Research Article

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

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Abstract

Antimicrobial resistance (AMR) burden in *Escherichia coli* along the 90 km stretch of Vembanad Lake, Kerala, India was assessed. Seventy-seven percent of water samples drawn from 35 different stations of the Lake harbored *E. coli*. Antibiotic susceptibility test performed on 116 *E. coli* isolates revealed 81% were resistant to ≥ one antibiotic with 39 AMR profiles, 30% multidrug resistant, 32% extended spectrum β lactamase (ESBL) producers as per CLSI. The probability of isolating cefotaxime resistant *E. coli* was the highest 0.7 (P ≤ 0.05) in the Lake. Genetically diverse ESBL types *bla*<sub>TEM</sub>-116, *bla*<sub>CTX-M-152</sub>, *bla*<sub>CTX-M-27</sub>, *bla*<sub>CTX-M-55</sub>, *bla*<sub>CTX-M-205</sub>, and *bla*<sub>SHV</sub>-27 were identified. Molecular typing (ERIC PCR, MLST and PBRT) confirmed the diversity among *E. coli* between and within the stations. ST11439 and Single and Double Loci Variants of ST443, ST4533 were identified in Multi locus sequence typing (MLST) analysis. Inc plasmids (B/O, F, W, I1, FIIA, HI1, P-1α, K/B and N) identified in the Lake evidences the transmission potential. Low multiple antibiotic resistance index (average < 0.2) indicating lower risk to the human population albeit, an emerging concern of ESBL resistance in the Lake. The occurrence of genetically variant ESBL *E. coli* in Vembanad Lake signals health hazards and necessitates pragmatizing strategic control measures.

Keywords: Vembanad Lake; AntimicrobialResistance; Extended Spectrum Beta Lactamase *Escherichia coli*; Water; Molecular typing
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 (Schroeder 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 (Thanemukham 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 phoAgene-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

Phenotypic antimicrobial susceptibility testing (AST) was carried out by Disc Diffusion Assay (DDA) with 15 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
Agar (MHA) (BD Difco). The AMR pattern was determined in accordance with CLSI (CLSI 2019) and WHONET software version 5.6 (Stelling and O’Brien 1997).

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

Isolates which produced zone diameter of ≤17mm, ≤22mm, ≤27mm, ≤25mm, and ≤27mm against cefpodoxime (10µg), ceftazidime (30µg), aztreonam (30µg), and cefotaxime (30µg), respectively were considered as presumptive ESBL producers (CLSI 2019). These presumptive ESBL isolates (n=83) were further tested in combined disk diffusion (CDD) assay, a confirmatory test with clavulanic acid (10 µg) in MHA agar, and incubated at 35°C for 16 to 18h. Isolates showing ≥5mm zone diameter when clavulanate was combined with cefotaxime or ceftazidime were confirmed as ESBL producers. Those isolates without a significant effect of clavulanic acid and resistant to cefoxitin (zone diameter ≤14 mm) were considered as AmpC-producers (Jacoby 2009). MIC was 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**

*E. coli* isolates (n=94) that were phenotypically resistant to various antibiotics were further screened by PCR for very abundant and important AMR (AR) genes. Presumptive ESBL *E. coli* (n=83) which showed reduced susceptibility to cefpodoxime, ceftazidime, aztreonam, and cefotaxime were tested for *bla*<sub>CTX-M group 1</sub>, *bla*<sub>CTX-M group 2</sub>, *bla*<sub>CTX-M group 9</sub>-*bla*<sub>CTX-M group 8/25</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA-1-like</sub> genes specific for broad-spectrum β-lactamases and ESBL detection (Dallenne et al. 2010). *E. coli* (n=14) which showed reduced susceptibility to imipenem were tested for *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>NND</sub> genes specific for carbapenem resistance (Bush and Jacoby, 2010; Sahni et al. 2018). *E. coli* (n=23) showing phenotypic tetracycline resistance were tested for *tetA*and *tetB* genes for tetracycline resistance; isolates (n=6) with phenotypic chloramphenicol resistance was tested for *cat A* and *cat B* genes (Kim et al. 2013); *E. coli* (n=14) were tested for *sul1, sul2* and *dfrA* genes for folate pathway inhibitors resistance (Momtaz et al. 2012).

**ESBL allele identification by sequencing analysis**

*E. coli* which produced amplicons of a partial length corresponding to (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M group 1</sub>, *bla*<sub>CTX-M group 25</sub>, *bla*<sub>CTX-M group 2</sub>, *bla*<sub>CTX-M group 9</sub>) gene sequences were amplified as mentioned above (Dallenne et al. 2010) and products were purified by gel elution (Thermofisher Scientific) and outsourced for sequencing in an
automated sequencer (ABI 1377) at Agrigenome Pvt Lab (Kochi, India). Quality checks and sequence similarity was verified in NCBI [https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/].

MLST and eBurst analysis

MLST analysis was carried out for selected eight ESBL E. coli with differences in ESBL genes viz., blaTEM-116, blaCTX-M-23, blaCTX-M-55, blaCTX-M-152, blaCTX-M-205, and blaSHV-27 PCR amplification was carried out with 10X Extaq master mix (DSS Takara Bio India). The reactions for all the housekeeping genes viz., adk, fumC, gyrB, icd, mdh, purA, and recA were amplified as per Enterobase protocol (Wirth et al. 2006). The agarose gel extracted amplicons of various fragments were outsourced for Sanger sequencing at Agrigenome Pvt Lab (Kochi, India). After the quality check, the allele numbers for the gene fragments and sequence type for the ESBL E. coli were deduced from the public domain PubMLST [https://pubmlst.org/bigsdb?db=pubmlst_escherichia_seqdef]. eBurst analysis for the identified STs was carried out in Phyloviz software [https://online.phyloviz.net/] taking into account single and double locus variants of the identified clones.

Plasmid Based Replicon Typing

Plasmid characterization of the ESBL E. coli isolated from the water of Vembanad Lake was carried out by plasmid-based replicon typing (PBRT) (Carattoli et al. 2005; Johnson and Nolan, 2009). Three multiplex PCR reactions were performed for each ESBL E. coli and identified the replicon plasmid present in the ninety-four E. coli isolates. Modification in the use of 2X Phusion U Green multiplex PCR master mix (ThermoScientific) and the type of Inc Plasmid identified in the study was deduced (Johnson & Nolan, 2009).

ERIC PCR fingerprinting and Cluster analysis

ERIC – PCR reactions were performed in duplicate for each isolate in 25µl volume containing 3µl of E. coli genomic DNA, 2.5mM MgCl₂, 1U Taq polymerase, 0.2mM dNTPS, 1X PCR buffer, 1 µM of each primers (ERIC 1 and ERIC 2) and final volume adjusted with nuclease-free water. The reaction was carried out in 0.2 ml PCR tubes always in same thermal cycler (Nemoy et al. 2005; Mohapatra et al. 2007). Ninety four E. coli isolates that were resistant at least to one antibiotic were subjected to ERIC PCR analysis. The PCR product was visualized after electrophoresized in 3% agarose gel in maxi preparation with 120V for 3h and gel images were captured in the gel documentation system (Syngene). Phylogenetic tree was constructed in GelJ software after visually comparing
the banding pattern for 94 *E. coli* isolates. DNA ladder (100-bp) was used for the normalization. The phylogenetic
tree was constructed based on the similarity calculated by Pearson correlation between the fingerprints with the
tolerance of 1% and grouping of the fingerprints was carried out with the help of the algorithm unweighted-pair
group method using arithmetic averages (UPGMA) (Rasschaert et al. 2005).

**Estimation of MDR, MAR index and Epidemiological cut-off**

MDR was defined as non-susceptibility to at least one agent in three or more antimicrobial categories and
the MDR was determined (Magiorakos et al. 2012) and the Multiple Antibiotic Resistance (MAR) index was
estimated (Krumperman 1983). The Ecoff for the environment associated *E. coli* was determined as per the
normalized resistance interpretation method of Kronvall (2003) using http://www.bioscand.se/nri/. Isolates were
categorized as either Wild type (WT) or Acquired Resistant type or non-wild type (NWT) based on the Ecoff value
determined for each antibiotic.

**Statistics and cluster analyses**

Chi-square statistical analysis was carried out with SAS 9.3 for finding the association between cefoxitin,
cefotaxime, cefpodoxime, and ceftazidime to the other antibiotics tested. The binomial logistic regression model was
used to predict the probability for the isolate resistant to particular antibiotics under the study in Vembanad Lake
water and the binomial logistic regression is given below

\[
\text{logit}\left(\frac{p}{1-p}\right) = \beta_0
\]

where \(P\) is the probability of the isolate resistant to different antibiotics and \(\beta_0\) is intercept. The parameter \(\beta_0\) was
estimated by maximum likelihood method. The predicted probability value for the isolate to resistant to different
antibiotics is obtained from the formula given below

\[
p = \frac{\exp(\hat{\beta}_0)}{1 + \exp(\hat{\beta}_0)}
\]

Cluster analysis was carried out based on hierarchical cluster method for the parameters AMP10, CPM30,
CTX30, CX30, CAZ30, MRP10, GEN10, TE30, CIP5, COT25, C30 based on the AMR profiles. The values of
the antimicrobial resistance were plotted as 0 or 1 corresponding to the absence or presence of resistance
respectively. Binary Squared Euclidean Distance matrix was generated using AMR data between two cultures. The
dendrogram was generated based on the similarity matrix in SPSS software version 16.

Results and discussion

Prevalence of E. coli in the lake

The study is the first of its kind that established the AMR pattern in 35 stations of the Vembanad Lake, Kerala, India. E. coli was detected in 77% (27/35) of the sampled stations. After initial enrichment, primary and secondary screening, a total of 116 E.coli were detected from 27 different points in the Lake. All 116 isolates yielded specific amplicon in PCR targeted uid A (168bp) or phoA (999bp) genes and all of them belonged to biotype 1 (IMVC result: ++- -). The Alapuzha region of the Vembanad Lake water was comparatively safe in harboring E. coli (59%) compared to Kottayam (90%) and Ernakulam (100%).

Antimicrobial resistance (AMR) is a growing threat to the human population as it significantly curtails treatment options. Surface waters in aquatic bodies, owing to their microbial diversity and moving nature, play a considerable role in the emergence and transmission of AMR (Kittinger et al. 2016). Aquatic reservoirs have been described as hotspots for AMR emergence across the globe (Watts et al. 2017). Monitoring aquatic reservoirs for microbial quality and AMR is an indispensable tool for devising control strategies to protect human health. In this context, Vembanad the largest lake of India was assessed for AMR burden on the environmental. This is important to mitigate the AMR source. The present study corroborates with the earlier findings on the incidence of E. coli in selected areas of Vembanad Lake as 85.6–86.7% and 100% which attributed to the anthropogenic activity and seafood processing industries (Hatha et al. 2004; Chandran et al. 2008). Variations in the occurrence of E. coli in 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

Antibiotic resistance profiling revealed that all the 116 E. coli isolates were susceptible to Gentamicin, however, 81% of the E. coli isolates were resistant to a minimum of one and a maximum of nine antibiotics. A total of 94 AMR E.coli were isolated from the Lake. AMR was observed in 86% (43/50), 79% (34/43), and 74% (17/23) of the E. coli isolated from Kottayam, Alapuzha and Ernakulam regions of Vembanad Lake, respectively (Table S1). 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*; both between 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 increased 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 with reduced susceptibility to CTX, CAZ, CPD, and ATM, revealed that 37 were phenotypically confirmed as Class A ESBL producing *E. coli* (Kittinger et al. 2016) and were designated as CDD™ while the remaining 46 isolates that did not show increased zone size ≥5mm in CDD assay were designated as CDD (“). ESBL genes screening for CDD™ and CDD™ 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
intriguing. MIC level of phenotypic imipenem resistant isolates in disk diffusion assay (DDA) ranged between 0.19 and 0.25 µg/ml; the MIC observed was lower than the clinical resistance criterion of CLSI.

Tetracycline resistance mediated by tetA was very less in the present study compared to the aquaculture setting (Shivakumaraswamy et al. 2019). E. coli with phenotypic cefoxitin resistance in DDA showed an increase in zone diameter with clavulanic acid and hence, may not be ampC hyper producer (Jacoby, 2009). The identification of blaTEM positive non-ESBL producers phenotypically in the present study was reported earlier in the hospital patients (Bajpai et al. 2017), possibly due to the nonexpression of ESBL genes without the antibiotic pressure or due to the presence of TEM-1, TEM-2, and TEM-13 which are not ESBLs (Paterson & Bonomo, 2005). In CDD assay only 37 of the 83 isolates were phenotypically identified as ESBL producers but the genetic determination of antibiotic resistance genes revealed that the majority of the isolates harbored at least one of the ESBL variants. This may be due to several reasons such as the probability of possessing another variant of ESBL genes generally cannot be disregarded or could be the masking of additional enzymes such as AmpC β-lactamases or carbapenamases (Poulou et al. 2014) To confirm that sequencing analysis was carried out for the β-lactamase genes.

Molecular Typing of ESBL E. coli

ESBL allele identification by sequencing analysis

Sequencing analysis of partial genes of blaTEM, blaCTX-M group 9, blaCTX-M group 1, blaCTX-M group 8/25, blaCTX-M group 2, and blaSHV genes amplicon revealed that blaTEM genes belonged to blaTEM-1, and blaTEM-116; blaCTX-M group 9 belonged to blaCTX-M-27; blaCTX-M group 1 gene belonged to blaCTX-M-55; blaCTX-M group 8/25 belonged to blaCTX-M-152; blaCTX-M group 2 belonged to blaCTX-M-205 and blaSHV-27 belonged to blaSHV-27 and the results were summarized (Table3). Resistance mapping for the Vembanad Lake concerning the sampled locations and ESBL subtypes indicates that the majority of ESBL E. coli were nearer to the Kottayam region of the Lake and south of the Alappuzha part of the Lake had no CTX mediated ESBL producers (Fig. 2b).

Gene sequencing analysis revealed that blaTEM genes belonged to blaTEM-1, and blaTEM-116 which were identified earlier in the urban aquatic environments of India; 15 of the blaTEM were blaTEM-1 indicating it as the broad spectrum β-lactamase producer (non-ESBL). However, blaTEM-1 gene was carried in the majority of the blaCTX-M producing isolates of E. coli and hence, the isolates were ESBL producers (Paterson and Bonomo 2005; Singh et al.
et al. (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, Netherlands and Japan (Matsumura et al. 2015; Franzet al. 2015; Ghosh et al. 2017) and in rivers and lakes of Northwest China (Liu et al. 2018); *bla*$_{CTX}$-M group 1 gene belonged to *bla*$_{CTX}$-M :55 identified in Japan, Netherlands (Matsumura et al. 2015; Franz et al. 2015); *bla*$_{CTX}$-M group 9:25 belonged to *bla*$_{CTX}$-M :152, a novel variant form of *bla*$_{CTX}$-M :205 the particular ESBL type in India is not available in the public domain and probably this is the first 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 Urban riverine environment in India and in the community set up of Morocco (Barguigua et al. 2013; Mondal et al. 2019). There is no such study conducted in different geographical stations of the Vembanad Lake. In the context of the epidemiology, *bla*$_{CTX}$-M:55 is the second most common ESBL-encoding gene in Asian countries, and in the global epidemiology, the genotype *bla*$_{CTX}$-M:27 a single loci variant of *bla*$_{CTX}$-M:14 has slowly replaced other CTX-M genotypes although *bla*$_{CTX}$-M:14 and *bla*$_{CTX}$-M:15 are leading clones and *bla*$_{CTX}$-M:27 is now considered as a stable reservoir for the food animals in China (Bevan et al. 2017). In 27 occasions in the Lake, the ESBL *E. coli* co-existed with either *bla*$_{TEM}$:16, *bla*$_{CTX}$-M:27, *bla*$_{CTX}$-M:55, or *bla*$_{SHV}$:27 Co-existence of CTX-M types, TEM, and SHV were reported in India, Saudi Arabia, and Japan indicating the increased risk of treating these infections and co-evolving of two or three types of ESBL genes within an *E. coli* (Harada et al. 2013; Sharma et al. 2013; Hassan and Baha 2014). Resistance mapping of the ESBL types in relation to the sampling points has clearly identified that the Kottayam region only harboured *bla*$_{CTX}$-M:205.

In the Indian health care system, the major variants of ESBL producers harbored *bla*$_{TEM}$ followed by *bla*$_{CTX}$-M :1, *bla*$_{OXA}$, *bla*$_{SHV}$ and *bla*$_{CTX}$-M: group-2 (Gautam et al. 2019); *bla*$_{CTX}$-M group-1, *bla*$_{CTX}$-M:14, *bla*$_{CTX}$-M:15, *bla*$_{CTX}$-M:24, *bla*$_{CTX}$-M:27, *bla*$_{SHV}$:1 in Lake Zürich and Lake Thun, Switzerland (Abgottspon et al. 2014), and *bla*$_{CTX}$-M group 1, *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 Switzerland (Zurfluh et al. 2013). However, in the present study, *bla*$_{OXA}$-1 like was not detected, whereas, *bla*$_{TEM}$, and *bla*$_{CTX}$-M group 9 were dominant at 85.5% and 21.68%, respectively.

**Genotyping of *E. coli* by ERIC-PCR**

ERIC-PCR image analysis in Gel J software delineated the 94 isolates of *E. coli* into five major clusters. Out of five clusters (EC1-EC5), the cluster EC4 contained the maximum number of isolates (n=33) belonging to
Kottayam, Alappuzha, and Ernakulam region isolates; Cluster EC1 carried the majority of the Alappuzha region E. coli; Cluster EC2 carried Kottayam and Alappuzha region isolates only and cluster EC5 contained isolates belonging to Ernakulam and Kottayam regions. There existed diversity in the E. coli isolated from various sites in the Vembanad Lake but similarities also existed between and within the isolates from different geographical locations of the Lake. Within the clusters, several clades were formed that indicated very closely related E. coli isolates from different sites of the Lake (Fig. 3).

ERIC PCR analysis and clustering of the phenotypic AMR profile data revealed that the multidrug-resistant isolates from stations O and P were clustered along with the AC, stations I, and AD2 was grouped in the single cluster indicating the mixing of water of Kottayam and Alappuzha stations near the barrage. Among the 35 stations, 16 stations harbored blaCTX-M types of ESBL and higher number of ESBL were detected near the barrage region.

**Multi Locus Sequence Typing**

MLST analysis of the selected ESBL E. coli revealed that blaTEM-116, blaCTX-M-55, and blaSHV-27 belonged to new STs as a single locus variant of previously existing STs, and blaCTX-M-27 belonged to ST11439. ESBL E. coli belonging to blaCTX-M-152 and blaCTX-M-205 had entirely different allelic profiles with the later ST matched to only one fumC locus. eBurst analysis revealed that clones AD1 and X2 had the same profile for 6 loci, J2 and S1 had the same profile for 6 loci, others were distantly grouped in 5 different clonal complexes (Fig 5). X2 and AD1 were double locus variants (DLV) of ST 1049; F6 was a single locus variant (SLV) of ST3188; J5 and S1/J2 were SLV of ST4533 and ST3600 clones, respectively.

The ESBL E. coli clone ST 11439, SLV of ST4533, and ST10987 identified in the study do not have any clinical implications in human and animal sectors. However, the ESBL E. coli clone X2 and AD1 were Double locus variant (DLV) of ST 1049, the descendant of the Clonal Complex (CC) ST155 which has zoonotic potential were reported in sewage and drinking water of Kerala and Maharashtra in India (Salim et al. 2019; Rayasam et al. 2019). This clone ST155 has been recognized as the most important strain that has an intrinsic ability to acquire colistin resistance (Matamoros et al. 2017). Likewise, the SLV clone of ST443 belonging to clonal complex ST205 ESBL E. coli was identified in wild birds in Pakistan, Chile, Portugal, Sweden, and Switzerland (Guenther et al. 2011; Hernandez et al. 2013; Zurfluh et al. 2013; Mohsin et al. 2017; Atterby et al. 2017). However, these DLV of ST155
and ST205 were isolated as ESBL E. coli in the present study from the non-clinical environment which has anthropogenic activity as well as wild bird populations. In the present study, ESBL E. coli belonging to blaCTX-M was not linked to the ST131 clone of Germany and Japan (Matsumura et al. 2015; Ghosh et al. 2017).

Plasmid Based Replicon Typing

PBRT analysis revealed that thirty-three of the identified E. coli / ESBL E. coli carried different types of Inc Plasmids viz., B/O, F, W, I1, FIIA, HI1, P-1α, K/B, and N. Twenty patterns were observed in carrying these Inc plasmids. Eighteen of the E. coli carried B/O plasmid, followed by F plasmid. E. coli from eighteen stations harbored Inc plasmids. Eight stations in Kottayam harbored Inc plasmids in contrast to Ernakulam and Alappuzha, which harbored in 5 and 6 of their stations, respectively. IncB/O plasmid containing E. coli was present in 10 stations in the Lake (Table. S1).

In the present investigation, the very important mobile genetic element (MGE) i.e. plasmid was identified in 33 E. coli in the Lake water. ESBL E. coli isolates from the Kottayam stations carried multi-replicon plasmid (Table S1) as identified in surface water from watersheds in Northeast Georgia, USA, and elsewhere in the other clinical infectious conditions (Carattoli et al. 2008; Cho et al. 2019). Even though resistance plasmids of the same genetic types were observed in human and animal infections as well as in other environments and food, there exists a heterogeneous genotype when one or two molecular tools/methods were used together for subtyping (Lazarus et al. 2015). The same heterogeneity was identified in the present study as evidenced by different ESBL E. coli harboring various Inc Plasmids and when compared in MLST they were either SLV or DLV or an entirely new allelic profile (Carattoli 2009; Rozwandowicz et al. 2018).

Epidemiological cut off and MAR index

Mean MAR index for environmental E. coli was 0.14 (ranged from 0.0 to 0.6) and region-wise MAR index for Alapuzha, Ernakulam, and Kottayam regions were also below the risk criteria of 0.2 (Table S1). The majority of the sampling points in the Lake had a MAR index of less than 0.2 and only 25 isolates from 12 stations exceeded the MAR index of 0.2. The geographical points AB and AD of the Lake harboured more AMRisolates with >0.2 MAR 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 (Menon et 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
regions where antibiotics were inappropriately used (Riaz et al. 2011). Such an analysis exudes an idea of the number of bacteria showing antibiotic resistance in the risk zones of the study. Based on our findings certain locations in the Vembanad Lake, especially the Kottayam region, had MAR indices of >0.2 with more diverse ESBL genotypes were identified confirming that there was selective pressure in this part of the Lake, and the region is a tourist spot too (Davies and Brown 2016). However, the average of the region-wise MAR and overall MAR of Vembanad Lake did not exceed the threshold value of 0.2. The region in this study requires special attention for devising the control strategy.

Statistical and cluster analyses

The Chi-square analysis revealed that AMP resistance was significantly (P<0.01) associated with CTX, CPD, CAZ resistance; AMC with CPD resistance; CRO with CAZ; ATM with CTX, CAZ; IPM with CTX CPD; NAL with CPD; CIP with CPD; SXT, and CPD. However, TET and CHL resistance were not associated with FOX, CTX, CPD, and CAZ (P>0.05). Interpretation based on the logistic regression analysis revealed that the highest probability of *E. coli* in Vembanad Lake being resistant was towards cefotaxime (0.7) followed by ampicillin (0.3) as depicted in Table 1. Clustering of phenotypic AMR profile data produced 3 clusters with all the susceptible isolates grouped to a single cluster EC1 along with other fewer resistant isolates; EC2 cluster contained 15 isolates with multidrug resistance from all the three regions, EC3 cluster containing 4 isolates belonged to the Kottayam region of the Vembanad Lake and adjacent of the Alapuzha region with pattern matching to 7 antibiotics. Dendrogram with the isolate identity and station number is depicted in Fig. 4.

The present study has identified ESBL *E. coli* with genetic makeup *viz.*, ST11439- *bla*CTX,M-27 - IncF plasmid; STnew (SLV ST443) - *bla*SHV,27; STnew (SLV ST443) - *bla*CTX,M-55; STnew (SLV ST1049) - *bla*CTX,M-55 - Inc (B/O, HI1, I1, F); STnew (SLV ST1049) - *bla*CTX,M-27; STnew (SLV 4533) - *bla*TEM-116.

Healthy human and food-producing animals carried ESBL-producing strains of *E. coli* (Huijbers, P.M. et al. 2013). These resistant populations enter the aquatic environment and to the human chain increasing the risk of ESBL resistance transfer to the gut pathogens. Even though, Ecoff and MAR index determination provided the evidence for the presence of more WT strains rather than acquired resistance strains posing “low risk” to the population in the vicinity of the lake, the dissemination of ESBL-producing *E. coli* outside the health care setting
through water and food generally cannot be disregarded (Dhanji, H. et al. 2011). Surveillance of antibiotic resistance in water will be a valuable tool for the screening of resistance trends in the human population (Kwak et al. 2015) and the results of the present study indicate large diversity between the AMR profiles in E. coli isolated from various points of the Lake.

With the total population of 35 million, density of more than 859 km$^{-2}$ in the Lake vicinity that is three times as densely to rest of India, and the concomitant pressure on the natural resources, along with the additional pressure from floating population or tourism posesa serious threat to the public health. The lake Vembanad is microbially contaminated as evidenced by this study especially in the Kottayam regions and needs special attention for mitigation. The increasing anthropogenic pressure, chemical and microbiological pollution, the uncontrolled use of antimicrobial agents, and increased antibiotic consumption as well as the free movement of population and goods are the main factors facilitating the global invasion of bacteria with extremely high resistance to antibiotics. The presence of ESBL-producing E. coli with different subtypes in the water bodies (Vembanad Lake) meant for various activities viz., fisheries, agriculture including animal agriculture and tourism activities increase the risk factor of exposure and transmission to the human through water or food chain.

Conclusions

The quality assessment of the water in Vembanad Lake at thirty-five locations along the inland and coastal areas that encompass both freshwater and brackish waterparts of the whole lake revealed the prevalence of E. coli in 77% of the stations and the distribution of variant AMR and ESBL E. coli in the entire stretch of the Lake. The study also revealed the presence of MDR E. coli in different locations. The study also identified 39 different AMR patterns among the E. coli and 31.8% were extended-spectrum β-lactamase (ESBL) producers; with intra and inter-sample variations in AMR profiles. Despite different ESBL E. coli were identified, the MLST has shown that these ESBL E. coli are not related to epidemic clones of clinical infections. Epidemiological cut-off (Ecoff) and Multiple Antibiotic Resistance (MAR) index evidenced the predominance of wild type (WT) isolates than E.coli with acquired resistance in the Lake Vembanad indicating "low risk" to the population residing in the vicinities of the Lake. However, considering the continuous inflow of floating population of intracountry and international in the form of tourism, the high population density of the region exerting tremendous anthropogenic pressure on natural resources and so are the microbial pollutants with AMR can pose a serious threat not only human health but also, the
animals and the environment. This suggests for identification point and non-point sources of the fecal indicators (*E. coli*) by microbial and molecular source tracking tools.

**Declarations**

**Ethics approval and consent to participate**

Not Applicable

**Consent for publication**

Not Applicable

**Availability of data and materials**

Data generated from the research work viz., antimicrobial resistance, resistance gene, multi-locus sequencing, plasmid based replicon typing, ERIC PCR clusters are available in the repository of ICAR-CIFT, Microbiology Fermentation and Biotechnology Division.

**Competing interests**

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

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**Authors' contributions**

Murugadas Vaiyapuri, Madhusudana RaoBadireddy, Visnuvinayagam Sivam, Ravishankar Chandragiri Nagarajaraao and Mukteswar Prasad Mothadaka substantially contributed to conceptualization, designing of the work, analysis of data, drafting, and revision of the manuscript. Murugadas Vaiyapuri, Anna SherinPulithara Sebastian, Sandhya SoolamkandathVariem., ShaheerPeeralil., performed the experiments viz., isolation, identification of *E. coli*, determination of AMR and PCR analysis; Murugadas Vaiyapuri, Iris George, and Devi Sanjeev performed PBRT and MLST and eBurst analysis; Murugadas Vaiyapuri and ShaheerPeeralil has revised the
Figure and constructed the phylogenetic tree; Muthulakshmi Thandapani performed PCR for resistance genes; Radhakrishnan NairVasudevan, JoshyChalil George., Murugadas Vaiyapuri., Visnuvinayagam S., and Sheela Albert Mosesmade the sampling strategy and performed the statistical analysis.

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Appendix A. Supplementary data

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

References

Aarestrup FM, McDermott PF, Kahlmeter G (2007) Antimicrobial susceptibility testing—clinical break points and epidemiological cut-off values. The Community Reference Laboratory for Antimicrobial Resistance.

Abgottspon H, Nüesch-Inderbinen MT, Zurfluh K, Althaus D, Hächler H, Stephan R (2014) Enterobacteriaceae with extended-spectrum-and pAmpC-type β-lactamase-encoding genes isolated from freshwater fish from two lakes in Switzerland. Antimicrobial agents and chemotherapy 58(4):2482-4.

Al-Mossawi MAJ, Kadri M, Salem A, and Salama M (1982) Incidence of antibiotic resistant fecal coliforms in the coastal waters of Kuwait. Water, Air, and Soil Pollution 17, 141-149.

Atterby C, Börjesson S, Ny S, Järhult JD, Byfors S, Bonnedahl J (2017) ESBL-producing Escherichia coli in Swedish gulls—a case of environmental pollution from humans?. PloS one 12(12):e0190380.

Azam M et al. (2016) blaCTX-M-152, a Novel Variant of CTX-M-group-25, Identified in a Study Performed on the Prevalence of Multidrug Resistance among Natural Inhabitants of River Yamuna, India. Frontiers in microbiology 7, 176, doi:10.3389/fmicb.2016.00176.

Baird RB, Eaton AD, Rice EW, and Bridgewater L. (Eds.). (2017) Standard methods for the examination of water and wastewater. Washington, DC: American Public Health Association.

Bajpai T, Pandey M, Varma M, Bhatambare GS (2017) Prevalence of TEM, SHV, and CTX-M Beta-Lactamase genes in the urinary isolates of a tertiary care hospital. Avicenna journal of medicine 7(1):12.
Barguigua A, El Otmani F, Talmi M, Zerouali K, Timinouni M (2013) Prevalence and types of extended spectrum
β-lactamases among urinary Escherichia coli isolates in Moroccan community. Microbial pathogenesis
61:16-22.

Bevan ER, Jones AM, Hawkey PM (2017) Global epidemiology of CTX-M β-lactamases: temporal and
geographical shifts in genotype. Journal of antimicrobial chemotherapy 72(8):2145-55.

Bush K, Jacoby GA (2010) Updated functional classification of β-lactamases. Antimicrobial agents and
chemotherapy 54(3):969-76.

Byarugaba DK (2004) Antimicrobial resistance in developing countries and responsible risk factors. International
journal of antimicrobial agents 24(2):105-10.

Carattoli A. (2009). Resistance plasmid families in Enterobacteriaceae. Antimicrobial agents and
chemotherapy 53(6): 2227-2238.

Carattoli A. et al. (2005) Identification of plasmids by PCR-based replicon typing. Journal of microbiological
methods 63(3): 219-228.

Carattoli A. et al. (2008) Molecular epidemiology of Escherichia coli producing extended-spectrum β-lactamases
isolated in Rome, Italy. Journal of clinical microbiology 46(1):103-108.

Chandran A, Hatha AAM, and Varghese S (2008) Increased prevalence of indicator and pathogenic bacteria in
Vembanadu Lake: a function of salt water regulator, along south west coast of India. Journal of water and
health 6: 539-546.

Chandy SJ, Thomas K, Mathai E, Antonisamy B, Holloway KA, Stalsby Lundborg C (2013) Patterns of antibiotic
use in the community and challenges of antibiotic surveillance in a lower-middle-income country setting: a
repeated cross-sectional study in Vellore, South India. Journal of antimicrobial chemotherapy 68(1):229-
36.

Cho S et al. (2019) Genetic characterization of antimicrobial-resistant escherichia coli isolated from a mixed-use
watershed in northeast Georgia, USA. International journal of environmental research and public health
16(19): 3761.

CLSI (2019). Performance standards for antimicrobial susceptibility testing; Twenty-ninth informational
supplement. CLSI document M100-S29, Clinical and Laboratory Standards Institute, Wayne, PA
Dallenne C, Da Costa A, Decré D, Favier C, Arlet G (2010) Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. Journal of Antimicrobial Chemotherapy 65(3):490-5.

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

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

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

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

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

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

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

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

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

Harada, Y. et al. (2013)Clinical and molecular epidemiology of extended-spectrum β-lactamase-producing Klebsiellapneumoniae and Escherichia coli in a Japanese tertiary hospital. J Med MicroDiagn, 2127: 2161-703.
Harwood, V. J., et al. (2014) Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiology Reviews*, **38**, 1-40.

Hassan, H. & Baha A. (2014) Molecular characterization of extended-spectrum beta-lactamase producing Enterobacteriaceae in a Saudi Arabian tertiary hospital. *The Journal of Infection in Developing Countries*, **8.03**: 282-288.

Hassuna, N.A. et al. (2020) Molecular characterization of Extended-spectrum β-lactamase-producing *E. coli* recovered from community-acquired urinary tract infections in Upper Egypt. *Sci Rep* **10**, 2772. https://doi.org/10.1038/s41598-020-59772-z

Hatha, A.A., Chandran, A. & Rahiman, K.M. (2004) Prevalence of diarrheagenic serotypes of *Escherichia coli* in the Cochin estuary, along west coast of India. *Indian Journal of Marine Sciences*, **33**, 238-242.

Hawken, S.E. & Snitkin, E.S. (2019) Genomic epidemiology of multidrug-resistant Gram-negative organisms. *Annals of the New York Academy of Sciences*, **1435**, 39-56.

Hernandez, J., et al. (2013) Characterization and comparison of extended-spectrum β-lactamase (ESBL) resistance genotypes and population structure of *Escherichia coli* isolated from Franklin’s gulls (Leucophaeus pipixcan) and humans in Chile. *PLoS One*, **8**(9), e76150.

Huijbers, P.M. et al. (2013) Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*, **19**(6), E256-E259.

Jacoby, G.A. AmpC β-lactamases. (2009) *Clinical Microbiology Reviews*, **22**(1), 161-182.

Johnson, T. J., & Nolan, L. K. (2009) Plasmid replicon typing. In *Molecular Epidemiology of Microorganisms* (pp. 27-35). Humana Press, Totowa, NJ.

Kim, H. et al. (2017) Risk factors and molecular features of sequence type (ST) 131 extended-spectrum β-lactamase-producing *Escherichia coli* in community-onset bacteremia. *Scientific reports*, **7**(1), 1-8.

Kim, M. et al. (2013) Antibiotic resistance of bacteria isolated from the internal organs of edible snow crabs. *PLoS ONE*, **8**, 70887.

Kittinger, C. et al. (2016) Enterobacteriaceae isolated from the river Danube: antibiotic resistances, with a focus on the presence of ESBL and carbapenemases. *PloS one*, **11**, e0165820.
Kronvall, G. (2003) Determination of the real standard distribution of susceptible strains in zone histograms. *Int. J. Antimicrob. Agents*, 22, 7–13.

Kronvall, G., Kahlmeter, G., Myhre, E., & Galas, M. F. (2003) A new method for normalized interpretation of antimicrobial resistance from disk test results for comparative purposes. *Clinical microbiology and infection*, 9, 120-132.

Krumperman, P.H. (1983) Multiple antibiotic resistance indexing *Escherichia coli* to identify risk sources of faecal contamination of foods. *Applied and Environmental Microbiology*, 46, 165–170.

Kwak, Y. K., Colque, P., Byfors, S., Giske, C. G., Möllby, R. & Kühn, I. (2015) Surveillance of antimicrobial resistance among *Escherichia coli* in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? *International journal of antimicrobial agents*, 45(1), 25-32.

Lazarus, B., Paterson, D. L., Mollinger, J. L., & Rogers, B. A. (2015) Do human extraintestinal *Escherichia coli* infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. *Clinical Infectious Diseases*, 60(3), 439-452.

Liu, Haixia, et al. (2018) Molecular characteristics of extended-spectrum β-lactamase-producing *Escherichia coli* isolated from the rivers and lakes in Northwest China. *BMC microbiology*. 18, 125.

Liu, J., Zhao, Z., Orfe, L., Subbiah, M. & Call D. R. (2016) Soil-borne reservoirs of antibiotic-resistant bacteria are established following therapeutic treatment of dairy calves. *Environ. Microbiol*, 18, 557–564.

Lupo, A., Coyne, S. & Berendonk, T. U. (2012) Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in microbiology*, 3, 18.

Magiorakos, A.P. et al. (2012) Multidrug resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.*, 18(3), 268-281.

Matamoros, S. et al. (2017) Global phylogenetic analysis of *Escherichia coli* and plasmids carrying the mcr-1 gene indicates bacterial diversity but plasmid restriction. *Scientific reports*, 7(1), 1-9.

Matsumura, Yasufumi, et al. (2015) CTX-M-27-and CTX-M-14-producing, ciprofloxacin-resistant *Escherichia coli* of the H 30 subclonal group within ST131 drive a Japanese regional ESBL epidemic. *Journal of Antimicrobial Chemotherapy*, 70, 1639-1649.
Menon, N. N., Balchand, A. N. & Menon, N. R. (2000) Hydrobiology of the Cochin backwater system—a review. *Hydrobiologia*, **430**, 149-183.

Michael, I. et al. (2015) "Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water research* **47**, 957-995 (2013). Hufnagel, D.A., DePas, W.H. & Chapman, M.R. The biology of the *Escherichia coli* extracellular matrix. *Microbiology spectrum*, **3**, 249–267.

Mohapatra, B., R., Klaas, B. & Asit Mazumder. (2007) Comparison of five rep-PCR genomic fingerprinting methods for differentiation of fecal *Escherichia coli* from humans, poultry and wild birds. *FEMS microbiology letters*. **277**, 98-106.

Mohsin, M. et al. (2017) High prevalence of CTX-M-15-Type ESBL-producing *E. coli* from migratory avian species in Pakistan. *Frontiers in microbiology*, **8**, 2476.

Momtaz, H., E. Rahimi, S. & Moshkelani. (2012) Molecular detection of antimicrobial resistance genes in *E. coli* isolated from slaughtered commercial chickens in Iran. *Veterinarni Medicina*, **57**, 193–197.

Mondal, Aftab Hossain, et al. (2019) Prevalence and diversity of bla TEM, bla SHV and bla CTX-M variants among multidrug resistant *Klebsiella* spp. from an urban riverine environment in India. *International journal of environmental health research*. **29.2**, 117-129.

Morgan, R. C., Guerry, P. & Colwell, R. R. (1976) Antibiotic resistant bacteria in Chesapeake Bay. *Chesapeake Science*, **17(3)**, 216-219.

Murugadas, V., Joseph, T.C. & Lalitha, K.V. (2016) Distribution of pathotypes of *Escherichia coli* in seafood from retail markets of Kerala, India. *Indian J. Fish*, **63 (1)**, 152-155.

Nemoy, L. L. et al. (2005) Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates. *Journal of clinical microbiology*, **43(4)**, 1776–1781.

Paterson, D. L. & Bonomo, R. A. (2005) Extended-spectrum β-lactamases: a clinical update. *Clinical microbiology reviews*, **18**, 657-686.

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

Poulou, A. et al. (2014) Modified CLSI extended-spectrum β-lactamase (ESBL) confirmatory test for phenotypic detection of ESBLs among Enterobacteriaceae producing various β-lactamases. *Journal of clinical microbiology*, **52**, 1483-1489.
Queenan, A. M. & Bush, K. (2007) Carbatpenemases: the Versatile β-Lactamases. *Clinical Microbiology Reviews, 20*, 440-458.

Rasschaert, G. et al. (2005) Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of Salmonella enterica isolates. *Journal of clinical microbiology, 43(8)*: 3615-23. doi:10.1128/JCM.43.8.3615-3623.2005

Rayasam, S. D. et al. (2019) Extraintestinal pathogenic escherichia coli and antimicrobial drug resistance in a maharashtrian drinking water system. *The American journal of tropical medicine and hygiene, 100(5)*, 1101-1104.

Riaz, S., Faisal, M. & Hasnain, S. (2011) Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing Escherichia coli and Klebsiella species in Pakistan. *African Journal of Biotechnology, 10(33)*, 6325-6331.

Riedel, S. et al. (2019) A survey of AMR in Enterobacteriaceae isolated from the Chesapeake Bay and adjacent upper tributaries. *Microbiology Open, e839*.

Rozwandowicz, M. et al. (2018) Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy, 73(5)*, 1121-1137.

Sahni, R. D. et al. (2018) Extended-spectrum beta-lactamase producers: Detection for the diagnostic laboratory. *Journal of global infectious diseases, 10(3)*, 140.

Salim, A. et al. (2019) Draft Genome Sequence of an Escherichia coli Sequence Type 155 Strain Isolated from Sewage in Kerala, India. *Microbiology resource announcements, 8(27)*.

Schroeder, C.M. et al. (2002) Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. *Emerging infectious diseases, 8*, 1409-1414.

Selam, A.P. et al. (2012) Heavy metal assessment using geochemical and statistical tools in the surface sediments of Vembanad Lake, Southwest Coast of India. *Environmental monitoring and assessment, 184*, 5899-5915.

Sharma, Meeta, Sati Pathak, and Preeti Srivastava. (2013) Prevalence and antibiogram of Extended Spectrum β-Lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL producing Escherichia coli and Klebsiella spp. *Journal of clinical and diagnostic research: JCDR 7.10: 2173.*
Shivakumarswamy, S. K. et al. (2019) Phenotypic & genotypic study of antimicrobial profile of bacteria isolates from environmental samples. The Indian journal of medical research, 149, 232–239.

Singh, Nambram S., NeeljaSinghal, and Jugsharan S. Virdi. (2018) Genetic environment of blaTEM-1, blaCTX-M-15, blaCMY-42 and characterization of integrons of Escherichia coli isolated from an Indian urban aquatic environment. Frontiers in microbiology. 9, 382.

Stelling, J. M. & O’Brien, T. F. (1997) Surveillance of antimicrobial resistance: the WHONET program. Clin Infect Dis, 24 (1), 157–68.

Sukumaran, D.P., Durairaj, S. & Abdulla, M.H. (2012) Antibiotic resistance of Escherichia coli serotypes from Cochin estuary. Interdisciplinary perspectives on infectious diseases, Article ID 124879, 7.

Versalovic, J. et al. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in molecular and cellular biology. 5, 25-40.

Watts, J., Schreier, H., Lanska, L. & Hale, M. (2017) The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Marine drugs, 15, 158.

Wirth, T. et al. (2006) Sex and virulence in Escherichia coli: an evolutionary perspective. Molecular microbiology, 60(5), 1136-1151.

Zhang, H., Gao, Y. & Chang, W. (2016) Comparison of Extended-Spectrum β-Lactamase-Producing Escherichia coli Isolates from Drinking Well Water and Pit Latrine Wastewater in a Rural Area of China. BioMed research international, 4343564.

Zurfluh, K., Hächler, H., Nüesch-Inderbinen, M. & Stephan, R. (2013) Characteristics of extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. Appl. Environ. Microbiol., 79(9), 3021-3026.
Fig. 1. Frequencies of Antimicrobial Resistance in *E. 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- Amoxicillin/Clavulanic 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.

Fig. 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.

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

The blue pins denote the Ernakulam Region, green pins denote Ernakulam 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.

Fig. 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.

Fig. 4. Dendrogram of phenotypic AMR profile of *E. coli* isolates from different stations of Vembanad Lake

Fig. 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.

Table 1. Resistant (R) and Wild Type (WT) *E. coli* isolated from Vembanad Lake, Kerala, India

Table 2. Variations in AMR patterns of Extended Spectrum β-lactamase *Escherichia coli* (ESBL) and other *E. coli* isolated from Vembanad Lake, Kerala, India
Table 3. Distribution of ESBL genes in *E. coli* isolated from Vembanad Lake

Supplementary file

Resistance mapping and genetic diversity of ESBL *Escherichia coli* isolated from largest fresh cum brackish water of the Vembanad Lake, Kerala, India