Functional molecular infection epidemiology of drug resistant Uropathogenic *Escherichia coli* (UPEC)

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By

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DECLARATION

The research work presented in this thesis entitled **"Functional molecular infection epidemiology of drug resistant Uropathogenic *Escherichia coli* (UPEC)"**, has been carried out by me at the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, under the guidance of Dr. Niyaz Ahmed, Associate Professor. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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CERTIFICATE

This is to certify that **Mr. Arif Hussain** has carried out the research work embodied in the present thesis under the supervision and guidance of Dr. Niyaz Ahmed, Associate Professor, for a full period prescribed under the Ph.D. ordinance of this University. We recommend this thesis entitled **"Functional molecular infection epidemiology of drug resistant Uropathogenic *Escherichia coli* (UPEC)"** for submission for the degree of Doctor of Philosophy of this University.

Dr. Niyaz Ahmed
Research Supervisor

Head,
Department Biotechnology and Bioinformatics

Dean,
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Dedication

This work is dedicated to the fond memories of Janab Abdus Samad, whose sufferings and ultimate demise due to urosepsis motivated us to initiate this research program.

Acknowledgement

With praise to almighty Allah and His blessings and peace upon the final prophet,

I would like to thank my family members, especially my parents for always believing in me, for their constant love and support. I also thank my extended family particularly my uncle and aunt for their great help and compassion.

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Arif Hussain

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Appendix i

List of abbreviation

ATCC	American Type Culture Collection
CLSI	Clinical Laboratory Standards Institute
BLAST	Basic Local Alignment Search Tool
ESBL	Extended Spectrum β-Lactamase
AmpC	AmpC β-Lactamase
MIC	Minimum Inhibitory Concentration
MDR	Multi-Drug Resistance
PMQR	Plasmid-Mediated Quinolone Resistance
VF	Virulence Factor
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
PFGE	Pulsed-Field Gel Electrophoresis
MLST	Multilocus Sequencing Typing
PBRT	PCR-Based Replicon Typing
Inc	Incompatibility
Rep	Replicon
kb	Kilobases
bp	Base pairs
kV	Kilovolts
V/cm	Volts per centimetre
xg	rcf-Relative Centrifugal Force
L	Liters
ml	Millilitres
mm	Milimeters
µl	Microlitres
M	Molar
mM	Millimolar
µM	Micromolar

mg	Milligrams
ng	Nanograms
LB	Luria-Bertani broth
MH	Mueller Hinton
SDS	Sodium Dodecyl Sulphate
SKG	SeaKem® Gold Agarose
PK	Protease K
TSA	Tryptic Soy Agar
EDTA	Ethylene Diamine Tetra Acid
TE	Tris-EDTA
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
MgSO4	Magnesium Sulphate
MgCl2	Magnesium Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ddH2O	Double Distilled Water
STEC	Shiga Toxin-Producing *E. coli*
EPEC	Enteropathogenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EAEC	Enteroaggregative *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection
HUS	Hemolytic-Uremic Syndrome
CTR	Ceftriaxone
CAZ	Ceftazidime
CPM	Cefipime

AMC	Amoxicillin/Clavulanic Acid
TZP	Piperacilling/Tazobactam
MER	Meropenem
CIP	Ciprofloxacin
SXT	Trimethoprim/sulfamethoxazole
GEN	Gentamicin
DOX	Doxycycline
TIG	Tigecycline
FOF	Fosfomycin
COL	Colistin
NIT	Nitrofurantoin

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Chapter 1

General Introduction

1.1 *Enterobacteriaceae*

Escherichia coli (*E. coli*) belong to a large family of bacteria known as *Enterobacteriaceae.* Members of the *Enterobacteriaceae* share certain common features such as they are rod-shaped and typically 1-5μm in length, are Gram-negative, facultative anaerobes that ferment sugars to produce acids and other by-products (Donnenberg 2000). Most *E. coli* have peritrichous flagella. Majority of the members of this family form part of normal gut microflora; apart from this they are also found in water/soil or as parasites on different animals and plants (Farmer III 2007). *E. coli* is one of the most important members of *Enterobacteriaceae* due to its biological significance to humans (J.H. and ABopp 2007). The commensal bacteria benefit the host by protecting their intestines from exogenous pathogens by a phenomenon called colonization resistance (Lawley and Walker 2013). In addition, commensal bacteria can also indirectly control infecting pathogens by stimulating host immunity in the intestines by a phenomenon termed immune-mediated colonization resistance (Buffie and Pamer 2013). Indiscriminate usage/underdosage of antibiotics leads to killing of commensal bacteria which then abrogates the protective/defensive mechanisms enabled by the commensal microorganisms (Buffie and Pamer 2013).

1.2 *Escherichia coli*

E. coli is a versatile organism which typically colonizes the gastrointestinal tract (GI) and makes up part of the normal intestinal microflora. Infants, soon after few hours of their birth, get their gastrointestinal tract colonized with *E. coli* (Kosek, Bern and Guerrant 2003). Therefore, the primary habitat of *E. coli* is the vertebrate gut and its primary niche is the mucous layer of the colon (Pinheiro da Silva et al. 2007); hence, *E. coli* has co-evolved with its human host. Although it is the predominant facultative anaerobe of the human intestinal microflora living in symbiosis with its host, it comprises only about 1%

of the total bacterial biomass (Guarner and Malagelada 2003). The lower GI tract has high turnover rate and constantly sheds epithelial cells and many bacteria and due to this, *E. coli* is often present in human and animal feces and thus leads to contamination (Simon and Gorbach 1984). Although, there are high chances of recombination events in the populous niches of gut, its population structure is said to be predominantly clonal in nature (Ochman and Selander 1984). In terms of niche adaptation, *E. coli* is very versatile and can adapt to different niches, even the commensal *E. coli* can adapt and evolve into pathogenic ones (Tenaillon et al. 2010).

1.3 *E. coli* pathotypes

E. coli comprises of non-pathogenic commensal isolates that form part of normal gut flora of humans and animals. In addition to this, there are several well adapted variants (clones) of *E. coli* that have evolved specific virulence mechanisms, that make them capable of colonizing new niches and inflict broad spectrum of infections in sites other than the intestines. This successful selection of virulence factors enables them to become specific "pathotypes". The *E. coli* pathotypes responsible for extraintestinal infections are termed as extraintestinal pathogenic *E. coli* (ExPEC) and those responsible for enteric diseases are called intestinal pathogens (Russo and Johnson 2000). There are six well described categories among the intestinal pathogens, they are: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper 1998). Among the extraintestinal pathogenic *E. coli,* the uropathogenic *E. coli* (UPEC) is the major player in causing extraintestinal colonization culminating in urinary tract infections. ExPEC also include pathotypes such as neonatal meningitis associated *E. coli* (NMEC), sepsis associated *E. coli* (SEPEC) and avian pathogenic *E. coli* (APEC) that cause respiratory infections, pericarditis and septicaemia of poultry (Dho-Moulin and Fairbrother 1999, Donlan and Costerton 2002). The different ExPEC pathotypes tend to be largely clonal by sharing their antigenic and virulence features. All ExPEC pathotypes are extracellular with only a few exceptions (Ewers et al. 2007).

1.4 Genetic diversity and population structure of *E. coli*

The population structure of *E. coli* is shaped by multiple factors prevailing in primary (host) and secondary habitats (environment). Most *E. coli* infections are believed to originate from human flora (Tenaillon et al. 2010). Moreover, the species *E. coli* encompasses enormous diversity of strains which makes it tremendously difficult to clearly distinguish the potential ExPEC strains among *E. coli* isolates of gut microflora in healthy individuals by any simple method (Bailey et al. 2010).

There are 4 major tools currently available for distinguishing different groups and lineages of *E. coli* and they are:

1. *Serotyping:* to group closely related strains by reacting it to the specific antisera (Orskov and Orskov 1992).
2. *Multilocus enzyme electrophoresis:* to characterize isolates by the relative electrophoretic mobility of several housekeeping cellular enzymes (Selander et al. 1986).
3. *Multilocus sequence typing (MLST):* the alleles of several housekeeping genes are determined to obtain an allelic profile that defines the sequence type (ST). There are currently 3 MLST schemes available for *E. coli;* Institut Pasteur's MLST scheme (www.pasteur.fr), Mark Achtman's MLST scheme (mlst.ucc.ie) and T. Whittam's MLST scheme (www.shigatox.net) (Larsen et al. 2012).

4. *Phylogrouping by triplex PCR:* this approach allows strains to be assigned to one of the four main phylogenetic groups (A, B1, B2 and D) by analysing PCR results of three DNA markers (Clermont, Bonacorsi and Bingen 2000).

   However although not simple, the best tool to decipher the genetic identity of *E. coli* is the whole-genome profiling by next-generation sequencing technologies (Tenaillon et al. 2010).

1.5 Urinary tract infections

Urinary tract infections (UTIs) are among the most common infectious diseases of humans and the most common nosocomial infections. UTI causes substantial morbidity and mortality if not optimally treated. There are estimates that at least 40-50% of women and 5% of men suffer with UTI in their lifetime. UTIs are very common among females with up to 60% of women encountering at least one episode in their lifetime (Totsika et al. 2012). Management of UTI accounts for the annual expenditure of more than 3 billion dollars in United States alone. About, 150 million cases of urinary tract infections were recorded annually as estimated (Totsika et al. 2012, Nickel 2005). However, there are not many studies on UTI from India. Uropathogenic *E. coli* (UPEC) is the most common etiological agent responsible for UTI; it belongs to a class of ExPEC category (Jadhav et al. 2011). They often originate from fecal flora and enter the urinary tract *via* colonization of the genital organs and peri-urethral area. They then ascend to bladder causing classical symptoms of cystitis (i.e., pain, frequency and urgency of urination). UPEC can proceed from bladder *via* ureters to establish a secondary infection called pyelonephritis which possibly causes irreversible kidney damage, sometimes leading to kidney failure (Scholes et al. 2005).

1.6 Virulence-associated factors of Uropathogenic *E. coli*

UPEC strains encode a number of virulence factors that are present on certain mobile genetic elements such as Pathogenicity Islands (PAIs), which help them survive the hostile environment of gut as well as initiate and cause an extraintestinal infection (Dobrindt et al. 2002). Basically, the biological features of UPEC strains that contribute to their role in pathogenesis include haemolysin and aerobactin production, expression of several adhesins such as P fimbriae, secretion of toxins like cytotoxic necrotizing factor and capsule production. There are basically two groups of virulence factors: (i) Adherence factors; these factors are associated with bacterial cell surface and (ii) Secreted virulence factors; these are secreted out and exported to the site of action (Johnson 1991).

There are several bacterial properties such as those listed in table 1 (Pitout 2012); some are fairly well established as virulence factors in *E. coli* implicated in UTI. These factors were identified based on epidemiological studies involving *E. coli* obtained from UTI cases wherein these factors were overrepresented. These are only the fraction of the total virulence mechanisms present in clinical *E. coli* strains, it is believed there could be many unidentified virulence factors (Johnson 1991) that remain hither to unexplored.

Table1: List of Uropathogenic *E. coli* virulence factors (Pitout 2012).

ADHESINS	
F10 papA	P fimbriae subunit variant
papC papACEFG	genes of P fimbriae operon
papEFG papACEFG	genes of P fimbriae operon
sfa/foc	S or F1C fimbriae
focG	F1C fimbriae adhesion
Iha	Adhesion siderophore
fimH	Type 1 fimbriae
Tsh	temperature sensitive hemagglutinin
Hra	Heat-resistant agglutinin
afa/draBC	Dr-binding adhesins

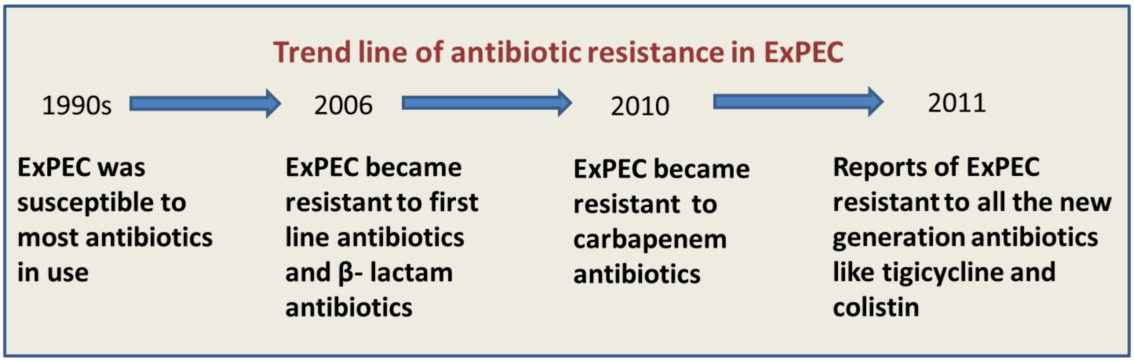
TOXINS	
hlyD	α-Hemolysin
Sat	Secreted autotransporter toxin
Pic	Serine protease
Vat	Vacuolating toxin
cnf1	Cytotoxic necrotizing factor

SIDEROPHORES	
iroN	Salmochelin (siderophore) receptor
fyuA	Yersiniabactin (siderophore) receptor
ireA	Siderophore receptor
iutA	Aerobactin (siderophore) receptor

CAPSULES	
kpsM II	kpsM II kpsM II group 2 capsule
K1	K1 group 2 capsule variants
K2	K2 group 2 capsule variants
K5	K5 group 2 capsule variants
KPSMTIII	Group 3 capsule

MISCELLANEOUS	
Usp	Uropathogenic-specific protein
traT	Serum resistance-associated
ompT	Outer membrane protease T
Iss	Increased serum survival
H7	fliC Flagellin variant
malX	Pathogenicity island marker

1.7 Antimicrobial resistance in Uropathogenic *E. coli*



Urinary tract infection (UTI) is one of the easily treatable diseases but its management is complicated due to widespread antimicrobial resistance in UPEC strains. Antimicrobial resistance has emerged as a result of indiscriminate use of cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole which are often administered to treat community and nosocomial infections caused by *E. coli* (Gundogdu et al. 2011). One of the best examples of the development of antibiotic resistance and its consequence are most evident in the form of β-lactamase production which includes the extended-spectrum-β-lactamases (ESBLs e.g., CTX-M-type), plasmid-mediated AMP C β-lactamases (e.g., CMY types) and carbapenemases. The acquisition of antimicrobial resistance is thought to occur by horizontal gene transfer because many of these resistance genes are located on conjugative plasmids, transposases, insertion sequences and integrases (Pitout 2012).

Table 2: Major β-lactam antibiotic classes in UPEC (Pitout 2012)

Enzymes	Class	Examples	Inhibited by
Extended-spectrum β-lactamases (ESBLs)	Class A	CTX-M, TEM, SHV	Clavulanic acid Tazobactam Sulbactam
Plasmid-mediated AmpC β-lactamases	Class C	CMY, FOX, ACT, MOX, ACC, DHA	Boronic acid
Metallo-β-lactamases (MBLs)	Class B	IMP, VIM, NDM	Metal chelators, e.g., EDTA and dipicolinic acid
KPC carbapenemases	Class A	KPC	Clavulanic acid (weak) Tazobactam Boronic acid
OXA-β-lactamases	Class D	OXA-48, -181	Clavulanic acid (weak) Nacl

1.8 Pandemic clonal groups in *Escherichia coli*

Recent reports have estimated that approximately 10-20% of all *E. coli* associated with UTIs can be caused by clonal groups of *E. coli* (Manges et al. 2008). In the past decade, many epidemiological studies have highlighted the pandemic spread of antimicrobial resistance in *E. coli*, particularly, the CTX-M-15 type extended spectrum-β-lactamase enzymes (Chandramohan and Revell 2012). The emergence and pandemic spread of antimicrobial resistance was corroborated with the finding that certain sequence types such as ST38, ST131, ST405, and ST648 were involved in clonal outbreaks of *E. coli* clinical infections. Among these clonal groups, there are some which are detected in multiple countries across different continents such as sequence type ST131. Moreover, ST131 also cause cross infections in companion animals implying their zoonotic potential (Pitout 2012, Hussain et al. 2012)

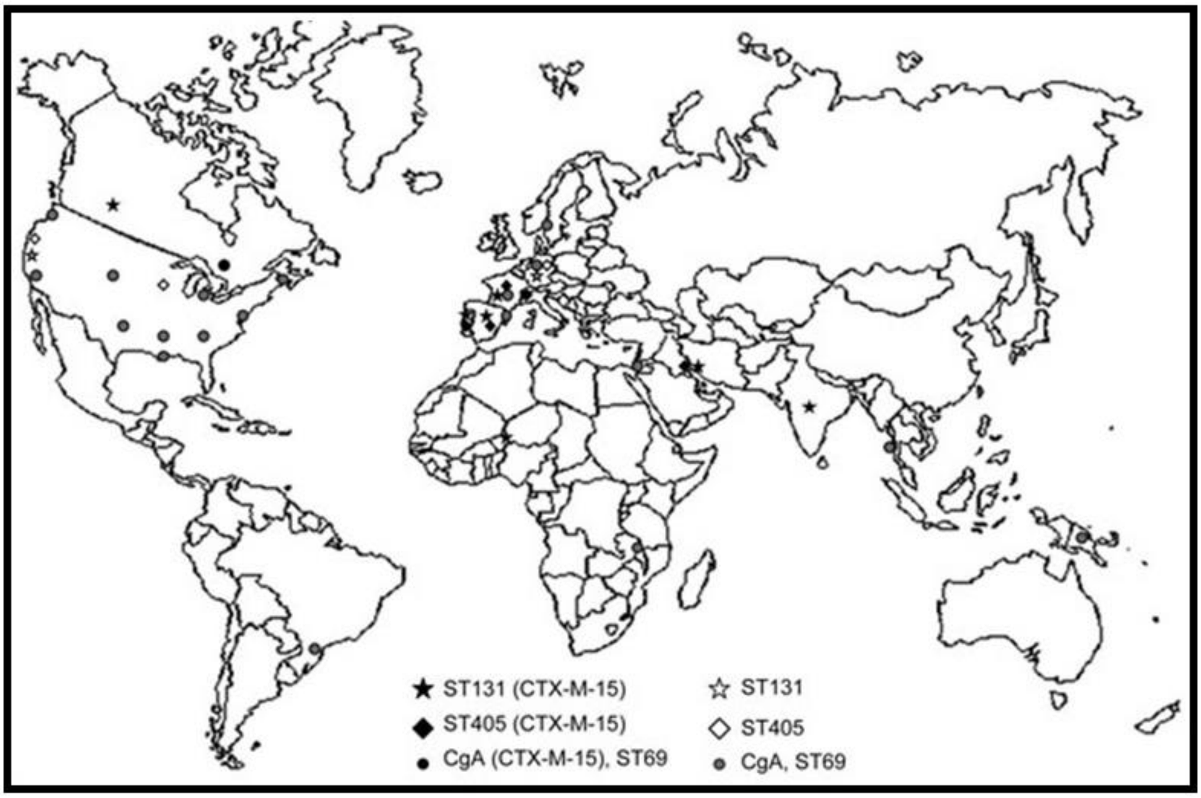


Figure 1: Geographic distribution of epidemic *E. coli* clonal groups associated with CTX-M-15 ESBL enzyme (http://web.mpiib-berlin.mpg.de/mlst/dbs/*E. coli*).

1.9 Scientific rationale and objectives

E. coli is an important cause of several extraintestinal infections, particularly the UTIs. Presence of several putative virulence genes has been strongly associated with the pathogenicity of UPEC isolates, which include: adhesions, toxins, lipopolysaccharides and protectins. These virulence factors are usually carried on pathogenicity islands and on other extra-chromosomal DNA. Until late 1990s, UPEC isolates were fairly susceptible to even first line antimicrobials but several surveillance studies in the last decade identified high rates of resistance to non-β-lactam antibiotics such as cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole. Resistance to newer β-lactamases such as AMP C β-lactamases, CTX-M-types and carbapenemase were pronounced among UPEC isolates. The phylogenetic groups B2 and D predominate among the UPEC isolates which are genetically highly diverse. Nevertheless, reports of their evolving clonally in the form of successful international sequence types such as ST131, ST405, ST38 is increasing which entails worldwide dissemination of important ESBL enzymes in strains implicated in different infections. Further studies are needed to fully understand the population structure and underlying biological and environmental factors that favour and select the most successful lineages associated with high virulence and drug resistant *E. coli* in certain high endemic countries such as India. There are no exhaustive data emanating from this country which is grappling with tremendous infection burden in recent times. Study of UPEC from India assumes a high significance and priority mainly due to the facts that: a) Indian strains of ExPEC including UPEC remain hitherto understudied; b) epidemiological context of UPEC infections has not been dissected in the light of tremendous host diversity, poor sanitation and community hygiene and widespread abuse of antibiotics.

The spread of highly virulent pandemic ExPEC strains such as *E. coli* ST131 and the emergence of newer and novel extended spectrum β-lactamase variants like CTX-M-15 and NDM-1 enzymes along with the inferences drawn from data on returned travellers from Asian countries, provide evidence of pandemic clonal dissemination. Given this, it is urgently needed to determine epidemiological significance of strains and isolates obtained from Indian hospitals. To determine this, an extensive estimation of the virulence potential, prevalence of antimicrobial resistance and understanding of the population biology of UPEC organisms is required.

Given the above rationale, we strived to study the population biology, phylogenetic affinities and molecular basis of ESBL and antimicrobial resistance of UPEC from an endemic UTI setting in India with a view to understand the most significant lineages causing UTI. To achieve these aims, the following specific objectives were framed:

- To organize and develop a large collection of UPEC (n=337) and stool *E. coli* (n=50) isolates and to gain first insights in to virulence, drug resistance and phylogenetic affiliations and to compare the genetic diversity with strains from global sources

- Identification of internationally significant clones within the strain collection and their comprehensive analysis using various phenotyping and genotyping tools
- Whole genome sequencing of a pandemic, multivirulent/multiresistant *E. coli* strain (ST131) NA114 by next generation sequencing technology
- To comprehensively characterize the identified clones and measure their possible association with different phenotypic and genotypic profiles

Chapter 2

Virulence Characteristics and Genetic Affinities of Multiple Drug Resistant Uropathogenic *Escherichia coli* from a Semi Urban Locality in India

Part of this chapter was published as**:** Jadhav S, **Hussain A**, Devi S, Kumar A, Parveen S, Gandham N, et al. Virulence characteristics and genetic affinities of multiple drug resistant uropathogenic *Escherichia coli* from a semi urban locality in India. PloS one. 2011; 6(3):e18063.

2.2 Introduction

Urinary tract infections (UTI) are the second most common human infections and are mainly caused by uropathogenic *E. coli* (UPEC). *E. coli* strains can be assigned to one of the four main phylogroup; A, B1, B2, and D, depending on the combination of results for three genetic markers *chuA*, *yjaA* and DNA fragment *TspE4* (Clermont et al. 2000). Different studies have demonstrated that these phylogroups differ in the presence of virulence factor, ecological niches and life history. The severity of UTI depends both on the virulence of the bacteria and the susceptibility of the host (Fowler, Latta and Stamey 1977). UPEC harbor numerous virulence factors including alpha-hemolysin, cytotoxic necrotizing factor, adhesins and iron acquisition systems. These factors support their ability to adhere to uroepethelial cells, help resist the bactericidal effect of serum and augment cell surface hydrophobicity thereby leading ultimately to tissue damage (Dobrindt 2005, Johnson and Russo 2005).

Adherence to the urinary tract mucosa might protect bacteria from urinary lavage and in turn augment their ability to survive and invade renal tissues (Hagan and Mobley 2007). Specific adhesion is mediated by certain adhesins which can be differentiated based on their receptor binding specificity. P fimbriae that are encoded by the *E. coli* pap (pyelonephritis-associated pilus) operon are the most important mannose-resistant adhesins, although they are expressed by only a limited number of *E. coli* serotypes. Hemolysin production is another important virulence property of UPEC. Hemolysins inflict direct cytotoxic effects on renal epithelium resulting in scaring. Alpha-hemolysin is described to be a lethal factor with dermonecrotic effects and is antigenic in nature. Also, hemolysins are toxic to a series of host tissues and cells including RBCs, leucocytes, epithelial and endothelial cells. The frequency of isolation of hemolytic *E. coli* significantly associates with the severity of the infection (Blanco et al. 1992) (Blanco et al. 1995).

Bacteria are lysed by normal serum due to the activity of the complement system. The alternate pathway of complement activation is potentially important than the classical pathway. Bacterial resistance to killing by serum results from individual or combined effects of capsular polysaccharides, lipopolysaccharides and surface proteins (Leffler and Svanborg-Eden 1981). Given this generic virulence 'arsenal' of UPEC, strains from different geographical regions pose different disease severity and should be genetically different.

To screen for the molecular determinants of the above mentioned virulence attributes among UPEC in the western Indian region, we analysed 150 human clinical isolates. We intended to identify the virulence gene profiles of UPEC (which have different frequencies in different disease conditions ranging from asymptomatic bacteriuria to chronic pyelonephritis). In addition, increasing antimicrobial resistance in bacterial pathogens is of major concern as it can vary according to geographical and regional situations (Mathai et al. 2001, Karlowsky et al. 2001). It is very relevant to ensure the appropriate therapy based on full knowledge of the organisms that cause UTI and their antibiotic susceptibility profiles (Sahm et al. 2001). Therefore, it is necessary to do bacteriological testing also with reference to extended-spectrum beta-lactamase (ESBL) producers with resistance to beta-lactam antibiotics, including third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. Not much information on ESBL producing organisms causing UTI is available from India.

2.3 Materials and Methods

2.3.1 Ethics statement

Written informed consents were obtained from all patients and healthy controls for the use of their strains which were cultured as part of compulsory diagnostic screening. Study protocols were approved by the institutional ethics committee of Dr. D. Y. Patil Medical College, Pune and by the Institutional Biosafety Committee of the School of Life Sciences of the University of Hyderabad, India.

2.3.2 Study population and strains

One hundred and fifty *E. coli* isolates were obtained from urine samples of human patients in cell counts of 10^5CFU/ml. Fifty fecal *E. coli* isolates (that were used as controls), were isolated from the feces of healthy individuals who had reported for routine health checkup between January 2009–March 2010 at Dr. D. Y. Patil Medical College and Hospital, Pimpri, Pune, India. Patient background and provisional diagnosis of the infection were obtained from hospital records. Identification of isolates was done using standard microbiological techniques (Quackenbush and Falkow 1979). All strains were stored in 15% glycerol-supplemented Luria- Bertani medium at -80˚C (refer Appendix i).

2.3.3Antimicrobial susceptibility test

The antimicrobial susceptibility testing was carried out on Mueller Hinton agar by disc diffusion method using the following antimicrobial substances (Penders and Verstraete 2006): amoxicillin (10 mg), ceftazidime (30 mg), ciprofloxacin (10 mg), co-trimoxazole (25 mg), gentamicin (10 mg), nitrofurantoin (300 mg), nalidixic acid (30 mg), and tetracycline (30 mg). Multi-drug resistant (MDR) strains were defined as those which showed resistance to three or more antimicrobial substances. Extended-spectrum beta-lactamase (ESBL) production was detected by the double disk synergy

(DDS) test as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Its presence was assayed using the antibiotic disks comprising of ceftazidime and ceftazidime/ clavulanic acid (30/10 mg). An isolate was graded ESBL producer when its zone of inhibition varied by ≥5 mm among at least one of the combination disks and its coordinate comprising of standard antibiotic disk. *E. coli* ATCC 25922 was used as a negative control while *K. pneumoniae ssp. pneumoniae* ATCC 700603 served as positive control (Tankhiwale et al. 2004).

2.3.4 Phylogenetic group determination

The phylogenetic group of each strain was determined according to a PCR-based method as described by Clermont etal., (Clermont et al. 2000). In brief, PCR was performed on DNA templates obtained by boiling lysis of bacteria. The primer pairs used were ChuA.1 (5′-GACGAACCAACGGTCAGGAT-3′)/ ChuA.2 (5′-TGCCGCCAGTACCAAA GACA-3′), YjaA.1 (5′-TGAAGTGTCAGGAGACGCTG-3′)/ YjaA.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′) and TspE4C2.1 (5′-GAGTAATGTCGGGGC ATTCA-3′)/ TspE4C2.2 (5′-CGCGCCAACAAAGTATTACG-3′). The PCR conditions were as follows: denaturation for 3min at 94˚C, 30 cycles of 30 sec at 94˚C and 30 sec at 55˚C and a final extension step of 10min at 72˚C. Each strain was tested thrice and then assigned to one of the four phylogroup based on the amplification results of the three DNA markers as follows: *chuA*+, *yjaA*+, group B2; *chuA*+, *yjaA*−, group D; *chuA*−, TspE4.C2+, groupB1; *chuA*−, TspE4.C2−, group A.

2.3.5 PCR- based virulence gene profiling

The presence of virulence genes was investigated by simplex PCRs. The template DNA were obtained from the pure culture of *E. coli* isolates grown overnight on macConkey agar plates, a loop full of colony was added to 100µl of milliQ, boiled at 95˚C for 15 min, centrifuged and the supernatant was used as PCR template. Reference strains such as CFT073 and RS218 obtained from German laboratories were used as positive controls. PCR amplification was carried out in 20µl reaction mixture consisting of 1X Taq buffer, 200µM dNTPs, and primers of 0.2PM each with 200ng of template DNA. The PCR cycling conditions were as follows: denaturation at 94˚C (30 cycles, 45 seconds each), annealing at 63˚C (30 cycles, 45 seconds each) and strand elongation at 72˚C (45 seconds each). The PCR products were detected by electrophoresis and visualized under UV gel documentation unit (Rodriguez-Siek et al. 2005b, Ewers et al. 2009).

2.3.6 Statistical Analysis

Chi square test was used to compare the occurrence of virulence markers in cases and controls. P values less than 0.05 were considered significant.

2.4 Results and Discussion

2.4.1 Incidence of UTI in relationship with gender and socioeconomic status

In this study we observed a higher proportion of UTI in females (64%) than in males (36%). This is understandable due to the anatomy and is a consistent trend worldwide. Peak in the incidence of UTI was observed in the age groups 11–21 and 60–71 years. Among these, elderly patients are likely predisposed to conditions such as urinary tract obstruction, poor bladder emptying, and diabetes mellitus, etc. These factors favor colonization of bacteria and play an important role in UTI. Other studies have also reported similar findings (Gales et al. 2002, Ulleryd 2003). India has a large infection burden and the genito-urinary infections are very prominent. This may be due to a less affordable personal/ community hygiene for some of the economically backword populations. We targeted these groups and analyzed strains obtained from such communities who reported for the UTI in periurban, Pune. As such there is not much information available from India on the genetic and phenotypic diversity of UPEC. This information should therefore be construed as the first systematic analysis performed on a diverse type of patients/samples.

2.4.2 Antimicrobial susceptibility profiles of UPEC

Antimicrobial susceptibility testing was carried out on all the clinical isolates. The majority of isolates were sensitive to nitrofurantoin - 86 (57.3%) followed by ciprofloxacin - 78 (52%) and nalidixic acid - 74 (49%). This study showed high resistance to amoxicillin - 101 (67.3%), tetracycline - 92 (61.3%), and cefotaxime - 68 (45.3%) (Figure 2). Upon testing for ESBL, we found that 32 (21.3%) were ESBL producers. A high proportion (31.3%) of these ESBL-producing isolates was belonging to the serotype O25. Four of these were positively tested for the subgroup O25b, which has been linked to a clonally related group of highly virulent, multiresistant *E. coli* strains (B2-O25b-ST131- CTX-M-15) that are emerging among humans and animals worldwide (Ewers et al. 2010, Nicolas-Chanoine et al. 2008). Further studies, i.e. multi-locus sequence typing (MLST), macrorestriction analysis, and determination of beta-lactamase enzymes are needed to unravel the phylogenetic relatedness of these O25-ESBL-producing Indian isolates to the worldwide recognized clonal groups and to characterize the remaining multi-resistant isolates observed. ESBL isolates were frequently (65.6%) associated with a hemolytic phenotype, with a higher rate than the non-ESBL-producing UPEC strains (58.5%). Hence, although it is a commonly accepted fact that bacterial pathogens acquire resistance determinants and express a multi-resistant phenotype at the cost of their virulence properties, the frequent observation of hemolytic UPEC among our strain collection underlines the possible emergence of highly virulent multi-resistant strains in this area.

It is particularly worrisome that more than half of the ESBL producers (53.1%) in our collection were resistant to ciprofloxacin suggesting that they may be resistant to all available fluoroquinolones- the drug of choice for treatment of infections caused by ESBL-producing strains. Overall, nitrofurantoin and ciprofloxacin were found superior to amoxicillin and co-trimaxozole. None of the uropathogens from this study area were 100% susceptible to any of the antimicrobial substances used. other studies have shown a sensitivity rate of 95–100% to nitrofurantoin (Kumar et al. 2013). But in our study, nitrofurantoin showed only 57.3% sensitivity; the reason for this low sensitivity and high resistance to almost all antimicrobials in the study area may indicate difference in antimicrobial usage, infection control practices and other unrecognized factors including genetic propensity of these strains to accumulate mutations conferring MDR phenotypes. Periodic review and formulation of antibiotic policy are needed to control acquisition of drug resistance.

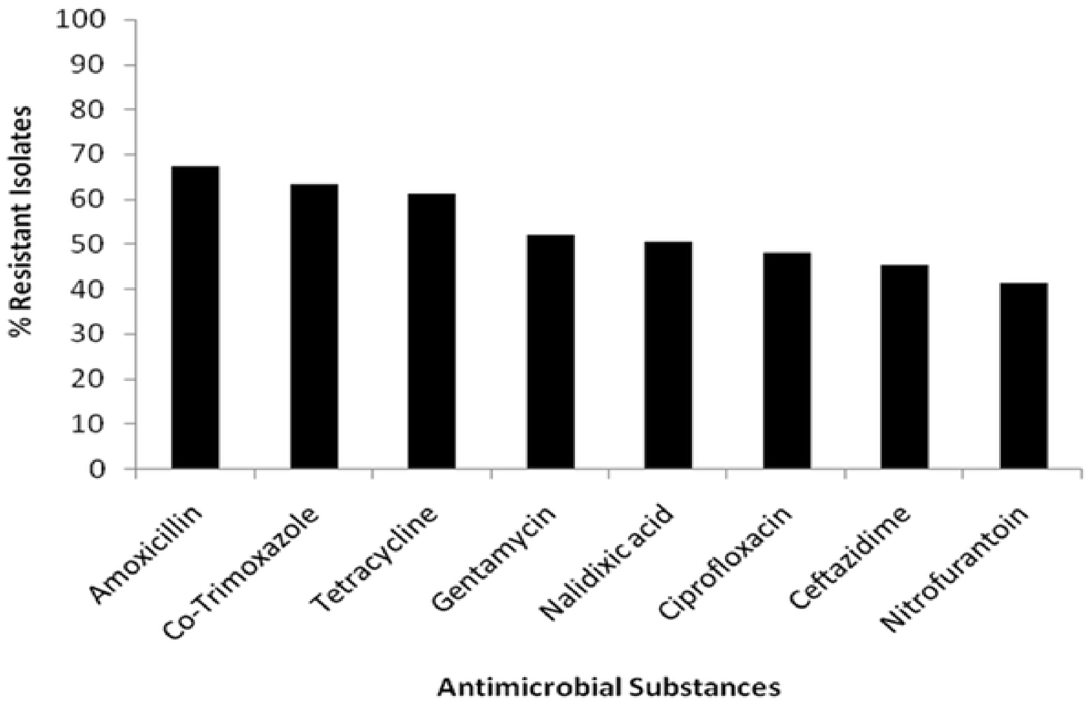


Figure 2: The antimicrobial susceptibility and resistance pattern of 150 UPEC isolates.

2.4.3 Phylogenetic grouping

Using the triplex PCR we assigned 150 clinical and 50 stool *E. coli* isolates into four phylogroups - A, B1, B2, and D. classification of stool *E. coli* isolates in phylogenetic group revealed that group A was the most common (60%), followed by group D (24%), B2 (11.11%), and B1 (4.4%). Among the 150 UPEC isolates, group B2 was the most frequent (54.4%) followed by group A (21.6%), group D (19.4%), and B1 (4.5%). Phylogenetic group B2 was seen to be strongly associated with clinical UPEC strains that is, 54% of clinical population belongs to B2. In contrast, only 11% of stool isolates were affiliated to B2 phylogroup indicating that B2 group is uncommon among stool *E. coli* isolates. However, 60% of stool *E. coli* isolates were belonging to group A, also group A was also the second most predominant phylogroup in the UPEC collection (figure 3). It is reported earlier that the phylogroup A and B1 mainly consist of commensal strains found in the intestine of human and animals, as well as environment samples. In contrast, phylogroup B2 and to a lesser extent group D consist of pathogenic strains that normally have virulence associated genes. Our study confirmed that B2 group is strongly associated with the extraintestinal infections, such as UTI. We also observed an unusual case where phylogenetic group A, which was thought to be only associated with commensal bacteria, is also being found to be significantly associated with UPEC, this further explains to us the results of virulence profiling wherein we found less density of virulence in UPEC isolates that is because the second majority among UPEC isolates is the phylogroup A.

2.4.4 Virulence gene profiles

Out of 150 *E. coli* strains 142 strains were found to harbour at least one virulence gene. That is 97% of total population were positive for one virulence gene at least. The prevalence of genes within the total population for *pap, sfa, afa, hly, aer,* and *cnf* were found to be 36%, 55%, 61%, 10%, 69%, and 46% respectively. And the prevalence of genes in fecal isolates were - pap 12%, *sfa* 89%, *afa* 36%, *hly* 14%, *aer* 78% and *cnf* 19.5% (figure 4). When we compared the occurrence of virulence genes between clinical and stool isolates, overall, the clinical *E. coli* isolates harboured more virulence markers in terms of co-occurrence and virulence score for different virulence genes. Among the six genes ExPEC associated virulence factors; *pap, afa* and *cnf* were strongly associated with the UPEC isolates in comparison to the fecal isolates, suggesting an important role in causing UTI. Our finding also suggests that, the gut of healthy people is also colonized with a small proportion of UPEC isolates as demonstrated by the presence of multiple virulence genes in a fraction of stool *E. coli* isolates. This is of great health concern given the insufficient personal and community hygiene in most of the economically backward communities in India. Further studies for better understanding of the interaction of different virulence factors at molecular level are necessary as most urovirulent strains express multiple virulence factors simultaneously. We believe that the methods of detection of the above mentioned virulence markers are reasonably easy and screening them in a clinical microbiology laboratory is a worthwhile exercise.

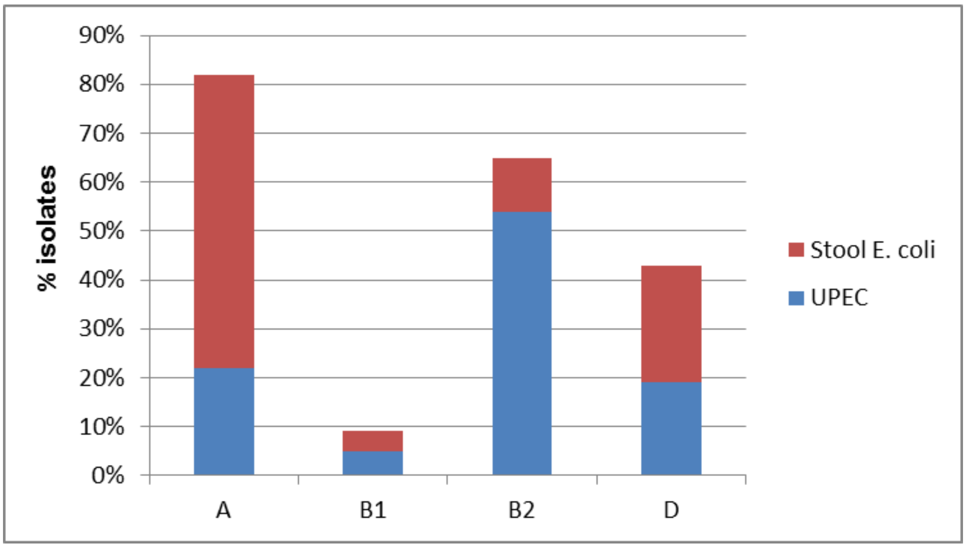


Figure 3: Distribution of 150 UPEC (clinical) and 50 stool (commensal) *E. coli* isolates among the four phylogroups.

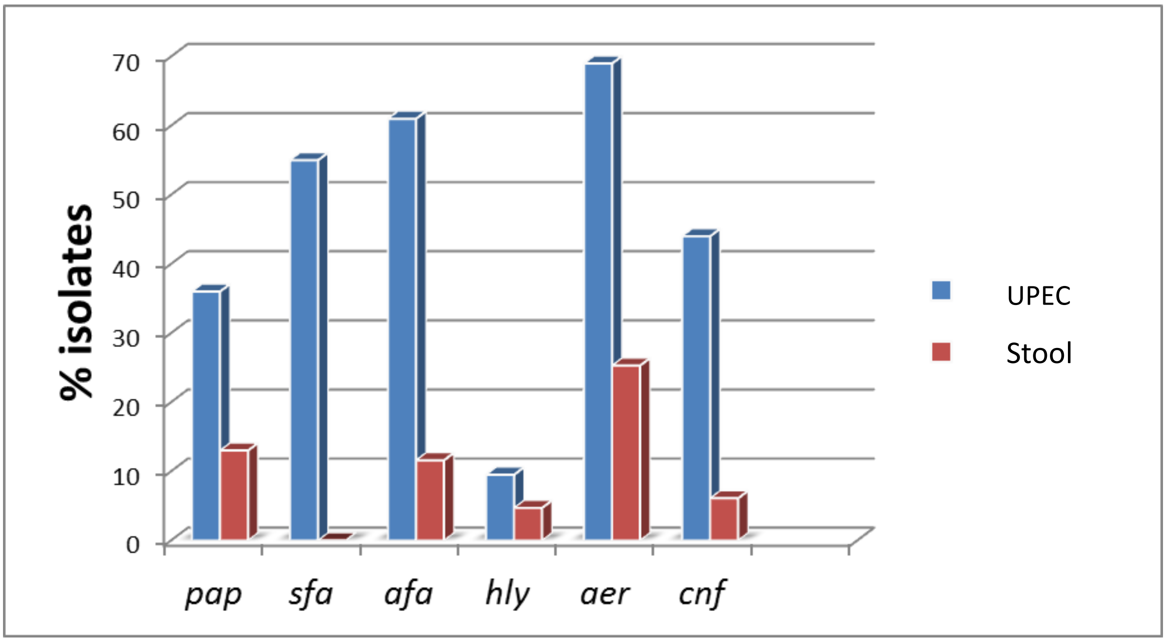


Figure 4: Distribution of the 6 ExPEC associated virulence genes among 150 UPEC and 50 stool *E. coli* isolates.

2.5 Conclusion

In conclusion, our observations form an important baseline data-set towards understanding the virulence properties, antibiotic resistance profiles and genetic diversity of UPEC from India. We hope that such observations will be more meaningful towards systematically unraveling the population genetic structure of UPEC and their propensity to spread, or emerge with multi drug resistance phenotypes in new epidemiological territories. In the backdrop of this work, future studies involving decipherment of MDR mechanisms, lineage tracking based on MLST and molecular epidemiology and infection biology of Indian UPEC at greater details. On the clinical side, it will be possible to ascertain reservoirs of infection which could be important in understanding the mechanisms of chronic and recurrent UTI in a community setting and how they are maintained within or in the vicinity of different human populations of a multicultural and densely populated country such as India.

Chapter 3

Multiresistant Uropathogenic *Escherichia coli* from a Region in India Where Urinary Tract Infections Are Endemic: Genotypic and Phenotypic Characteristics of Sequence Type 131 Isolates of the CTX-M-15 Extended-Spectrum-β-Lactamase-Producing Lineage

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3.1 Introduction

Escherichia coli is a universal commensal bacterium causing infections in humans and animals and serves as a common cause of urinary tract infections (UTI) and bacteremia in humans (Paterson and Bonomo 2005). In addition, this group of strains, designated extraintestinal pathogenic *E. coli* (ExPEC), causes a variety of infections at extraintestinal sites ranging from the biliary system to the central nervous system. These infections are prevalent both in nosocomial and in community settings (Pitout and Laupland 2008). UTI, although treatable, is now becoming increasingly tough to control because of rampant antimicrobial resistance in the *Enterobacteriaceae* family, particularly in *E. coli* (Paterson and Bonomo 2005). As a result, these organisms are responsible for significant social and economic burdens for the communities and public health departments (Harding and Ronald 1994). In the past decade, there has been a dramatic increase in the identification of *E. coli* strains with CTX-M enzymes, a new group of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) that have replaced classical TEM- and SHV-type ESBLs in many countries (Livermore et al. 2007). There are more than 80 variants described in the CTX-M group of enzymes that are the primary cause of resistance to expanded-spectrum cephalosporins (Canton and Coque 2006). Currently, the most widely distributed CTX-M enzyme is CTX-M-15, which was first detected in *E. coli* from India in 2001 (Karim et al. 2001). One of the reasons for widespread occurrence of antibiotic-resistant *E. coli* in communities from multiple locales is thought to be due to the dissemination of clonal organisms harboring antimicrobial resistance genes (Clermont et al. 2000, Johnson et al. 2009, Literacka et al. 2009).

Recent studies using MLST explored the population biology of ESBL-producing *E. coli* and uncovered emergence of an apparently dominant clone of CTX-M-15-

producing *E. coli* carrying high levels of virulence-associated genes (VAGs); this was designated sequence type 131 (ST131), occurring in many different countries, and was thus recognized as a pandemic ExPEC clone (Rogers, Sidjabat and Paterson 2011). It has been shown that this group (ST131) of ESBL-producing *E. coli* strains, in addition to being resistant to most beta-lactam antibiotics, is frequently resistant to aminoglycosides and fluoroquinolones (Lau et al. 2008b). Also, their spread posed a significant threat to human health, as they entail serious therapeutic challenges due to their ability to withstand the effect of different classes of antimicrobial agents. Moreover, the prevention and control of the transmission of uropathogenic *E. coli* infections are limited by poor understanding of the population genetics and virulence/resistance genotypes of these pathogens (Jadhav et al. 2011). The endemic potential and ability of particular lineages of antibiotic-resistant *E. coli* to disseminate and cause disease are seldomly studied in countries such as India, where recent surveys have identified prevalence of ESBL producer groups to be up to 70 to 90% of the total *Enterobacteriaceae* reported, although this figure may be based on studies with biased sampling; nevertheless, they indicate a serious problem(Hawkey 2008, Heddini et al. 2009). Moreover, it was demonstrated that there exists a great propensity of transmission of multiresistant clones from humans to animals and vice versa (Ewers et al. 2012, Ewers et al. 2009).

In appreciation of the above-described issues, we designed a pilot study to investigate the prevalence and to determine the virulence and antimicrobial properties of the ST131 clones present among clinical *E. coli* isolates cultured from the urine of infected patients attending a tertiary care hospital in Pune, India. We believe this study is important in the backdrop of increased occurrence of carbapenem resistance genes in *Enterobacteriaceae*, especially $bla_{\mathrm{NDM\text{-}1}}$ in the Indian subcontinent (Kumarasamy et al. 2010), and for looking into the possibility of their dissemination being associated with highly virulent/resistant clones.

3.2 Materials and methods

3.2.1 Bacterial isolates, O typing, and antimicrobial susceptibility testing

A total of 100 clinical isolates of *E. coli* from patients with UTI were initially used that were recovered from urine samples of human patients giving a viable count of >10^5 CFU/ml. These isolates were received from the microbiology department of a hospital in Pune (refer Appendix i). Seven European ST131 ESBL *E. coli* isolates archived at the Institute of Microbiology and Epizootics (IMT), Free University Berlin, were also obtained for pulsed-field gel electrophoresis (PFGE). The ESBL production was confirmed phenotypically using the clinical and laboratory standards institute (CLSI) criteria for ESBL screening. O typing of ESBL-positive *E. coli* strains was done by a recently described molecular approach based on allele-specific PCR, targeting the *rfb*O25b subgroup gene locus (Clermont et al. 2009b). After this stage, only 16 out of the above-described 100 *E. coli* strains were used for further assays, described below. Susceptibility to the following non-beta-lactam molecules was assessed by the disc diffusion procedure: ciprofloxacin, chloramphenicol, gentamicin, sulfamethoxazole-trimethoprim, and tetracycline. Isolates were defined as resistant or susceptible according to CLSI guidelines.

3.2.2 MLST and phylogenetic grouping

Identification of *E. coli* phylogenetic groups was performed using the multiplex PCR-based method of Clermont et al. (Clermont et al. 2009b). Multilocus sequence typing (MLST) was performed as described previously (Wirth et al. 2006). Gene amplification and sequencing were performed by using primers specified at the *E. coli* MLST website (**http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli**). Sequences were analyzed by the software package Ridom SeqSphere 0.9.19 (**http://www3.ridom.de/seqsphere**), and sequence types were determined accordingly.

3.2.3 Antimicrobial resistance gene detection

PCR amplification and sequencing were used to test for the presence of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ genes among the phenotypically ESBL-positive strains (Rodriguez-Siek et al. 2005a). PCR was used to determine the presence of other antibiotic resistance genes, such as those conferring resistance to tetracycline [namely, *tet*(A), *tet*(B), *tet*(C)], to sulfonamides (*sul1*, *sul2*), to streptomycin (*strA*, *strB*), to aminoglycosides (*aadA1*-like, *aac4*), and to some other plasmid-mediated quinolone resistance determinants, such as *aac*(6′)*-Ib*, *qnrA*, *qnrB*, and *qnrS* (Bert, Branger and Lambert-Zechovsky 2002a, Bertrand et al. 2006b, Boerlin et al. 2005a, Grimm et al. 2004, Park et al. 2006b). The 16 ESBL-producing ST131 *E. coli* isolates were screened by PCR for the presence of *intl1* and *intl2* genes, encoding class 1 and 2 integrases with the help of primers described elsewhere (Skurnik et al. 2005a). To identify the gene cassettes, a PCR and subsequent sequence analysis were performed on the isolates that were positive for *intl1*, using primer pairs defined earlier (Maguire et al. 2001).

3.2.4 Carbapenemase detection

Imipenem and meropenem susceptibility testing was performed using the disk diffusion method in accordance with the CLSI criteria. The modified Hodge test was performed on all the 16 isolates on Mueller-Hinton agar (Anderson et al. 2007b). After a12-h incubation, the plates were observed for clover leaf-type pattern at the junction of the test organism and the standard strain within the zone of inhibition of the carbapenem disc. Briefly, the Mueller-Hinton agar plate was inoculated with a dilution of a 0.5 McFarland suspension of *E. coli* ATCC 25922 and streaked with a swab. A 10-µg ertapenem disk was placed in the center of a petri dish, and each test isolate was streaked from the disk to the edge of the dish. *Klebsiella pneumoniae* (ATCC BAA-1705) and *E. coli* (ATCC 25922) were used as positive and negative controls, respectively. Four carbapenemase genes, *bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{VIM}$, and *bla*$_{NDM-1}$, were amplified as described previously (Bertrand et al. 2006b, Pfeifer et al. 2011a).

3.2.5 PFGE analysis

XbaI PFGE analysis was used to explore the possible clonal nature of the 16 Indian ST131 isolates in comparison with seven European ST131 strains to construct a dendrogram. The analysis was performed using a CHEF DRIII system (Bio-Rad, Munich, Germany). Cluster analysis of dice similarity indices based on the unweighted pair group method with arithmetic mean (UPGMA) was used to generate a dendrogram describing the relationships among PFGE profiles. Isolates were considered to be belonging to the same PFGE cluster if their Dice similarity index was ≥85% (Carrico et al. 2005, Martinez et al. 1998).

3.2.6 Conjugal transfer and plasmid analysis

Conjugative transfer of ESBL genes was tested by broth mating experiments by using a plasmid-free, sodium azide-resistant *E. coli* J53Azi^R strain as a recipient. The strains were grown to the exponential phase and then mixed in the ratio of 1:2 (donor:recipient). Transconjugants were selected on Endo agar containing 100 µg/ml sodium azide and 4 µg/ml cefotaxime. Potential transconjugants were verified by PCRs, and the presence of relevant *bla* genes and cotransfer of resistance determinants were confirmed by amplifying the respective genes in the transconjugants as described above (Ewers et al. 2004). The number and approximate sizes of the plasmids in each of the 16 ST131 isolates and the respective transconjugants were determined along with four reference plasmids of known sizes (*E. coli* [RS478], 272 kb; *E. coli* [x-109/97], 19 kb; *Salmonella* *enterica* serovar Typhimurium, 91.2 kb; *E. coli*[RS478], 170.24 kb) by using a modified version of the methodology previously described by Kado and Liu (Kado and Liu 1981). This was followed by separation of plasmids by electrophoresis in 0.4% agarose gels made with 1× Tris-EDTA (TE) buffer. Gels were run at 20 V/cm for 8 h, stained with ethidium bromide, and photographed. Plasmids were assigned to major plasmid families by PCR-based replicon typing (PBRT) (Carattoli et al. 2005).

3.2.7 Serum sensitivity and biofilm formation assay

Serum sensitivity assay was performed on three clinical strains and their transconjugants as previously described (Dozois et al. 1992). Briefly, the overnight bacterial cultures were washed and suspended in 1 ml phosphate-buffered saline (PBS); 50 μl of this suspension was added to 450 μl of serum, and 100 μl from each well was taken out for CFU determination before and after incubation at 37°C in a shaker incubator. Strains IMT10740 and IMT5053 were used as positive and negative controls, respectively. Biofilm formation assays were performed on 15 clinical strains and their conjugant strains by using a modified version of a previously described method (Naves et al. 2008). Strains were grown overnight in LB broth, and the cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh M63 medium. Then, 200 μl of aliquots was placed in wells of polystyrene microtiter plates and incubated for 48 h at 28°C without shaking. Afterward, ODs were read at 600 nm, the wells were washed thrice, and the staining of adhered bacteria was performed with 0.1% crystal violet (solubilized in ethanol) followed by reading the OD at 570 nm. Biofilm measurements were calculated using the formula as previously described (Naves et al. 2008).

3.2.8 Virulence gene typing

All the 16 ST131 isolates were investigated by multiplex PCR for the presence of 38 virulence-associated genes (VAGs) corresponding to the extraintestinal pathogenic *E. coli* based on the targets that have been previously described (Rodriguez-Siek et al. 2005b, Johnson, Johnson and Nolan 2006, Bingen et al. 1998). The genes tested correspond to the main classes of extraintestinal VAGs; adhesin, toxin, iron capturing system, and protectin/invasin.

3.2.9 Statistical analysis

The student's *t* test was performed using GraphPad Prism 5.0 software. The *P* value was calculated for each experiment conducted in triplicates.

3.3 Results

3.3.1 Isolation and antimicrobial resistance profiling

One hundred *E. coli* samples were obtained between January 2009 and October 2009. A total of 23 (23%) ESBL-producing isolates were detected phenotypically using the CLSI criteria for ESBL screening and disk confirmation test. These 23 isolates were then subjected to O typing that revealed 16 (70%) out of 23 ESBL-producing strains to be positive for the *rfbO25b* subgroup, indicating the presence of 16 CTX-M-15-O25b-ST131 group strains. Of these 16 strains, 10 were from male patients with an average age of 40 years who were diagnosed with prostatitis and pyelonephritis. To assess multidrug resistance of the 16 ST131 isolates, susceptibility to non-beta-lactam antimicrobials was tested. Ninety-four percent of the isolates were resistant to tetracycline, 81% to ciprofloxacin, and 69% to gentamicin and sulfamethoxazole-trimethoprim (TMP-SMX), but only 6% to chloramphenicol. Out of the16 isolates, 12 (75%) were resistant to at least three classes of antimicrobials, and therefore a total of 12 multidrug-resistant (MDR) ESBL ST131 strains were finally identified (Fig. 5).

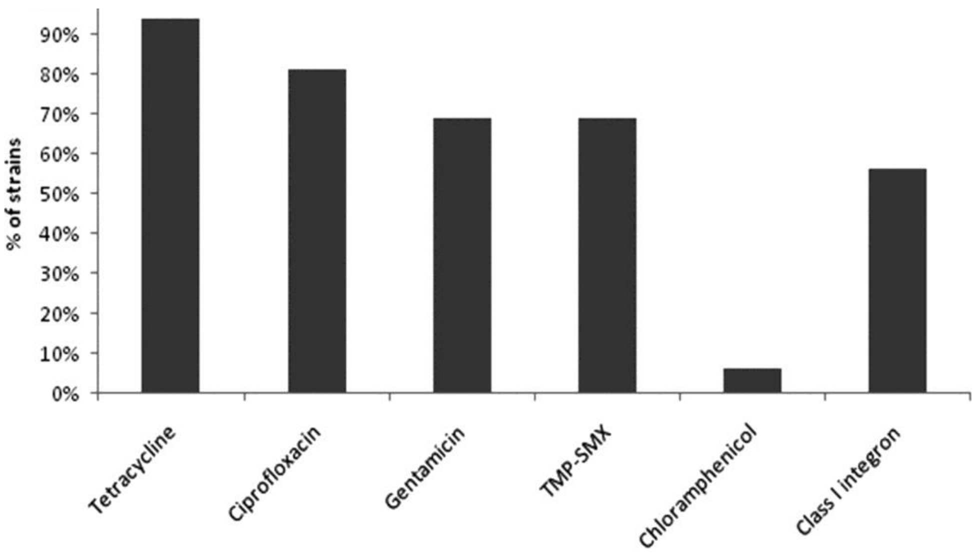

Figure 5: The percentages of 16 ST131 CTX-M-15-producing *E. coli* isolates resistant to five different non-beta-lactam antibiotics and the percentages of isolates positive for class 1 integron.

3.3.2 Genetic relationships and plasmid profiles

All of the 16 ESBL strains positive for the *rfbO25b* locus were allocated to phylogenetic group B2 on the basis of triplex PCR. MLST analysis of these 16 strains showed that they belonged to the ST131 clone. To obtain a finer resolution of clonal relationships among the 16 Indian ST131 *E. coli* strains and seven ST131 strains from European origin, a PFGE analysis was performed (Fig. 6). Owing to their common genetic background, the ST131 isolates exhibited more homogenous PFGE profiles. All the ST131 strains tested by us formed five small clonal groups (groups 1 to 5) as documented at the 85% similarity level. Three of the five PFGE groups (groups 1, 4, 5) corresponded consistently to the geographic/host origin, whereas two groups (groups 2 and 3) carried strains both from the Indian and European origins. Overall, 10 out of the16 $bla_{\text{CTX-M}}$ (Indian) ST131 isolates were found to be relatively clonal, and also three out of seven European strains clustered closely with the Indian strains, indicating their genetic affinities despite geographic barriers and different host origins. This highlights the fact that they are an epidemiologically important clone appearing/emerging in different continents.

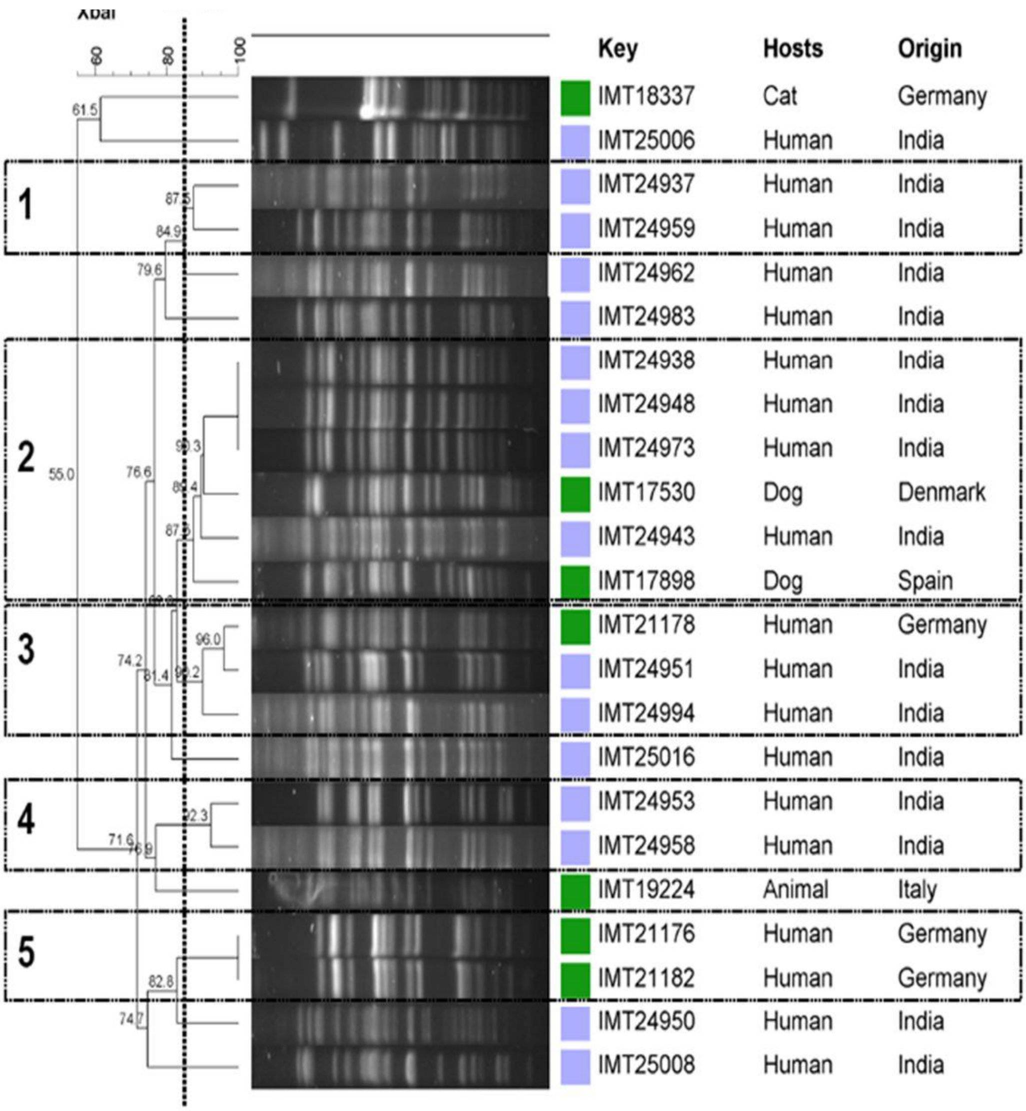

Figure 6: Dendrogram based on PFGE profiles of 16 Indian and 7 European ESBL-producing ST131 *E. coli* isolates.

Table 3: Results of different antimicrobial resistance gene screening, other than ESBL genes. In addition, the 16 ESBL ST131 strains were screened for the following antimicrobial resistance genes; *tet*B *tet*C, *sul3*, *qnrA*, *qnrB*, *qnrS*, *aadA*, *aaC4* and were found to be absent in all the 16 strains.

Strain No.	Uniform code	***sul*1**	***sul*2**	***tet*A**	***str*A**	***str*B**	***bla*$_{\mathbf{OXA}}$ group 1**	***acc(6´)-Ib-cr***
IMT24938	2	+	+	+	+	-	-	+
IMT24948	3	-	-	+	+	-	-	-
IMT24958	8	+	+	+	+	+	+	+
IMT24962	14	+	+	+	+	+	+	+
IMT24983	17	+	+	+	+	-	+	-
IMT25006	18	+	+	+	+	+	+	+
IMT24937	20	+	-	+	+	-	+	+
IMT24943	25	+	+	-	+	-	+	+
IMT24950	26	-	-	+	+	+	+	+
IMT24951	29	+	+	+	+	-	+	+
IMT24953	41	+	-	+	+	-	+	+
IMT24959	51	+	+	+	+	-	-	+
IMT24973	62	-	-	+	+	+	-	-
IMT24994	72	+	+	+	+	-	+	+
IMT25008	75	+	+	+	+	-	-	+
IMT25016	83	+	-	+	+	-	+	-

From a total of 16 CTX-M-producing isolates, conjugation to *E. coli* J53Azi^{R} was demonstrated in 15 strains, wherein larger plasmids ranging in sizes from 120 kb to 272 kb were transferred or exchanged. In these strains, all the antimicrobial resistance genes as reported in the parental strains along with the *bla*$_{\text{CTX-M-15}}$gene were detected on IncFIA conjugative plasmid by PCR. The parental clinical strains carried up to six plasmids, most of them displayed two larger plasmids of around 120 kb and/or 272 kb (data not shown); plasmid analysis among transconjugants revealed larger plasmids of >100 kb, except for one strain which revealed an identical profile. The plasmids belonged to the narrow host range incompatibility group IncF; 15 isolates contained the FIA replicon, 13 isolates had the IncFIB replicon, while one isolate harboured P, Y, and A/C replicons in addition to FIA and FIB replicons.

All the 16 ST131 isolates were positive for $bla_{\text{CTX-M}}$ genes (CTX-M-15); additionally, eight (50%) of these isolates also produced TEM-1. bla_{SHV} was absent in all, and the bla_{OXA} group 1 was present in 11 (68.7%) of the 16 Indian isolates. Only one strain (6.3%) was positive for bla_{OXA} group 2. The gene variant *aac (6')-Ib-cr*, which has been associated with fluoroquinolone resistance, was detected in 12 (75%) ST131 strains as determined by digestion of the amplicons with BsegI. No other plasmid-mediated quinolone resistance genes (*qnrA, qnrB,* and *qnrS*) were detected. Other resistance genes detected among ST131 isolates are shown in Table 3. Screening of integron classes revealed that 9 (56.3%) out of 16 ESBL producers contained the intl1 gene, whereas the intl2 gene, which is specific for class 2 integron, was absent in all 16 isolates. The gene cassette arrangement detected among the intl1-positive isolates was dfrA12-aadA2 (for two isolates) and dfrA17-aadA5 (for six isolates). All the ST131 isolates were found to be carbapenem sensitive by the disc diffusion method. The four carbapenemase genes, $bla_{\text{OXA-48}}$, bla_{KPC}, bla_{VIM}, and $bla_{\text{NDM-1}}$, were also absent.

3.3.3 Profiling of virulence genotypes/phenotypes

Serum resistance assay of the three clinical strains (IMT24943, IMT24951, IMT24973) versus their transconjugants along with the strain J53 revealed remarkable increase in serum resistance (Fig. 7) by the laboratory strain J53 alone and J53 with ESBL plasmids from clinical strains ($P = 0.0001$ for each pair). J53 with ESBL plasmids (transconjugants) displayed almost similar or increased resistance compared to that of their parental strains. The pattern of biofilm formation observed in cases of the clinical isolates, transconjugants, and the empty host J53 is shown in Fig. 8. Basically, there were no prominent differences observed (with respect to biofilm formation) between the empty J53 host strain and J53 with ESBL plasmids. It may be concluded that there is reduced influence of plasmids on biofilm formation; alternatively, the basic capacity of the wild-type strains to produce a biofilm is simply too low to identify significant differences in transconjugants.

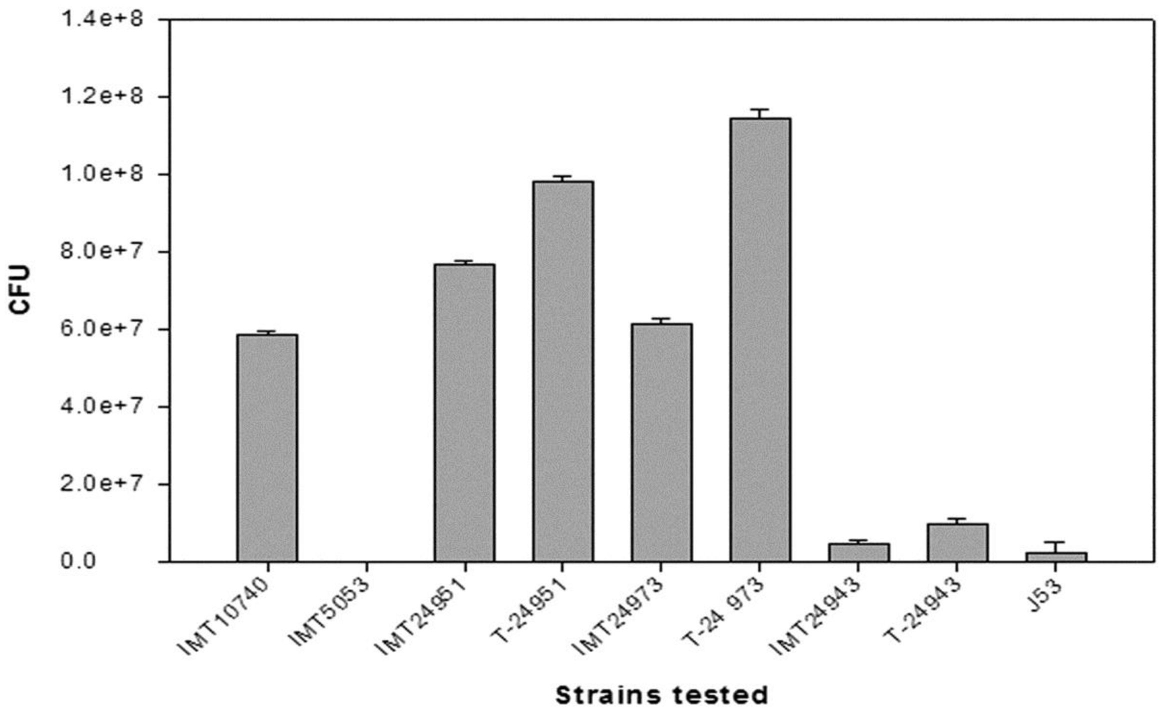

Figure 7: Serum resistance assays of three parental (IMT) ST131 ESBL-producing *E. coli* isolates and each transconjugant (T).

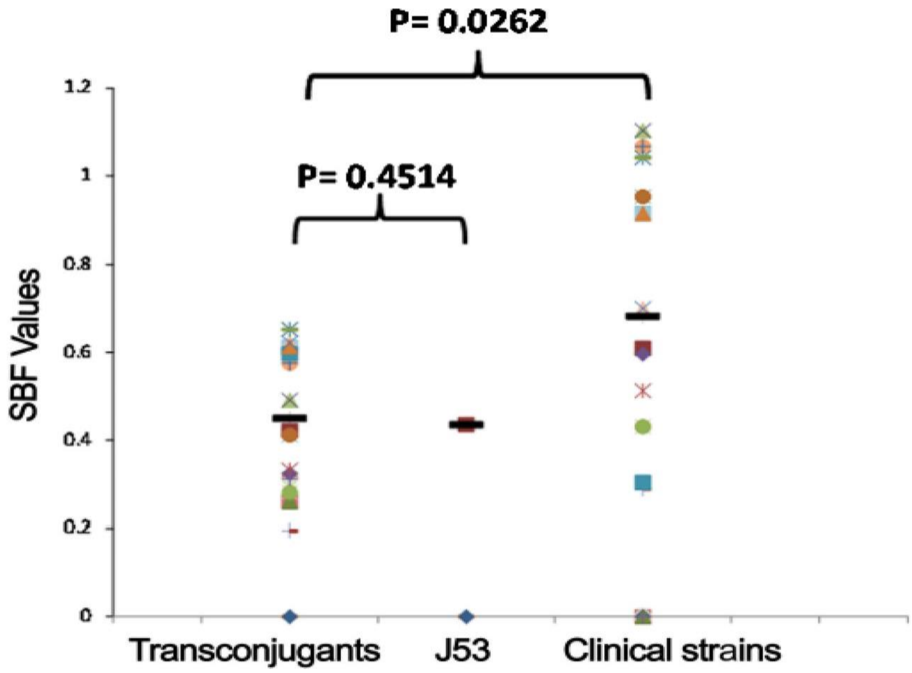

Figure 8: In vitro biofilm formation characteristics of transconjugants, clinical strains, and the plasmid-free host J53.

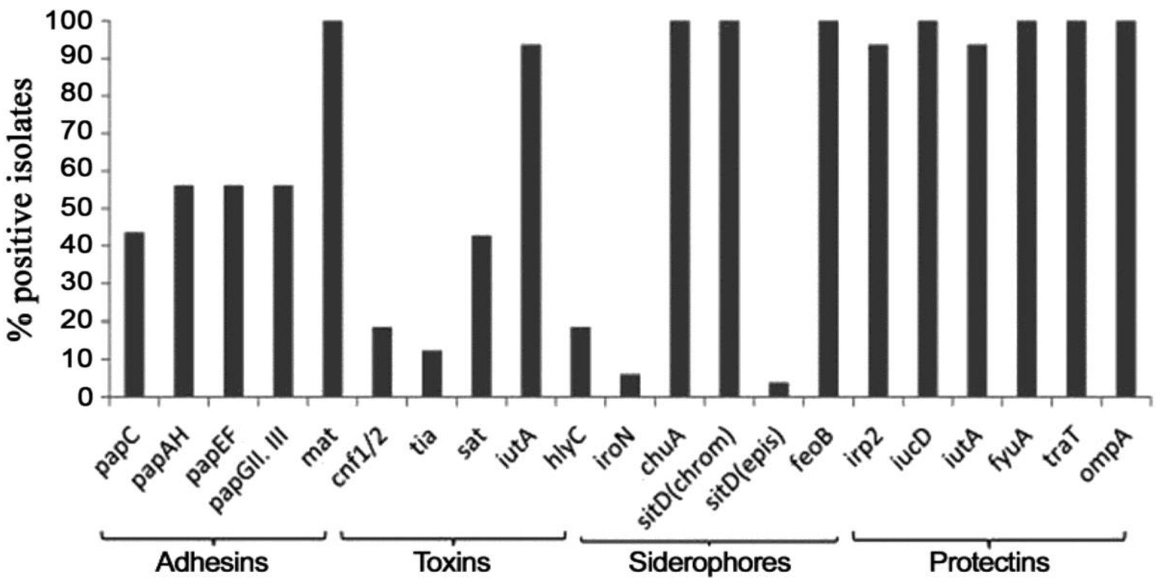

Figure 9: Virulence scores of 16 ST131 UPEC isolates for 23 genes out of 38 extraintestinal virulence-associated genes assessed by multiplex PCRs.

Virulence profiles were determined (Fig. 9) for the 16 ST131 clinical isolates to get an idea of the extent of within-group diversity and the virulence potential of this clonal group. Out of 38 extraintestinal VAGs, 23 were detected at least once, with their prevalence ranging from as low as 6.3% with respect to *iroN* (catecholate siderophore) to 100% presence of the following genes: *chuA* (gene for heme transport), *traT* (serum resistance), *sitD* (salmonella iron transport gene), *ompA* (outer membrane protein), *iucD* (aerobactin), *sat* (secreted auto transporter toxin), *fyuA* (*Yersinia* siderophore receptor), *mat* (meningitis-associated fimbriae), and *feoB* (iron transporter). In general, the virulence gene profile similarity was high among the 16 ST131 isolates. The three identical (IMT24938, IMT24948, IMT24973) isolates seen in group 2 of the PFGE dendrogram exhibited an almost identical virulence profile. Relatively, the isolates within the clusters were more similar in their virulence profiles than the strains from adjacent clusters and singleton strains.

4.1 Discussion

The worldwide occurrence of *E. coli* with CTX-M extended-spectrum beta-lactamases partly reflects the spread of clonal lineages, notably ST131, and hence we sought to identify the ST131 clone of bacteria among the ESBL-positive *E. coli* from the peri-urban areas of Pune, India, and to compare them with the isolates of European origin. The emergence of new MDR (extraintestinal) strains of *E. coli* spreading through populations with the capacity to evolve continuously appears to pose a significant public health threat. A great deal of attention is required in this direction. To get a deeper understanding of the underlying resistance genotypes and the mechanisms thereof, it was intended to characterize these clinical isolates in detail with regard to their ESBL production capacity and other antimicrobial resistance features. We also sought to find out whether there is any relationship between ESBL plasmids and virulence.

Our analysis of 100 rigorously selected *E. coli* isolates found that the clonal group ST131 was prevalent particularly among the ESBL isolates. We could confirm the presence of a total of 16 ST131 isolates by MLST in just a small collection of 100 isolates, suggesting a moderately high level of abundance. They were all affiliated to the phylogenetic group B2, which extends to most of the extraintestinal pathogenic *E. coli*. The representation of ST131 isolates was found to be more among male patients; this explains the enhanced capability of ST131bacteria to establish ascending urinary tract infections (pyelonephritis and prostatitis) despite the male anatomical barriers.

In agreement with other reports, most of the ST131 ESBL producers from this study were determined to be multidrug resistant and displayed high coresistance rates toward tetracycline, fluoroquinolones, sulfamethoxazole-trimethoprim, and gentamicin. Most of the isolates remained susceptible to chloramphenicol, which is effective against a wide variety of Gram-positive and Gram-negative bacteria. Consistent with most of the studies, our ST131 isolates were also highly resistant to fluoroquinolones (81.3%) and trimethoprim-sulfamethoxazole (68.8%).

Transconjugants were created in *E. coli* J53 from 15 (93.8%) out of the 16 strains. It was demonstrated that all of the transferred plasmids were ESBL plasmids. This proved their readily transferable nature with a strong potential for spreading of the ESBL and antimicrobial-resistant genes among bacterial populations. An antimicrobial resistance phenotype similar to the clinical strains was seen in transconjugants, and hence, this observation validates that the genes encoding ESBLs were located on transferable plasmids that harboured genes encoding resistance to several other classes of antimicrobials. Further, we sought to find out whether there was any relationship between the ESBL plasmids and virulence, and surprisingly, we found that the ESBL plasmids not only carried antimicrobial resistance genes but also conferred virulence and survival advantage to the recipient bacteria against the bactericidal activity of sera. However, when we further investigated these plasmids for their role in another virulence attribute, the biofilm formation, we didn't observe any positive influence of ESBL plasmids on biofilm formation. This could simply be due to the regulatory mechanisms involved in biofilm formation that were active on the chromosomal part of the bacteria. Further study of biofilm formation and other virulence features of these plasmids with the plasmid-cured strains and their comparison with parental clinical strains will be important for understanding the relationship between virulence phenotypes and ESBL plasmids.

The CTX-M beta-lactamases have been recognized worldwide as an important mechanism of resistance to cephalosporins (cefotaxime and ceftriaxone) used for Gram-negative pathogens (Canton and Coque 2006). We found that all of the 16 ST131 strains harboured the CTX-M-15 allele. Our finding was in agreement with most of the previously published works reporting a strong association of ST131 strains with CTX-M-15-type enzymes, indicating that they are frequently carried in “aggressive” lineages with high virulence genotypes (Canton and Coque 2006). Similar to the earlier studies, the CTX-M-15 allele was located on plasmids with replicon type FII-FIA, whereas the other replicon types, such as FIB, A/C, P, and Y, which were detected in the clinical wild-type strains, were absent in the transconjugants obtained from them (Clermont et al. 2008, Ewers et al. 2010).

Plasmid-mediated quinolone resistance (PMQR) has become an important emerging issue. The most common PMQR gene observed in this study was *aac(6′)-Ib-cr* (81%). The association of *aac(6′)-Ib-cr* with CTX-M-producing *E. coli* is not surprising, as many studies have shown that it is often harboured on the same plasmids as the $bla_{\text{CTX-M}}$ gene

(Park et al. 2006a, Pitout et al. 2007, Robicsek et al. 2006). We did not detect any other PMQR genes, like *qnrA*, *qnrB*, and *qnrS*, which have also been shown to be associated with CTX-M-producing *E. coli* in recent studies. However, a number of other studies reported that these genes are rather uncommon among CTX-M-producing *E. coli*, and hence our study is in agreement with the observations reported elsewhere (Park et al. 2006a, Pitout et al. 2007, Robicsek et al. 2006). The fluoroquinolone resistance phenotype among these 16 ST131 strains also could be due to mutations within the genes, such as *gyrA* and *parC* (Chang et al. 2011), which has not been determined by us.

Several studies have investigated the distribution of integrons in uropathogenic *E. coli* (UPEC) and have established a strong association between the presence of integrons and antimicrobial resistance in MDR and single-drug-resistant *E. coli* strains (Blahna et al. 2006, Rao et al. 2006). While the analysis of integron-encoded integrases indicated that class 1 integron was the principal integron class in the 16 Indian ESBL strains, no class 2 integron-encoded integrases were detected; these integrons were plasmid encoded and transmissible, as indicated by conjugation experiments. These results are similar to a recent report on antimicrobial resistance in Uropathogenic *E. coli* from Europe and Canada (Blahna et al. 2006). We observed a higher frequency of *sul1* than *sul2*. Sulfonamides are regarded as highly important antimicrobial agents for the treatment of *E. coli* infections, and the presence of sulfonamide resistance can lead to treatment failure in cases of UTI (Blahna et al. 2006). The association of ESBL genes with other resistance determinants (such as genes conferring resistance to sulfonamides, tetracycline, and aminoglycoside) were observed in this study, as the candidate resistance genes are often carried on the same plasmid, as demonstrated by similar antimicrobial profiles of transconjugants (Johnson et al. 2009, Rodriguez et al. 2009, Rogers et al. 2011). These results prove that the ESBL genes continue to evolve and harbor an increasing range of resistance determinants (Harish et al. 2007, Kumarasamy et al. 2010).

Our isolates were checked for susceptibilities to carbapenems, and it was observed that all 16 ST131 ESBL producers were highly susceptible. However, a recent study has highlighted the emergence and widespread distribution of NDM-1 in MDR *Enterobacteriaceae* in different parts of India, Pakistan, and the United Kingdom (Kumarasamy et al. 2010). The reason for this contrasting observation may be the geographic variation that is subject to carbapenem usage, biased sampling, or the small sample size of our study. Nevertheless, it can be said that the association of carbapenemase enzymes with ST131 strains is not widespread, as yet. Plasmids that were isolated from transconjugants were found to be larger than 100 kb, implying that the ESBL plasmids in clinical strains are >100 kb in size. However, the donor strains harboured more than one plasmid, ranging in size from 8.5 kb to 272 kb. Genotyping by PFGE showed that the clinical strains were genetically clonal, as they were sourced from a single hospital and from one geographical area. The three indistinguishable Indian strains on PFGE fingerprints were isolated 1 month apart; two strains were from the same ward, and the third one came from a different ward. This possibly suggests a nosocomial spread. The clustering of three European strains with the Indian strains also supports a possible clonal nature of ST131 strains. This observation is in corroboration with two recent reports (Clark et al. 2012, Lavigne et al. 2012) that have shown sequence similarity of one of our ST131 isolates, NA114, from India to the European isolates (Avasthi et al. 2011); this perhaps points to a somewhat conserved genomic structure of a few ST131 strains of European origin. However, on the other hand, we saw some degree of heterogeneity in the PFGE profiles among the Indian strains and between the European ST131 strains which could possibly be due to the high rates of recombination within the accessory genome of this species. Another worrying fact is that these ESBL-producing strains have a high background of virulence. This resistance-virulence combination might impart ST131 a competitive advantage over other *E. coli* strains.

In conclusion, our study is perhaps the first one to identify and characterize ST131 *E. coli* in UPEC populations from an Indian setting. This study demonstrates the complexity of the acquisition and spread of MDR phenotypes that could pose difficulties in treating serious Gram-negative infections. The observation that the ESBL plasmids are readily transferrable might present another problem of rapid acquisition of MDR genes by the docile strains. The PFGE profiles, virulence factors, and antimicrobial resistance pattern of these ST131 isolates exhibit that the strains from this geographic location were homogenous pathogens. The transfer of virulence phenotype due to ESBL plasmids might lead to an increase in acquisition of virulence among environmental and commensal bacteria. Therefore, the emergence and dissemination of this particular lineage of *E. coli* in Indian infection settings is a cause of concern and points to a need to identify their origins, reservoirs, and transmission pathways so that better prevention strategies are designed. Having said this, we would like to carry out further studies on a large number of *E. coli* isolates from different places within India to have an exhaustive opinion on epidemiology, evolution, and pathogen biology. Whole-genome sequencing of these strains, including their plasmids, would be instrumental to gain more insights into the molecular mechanisms imparting fitness or survival advantage to these ST131 lineages over other pathogens. Finally, on the public awareness and policy front, while antimicrobial resistance is projected as an important public health concern in China (Heddini et al. 2009), India also deserves such attention given the widespread occurrence of new beta-lactamase genes, such as $bla_{\text{CTX-M}}$ and $bla_{\text{NDM-1}}$, amidst the absence of a proper antibiotic policy based on epidemiological knowledge entailing socioeconomic and bio-geo-climatic factors that favour rapid emergence of antimicrobial resistance.

Chapter 4

Whole genome sequencing of a pandemic; multivirulent/multiresistant *Escherichia coli* strain (ST131) NA114 by next generation sequencing technology

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4.1 Introduction

Pathogenic *Escherichia coli* constitutes a significant threat to public health, and the emergence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* with high virulence potential is alarming (Kaper 2005, Kumarasamy et al. 2010, Livermore 2009, Miriagou et al. 2010, Rogers et al. 2011). Comparative genomics holds significant promise in understanding the genome organization of such bacteria and thereby identifying coordinates highly relevant in the development of intervention strategies (Ahmed et al. 2008) . Our group has recently studied Uropathogenic *E. coli* (UPEC) from the western Indian city of Pune (Jadhav et al. 2011, Lau et al. 2008a), whereupon strain NA114 emerged as an ideal representative of the entire Pune collection. The three major characteristics of strain NA114 that make it epidemiologically and clinically significant are its affiliation with serogroup O25, its placement in phylogenetic group B2, and its sequence type, ST131 (Lau et al. 2008a). The latter denotes a pandemic clone frequently associated with community-acquired antimicrobial-resistant infections (Rogers et al. 2011). Motivated by these facts, we performed complete in-depth sequencing, annotation, and analysis of the genome of UPEC strain NA114, which was originally obtained from the urine of a 70-year-old male patient with prostatitis from Pune. Antibiotic sensitivity tests revealed that it was a multidrug-resistant strain refractory to several common antibiotics and was an ESBL producer (Jadhav et al. 2011).

4.2 Genomic DNA preparation and quality check

Genomic DNA for whole genome sequencing was prepared using Qiagen Blood and Tissue DNAeasy kit (Qiagen GmbH, Germany). The quality of DNA was checked by running the samples in 0.8 % agarose gel and by measuring its concentration in a nanodrop (ND-200) and the quality of DNA was also checked by loading it on an agarose gel. The concentration of DNA was found to be between 150- 250 ng/ul for all the samples, the DNA samples were then pooled to give a final concentration of > 10 µg.

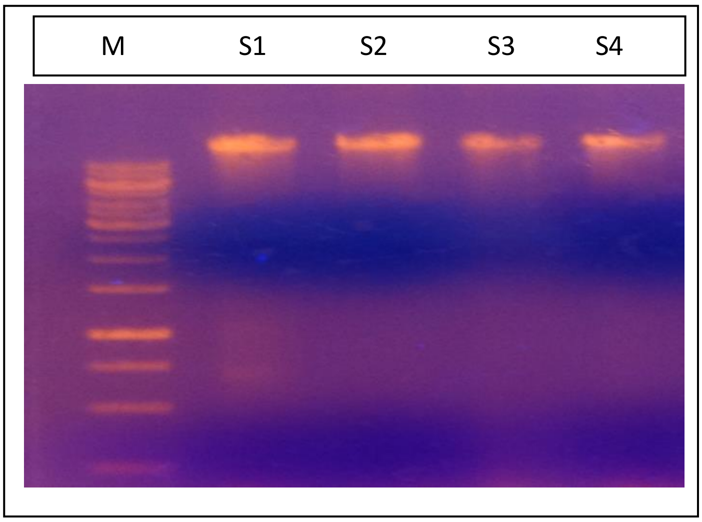

Figure 10: S1-S4; Genomic DNA preparation of strain NA114 and its quality check.

4.3 Sequencing chemistry

The DNA sample was processed for sequencing by illumina genome analyser which involves 3 steps in its chemistry; i) Library preparation, ii) Cluster generation and iii) Sequencing by synthesis.

The genome sequencing was performed on massive amounts of parallel strands of 100 of millions of fragments of DNA, for this first the genomic DNA was fragmented by sonication, the sheared ends are repaired by adenylation and adapter oligos are ligated to both the ends of the fragments. These fragments are then size selected and purified and then amplified to enrich the fragments. The second step was the cluster generation on the flow cell wherein, single molecules are isothermally amplified to prepare them for the high throughput sequencing. The eight channel genome analyser flow cell has dense amount of oligos attached on its surface, these oligos bind to adapters ligated to the library fragments. Single DNA molecules hybridize to the lawn of oligos, bound fragments are extended to create copies, and these copies are covalently bound to the flow cell surface, each library fragment was clonally amplified through a series of extension and isothermal bridge amplifications resulting in hundreds of millions of unique clusters. The reverse strands were cleaved and sequencing primers were hybridized to the DNA templates. After cluster generation the library was ready for sequencing, on the genome analyser millions of clusters were sequenced simultaneously. The DNA templates were sequenced base to base and parallel using fluorescently labelled reversible terminated nucleated, all 4 bases compete with each other for the template, this natural competition ensures the highest accuracy. After each round of synthesis the clusters are excited with lasers emitting the colour that identifies the newly added base. The fluorescent label and the blocking group are then removed allowing for the addition of next base. This way, the Illumina Genome Analyzer (GA2x, pipe51line version l.6) generated 54 bp paired-end sequence reads, amounting to 8 gigabytes of data with an insert size of 300bp and 500X genome coverage.

4.4 Genome assembly pipeline

The raw reads were filtered for primer contamination, enrichment PCR primer contamination and bad quality reads using FASTX toolkit. Reads with quality threshold less than 20 were removed or trimmed accordingly. Denovo Assembly of high quality filtered reads was carried out using Velvet denovo assembler. Velvet was run for a series of k-mer values and an optimal k-mer of 31 was chosen based on comparison of different parameters like N-50, number of nodes, maximum contig size, genome size and number of reads being used (Zerbino and Birney 2008). A reference based approach is then followed to order and arrange the contigs. Assembled contigs were compared with the database to find the closest relative organism to be selected as reference. Mauve was used to re-order the contigs according to the selected reference genome (*E. coli* SE15) (Toh et al. 2010). The genome alignment tools, BLAT (Kent 2002) and MUMmer (Kurtz et al.

2004) were also used to validate the aligned contigs. The genome was annotated with the help of RAST server (Aziz et al. 2008) and putative CDSs were identified by comparing outputs from Glimmer (Delcher et al. 1999), Genemark (Besemer and Borodovsky 2005) and EasyGene (Larsen and Krogh 2003). Artemis (Rutherford et al. 2000) was used to glean the following details of the genome.

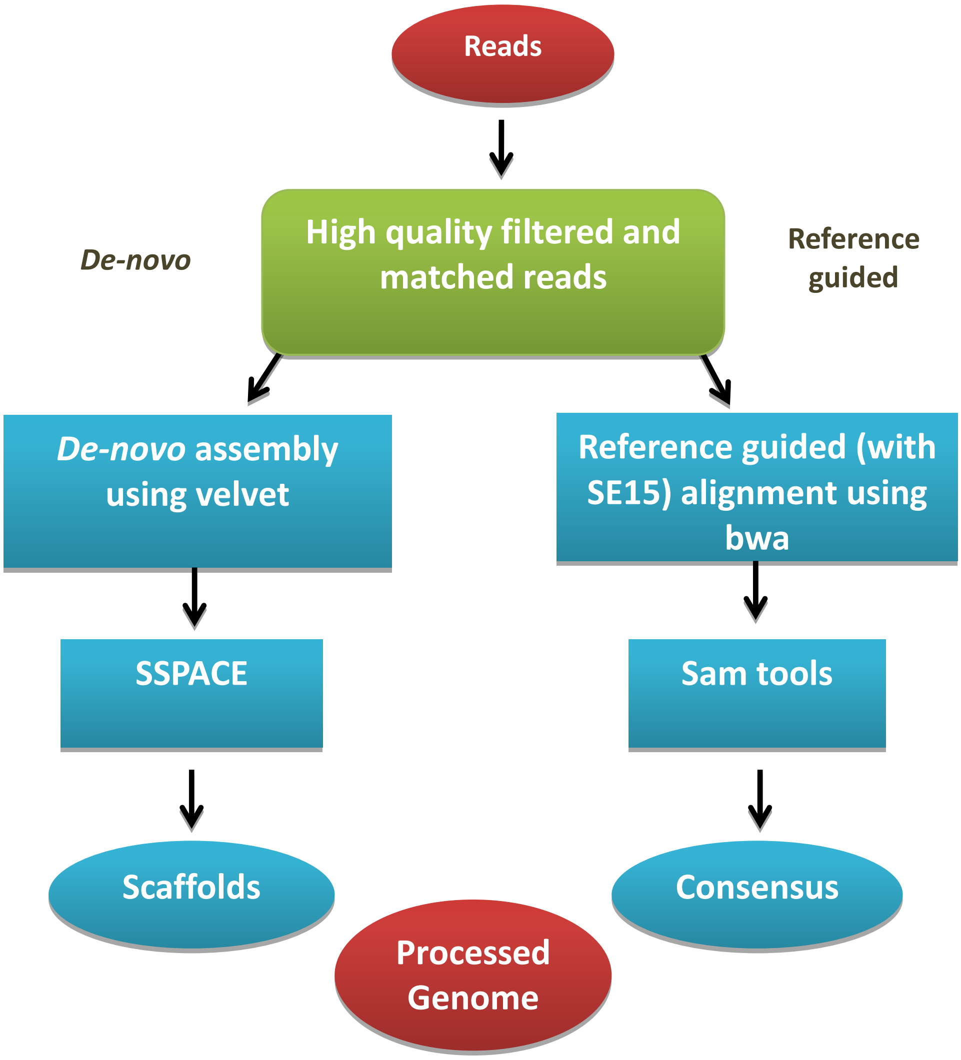

Figure 11: Genome assembly pipeline employed for assembling the NA114 genome.

4.5 Genome statistics

The size of the NA114 chromosome was 4,935,666 bp with a G+C content of 51.16% and a coding percentage of 88.4% with 4,875 protein coding sequences with an average length of 901 bp. The genome revealed 67 tRNA and 3 rRNA genes. We also found several virulence genes, including *iha*, *sat*, *fimH*, *kpsM*, *iutA*, and *malX*, which correspond to the genes of *E. coli* CFT073. In addition, genes corresponding to another UPEC strain, UTI89, such as *fyuA* and *usp* etc., were located. PCR-based analysis showed that this strain carried multiple virulence genes infrequently described in a clone of this type, including *sfa*, *aer*, *cnf*, and an intact polyketide synthase (*pks*) island. *E. coli* NA114 also contains other virulence factors, such as *pap*, *fim*, and genes for iron uptake systems such as the hemin uptake system and the yersiniabactin siderophore (*ybt*). In addition to a 4.935-Mb chromosomal genome, strain NA114 also harboured a single plasmid of 3.5 kb which has yet to be analyzed with regard to its replicon type and resistance gene profiles, if it has any.

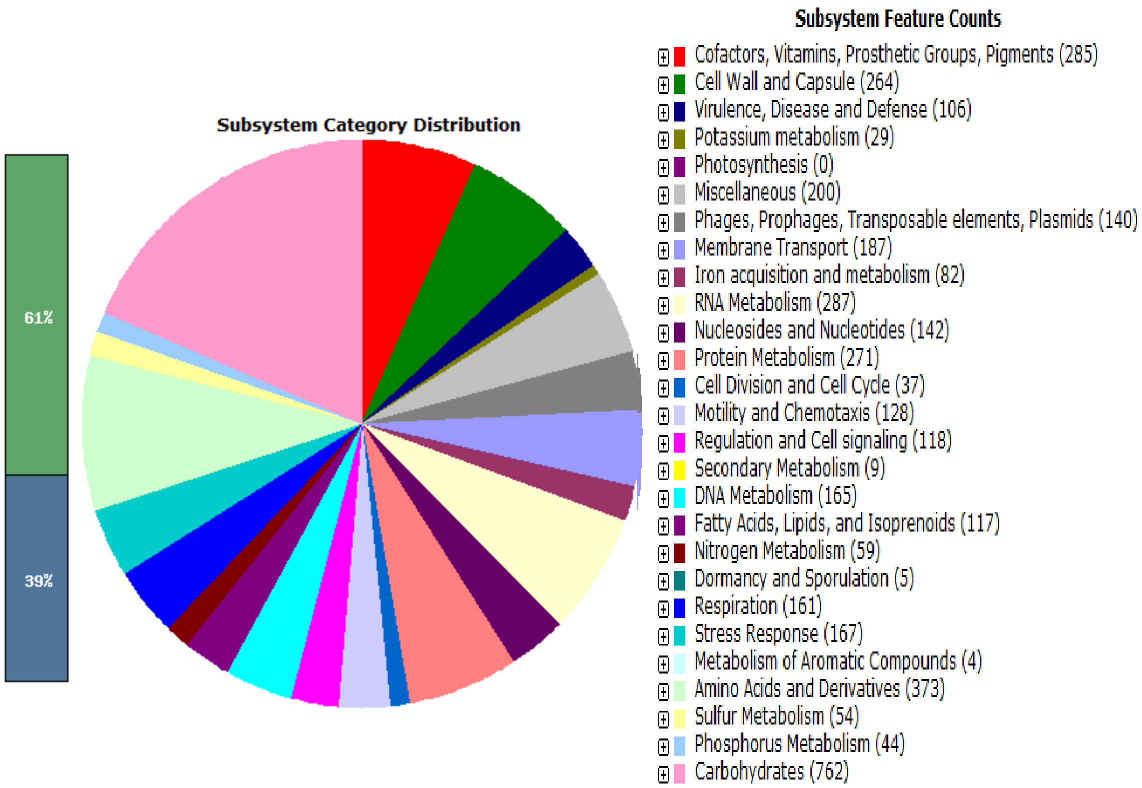

Figure 12: Different subsystems features of NA114 generated by RAST.

4.6 Major observations

This was the first ST131 genome submitted in Genbank from the whole of Asia. This genome enhanced the capacity for rapid qualitative and quantitative comparative studies. For this reason since its publication in 2011, the genome of NA114 has been widely used by researches around the globe as demonstrated by the large number of citations of the original paper, this reflects the continuous use of this genome in many laboratories around the world. Researchers around the world have used this genome in their comparisons, their observations (Clark et al. 2012; Lavigne et al. 2012; Paul et al. 2012) together with our analysis have been summarised as follows

- Interestingly, no novel genomic islands were discovered when compared with other strains like UTI18 and SE15
- They were found to be highly homogenous in their virulence and their antimicrobial resistance properties
- Comparison of NA114 (ST131) with ST131 strains from other countries displayed close genomic similarities meaning they are highly homogenous
- The diversity observed between NA114 and other ST131 is majorly due to homologous recombination and point mutations
- FimH tip adhesin was identified to be under high positive selection

Together the data from our and other studies provided evidence for the circulation of a genetically monomorphic *E. coli* ST131 clone as a dominant strain, isolated from unrelated clinical cases of extraintestinal infections. To our knowledge, this is the first time such a phenomenon has been reported for an ST of *E. coli*. These observations and the comparative genomic studies emanating there from could be extremely useful both in improving our fundamental understanding of multidrug resistance mechanisms encoded by UPEC and in the design of effective drugs to control and manage the alarming health hazards caused by ESBL-producing bacteria in both the developing and developed parts of the world.

4.7 Nucleotide sequence accession number: The genome sequence of *E. coli* NA114 has been deposited in GenBank under accession no. **CP002797.**

Chapter 5

Association of different genotypic and phenotypic profiles with clinical ST131, non-ST131 and stool non-ST131 *Escherichia coli* isolates

5.1 Introduction

Recent studies have reported that around 80% of UTIs are caused by ExPEC (Stamm 1991). These bacteria are becoming increasingly resistant to front-line antibiotics like ciprofloxacin and trimethoprim which are frequently reported from countries all over Europe, America and much of Asia (Foxman 2010). Moreover, the increasing incidence of extended- spectrum-β-lactamases (ESBLs) in ExPEC, mainly the non –TEM/SHV ESBLs such as the CTX-M enzymes has become a serious clinical problem globally, particularly in the past decade (Canton and Coque 2006). Also, since the time of the first report on detection of CTX-M producing *Enterobacteriaceae* (Karim et al. 2001), several surveys have reported the presence of ESBLs in clinical isolates leading to an understanding that an endemic situation of CTX-M ESBL is prevailing in many parts of India (Paterson and Bonomo 2005). In fact, this scenario has compelled clinicians to go for widespread use of 'last line antibiotics' such as carbapenems to treat various life threatening infections. Consequently, such a selection pressure shaped bacteria (also known as superbugs) with novel genes that could degrade even the carbapenem group of antibiotics, the best example being NDM-1 gene (Hawkey 2008, Harish et al. 2007). Following the first description of NDM-1 in *Klebsiella pneumoniae* in 2008, a variety of bacterial species positive for NDM carbapenemases were reported globally (Kumarasamy et al. 2010). The emergence of antimicrobial resistant 'superbugs' is thus a global problem. However, the circumstances predominating in developing countries particularly pose serious concerns (Mitka 2013) despite the fact that there is no credible scientific evidence to believe NDM-1 gene had its origins in India.

The increase in the prevalence of multidrug resistant bacteria in recent years has posed greatest risk to public health (Vento and Cainelli 2010, Wise and Piddock 2010). Moreover, the dissemination of clonal organisms carrying heavy antibiotic resistance background has aggravated the problem (Canton, Coque and Baquero 2003). The strain with sequence type 131 (ST131) is one such pandemic clone that is rapidly and boundlessly disseminating in different countries across continents (Hussain et al. 2012). These clonal pathogens are highly homogenous in their virulence and their antimicrobial resistance properties (Rogers et al. 2011). PFGE analysis revealed that they are clonal as they are mostly similar at 85% similarity (Lavigne et al. 2012). Phylogenetic analysis based on whole genome data confirmed that the ST131 clones are genetically monomorphic in nature (Clark et al. 2012). *E. coli* ST131 has emerged globally as an important pathogen causing urinary tract and bloodstream infections within communities and hospitals (Totsika et al. 2011). This clonal group is also responsible for the recent worldwide spread of CTX-M-15 ESBL types (Coque et al. 2008), which are known to frequently harbour fluoroquinolone resistance and hence, offer very limited treatment options (Johnson et al. 2010). Clinical findings also suggest that the strains belonging to this group are highly virulent and have a fitness advantage (Gibreel et al. 2012). If this is the case, then the association of newer antibiotic resistances like NDM-1 with *E. coli* ST131 (Bonnin et al. 2012) would have grave complications in the form of pandemics and life threatening treatment failures.

The dissemination of resistance among bacteria is mainly thought to be due to mobile genes present on self-transmissible plasmids (Carattoli 2009). However, a large cache of antibiotic resistant bacterial clones goes undetected as they often take refuge as normal human flora in the gut of humans and animals. Moreover, the commensal bacteria are at the risk of exposure to orally ingested antibiotics this way; they possibly possess high antimicrobial resistance genes and facilitate the spread of such genes to other strains of bacteria (Mazurek et al. 2013, Verraes et al. 2013).

Our previous studies from India have reported important initial data on clinical isolates of *E. coli* (Hussain et al. 2012). However, a thorough comparative work describing ST131 ESBL producing *E. coli* and other non-ST131 ESBL *E. coli* has not been reported so far. Therefore, the objectives of this study were to assess the diversity, distribution and transmissibility of antimicrobial and ESBL profiles in commensal, ST131 and other non-ST131 clinical *E. coli* isolates. We also attempted to determine their phylogeny and genetic diversity using PCR-based approaches. The virulence attributes of the three categories of isolates were determined by genotyping as well as phenotypic methods.

5.2 Materials and methods

5.2.1 Sample collection and screening for ST131 strains

A total of 350 *E. coli* isolates were collected from symptomatic UTI patients from a major tertiary hospital (Dr. D. Y. Patil Medical College and hospital, Pimpri, Pune, India) between January 2009 and March 2011. Fifty stool *E. coli* isolates were also isolated from feces of healthy individuals who had reported for routine health checkup at the aforementioned hospital. All isolates were tested for purity and were identified by standard laboratory methods (refer Appendix i). O types of all *E. coli* strains were initially determined with a molecular approach based on allele-specific PCR, targeting the *rfbO25* subgroup gene locus (Clermont et al. 2009a). ST131 strains were further confirmed by PCR-based detection of the *pabB* allele as described earlier (Clermont et al. 2009a). After this screening procedure, only 83 out of 350 and 49 fecal *E. coli* isolates were finally selected for further studies. That is, a total of 132 *E. coli* isolates were subjected to thorough investigation as described below.

5.2.2 Antibiotic susceptibility testing

Antibiotic susceptibility profiles were obtained for all *E. coli* isolates by using standard disc diffusion method on Mueller-Hinton Agar. The CLSI recommendations were followed for antimicrobial susceptibility testing (Hussain et al. 2012). The antibiotic panel used was as follows: gentamicin (120µg); ciprofloxacin (5µg); nalidixic acid (30µg); chloramphenicol (30µg); sulfamethoxazole/trimethoprim (25µg); tetracycline (30µg). All strains were tested for the ESBL production phenotype using the method given in CLSI guideline M31-A3 (Schissler et al. 2009). Metallo-β-lactamase (MBL) production was determined phenotypically by combined- disk test, where two disks, one containing IPM-EDTA and the other with IPM alone were placed 25mm apart. An increase in zone diameter of >7mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL. The modified Hodge test was also performed to detect carbapenamase production (Anderson et al. 2007a).

5.2.3 Antimicrobial resistance gene testing

The presence of genes encoding antimicrobial resistance was determined by PCR amplification of respective genes. PCR amplification of the bla_{CTXM} bla_{TEM} and bla_{SHV} genes was carried out on all *E. coli* isolates using PCR conditions and primers previously described (Rodriguez-Siek et al. 2005b). The presence of other antibiotic resistance genes such as those conferring resistance to tetracycline, namely *tet(A), tet(B), tet(C)*; to sulfonamides *(sul1, sul2, sul3)*; to streptomycin *(strA, strB)*; to aminoglycosides *(aadA1-like, aac4)*; to trimethoprim *(dhfr, dfr)* and some other plasmid-mediated quinolone resistance determinants such as *aac (6')-lb* was determined by PCR (Bertrand et al. 2006a, Bert, Branger and Lambert-Zechovsky 2002b, Boerlin et al. 2005b, Ewers et al. 2004, Jouini et al. 2007, Robicsek et al. 2006). Four well known carbapenemase genes, $bla_{\mathrm{OXA\text{-}48,}}$ $bla_{\mathrm{KPC,}}$ bla_{VIM} and $bla_{\mathrm{NDM\text{-}1}}$ were also amplified as described previously (Pfeifer et al. 2011b, Bertrand et al. 2006a). All the *E. coli* isolates were screened by PCR for the presence of intergron-associated *intl1* gene encoding class 1 integrase, with the help of primers described elsewhere (Skurnik et al. 2005b).

5.2.4 Conjugation and plasmid analysis

Conjugative transfer of ESBL genes was tested by broth-mating experiments using plasmid-free, sodium azide resistant *E. coli* as recipients (Ewers et al. 2004). Putative transconjugants were selected on EMB agar plates containing sodium azide (100µg/ml) and cefotaxime (4µg/ml) and these were checked by PCRs for the presence of relevant *bla* genes. The number and size of plasmids were determined by using a modified version of the methodology as previously described (Kado and Liu 1981). Plasmids were classified into two major replicon types according to their incompatibility groups by using a PCR-based replicon-typing scheme (Carattoli et al. 2005).

5.2.5 Biofilm formation

Biofilm formation by *E. coli* isolates from three groups was assayed by a 96-well polystyrene microtiter plate in the static biofilm model, as described in our previous study (Hussain et al. 2012).

5.2.6 Zebra fish lethality assay

Zebra fish were anesthetized by immersion in water containing 168µg/ml of tricaine (Sigma) and then intraperitoneally injected with 10 µl of PBS containing *E. coli* (1x 10^6 CFU/ml) using a 26.5 guage syringe (Becton-Dickinson). After injection, the fish were returned to water tanks immediately. The fish were closely monitored for mortality every 3 hours, upto 1 week as described earlier (Chao et al. 2010).

5.2.7 Phylogenetic grouping and virulence gene identification

All the isolates were tested for the determination of their phylogenetic groups using PCR according to the method described by Clermont *et al*. (Clermont et al. 2000). All the *E.*

coli isolates were screened for 6 major ExPEC associated virulence genes by PCR as described previously (Rodriguez-Siek et al. 2005b, Bingen et al. 1998) Virulence score for each isolate was determined based on the number of virulence genes detected. The virulence genotype similarity between isolates was determined by calculating the number of shared virulence genes divided by the total number of virulence genes probed.

5.2. 8 Metabolic profiling

The three categories of *E. coli* isolates were tested against 36 different substrates to check their metabolizing abilities by KB009 Hi carbohydrate Kit (Hi-media, Mumbai). 50µl of inoculum was added into the wells of the strip. Upon incubation, the organisms underwent metabolic changes which were indicated by spontaneous colour change in media due to change of pH.

5.2.9 Genotyping by ERIC-PCR

E. coli strains were typed by ERIC-PCR. Crude DNA obtained from the *E. coli* isolates by direct cell lysis were analysed by ERIC sequence based PCR with the ERIC1R (5-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers; PCR was performed as previously described (Versalovic et al. 1991). In brief, ERIC-PCR was performed using the following parameters: denaturation at 94°C for 30s, annealing at 50°C for 1min and extension at 72°C for 8 min for 30 cycles; this was followed by a final extension at 72°C for 10 min. Amplicons were separated on a 1.5% agarose gel containing ethidium bromide (5 µg/mL) at 50 V for 3 hours. The banding pattern of 126 *E. coli* isolates was analysed using Bionumerics^{TM} software (Applied Maths, St-Martens-Latem, Belgium). Bands were scored by Dice similarity indices based on the unweighted paired group method with arithmetic mean (UPGMA) and dendrograms were generated to reveal the relationships among ERIC profiles.

5.3 Results and discussion

5.3.1 ST131 status and patient demography

A total of 350 *E. coli* isolates were screened with a pair of primer for *pabB* fragment that is specific for the ST131 lineage. Of these, 43 isolates (12.2%) were positive for ST131 PCR. These ST131 strains were additionally tested for *rfbO25b* locus by PCR and it was confirmed that these strains in fact belonged to ST131. None out of the 43 fecal isolates was found to be associated with ST131 lineage. Among the 43 ST131 *E. coli* isolates, 26 (60%) were from female patients with mean age of 38 years, 17 (40%) were from male patients with mean age of 48 years. Whereas, among the 40 non-ST131 *E. coli* isolates, 21 (52%) were from female patients and 19 (47%) were from male patients with mean age of 35 and 45 years respectively. Male patients (for both ST131 and non-ST131 isolates) were more likely to develop prostatitis than female patients with common UTI. Among the different medical wards, the majority of ST131 samples came from the surgical ward (27%). Rest of the ST131 isolates ranging between 2-25% came from different wards of the hospitals. Whereas the non-ST131 isolates were originated only from non-surgical wards.

5.3.2 Phenotypic and molecular screening for antimicrobial resistance

A total of 126 isolates (comprising 3 groups of 40±3 randomly selected isolates each) that were (i) ST131 *E. coli*; (ii) non-ST131 *E. coli*; (iii) fecal *E. coli* underwent antimicrobial susceptility testing. Disk diffusion susceptibility testing entailing 6 antibiotics belonging to five different non-β-lactam classes showed that the resistance rates were high among all *E. coli* isolates regardless of their ST131 or clinical status (Table 4). The antimicrobial resistance rates for the three groups of strains, namely, ST131, non-ST131 and fecal isolates respectively, were as follows: ciprofloxacin 90%, 92%, 76%; tetracycline 63%, 75%, 86%; gentamicin 60%, 50%, 42%; cotrimoxazole 63%, 75%, 86%; and chloramphenicol 40%, 33%, 25%. *E. coli* isolates within the 3 groups showed nearly equal resistance rate (95%) towards Nalidixic acid. ST131 and non-ST131 isolates including the fecal isolates did not differ significantly for resistance prevalence for any of the particular antibiotics tested. Aggregate resistance scores (resistance to all the 6 antibiotics) although differed marginally among the 3 groups, were highest among the ST131 isolates (35%) and lowest among fecal isolates (14%).

Among the three groups of strains, the proportions of multidrug-resistant (MDR) isolates were as follows: ST131 and fecal isolates 91% each, non ST-131 isolates 95%. With regard to the ESBL production, 37 (86%) of the 43 ST131 isolates, 39 (91%) of 43 fecal and 33 (83%) of 40 non-ST131 isolates were ESBL-producers. The rate of ESBL production among the three groups differed slightly with higher ESBLs in fecal isolates followed by ST131 and then non-ST131 isolates (Table 4).

Table 4: Contribution of ST131, non-ST131 and stool *E. coli* isolates to overall antimicrobial resistance rates (total percentage).

Resistant phenotype		Overall prevalence of resistant phenotype in population, %	Estimated fraction due to ST131clinical isolates	Estimated fraction due to non-ST131 clinical isolates	Estimated fraction due to fecal strains
Antimicrobial class	Specific trait				
Quinolone/ fluoroquinolone	Ciprofloxacin	76	0.40	0.25	0.34
	Nalidixic acid	85	0.38	0.23	0.38
Sulfonamide/ trimethoprim	Cotrimaxazole	68	0.33	0.23	0.43
Aminoglycosides	Gentamycin	45	0.47	0.21	0.31
Phenicols	Chloramphenicol	28	0.51	0.17	0.31
Tetracyclines	Tetracycline	67	0.35	0.27	0.37
ESBL phenotype		87	0.33	0.30	0.35
MBL phenotype		20	0.48	0.52	0.00
Multidrug resistance		92	0.33	0.32	0.33

Table 5: Molecular determination of antimicrobial resistance in 126 *E. coli* isolates.

		No. (%) of isolates		
Antibiotic class	Specific trait	ST131 *E. coli* isolates (group1; n=43)	non-ST131 *E. coli* isolates (group2; n=40)	Fecal *E. coli* isolates (group3; n=43)
Tetracyclines	*tet (A)*	24 (56)	17 (43)	7 (16)
	tet (B)	5 (12)	22 (55)	10 (23)
Aminoglycosides	*Str A*	33 (77)	19 (48)	24 (56)
	Str B	15 (35)	7 (18)	16 (37)
Fluoroquinolone	*aac(6')-lb-cr*	36 (84)	17 (43)	36 (84)
Sulfonamides	*Sul1*	20 (47)	17 (43)	10 (23)
	Sul2	5 (12)	12 (30)	22 (51)
Trimethoprim	*Dfr*	18 (42)	7 (18)	21 (49)
	Dhfr	15 (35)	3 (8)	5 (12)

Table 6: Antimicrobial resistance scores and range towards 6 non-β-lactum antibiotics among 6 groups of strains as defined by the possible combination of ESBL-CTX-M-15, ESBL-fluoroquinolone^R and CTX-M-15- fluoroquinolone^R, including for MBL$^+$ and MBL$^-$ strains.

		Total Median Score		ST131 Median Score		Non-ST131 Median score		Fecal isolates Median Score	
Subset	Subset definition	No.	(range)	No.	(range)	No.	(range)	No.	(range)
1	ESBL$^+$,CTX-M-15$^+$	88	4(1-6)	35	5(2-6)	18	4(2-6)	35	4(2-5)
2	ESBL$^+$ CTX-M-15$^-$	41	4(1-6)	15	4(3-6)	2	3(3)	24	5(1-6)
3	ESBL$^-$, fluoroquinole^R	10	4(3-6)	4	3(3-4)	5	4(4-6)	1	0(5)
4	ESBL$^-$, fluoroquinolone^S	7	1(0-3)	2	0(0)	2	2(1-3)	3	1(0-3)
5	CTX-M-15$^+$, fluoroquinolone^R	69	4(2-6)	38	5(2-6)	20	4(2-6)	11	4(2-5)
6	CTX-M-15$^+$, fluoroquinolone^S	7	3(0-4)	3	3(0-3)	1	0(3)	3	3(3-4)
7	MBL$^+$	25	5(2-6)	12	5(2-6)	13	4(2-6)	0	0
8	MBL$^-$	58	4(0-6)	31	4(0-6)	27	4(1-6)	0	0

Table 7: Phylogenetic and virulence characteristics of 126 *E. coli* isolates comprising 43 ST131, 40 non-ST131 and 43 fecal strains.

No. (%) of isolates with trait				
Specific trait	Total (n=126)	ST131 (n=43)	Non-ST131 (n=40)	Fecal strain (n=43)
Phylogenic group				
Group A	34(27)	0(0)	9(23)	25(58)
Group B1	10(8)	0(0)	8(20)	2(5)
Group B2	62(49)	43(100)	15(38)	4(9)
Group D	18(14)	0(0)	8(20)	10(23)
Virulence genes				
Afa	16(13)	7(16)	1(3)	8(19)
sfa/foc	4(3)	4(9)	0(0)	0(0)
papA	45(36)	29(67)	12(30)	4(9)
Aer	55(44)	9(23)	9(23)	14(33)
hlyD	11(9)	2(5)	2(5)	3(7)
Cnf	4(3)	1(3)	1(3)	0(0)

The prevalence of the major antimicrobial resistance genes in our isolates for tetracycline, aminoglycosides, fluoroquinolones, sulfonamides and trimethoprim are reported in Table 5. All 126 isolates were negative for *sul 3*, *aac* and *tet (C)* genes. The correlation between resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was high for fluoroqinolones (94% agreement), cotrimoxazole (90% agreement), and aminoglycosides (88% agreement), whereas, the agreement between resistance to tetracycline and the presence of *tet(A)* and *tet(B)* was poor (60% agreement). The agreement between the ESBL genotypes and ESBL positive phenotype in ST131 and fecal isolates was 100% and for non-ST131 it was 84%. The main gene contributing for ESBL phenotype among ST131 and fecal strains was $bla_{\text{CTXM-15}}$ whereas for non-ST131 it was $bla_{\text{CTXM-15}}$ together with bla_{TEM}. The $bla_{\text{CTXM-15}}$ was found to be strongly associated with ST131 isolates (93%). However, a similar trend was also found for fecal strains. The possible combinations of ESBL-CTX-M-15, ESBL-fluoroquinole^{R} and CTX-M-15–fluoroquinole^{R} gave rise to 6 subgroups with 2 subgroups per combination (Table 6).

Among these, the dual positive subgroup ($ESBL^+$,CTX-M-15^+) was the most predominant (70%), followed successively by the CTX-M-15^+-fluoroquinolone^R subgroup (55%), the $ESBL^+$ CTX-M-15^- subgroup (33%), the $ESBL^-$ fluoroquinole^R subgroup (8%) and the $ESBL^-$ fluoroquinolone^S and CTX-M-15^+ fluoroquinolone^S subgroups with a low prevalence of (6%) each (Table 6). Within these subgroups, the ST131 strains contributed more or less equal number of representatives to each of the 6 subgroups as that of the fecal strains followed by non-ST131 strains. The prevalence of MBL (metallo beta-lactamase) positive strains was only 20% (25 out of a total 126 isolates) having a median and resistance range higher than that of MBL negative isolates (Table 6). Overall, resistance to antimicrobial agents other than those specified (Table 6) was common and differed insignificantly between the three groups.

5.3.3 Conjugation and plasmid analysis

Plasmid analysis revealed that the three categories of strains contained up to seven plasmids per strain and no categories of strains were harbouring any particular set of plasmid of any predefined size. For identification of plasmids that were responsible for ESBL production in clinical strains, the strains were transconjugated with the laboratory strain J53 which do not harbour plasmids of its own. We were successful in getting 13 bacterial plasmids transferred to J53 strain by *in vitro* conjugation. All the transconjugants were subjected to antibiotic susceptibility test and ESBL production test by double disc synergy method. We could confirm that all the 13 transconjugants contained ESBL plasmid as detected by the above tests and 9 out the 13 transconjugants were also checked for their ability to confer co-resistance to other classes of antibiotics. As observed, most of the transconjugant strains were found to confer resistance to one or a maximum of three antibiotics but none of these transconjugants were co-resistant to all the resistant phenotypes as compared to that of their parent clinical strains.

5.3.4 Biofilm formation

Biofilm formation is a crucial activity of many pathogenic organisms which helps them to cumulatively release virulence associated products (or metabolites) and also helps them to resist the effectiveness of antibiotics by preventing it to reach individual bacteria. We sought to check the biofilm forming ability of the three categories of strains we recruited and found that the ST131 strains were superior in their ability to form biofilm. This was followed by non-ST131 strains. The faecal strains were weak biofilm formers compared to ST131 and non-ST131 strains. This indicates that biofilm formation could possibly contribute to their resilience and predominance that offers them an edge over other bacterial lineages; other studies have also shown that ST131 strains from global sources harbour virulence factors that potentially associate with *in vitro* biofilm formation (Naves et al 2008; Hancock et al 2008).

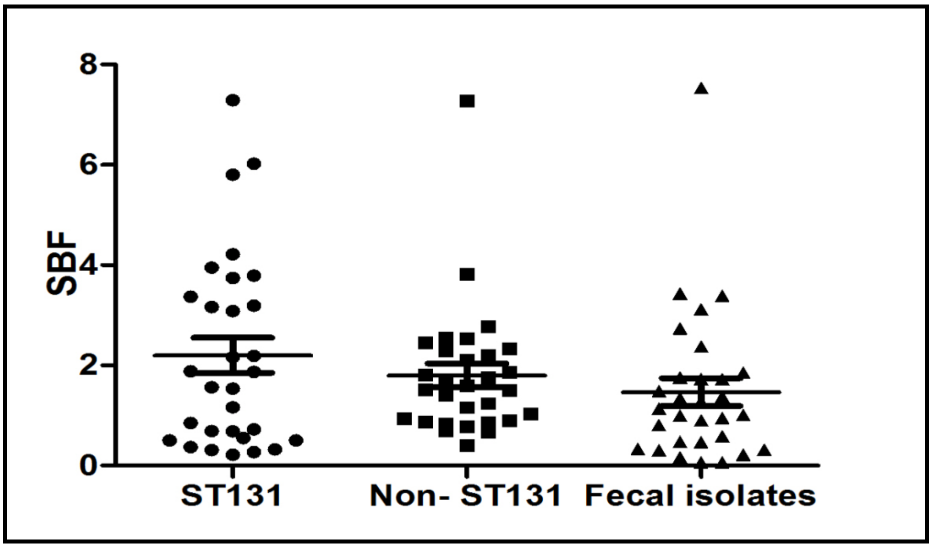

Figure 13: Comparison of specific biofilm formation capacity among ST131, non-ST131 and stool *E. coli* isolates. The mean value of each population is indicated by a dark line.

5.3.5 Zebra fish lethality assay

The zebra fish infection model is considered as a valuable tool to resolve diverse virulence phenotypes of closely related ExPEC strains. Therefore, to get insights into the virulence potential of ST131 strains and compare it with non-ST131 and stool *E. coli* isolates, we carried out an *in vivo* infection assay in zebra fish. In this study, we injected 10 µl of bacterial inoculum into the peritoneal cavity of zebra fish. Three bacterial strains per category were tested; each bacterial strain was injected into 4 replicates of fishes. The control fish was injected only with PBS. The control fish were all healthy and survived even after 5 days post injection. The ST131 strains were significantly virulent than the non-ST131 clinical and stool strains. Almost 90% of zebra fish injected with ST131 strains were killed within 24 hours post injection. The lethality of stool *E. coli* isolates was moderate followed by non-ST131 clinical isolates. Our data reveal that the ST131 strains were highly virulent in zebra fish. This observation is consistent with the presence of several classical virulence factors (such as *hlyA*, *cnf and aer*) in our ST131 isolates. Virulence in stool *E. coli* isolates could be accounted partly for the presence of *hlyA* and *aer* genes; in addition, production of other unknown toxins or virulence factors cannot be ruled out.

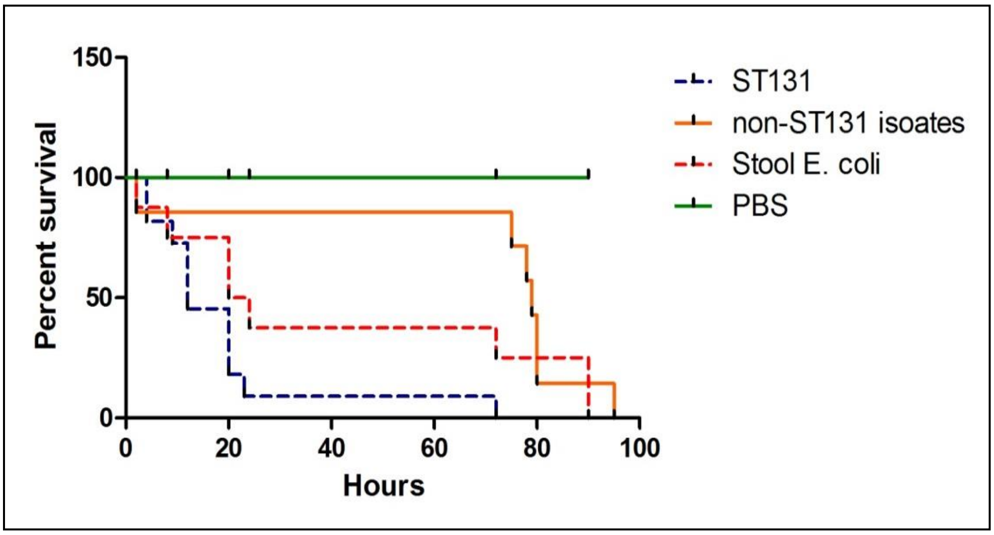

Figure 14: The survival rates of adult zebra fish infected with *E. coli* isolates belonging to 3 different categories. Each experimental group contained 12 zebra fish and each experiment was performed in duplicates.

5.3.6 Phylogenetic grouping and virulence gene identification

All the 126 strains (comprising 43 ST131 strains, 40 non-ST131 strains and 43 fecal strains) formed four distinct phylogenetic groups (A, B1, B2 and D) as shown in Table 8. Among the total population, group B2 was most prevalent phylogenetic group corresponding to 49% of the isolates followed in prevalence by other groups: A (27%), D (14%) and B1 (8%) (Table 7). The ST131 isolates were all from group B2 (accounting for 43 (70%) of the total 62 B2 isolates). Whereas, the non-ST131 and fecal isolates exhibited diverse phylogenetic groups with B2 and A being predominant, respectively. Although the non-ST131 isolates were likely to be from B2 group (38%) the phylogenetic group distribution was not significantly associated with CTX-M-15 positive and or CTX-M-15 negative isolates. In contrast, the fecal isolates were predominantly from group A, followed by groups D, B2 and B1. Overall, the distribution of isolates into phylogroups varied significantly among three groups of *E. coli* isolates (except for group D to which both non-ST131 and fecal isolates affiliated in equal proportion).

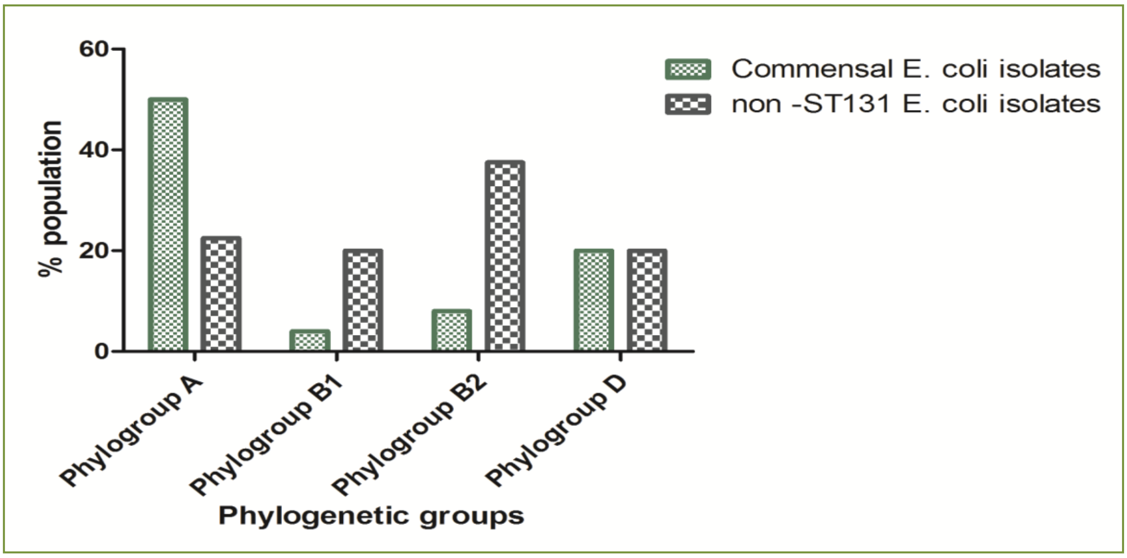

Figure 15: Phylogenetic groups among non-ST131 and stool *E. coli* isolates, all ST131 *E. coli* belonged to phylogroup B2.

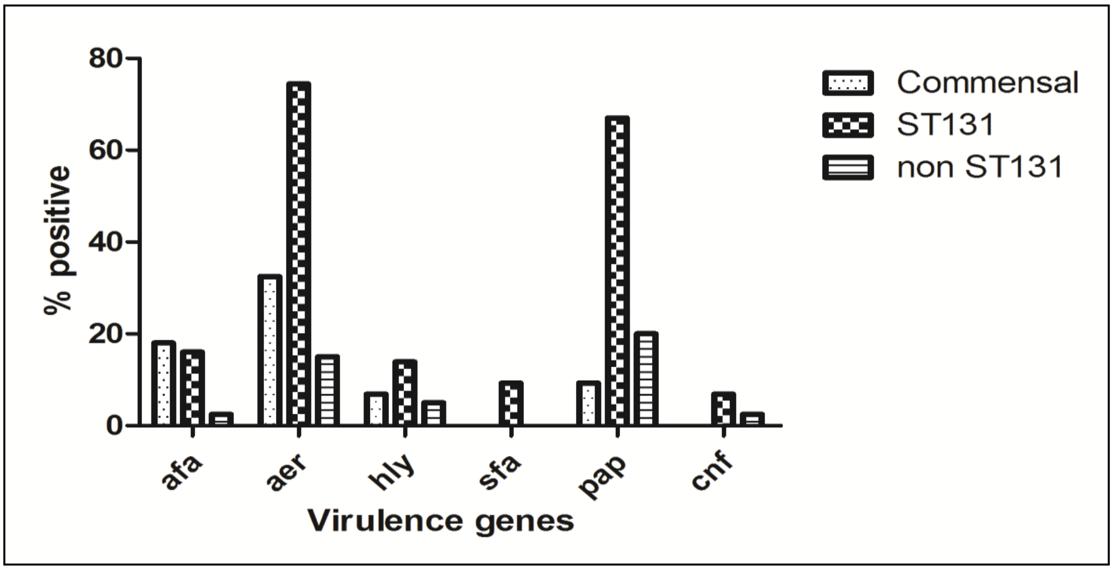

Figure 16: Virulence factors among ST131, non-ST131 and stool *E. coli* isolates.

Genotypes for 6 ExPEC–associated virulence markers were determined for all the 126 isolates comprising 3 groups as defined by ST131 type and clinical status. All the 6 virulence genes explored were at least detected in 1 isolate. Among the three groups, the median of virulence score and the virulence range values increased progressively from a high of 2 (0-5) for ST131 isolates to a low of 1 (0-1) for fecal isolates with the intermediate score and range of 1 (0-3) for non-ST131 isolates. The ST131 isolates harboured a slightly higher and discrete virulence profile than that of non-ST131 and fecal isolates (Table 8). In the ST131 group, an aggregate of 52 isolates was positive for the 6 different virulence markers, whereas there were 25 and 29 positive isolates within non-ST131 and fecal groups. Particularly, the *sfa/foc* (encoding S fimbria and FIC fimbriae) and *pap* (encoding type 1 fimbrial adhesin) were predominant in ST131 strains. Coinciding with greater prevalence of a few ExPEC–associated virulence markers among ST131 isolates, a significant proportion of ST131 isolates (58%) than non-ST131 and fecal isolates (5% each) were technically falling in ExPEC category. Moreover, among the ST131 isolates, presence of CTX-M-15 was significantly associated with ExPEC status (98%).

5.3.7 Metabolic profiling

To find an alternative explanation for the success of prevalent ST131 *E. coli* we compared the carbohydrate utilization among the three categories of *E. coli*. 35 API test reagents were used to perform metabolic profiling (carbohydrate utilization) on 6 ST131, 6 clinical non-ST131 and 6 stool non-ST131 *E. coli*. The aggregate utilization percentages for ST131, non-ST131 and stool non-ST131 *E. coli* were 53%, 52% and 49%, respectively. Although not statistically significant, the Bioscore of ST131 was slightly higher than the non-ST131 *E. coli*; similarly none of the biochemical traits was significantly associated with ST131 clones. However, they exhibited a fair trend in their ability to utilize two substrates: salicin and sorbose.

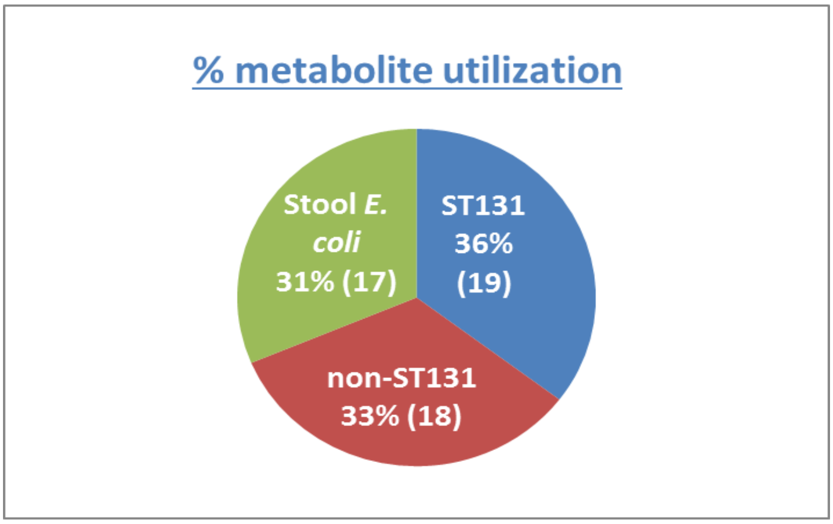

Figure 17: Aggregate Bio-scores for utilization of 35 different carbon sources, among ST131, non-ST131 and fecal *E. coli*.

4.3.8 Genotyping

ERIC-PCR based dendrogram of 126 *E. coli* isolates could be broadly classified into three clusters as defined by the ability of same category of strains to cluster together, although the three categories did not form distinct clusters with respect to ST131 and or clinical status. Nevertheless, the ST131 strains were more clonal and genetically similar followed by non-ST131strains and then fecal strains. These observations confirm our previous results that the ST131 strains are evolving clonally whereas the non-ST131fecal isolates were genetically diverse with non-ST131 clinical strains being intermediate in their genetic heterogeneity.

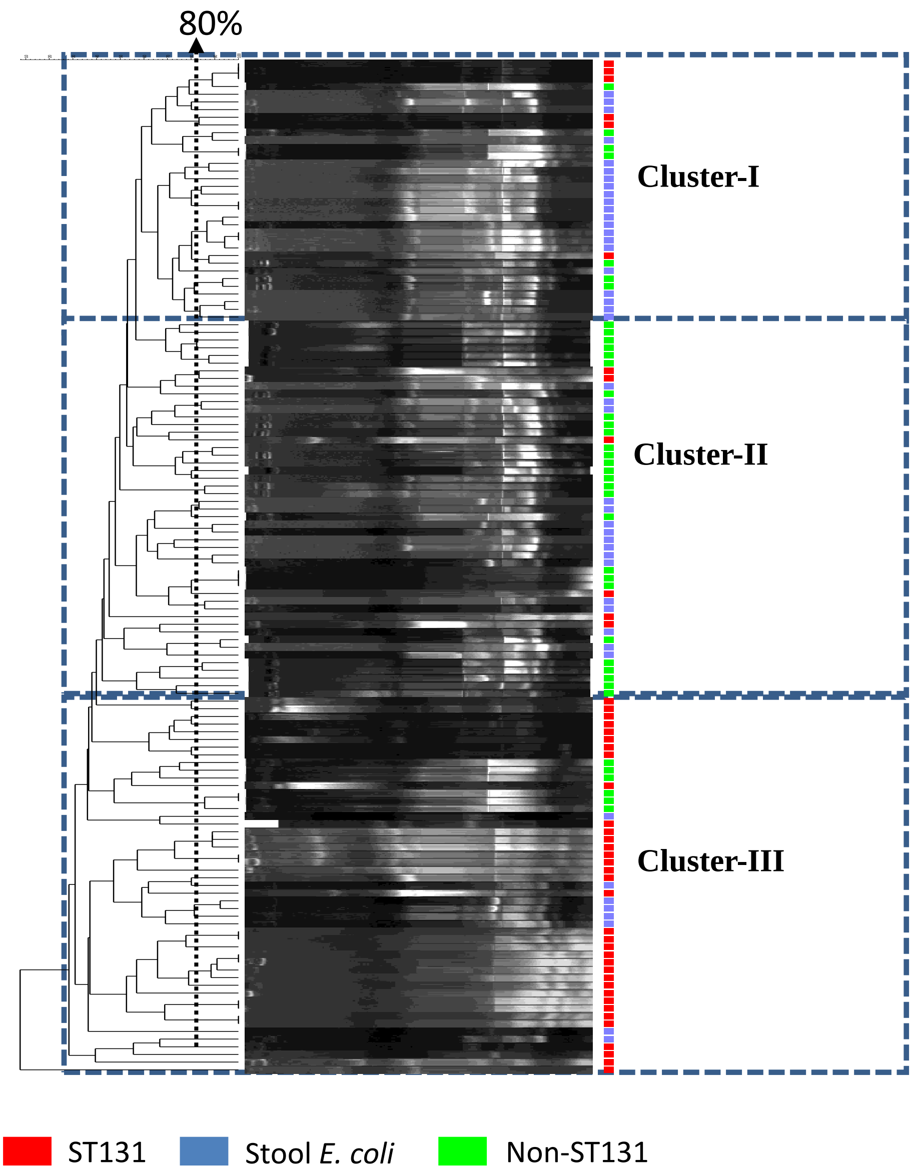

Figure 18: ERIC-PCR based dendrogram of 132 *E. coli* isolates comprising 47 ST131, 41non-ST131 and 43 stool *E. coli* isolates as produced by UPGMA algorithm based on Dice similarity coefficients. Cluster I harbours maximum stool *E. coli* isolates, cluster II mainly comprises non-ST131 clinical isolates and cluster III is mainly populated with ST131 isolates.

Chapter 6
Summary and epilogue

6.1 Summary and epilogue

ExPEC are of significant health concern. The emergence of drug resistant *E. coli* with high virulence potential is alarming. Lack of sufficient data on transmission dynamics, virulence spectrum and antimicrobial resistance of certain pathogens such as the UPEC from countries with high infection burden, such as India, hinders the infection control and management efforts. To begin with, we extensively genotyped and phenotyped a collection of 150 UPEC obtained from patients belonging to a semi-urban, industrialized setting near Pune, India. The isolates representing different clinical categories were analyzed in comparison with 50 commensal *E. coli* isolates from India as well as 50 ExPEC strains from a German collection. Virulent strains were identified based on the presence of six extraintestinal virulence factors. We generated antimicrobial resistance profiles for all the clinical isolates and carried out phylogenetic analysis based on a triplex-PCR. *E. coli* from urinary tract infection cases expressed higher percentage of virulence score and multiple virulence genes. Among the six genes, ExPEC associated virulence factors; *pap, afa* and *cnf* were strongly associated with UPEC isolates in comparison to the fecal isolates suggesting an important role in the etiology of UTI. Phylogenetic group B2 was seen to be strongly associated with clinical UPEC strains, that is, 54% of clinical population belonged to phylogroup B2. In contrast, only 11% of stool isolates were affiliated to B2 phylogroup indicating that the B2 group is uncommon among stool *E. coli* isolates in India. Moreover, clinical isolates exhibited highest resistance against amoxicillin (67.3%) and least against nitrofurantoin (57.3%). We also observed that 31.3% of UPEC were extended-spectrum beta-lactamase (ESBL) producers belonging to serotype O25, of which four were also positive for O25b subgroup that is linked to B2-O25b-ST131-CTX-M-15 virulent/multiresistant type.

E. coli sequence type 131 (O25b:H4), associated with the CTX-M-15 extended-spectrum-beta-lactamases (ESBLs), and linked predominantly to the community-onset antimicrobial-resistant infections, has globally emerged as a public health concern. However, scant attention is given to the understanding of the molecular epidemiology of these strains in high-burden countries such as India. Of the 100 clinical *E. coli* isolates obtained by us from a setting where urinary tract infections are endemic, 16 ST131 *E. coli* isolates were identified by multilocus sequence typing (MLST). Further, genotyping and phenotyping methods were employed to characterize their virulence and drug resistance patterns. All the 16 ST131 isolates harboured CTX-M-15 gene, and half of them also carried TEM-1; 11 of these were positive for bla_{OXA} groups 1 and 12 for *aac(6)Ib-cr*. At least 12 isolates were refractory to four non-beta-lactam antibiotics: ciprofloxacin, gentamicin, sulfamethoxazole-trimethoprim, and tetracycline. Nine isolates carried the class 1 integron. Plasmid analysis indicated a large pool of up to six plasmids per strain with a mean of approximately three plasmids. Conjugation and PCR-based replicon typing (PBRT) revealed that the spread of resistance was associated with the FIA incompatibility group of plasmids. Pulsed-field gel electrophoresis (PFGE) and genotyping of the virulence genes showed a low level of diversity among these strains. The association of ESBL-encoding plasmid with virulence was demonstrated in transconjugants by serum assay. None of the 16 ST131 ESBL-producing *E. coli* strains were known to synthesize carbapenemase enzymes. Our study reports a snapshot of the highly virulent/multiresistant clone ST131 of uropathogenic *E. coli* from India. Further the ST131 genotypes from this region are clonally evolved and are strongly associated with the CTX-M-15 enzyme, carry a high antibiotic resistance background, and have emerged as an important cause of community-acquired urinary tract infections. We performed complete in-depth sequencing, annotation, and analysis of the genome of

UPEC strain NA114 which is a prototype ST131strain that was originally obtained from the urine of a 70-year-old male patient with prostatitis. Several virulence genes, including *iha, sat, fimH, kpsM, iutA,* and *malX*, which correspond to the genes of *E. coli* CFT073 were identified and annotated in the genome. In addition, genes corresponding to another UPEC strain UTI89 such as *fyuA* and *usp* were detected in the genome of UPEC strain NA114.

The pandemic dissemination of the *E. coli* strains with a highly virulent, multidrug-resistant phenotype laced with CTX-M-15 extended-spectrum β-lactamase (ESBL) plus sequence type 131 (ST131) has become a major global health concern. The virulence, antimicrobial resistance and genetic fingerprints of ST131 isolates differ from those of non-ST131 and fecal isolates. For this, 126 *E. coli* isolates that constituted 3 groups of random isolates per group [(i) 43 ST131 *E. coli*; (ii) 40 non-ST131 *E. coli*; (iii) 43 fecal *E. coli*] collected from a major tertiary hospital in Pune (January 2009-March 2011) were analyzed. All isolates were screened for *pabB* gene specific for ST131 clones. Isolates were phlyogrouped by multiplex PCR and screened by PCR for 6 different ExPEC-associated virulence factors. All isolates were typed by ERIC-PCR based fingerprinting to establish genetic relatedness. Out of the 126 isolates analysed, 43 were identified as ST131 type and 83 were non-ST131 (40 clinical non-ST131 strains and 43 non-ST131 fecal isolates). Resistance to all the 6 antimicrobials was highest among the ST131 isolates (35%) and lower among fecal isolates (14%). Resistance rates with respect to individual antibiotics were more or less similar among the three groups. CTX-M-15 was strongly associated with ESBL phenotype among ST131 and fecal isolates (100%). It was 84% among non-ST131 isolates. A total of 25 (20%) from 126 isolates were metallo beta-lactamase (MBL) positive. The ST131 isolates harboured a slightly higher and discrete virulence profile than that of the non-ST131 and fecal isolates with the median and virulence ranges of 2(0-5) for ST131, 1 (0-3) for non-ST131 and 1 (0-1) for fecal *E. coli* isolates. Majority of the ST131 clones clustered together by ERIC fingerprinting with ≥ 60% similarity and few out-groups. The non-ST131 and fecal isolates often formed co-clusters with ≥ 60% similarity. We did not find any significant association of antimicrobial resistance, virulence profiles and metabolic potential with ST131 lineage that could play an important role in the fitness of this clone in comparison to other *E. coli* strains. Nevertheless, the ST131 clones were moderately resistant and virulent and were metabolically slightly superior when compared to the other two categories of *E. coli* isolates. These traits dissected by us, along with other unknown factors might have contributed to the success of ST131 clone in becoming a significant human pathogen implicated in most of the UTI infections globally.

Given these findings, it will be appropriate to propose for the development of a fool proof knowledgebase about the functional molecular infection epidemiology of UPEC based on the initial snapshot of the characteristics and genotypes of Indian strains. In future, it will be possible to decipher the population genetics by employing whole genome sequencing and comparative analysis of some of the potentially emerging isolates from our collection. Whole genome analysis of such strains would also explain the biological basis for their successful parasitism as a function of their high infection rates and ability to disseminate globally. Comparative analysis of such clinically relevant strains in juxtaposition with commensal strains would help in identification of molecular targets useful in typing/differentiating such strains. Moreover, observations emanating from such studies could define potential vaccine candidates.

On the other hand, these well characterized isolates could be possibly harnessed further to study their infection biology. Although, *E. coli* are increasingly implicated in severe sepsis related cases, which is basically due the phenomenon of urosepsis (entry of *E. coli* in systemic blood circulation through inflammation of kidneys), there are very sparse data on the molecular basis of *E. coli* induced sepsis, despite, it's etiological role being well established. Having this said, it will be pertinent to further characterize these strains by *in vitro* and *in vivo* infection experiments and to dissect out the infection responses. This way, we could perhaps identify biomarkers of *E. coli* induced sepsis, and also could discover the novel molecular determinants of pathogenesis (sepsis) other than the bacterial lipopolysaccharides.

Appendix i

Epidemiological setting, study population and clinical material

Patient Demographics and *E. coli* Strain Isolation in Pure Culture

India has a large infection burden and the genito-urinary infections are very prominent. This may be due to less affordable personal and or community hygiene for some of the economically backward populations. We targeted mostly these groups and analyzed strains obtained from such communities who reported for the UTI in Periurban Pune. As such there is not much information available from India on the genetic and phenotypic diversity of UPEC (Jadhav et al. 2011). Moreover, the epidemiology of multivirulet/multi resistant strains in Asian countries has been inferred mostly from studies of returned travelers, no data is available on the epidemiology of these strains from developing nations particularly from India. This reflects the need for investigating the epidemiological significance of the pathogens which is achieved by having a full understanding of the population biology of the UPEC strains (Pitout et al. 2009). Therefore, this information should therefore be construed as the first systematic analysis performed on a diverse type of patients/samples.

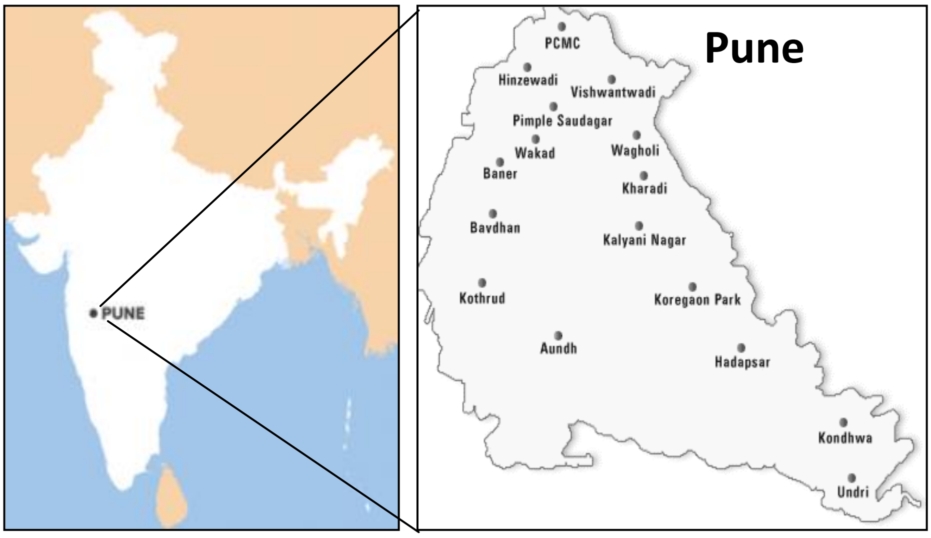

Figure 19: Urinary tract infections cases are common among adults and paediatrics residing in the reporting city of India: We carried out a retrospective analysis of *E. coli* isolates causing UTI, obtained from two hospitals between Jan 2009- Dec 2011.

Pune is an eighth largest metropolis. It is located in a periurban plane also many hospital reports indicate that UTI are endemic in this region (Jadhav et al. 2011, Hussain et al. 2012) and therefore represents one of the best settings to carry out the retrospective analysis of UTI infections; we have our collections centres in two of the hospitals in Pune. The study population was drawn in from two settings, one in the microbiology laboratory at a major tertiary hospital Dr. D.Y. Patil Medical Hospital in Pune and from another microbiology laboratory from Bharath Hospital, Pune, India.

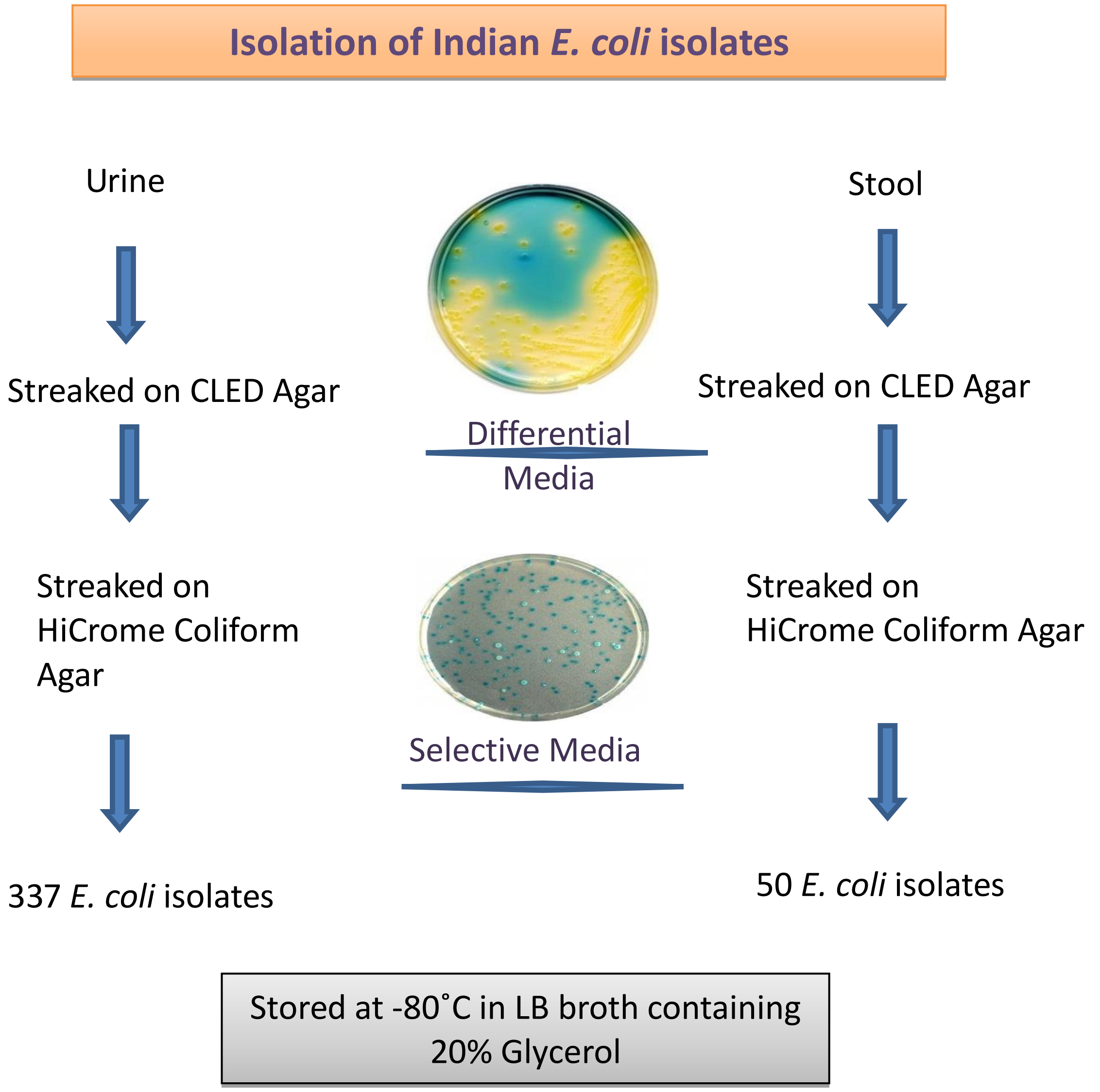

Figure 20: General procedure followed to isolate UPEC and stool *E. coli* isolates, the samples are collected in different batches as described in the respective chapters.

In total 337 UPEC isolates and 50 stool *E. coli* isolates were collected in different batches Of these 337 isolates, 134 were recovered between Jan 2009- Dec 2009 and 170 isolates being recovered in 2010 another 33 isolates were collected in 2011 and all the 50 stool isolates *E. coli* were recovered in 2009 from healthy individuals who provided samples for routine health check-ups. Out of 337 *E. coli* isolates 143 were from male patients with a mean age of 42 years whereas 194 out of 337 were from female patients with a mean age of 37 years.

In order to minimize the possibility of collecting non-UPEC strains, the selection was limited to urine samples that showed pure culture with a significant total count (10^5 CFU/ml). Urine samples were plated onto CLED agar plates (Oxoid, Ltd, UK), which were incubated at 37˚C for 18 hrs in a normal atmosphere. To maximize the chances of collecting *E. coli*, all lactose fermenting strains were further examined using chromogenic media Chrom *E. coli* agar (Himedia) as per manufacturer's directions, on which *E. coli* are identified based on the ability to produce β–glucuronidase (*Escherichia coli*: luxuriant blue, *Klebsiella pneumonia:* luxuriant colourless, *Salmonella enteritidis*: luxuriant colourless). Single blue colony (*E. coli*) was picked and then streaked on EMB agar to conform its identity on the basis of formation of green metallic sheen. The *E. coli* pure cultures were finally stored in -80 in LB broth containing 20 % glycerol.

This study included all those patients for whom UTI was suspected and were recommended for urine culture, both pediatrics and adults patients of both sexes were included and both inpatients and out patients were included. Information on patient's age, gender and the provisional diagnosis report was obtained along with sample.

Table 8: 58 ExPEC DNA samples from various animal and human specimens from global sources.

German DNA samples	
Pathotypes	**No. of isolates**
Human UPEC	24
Dog UPEC	2
Meningitis (Human)	1
Avian Pathogenic *E. coli*	31
Total	58

In addition to the Indian UPEC samples, DNA samples of fifty eight ExPEC isolates from Germany and from global sources archived at the Institute of Microbiology and Epizootics (IMT), Free University Berlin; were employed in this study, these isolates represented various animal and human specimens, including 24 UPEC and one archetypical new-born meningitis strain RS218 from humans, two UPEC from dogs and 31 avian pathogenic *E. coli* (APEC) from different birds. These DNA samples have been employed in strain genotyping studies.

The general procedure and the total number of *E. coli* isolated from urine and stool samples has been described above, the exact number and category of *E. coli* employed in each study has been described separately in the material and methods section of respective chapters.

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Virulence Characteristics and Genetic Affinities of Multiple Drug Resistant Uropathogenic *Escherichia coli* from a Semi Urban Locality in India

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Abstract

Extraintestinal pathogenic *Escherichia coli* (ExPEC) are of significant health concern. The emergence of drug resistant *E. coli* with high virulence potential is alarming. Lack of sufficient data on transmission dynamics, virulence spectrum and antimicrobial resistance of certain pathogens such as the uropathogenic *E. coli* (UPEC) from countries with high infection burden, such as India, hinders the infection control and management efforts. In this study, we extensively genotyped and phenotyped a collection of 150 UPEC obtained from patients belonging to a semi-urban, industrialized setting near Pune, India. The isolates representing different clinical categories were analyzed in comparison with 50 commensal *E. coli* isolates from India as well as 50 ExPEC strains from Germany. Virulent strains were identified based on hemolysis, haemagglutination, cell surface hydrophobicity, serum bactericidal activity as well as with the help of O serotyping. We generated antimicrobial resistance profiles for all the clinical isolates and carried out phylogenetic analysis based on repetitive extragenic palindromic (rep)-PCR. *E. coli* from urinary tract infection cases expressed higher percentages of type I (45%) and P fimbriae (40%) when compared to fecal isolates (25% and 8% respectively). Hemolytic group comprised of 60% of UPEC and only 2% of *E. coli* from feces. Additionally, we found that serum resistance and cell surface hydrophobicity were not significantly ($p = 0.16/p = 0.51$) associated with UPEC from clinical cases. Moreover, clinical isolates exhibited highest resistance against amoxicillin (67.3%) and least against nitrofurantoin (57.3%). We also observed that 31.3% of UPEC were extended-spectrum beta-lactamase (ESBL) producers belonging to serotype O25, of which four were also positive for O25b subgroup that is linked to B2-O25b-ST131-CTX-M-15 virulent/multiresistant type. Furthermore, isolates from India and Germany (as well as global sources) were found to be genetically distinct with no evidence to espouse expansion of *E. coli* from India to the west or *vice-versa*.

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Introduction

Urinary tract infections (UTI) are the second most common human infections and are mainly caused by uropathogenic *E. coli*. The severity of UTI depends both on the virulence of the bacteria and the susceptibility of the host [1]. UPEC harbor numerous virulence factors including alpha-hemolysin, cytotoxic necrotizing factor, adhesins and iron acquisition systems. These factors support their ability to adhere to uroepethelial cells, help resist the bactericidal effect of serum and augment cell surface hydrophobicity thereby leading ultimately to tissue damage [2,3,4,5].

Adherence to the urinary tract mucosa might protect bacteria from urinary lavage and in turn augment their ability to survive and invade renal tissues [6]. Specific adhesion is mediated by certain adhesins which can be differentiated based on their receptor binding specificity.

P fimbriae that are encoded by the *E. coli pap* (pyelonephritis-associated pilus) operon are the most important mannose-resistant adhesins, although they are expressed by only a limited number of *E. coli* serotypes. The most rampant P-fimbriated serotypes of the UPEC strains revealed one of the six O groups O1, O2, O4, O6, O7, and O18 [6,7,8].

Hemolysin production is another important virulence property of UPEC. Hemolysins inflict direct cytotoxic effects on renal epithelium resulting in scaring. Alpha-hemolysin is described to be a lethal factor with dermonecrotic effects and is antigenic in nature. Also, hemolysins are toxic to a series of host tissues and cells including RBCs, leucocytes, epithelial and endothelial cells. The frequency of isolation of hemolytic *E. coli* significantly associates with the severity of the infection [7,8].

The importance of cell surface hydrophobicity as a virulence attribute (that facilitates bacterial adherence to mammalian cells) is known for nearly a century now, thanks to the pioneering studies of Mudd and Mudd [4]. It is an important factor helping *E. coli* to adhere to various surfaces for colonization. Bacteria are lysed by normal serum due to the activity of the complement system. The alternate pathway of complement activation is potentially important than the classical pathway. Bacterial resistance to killing by serum results from individual or combined effects of capsular polysaccharides, lipopolysaccharides and surface proteins [4]. Given this generic virulence 'arsenal' of UPEC, strains from different geographical regions pose different disease severity and should be genetically different.

To screen for the above mentioned virulence attributes and to find the most predominant serotypes among UPEC in the western Indian region, we analyzed 150 human clinical isolates. We intended to classify them epidemiologically into different serotypes and to obtain virulence marker profiles of UPEC (which have different frequencies in different disease conditions ranging from asymptomatic bacteriuria to chronic pyelonephritis). In addition, increasing antimicrobial resistance in bacterial pathogens is of major concern as it can vary according to geographical and regional situations [9,10,11,12]. It is very relevant to ensure the appropriate therapy based on full knowledge of the organisms that cause UTI and their antibiotic susceptibility profiles. Therefore, it is necessary to do bacteriological testing also with reference to extended-spectrum beta-lactamase (ESBL) producers with resistance to beta-lactam antibiotics, including third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. Not much information on ESBL producing organisms causing UTI is available from India.

The present work was essentially carried out as an important precursor of a larger study aimed at understanding the transmission dynamics, population genetic structure and virulence mechanisms of UPEC from India. Herein, we present a much needed snapshot comprising of the virulence characteristics and antimicrobial resistance patterns of the available UPEC strains from western India representing the clinical conditions such as symptomatic UTI, bacteriuria, pyelonephritis, cystitis, prostatitis, septicemia and pyrexia of unknown origin (PUO). We sought to define the clinical correlates of different virulence factors and their association with various biological and life-style related factors of the host. In addition, we used rep-PCR based DNA profiling to know the genetic affinities of our isolates and to explore if this simple PCR based genetic analysis helps in understanding their spread patterns indicative of their diverse or clonal relationships. Also, we attempted to use this technique to know whether the bacterial isolates have any geographic inclination and how similar or different they are when compared to some of the well characterized isolates from western countries such as Germany.

Materials and Methods

Ethics statement

Written informed consents were obtained from all patients and healthy controls for the use of their strains which were cultured as part of compulsory diagnostic screening. Study protocols were approved by the institutional ethics committee of Dr. D. Y. Patil Medical College, Pune and by the Institutional Biosafety Committee of the School of Life Sciences of the University of Hyderabad, India.

Study population and strains

One hundred and fifty *E. coli* isolates were obtained from urine samples of human patients in cell counts of 10^5/ml. Fifty fecal *E. coli* isolates (that were used as controls), were isolated from the feces of healthy individuals who had reported for routine health checkup between January 2009–March 2010 at Dr. D. Y. Patil Medical College and Hospital, Pimpri, Pune, India. Patient background and provisional diagnosis of the infection were obtained from hospital records (Table S1). Identification of isolates was done using standard microbiological techniques [13,14,15]. All strains were stored on 15% glycerol-supplemented Luria-Bertani medium at $-80°$C.

DNA samples

DNA samples of fifty ExPEC isolates from Germany and from global sources [16] were obtained; these represented various animal and human specimens, including 17 UPEC and archetypical newborn meningitis strain RS218 from humans, two UPEC from dogs and 31 avian pathogenic *E. coli* (APEC) from different birds. These strains were used for repetitive extragenic palindromic (rep) PCR together with the aforementioned human clinical isolates.

Antimicrobial susceptibility test

The antimicrobial susceptibility testing was carried out on Mueller Hinton agar by disc diffusion method using the following antimicrobial substances [17]: amoxicillin (10 µg), ceftazidime (30 µg), ciprofloxacin (10 µg), co-trimoxazole (25 µg), gentamicin (10 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), and tetracycline (30 µg). Multi-drug resistant (MDR) strains were defined as those which showed resistance to three or more antimicrobial substances. Extended-spectrum beta-lactamase (ESBL) production was detected by the double-disk synergy (DDS) test as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Its presence was assayed using the antibiotic disks comprising of ceftazidime and ceftazidime/clavulanic acid (30/10 µg). An isolate was graded ESBL producer when its zone of inhibition varied by $\geq$5 mm among at least one of the combination disks and its coordinate comprising of standard antibiotic disk. *E. coli* ATCC 25922 was used as a negative control while *K. pneumoniae* ssp. *pneumoniae* ATCC 700603 served as positive control [18,19].

Phenotypic assays to determine virulence factors

a) Alpha-hemolysin production. The detection of alpha-hemolysin was performed by analysing the hemolytic zone observed after overnight growth at 37°C on sheep blood (5%) agar [20].

b) Haemagglutination. A suspension of human A +ve blood and PBS was mixed on a VDRL (venereal diseases research laboratory - test) cavity slide with a single colony of *E. coli*. After incubation on rotor at room temperature for some minutes, agglutination was seen. Similarly, haemagglutination was carried out in the presence of D-mannose. An ATCC *E. coli* 25922 strain was used as a negative control for mannose sensitive haemagglutination assay and a known strain of *E. coli* repeatedly giving positive was taken as control for the assay [21].

c) Cell Surface Hydrophobicity. Bacteria were tested for their hydrophobic property by using different molar concentrations of ammonium sulphate in VDRL tile; 40 µl of bacterial suspension in PBS was added in each of the wells containing 1 M, 1.4 M and 2 M ammonium sulphate. Clumps were seen by naked eyes. Strains were considered hydrophobic, if they aggregated in the PBS concentration of $\leq$1.4 M [21].

d) Serum Bactericidal Assay. Bacteria were diluted in Hank's balanced salt solution to get appropriate dilutions and mixed with human serum in a sterile tube and incubated in a water bath at 37°C and inoculated on nutrient agar plates at 0 hr, 1 hr, 2 hr of incubation in water bath. Growth at 0 hr was taken as control. *E. coli* was considered sensitive if count dropped to 1% and was considered resistant if >90% of the organisms survived after 2 hrs of incubation. An *E. coli* isolate that was consistently serum resistant and a one which was consistently sensitive were used as positive and negative controls respectively [22].

Serotyping

Typing of somatic antigens was performed at the National Salmonella and *Escherichia* Centre, Central Research Institute, Kasauli, India, using antisera against O antigens - O1 to O173. O25 positive *E. coli* strains were further subjected to genotyping by a recent method based on allele-specific PCR targeting the *rfb*O25b subgroup gene locus [23].

Repetive extragenic palindromic PCR

DNA sequences of primers used for Rep - fingerprinting were as follows: Forward Rep2a2: 5′-ACGGCTTATCGGGCCTA-3′, Reverse Rep1Ra1:5′-GCGACGGCATCAGGC-3′. PCR amplification was carried out in 20 µl reaction mixture consisting of 10× Taq buffer, 25 mM $MgCl_2$, 10 mM dNTP, 100% DMSO, 100 pM of each primer and 2 U of Taq DNA polymerase and 5 µl of template. The PCR conditions were 95°C for 7 min, followed by 30 cycles of DNA amplification consisting of 45 s at 95°C, 1 min at 40°C and 8 min at 65°C followed by 16 min incubation at 65°C. The amplicons were run on 1.5% agarose gels and were analyzed by Bionumerics® software [24,25].

Detection of O25b subgroup strain by PCR

The newly described O25b O type *E. coli* were detected by using the following primers gndbis.f (5′ATACCGACGACGCC-GATCTG-3′) and rfbO25b.r (5′TGCTATTCATTATGCG-CAGC-3′). Annealing temperature of 60°C was used to generate a PCR product of 300 bp with the conditions as previously described [23].

Statistical Analysis

Chi square test was used to compare the occurrence of virulence markers in cases and controls. P values less than 0.05 was considered significant.

Results and Discussion

Incidence of UTI in relationship with gender and socioeconomic status

In this study we observed a higher proportion of UTI in females (64%) than in males (36%). This is understandable due to the anatomy and is a consistent trend worldwide. Peak in the incidence of UTI was observed in the age groups 11–21 and 60–71 years. Among these, elderly patients are likely predisposed to conditions such as urinary tract obstruction, poor bladder emptying, and diabetes mellitus, etc. These factors favor colonization of bacteria and play an important role in UTI. Other studies have also reported similar findings [26,27]. India has a large infection burden and the genito-urinary infections are very prominent. This may be due to a less affordable personal/ community hygiene for some of the economically backword populations. We targeted these groups and analyzed strains obtained from such communities who reported for the UTI in periurban Pune. As such there is not much information available from India on the genetic and phenotypic diversity of UPEC. This information should therefore be construed as the first systematic analysis performed on a diverse type of patients/samples.

Virulence characteristics of UPEC from India

a) Type 1 (MSHA) and P fimbriae (MRHA). In the present study, 45% of the UTI isolates and 25% of fecal isolates showed the presence of type 1 (Mannose sensitive hemagglutination - MSHA) fimbriae. Thus the difference among UTI and fecal isolates was not statistically significant. It was found that 40% of UTI isolates and 8% of *E. coli* isolates from the controls exhibited P fimbriae (Mannose resistant hemagglutination -MRHA). The difference was statistically significant (P = 0.0002). Expression of type 1 fimbriae was more discerned in isolates from the cases of simple UTI and cystitis. In our observation, maximum P fimbriae positive isolates were highly associated with simple UTI, while only 10% of such isolates caused pyelonephritis. As such there is no relation among the occurrence or severity of symptoms/the site of infection and fimbrial expression (of P or type1) in case of bacterial isolates from urine [28].

Our observation was in agreement with another study [29], wherein the presence of type 1 fimbriae was seen in 71% of isolates of *E. coli* from UTI and in 60% of isolates of *E. coli* from the control fecal flora. Taken together, UTI associated isolates did not show any significant expression of MSHA compared to controls; however, Najar *et al.* (2007) [29] showed an overall higher expression of type 1 fimbriae in both the UTI isolates and controls. Many studies have demonstrated the presence of P fimbriae as maximum in UTI isolates than in fecal isolates of healthy persons [29,30].

Although, since we looked only at phenotypes of the isolates *in vitro*, it is difficult to dwell upon the real significance of the fimbrial expression patterns *vis a vis* the niche tropism (upper or lower UTI) and its impact on different outcomes of infection – these are largely the phenomena due to microbial mechanisms operating *in vivo*. Normally, the expression of P fimbriae is increased among isolates associated with lower urinary tract infections, basically resembling an '*in vivo*' situation as there is an interplay between type1-fimbriae and P-fimbriae expression ("switch-off" of Type 1 fimbriae leads to increased motility of bacteria and finally to binding of P-fimbrial adhesin to receptors on kidney epithelial cells). The higher or lower expression itself might therefore only give an idea about the quantitative occurrence of P fimbriae, but not about the different adhesion subunits which would be more relevant with respect to upper/lower UTI infections.

b) Hemolysin. Production of alpha hemolysin was observed in 60% of UPEC from the cases while only 2% of *E. coli* isolates from feces produced alpha hemolysin (P = 0.0001). Other studies have also reported similar findings [31]. In our observation, hemolysin was highest in pyelonephritis, lower in cystitis and least in asymptomatic bacteriuria.

c) Serum resistance and cell surface hydrophobicity. Serum resistance and cell surface hydrophobicity markers were not significantly present in *E. coli* isolates from patients compared to control fecal isolates: 55% and 76% of urinary *E. coli* isolates showed serum resistance and cell surface hydrophobicity respectively and the control isolates showed 40% and 70% for serum resistance and cell surface hydrophobicity respectively.

The difference in both the cases was not statistically significant (p = 0.16/p = 0.51). This trend has been reported earlier and our study confirmed the same for Indian isolates [32].

Occurrence of virulence factors in UPEC strains confirms the association of UPEC with urinary pathogenicity. It was interesting

Table 1. Occurrence of virulence phenotypes in *E. coli* isolates from cases and controls.

Virulence phenotypes	Number of isolates from cases (%) total cases = 150	Number of isolates from controls (%) total controls = 50	P-value
MRHA	60 (40.0)	4 (8.0)	0.0002
MSHA	68 (45.3)	12 (24.0)	0.406
Hemolysin	90 (60.0)	1 (2.0)	0.00001
SR	83 (55.3)	20 (40.0)	0.16
CSH	120 (80.0)	35 (70.0)	0.51

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to note that UPEC with virulence factors were significantly more prevalent in patient groups than in controls (Table 1). Such a significant difference was noticed earlier, indicating that several virulence factors act synergistically to cause infection of the urinary tract [33]. Nevertheless, it was interesting that 8% of the isolates (12/150) from infection cases did not reveal any virulence markers under investigation. These were the isolates from complicated UTI. It appears that these isolates, though devoid of any virulence factors as probed by us, were able to localize in the deeper tissues of a compromised urinary tract where scarcely any drug reaches in therapeutic quantum. It may also be due to the reason that they might possess virulence markers/mechanisms other than those tested by us.

We screened 19 mucoid UPEC isolates by phenotypic and biochemical characterization and found that all were from patient groups and were capsulated. Out of the 19 mucoid UPEC, 11 were capsulated and serum resistant. Capsule confers serum and phagocyte resistance and this could be attributed to sialic acid residues that subvert the ability of bacterial surface to activate complement by alternative pathway thereby augmenting the virulence potential of such pathogens. However, eight of the mucoid strains were susceptible to serum, perhaps due to the non capsular factors and this might have a role in serum resistance.

Antimicrobial susceptibility profiles of UPEC

Antimicrobial susceptibility testing was carried out on all the clinical isolates. The majority of isolates were sensitive to nitrofurantoin - 86 (57.3%) followed by ciprofloxacin - 78 (52%) and nalidixic acid - 74 (49%). This study showed high resistance to amoxicillin - 101 (67.3%), tetracycline - 92 (61.3%), and cefotaxime - 68 (45.3%) (Figure 1). Upon testing for ESBL, we found that 32 (21.3%) were ESBL producers. A high proportion (31.3%) of these ESBL-producing isolates was belonging to the serotype O25. Four of these were positively tested for the subgroup O25b (Figure 2), which has been linked to a clonally related group of highly virulent, multiresistant *E. coli* strains (B2-O25b-ST131-CTX-M-15) that are emerging among humans and animals worldwide [34,35]. Further studies, i.e. multi locus sequence typing (MLST), macrorestriction analysis, and determination of beta-lactamase enzymes are needed to unravel the phylogenetic

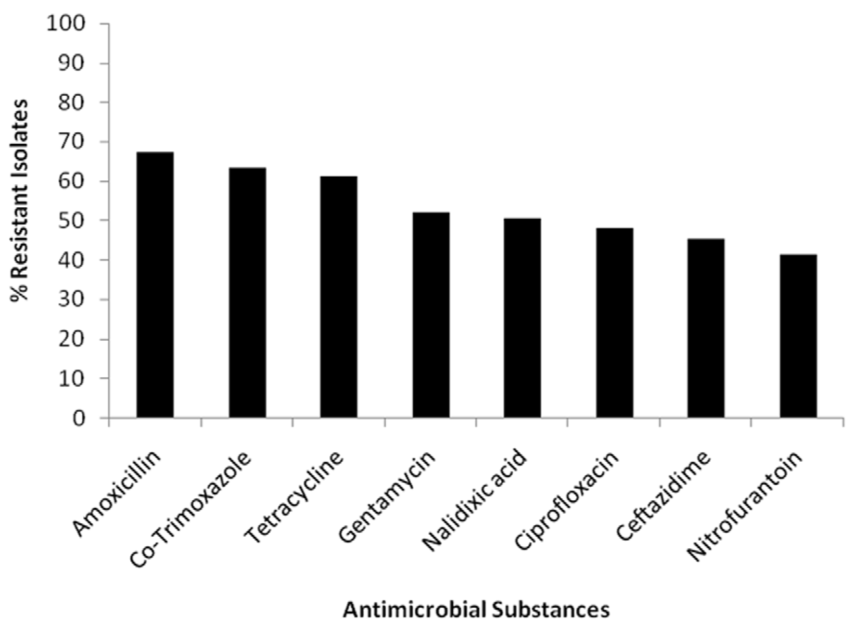

Figure 1. The antimicrobial susceptibility and resistance pattern of 150 UTI isolates from India. Nitrofurantoin was consistently the most active (59%) of the systemically active antimicrobials, with ceftazidime, giving similar results (54.7%). These were followed by ciprofloxacin (52%), nalidixic acid (49%) and gentamicin (48%), but sensitivity to amoxicillin was found to be low (32.7%). Sensivity profiles of Co-trimoxazole (36.7%) and tetracycline (38.7%) were quite similar.
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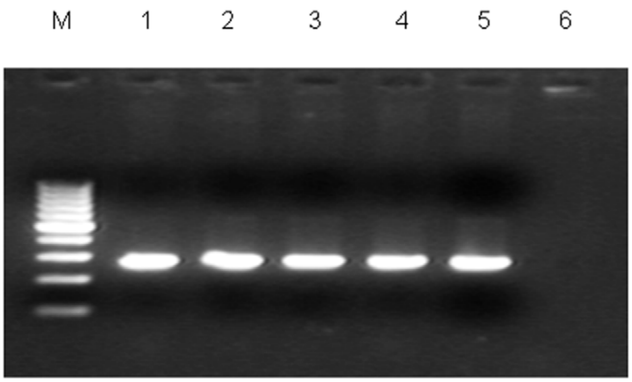

Figure 2. PCR based detection of O25b subgroup that is linked to the B2-O25b-ST131-CTX-M-15 clonal group of strains. Among 32 ESBL producers in our collection (n = 150) four were positive for O25b subgroup (Lanes 1 to 4); lane 5, positive (strain showing consistently positive); lane 6, negative; M, 100 bp DNA ladder.
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relatedness of these O25-ESBL-producing Indian isolates to the worldwide recognized clonal groups and to characterize the remaining multiresistant isolates observed. ESBL isolates were frequently (65.6%) associated with a hemolytic phenotype, with a higher rate than the non-ESBL-producing UPEC strains (58.5%). Hence, although it is a commonly accepted fact that bacterial pathogens acquire resistance determinants and express a multiresistant phenotype at the cost of their virulence properties, the frequent observation of hemolytic UPEC among our strain collection underlines the possible emergence of highly virulent multiresistant strains [36] in this area.

It is particularly worrisome that more than half of the ESBL producers (53.1%) in our collection were resistant to ciprofloxacin suggesting that they may be resistant to all available fluoroquinolones - the drug of choice for treatment of infections caused by ESBL-producing strains. Overall, nitrofurantoin and ciprofloxacin were found superior to amoxicillin and co-trimoxazole. None of the uropathogens from this study area were 100% susceptible to any of the antimicrobial substances used. Prior studies have shown a sensitivity rate of 95–100% to nitrofurantoin [37]. But in our study, nitrofurantoin showed only 57.3% sensitivity; the reason for this low sensitivity and high resistance to almost all antimicrobials in the study area may indicate difference in antimicrobial usage, infection control practices and other unrecognized factors including genetic propensity of these strains to accumulate mutations conferring MDR phenotypes. Periodic review and formulation of antibiotic policy are needed to control acquisition of drug resistance. Further studies for better understanding of the interaction of different virulence factors at molecular level are necessary as most urovirulent strains express multiple virulence factors simultaneously. We believe that the methods of detection of the above mentioned virulence markers are reasonably easy and screening them in a clinical microbiology laboratory is a worthwhile exercise.

Table 2. Distribution of various O-antigens in clinical isolates of *E. coli.*

O-Antigen	Pyelonephritis	Prostatitis	Cystitis	Simple UTI	Septicaemia	PUO	Total
O1	-	1	-	5	2	-	8
O2	-	1	-	1	-	-	2
O9	-	-	-	-	-	-	0
O14	1	-	-	-	-	-	1
O20	1	-	-	-	1	1	3
O25	6	7	11	15	2	4	45
O44	1	-	-	2	1	-	4
O45	-	-	1	-	-	-	1
O60	1		1	4	1	-	7
O64	-	-	1	1	-	-	2
O76	-	-	1	3	-	-	4
O79	-	1	-	-	-	-	1
O84	-	-	-	1	-	-	1
O102	3	1	-	8	-	1	13
O116	-	-	-	1	-	-	0
O120	2	2	3	6	-	1	14
O130	-	1	-	1	-	-	2
Rough	1		1	2	2	-	6
O 95	1	-	-	-	-	-	1
O100	-	-	-	1	-	-	1
Non- viable	-		1		-	-	1
Non *E. coli*	-	1	1	2	-	1	5
Untypeable	5	-	3	13	3	4	28
Total	22	15	24	65	12	12	150

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Serotyping and genotyping

In our study, as many as 19 different serotypes (O1, O2, O9, O14, O20, O25, O44, O45, O60, O64, O76, O79, O84, O102, O116, O120, O130, O95, O100) were observed among the cultured UPEC. Out of these, 27 *E. coli* were untypeable; six expressed a rough O antigen and five were *Escherichia* species (non-*E. coli*; NEC). A majority of *E. coli* isolates expressed O antigens - O25 (31.9%), O102 (9.7%), O120 (8.3%), and O1 (5.6%). Serotype O25 was predominantly found to be isolated from patients with pyelonephritis, prostatitis, cystitis and simple UTI. Renal failure associated isolates mainly belonged to O25 and O102 serotypes (Table 2).

To determine the relatedness of the strains, a dendrogram based on rep-PCR based fingerprint data was constructed in Bionumerics®. For this study, the clinical strains from India and 50 German isolates were used while the fecal isolates were excluded as the aim of this experiment was not to prove pathogenicity of the ExPEC but to see how diverse and clonal they are and to analyze Indian isolates in juxtaposition with German strains. Apart from the occurrence of singleton strains, the dendrogram obtained could be broadly classified into 5 major clusters: Indian-German cluster I (n = 15 strains), Indian cluster I (n = 63), mixed cluster I (n = 17) and mixed clusters IIa/IIb (n = 30), which include strains from all the three locales, as depicted in Figure 3. All the strains that clustered under Indian branch (exceptionally harboring one isolate from Germany) revealed very diverse pattern, except for a few ones - it appears that the bacteria we cultured were very diverse and the ones which were close in the cluster have no phenotypic resemblances with each other in terms of antimicrobial resistance profile or serotype or other virulence markers. This may be due to the reason that the UPEC in this study area could be highly diverse at sub-species and serotype levels.

The mixed cluster I contained a group of 17 bacterial isolates from India, Germany and from global sources. This cluster predominantly comprises of APEC from cases of septicemia in chicken or goose, while the remaining seven strains were human UPEC, including strain CFT073, or they were septicemia-associated strains (SEPEC). The mixed cluster II was subdivided into two subclusters- subcluster IIa (n = 10) and subcluster IIb (n = 20). The subcluster IIa largely encompasses APEC. Eight out of ten members of this cluster were APEC and the rest were UTI pathogens from humans. In the subcluster IIb, human isolates as well as isolates from animals were grouped together. There was no discrete band pattern between strains isolated from different sources; out of twenty strains in subcluster IIb nine were APEC, seven were UPEC of humans and two from dog, one was a human SEPEC and finally the archetypical NMEC strain RS218 grouping within this subcluster. From this analysis, we may conclude that the technique of rep-PCR is not resolving enough to distinguishing strains that portray similar phenotypic markers; nevertheless the Indian ExPEC population was clearly distinguishable from German ExPEC. We also found that the isolates from different animal sources were more or less grouping into distinct clusters, indicating that this technique can also be used to find the sources of infection in a reservoir such as a water body.

In conclusion, our observations form an important baseline data-set towards understanding the virulence properties, antibiotic resistance profiles and genetic diversity of UPEC from India. We hope that such observations will be more meaningful towards systematically unraveling the population genetic structure of UPEC and their propensity to spread, or emerge with multi drug resistance phenotypes in new epidemiological territories. In the backdrop of this work, future studies involving decipherment of MDR mechanisms, lineage tracking based on MLST [16] and

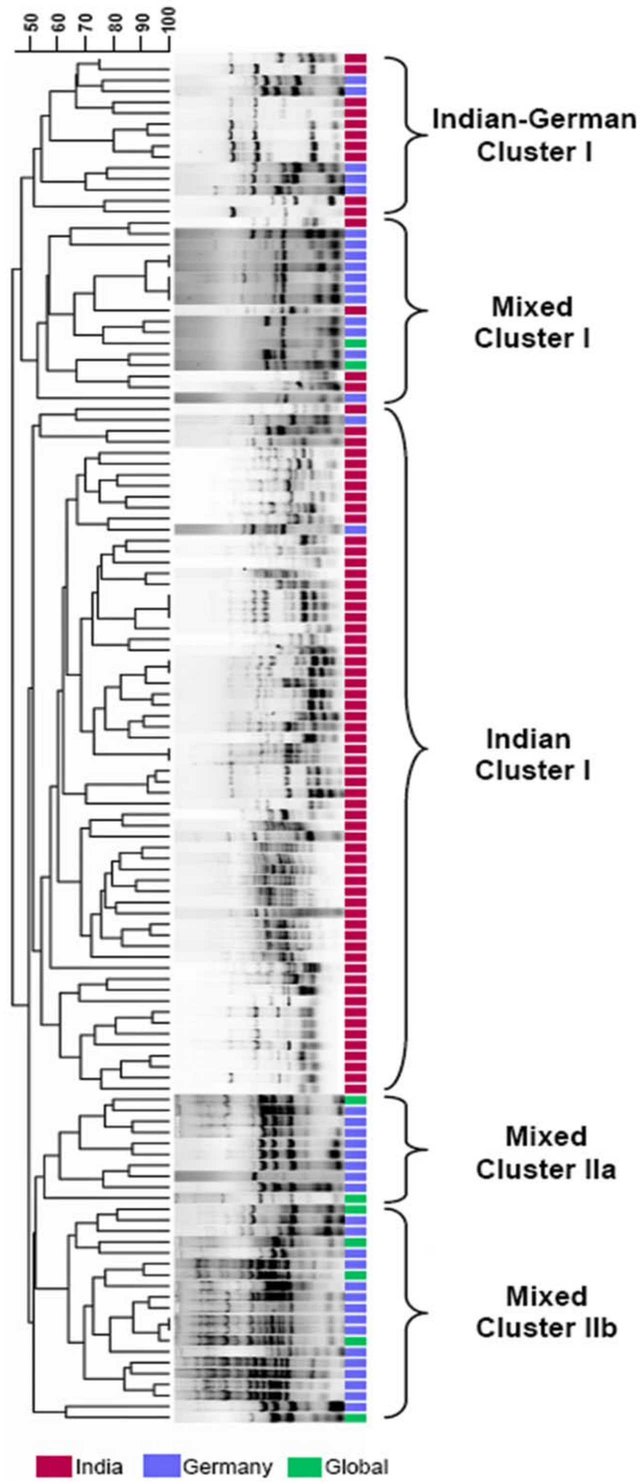

Figure 3. Dendrogram based on rep-PCR, developed in Bionumerics® revealed genetic relationships of *E. coli* representing Indian clinical isolates and ExPECs from global sources. Different geographic clusters are labeled. Upper scale denotes genetic distance.
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whole genome sequencing of representative isolates from different MLST clades [2] shall be imminent. Further, the 'omics' inspired studies will ultimately help understanding of the functional molecular epidemiology and infection biology of Indian UPEC at greater details. On the clinical side, it will be possible to ascertain reservoirs of infection which could be important in understanding the mechanisms of chronic and recurrent UTI [38] in a community setting and how they are maintained within or in the vicinity of different human populations of a multicultural and densely populated country such as India.

Supporting Information

Table S1 Clinical types, antibiogram, serotype and virulence characteristics of the UPEC isolates from Pune, India.
(DOC)

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Author Contributions

Conceived and designed the experiments: NA. Performed the experiments: SJ AH SD SP. Analyzed the data: AK CE NA. Contributed reagents/materials/analysis tools: NG LHW. Wrote the manuscript: CE NA.

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Genome of Multidrug-Resistant Uropathogenic *Escherichia coli* Strain NA114 from India▽

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Uropathogenic *Escherichia coli* (UPEC) causes serious infections in people at risk and has a significant environmental prevalence due to contamination by human and animal excreta. In developing countries, UPEC assumes importance in certain dwellings because of poor community/personal hygiene and exposure to contaminated water or soil. We report the complete genome sequence of *E. coli* strain NA114 from India, a UPEC strain with a multidrug resistance phenotype and the capacity to produce extended-spectrum beta-lactamase. The genome sequence and comparative genomics emanating from it will be significant in understanding the genetic makeup of diverse UPEC strains and in boosting the development of new diagnostics/vaccines.

Pathogenic *Escherichia coli* constitutes a significant threat to public health, and the emergence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* with high virulence potential is alarming (14, 16, 20, 22, 23). Comparative genomics holds significant promise in understanding the genome organization of such bacteria and thereby identifying coordinates highly relevant in the development of intervention strategies (1). Our group has recently studied uropathogenic *E. coli* (UPEC) from the western Indian city of Pune (11), whereupon strain NA114 emerged as an ideal representative of the entire Pune collection. The three major characteristics of strain NA114 that make it epidemiologically and clinically significant are its affiliation with serogroup O25, its placement in phylogenetic group B2, and its sequence type, ST131 (19). The latter denotes a pandemic clone frequently associated with community-acquired antimicrobial-resistant infections (23). Motivated by these facts, we performed complete in-depth sequencing, annotation, and analysis of the genome of UPEC strain NA114, which was originally obtained from the urine of a 70-year-old male patient with prostatitis from Pune. Antibiotic sensitivity tests revealed that it was a multidrug-resistant strain refractory to several common antibiotics and was an ESBL producer (11).

(This work constitutes part of the unpublished doctoral work of Arif Hussain.)

The genome sequence was determined by Illumina Genome Analyzer (GA2x, pipeline ver. 1.6) and consisted of sequence traces equivalent to 8 gigabytes of data, encompassing 54-bp paired-end reads with an insert size of 300 bp, and the genome coverage achieved was 500×. The sequence was assembled using Velvet (26), and the contigs were ordered with respect to the best-aligned positions compared to the reference genome of *E. coli* SE15 (25) using Mauve (5, 6). The genome alignment tools BLAT (15) and MUMmer (17) were also used to validate the aligned contigs. The genome was annotated with the help of the RAST server (2), and putative CDSs were identified by comparing outputs from Glimmer (7), Genemark (4), and EasyGene (18). Artemis (24) was used to glean the following details of the genome.

The size of the NA114 chromosome was 4,935,666 bp with a G+C content of 51.16% and a coding percentage of 88.4% with 4,875 protein coding sequences with an average length of 901 bp. The genome revealed 67 tRNA and 3 rRNA genes. We also found several virulence genes, including *iha*, *sat*, *fimH*, *kpsM*, *iutA*, and *malX*, which correspond to the genes of *E. coli* CFT073 (9). In addition, genes corresponding to another UPEC strain, UTI89, such as *fyuA* and *usp* etc., were located. PCR-based analysis showed that this strain carried multiple virulence genes infrequently described in a clone of this type, including *sfa*, *aer*, *cnf*, and an intact polyketide synthase (*pks*) island (12). *E. coli* NA114 also contains other virulence factors, such as *pap*, *fim*, and genes for iron uptake systems such as the hemin uptake system and the yersiniabactin siderophore (*ybt*). In addition to a 4.935-Mb chromosomal genome, strain NA114 also harbored a single plasmid of 3.5 kb which has yet to be analyzed with regard to its replicon type and resistance gene profiles, if it has any.

These observations and the comparative genomic studies emanating therefrom could be extremely useful both in improving our fundamental understanding of multidrug resistance mechanisms encoded by UPEC and in the design of effective drugs to control and manage the alarming health hazards caused by ESBL-producing bacteria in both the developing and developed parts of the world.

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Nucleotide sequence accession number. The genome sequence of *E. coli* NA114 has been deposited in GenBank under accession no. CP002797.

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Multiresistant Uropathogenic *Escherichia coli* from a Region in India Where Urinary Tract Infections Are Endemic: Genotypic and Phenotypic Characteristics of Sequence Type 131 Isolates of the CTX-M-15 Extended-Spectrum-β-Lactamase-Producing Lineage

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***Escherichia coli* sequence type 131 (O25b:H4), associated with the CTX-M-15 extended-spectrum beta-lactamases (ESBLs) and linked predominantly to the community-onset antimicrobial-resistant infections, has globally emerged as a public health concern. However, scant attention is given to the understanding of the molecular epidemiology of these strains in high-burden countries such as India. Of the 100 clinical *E. coli* isolates obtained by us from a setting where urinary tract infections are endemic, 16 ST131 *E. coli* isolates were identified by multilocus sequence typing (MLST). Further, genotyping and phenotyping methods were employed to characterize their virulence and drug resistance patterns. All the 16 ST131 isolates harbored the CTX-M-15 gene, and half of them also carried TEM-1; 11 of these were positive for *bla*$_{\mathrm{OXA}}$ groups 1 and 12 for *aac(6′)-Ib-cr*. At least 12 isolates were refractory to four non-beta-lactam antibiotics: ciprofloxacin, gentamicin, sulfamethoxazole-trimethoprim, and tetracycline. Nine isolates carried the class 1 integron. Plasmid analysis indicated a large pool of up to six plasmids per strain with a mean of approximately three plasmids. Conjugation and PCR-based replicon typing (PBRT) revealed that the spread of resistance was associated with the FIA incompatibility group of plasmids. Pulsed-field gel electrophoresis (PFGE) and genotyping of the virulence genes showed a low level of diversity among these strains. The association of ESBL-encoding plasmid with virulence was demonstrated in transconjugants by serum assay. None of the 16 ST131 ESBL-producing *E. coli* strains were known to synthesize carbapenemase enzymes. In conclusion, our study reports a snapshot of the highly virulent/multiresistant clone ST131 of uropathogenic *E. coli* from India. This study suggests that the ST131 genotypes from this region are clonally evolved and are strongly associated with the CTX-M-15 enzyme, carry a high antibiotic resistance background, and have emerged as an important cause of community-acquired urinary tract infections.**

Escherichia coli is a universal commensal bacterium causing infections in humans and animals and serves as a common cause of urinary tract infections (UTI) and bacteremia in humans (43). In addition, this group of strains, designated extraintestinal pathogenic *E. coli* (ExPEC), causes a variety of infections at extraintestinal sites ranging from the biliary system to the central nervous system. These infections are prevalent both in nosocomial and in community settings (46). UTI, although treatable, is now becoming increasingly tough to control because of rampant antimicrobial resistance in the *Enterobacteriaceae* family, particularly in *E. coli* (43, 52). As a result, these organisms are responsible for significant social and economic burdens for the communities and public health departments (24).

In the past decade, there has been a dramatic increase in the identification of *E. coli* strains with CTX-M enzymes, a new group of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) that have replaced classical TEM- and SHV-type ESBLs in many countries (38). There are more than 80 variants described in the CTX-M group of enzymes that are the primary cause of resistance to expanded-spectrum cephalosporins (8). Currently, the most widely distributed CTX-M enzyme is CTX-M-15, which was first detected in *E. coli* from India in 2001 (34, 46). One of the reasons for widespread occurrence of antibiotic-resistant *E. coli* in communities from multiple locales is thought to be due to the dissemination of clonal organisms harboring antimicrobial resistance genes (13, 30, 37).

Recent studies using MLST explored the population biology of ESBL-producing *E. coli* and uncovered emergence of an apparently dominant clone of CTX-M-15-producing *E. coli* carrying high levels of virulence-associated genes (VAGs); this was designated sequence type 131 (ST131), occurring in many different countries, and was thus recognized as a pandemic ExPEC clone (51). It has been shown that this group (ST131) of ESBL-producing *E. coli* strains, in addition to being resistant to most beta-lactam antibiotics, is frequently resistant to aminoglycosides and fluoroquinolones (36). Also, their spread posed a significant threat to human health, as they entail serious therapeutic challenges due to their ability to withstand the effect of different classes of antimicrobial agents. Moreover, the prevention and control of

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the transmission of uropathogenic *E. coli* infections are limited by poor understanding of the population genetics and virulence/resistance genotypes of these pathogens (28).

The endemic potential and ability of particular lineages of antibiotic-resistant *E. coli* to disseminate and cause disease are seldomly studied in countries such as India, where recent surveys have identified prevalence of ESBL producer groups to be up to 70 to 90% of the total *Enterobacteriaceae* reported, although this figure may be based on studies with biased sampling; nevertheless, they indicate a serious problem (26, 27). Moreover, it was demonstrated that there exists a great propensity of transmission of multiresistant clones from humans to animals and vice versa (19, 20).

In appreciation of the above-described issues, we designed a pilot study to investigate the prevalence and to determine the virulence and antimicrobial properties of the ST131 clones present among clinical *E. coli* isolates cultured from the urine of infected patients attending a tertiary care hospital in Pune, India. We believe this study is important in the backdrop of increased occurrence of carbapenem resistance genes in *Enterobacteriaceae*, especially $bla_{\mathrm{NDM-1}}$ in the Indian subcontinent (35), and for looking into the possibility of their dissemination being associated with highly virulent/resistant clones.

MATERIALS AND METHODS

Bacterial isolates, O typing, and antimicrobial susceptibility testing. A total of 100 clinical isolates of *E. coli* from patients with UTI were initially used that were recovered from urine samples of human patients giving a viable count of $>10^5$ CFU/ml. These isolates were received from the microbiology department of a hospital in Pune. Seven European ST131 ESBL *E. coli* isolates archived at the Institute of Microbiology and Epizootics (IMT), Free University Berlin, were also obtained for pulsed-field gel electrophoresis (PFGE). The ESBL production was confirmed phenotypically using the clinical and laboratory standards institute (CLSI) criteria for ESBL screening (16). O typing of ESBL-positive *E. coli* strains was done by a recently described molecular approach based on allele-specific PCR, targeting the *rfb*O25b subgroup gene locus (14). After this stage, only 16 out of the above-described 100 *E. coli* strains were used for further assays, described below. Susceptibility to the following non-beta-lactam molecules was assessed by the disc diffusion procedure: ciprofloxacin, chloramphenicol, gentamicin, sulfamethoxazole-trimethoprim, and tetracycline. Isolates were defined as resistant or susceptible according to CLSI guidelines (16).

MLST and phylogenetic grouping. Identification of *E. coli* phylogenetic groups was performed using the multiplex PCR-based method of Clermont et al. (13). Multilocus sequence typing (MLST) was performed as described previously (54). Gene amplification and sequencing were performed by using primers specified at the *E. coli* MLST website (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). Sequences were analyzed by the software package Ridom SeqSphere 0.9.19 (http://www3.ridom.de/seqsphere), and sequence types were determined accordingly.

Antimicrobial resistance gene detection. PCR amplification and sequencing were used to test for the presence of bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{OXA} genes among the phenotypically ESBL-positive strains (49). PCR was used to determine the presence of other antibiotic resistance genes, such as those conferring resistance to tetracycline [namely, *tet*(A), *tet*(B), *tet*(C)], to sulfonamides (*sul1*, *sul2*), to streptomycin (*strA*, *strB*), to aminoglycosides (*aadA1*-like, *aac4*), and to some other plasmid-mediated quinolone resistance determinants, such as *aac*(6′)-*Ib*, *qnrA*, *qnrB*, and *qnrS* (4, 3, 7, 23, 32, 42, 48). The 16 ESBL-producing ST131 *E. coli* isolates were screened by PCR for the presence of *intl1* and *intl2* genes, encoding class 1 and 2 integrases with the help of primers described elsewhere (53). To identify the gene cassettes, a PCR and subsequent sequence analysis were performed on the isolates that were positive for *intl1*, using primer pairs defined earlier (39).

Carbapenemase detection. Imipenem and meropenem susceptibility testing was performed using the disk diffusion method in accordance with the CLSI criteria (17). The modified Hodge test was performed on all the 16 isolates on Mueller-Hinton agar (1). After a 12-h incubation, the plates were observed for clover leaf-type pattern at the junction of the test organism and the standard strain within the zone of inhibition of the carbapenem disc. Briefly, the Mueller-Hinton agar plate was inoculated with a dilution of a 0.5 McFarland suspension of *E. coli* ATCC 25922 and streaked with a swab. A 10-μg ertapenem disk was placed in the center of a petri dish, and each test isolate was streaked from the disk to the edge of the dish. *Klebsiella pneumoniae* (ATCC BAA-1705) and *E. coli* (ATCC 25922) were used as positive and negative controls, respectively. Four carbapenemase genes, $bla_{\mathrm{OXA\text{-}48}}$, bla_{KPC}, bla_{VIM}, and $bla_{\mathrm{NDM\text{-}1}}$, were amplified as described previously (4, 44).

PFGE analysis. XbaI PFGE analysis was used to explore the possible clonal nature of the 16 Indian ST131 isolates in comparison with seven European ST131 strains to construct a dendrogram. The analysis was performed using a CHEF DRIII system (Bio-Rad, Munich, Germany). Cluster analysis of dice similarity indices based on the unweighted pair group method with arithmetic mean (UPGMA) was used to generate a dendrogram describing the relationships among PFGE profiles. Isolates were considered to be belonging to the same PFGE cluster if their Dice similarity index was ≥85% (10, 40).

Conjugal transfer and plasmid analysis. Conjugative transfer of ESBL genes was tested by broth mating experiments by using a plasmid-free, sodium azide-resistant *E. coli* J53Azi^{R} strain as a recipient. The strains were grown to the exponential phase and then mixed in the ratio of 1:2 (donor:recipient). Transconjugants were selected on Endo agar containing 100 μg/ml sodium azide and 4 μg/ml cefotaxime. Potential transconjugants were verified by PCRs, and the presence of relevant *bla* genes and cotransfer of resistance determinants were confirmed by amplifying the respective genes in the transconjugants as described above (22). The number and approximate sizes of the plasmids in each of the 16 ST131 isolates and the respective transconjugants were determined along with four reference plasmids of known sizes (*E. coli* [RS478], 272 kb; *E. coli* [x-109/97], 19 kb; *Salmonella enterica* serovar Typhimurium, 91.2 kb; *E. coli* [RS478], 170.24 kb) by using a modified version of the methodology previously described by Kado and Liu (33). This was followed by separation of plasmids by electrophoresis in 0.4% agarose gels made with 1× Tris-EDTA (TE) buffer. Gels were run at 20 V/cm for 8 h, stained with ethidium bromide, and photographed. Plasmids were assigned to major plasmid families by PCR-based replicon typing (PBRT) (9).

Serum sensitivity and biofilm formation assay. Serum sensitivity assay was performed on three clinical strains and their transconjugants as previously described (18). Briefly, the overnight bacterial cultures were washed and suspended in 1 ml phosphate-buffered saline (PBS); 50 μl of this suspension was added to 450 μl of serum, and 100 μl from each well was taken out for CFU determination before and after incubation at 37°C in a shaker incubator. Strains IMT10740 and IMT5053 were used as positive and negative controls, respectively. Biofilm formation assays were performed on 15 clinical strains and their conjugant strains by using a modified version of a previously described method (41). Strains were grown overnight in LB broth, and the cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh M63 medium. Then, 200 μl of aliquots was placed in wells of polystyrene microtiter plates and incubated for 48 h at 28°C without shaking. Afterward, ODs were read at 600 nm, the wells were washed thrice, and the staining of adhered bacteria was performed with 0.1% crystal violet (solubilized in ethanol) followed by reading the OD at 570 nm. Biofilm measurements were calculated using the formula as previously described (41).

Virulence gene typing. All the 16 ST131 isolates were investigated by multiplex PCR for the presence of 38 virulence-associated genes (VAGs) corresponding to the extraintestinal pathogenic *E. coli* based on the targets

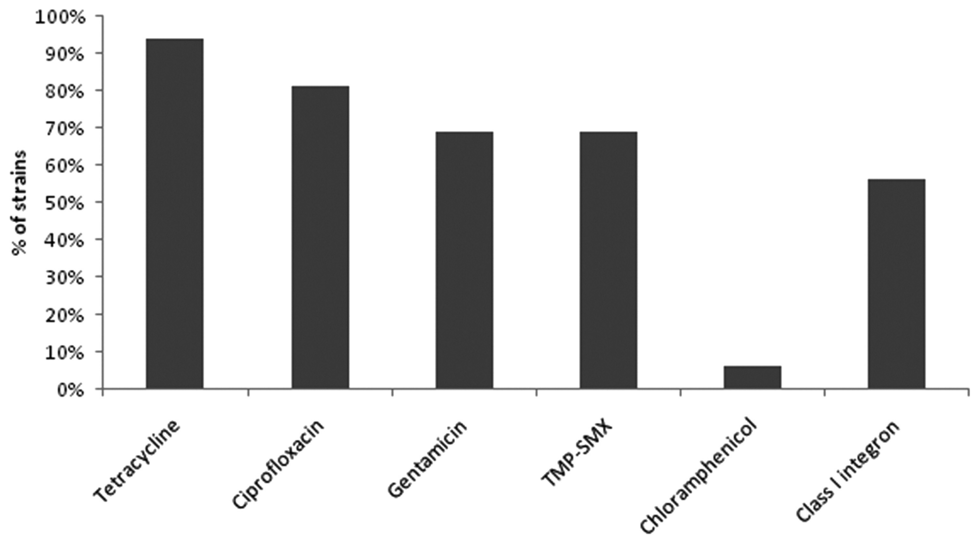

FIG 1 The percentages of 16 ST131 CTX-M-15-producing *E. coli* isolates resistant to five different non-beta-lactam antibiotics and the percentages of isolates positive for class 1 integron. Antimicrobial susceptibility testing was performed using the disk diffusion method according to CLSI criteria.

that have been previously described (49, 31, 5, 20). The genes tested correspond to the main classes of extraintestinal VAGs; adhesin, toxin, iron capturing system, and protectin/invasin.

Statistical analysis. The student's *t* test was performed using GraphPad Prism 5.0 software. The *P* value was calculated for each experiment conducted in triplicates.

RESULTS

Isolation and antimicrobial resistance profiling. One hundred *E. coli* samples were obtained between January 2009 and October 2009 (28). A total of 23 (23%) ESBL-producing isolates were detected phenotypically using the CLSI criteria for ESBL screening and disk confirmation test. These 23 isolates were then subjected to O typing that revealed 16 (70%) out of 23 ESBL-producing strains to be positive for the *rfbO25b* subgroup, indicating the presence of 16 CTX-M-15-O25b-ST131 group strains. Of these 16 strains, 10 were from male patients with an average age of 40 years who were diagnosed with prostatitis and pyelonephritis. To assess multidrug resistance of the 16 ST131 isolates, susceptibility to non-beta-lactam antimicrobials was tested. Ninety-four percent of the isolates were resistant to tetracycline, 81% to ciprofloxacin, and 69% to gentamicin and sulfamethoxazole-trimethoprim (TMP-SMX), but only 6% to chloramphenicol. Out of the16 isolates, 12 (75%) were resistant to at least three classes of antimicrobials, and therefore a total of 12 multidrug-resistant (MDR) ESBL ST131 strains were finally identified (Fig. 1).

Genetic relationships and plasmid profiles. All of the 16 ESBL strains positive for the *rfbO25b* locus were allocated to phylogenetic group B2 on the basis of triplex PCR. MLST analysis of these 16 strains showed that they belonged to the ST131 clone. To obtain a finer resolution of clonal relationships among the 16 Indian ST131 *E. coli* strains and seven ST131 strains from European origin, a PFGE analysis was performed (Fig. 2). Owing to their common genetic background, the ST131 isolates exhibited more homogenous PFGE profiles. All the ST131 strains tested by us formed five small clonal groups (groups 1 to 5) as documented at the 85% similarity level. Three of the five PFGE groups (groups 1, 4, 5) corresponded consistently to the geographic/host origin, whereas two groups (groups 2 and 3) carried strains both from the Indian and European origins. Overall, 10 out of the16 *bla*$_{\text{CTX-M}}$ (Indian) ST131 isolates were found to be relatively clonal, and also three out of seven European strains clustered closely with the Indian strains, indicating their genetic affinities despite geographic barriers and different host origins. This highlights the fact that they are an epidemiologically important clone appearing/emerging in different continents.

From a total of 16 CTX-M-producing isolates, conjugation to *E. coli* J53Azi^{R} was demonstrated in 15 strains, wherein larger plasmids ranging in sizes from 120 kb to 272 kb were transferred or exchanged. In these strains, all the antimicrobial resistance genes as reported in the parental strains along with the *bla*$_{\text{CTX-M-15}}$ gene were detected on IncFIA conjugative plasmid by PCR. The parental clinical strains carried up to six plasmids, most of them displayed two larger plasmids of around 120 kb and/or 272 kb (data not shown); plasmid analysis among transconjugants revealed larger plasmids of $>$100 kb, except for one strain which revealed an identical profile. The plasmids belonged to the narrow host range incompatibility group IncF; 15 isolates contained the FIA replicon, 13 isolates had the IncFIB replicon, while one isolate harbored P, Y, and A/C replicons in addition to FIA and FIB replicons.

All the 16 ST131 isolates were positive for *bla*$_{\text{CTX-M}}$ genes (CTX-M-15); additionally, eight (50%) of these isolates also produced TEM-1. *bla*$_{\text{SHV}}$ was absent in all, and the *bla*$_{\text{OXA}}$ group 1 was present in 11 (68.7%) of the 16 Indian isolates. Only one strain (6.3%) was positive for *bla*$_{\text{OXA}}$ group 2. The gene variant *aac(6′)-Ib-cr*, which has been associated with fluoroquinolone resistance, was detected in 12 (75%) ST131 strains as determined by digestion of the amplicons with BsegI. No other plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, and *qnrS*) were detected. Other resistance genes detected among ST131 isolates are shown in Table 1. Screening of integron classes revealed that 9 (56.3%) out of 16 ESBL producers contained the *intl1* gene, whereas the *intl2* gene, which is specific for class 2 integron, was absent in all 16 isolates. The gene cassette arrangement detected among the *intl1*-

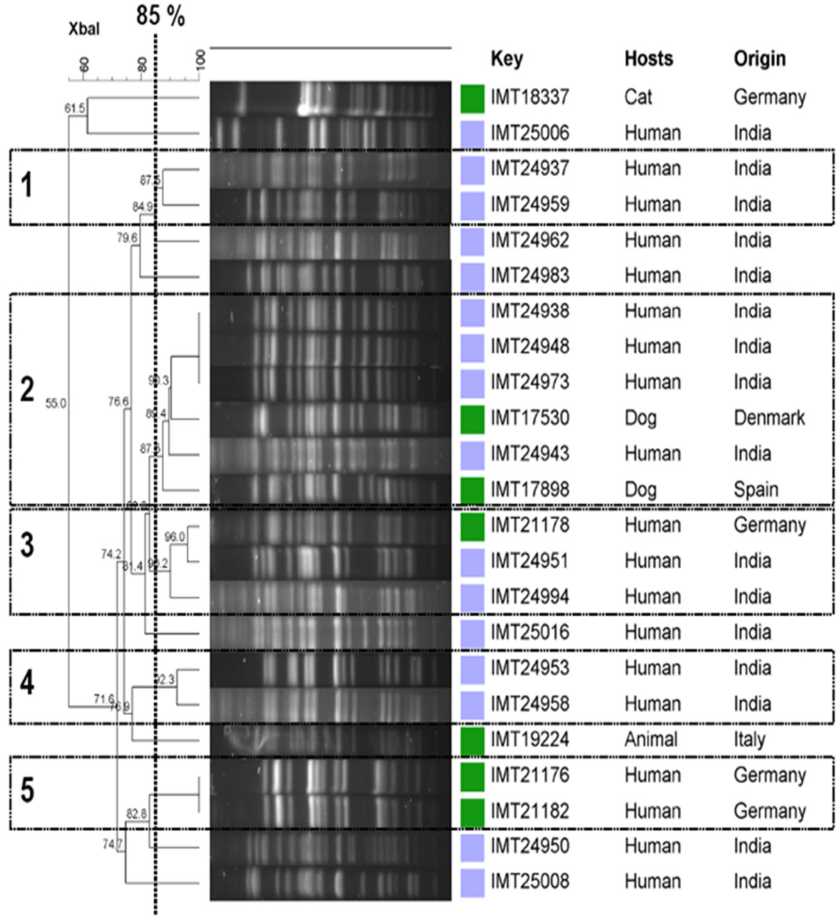

FIG 2 Dendrogram based on PFGE profiles of 16 Indian and 7 European ESBL-producing ST131 *E. coli* isolates. The dendrogram for the 23 isolates as produced by the UPGMA algorithm based on the Dice similarity coefficient included five PFGE groups as defined based on ≤85% similarity of PFGE profiles. Different geographically distinct isolates are accordingly labeled. The blue boxes indicate strains from India; all European origin strains are labeled with green boxes.

positive isolates was *dfrA12-aadA2* (for two isolates) and *dfrA17-aadA5* (for six isolates). All the ST131 isolates were found to be carbapenem sensitive by the disc diffusion method. The four carbapenemase genes, bla_{OXA-48}, bla_{KPC}, bla_{VIM}, and bla_{NDM-1}, were also absent.

Profiling of virulence genotypes/phenotypes. Serum resistance assay of the three clinical strains (IMT24943, IMT24951, IMT24973) versus their transconjugants along with the strain J53 revealed remarkable increase in serum resistance (Fig. 3) by the laboratory strain J53 alone and J53 with ESBL plasmids from clinical strains (P = 0.0001 for each pair). J53 with ESBL plasmids (transconjugants) displayed almost similar or increased resistance compared to that of their parental strains. The pattern of biofilm formation observed in cases of the clinical isolates, transconjugants, and the empty host J53 is shown in Fig. 4. Basically, there were no prominent differences observed (with respect to biofilm formation) between the empty J53 host strain and J53 with ESBL plasmids. It may be concluded that there is reduced influence of plasmids on biofilm formation; alternatively, the basic capacity of the wild-type strains to produce a biofilm is simply too low to identify significant differences in transconjugants.

Virulence profiles were determined (Fig. 5) for the 16 ST131 clinical isolates to get an idea of the extent of within-group diversity and the virulence potential of this clonal group. Out of 38 extraintestinal VAGs, 23 were detected at least once, with their prevalence ranging from as low as 6.3% with respect to *iroN* (catecholate siderophore) to 100% presence of the following genes: *chuA* (gene for heme transport), *traT* (serum resistance), *sitD* (salmonella iron transport gene), *ompA* (outer membrane protein), *iucD* (aerobactin), *sat* (secreted autotransporter toxin), *fyuA* (*Yersinia* siderophore receptor), *mat* (meningitis-associated fimbriae), and *feoB* (iron transporter). In general, the virulence gene profile similarity was high among the 16 ST131 isolates. The three identical (IMT24938, IMT24948, IMT24973) isolates seen in group 2 of the PFGE dendrogram exhibited an almost identical virulence profile. Relatively, the isolates within the clusters were more similar in their virulence profiles than the strains from adjacent clusters and singleton strains.

DISCUSSION

The worldwide occurrence of *E. coli* with CTX-M extended-spectrum beta-lactamases partly reflects the spread of clonal lineages, notably ST131, and hence we sought to identify the ST131 clone of bacteria among the ESBL-positive *E. coli* from the peri-urban areas of Pune, India, and to compare them with the isolates of European origin. The emergence of new MDR (extraintestinal)

TABLE 1 Results of different screenings of antimicrobial resistance genes other than ESBL genes[a]

Strain no.	Uniform code	Result *sul1*	*sul2*	*tet*(A)	*str*(A)	*str*(B)	bla_{OXA} group 1	*acc(6′)-Ib-cr*
IMT24938	2	+	+	+	+	−	−	+
IMT24948	3	−	−	+	+	−	−	−
IMT24958	8	+	+	+	+	+	+	+
IMT24962	14	+	+	+	+	+	+	+
IMT24983	17	+	+	+	+	−	+	−
IMT25006	18	+	+	+	+	+	+	+
IMT24937	20	+	−	+	+	−	+	+
IMT24943	25	+	+	−	+	−	+	+
IMT24950	26	−	−	+	+	+	+	+
IMT24951	29	+	+	+	+	−	+	+
IMT24953	41	+	−	+	+	−	+	+
IMT24959	51	+	+	+	+	−	−	+
IMT24973	62	−	−	+	+	+	−	−
IMT24994	72	+	+	+	+	−	+	+
IMT25008	75	+	+	+	+	−	−	+
IMT25016	83	+	−	+	+	−	+	−

[a] The 16 ESBL ST131 strains were screened for the *tet*(B), *tet*(C), *sul3*, *qnrA*, *qnrB*, *qnrS*, *aadA*, and *aaC4* antimicrobial resistance genes, which were found to be absent in all 16 strains. +, present; −, absent.

strains of *E. coli* spreading through populations with the capacity to evolve continuously appears to pose a significant public health threat. A great deal of attention is required in this direction. To get a deeper understanding of the underlying resistance genotypes and the mechanisms thereof, it was intended to characterize these clinical isolates in detail with regard to their ESBL production capacity and other antimicrobial resistance features. We also sought to find out whether there is any relationship between ESBL plasmids and virulence.

Our analysis of 100 rigorously selected *E. coli* isolates found that the clonal group ST131 was prevalent particularly among the ESBL isolates. We could confirm the presence of a total of 16 ST131 isolates by MLST in just a small collection of 100 isolates, suggesting a moderately high level of abundance. They were all affiliated to the phylogenetic group B2, which extends to most of the extraintestinal pathogenic *E. coli*. The representation of ST131 isolates was found to be more among male patients; this explains the enhanced capability of ST131bacteria to establish ascending urinary tract infections (pyelonephritis and prostatitis) despite the male anatomical barriers.

In agreement with other reports, most of the ST131 ESBL producers from this study were determined to be multidrug resistant and displayed high coresistance rates toward tetracycline, fluoroquinolones, sulfamethoxazole-trimethoprim, and gentamicin. Most of the isolates remained susceptible to chloramphenicol, which is effective against a wide variety of Gram-positive and

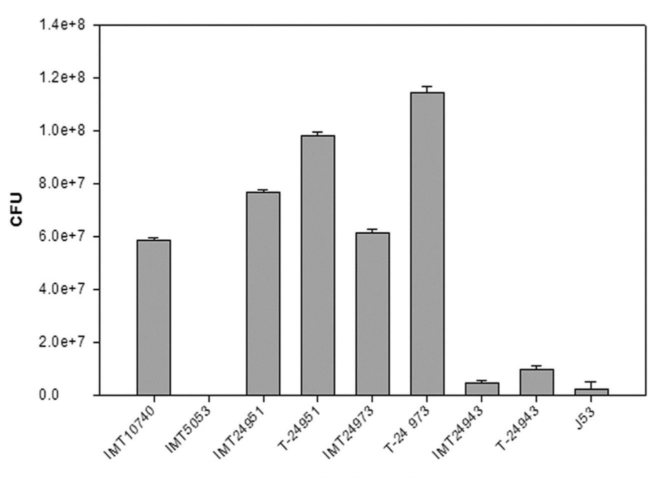

FIG 3 Serum resistance assays of three parental (IMT) ST131 ESBL-producing *E. coli* isolates and each transconjugant (T). The assays were performed for 3 h. Samples were taken in triplicates at time zero and at each hour. The graph is based on the results of three assays for each strain. Results are shown as CFU viability. Error bars indicate standard deviations.

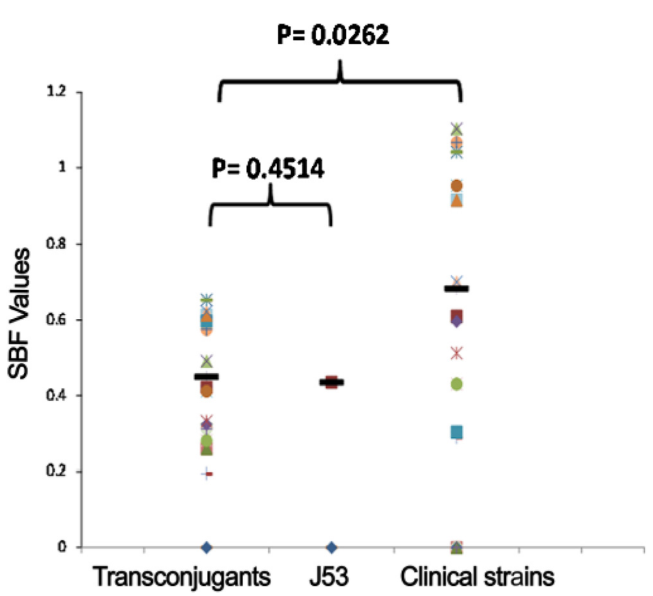

FIG 4 *In vitro* biofilm formation characteristics of transconjugants, clinical strains, and the plasmid-free host J53. The black bar indicates mean values. The *t* test analysis represents the differences between the transconjugants and clinical strains to be highly significant, whereas the difference between transconjugants and J53 was found not to be significant.

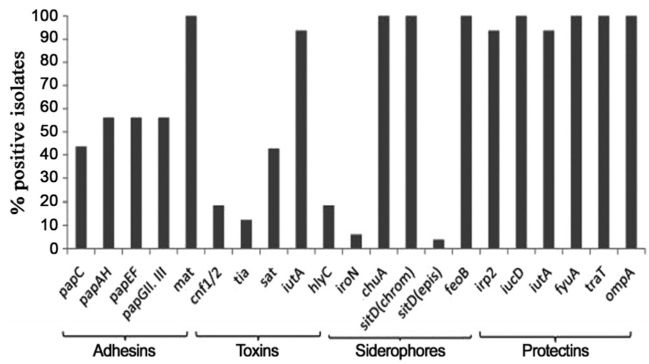

FIG 5 Virulence scores of 16 ST131 UPEC isolates for 23 genes out of 38 extraintestinal virulence-associated genes assessed by multiplex PCRs.

Gram-negative bacteria. Consistent with most of the studies, our ST131 isolates were also highly resistant to fluoroquinolones (81.3%) and trimethoprim-sulfamethoxazole (68.8%) (4).

Transconjugants were created in *E. coli* J53 from 15 (93.8%) out of the 16 strains. It was demonstrated that all of the transferred plasmids were ESBL plasmids. This proved their readily transferable nature with a strong potential for spreading of the ESBL and antimicrobial-resistant genes among bacterial populations. An antimicrobial resistance phenotype similar to the clinical strains was seen in transconjugants, and hence, this observation validates that the genes encoding ESBLs were located on transferable plasmids that harbored genes encoding resistance to several other classes of antimicrobials. Further, we sought to find out whether there was any relationship between the ESBL plasmids and virulence, and surprisingly, we found that the ESBL plasmids not only carried antimicrobial resistance genes but also conferred virulence and survival advantage to the recipient bacteria against the bactericidal activity of sera. However, when we further investigated these plasmids for their role in another virulence attribute, the biofilm formation, we didn't observe any positive influence of ESBL plasmids on biofilm formation. This could simply be due to the regulatory mechanisms involved in biofilm formation that were active on the chromosomal part of the bacteria. Further study of biofilm formation and other virulence features of these plasmids with the plasmid-cured strains and their comparison with parental clinical strains will be important for understanding the relationship between virulence phenotypes and ESBL plasmids.

The CTX-M beta-lactamases have been recognized worldwide as an important mechanism of resistance to cephalosporins (cefotaxime and ceftriaxone) used for Gram-negative pathogens (8). We found that all of the 16 ST131 strains harbored the CTX-M-15 allele. Our finding was in agreement with most of the previously published works reporting a strong association of ST131 strains with CTX-M-15-type enzymes, indicating that they are frequently carried in "aggressive" lineages with high virulence genotypes (8). Similar to the earlier studies, the CTX-M-15 allele was located on plasmids with replicon type FII-FIA, whereas the other replicon types, such as FIB, A/C, P, and Y, which were detected in the clinical wild-type strains, were absent in the transconjugants obtained from them (15, 21).

Plasmid-mediated quinolone resistance (PMQR) has become an important emerging issue. The most common PMQR gene observed in this study was *aac(6′)-Ib-cr* (81%). The association of *aac(6′)-Ib-cr* with CTX-M-producing *E. coli* is not surprising, as many studies have shown that it is often harbored on the same plasmids as the $bla_{\text{CTX-M}}$ gene (42, 45, 48). We did not detect any other PMQR genes, like *qnrA*, *qnrB*, and *qnrS*, which have also been shown to be associated with CTX-M-producing *E. coli* in recent studies. However, a number of other studies reported that these genes are rather uncommon among CTX-M-producing *E. coli*, and hence our study is in agreement with the observations reported elsewhere (42, 45, 48). The fluoroquinolone resistance phenotype among these 16 ST131 strains also could be due to mutations within the genes, such as *gyrA* and *parC* (11), which has not been determined by us.

Several studies have investigated the distribution of integrons in uropathogenic *E. coli* (UPEC) and have established a strong association between the presence of integrons and antimicrobial resistance in MDR and single-drug-resistant *E. coli* strains (6, 47). While the analysis of integron-encoded integrases indicated that class 1 integron was the principal integron class in the 16 Indian ESBL strains, no class 2 integron-encoded integrases were detected; these integrons were plasmid encoded and transmissible, as indicated by conjugation experiments. These results are similar to a recent report on antimicrobial resistance in uropathogenic *E. coli* from Europe and Canada (6). We observed a higher frequency of *sul1* than *sul2*. Sulfonamides are regarded as highly important antimicrobial agents for the treatment of *E. coli* infections, and the presence of sulfonamide resistance can lead to treatment failure in cases of UTI (6). The association of ESBL genes with other resistance determinants (such as genes conferring resistance to sulfonamides, tetracycline, and aminoglycoside) were observed in this study, as the candidate resistance genes are often carried on the same plasmid, as demonstrated by similar antimicrobial profiles of transconjugants (30, 50, 51). These results prove that the ESBL genes continue to evolve and harbor an increasing range of resistance determinants (25).

Our isolates were checked for susceptibilities to carbapenems, and it was observed that all 16 ST131 ESBL producers were highly susceptible. However, a recent study has highlighted the emergence and widespread distribution of NDM-1 in MDR *Enterobacteriaceae* in different parts of India, Pakistan, and the United Kingdom (35). The reason for this contrasting observation may be the geographic variation that is subject to carbapenem usage, biased sampling, or the small sample size of our study. Nevertheless, it can be said that the association of carbapenemase enzymes with ST131 strains is not widespread, as yet. Plasmids that were isolated from transconjugants were found to be larger than 100 kb, implying that the ESBL plasmids in clinical strains are >100 kb in size. However, the donor strains harbored more than one plasmid, ranging in size from 8.5 kb to 272 kb. Genotyping by PFGE showed that the clinical strains were genetically clonal, as they were sourced from a single hospital and from one geographical area. The three indistinguishable Indian strains on PFGE fingerprints were isolated 1 month apart; two strains were from the same ward, and the third one came from a different ward. This possibly suggests a nosocomial spread. The clustering of three European strains with the Indian strains also supports a possible clonal nature of ST131 strains. This observation is in corroboration with two recent reports (12, 29) that have shown sequence similarity of one of our ST131 isolates, NA114, from India to the European isolates (2); this perhaps points to a somewhat conserved genomic structure of a few ST131 strains of European origin. However, on the other hand, we saw some degree of heterogeneity in the PFGE profiles among the Indian strains and between the European ST131 strains which could possibly be due to the high rates of recombination within the accessory genome of this species. Another worrying fact is that these ESBL-producing strains have a high background of virulence. This resistance-virulence combination might impart ST131 a competitive advantage over other *E. coli* strains.

In conclusion, our study is perhaps the first one to identify and characterize ST131 *E. coli* in UPEC populations from an Indian setting. This study demonstrates the complexity of the acquisition and spread of MDR phenotypes that could pose difficulties in treating serious Gram-negative infections. The observation that the ESBL plasmids are readily transferrable might present another problem of rapid acquisition of MDR genes by the docile strains. The PFGE profiles, virulence factors, and antimicrobial resistance pattern of these ST131 isolates exhibit that the strains from this geographic location were homogenous pathogens. The transfer of virulence phenotype due to ESBL plasmids might lead to an increase in acquisition of virulence among environmental and commensal bacteria. Therefore, the emergence and dissemination of this particular lineage of *E. coli* in Indian infection settings is a cause of concern and points to a need to identify their origins, reservoirs, and transmission pathways so that better prevention strategies are designed. Having said this, we would like to carry out further studies on a large number of *E. coli* isolates from different places within India to have an exhaustive opinion on epidemiology, evolution, and pathogen biology. Whole-genome sequencing of these strains, including their plasmids, would be instrumental to gain more insights into the molecular mechanisms imparting fitness or survival advantage to these ST131 lineages over other pathogens. Finally, on the public awareness and policy front, while antimicrobial resistance is projected as an important public health concern in China (27), India also deserves such attention given the widespread occurrence of new beta-lactamase genes, such as $bla_{\mathrm{CTX\text{-}M}}$ and $bla_{\mathrm{NDM\text{-}1}}$, amidst the absence of a proper antibiotic policy based on epidemiological knowledge entailing socioeconomic and bio-geo-climatic factors that favor rapid emergence of antimicrobial resistance.

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