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ORIGINAL ARTICLE

Molecular Characterization and Bio-Computational Analysis of Multi-Drug Resistant *E. coli* Isolated From Clinical Samples

Jahanara Kudsi [1], Prabhurajeshwar C [1] and Kelmani Chandrakanth R *
[1]*Medical Biotechnology and Phage Therapy Laboratory,
Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga-585 106, Karnataka, India.
Department of Microbiology, Gulbarga Institute of Medical Sciences (GIMS)
Gulbarga-585105, Karnataka, India
*** Corresponding author's** E-mail: : ckelmani@gmail.com

ABSTRACT
The predominance of multidrug-resistance (MDR) Escherichia coli strains producing β-Lactamase enzyme is a budding issue over the globe. Strain composing is an epidemiologically vital method not just to detect the cross transmission of nosocomial pathogens however in addition for deciding the source of contamination. The present assessment was directed to comprehend the clonal relationship among different β-Lactamase-producing MDR E. coli strains utilizing Enterobacterial repetitive intergenic consensus (ERIC) polymerase chain response (PCR). At 60% comparability cut-off esteem, the dendogram examination demonstrated that there was a sum of 14 remarkable groups of ERIC (CL-1 - CL-14) inside the 41 E. coli strains, which revealed the hereditary assorted diversity existing between them.
Keywords: *β-lactamases, ERIC -polymerase chain reaction, Escherichia coli, Multidrug Resistance*

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INTRODUCTION

Escherichia coli (E. coli) is a Gram-negative, facultative anaerobic, coliform bacterium of the class *Escherichia* that is normally found in the lower digestive tract of warm-blooded living beings (endotherms). Most *E. coli* strains are resistance, however some serotypes can cause genuine nourishment harming in their hosts, and are at times in charge of item reviews because of sustenance contamination. The resistance strains are a part of the ordinary vegetation of the gut, and can profit their hosts by creating vitamin K2, and avoiding colonization of the digestive tract with pathogenic microscopic organisms, having a harmonious relationship. *E. coli* is ousted into nature through faecal matter. The bacterium develops enormously in crisp faecal matter under oxygen consuming conditions for 3 days, however its numbers decay gradually afterwards.

E. coli and other facultative anaerobes constitute around 0.1% of gut flora, and fecal– oral transmission is the real course through which pathogenic strains of the bacterium cause sickness. Cells can get by outside the body for a constrained measure of time, which makes them potential pointer living beings to test ecological examples for faecal contamination. A developing assemblage of research, however, has inspected naturally relentless *E. coli* which can get by for broadened periods outside a host.

The bacterium can be developed and refined effectively and reasonably in a research centre setting, and has been seriously examined for more than 60 years. *E. coli* is a chemoheterotroph whose synthetically characterized medium must incorporate a source of carbon and energy [1]. *E. coli* is the most broadly considered prokaryotic model living organism, and an essential species in the fields of biotechnology and microbiology, where it has filled in as the host life form for the major of work with recombinant DNA. Under ideal conditions, it takes up to 20 minutes to replicate.

Advances in exploratory and computational innovations for biosciences have been altering natural research. The last quite a few years have seen the advancement , development of a few exceptional trial

strategies, for example, DNA sequencing procedure, DNA microarray [2], substantial scale two-dimensional protein gel electrophoresis [3]. Rising up out of the application of these advances is another method of science, the frameworks science, which accentuates an all encompassing comprehension of how organic frameworks work [4, 5].

As showed by the successful sequencing of in excess of 1000 genomes of regular plasmids, organelles, infections and viroids, microscopic organisms, plants, and creatures, including mouse [6] and human [7,8], there are barely any, innovative jumps in acquiring the hereditary data of practically any living being. Notwithstanding its suggestions for simple applications, such data guarantees to convey us more like a complete understanding on how the hereditary data in a genome decides the phenotype of a life form or a cell in a specific situation.

Coherent subsequent stages in this subject are to recognize the qualities in a genome and to decide their capacities, specifically, by clarifying what items these qualities create and how these items associate with each other. For a specific organic framework, these downstream examinations can be of importance more unpredictable than sequencing the genome. To be sure, they require a wide range of devices to describe singular quality items by utilizing biochemical, biophysical, or hereditary systems or a vast set of such sub-atomic segments by profiling quality articulation at the mRNA level-utilizing DNA microarray [2] or at the protein level utilizing two-dimensional protein gels [3] or mass spectrometry [9-11]. Likewise, other high-throughput systems, for example, yeast two-half and half investigation (11), have been effectively connected to examine cooperation's between quality items at an expansive scale [12, 13].

Strain typing is an epidemiologically essential instrument not just to detect the cross transmission of nosocomial pathogens yet in addition for deciding the source of infection. Accessible sub-typing strategies for *E. coli* incorporate pulse field gel electrophoresis (PFGE), plasmid profiling, ribotyping and polymerase chain response (PCR) - based typing strategies, for example, self-assertive prepared PCR, repetitive extragenic palindromes, Enterobacterial repetitive intergenic consensus (ERIC). As it is the direct, simple, low per strain cost and restricted requirement for specific research facility hardware supports the utilization of ERIC-PCR over other methods [15]. The present investigation was conveyed out to decide the hereditary assorted variety of various β-lactamase creating multidrug-resistance (MDR) *E. coli* strains utilizing ERIC-PCR in a clinic setup.

MATERIAL AND METHODS

Isolation of *E.coli* Isolates from clinical samples

Total 150 strains of *E.coli* from 170 different clinical samples like urine, stool, blood, pus etc were isolated. The clinical samples were collected from hospitals and diagnostic centres in Kalaburagi region over a period of three months in 2013. Identification was done by culture on EMB agar [24]. Isolation of strains was done by conventional morphological, cultural and biochemical characterisation. Standard strain of *E.coli* MTCC 443 was obtained from Medical and Phage Therapy Laboratory, Department of Biotechnology, Gulbarga University, Kalaburagi.

Identification of Multi Drug Resistance (MDR) *E. coli* isolates

Multi Drug Resistance is defined as resistance to at least three of the antimicrobials tested. The Multi Drug Resistance (MDR) character of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics [16, 17].

Molecular Characterization of Multidrug Resistant

DNA extraction

DNA was isolated from bacterial cells using DNA purification kit (DNA purification kit, Himedia Mumbia). The purified DNA was stored at -20°C. The samples were run on agarose gel and stained with ethidium bromide. The stained gel was examined for presence of bands under UV-light using molecular weight marker. [18].

Polymerase Chain Reaction (PCR) Amplification of MDR gene from *E.coli*

The PCR analysis was targeted to detect *blaCTX-M* responsible for cefotaxime mediated ESBL positive *E.coli.* The amplification condition was carried out using 20µl reaction cocktail thermal cycler (Eppendorf) (Table: 1) and PCR programme was standardized for 30 cycles and performed using modified conditions as in table: 2[19]. The amplified product was run to 1% (W/V) agarose gel electrophoresis with 1kb DNA as a standard molecular weight marker and electrophoretic profile was cited using photo gel documentation system (Velber Lourmat, France) and photograph was done.

The PCR primers sequences were obtained from published primer sequences or previously reported primers as shown in the table: 3, which are resistant to cephalosporin drugs [20].

Analysis of Genetic Relatedness of ERIC-PCR

Clonal distributions of Multidrug resistant strains were studied by enterobacterial repetitive intergenic consequences (ERIC)-PCR genotyping. ERIC-PCR amplification was carried out with conserved primers as described by Versalovic 1991 with some modifications.

The pair wise matrix of genetic distance was employed to draw their precise relationships between ESBL resistant *E.coli* isolates and for the cluster analysis for grouping the isolates based on the dendogram produced by unweighted Pair Group Method with Arithmetic Average (UPGMA) of phylogeny interface packages (PHYLIP package) [22].

Biocomputational Studies

Docking studies with *CTX-M9* structure with Cefotaxime

Computers and programs using software's were used to predict or stimulate the probable reaction between two molecules based on their 3 dimensional structures, the active site in *CTX-M 9* protein which can interact with β-lactam antibiotics including cefotaxime [23]. The PDB 3HLW protein structure was downloaded from PDB structure database in PDB file format. The cefotaxime structure was downloaded from NCBI-PubChem Compound. The software version Hex 6.3 was used for the docking of the protein structure (ligand) and the software Marvin Sketch was used for the sketching of the antibiotic structure.

The Cefotaxime antibiotic finds the binding cavity in the *CTX-M 9* protein and automatically binds (docks) with the receptor molecule (*CTX-M9*). The docking energy (E-total) is in negative form, the efficiency to binding of Cefotaxime antibiotic to the protein (*CTX-M9*) was high. Similarly, the ligand (Inhibitor) was docked into each enzyme structure. Further, the software displays binding energy in the form of E-total (Energy total) and calculates in terms of K.cal/mole.

RESULTS

Isolation and Identification of *E.coli* isolates

Total 120 *E.coli* strains were isolated from total of 170 clinical samples; collected isolates were identified on Macconkey agar as differential media; single isolated colonies were streaked on Eosin Methylene Blue (EMB) agar. The colonies with characteristic features of isolates on EMB with green colonies metallic sheen were confirmed as *E.coli* [24].

Molecular Characterization of Multiple Drug Resistance *E.coli* isolates

Isolation of Genomic DNA

Total of 20 ESBL producing *E.coli* isolates selected for genomic DNA. The presence of genomic DNA confirmed on the banding pattern and spectrophotometric analysis.

Genotypic detection of Cefotaxime (*blaCTX-M*) by Polymerase Chain Reaction

To confirm the presence cephalosporins resistant gene (*CTX-M gene*) of *E.coli* isolates were selected based on ESBL detection test pattern, they were evaluated by PCR amplification on agarose gel electrophoresis. The amplification results were positive for all strains, emphasizes the presence of *CTX-M* gene and size of the amplified products as shown in the Figure 1.

Analysis of Genetic Relatedness of ERIC-PCR

The genetic diversity analysis of 33 non-repeated randomly collected β-lactamase-producing *E. coli* isolates was carried out by ERIC-PCR fingerprint method using ERIC-IR and ERIC-2 primers. The fingerprints obtained from the ERIC typing of the 33 MDR *E. coli* isolates illustrated a DNA banding profile consisting of amplified bands ranging from 1 to 7 having size 150 bp to 2500 bp **(**Figure 2 A and B **).** At 60 per cent similarity cut-off value of the dendogram analysis confirms that there were a total of 14 unique clusters of ERIC; CL-1 to CL-14 within the 33 *E. coli* isolates, exhibiting genetic diversity (Figure 22**).** Five clusters; CL-1, CL-2, CL-6, CL-8 and CL-14 represented more than four isolates while the rest nine clusters contained less number of isolates varying from 1 to 3. Some isolates showing similar ERIC profile, four from CL-1 (S19, S16, S11 and S13), three from CL-8 (S15, S1 and S5) and three from CL-12 (S9, S2 and S4), which indicated clonal similarities between the isolates.

Biocomputational Studies

The drug cefotaxime as well as inhibitors clavulanic acid were docked into enzyme-structures (cefotaximase). Schrodinger analysis exhibited the binding of *CTX-M-9* with ligands (cefotaxime, clavulanic acid) showed reasonably higher glide (negative) energy (Table 4). As compare to the previous similar studies, clavulanic acid was found to be the more efficient inhibitors for *CTX-M-9* as compared with other inhibitors.

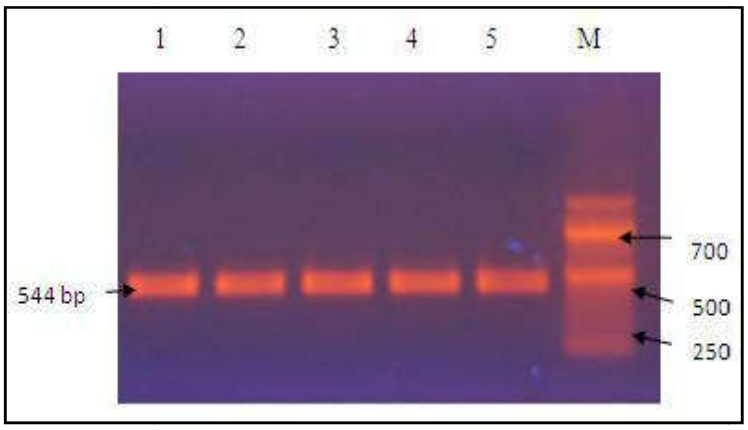

Figure 1: PCR Amplification of bla-CTX-M gene

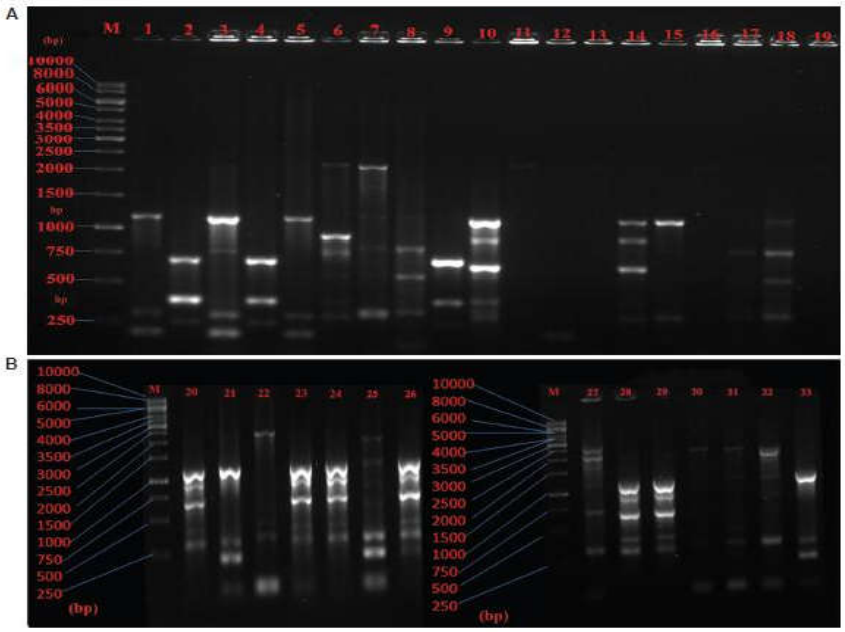

Figure 2: Agarose gel electrophoresis of enterobacterial repetitive intergenic consensus chain reaction (ERIC-PCR) generated DNA of 1-23 Escherichia coli isolates, M: Marker of 1kb

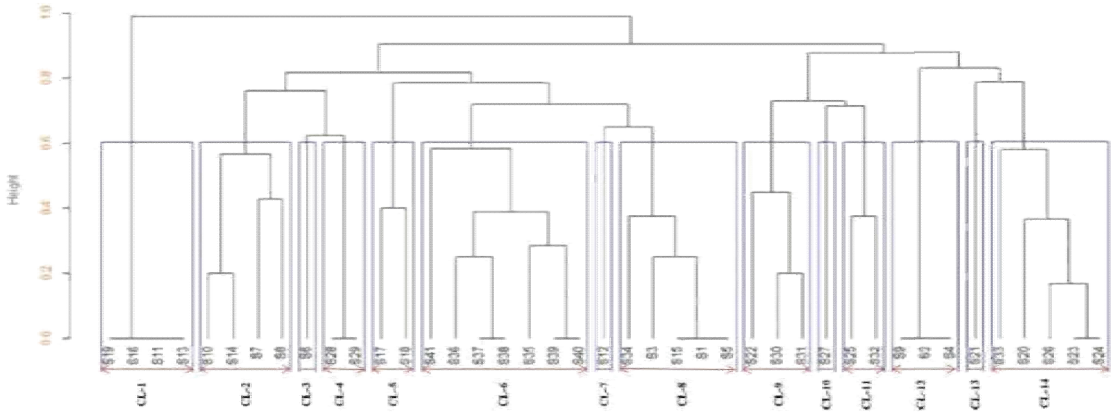

Figure 3. Dendogram of *Escherichia coli* isolates at 60 per cent similarity cut-off value analysis, it showed 14 unique clusters. CL: Cluster.

Table: 1 Master Mix of PCR reaction Mixture of *blaCTX-M* gene

Constituents	Quantity (µl)	Final Concentration
Template DNA	2.0	25 ng
10X Taq DNA assay buffer	2.0	1X
dNTPs	0.4	200 µM each dNTPs
Primer	2.0	20 picomoles
Taq DNA polymerase	0.3	1 Unit
Sterile water	13.3	20 µl

Table: 2 Programme for blaCTX-M gene Amplification

Process	Temp (^{0}C)	Time	No. of Cycles
Initial Denaturation	94	2 min	1
Denaturation	92	1 min	30
Annealing	64	1 min	
Extension	74	2 min	
Final Extension	74	10min	1
Storage	4	Infinite	

Table: 3 Sequence details of *bla*CTX-M gene primers

Primers	Primers Sequence	Primer Length	Reference
*bla*CTX-M (Forward)	5^1-TCCCGCAGATAAATCACC-3^1	18	
*bla*CTX-M (Reverse)	3^1-ATGTGCAGYACCAGTAARGT-5^1	20	

Table 4: Interaction energies of the docked with CTX-M enzyme Antibiotic and inhibitor complexes

Name of the gene variant	Cefotaxime (Antibiotic)	Clavulanic Acid (Inhibitors)
PDB ID of Template retrieved for docking	1 YLJ	
CTX-M 9	Glide energy = - 29.787	Glide energy = -22.430
	Glide gScore = -3.502	Glide gScore =-4.683

DISCUSSION

Extended-spectrum beta-lactamase (ESBL) producing strains of *Enterobacteriaceae* have emerged as a big challenge in the hospitalized patients as well as in the community where *E. coli* isolates as high as 61% have been found to be the ESBL producers, Extended-spectrum β-lactamases (ESBLs) enzymes that confer resistance to a broad range of β-lactams. Treatment of extended spectrum beta-lactamase (ESBL) producing strains of *Enterobacteriaceae* has emerged as a major challenge in hospitalized as well as community based patients[25] .In developing country like India, with scarce economic resources it is necessary for proper evaluation of antibiotic effectiveness is necessary. India is among the nations with the highest burden of bacterial infections and the crude mortality from the infectious diseases is about 417 persons per 100,000 [26]. In 2010, India was the world's largest consumer of antibiotics for human health with 12.9 x 109 units of antibiotic consumption (~10.7 units per person) [27]. Global antibiotic consumption index was reportedly high among the BRICS countries, i.e., Brazil, Russia, India, China, and South Africa during the period 2000–2010. Among the BRICS nations, 23% of the retail antibiotics sales were attributable to India [28]. The β-lactam antibiotics, especially the cephalosporin's and b-lactam-b-lactamases inhibitor combinations are major drug classes used to treat infections caused by *E. coli* [1]. Most of ESBLs are mutants of TEM and SHV enzymes, but CTX-M enzymes are the newly emerging ESBLs [29] and are increasingly prevalent worldwide among *E.coli* bacteria. CTX-M ESBLs is identified as a rapidly growing family of enzyme that selectively prefers to hydrolyze cephotaxime and most of them are active against ceftazidime [30]. Therefore in the present study we are predominantly focusing on prevalence of CTX-M ESBL producing *Escherichia coli* in our region.

β-lactamase inhibitors are designed to inhibit or destroy the effectiveness of β-lactamase enzymes, they form an irreversible acyl enzyme complex by a covalent bond during the catalysis reaction with the β-lactamase, which leads to activity loss of the enzyme. This approach aims to identify compounds that

interact with a bimolecular target and consequently to develop a structure-based design for the improvement of the activity and the selectivity of these antibacterial compounds, thus computational biology or *In Silico* approach is developing day by day with refinement. It is becoming a promising field and with the help of programming tools the time and cost of biological work related to drug discovery, molecular interaction is reducing. One advanced method is the docking of the drug molecule or ligand or inhibitor with the target.

About 150 isolates were isolated by standard methods and antibiotic susceptibility profiling was performed. Total 120 strains i.e., 80% isolates were MDR strains, the rate of resistance to ß-lactam/ß-lactamase inhibitor in this study was nearly similar as recorded earlier [31]. But lower than that recorded by Diaz *et al.* 2010, who reported 88.6% of the isolate were susceptible to piperacillin/tazobactam, while 69.4% were susceptible to amoxicillin/clavulanates. The same ESBL positive strains were used for the amplification of *CTX-M* gene. ERIC-PCR analysis of 33 isolates which carried genes for ESBLs were genetically diverse and comprised a heterogeneous population with a total 14 ERIC-PCR clusters as presented in figure 20. A similar investigation was given by Durmaz *et al.* [32]. On ESBL-producing quinolone-resistant clinical *E. coli* isolates. On the contarary Shakil *et al.,* [34] reported clonal similarity among four *blaCTX-M*-15-positive *E. coli* isolates from NICU patients by ERIC-PCR and assigned them to be temporarily responsible for clonal outbreak. From the present study, it can be concluded that ERIC-PCR-based strain typing of β-lactamase producing MDR *E. coli* collected from various clinical samples exhibited a considerable amount of clonal heterogeneity.

Docking studies of CTX-M-9 protein (cephotaximase enzyme) with drug cephotaxime and inhibitor clavulanic acid showed reasonably higher glide (negative) energy. Higher glide (negative) energy is considered as an indicator of effective binding of an enzyme-inhibitor-complex and Schrodinger analysis exhibited the binding of *CTX-M-9* with ligand (cephotaxime, clavulanic acid) Among the previous similar studies on enzyme inhibitor-complexes, the complex involving clavulanic acid displayed comparatively high interaction energy [35]. Hence clavulanic acid was found to be the more efficient inhibitors for *CTX-M-9* as compared with other inhibitors.

In conclusion these interactions data will become supportive to the researchers for the future development of a versatile CTX-M resistant antibiotic/inhibitor. It is necessary to mention that the research presented here is a sparse and mainly in silico. More experimental studies are needed to validate the findings presented above.

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CONFLICT OF INTEREST STATEMENT

We declare that no conflict of interest.

AUTHORS CONTRIBUTION

First and Second author is responsible for carrying out the research work, data analysis and optimization of experimental work and Corresponding author is responsible for research planning executing and providing valuable inputs and in writing manuscript.

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