial agents 24(2):105-10.

499

500   Carattoli A. (2009). Resistance plasmid families in Enterobacteriaceae. Antimicrobial agents and

501   chemotherapy *53*(6): 2227-2238.

502   Carattoli A. et al. (2005) Identification of plasmids by PCR-based replicon typing. Journal of microbiological

503   methods 63(3): 219-228.

504   Carattoli A. et al. (2008) Molecular epidemiology of Escherichia coli producing extended-spectrum β-lactamases

505   isolated in Rome, Italy. Journal of clinical microbiology 46(1):103-108.

506   Chandran A, Hatha AAM, and Varghese S (2008) Increased prevalence of indicator and pathogenic bacteria in

507   Vembanadu Lake: a function of salt water regulator, along south west coast of India. Journal of water and

508   health 6: 539-546.

509   Chandy SJ, Thomas K, Mathai E, Antonisamy B, Holloway KA, Stalsby Lundborg C (2013) Patterns of antibiotic

510   use in the community and challenges of antibiotic surveillance in a lower-middle-income country setting: a

511   repeated cross-sectional study in Vellore, South India. Journal of antimicrobial chemotherapy 68(1):229-

512   36.

513   Cho S et al. (2019) Genetic characterization of antimicrobial-resistant escherichia coli isolated from a mixed-use

514   watershed in northeast Georgia, USA. International journal of environmental research and public health

515   16(19): 3761.

516   CLSI (2019). Performance standards for antimicrobial susceptibility testing; Twenty-nineth informational

517   supplement. CLSI document M100-S29, Clinical and Laboratory Standards Institute, Wayne, PA

518 Dallenne C, Da Costa A, Decré D, Favier C, Arlet G (2010) Development of a set of multiplex PCR
519   assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae.
520   Journal of Antimicrobial Chemotherapy 65(3):490-5.

521 Davis R, Brown PD (2016) Multiple antibiotic resistance index, fitness and virulence potential in respiratory
522   Pseudomonas aeruginosa from Jamaica. Journal of medical microbiology 65(4):261-71.

523 Dhanji H, Patel R, Wall R, Doumith M, Patel B, Hope R, Livermore DM, Woodford N (2011). Variation in the
524   genetic environments of bla CTX-M-15 in Escherichia coli from the faeces of travellers returning to the
525   United Kingdom. Journal of antimicrobial chemotherapy 66(5):1005-12.

526 Farkas A, Bocoş B, Butiuc-Keul A (2016) Antibiotic resistance and intI1 carriage in waterborne Enterobacteriaceae.
527   Water, Air, & Soil Pollution 227(7):1-1.

528 Franz E et al. (2015) Pathogenic *Escherichia coli* producing Extended-Spectrum β-Lactamases isolated from
529   surface water and wastewater. Sci Rep 5: 14372.

530 Gautam V et al. (2019) Molecular characterization of extended-spectrum β-lactamases among clinical isolates of
531   *Escherichia coli* & *Klebsiella pneumoniae*: A multi-centric study from tertiary care hospitals in India.
532   Indian Journal of Medical Research 149: 208-215.

533 Ghosh, Hiren, et al. (2017) blaCTX-M-27–encoding Escherichia coli sequence type 131 lineage C1-M27 clone in
534   clinical isolates, Germany. Emerging infectious diseases 23, 1754.

535 Godambe LP, Bandekar J, & Shashidhar R (2017) Species specific PCR based detection of *Escherichia coli* from
536   Indian foods. 3 Biotech, 7(2): 130.

537 Guenther S, Ewers C, & Wieler LH (2011) Extended-spectrum beta-lactamases producing E. coli in wildlife, yet
538   another form of environmental pollution?. Frontiers in microbiology 2: 246.

539 Haldar, R., Khosa, R. &Gosain, A. K. (2019) Impact of Anthropogenic Interventions on the Vembanad Lake
540   System. In *Water Resources and Environmental Engineering I* (pp. 9-29). Springer, Singapore.

541 Harada, Y. et al. (2013)Clinical and molecular epidemiology of extended-spectrum β-lactamase-producing
542   Klebsiellapneumoniae and Escherichia coli in a Japanese tertiary hospital. *J Med MicrobDiagn*, **2.127**:
543   2161-703.

544 Harwood, V. J., et al.(2014)Microbial source tracking markers for detection of fecal contamination in environmental

545   waters: relationships between pathogens and human health outcomes. *FEMS microbiology reviews,* **38**, 1-

546   40.

547 Hassan, H. & Baha A. (2014)Molecular characterization of extended-spectrum beta-lactamase producing

548   Enterobacteriaceae in a Saudi Arabian tertiary hospital. *The Journal of Infection in Developing*

549   *Countries,* **8.03**: 282-288.

550 Hassuna, N.A. et al. (2020)**M**olecular characterization of Extended-spectrum β lactamase- producing *E.*

551   *coli* recovered from community-acquired urinary tract infections in Upper Egypt. *Sci Rep* **10,** 2772.

552   https://doi.org/10.1038/s41598-020-59772-z

553 Hatha, A.A., Chandran, A. &Rahiman, K.M. (2004)Prevalence of diarrhegenic serotypes of *Escherichia coli* in the

554   Cochin estuary, alon*g west coast of India. Indian Journal of Marine Sciences*, **33**, 238-242.

555 Hawken, S.E. &Snitkin, E.S. (2019)Genomic epidemiology of multidrug-resistant Gram-negative organisms. *Annals*

556   *of the New York Academy of Sciences*.**1435,** 39-56.

557 Hernandez, J., et al. (2013)Characterization and comparison of extended-spectrum β-lactamase (ESBL) resistance

558   genotypes and population structure of Escherichia coli isolated from Franklin's gulls

559   (Leucophaeuspipixcan) and humans in Chile. *PLoS One*, *8*(**9**)**,** e76150.

560 Huijbers, P.M. et al. (2013)Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans

561   living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*, **19(6)**,

562   E256-E259.

563 Jacoby, G.A. AmpC β-lactamases. (2009)*Clinical microbiology reviews*, **22(1)**, 161-182 .

564 Johnson, T. J., & Nolan, L. K. (2009)Plasmid replicon typing. In *Molecular Epidemiology of Microorganisms* (pp.

565   27-35). Humana Press, Totowa, NJ.

566 Kim, H. et al.(2017)Risk factors and molecular features of sequence type (ST) 131 extended-spectrum β-lactamase-

567   producing Escherichia coli in community-onset bacteremia. *Scientific reports*, *7*(**1**), 1-8.

568 Kim, M. et al. (2013)Antibiotic resistance of bacteria isolated from the internal organs of edible snow

569   crabs. *PLoSONE,* **8**,70887.

570 Kittinger, C. et al. (2016)Enterobacteriaceae isolated from the river Danube: antibiotic resistances, with a focus on

571   the presence of ESBL and carbapenemases. *PloS one*, **11**, e0165820.

572 Kronvall**,** G**.** (2003)Determination of the real standard distribution of susceptible strains in zone histograms. *Int. J.*

573 *Antimicrob. Agents,***22**,7–13.

574 Kronvall, G., Kahlmeter, G., Myhre, E., & Galas, M. F. (2003)A new method for normalized interpretation of

575 antimicrobial resistance from disk test results for comparative purposes. *Clinical microbiology and*

576 *infection*, **9**, 120-132.

577 Krumperman, P.H. (1983)Multiple antibiotic resistance indexing *Escherichia coli* to identify risk sources of faecal

578 contamination of foods. *Applied and Environmental Microbiology,* **46,** 165–170.

579 Kwak, Y. K., Colque, P., Byfors, S., Giske, C. G., Möllby, R. &Kühn, I. (2015)Surveillance of antimicrobial

580 resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance

581 trends in the society?.*International journal of antimicrobial agents*, **45(1)**, 25-32.

582 Lazarus, B., Paterson, D. L., Mollinger, J. L., & Rogers, B. A. (2015)Do human extraintestinal Escherichia coli

583 infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A

584 systematic review. *Clinical Infectious Diseases*, **60(3)**, 439-452.

585 Liu, Haixia, et al. (2018)Molecular characteristics of extended-spectrum β-lactamase-producing Escherichia coli

586 isolated from the rivers and lakes in Northwest China. *BMC microbiology.* **18**, 125.

587 Liu, J., Zhao, Z., Orfe, L., Subbiah, M. & Call D. R. (2016)Soil-borne reservoirs of antibiotic-resistant bacteria are

588 established following therapeutic treatment of dairy calves. *Environ. Microbiol,* **18**, 557–564.

589 Lupo, A., Coyne, S. &Berendonk, T. U. (2012)Origin and evolution of antibiotic resistance: the common

590 mechanisms of emergence and spread in water bodies. *Frontiers in microbiology*, **3**, 18.

591 Magiorakos, A.P. et al. (2012)Multidrug resistant, extensively drug-resistant and pandrug-resistant bacteria: an

592 international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol.*

593 *Infect,* **18(3)**, 268-281.

594 Matamoros, S. et al.(2017)Global phylogenetic analysis of Escherichia coli and plasmids carrying the mcr-1 gene

595 indicates bacterial diversity but plasmid restriction. *Scientific reports*, **7(1),** 1-9.

596 Matsumura, Yasufumi, et al. (2015)CTX-M-27-and CTX-M-14-producing, ciprofloxacin-resistant Escherichia coli

597 of the H 30 subclonal group within ST131 drive a Japanese regional ESBL epidemic. *Journal of*

598 *Antimicrobial Chemotherapy.* **70**, 1639-1649.

599   Menon, N. N., Balchand, A. N. & Menon, N. R. (2000)Hydrobiology of the Cochin backwater system–a

600   review. *Hydrobiologia*, **430**, 149-183.

601   Michael, I. et al. (2015)"Urban wastewater treatment plants as hotspots for the release of antibiotics in the

602   environment: a review. *Water research* **47**, 957-995 (2013).Hufnagel, D.A*., DePa*s, W.H. & Chapman,

603   M.R. The biology of the *Escherichia coli* extracellular matrix. *Microbiology spectrum*, **3**, 249–267.

604   Mohapatra, B, R., Klaas, B. &AsitMazumder. (2007)Comparison of five rep-PCR genomic fingerprinting methods

605   for differentiation of fecal Escherichia coli from humans, poultry and wild birds. *FEMS microbiology*

606   *letters.* **277**, 98-106.

607   Mohsin, M. et al.(2017)High prevalence of CTX-M-15-Type ESBL-producing E. coli from migratory avian species

608   in Pakistan. *Frontiers in microbiology*, **8,** 2476.

609   Momtaz, H., E. Rahimi, S. &Moshkelani. (2012)Molecular detection of antimicrobial resistance genes in *E. coli*

610   isolated from slaughtered commercial chickens in Iran. *VeterinarniMedicina,* **57***,* 193–197.

611   Mondal, Aftab Hossain, et al. (2019)Prevalence and diversity of bla TEM, bla SHV and bla CTX-M variants among

612   multidrug resistant Klebsiella spp. from an urban riverine environment in India. *International journal of*

613   *environmental health research.* **29.2**, 117-129.

614   Morgan, R. C., Guerry, P. & Colwell, R. R. (1976)Antibiotic resistant bacteria in Chesapeake Bay. *Chesapeake*

615   *Science,* **17(3),** 216-219.

616   Murugadas, V., Joseph, T.C. & Lalitha. K.V. (2016)Distribution of pathotypes of *Escherichia coli* in seafood from

617   retail markets of Kerala, India. *Indian J. Fish*, **63 (1)**, 152-155.

618   Nemoy, L. L. et al.(2005)Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of

619   extended-spectrum beta-lactamase-producing Escherichia coli isolates. *Journal of clinical*

620   *microbiology*, **43(4)**, 1776–1781.

621   Paterson, D. L. &Bonomo, R. A. (2005)Extended-spectrum β-lactamases: a clinical update. *Clinical microbiology*

622   *reviews*, **18**, 657-686.

623   Patterson, J. E. (2000)Extended-spectrum beta-lactamases. In *Seminars in respiratory infections*, 15, 299-307.

624   Poulou, A. et al. (2014)Modified CLSI extended-spectrum β-lactamase (ESBL) confirmatory test for phenotypic

625   detection of ESBLs among Enterobacteriaceae producing various β-lactamases. *Journal of clinical*

626   *microbiology*, **52**, 1483-1489.

627 Queenan, A. M. & Bush, K. (2007)Carbapenemases: the Versatile β-Lactamases. *Clinical Microbiology Reviews*,

628   **20,** 440-458.

629 Rasschaert, G. et al. (2005)Comparison of five repetitive-sequence-based PCR typing methods for molecular

630   discrimination of Salmonella enterica isolates. *Journal of clinical microbiology,* **43,8**: 3615-23.

631   doi:10.1128/JCM.43.8.3615-3623.2005

632 Rayasam, S. D. et al.(2019)Extraintestinal pathogenic escherichia coli and antimicrobial drug resistance in a

633   maharashtrian drinking water system. *The American journal of tropical medicine and hygiene*, *100*(**5**)**,**

634   1101-1104.

635 Riaz, S., Faisal, M.&Hasnain, S. (2011)Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR)

636   calculation of extended spectrum β-lactamase (ESBL) producing Escherichia coli and Klebsiella species in

637   Pakistan. *African Journal of Biotechnology*, **10(33)**, 6325-6331.

638 Riedel, S. et al. (2019)A survey of AMR in Enterobacteriaceae isolated from the Chesapeake Bay and adjacent

639   upper tributaries. *Microbiology Open*, e839.

640 Rozwandowicz, M. et al. (2018)Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of

641   Antimicrobial Chemotherapy*, *73*(5), 1121-1137.

642 Sahni, R. D. et al.(2018)Extended-spectrum beta-lactamase producers: Detection for the diagnostic laboratory.

643   *Journal of global infectious diseases*, **10(3)**, 140.

644 Salim, A. et al.(2019)Draft Genome Sequence of an Escherichia coli Sequence Type 155 Strain Isolated from

645   Sewage in Kerala, India. *Microbiology resource announcements*, *8*(**27**).

646 Schroede*r,* C.M. et al. (2002)*Antimicrobial resistance o***f** *Escherichia coli* O26, O103, O111, O128, and O145 from

647   animals and humans. *Emerging infectious diseases*, **8,** 1409-1414.

648 Selvam, A.P. et al.(2012) Heavy metal assessment using geochemical and statistical tools in the surface sediments

649   of Vembanad Lake, Southwest Coast of India. *Environmental monitoring and assessment, 184, 5899-5915*.

650 Sharma, Meeta, Sati Pathak, and Preeti Srivastava. (2013)Prevalence and antibiogram of Extended Spectrum β-

651   Lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL

652   producing Escherichia coli and Klebsiella spp. *Journal of clinical and diagnostic research: JCDR* 7.10:

653   2173.

654 Shivakumaraswamy, S. K. et al.(2019)Phenotypic & genotypic study of antimicrobial profile of bacteria isolates

655   from environmental samples. *The Indian journal of medical research*, **149**, 232–239.

656 Singh, Nambram S., NeeljaSinghal, and Jugsharan S. Virdi. (2018)Genetic environment of blaTEM-1, blaCTX-M-

657   15, blaCMY-42 and characterization of integrons of Escherichia coli isolated from an Indian urban aquatic

658   environment. *Frontiers in microbiology.* **9,** 382.

659 Stelling, J. M. & O'Brien, T. F. (1997)Surveillance of antimicrobial resistance: the WHONET program. *Clin Infect*

660   *Dis*, **24 (1)**,157–68.

661 Sukumaran, D.**P.,**Durairaj, S. & Abdulla, M.H. (2012)Antibiotic resistance of *Escherichia coli serotypes* from

662   Cochin estuary. *Interdisciplinary perspectives on infectious diseases*, Article ID 124879, **7**.

663 Versalovic, J. et al. (1994)Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain

664   reaction. *Methods in molecular and cellular biology.* **5**, 25-40.

665 Watts, J., Schreier, H., Lanska, L. &Hale, M.(2017)The rising tide of antimicrobial resistance in aquaculture:

666   sources, sinks and solutions. *Marine drugs*, **15,** 158.

667 Wirth, T.et al.(2006)Sex and virulence in Escherichia coli: an evolutionary perspective. *Molecular*

668   *microbiology*, **60**(**5**), 1136-1151.

669 Zhang, H., Gao, Y. & Chang, W.(2016) Comparison of Extended-Spectrum β-Lactamase-Producing Escherichia

670   coli Isolates from Drinking Well Water and Pit Latrine Wastewater in a Rural Area of China. *BioMed*

671   *research international*, 4343564.

672 Zurfluh, K., Hächler, H., Nüesch-Inderbinen, M. & Stephan, R. (2013) Characteristics of extended-spectrum β-

673   lactamase-and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland.

674   *Appl. Environ. Microbiol.*, **79(9)**, 3021-3026.

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682 **Fig.1. Frequencies of Antimicrobial Resistance in*E. coli* isolated from Vembanad Lake**

683 Note: CTX- Cefotaxime; AMP- Ampicillin; TCY- Tetracycline; CRO- Ceftriaxone; NAL- Nalidixic acid; CAZ-
684 Ceftazidime; IPM- Imipenem; ATM- Aztreonam; SXT- Trimethoprim/ Sulfamethoxazole; AMC-
685 AmoxicillinClavulanic acid; CPD- Cefpodoxime; FOX- Cefoxitin; CIP- Ciprofloxacin; CHL- Chloramphenicol;
686 GEN- Gentamicin. Bar represent frequencies with standard error obtained after zone diameter analysis in
687 WHONET 5.6 software.

688

689 **Fig.2a. The location map of Vembanad Lake, Kerala, India**

690 The figure denotes the stations covered in the Lake encompassing three districts of Kerala viz., Ernakulam,
691 Kottayam and Alappuzha. North end at Munambam in Ernakulam District and South end at Rajiv Boat Jetty of
692 Alappuzha District.

693

694 **Fig. 2b. Resistance mapping in sampled stations of Vembanad Lake**

695 The blue pins denote the Ernakulam Region, green pins denoteErankulam region and pink pins denote Kottayam
696 regions of the Vembanad Lake. Black circle denote the stations with MAR index > 0.2 and other locations marked
697 with ESBL or β-lactamase types.

698

699 **Fig.3.ERIC-PCR fingerprint patterns of *E. coli* isolates from different stations of Vembanad Lake .**

700 ERIC-PCR banding pattern were clustered with the aid of GelJ software and tree was constructed using Pearson
701 correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA). Five major clusters
702 were defined from groups formed with similarity.

703 **Fig.4. Dendrogram of phenotypic AMR profile of *E. coli* isolates fromdifferent stations of Vembanad Lake**

704 **Fig.5. Minimal spanning tree from eBurst analysis**

705 Nodes with different colours were the closest clonal complexes chosen for the analysis. Blue rings highlighted are

706 the ESBL *E. coli* taken for the analysis. Node with blue were STs chosen for analysis including the tested

707 isolates. Numbering inside the nodes indicates the ST number. Numbers in the line connecting nodes

708 denotes the allele numbers.

709 **Table1. Resistant (R) and Wild Type (WT) *E. coli* isolated from Vembanad Lake, Kerala, India**

710

711 **Table 2. Variations in AMR patterns of Extended Spectrum β-lactamase *Escherichia coli* (ESBL) and other**

712 ***E. coli* isolated from Vembanad Lake, Kerala, India**

713

714   **Table 3. Distribution of ESBL genes in *E. coli* isolated from Vembanad Lake**

715

716   **Supplementary file**

717   **Resistance mapping and genetic diversity of ESBL*Escherichia coli* isolated from largest fresh cum brackish**

718   **water of the Vembanad Lake, Kerala, India**

Figures
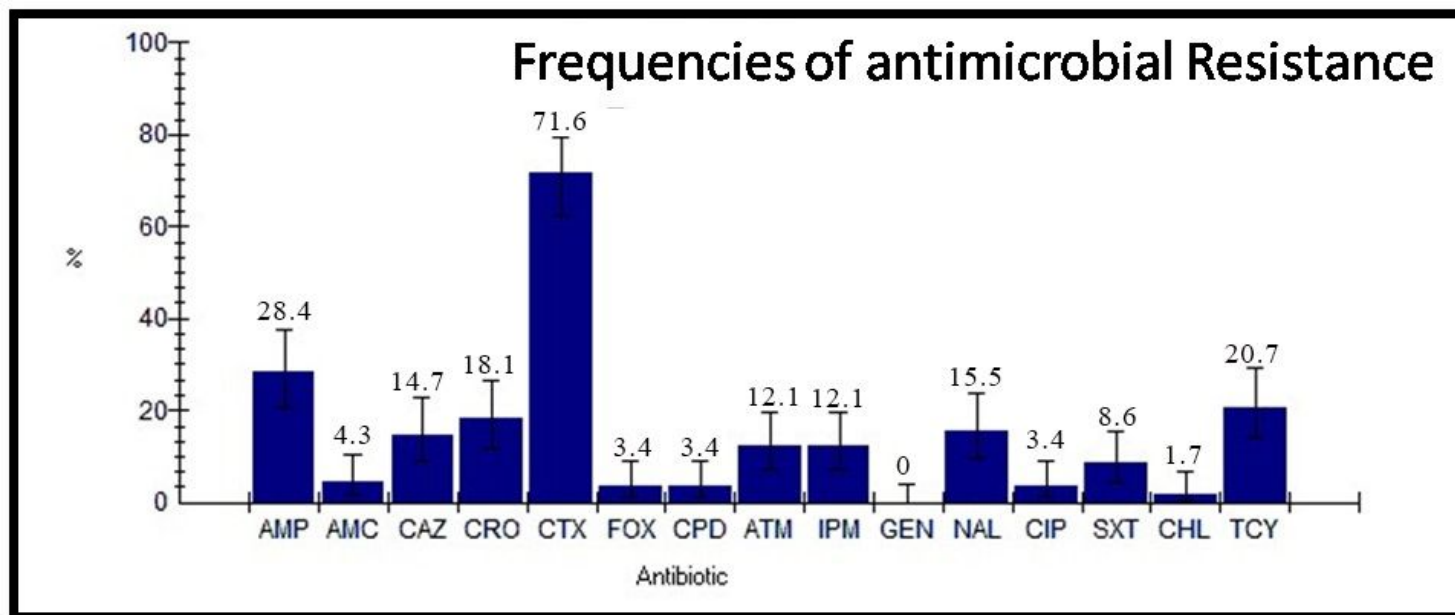
Figure 1

Frequencies of Antimicrobial Resistance inE. coli isolated from Vembanad Lake Note: CTX- Cefotaxime; AMP- Ampicillin; TCY- Tetracycline; CRO- Ceftriaxone; NAL- Nalidixic acid; CAZ- Ceftazidime; IPM- Imipenem; ATM- Aztreonam; SXT- Trimethoprim/ Sulfamethoxazole; AMC- AmoxicillinClavulanic acid; CPD- Cefpodoxime; FOX- Cefoxitin; CIP- Ciprofloxacin; CHL- Chloramphenicol; GEN- Gentamicin. Bar represent frequencies with standard error obtained after zone diameter analysis in WHONET 5.6 software.
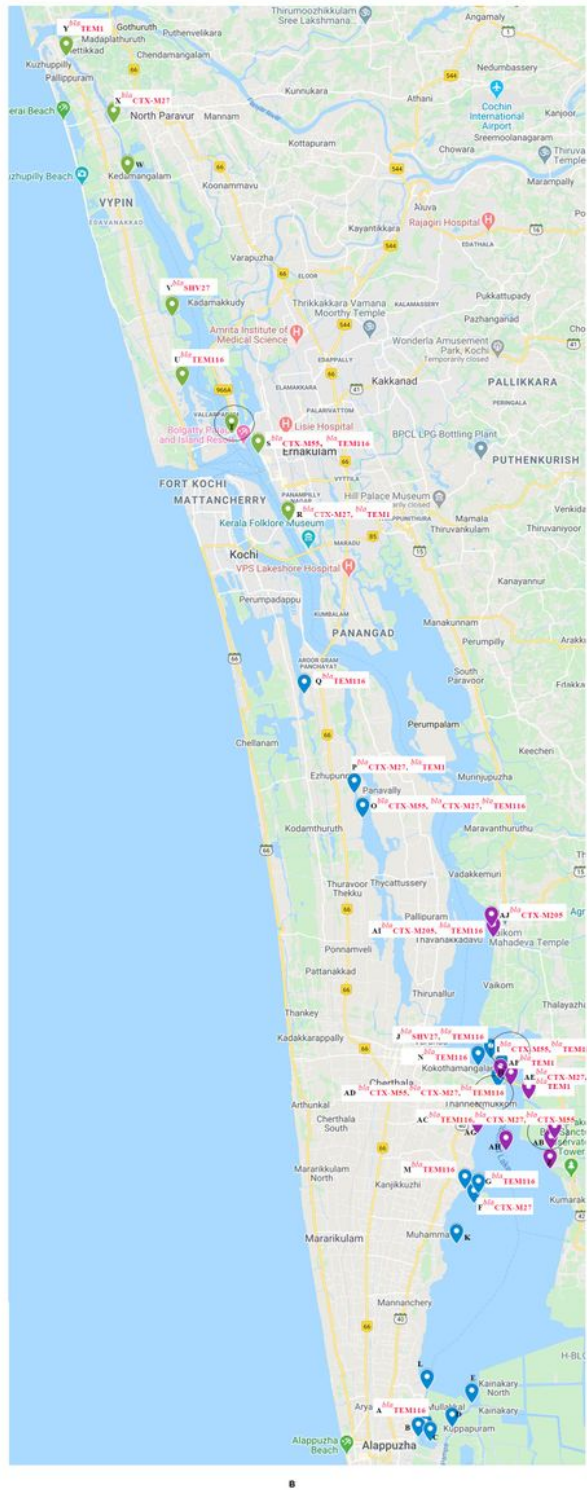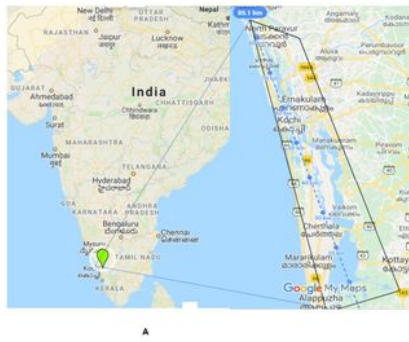
Figure 2

2a. The location map of Vembanad Lake, Kerala, India The figure denotes the stations covered in the Lake encompassing three districts of Kerala viz., Ernakulam, Kottayam and Alappuzha. North end at Munambam in Ernakulam District and South end at Rajiv Boat Jetty of Alappuzha District. . 2b. Resistance mapping in sampled stations of Vembanad Lake The blue pins denote the Ernakulam Region, green pins denoteErankulam region and pink pins denote Kottayam regions of the Vembanad Lake. Black

circle denote the stations with MAR index > 0.2 and other locations marked with ESBL or β-lactamase types. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
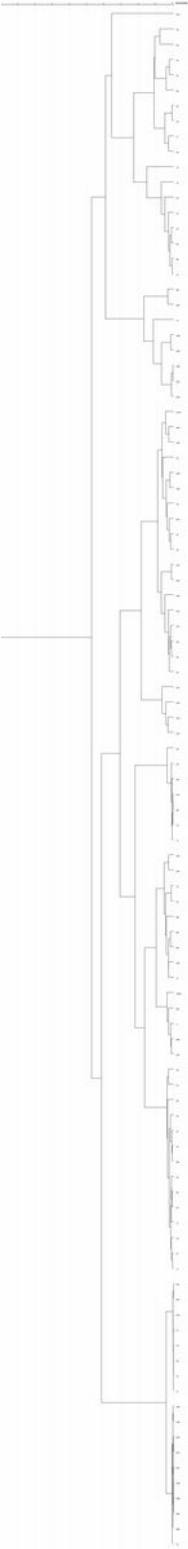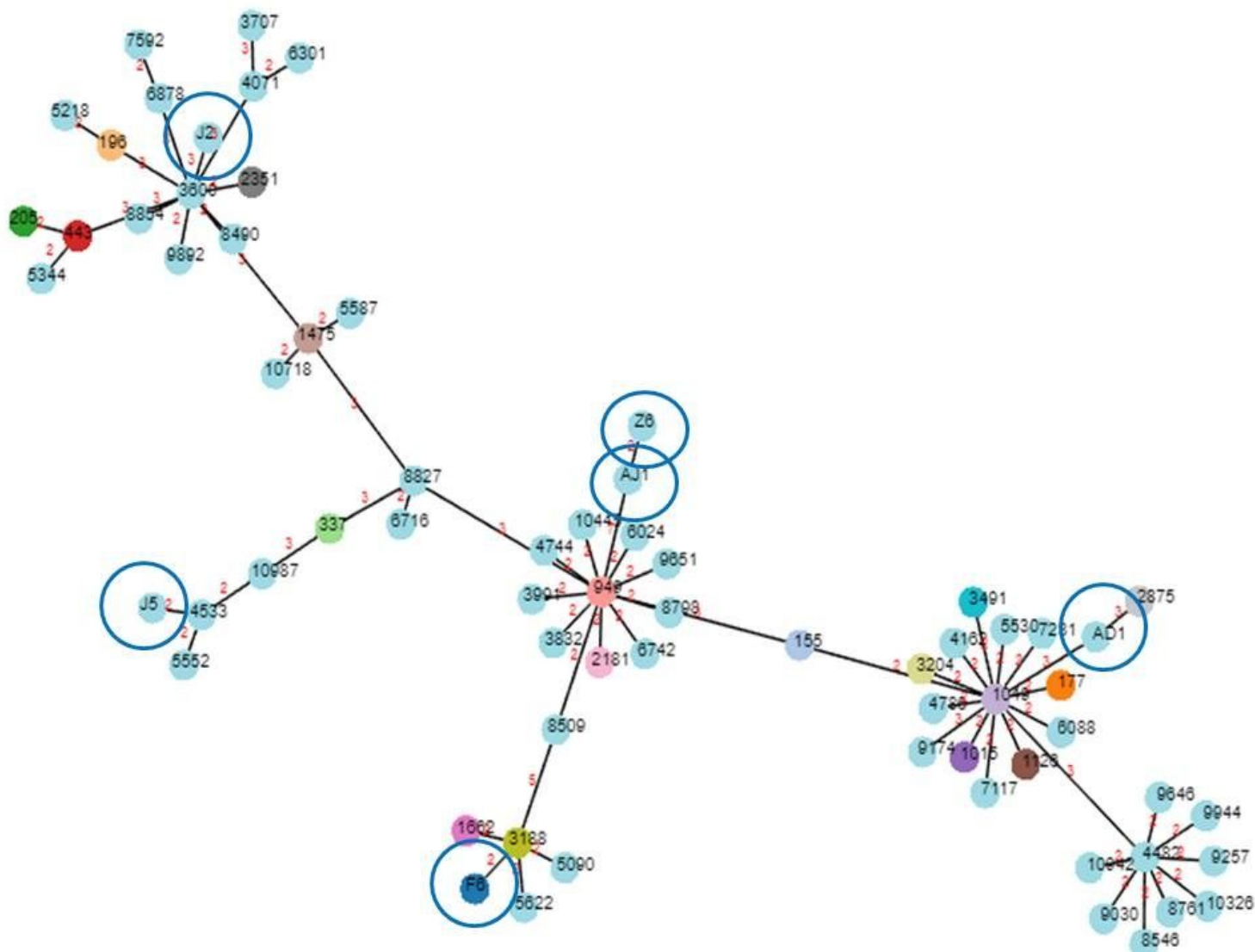

Figure 3

ERIC-PCR fingerprint patterns of E. coli isolates from different stations of Vembanad Lake . ERIC-PCR banding pattern were clustered with the aid of GelJ software and tree was constructed using Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA). Five major clusters were defined from groups formed with similarity.

Figure 4

Dendrogram of phenotypic AMR profile of E. coli isolates fromdifferent stations of Vembanad Lake

Figure 5

Minimal spanning tree from eBurst analysis Nodes with different colours were the closest clonal complexes chosen for the analysis. Blue rings highlighted are the ESBL E. coli taken for the analysis. Node with blue were STs chosen for analysis including the tested isolates. Numbering inside the nodes indicates the ST number. Numbers in the line connecting nodes denotes the allele numbers.

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