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Original Article

Molecular characterization of CTX-M type Extended Spectrum Beta Lactamase producing *E. coli* isolated from humans and the environment

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Abstract

Purpose: *Escherichia coli* is a common pathogen causing community- and hospital-acquired infections. The infections caused by the Extended Spectrum Beta Lactamase (ESBL) enzymes-producing *E. coli* hinder antibiotic treatment. **Materials and Methods:** Plasmid DNA samples were subjected to PCR specific for TEM, SHV and CTX-M genes obtained from 110 *E. coli* strains isolated from hospitalized patients, healthy individuals and environment in Vellore, South India**. Results:** Among the 110 isolates tested, 21.8% were positive for TEM and 2.7% positive for SHV and 91.8% positive for CTX-M. The proportion of CTX-M positive *E. coli* was not statistically different between the study groups. Nineteen of 20 strains were CTX-M-15 type and the other was CTX-M-14 type. The phylogenetic analysis of 19 strains clustered with the pandemic CTX-M-15-ST131 strain, indicating this as an evolving global problem for antibiotic therapy. The geomapping of samples indicated 'hotspot' areas of healthy individuals, patients and the environmental samples. **Conclusion:** The spatial presentation of GIS mapping allowed identification of clustering among patients and healthy individuals and contaminated environmental points.

Key words: *Extended spectrum β lactamase, E. coli, PCR, geomapping*

Introduction

Extended Spectrum β-Lactamase (ESBL)-mediated antibiotic resistance is the rapidly growing mode of resistance observed in bacteria notably among the Gram-negative pathogens. Chiefly, the TEM and SHV type ESBL are known to be wide spread, causing many infections.[1] The beginning of this new century marked a massive distinction in the distribution of ESBLs by the emergence of CTX-M, a new ESBL type. CTX-M was found different from the classical TEM and SHV, as this gene originated in a clinically insignificant genus *Kluyvera* spp., present in the environment.[2] Ever since its earliest appearance, CTX-M has spread rapidly all across the globe to the extent of even replacing the other ESBL types.

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The incessant spread and global prevalence of ESBL resistance poses a serious threat to the human health, as it is responsible for the narrowing of therapeutic options pertaining to infections. The Asia–Pacific region, particularly India, China and Thailand are marked high-risk countries because of the increased rates of infections, both hospital- and community-acquired, caused by ESBL-producing *E. coli* and *K. pneumonia.*[3] India, with the prevalence >80%, is now said to be the capital of ESBL-producing strains. There is an urgent need for information from continuous surveillance to obtain information that will help formulate appropriate antibiotic policy to manage the growing drug resistance problem. Geographical information system (GIS) has been applied to several communicable diseases, but there has been little work conducted on its use in identifying the spread of drug resistant pathogens, particularly in India. Modelling ESBL pathogen transmission facilitates the understanding of spatial and temporal patterns of ESBL *E. coli* infection in patients, healthy population and in the environment.

We attempted to characterize the distinct ESBL types (TEM, SHV and CTX-M) produced by *E. coli* isolated from different groups of humans and the environment. We also aimed to provide insights into the geographic distribution of ESBL among different study groups and environment on a citywide scale.

Materials and Methods

Vellore district lies at a latitude of 12°15/to 13°15/North and at a longitude of 78°20/to 79°50/East in Tamil Nadu state, India. The district has a large geographical area of 6077 km^2. The Vellore metropolitan area is a considerably

small but crowded city with a total population of 481,966 according to the census. The study hospital, which is located at a rural area about 8 km away from the municipal limit, is a 200 bed multi-specialty hospital serves patients from rural, peri-uban and contiguous urban areas of Vellore district.

In our previous study (George *et al*. In Press), 363 *E. coli* strains were isolated from stool samples from patients, healthy volunteers (adults and infants) and environmental samples. The patient samples were collected at over a period of 14 months. Environmental samples were collected from sewage drains, public toilets, slaughterhouses and markets. Samples from the environment include water samples and swab samples. Water samples were collected from different sewage drains and open and stagnant water bodies present in Vellore city. Environmental swab samples were collected from public toilets, market places and slaughterhouses in Vellore. The hospital environment was studied by collecting swab samples from wards, procedure rooms and ICUs of the study hospital. Samples from the hospital sewage were also included in the study.

Clean, sterile falcon tubes were used for sample collection; 50 ml of water was collected by dipping the falcon tube horizontally against the water flow. The tubes were then sealed and transported to the laboratory in an icebox.

Sterile cotton swabs with long handles were used for sampling. The swabs from public toilet include samples from the basin, the foot/thigh rest and the toilet floor. Swabs were also collected from the cutting boards and utensils used in the meat markets and slaughterhouses. The swabs were dipped in sterile normal saline in case of dry surface sampling. After collection, the swabs were directly placed in tubes containing sterile MacConkey broths and transported to the laboratory in an icebox.

Among these, 124 were identified as ESBL positive by double disk synergy test. In this study, strains (n = 110) which we were able to revive from stored culture was used for the PCR assay. The PCR assay was targeted for the presence of TEM, SHV and CTX-M genes with specific primers.

PCR for TEM, SHV and CTX-M genes

The plasmid DNA was extracted from all ESBL-producing *E. coli* isolates using plasmid mini kit (HiYield plasmid mini kit, Real Biotech Corporation, Banqiao, Taiwan). The extracted Plasmid DNA was stored at -20°C until use.

All the plasmid DNA samples were subjected to uniplex PCR using primer sets each specific for TEM, SHV and CTX-M genes. The primers used in this study are indicated in [Table 1]. The cycling conditions used for TEM include an initial denaturation at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 15 seconds, primer annealing at 55°C for 30 seconds and primer extension at 72°C for 45 seconds and a final extension at 74°C for 10 minutes. For SHV and CTX-M, initial denaturation at 95°C for 3 minutes, 30 cycles of denaturation at 95°C for 1 minute, primer annealing at 52°C for 40 seconds and primer extension at 72°C for 1 minute and final extension at 72°C for 5 minutes were used. A standard strain *E. coli* A-2-23 lodging TEM, SHV and CTX-M genes (Kind courtesy Dr. Anand Manoharan, Christian Medical College and Hospital, Vellore, India) was used as positive control in all PCR assays. PCR reagents were procured from (PCR Master Mix, Thermo Fisher Scientific, PA, USA). Negative controls replacing the template with nuclease-free water were included in every assay. The PCR assays were carried out in Eppendorf thermal cycler (Mastercycler® personal 5332, Hamburg, Germany). Standard precautions were employed for PCR testing such as flow through, disposable plastic ware and gloves, filter blocked tips and dedicated micropipettes, as previously described in standard protocols.

An aliquot of 5 μL amplicon was analyzed by gel electrophoresis in 2% agarose (Sigma, MO, USA) prepared in Tris-Borate-EDTA buffer containing 0.5 μg/mL of ethidium bromide (Sigma, MO, USA). The gels were examined in a gel documentation system (Genei, Bangalore, India) for specific amplification at 1080 bp TEM product, 471 bp SHV product and 544 bp CTX-M product.

DNA sequencing and analysis

Twenty CTX-M PCR positive samples were selected through statistical random table method. The 544-bp PCR amplicons were sequenced through a commercial firm (Scigenom, Cochin, Kerala, India) using the primers used for amplification. Bidirectional sequencing was carried out for the amplified DNA. The two sequences were aligned to obtain a single positive strand and subjected to BLAST analysis. The sequences were submitted in GenBank and accession numbers were obtained. The MEGA 5.2 software program was used for ClustalW alignment of the partial CTX-M gene nucleotide sequences. Phylogenetic tree were generated by Maximum Likelihood method and Tamura-Nei model using the same program from the aligned nucleotide sequences. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by applying bootstrap resampling (n = 1000 replicates).

Initially, all 20 sequences were compared with each other and a phylogenetic tree was computed. The subtype was identified for all 20 sequences by comparison with existing GenBank sequences database. In addition, sequences from GenBank of each subtype were identified from different countries and compared towards scrutiny of genetic diversity. All 20 sequences were subsequently compared with selected 35 CTX-M gene sequences. The

sequences were selected from 13 different countries from continents of Asia, Africa, Europe and South America for analysis. The sequences were clustal aligned and a dendrogram was plotted for analysis.

Geomapping

The geographic coordinates (latitude and longitude) of each point of sampling and residential location of patients were identified manually using Google® Map. Among 110 positive samples, geographic coordinates of 109 (100 patients and 9 environmental sampling locations) were geo-coded in Epi Info 7.1.2 software program along with patient groups and ESBL types of each sample. One patient was eliminated because of vague description of location and could not be confirmed. A thematic map was then established using the base map as the background. Patient groups, healthy individuals and environmental sampling locations were imposed as different layers on it.

Statistical analysis

The statistical analysis was carried out using Epi Info 6.04 d software program for calculation of Confidence intervals and Chi square test for comparison between groups.

Results

One hundred and ten isolates were examined using type specific PCR as described above. The PCR results of 110 isolates indicated 24 (21.8%; mid-p 95% CI: 14.85-30.26) positive for TEM and 3 (2.7%; 0.7-7.2) positive for SHV and 101 (91.8%; 85.52-95.94) positive for CTX-M. A representative gel analysis for each PCR assay is shown in [Figure 1]. The details of the distribution of these ESBL types under each study category are given in [Table 2]. The results also shows that none of the strains harboured all three types together in their plasmids and only one strain was seen without any of these three type of ESBL. The prevalence of CTX-M gene carrying plasmid was higher in all the study groups, but no significant difference was observed in the prevalence of this gene in healthy individuals compared with patients' and environmental isolates of *E. coli*. The TEM gene carrying plasmid was next common to CTX-M and found to be significantly higher in *E. coli* obtained from patients compared to healthy individuals (P = 0.014). Frequency of TEM and CTX-M gene carrying plasmid(s) together among all the study groups was not significantly different between the groups [Table 2].

Table 1: Primers used in the PCR for ESBL genes

Target gene	Forward and reverse primers	Product size (bp)	Reference
*bla*TEM	ATAAAATTCTTGAAGACGAAA; GACAGTTACCAATGCTTAATCA	1080	29
*bla*SHV	TCAGCGAAAAACACCTTG; TCCCGCAGATAAATCACC	471	30
blaCTX-M	ATGTGCAGYACCAGTAARGTKATGGC; TGGGTRAARTARGTSACCAGAAYCAGCGG	544	31

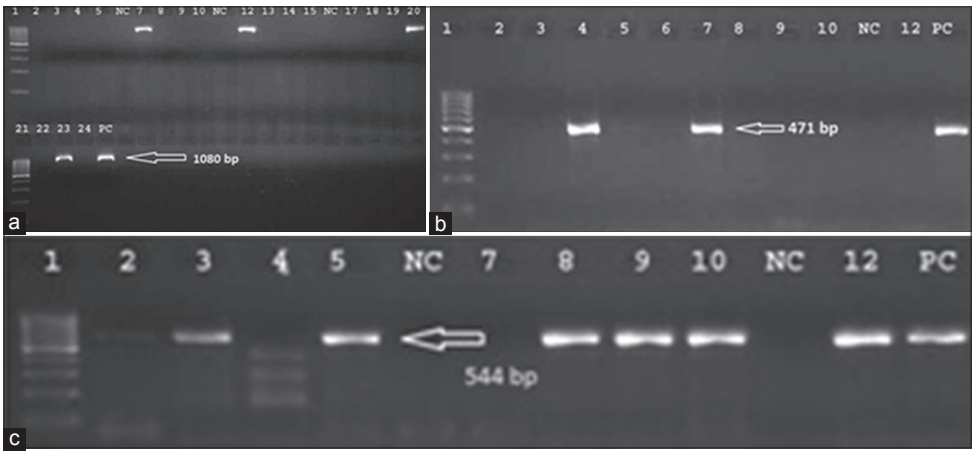

Figure 1: Gel picture showing the results of PCR amplification of TEM, SHV and CTX-M genes (a) Analysis of PCR amplicons on a 2% agarose gel indicating Lane 1 - 100-bp DNA ladder; Lanes 7, 12, 20 & 23 – patient samples positive for TEM gene (1080 bp); PC - Positive control; NC - Negative control (b) Analysis of PCR amplicons on a 2% agarose gel indicating Lane 1 - 100-bp DNA ladder; Lanes 4 & 7 - patient samples positive for SHV gene (471 bp); PC - Positive control; NC - Negative control (c) Analysis of PCR amplicons on a 2% agarose gel indicating Lane 1 – 100-bp DNA ladder; Lanes 3, 5, 8, 9, 10 & 12 - patient samples positive for CTX-M gene (544 bp); PC - Positive control; NC - Negative control

The partial sequences from statistically selected CTX-M positive strains were submitted to GenBank and the accession numbers are given below.

GenBank accession numbers

KF448072, KF448073, KF448074, KF448075, KF448076, KF448077, KF561448, KF561449, KF561450, KF561451, KF561452, KF561453, KF561454, KF561455, KF561456, KF561444, KF561445, KF561446, KF561447, KF561457.

These 20 sequences when subjected to BLAST search had maximum identity to CTX-M type β-lactamase gene and fell into two major lineages: CTX-M-14 and CTX-M-15. Among the 20 sequences, 19 were CTX-M-15 (indicated with dark circles) and one was CTX-M-14 (indicated with dark square; [Figure 2]. On BLAST analysis, all CTX-M-15 sequences were found to have a high level of similarity to a pandemic, multidrug resistant *E. coli* O25b-ST131 strain. [CTX-M-type ESBL-producing *E. coli* with sequence type 131 belonging to the O25b serogroup and the B2 phylogenetic group (ST131-O25b)]. Our strains are now referred to as ST-131-like CTX-M-15, because further confirmatory test such as multilocus sequence typing (MLST) for 7 housekeeping genes was not carried out.

Table 2: Incidence of TEM, SHV and CTX-M in different study groups

ESBL type	Patients (*n*=36) (%)	Healthy subjects (*n*=65) (%)	Environment (*n*=9) (%)
TEM	10 (37.7)	11 (16.9)	3 (33.3)
SHV	0	1 (1.5)	2 (22.2)
CTX-M	32 (88.8)	62 (95.3)	7 (77.8)
TEM and SHV	0	0	0
CTX-M and TEM	7 (19.4)	9 (13.8)	3 (33.3)
SHV and CTX-M	0	1 (1.5)	1 (1.5)
All three	0	0	0
None	1 (2.7)	0	0

The nucleotide comparison of 55 partial CTX-M gene sequences that includes 20 sequences from our study revealed 5 major phylogenetic clusters. Cluster I with 25 sequences had 10 CTX-M-15 sequences reported from our study and 15 from countries such as India, Uganda, Ireland, Brazil, Germany and China. In Cluster II, among the 10 sequences clustered together, 4 were from our study and 6 were from India, Nepal and Iran. In Cluster III, one CTX-M-14 sequence identified in our study was homogeneously clustered with 10 other CTX-M-14 sequences reported from Germany, China, Spain, Russia and Hong Kong. Cluster IV with 7 sequences clustered with

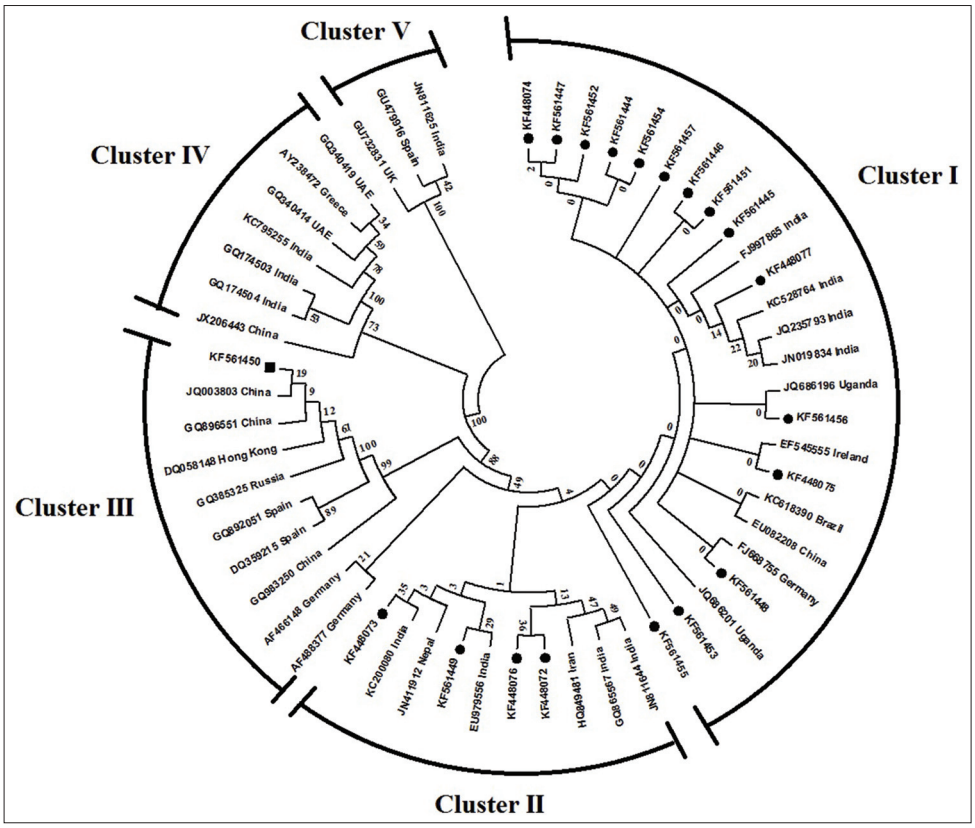

Figure 2: A dendrogram showing partial CTX-M gene sequence divergence of ESBL-positive *E. coli*. Bootstrap values were calculated for 1000 replications

sequences reported from UAE, India, China and Greece. Cluster V was with 3 sequences reported from India, Spain and UK.

Out of 109 samples tested for geographic distribution through geocoding technology, 83 were from the city. The sampling sites and Tamil Nadu Map indicating location of positive isolates recovered in this study were shown in [Figures 3a and b], respectively. Of these, 10 were patients (shown in red), 65 were healthy adults and infants (shown in green) and 6 were environmental samples (shown in blue). The other 26 positive samples are patients from outside our study area and therefore are not shown in the map. The results identified a significant clustering of patients and healthy individuals to sewage points indicating acquisition of ESBL-positive *E. coli* from environmental sources. All ESBL-positive *E. coli*-shedding patients were from the same location and were much closer (0.1-2 km) to certain environmental points (polluted water bodies such as open sewage drains and public toilets; [Figure 3c]. The healthy adults and infants positive for ESBL-producing *E. coli* were also found to appear to cluster in each area. A large number of healthy individuals positive for ESBL were physically living nearer to the environmental contamination points, whereas those who lived further away had low carriage of ESBL-producing *E. coli*.

Discussion

Our study was aimed to understand the frequency of faecal carriage of TEM, SHV and CTX-M type ESBL-producing *E. coli* among the hospitalised patients, healthy adults and infants in the community and environmental sources and study the geographic distribution of ESBL among different study groups and environment on a citywide scale.

The PCR results of ESBL *E. coli* indicated 21.8% of TEM, 2.7% of SHV and 91.8% of CTX-M. The understanding from the various reports worldwide is that CTX-M-15 and CTX-M-14 are the most important types posing a threat to human and animal health.[4] In our study, the PCR results obtained makes us infer that CTX-M was the predominant type of ESBL plasmid carried by *E. coli* isolated from all the study groups. In a recent study in north-western India by Kaur and Aggarwal,[5] 45.8% isolates were found to be ESBL producers. The multiplex PCR for TEM, SHV and CTX-M showed 59.32% of *E. coli* possessed the CTX-M genes. Among the Klebsiella isolates, a majority were co producers of the ESBL genes; either 2 or all 3 genes co-existed.[5] Roy *et al*.[6] from east India reported CTX-M-15 in all ESBL-positive isolates from patients with neonatal septicaemia. Hussain *et al*.[7] from Hyderabad, in an adjacent state to us in south India reported 16 ST131

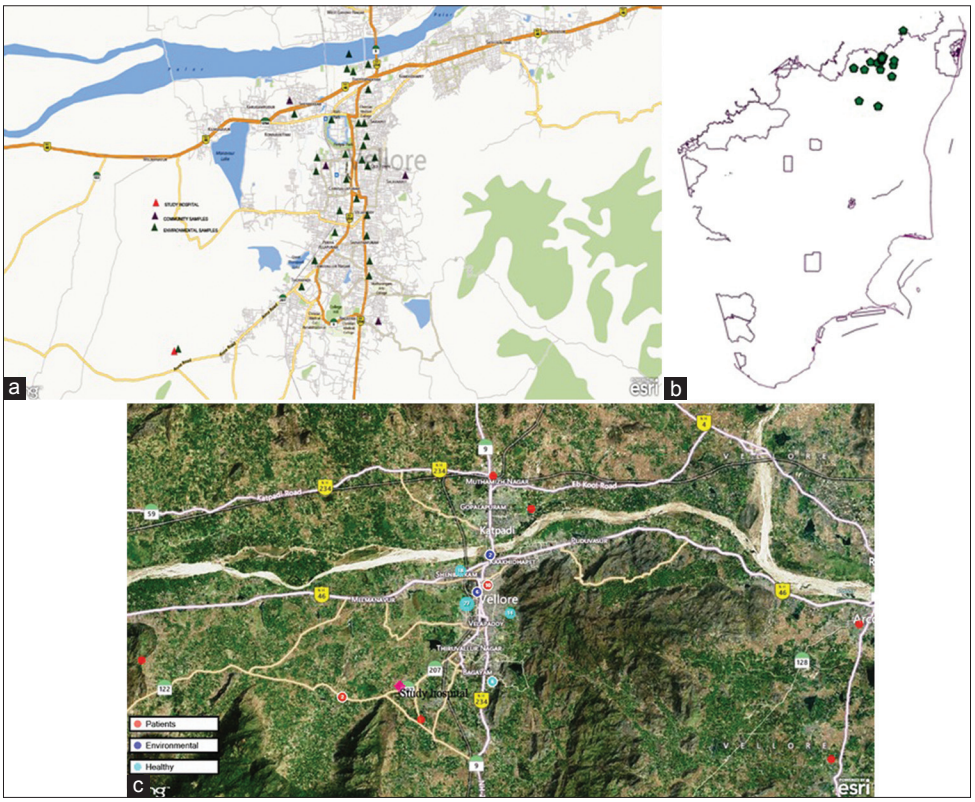

Figure 3: (a) A street map of Vellore showing the sites of sample collection for the study (b) Map of Tamil Nadu (South India). Dots indicate location of positive isolates recovered in this study (c) The satellite view of the area showing the distribution of ST-131-like CTX-M isolates from the study groups. The number of strains in each area is indicated in the colour-coded circle of each group

E. coli isolates identified by MLST among 100 clinical *E. coli* isolates and all 16 ST131 isolates harboured the CTX-M-15 gene. Another study[8] from south India reported 79.4% of phenotypic positive nosocomial isolates positive for CTX-M genes, of which 63.7% were *E. coli* and all were positive for the CTX-M-1 group. These reports document evidence of the high prevalence of CTX-M type ESBL in almost all parts of India and highlight the escalating antimicrobial resistance and the likely switch over to CTX-M-15 type ESBL in India. Comparison of our study CTX-M ESBL-producing *E. coli* sequences with sequences reported from Southeast Asian countries other than India was limited as there were a few GenBank submissions only from Thailand and Nepal. The Thailand strain matched with 100% identity with our 14 sequences and 99% with 5 other sequences. Our CTX-M-14 strain was non-identical to this Thailand strain. The Nepal strain included in the phylogenetic analysis shown in [Figure 2] was clustered along with our 4 CTX-M-15 sequences in cluster II.

This current study gives the understanding of the CTX-M-producing *E. coli* faecal carriage in patients and healthy individuals and presence of these strains in the environment. The ESBL strains from the patients harboured CTX-M (88%) and TEM (37%) indicating that 19% of the strains had both the enzymes. The retention of the TEM genes in the hospital strains indicates the character of TEM thriving in hospital settings. In comparison to our study groups, a statistical significant difference was found in the distribution of TEM but not in CTX-M among healthy individuals and patients. Altinkum *et al*.[9] have reported no significant difference in the distribution of ESBL types among hospitalized and in-patients. In our study, CTX-M and TEM gene-carrying plasmid (s) was found in patients (19.4%), healthy individuals (13.8%) and CTX-M (33.3%). The difference between patients and healthy individuals however was found insignificant. None of the strains harboured all three together, and one strain did not harbour any of the three genes. In this study, no attempt was made to characterize the transmissibility of the resistance plasmids using recipient strains.

The strains from the healthy individuals had the highest rate of CTX-M (95.3%) suggesting the easy spread of these strains in the community. This is the an important finding of this study because the high rate intestinal carriage of CTX-M *E. coli* may lead to the sudden outbreaks of infections caused by them in a hospital setting. A six-centred Indian study recently indicates the co-occurrence of these genes among *E. coli* strains isolated from clinical specimens (TEM and CTX-M predominantly in *E. coli* (39.2%); TEM, SHV and CTX-M together (42.6%) among the Klebsiella spp.).[10] Horton *et al*.[11] observed high level of faecal carriage of CTX-M *E. coli* in animals that has implications on environmental contamination and food production. Our study also observed high CTX-M-producing *E. coli* (77%) among the environment strains indicating their dissemination and survival. It is very clear through the results of the study that TEM and especially SHV enzymes are being replaced by the CTX-M enzymes over a period of time. The reason behind this scenario remains to be unravelled; the high mobilizing property of CTX-M gene may be one of the reasons. This phenomenon may be due to the unusual dissemination of the blaCTX-M genes achieved by fast mobilizing plasmids and transposons, which acquires high rates of clonal survival success. CTX-M ESBL in *E. coli* is an important cause of urinary tract infections, neonatal meningitis, enteric and other systemic infections in humans.[12] This acquired attribute of the strains poses a serious clinical challenge.

The CTX-M-15 sequences identified in our study were all closely associated with the pandemic *E. coli* O25b-ST131 a multidrug-resistant clone, predominantly causing community-related infections and rapidly emerging all over the globe.[13] The ST131 *E. coli* was reported to have high tendency to cause urinary sepsis compared to non-ST131, because it harbours broad range of virulence and resistance genes. The ST131 has been shown to have ability for high intestine colonization and urinary tract infection because it displays greater fitness in pathogenesis, colonization or in transmission.[14] In our study, the ST131-like CTX-M-15 suggests enhanced capacity to persist in the environment, colonize and subsequently to cause infectious diseases in humans. In consistence with previous reports,[15] our strains showed resistance to almost all generations of cephalosporins including cefazolin, cephalexin, cefotetan, cefaclor, ceftazidime, cefotaxime, cepfodoxime, ceftriaxone, cefixime and ampicillin and susceptibility to carbapenems and tigecycline (data not shown). Previously, ST131 has been shown to have the ability to cause severe infection in otherwise healthy individuals.[16] In our study, the ST131-like CTX-M-15 strains were found predominantly in healthy individuals (95.3%) as well as in patients (88.8%) and environmental samples (77.9%).

ESBL 'hotspots' were easily visualised, as were prevalence trends in neighbourhoods. Pre-existing poor water and sanitation situation, crowded conditions in peri-urban slums and rural areas of Vellore district have complicated the implementation of health measures. Molecular epidemiological studies of this strain could give useful information on the 'hotspot' areas. Future wider implementation of such mapping analysis might lead to an improved medical response through targeted outreach, water chlorination and timelier advocacy. The accessible spatial information can be used to better plan for infection management, antibiotic policy and determination of geographical barriers to health service access.

A combination of geographic and prevalence data shows identification of most likely modes of transmission and spatial risk factors, in our case proximity of polluted water

bodies could be used for environmental niche mapping patterns. Faecally shed *E. coli* can remain in contaminated water bodies for up to 50 days at 25°C regardless of type of water sources.[17] The dataset used in this study though is limited by the small sample size has shown the possible ESBL-producing *E. coli* transmission. Further research is required that incorporates a larger number of samples from hospitals and the community to determine the pattern of spread. Geographical Information System provides opportunities to identify 'hotspot' regions of multidrug-resistant pathogens such as ESBL-producing *E. coli* and further develop public health strategies for the control of infectious diseases caused by them.

In conclusion, this cross-sectional study carried out over a period of 14 months documents that there is a high prevalence of CTX-M-producing *E. coli* associated with the gut flora of patients and healthy individuals and they are observed in the environment. Significantly, the CTX-M gene positive strains carried by healthy individuals showed possible acquisition from the environmental sources. The phylogenetic analysis of 19 strains from our study clustered with the pandemic CTX-M-15-ST131 strain indicating this as an evolving global problem for antibiotic therapy.

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