

**Functional molecular infection epidemiology of  
extraintestinal pathogenic *Escherichia coli* with  
major focus on isolates from skin and soft tissue  
infections and NDM harboring carbapenem  
resistant strains**

**Thesis submitted to the University of Hyderabad  
for the degree of**

**DOCTOR OF PHILOSOPHY**

By

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**December, 2016**

# University of Hyderabad

(A Central University by an Act of Parliament)

Department of Biotechnology and Bioinformatics

School of Life Sciences

P.O. Central University, Gachibowli, Hyderabad-500046



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## DECLARATION

The research work presented in the thesis entitled “**Functional molecular infection epidemiology of extraintestinal pathogenic *Escherichia coli* with major focus on isolates from skin and soft tissue infections and NDM harboring carbapenem resistant strains**”, 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 Prof. Dr. Niyaz Ahmed. 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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Date:  
30/12/2016

## CERTIFICATE

This is to certify that the thesis entitled **“Functional molecular infection epidemiology of extraintestinal pathogenic *Escherichia coli* with major focus on isolates from skin and soft tissue infections and NDM harboring carbapenem resistant strains”** submitted by Mr. Amit Ranjan bearing registration number 10LTPM03 in partial fulfillment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:

1. Genomic and functional portrait of a highly virulent, CTX-M-15-producing H30-Rx subclone of *Escherichia coli* sequence type (ST) 131 (ISSN Number 0066-4804, Antimicrobial Agents and Chemotherapy (AAC) 2015).
2. Molecular Epidemiology and Genome Dynamics of New Delhi Metallo- $\beta$ -Lactamase-Producing Extraintestinal Pathogenic *Escherichia coli* Strains from India (ISSN Number 0066-4804, Antimicrobial Agents and Chemotherapy (AAC) 2016).

B. Presented in the following conferences:

1. Bioquest 2015 held at University of Hyderabad, on 23rd and 24th September 2015 (National).
2. 17th International congress on Infectious Diseases (ICID) conference held from 02 to 05 March, 2016 at HICC Hyderabad, India (International).
3. 56th Annual conference of Association of Microbiologists of India (AMI) held from 07 to 10 December, 2015 at JNU Convention Centre , New Delhi, India (International).

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***This work is dedicated to my  
beloved father whose entire life  
has inspired me to work harder  
and harder.....***



# Acknowledgement

*First of all I would like to acknowledge my mentor Prof. Dr. Niyaz Ahmed for his constant support, guidance and encouragement which has lead me to complete this work. His words has always inspired and motivated me both at professional as well as personal frontier to groom myself. I am really thankful to him for being open to new ideas and the freedom he provided me to carry out my research work.*

*I would also thank the doctoral committee members Dr. Insaf Ahmed Qureshi and Dr. Nooruddin Khan for their constant support and valuable suggestions during the entire thesis work.*

*Prof. Dr. L H Wieler and Mr. Torsten Semmler are acknowledged for being one of most supporting persons and hosting me at Freie University and Robert Koch Institute, Berlin for my stay of six months under Erasmus+ program. Dr. Sebastian Gunther needs to be specially acknowledged for the constant guidance and infrastructure during my stay at FU Berlin.*

*Dr. Savita Jadhav is highly acknowledged for the provision of clinical samples from D. Y Patil hospital, Pune for the research work.*

*I would like to thank the present and past heads of Department of Biotechnology and Bioinformatics and present and past deans of*

*School of Life sciences for use of the departmental/School facilities.*

*I would also like to acknowledge all the departmental and school non teaching staffs specially Rahul, Rajshekhar, Shekhar, Narayana, Murthy, Arun for their help in doing all the official works in the department/school throughout this period of work.*

*All the present and past members of Pathogen Biology Laboratory are highly acknowledged for creation of the wonderful environment in lab and outside which has helped in working stress free all throughout my stay in Hyderabad. The senior lab members Dr. Shivendra, Dr. Suhail, Dr. Ashutosh, Mr. Kishore and others have been so great from the very first day that it never felt, I was new to the group and has trained me in different ways leading me to carry out my research and other activities in the lab. Dr Arif, Dr. Nishant and Sabiha need a special mention for their help in my work as well as being always there for the work discussions. Dr. Savita has always been supportive and helpful besides being part of all our fun time and I express heartfelt thanks to her. I would like to thank Dr. Ramani, Dr. Narender, Dr. Vidyullatha, Dr. Majid, Shankar, Priya, Shamsul, Sumeet, Naveen, Shveta and Majjid for helping me one way or the other during this work. I would also thank the help of M.Sc project students Agnismita and Arya working with me during the period.*

*I am extremely indebted to my roommate and my best friend Deepak Babu for all the fond memories and being always with me in my*

*thick and thin times. It is extremely unimaginable how my times would have been without him.*

*I am also thankful to other friends in the university and department: Nidhi, Dr. Prashant, Nikhil, Bramhini, Daniel and Nisha.*

*I am extremely blessed to have a lovely family and would like to thank all, specially my mother for her love, care and faith in me which has boosted me always and helped me to carry out my work with positive note.*

*I would like to thank each and every individual for the slightest of help they would have done during the entire work.*

*I acknowledge the funding support from Indian Council of Medical Research (ICMR) for the support as Junior and Senior research fellowship.*

*Last but the most important one, I would like to thank the Almighty for the life and providing the opportunity to present this work in front of the world.....*

*Amit*

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## *Abbreviations*

|                |                                                    |
|----------------|----------------------------------------------------|
| APEC           | Avian pathogenic <i>Escherichia coli</i>           |
| CFs            | Colonization factors                               |
| CDDEP          | Center for disease dynamics, economics and policy  |
| CLSI           | Clinical and Laboratory Standards Institute        |
| DAEC           | Diffusely adherent <i>Escherichia coli</i>         |
| ESBL           | Extended spectrum of beta-lactamase                |
| ExPEC          | Extraintestinal pathogenic <i>Escherichia coli</i> |
| EPEC           | Enteropathogenic <i>Escherichia coli</i>           |
| ETEC           | Enterotoxigenic <i>Escherichia coli</i>            |
| <i>E. coli</i> | <i>Escherichia coli</i>                            |
| EAEC           | Enteraggregative <i>Escherichia coli</i>           |
| EHEC           | Enterohaemorrhagic <i>Escherichia coli</i>         |
| GI tract       | Gastro-intestinal tract                            |
| InPEC          | Intestinal pathogenic <i>Escherichia coli</i>      |
| LT             | Heat labile enterotoxin                            |
| LPS            | Lippopolysaccharide                                |
| MPEC           | Mammary associated <i>Escherichia coli</i>         |
| MDR            | Multidrug resistance                               |

|                       |                                                             |
|-----------------------|-------------------------------------------------------------|
| <b>MBL</b>            | <b>Metallo beta-lactamase</b>                               |
| <b>NDM</b>            | <b>New Delhi metallo-beta-lactamase</b>                     |
| <b>NMEC</b>           | <b>Neonatal meningitis <i>Escherichia coli</i></b>          |
| <b>PAHO</b>           | <b>Pan American health organization</b>                     |
| <b>PI<sub>3</sub></b> | <b>Phosphatidyl inositol 3</b>                              |
| <b>STEC</b>           | <b>Shigatoxigenic <i>Escherichia coli</i></b>               |
| <b>SSTI</b>           | <b>Skin and soft tissue infection</b>                       |
| <b>SePEC</b>          | <b>Sepsis associated pathogenic <i>Escherichia coli</i></b> |
| <b>ST</b>             | <b>Heat stable enterotoxin</b>                              |
| <b>SIRS</b>           | <b>Severe inflammatory response syndrome</b>                |
| <b>UTI</b>            | <b>Urinary tract infection</b>                              |
| <b>UPEC</b>           | <b>Uropathogenic <i>Escherichia coli</i></b>                |
| <b>WHO</b>            | <b>World health organization</b>                            |

# Chapter 1:

## Introduction and state of the art



## ***Escherichia coli***

*Escherichia coli* (*E. coli*) is one of the most versatile and common microorganisms. Under normal conditions it resides as commensal organism in gastrointestinal (GI) tracts of all mammals and even in some cold blooded animals which reside at relatively warmer conditions (Kaper et al. 2004, Conway and Cohen 2015). *E. coli* is acquired within few hours after birth and persists lifelong inside the GI tract (Kaper et al. 2004). This Gram negative, facultative anaerobe exploits various alternative metabolic pathways and is of immense importance as it competes with different microorganisms for nutrients as well as niche in the gut and hence acts as an initial line of defense against other invading enteric pathogens (Sweeney et al. 1996, Conway and Cohen 2015). Besides this, *E. coli* also exists in the environment and is often recovered from contaminated water and food (Ishii and Sadowsky 2008). Further, it displays its ubiquitous nature as it is able to survive in water, algal surfaces and soils of tropical, subtropical and even temperate regions. At present *E. coli* stands as the most studied model organism and is employed in several of the laboratory based techniques involved in the studies related to genetic engineering and basic biology (Blount 2015). Apart from this, the pathogenic forms of this usually harmless, commensal organism are the cause of various types of diseases associated with different hosts including humans, mammals, birds etc. and destined at various anatomical sites including both the normal intestinal as well as non-intestinal sites (Kaper et al. 2004). The process of metamorphosis of commensal microorganism into a pathogenic one has traditionally been through uptake of virulence gene repertoires in *E. coli*'s core or pan genome through the acquisition either being phage based, plasmid based, or even mediated through the pathogenic islands etc. (Fig 1) and thus leading to the generation of different pathotypes (Ahmed et al. 2008).

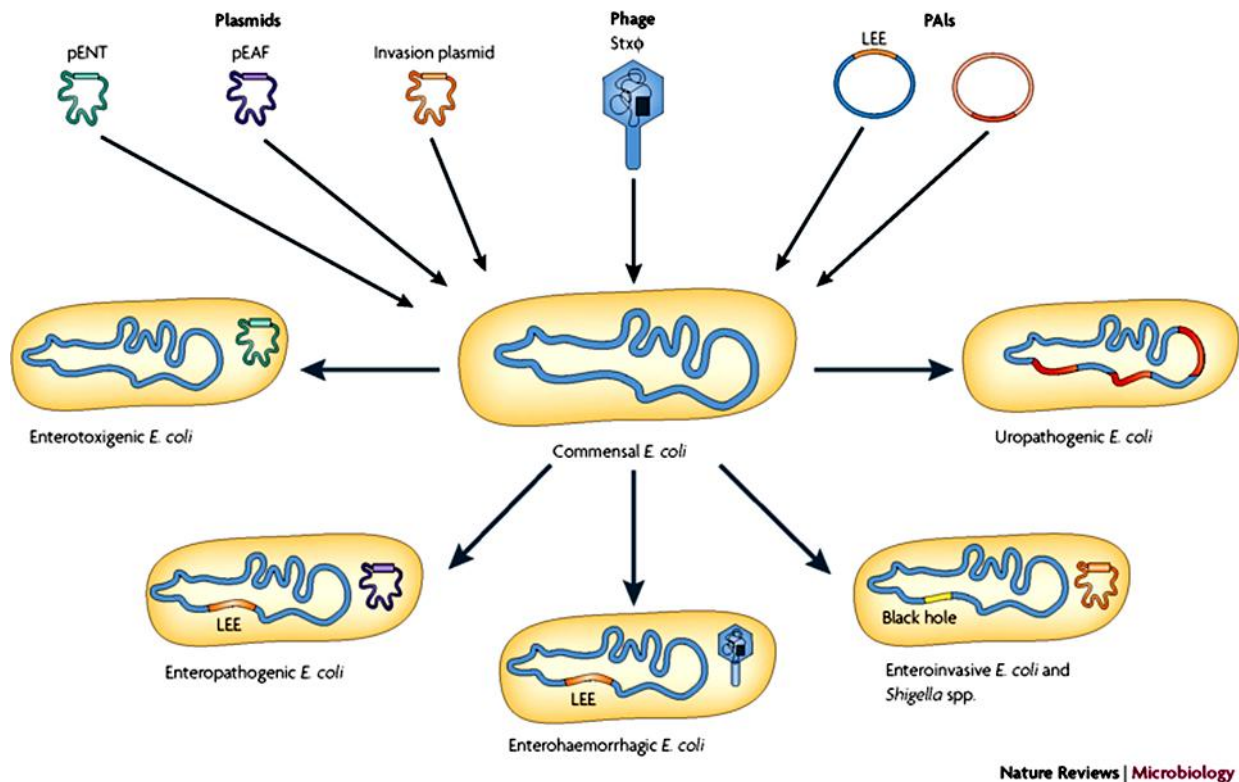


**Fig.1 Evolution of different pathotypes of *E. coli* and the mechanism involved**

(Ahmed et al. 2008)

### ***E. coli* classification**

In general, *E. coli* is divided into two broad categories based on its pathogenicity i.e. non-pathogenic *E. coli* and pathogenic *E. coli*. Pathogenic *E. coli* can further be subdivided into two major groups based on the sites of infections; such as intestinal pathogenic *E. coli* (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Kaper et al. 2004). The intestinal *E. coli* can again be subdivided into six different forms. ExPEC are divided into different forms on the basis of the diseases or hosts or sites it inflicts and are majorly into four pathotypes but some recent ExPEC types are also reported. Fig 2 depicts a brief outline of classification of *E. coli*.

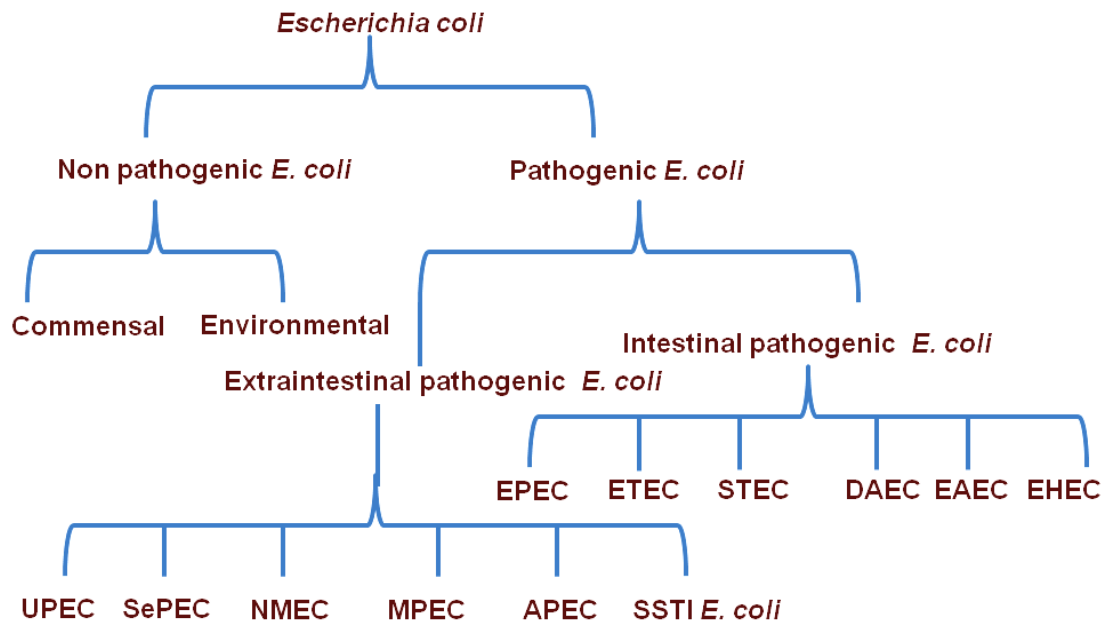


**Fig.2 Schematic representation of *E. coli* classification**

### **Commensal *E. coli***

Commensal *E. coli* are normally the inhabitants of the intestines of higher animals (Wang et al. 2009, Blyton et al. 2013). They do not cause any clinical outcomes in normal conditions except in immunocompromised persons and live symbiotically in association with various other members of intestinal microbiota (Kaper et al. 2004). They are very successful competitors against the enteric pathogens for both nutrients and space in the GI tract and thus ensure protection against them. In general they lack the virulence properties and are typically associated with B1 and A phylogroup of *E. coli* classification (Dobrindt 2005).

### **Environmental *E. coli***

Environment as such is not considered to be the natural habitat of *E. coli* but the organism is isolated from there. Environment often serves as reservoir of subsequent infections in cases involving faeco-oral route of transmission (Brennan et al. 2010). Further, the enumeration of coliform bacteria in water forms an essential approach to monitor the quality of water (van

Elsas et al. 2011). Given hostile growth conditions in soil these organisms often lead a challenging life which helps them acquire some new traits making them even more capable to adapt different niches and increase their host spectrum as well as infection capabilities.

### **Intestinal Pathogenic *E. coli* (InPEC)**

Intestinal pathogenic *E. coli* are associated with various clinical manifestations in the intestines or GI tract of humans and mammals. They are further divided into six different pathotypes based on the nature of diseases they are associated with and type of virulence factors they harness (Kaper et al. 2004, Chen and Frankel 2005).

### **Enteropathogenic *E. coli* (EPEC)**

EPEC was the first pathotype that was known to cause diarrhea in 1945 in UK (Chen and Frankel 2005). The pathotype was isolated only from the diseased children and was absent in healthy individuals. The EPEC infections possess in particular unique characteristics of attaching to intestinal epithelial lining (adhesins) and then inducing changes in the cytoskeleton; these properties are mostly encoded by a 35-kb PAI (LEE) thereby inducing a cascade of events which ultimately results into secretion of ions, increase in permeability in intestine, inflammation etc (McDaniel et al. 1995).

### **Enterohaemorrhagic *E. coli* (EHEC)**

EHEC was first reported in 1982 in humans as cause of several clinical complications namely the bloody diarrhea, normal diarrhea and haemolytic uremic syndrome (Kaper and O'Brien 2014). Most of the earlier cases of EHEC are related to serotype O157:H7 but several other serotypes are also reported. The pathogenesis mainly involves Stx (verocytotoxin) and in some cases locus for enterocyte effacement (LEE) or both (Nguyen and Sperandio 2012, Nataro and Kaper 1998).

### **Enterotoxigenic *E. coli* (ETEC)**

ETEC is the pathotype responsible for watery diarrhea that is self-limiting but sometimes is quite severe in case of children and travelers in developing nations (Qadri et al. 2005). The colonization is mediated by a vast variety of colonization factors (CFs) in humans and K88 and K99 in case of animals (Jin and Zhao 2000). ETEC secretes either a heat labile (LT) or heat stable enterotoxin (ST) or sometimes both. These toxins resemble closely to cholera toxin both in structure as well as functions (Spangler 1992).

### **Enteroinvasive *E. coli* (EIEC)**

EIEC are associated with various complications such as invasive inflammatory colitis, dysentery, watery diarrhea etc (Kaper et al. 2004). The symptoms are similar to other pathotypes and outcome is largely by the virulence plasmid which encodes type III secretion system and often contains several insertion sequences (Tran Van Nhieu et al. 2000). This pathotype is invasive and its virulence is the result of a virulent plasmid and several other factors encoded on its chromosome (Kaper et al. 2004).

### **Enteraggregative *E. coli* (EAEC)**

EAEC are one of the emerging pathotypes and the second most common cause of diarrhea in adults and children, mostly in travelers in all countries irrespective of development status. Mostly the diarrhea is watery but in certain cases mucus or blood might be accompanied (Okhuysen and Dupont 2010). The EAEC colonizes and might cause inflammation of colon but this pathotype is described to be not secreting LT or ST (Kaper et al. 2004). EAEC adheres to the surface in stacked brick configuration and causes clinical complications by virtue of its virulence factors and toxins (Nataro and Kaper 1998, Cieczulin et al. 1997).

### **Diffusely Adherent *E. coli* (DAEC)**

DAEC are characterized by their unique phenomenon of diffuse adherence to HEp-2 cells and majority of them inherit F185 which is a type of fimbrial adhesin (Nataro and Kaper 1998, Bilge et al. 1989). After attachment to surface, the signal cascade is activated using different cell surface associated and intracellular signaling molecules leading to activation of PI-3 kinases (Peiffer et al. 1998). This is associated with diarrhea in children less than 12 years of age and in certain cases it might act as a trigger of inflammatory bowel disease (Giron et al. 1991).

### **Extraintestinal pathogenic *E. coli* (ExPEC)**

ExPEC is the common designation for all the pathotypes which are associated with infections other than the intestinal sites. These include the infections in urinary tract, blood stream, skin, soft tissues, meninges of newborns, mammary glands, different organs in birds etc (Ron 2006). These pathotypes are responsible for wide spectrum of diseases and are characterized into different subtypes. The virulence capacities within these pathotypes vary and they mostly belong to B2 and D phylogroup but nevertheless other phylogroups are also isolated from some clinical cases associated with ExPEC (Bingen et al. 1998, Picard et al. 1999).

### **Neonatal Meningitis *E. coli* (NMEC)**

NMEC pathotype is the normal inhabitant of GI tract and one of the most common cause of bacterial meningitis (Dawson et al. 1999). This bacterium is often acquired from mother parentally and then enters the brain crossing blood brain barrier and leading to severe complications in the form of neurological disorders (Unhanand et al. 1993). This pathotype has different virulence mechanisms such as capsule for protection, ompA for immune

evasion, FimH for adhesion etc; by virtue of these it protects itself and is able to induce clinical complications in brain (Wooster et al. 2006, Kim et al. 2003, Kim 2008).

### **Avian Pathogenic *E. coli* (APEC)**

Avian pathogenic *E. coli* is the pathotype which causes infections in birds such as chicken, turkey, etc. These infections include aerocolitis, polyserositis, septicemia, colibacillosis and others (Dho-Moulin and Fairbrother 1999). The pathogens associated with infections have diverse capabilities and cause various types of infections, both localized and systemic. The pathogenicity and mechanism of APEC infections is still not fully understood but these virtues are attributed to wide varieties of virulence genes which overlap with different ExPEC types (Rodriguez-Siek et al. 2005, Maluta et al. 2014).

### **Sepsis associated *E. coli* (SePEC)**

SePEC is one of the most severe pathotype that causes sepsis/bacteremia which often leads to the damage of tissues/organs, severe inflammatory response syndrome (SIRS) and sometimes even death. This pathotype can originate from any form of infection and finally enters bloodstream and spreads throughout the body (Conceicao et al. 2012). The cases of sepsis infections are very adverse and reported in high numbers requiring continuous monitoring. The pathogenic potential of this pathotype still remains elusive and often has commonality with other ExPEC pathotypes (Poolman and Wacker 2016).

### **Mammary pathogenic *E. coli* (MPEC)**

MPEC is one of the newest pathotypes which is not considered separately but there is a growing demand to consider this pathotype in a separate group (Shpigel et al. 2008). These pathogens cause mastitis in cattle and are one of the frequent problems faced in dairy farms. Only few reports are available for this pathotype which shows high diversity among the underlying strains in terms of their adhesins, LPS etc (Blum et al. 2015). This possibility of

wide variation is particularly due to different niches where they colonize unlike other pathotypes (Richards et al. 2015).

### **Skin and soft tissue infection associated (SSTI) *E. coli***

SSTI *E. coli* pathotype is also one of the newest pathotypes and is commonly implicated in SSTI. This pathotype is very sparsely studied but has high virulence potential in terms of affiliation to B2 and D phylogroups (Petkovsek et al. 2009). The organisms of this pathotype are often self limiting but in certain cases they might lead to severe complications requiring antibiotic therapy. The pathobiology of SSTI has not been explored properly till date and needs to be investigated in detail.

### **Uropathogenic *E. coli* (UPEC)**

This pathotype which gains access to the urinary tract through ascending infection and causes clinical outcomes such as those ranging from non symptomatic UTI to life threatening cases of urosepsis is termed as UPEC (Stamm 1991, Totsika et al. 2012). UPEC strains have wide varieties of virulence factors which enable them to cause different complications as above (Wiles et al. 2008, Flores-Mireles et al. 2015). UPEC is the most studied pathotype among ExPEC and are majorly affiliated to B2 and D phylogroups of Clermont's phylogrouping. Apart from virulence, the growing multi drug resistance among them is a major challenge in their treatment and management.

### **Diseases Associated with ExPEC**

ExPEC are associated with wide varieties of infections in humans, birds, cattle, companion animals etc. These infections might appear as mild as skin and soft tissues infections (which are mainly self-limiting but in certain cases may need antibiotic therapy) and also as some of the severe forms such as sepsis which leads to tissue damage and death in extreme cases. Colisepticemia and mastitis are few of the diseases which are encountered in poultry and

dairy industries causing huge economic losses (Johnson et al. 2008). In humans, urinary tract infections are quite common and have substantial impact in terms of losses to economy and quality of life (Ewers et al. 2007). The infections associated with ExPEC pathotypes are as follows:

### **Skin and soft tissue infections (SSTI)**

SSTI are one of the most common forms of infections encountered in daily life of most of the individuals. Many microorganisms are isolated from these infections which majorly include *S. aureus* or other Gram positive bacteria (Ki and Rotstein 2008). In almost all the cases *E. coli* is also isolated from these infections but the studies related to its association remain scarce, with only limited data available for SSTI *E. coli* (Petkovsek et al. 2009) and hence they need to be interpreted in clearer terms in present time.

### **Sepsis**

Sepsis is one of the most severe forms of infections in which the patient suffers from severe inflammatory response (SIRS) from the body in the event of an established infection (Lever and Mackenzie 2007). It is the leading cause of death worldwide with a death toll of about 300 per 100000 in US (Mayr et al. 2014). Among the various causative agents, 62 % of all cases were reported due to Gram negative infections by EPIC II, among which 16% were associated with *E. coli* (Vincent et al. 2009, Mayr et al. 2014). The genitourinary tract is involved in about 10% of males and 18% in females with mortality up to ~7-9% (Esper et al. 2006). As *E. coli* is a major organism in UTIs, it is also a leading cause for UTI derived sepsis. Besides this, nearly 20% of the cases of bacteria are not site specific while about 7-9% are due to wound/soft tissue infections (Mayr et al. 2014).

## **Poultry diseases**

*E. coli* infections are the most common infections in poultry farms infecting chickens of nearly all ages. The underlying diseases include the infections of oviduct, yolk sac, subcutaneous layer, eye balls, joints, bone marrow and systemic infection (coli septicemia) etc. Thus, *E. coli* is one of the biggest threats to chickens, turkey, ducks etc. and accounts for huge losses in terms of revenue (Dho-Moulin and Fairbrother 1999). Besides this, they also pose threat to human health as they might act as reservoir to human infections as the avian pathogenic strains show various commonalities with the strains causing human infections in terms of virulence factors and thus having zoonotic potential (Ewers et al. 2007, Nandanwar et al. 2014).

## **Meningitis**

NMEC pathotype is associated with meningitis in newborns leading to neurological sequel or abnormal development and can even be fatal if left untreated (Bonacorsi and Bingen 2005). The victims of this infection are usually less than four weeks but in certain cases they may be 6-8 weeks also. Early therapy and development of treatment strategies over time has decreased fatality from 50% (1970s) to less than 10% (2010) (Uchida et al. 2011). The rate of such infections in developing countries is even higher in present times and needs serious attention.

## **Mastitis**

Milk is an essential dietary component worldwide and its production is related to the health of cattle and hygiene of the farms. Mastitis is the most common infection of the mammary tissues due to which the milk production is reduced (Suojala et al. 2013). Substantial revenue allocations need to be infused to maintain hygienic conditions in order to prevent such infections in which *E. coli* is one of the most commonly implicated organism. According to

an estimate, about 61- 97 Euros on an average are lost due to mastitis worldwide per cow (Hogeveen et al. 2011). The developing countries have even higher infection load due to poor farm facilities and improper rearing of cattle.

### **Urinary tract infections (UTI)**

Urinary tract infections are most common forms of ExPEC infections worldwide resulting in a huge loss of economy due to morbidity and mortality associated with it. The clinical stages ranging from non symptomatic UTI to symptomatic UTI (Urethritis) then cystitis when the bacteria infect and colonize bladder, further progressing to kidney causing pyelonephritis and lastly invading into the blood stream to cause urosepsis; the latter often leads to kidney injury and organ failures. The brief outline of the UTI and its clinical stages is depicted in Fig 3. *E. coli* being the primary organism in UTI accounts for about 80-90% of infections and according to an estimate every woman might have at least one episode of UTI in their lifetime (Foxman and Brown 2003, Wang et al. 2013). It is the most common in females due to anatomical predisposition but is also prevalent in old aged men (Foxman 2003). In developing countries like India it is quite common infection due to the socio-economic reasons as well as due to less developed healthcare and sanitation facilities and therefore needs honest attention as it adversely affects the quality of life besides impacting economically.

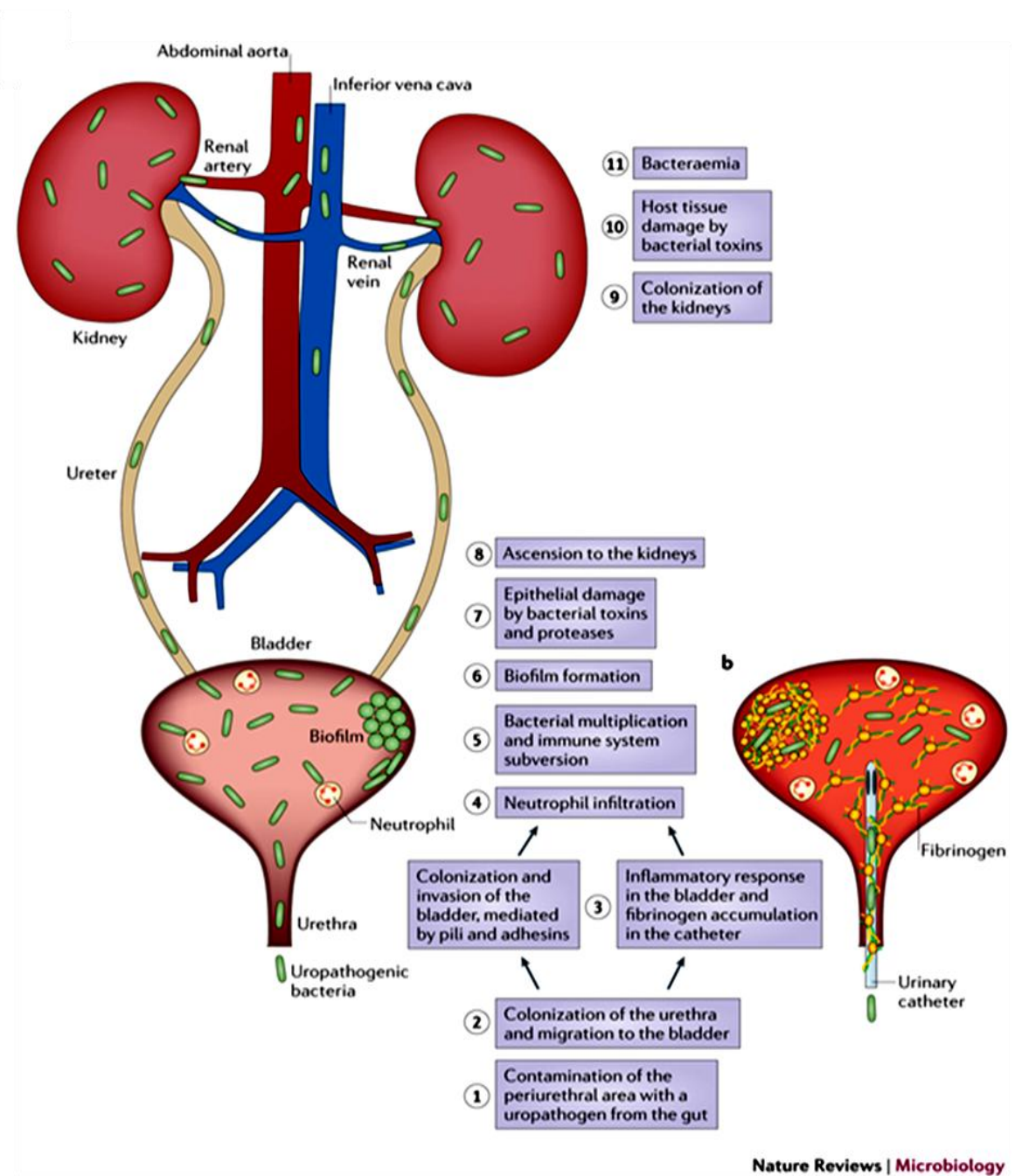


Fig.3 An overview of Urinary tract infection and its progression to sepsis in normal and catheter based infection modules (Flores-Mireles et al. 2015)

## Virulence factors

In order to have capabilities of infections in wide varieties of hosts and different anatomical sites, an organism must be equipped with a wide range of virulence associated genes or factors (Johnson 1991). *E. coli* enjoys this wide spectrum of virulence factors which can be classified into four major classes based on the activities they perform during the successful colonization/ infection.

- a) Adhesins and invasins
- b) Toxins
- c) Protectants
- d) Siderophores and iron acquisition system

By the virtue of adhesins, these pathogens first attach to the surface of epithelial cells as an initial stage for colonization. In certain cases, the organism further invades epithelial cells thus recruiting immune cells at the site and also leading to an ascending infection. Once the niche is established, the organism secretes toxins which are capable of inducing damage to the host epithelial cells and thus making their niche even more favorable (Flores-Mireles et al. 2015). As iron plays an important role for both host and the pathogen, there is always a competition for it (Gorlina 1985, Skaar 2010). In order to win this competition, ExPEC forms siderophores and also encodes various other iron acquisition systems (Gao et al. 2012). To protect themselves from the deleterious effect of serum and immune system they secrete certain protectants. In addition, biofilm formed at the infection site further helps them in protection as well as creates an area of high genetic exchange and drug inaccessibility (Beloin et al. 2008). Overall, the combined virtues of these virulence factors help ExPEC strains to cause infection at varied sites as well as in different hosts. Table1 represents a brief description of virulence factors and genes.

**Table1. Characterisation of virulenes factors of ExPEC (Köhler CD and Dobrindt U, 2011)**

| Functional category                              | Virulence factor                                                                                                                                                                                                                                                                                                     |
|--------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Adhesins and Invasins</b>                     | Type 1 fimbriae(Fim)<br>P fimbriae (Pap/Prf)<br>S/F1C fimbriae (Sfa/Foc)<br>N-acetyl d-glucosamine-specific fimbriae (Gaf)<br>M-agglutinin (Bma)<br>Bifunctional enterobactin receptor/adhesin (Iha)<br>Afimbrial adhesin (Afa)<br>Temperature sensitive hemagglutinin (Tsh)<br>Invasion of brain endothelium (IbeA) |
| <b>Siderophores and Iron acquisition systems</b> | Siderophore receptor (IreA)<br>Aerobactin (Iuc)<br>Yersiniabactin (Ybt)<br>Salmochelin (Iro)<br>Periplasmic iron binding protein (SitA)                                                                                                                                                                              |
| <b>Toxins</b>                                    | alpha-Hemolysin (HlyA)<br>Cytolethal distending toxin IV (CDT 1)<br>Cytotoxic necrotizing factor 1 (CNF-1)<br>Putative hemolysin (HlyF)<br>Colibactin (Clb)<br>Serine protease autotransporters Sat, Pic                                                                                                             |
| <b>Protectins</b>                                | Group II capsule incl. K1 capsule<br>Conjugal transfer surface exclusion protein (TraT)<br>Outer membrane protease T (OmpT)<br>Increased serum survival (Iss)<br>Colicin V (Cva)                                                                                                                                     |
| <b>Others</b>                                    | d-Serine deaminase (DsdA)<br>Maltose and glucose-specific PTS transporter subunit IICB (MalX)<br>Flagella                                                                                                                                                                                                            |

## Treatment of ExPEC infections

Treatment of ExPEC infections is majorly carried out with empirical antibiotics. The most important choice for these Gram negative infections are broad spectrum antibiotics such as IIIrd generation cephalosporins. The second most used antibiotics are the fluroquinolones. In certain cases, the choice of antibiotics also depends on the site of infection as like

Nitrofurantoin is obvious choice in terms of Urinary tract infections (Flores-Mireles et al. 2015). As there is evolution of multi drug resistant pathogens, treatment is very challenging and sometimes includes use of IVth generation cephalosporins (carbapenems). These antibiotics are termed as the last resort antibiotics due to their wide spectrum and less resistant phenotypes. These antibiotics include the carbapenems, colistin, azetronem etc. and in certain cases combinational therapies are also used depending on severity of infections (Papp-Wallace et al. 2011).

## **Major challenges in treatment**

At present the treatment of Gram negative *E. coli* infections poses two major challenges. These challenges are intriguing either due to the intrinsic evolutionary forces within the organism or by extrinsic forces such as antibiotic pressure, environmental stress, host factors etc. Thus, the two major challenges are the evolution of successful lineages within the ExPEc isolates and growing multi drug resistant strains limiting the treatment options as well as failures during the treatment.

## **Evolution of successful lineages**

The on going process of evolution within the pathogenic *E. coli* has lead to evolution of certain clones which have disseminated throughout the world as successful lineages (Petty et al. 2014). Some of the prominent and successful lineages are ST131, ST38, ST95, ST10, ST73, ST101, ST648, ST410 etc. The dual combination of virulence and multi drug resistance features such as that in sequence type ST131 has been the prime attraction throughout the world as this clone has disseminated globally and isolated from nearly all possible sources of infection (Pitout 2012, Petty et al. 2014). One in-depth study has further suggested the clonal diversification of this lineage to explain its better survival advantages and thus one of the reasons for global dissemination (Banerjee et al. 2013). ST131's initial

evolution was due to its ability to resist fluroquinolones but, rapid acquisition of CTX-M plasmids has projected it be the most successful clonal type of the present times (Ben Zakour et al. 2016). Besides this, there are also other sequence types such as ST648 which comprise of D phylogroup which contains mostly the ESBL producing strains (Ewers et al. 2014). ST95, ST73 etc are other successful lineages from poultry and urinary tract infections, respectively (Johnson et al. 2008, Lau et al. 2008). ST10 is reported to be mostly associated with commensal *E. coli* types (Manges and Johnson 2012).

### **Generation of multi drug resistance (MDR)**

The utility of antibiotics has always been important as antibiotic pressure is one of the driving forces in evolution of multidrug resistant organisms. *E. coli* is one of the microorganism which have undergone evolution by gradual increase in their genome size which might be due to multiple processes including horizontal gene transfers (Nikaido 2009). The *E. coli* strains conjugate easily and thus plasmids are one of the efficient means of exchange of genetic materials which majorly involve the resistance determinants (Johnson and Nolan 2009). Further, the acquisition through phages is also quite easy in case of *E. coli* and the different efflux pumps play significant roles in resistance development (Drulis-Kawa et al. 2012, Nikaido and Pages 2012). The indiscriminate use of antibiotics and self medication enhances this process and poses grave complications. Fig 4 briefly outlines the major forces that drive the evolution of multi drug resistant phenotypes in bacteria.

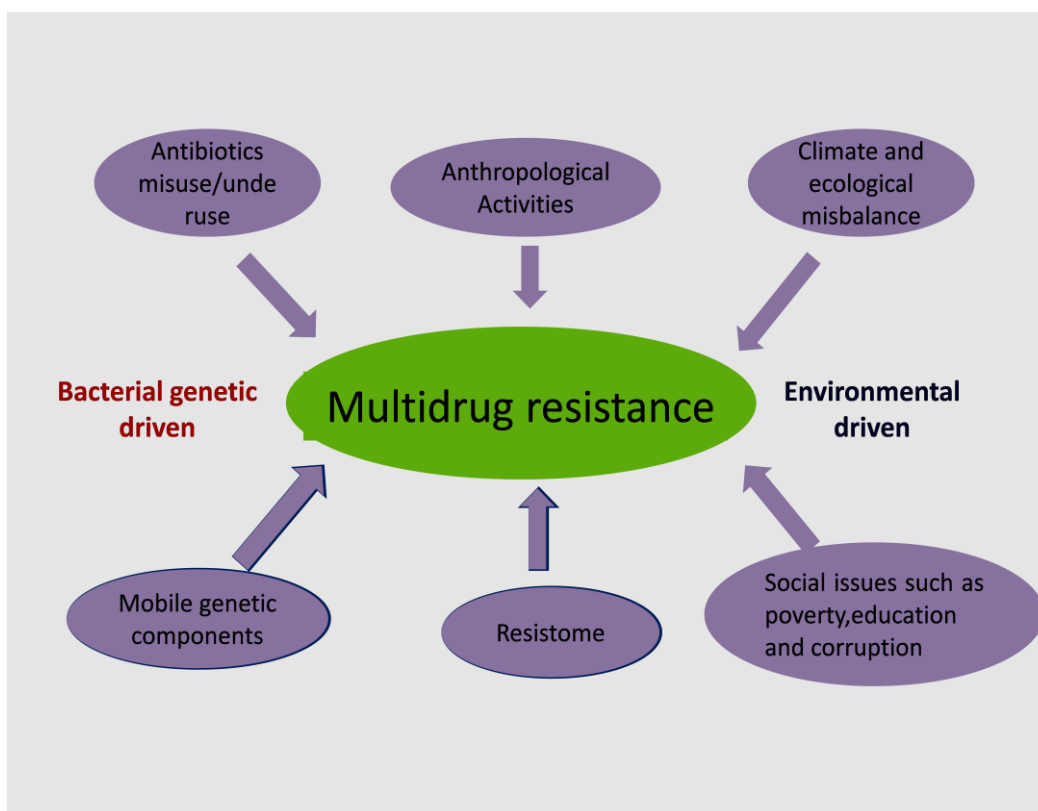


**Fig.4 Factors contributing to evolution of multidrug resistance phenotypes in bacteria**

At present *E. coli* are resistant to most of the commonly used antibiotics, including the beta lactam antibiotics such as cephalosporins, sulphaamides, fluoroquinolones, tri-methoprine, aminoglycosides, tetracyclines, macrolides etc (Hawkey 2008, Boyd et al. 2008, Tadesse et al. 2012, Foxman 2010). Thus, treatment of multidrug resistant organisms is quite challenging as very few antibiotics are left as choice for treatment. Particularly, India appears to be in a very hazardous situation as ~80% of infections are reported to be due to ESBL producers (Fig 5).

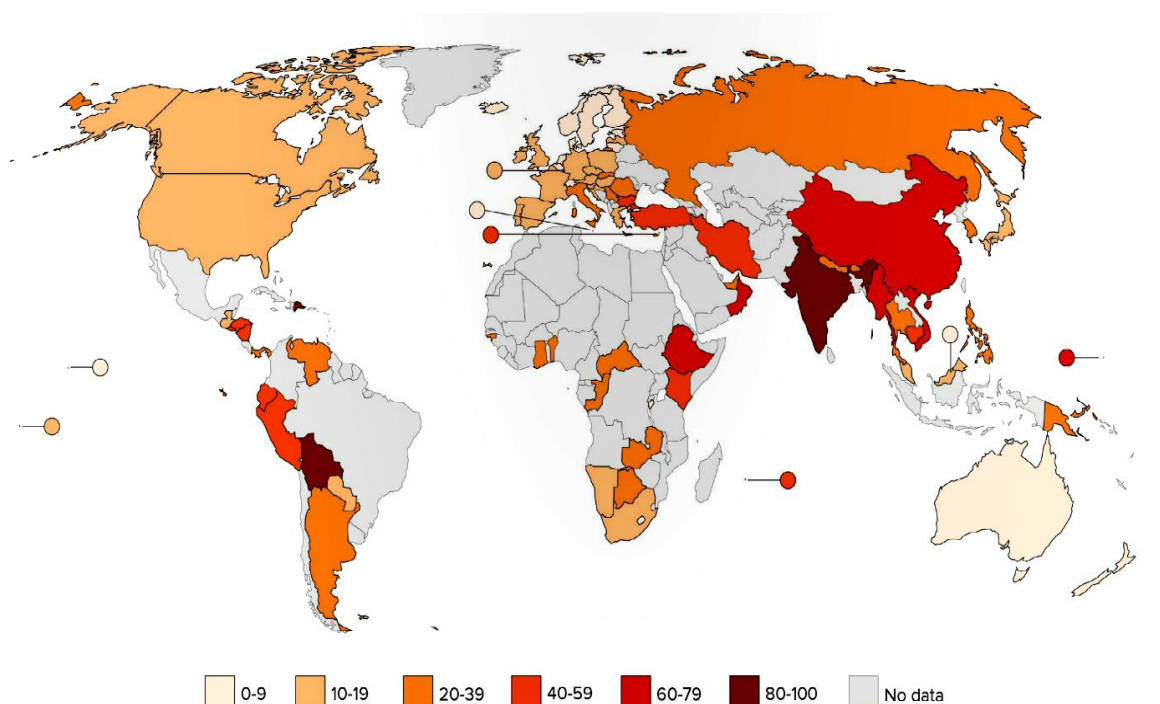


**Fig.5 Distribution of ESBL phenotypes around the world according to CDDEP 2015, WHO 2014 and PAHO**

### **ExPEC infections and scientific relevance of this study in the Indian context**

ExPEC infections are one of the most common infections worldwide. At present, India has the second largest population in the world. Thus, considering these factors, it is quite clear that infections of ExPEC would likely inflict severe impact on country's development. Among all the infections, urinary tract infections are the most prevalent in developing countries. In certain cases, ExPEC infections might lead to tissue and organ damage such as that of kidneys and in most severe cases it may lead to multi organ failure due to sepsis and ultimately death. Thus, it costs in terms of life/life quality of humans, livestock and poultry etc. as well as inflicts huge losses to the economy as enormous money is drained in management and treatment of these infections. These infections are more prevalent in India

due to lack of proper sanitary and healthcare facilities. Open defecation is another major cause for the spread of pathogens in the environment. The ease of access to antibiotics, self medication, lack of health education in addition to the non-effective antibiotic policies in India up-thrust these infections in this part of the world. Besides this, the hot and humid climatic conditions of India also act as one of the facilitators wherein these pathogens enjoy privilege of growing well and undergoing continuous evolution in terms of better adaptation. Previous studies from our group have shown UPEC infections to be endemic in this region and have revealed the presence of different lineages and genotypes of UPEC pathogens in India (Jadhav et al. 2011). Further, the presence of multidrug resistant clones of globally disseminated ST131 inheriting bla<sub>ctx-m-15</sub>, an ESBL enzyme, has also been observed and the similarities of this clone within the isolates of clinical, non clinical as well as faecal isolates has been demonstrated (Hussain et al. 2012, Hussain et al. 2014). With these observations, it appeared imperative to glean molecular epidemiology of these pathogens with deeper insights into biology of the ExPEC pathogens and to have a comprehensive view of the underlying mechanisms as well as the evolutionary snapshot of them. In order to understand this, the following objectives were laid down:

1. Functional molecular infection epidemiology of extraintestinal pathogenic *E. coli*
2. Characterization of a representative strain of highly virulent subclone from India
3. Molecular epidemiology of New Delhi metallo-beta-lactamase (NDM) positive, carbapenem resistant extraintestinal pathogenic *E. coli*

These objectives were carried out using rigorous phenotypic and functional analyses in conjunction with whole genome based sequence information and thus projecting a comprehensive view of ExPEC pathogens in the Indian context with respect to their phylogenetic grouping, virulence acumen, and multi drug resistant characteristics as well as comparative genomics was deduced.

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## Chapter 2:

### Objective 1

Functional molecular infection  
epidemiology of extraintestinal  
pathogenic *E. coli*

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This part is under communication as:

Comparative Genomics Reveal *Escherichia coli* skin and soft tissue infection isolates shares indispensable genetic and functional traits with isolates from other Extra-intestinal Infections



## Abstract

*Escherichia coli*, a pathogenic Gram-negative bacterium, is associated with a wide variety of diseases other than the intestinal infections such as urinary tract infections (UTIs), meningitis in neonates, septicemia, skin and soft tissue infections (SSTI), colisepticemia and thus broadly categorized as extra intestinal pathogenic *E. coli* (ExPEC). As an opportunistic pathogen responsible for cross infections, *E. coli* can also pose a high health risk of zoonotic diseases, although comparative genomic data on the functional and genetic coordinates for the ExPEC strains responsible for different types of infections are non-conclusive. In the present study, ExPEC *E. coli* from SSTI were characterized for virulence and drug resistance profiles and compared with those isolated from pyelonephritis and septicemia. Results revealed majority of the tested isolates to belong to pathogenic phylogroups B2 and D, while 67% of the isolates were multi-drug resistant (MDR) with 85% producing ESBL, and 6% metallo-beta-lactamase (MBL). *bla*<sub>ctx-m-15</sub> genotype was found to be associated with at least 70% of *E. coli* isolates under each category conferring resistance towards extended-spectrum of beta-lactam antibiotics. Whole genome sequencing and comparative genomics of eight ExPEC strains revealed two isolates NA633 and NA643 from SSTI infection to belong to pandemic sequence type ST131 while functional characteristics within three ExPEC pathotypes revealed equal capabilities of biofilm formation and to resist human serum. Despite of high similarities with respect to resistome, virulome, and sequence types specific genomic arrangements, strains from different infections lacked any disease-specific functional and genetic co-ordinates.

## Importance

Infections due to extra intestinal pathogenic *E. coli* (ExPEC) are of global concern as they imply huge public health budget in the management of healthcare facilities. The recent emergence of multi-drug resistance in pandemic *E. coli* clone ST131 is of primary interest among *E. coli* infections due to its global dissemination. In developing countries such as India, SSTI infections associated with *E. coli* are very marginally attempted. In this study, we employed genomic as well as phenotypic assays to deduce relationship among different ExPEC pathotypes and observed similarities between antibiotic resistances and virulence profiles. ST131 strains from SSTI infections were reported and revealed invariable genomic similarities across isolates of different disease conditions. Thus, the study represents functional molecular infection epidemiology insights of SSTI *E. coli* in comparison with other ExPEC pathotypes.

## Introduction

*Escherichia coli* is one of the most important cause of extra-intestinal infections with a potential to cause infection in almost any anatomical site and grouped into pathotypes such as uropathogenic *E. coli* (UPEC), septicemia associated *E. coli* (SePEC), skin and soft tissue infections associated *E. coli* (SSTI), neonatal meningitis causing *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC) (1, 2). UTI alone accounts for about 25% of all the cases of septic shock while soft tissue infections account for 10% of severe sepsis cases (3, 4). The clinical stages of UTI vary from non-symptomatic acute infections to sometimes severe septic shock. In the latter case, the infection progresses from urinary tract to bladder (cystitis), from bladder to kidneys (pyelonephritis), and finally into the blood stream (urosepsis). Skin and soft tissue infections (SSTI) are other major bacterial infections which are often self-limiting but severe cases may require hospitalization and parenteral antibiotic therapy. With the indiscriminate use of antibiotics and emergence of multi drug resistant organisms such as that of ST131 and carbapenem resistant organisms, treatment has become very challenging (5-7). Despite *E. coli* associated with the SSTI remains an important pathotype to be explored, to our knowledge only *E. coli* isolates from UTI have been previously characterized from India (8, 9). We designed this study to comprehensively characterize and deduce the functional molecular infection insight of *E. coli* associated with SSTI. We also determined the phylogenetic relationships, virulence profiles, antibiotic resistance patterns, and functional attributes, and finally whole genomes sequenced and analyzed the genomes of *E. coli* strains in comparison with septicemia and pyelonephritis isolates from same endemic setting of India.

## Results

### *E. coli* phylogenetic groups, Antimicrobial resistance and Virulence genotypes

Majority of the isolates belonged to virulent B2 (36%) and D (29%) phylogroups while 23% to A and rest 12% to B1 (Table1). Of the three disease types of *E. coli* confirmed, more than half of the isolates belonged to B2 and D phylogroups. In total, 73 (93%) isolates were found to be resistant to at least one of the six antimicrobial agents tested with the highest resistance towards ciprofloxacin (86%), followed by tetracycline (77%), cotrimoxazole (72%), gentamicin (31%), chloramphenicol (23%) and fosfomycin (5%). Overall, 67% of *E. coli* isolates were multi-drug resistant (MDR) with pyelonephritis, septicemia and SSTI *E. coli* contributing to 0.40, 0.32 and 0.26 fractions, respectively (Table S1). ESBL producing *E. coli* was found to be as high as 85%, and prevalence was found to be similar among all three disease types. Only 6% of the isolates were found to be MBL producers. Resistance gene profiling appeared in line with the phenotypic observations, with bla<sub>CTX-m-15</sub> as predominant genotype among all groups and NDM-1 was common among all MBL producers (table S1). Both *su1* and *su2* were observed in at least 71% of the isolates, in all three groups conferring resistance to sulphonamides, while plasmid based fluoroquinolone resistance gene (*aac(6')-Ib-cr*) occurred in 30% and 71% of the pyelonephritis and SSTI isolates.

The virulence profiling revealed that the *fimH* was present in ~90% of isolates in each category except for SSTI isolates (62%), the *papC* was prevalent in isolates belonging to septicemia and pyelonephritis. However, *afaB/C* and *sfaD/E* were found in varied proportion among all three groups. Among three toxin types, the *ctxA* was detected to a lesser extent among the pyelonephritis and septicemia isolates. Prevalence of protectins *traT* and *ibeA* was observed to be similar and iron acquisition genes *iroN* and *iucD* was comparable among all three groups (table1).

### ***Whole genome based resistome, virulome and comparative analysis***

Altogether, 12 strains comprising four from each of the three disease types were subjected to comparative genomic analysis to get a broader perspective of the complexity of these disease types of *E. coli*. Of the 12 genomes included in the present study, genome sequencing data from four strains were made available from our previous and ongoing studies, including two reference strains NA097 and NA114 (10, 11). The remaining eight strains were freshly subjected to whole genome sequencing (WGS). Whole genome based resistome profiles as analyzed confirmed these strains to be multi drug resistant. All strains demonstrated more or less similar combinations of resistance genes encoding for antibiotic efflux pumps, regulators, antibiotic inactivation and target modification genes. Similar to resistome, the virulome of these isolates also demonstrated little pathotype-specific variations. Further, *in-silico* MLST assigned two strains from SSTI (NA633 and NA643) to the pandemic ST131 clone while rest of the strains belonged to different sequence types (STs) such as ST38, ST68, ST405 and ST 617 (table S2).

Comparative genomic analyses of the WGS data using BLAST Ring Image Generator (BRIG) platform revealed sequence type-specific similarities wherein all five ST131 strains demonstrated similar arrangement of genomic regions while isolates belonging to other STs demonstrated dissimilar regions. This analysis negates any disease specific genomic features within these isolates.

### ***Functional virulence determination***

A total of 30 *E. coli* isolates comprising 10 from each category were analyzed for functional virulence determinants. Results revealed similar biofilm formation capabilities within all of the isolates in M63 medium after 48 hrs of incubation. Most of the isolates were found to be strong biofilm formers (Fig 1). Further, the isolates were also able to grow in human serum (50%). The capability to resist serum deleterious effect varied within isolates but differed

only insignificantly among the three disease types (Fig 1). These virulence assays demonstrated phenotypic virulence nature of these genetically diverse *E. coli* isolates.

## Discussion

Infections caused by extra intestinal pathogenic *E. coli* (ExPEC) are most common worldwide. In the present study, we observed that septicemia, pyelonephritis, and SSTI *E. coli* isolates belonged mostly to B2 and D phylogroups, which are considered to be associated with virulent *E. coli* groups(12, 13). These observations are similar to the findings of *Petkovsek et al* wherein SSTI *E. coli* were found to be of marked potential in respect to presence of virulence factors (VFs) (14) while *Ananias and Yano* have also shown various VFs to be common among different Sepsis associated *E. coli* (SePEC) isolates and are able to invade vero cell lines (15). The whole genome based virulome ascertained similar virulence repertoire complementing above observations.

Recent studies have reported considerable increase in multi-drug resistant (MDR) and extended spectrum beta lactamase (ESBL) genotypes in ExPEC associated with infections globally (16, 17). In the present study, majority (67%) of the ExPEC isolates were MDR, and CTX-M-15 was determined to be the predominant ESBL genotype (table S1 and 1). Furthermore, among the various combination of resistance genes and phenotypes, the dual combination of ESBL+ fluoroquinolone<sup>R</sup> was the most frequent, followed by CTX-M-15<sup>+</sup>, fluoroquinolone<sup>R</sup>, cotrimoxazole<sup>R</sup> and CTX-M-15<sup>+</sup>, fluoroquinolone<sup>R</sup>, cotrimoxazole<sup>R</sup>, tetracycline<sup>R</sup> (tableS3). The predominance of such large combinations of antimicrobial resistance genes implies presence of multiple plasmids carrying several antibiotic resistance genes. The resistome based on WGS reflects similar observations for the presence of multiple genes. MBL phenotype in all isolates was associated with the presence of NDM gene reflecting dissemination of carbapenim resistance in India via NDM (7).

ExPEC is known to be evolving and disseminating globally through clonal expansion having similar genetic architecture but also demonstrates different strain specific features (10, 18). In our study we observed similarities in virulence and resistance coordinates of different strains belonging to diverse sequence types (STs) such as ST131, ST38, ST68, ST405, and ST617. However, higher ST-specific commonality was observed among the strains rather than disease specific similarity (Fig 1). These observations provide a basis for the supposition that strains with diverse STs are capable of causing similar infection outcomes. Further, *E. coli* belonging to ST131 was found for the first time from SSTI infections in India.

In summary, our results suggest that the ExPEC isolates associated with pyelonephritis, septicemia, and SSTI have a considerable overlap with regard to phylogroups, virulence, drug resistance mechanisms, genomic and functional properties; and they didn't exhibit any significant association with any of the particular pathotype. Thus, the isolates belonging to different ExPEC pathotypes demonstrate equal predilection to cause varying types of infections.

## **Material and methods**

Please refer supplementary for detailed methodology.

### ***Bacterial strains***

A total of 78 isolates comprising 21 isolates from pus of patients suffering from different infections associated with skin and soft tissues in the year 2015 from DY Patil hospital, Pune and 57 *E. coli* isolates from our previous collection of January 2009 to December 2012 was employed for this study. All isolates were identified and preserved by standard laboratory methods.

### ***Phylogenetic grouping, virulence and resistance genotyping:***

PCR based phylogrouping, virulence genotyping and resistance gene profiling were performed using gene specific primers. Details may be found in supplementary file.

### ***Antimicrobial resistance and phenotypic virulence assay***

Extended spectrum beta lactamase (ESBL) production and antimicrobial resistance determination were performed by the standard disc diffusion method on Muller-Hinton agar (HiMedia) as per Kirby Bauer method and CLSI (Clinical and Laboratory Standards Institute) guidelines. Phenotypic virulence assays such as biofilm formation and serum resistance were performed as described previously (9, 10).

### ***Whole genome sequencing and comparative genomics***

Eight isolates from this study were sequenced and comparative genomics were performed with four other strains of our previous and ongoing studies to generate virulome, resistome and apply comparative genomics. The detailed methodology is described in supplementary section.

### ***Statistical analysis***

Statistical analysis for virulence and resistance genes was performed employing Chi square test and Mann Whitney test for serum resistance and biofilm formation.

### ***Accession numbers***

The accession numbers of the eight genomes sequenced for this study are MJDL00000000 (NA643), MJDK00000000 (NA635), MJDJ00000000 (NA633), JWHS00000000 (NA447), MJDI00000000 (NA267), MJGD00000000 (NA259), MKHD00000000 (NA056) and JSXL00000000 (NA057).

## Acknowledgements

We acknowledge funding received from Department of Biotechnology, Government of India [Ref.No.BT/HRD/NBA/34/01/2011(ix)] and support from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673) - functional molecular infection epidemiology, an initiative of the German Research Foundation (DFG) and the University of Hyderabad (India), of which NA and LHW served as speakers. NA is an Adjunct Professor at the Academy of Scientific and Innovative Research (ACSIR), India. AR would like to acknowledge the Indian Council of Medical Research (ICMR) for the fellowship.

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**Table 1:** Phylogenetic classification, virulence and antimicrobial resistance genotyping of extra intestinal pathogenic *E. coli* (ExPEC) strains (n=78)

|                                 |                      | Pyelonephritis<br><i>n</i> =30 (%) | Septicemia<br><i>n</i> =27 (%) | Skin and soft tissue<br>infections <i>n</i> =21 (%) |
|---------------------------------|----------------------|------------------------------------|--------------------------------|-----------------------------------------------------|
| Phylogenetic grouping           |                      |                                    |                                |                                                     |
| <b>A</b>                        |                      | 2 (7)                              | 8 (30)                         | 8 (38)                                              |
| <b>B1</b>                       |                      | 3 (10)                             | 4 (15)                         | 2 (10)                                              |
| <b>B2</b>                       |                      | 15 (50)                            | 7 (26)                         | 6 (29)                                              |
| <b>D</b>                        |                      | 10 (33)                            | 8 (30)                         | 5 (24)                                              |
| Virulence factors               |                      |                                    |                                |                                                     |
| <b>Toxins</b>                   | <i>sat</i>           | 16 (53)                            | 9 (33)                         | 6 (29)                                              |
|                                 | <i>usp</i>           | 10 (33)                            | 6(22)                          | 6 (29)                                              |
|                                 | <i>cvaC</i>          | 6 (20)                             | 6 (22)                         | 15 (71) *                                           |
| <b>Adhesins</b>                 | <i>fimH</i>          | 27 (90)                            | 24 (89)                        | 13 (62)*                                            |
|                                 | <i>papC</i>          | 28 (93)                            | 25 (93)                        | 5 (24) *                                            |
|                                 | <i>afaB/ C</i>       | 8 (27)                             | 1 (4)                          | 1 (05)                                              |
|                                 | <i>sfaD/E</i>        | 2 (7)                              | 2 (7)                          | 1 (05)                                              |
| <b>Protectins</b>               | <i>traT</i>          | 5 (17)                             | 4 (15)                         | 6 (29)                                              |
|                                 | <i>ibeA</i>          | 3 (10)                             | 3 (11)                         | 1 (05)                                              |
| <b>Iron acquisition</b>         | <i>iroN</i>          | 27 (90)                            | 21 (78)                        | 16 (76)                                             |
|                                 | <i>iucD</i>          | 19 (63)                            | 16 (59)                        | 15 (71)                                             |
| Antibiotic resistance profiling |                      |                                    |                                |                                                     |
| Antibiotic class                | Specific trait       |                                    |                                |                                                     |
| <b>ESBL</b>                     | <i>Ctxm-15</i>       | 21(70)                             | 24(89)                         | 17(81)                                              |
|                                 | <i>tem</i>           | 15(50)                             | 14(52)                         | 04(19)*                                             |
|                                 | <i>Ctxm+ tem</i>     | 11(37)                             | 12(44)                         | 04(19)                                              |
| <b>Metallo betalactamase</b>    | <i>ndm1</i>          | 0(0) <sup>nc</sup>                 | 1(04)                          | 5(24)*                                              |
| <b>Tetracyclines</b>            | <i>tetA</i>          | 20(67)                             | 17(63)                         | 08(38)                                              |
| <b>Aminoglycosides</b>          | <i>strA</i>          | 14(47)                             | 10(37)                         | 9(43)                                               |
| <b>Fluoroquinolone</b>          | <i>aac(6')-Ib-cr</i> | 9(30)                              | 13(48)                         | 15(71)                                              |
| <b>Sulfonamides</b>             | <i>sul1</i>          | 25(83)                             | 23(82)                         | 15(71)                                              |
|                                 | <i>sul2</i>          | 12(40)                             | 7(26)                          | 06(29)                                              |
| <b>Trimethoprim</b>             | <i>dhfr</i>          | 8(27)                              | 09(33)                         | 2(10)                                               |
| <b>Integrin</b>                 | <i>int1</i>          | 18(60)                             | 15(56)                         | 13(62)                                              |

\* denotes P<0.05 when compared to septicemia group

**Figure 1:**

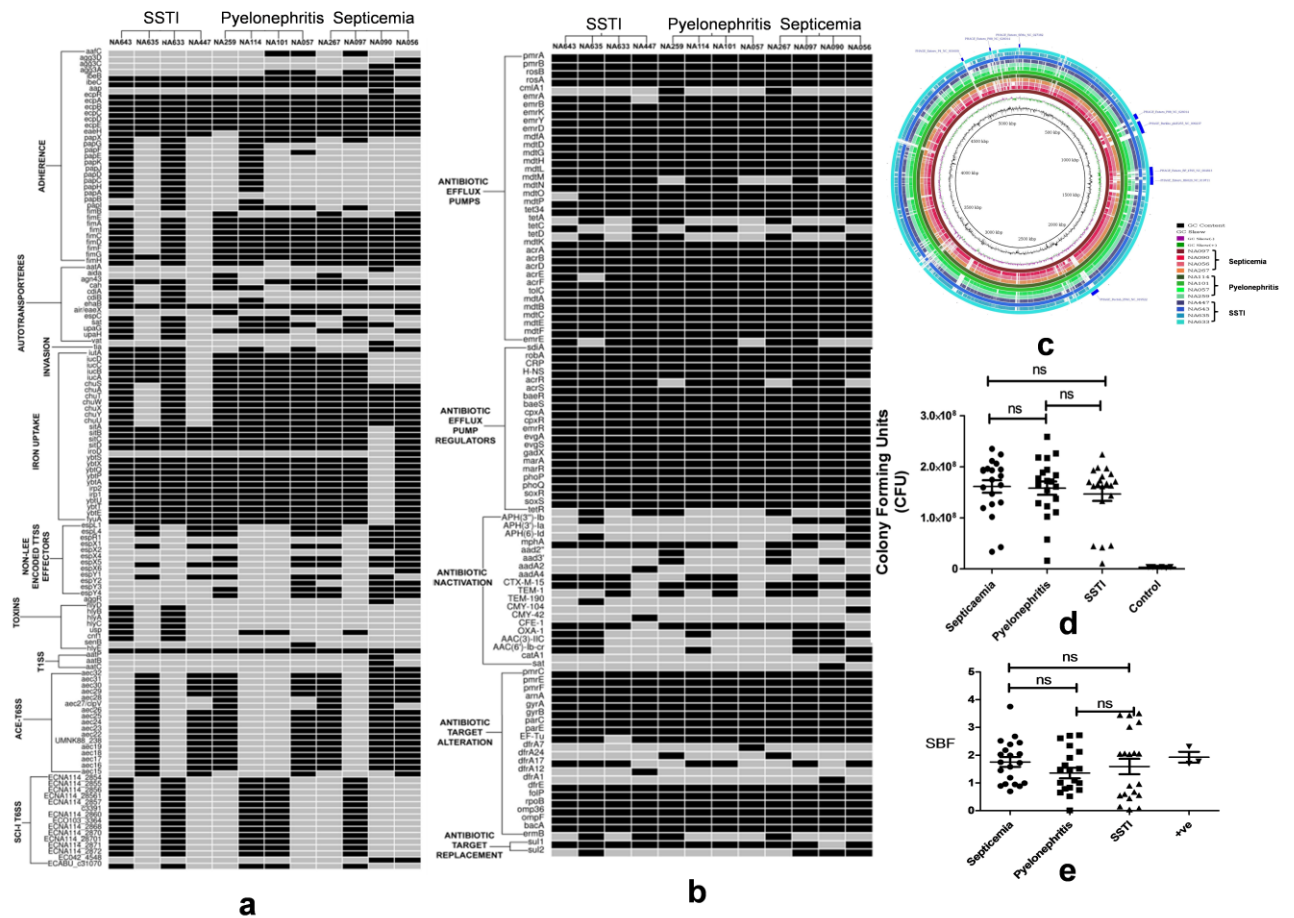


Fig.1 Heat map of virulome (a) and resistome (b) generated based upon the whole genome based comparative genomics of 12 strains comprising four strains from each infection groups, septicemia, pyelonephritis and SSTI. Black box represents the presence while white represents absence. 1c. BLAST Ring Image Generator (BRIG) images of 12 strains comprising of four strains from each category where each concentric ring represents one genome followed sequentially in groups as septicemia, pyelonephritis and SSTI isolates generated using NA097 septicemia strain as reference. 1d. serum resistance assay in 50% human serum and 1e. biofilm formation assay carried out on 10 strains from each category and repeated twice in technical triplicates. ns- non significant; SBF-specific biofilm formation

## Supplementary Material

### **Methods**

#### ***Preparation of DNA template and E. coli phylogenetic grouping:***

Template DNA was prepared using boiling lysis method. In brief, 100µl of the bacterial cultures were boiled at 95°C for 20 min and centrifuged at 6000rpm for 10 min. The supernatant obtained was used as a template. All *E. coli* isolates were classified into four major phylogenetic groups by multiplex PCR of 3 molecular markers; *chuA*, *yjaA* and *TspE4* (1). After electrophoresis (1.5% agarose), the gel images were captured and the isolates were assigned to the phylogenetic groups based on the dichotomous decision tree of *Clermont et al* (1).

#### ***ESBL screening and antimicrobial resistance***

ESBL production was confirmed phenotypically, using the guide line M31-A3, given by the clinical and laboratory standards institute (CLSI, 2008). Resistance to carbapenems was detected by the Etest (HiMedia) method and susceptibility was defined as per the breakpoints of the CLSI criteria. All *E. coli* isolates were also tested for their resistance against 6 major classes of non-β-lactam antibiotics by the standard disc agar diffusion method on Muller-Hinton agar (HiMedia) following the CLSI criteria. The following 6 antimicrobial discs (HiMedia) were used for the antimicrobial susceptibility test; ciprofloxacin (30µg), chloramphenicol (30µg), fosfomycin (200µg), gentamicin (10µg), sulfamethoxazole-trimethoprim (25µg), and tetracycline (30µg). Isolates showing resistance to three or more antimicrobial drugs were regarded as multidrug resistant (MDR).

#### ***Antimicrobial resistance gene testing***

PCR was used to detect β-lactamase genes (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-15</sub>) in ESBL positive *E. coli* isolates (2, 3). The presence of genes encoding carbapenemases was detected using various

primers (4). Major resistance genes, that were described to be associated with tetracycline<sup>R</sup> (*tetA*) sulphonamide<sup>R</sup> (*sul1* and *sul2*), aminoglycoside<sup>R</sup> (*strA*), the amino glycoside acetyl transferase (*aac (6')-lb-cr*), and trimethoprim<sup>R</sup> (*dhfr*) were determined by PCR using the primers and programs reported earlier (5, 6). The genes *int1* encoding class 1 integrase was also tested by PCR (7).

### ***Detection of virulence genes (VGs)***

All Indian *E. coli* were tested for 11 ExPEC virulence genes (VGs) presence that could possibly play close role in sepsis related pathophysiology. The genes targeted were related to the following four categories, (i) bacterial adhesins (*papC*, *fimH*, *afaB/C* and *sfaD/E*) ((8-11) ; (ii) toxins ( *usp*, *sat* and *cvaC*) (12-14); (iii) Protectants (*traT* and *ibeA*) (14, 15); and (iv) the iron acquisition system (*iroN* and *iucD*) (11, 16) and were determined using PCR protocols described previously.

### ***Whole genome sequencing and comparative genomics***

Paired end sequence data of the eight strains from Illumina MiSeq was used for the following in-silico analysis. NGS QC Toolkit (v2.3.3) (17) was used to filter out high quality reads followed by contig assembly using SPAdes Genome Assembler (v3.6.1) (18). Numbers of raw reads given out by the sequencer, respective read lengths, genome coverage obtained after filtering and total number of contigs were tabulated. The generated *denovo* contigs were further ordered and scaffolded using Contig-Layout-Authenticator (19) . Final draft genomes were obtained by merging the scaffolds using a series of N's. The draft genomes were then submitted to the RAST (20) server for annotation, and the genome statistics were obtained using ARTEMIS (21). The Sequence Type (ST) of each of these strains was found out by submitting the contigs to <https://cge.cbs.dtu.dk/services/MLST/>.

Twelve strains including eight strains from the current study and four from earlier reported and ongoing studies, belonging to different pathotypes were used for the following comparative analysis. BRIG (22) was used to visualize the genome variations within these twelve strains. GeneMarkS(23) was used to predict the protein sequences. BLASTp (24) analysis of the putative protein sequences was performed against virulence genes downloaded from the Virulence Factors Database (VFDB) (25) to obtain the virulence profiles of each of these strains. A particular virulence gene is considered to be present only if its BLASTp hit has an identity greater than 60% and query coverage greater than 85%. The presence-absence status of all such virulence genes in each of these strains was represented in the form of a heat map using R. A similar heat map using BLASTp for putative resistance associated genes from Comprehensive Antibiotic Resistance Database (CARD) (26) was generated.

### ***Phenotypic virulence determination***

Biofilm formation was carried out in M63 minimal medium for 48 hrs and serum bactericidal activity was assayed in 50% human serum for three hrs as reported previously (27, 28). Both the assays were performed on a subset of 10 isolates from each category twice in triplicates.

**Table S1:** Contribution of different pathotypes to phenotypic drug resistance and Multi Drug Resistance phenotypes

| Resistant phenotype       |                 | Overall prevalence of resistant phenotype in Indian population (in percentage) | Estimated fraction in Pyelonephritis (n=30) | Estimated fraction in Septicemia (n=28) | Estimated fraction in Skin and soft tissue infections (n=21) |
|---------------------------|-----------------|--------------------------------------------------------------------------------|---------------------------------------------|-----------------------------------------|--------------------------------------------------------------|
| Antimicrobial class       | Specific trait  |                                                                                |                                             |                                         |                                                              |
| Quinolone/fluoroquinolone | Ciprofloxacin   | 86                                                                             | 0.37                                        | 0.34                                    | 0.28                                                         |
| Sulfonamide/trimethoprim  | Cotrimaxazole   | 72                                                                             | 0.43                                        | 0.36                                    | 0.21                                                         |
| Aminoglycosides           | Gentamicin      | 31                                                                             | 0.33                                        | 0.41                                    | 0.25                                                         |
| Phenicols                 | Chloramphenicol | 23                                                                             | 0.39                                        | 0.50                                    | 0.11                                                         |
|                           | Fosphomycin     | 5                                                                              | 0.00                                        | 0.25                                    | 0.75                                                         |
| Tetracyclines             | Tetracycline    | 77                                                                             | 0.43                                        | 0.35                                    | 0.22                                                         |
| ESBL phenotype            | Cefotaxime      | 85                                                                             | 0.36                                        | 0.36                                    | 0.27                                                         |
| MBL phenotype             | Meropenem       | 06                                                                             | 0.00                                        | 0.16                                    | 0.83                                                         |
| Multidrug resistance      |                 | 67                                                                             | 0.40                                        | 0.32                                    | 0.26                                                         |

**Table S2:** Genome statistics of the eight strains sequenced under this study

| Strain | Accession Number | No. of Raw reads | Genome coverage (X) | No. of contigs(>=500bp) | Sequence Types | Genome size(bp) | No. of CDS | Coding % | No. of rRNAs | G+C content (%) | N50 value |
|--------|------------------|------------------|---------------------|-------------------------|----------------|-----------------|------------|----------|--------------|-----------------|-----------|
| NA643  | MJDL000000000    | 2136944          | 108.5               | 112                     | ST-131         | 5322063         | 5257       | 87.4     | 17           | 50.72           | 216749    |
| NA635  | MJDK000000000    | 1822696          | 91.8                | 122                     | ST-617         | 4948859         | 4769       | 87.2     | 12           | 50.78           | 164858    |
| NA633  | MJDJ000000000    | 1878242          | 94.6                | 81                      | ST-131         | 5275425         | 5212       | 87.7     | 17           | 50.68           | 265266    |
| NA447  | JWHS000000000    | 1500566          | 45                  | 282                     | ST-617         | 5091202         | 4990       | 85.7     | 15           | 50.68           | 52237     |
| NA267  | MJDI000000000    | 2092350          | 105                 | 164                     | ST-405         | 5393308         | 5229       | 86.8     | 13           | 50.51           | 118812    |
| NA259  | MJGD000000000    | 1758550          | 88.6                | 162                     | ST-405         | 5400321         | 5260       | 86.9     | 13           | 50.51           | 118812    |
| NA056  | MKHD000000000    | 1513364          | 78                  | 98                      | ST-68          | 5322471         | 5099       | 87.6     | 12           | 50.54           | 143398    |
| NA057  | JSXL000000000    | 1913156          | 57                  | 214                     | ST-38          | 5283771         | 5171       | 86.5     | 9            | 50.5            | 120832    |

**Table S3:** Prevalence of different possible combinations of ESBL, CTX-M-15, fluoroquinilone<sup>R</sup>, Cotrimoxazole<sup>R</sup> and Tetracycline<sup>R</sup> among Indian isolates

| Subset | Subset definition                                                                                          | Total Indian isolates (%) | Pyelonephritis <i>n</i> =30 (%) | Septicemia <i>n</i> =27 (%) | Skin and soft tissue infections <i>n</i> =21 (%) |
|--------|------------------------------------------------------------------------------------------------------------|---------------------------|---------------------------------|-----------------------------|--------------------------------------------------|
| 1      | ESBL <sup>+</sup> ,CTX-M-15 <sup>+</sup>                                                                   | 60(77)                    | 21(70)                          | 23(85)                      | 16(76)                                           |
| 2      | ESBL <sup>+</sup> CTX-M-15 <sup>+</sup> , tem <sup>+</sup>                                                 | 30(38)                    | 11(37)                          | 12(43)                      | 7(33)                                            |
| 3      | CTX-M-15 <sup>+</sup> , fluoroquinole <sup>R</sup>                                                         | 57(73)                    | 20(67)                          | 22(81)                      | 15(71)                                           |
| 4      | CTX-M-15 <sup>+</sup> , fluoroquinole <sup>R</sup> , cotrimoxazole <sup>R</sup>                            | 44(56)                    | 17(56)                          | 17(63)                      | 10(48)                                           |
| 5      | CTX-M-15 <sup>+</sup> , fluoroquinolone <sup>S</sup> cotrimoxazole <sup>R</sup>                            | 2(3)                      | 1(3)                            | 1(4)                        | 0(0)                                             |
| 6      | CTX-M-15 <sup>+</sup> , fluoroquinolone <sup>R</sup> cotrimoxazole <sup>S</sup>                            | 13(17)                    | 3(10)                           | 5(18)                       | 5(24)                                            |
| 7      | CTX-M-15 <sup>+</sup> , fluoroquinolone <sup>S</sup> , cotrimoxazole <sup>S</sup>                          | 3(4)                      | 0(0)                            | 1(4)                        | 2(10)                                            |
| 8      | CTX-M-15 <sup>+</sup> , fluoroquinole <sup>R</sup> , cotrimoxazole <sup>R</sup> ,tetracycline <sup>R</sup> | 39(50)                    | 17(56)                          | 16(57)                      | 6(29)                                            |
| 9      | CTX-M-15 <sup>+</sup> , fluoroquinole <sup>R</sup> , cotrimoxazole <sup>R</sup> ,tetracycline <sup>S</sup> | 4(5)                      | 0(0)                            | 0(0)                        | 4(19)                                            |
| 10     | CTX-M-15 <sup>-</sup> , fluoroquinolone <sup>S</sup>                                                       | 06(8)                     | 4(13)                           | 2(7)                        | 0 (0)                                            |

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# Chapter 3:

## Objective 2

### Characterization of a representative strain of highly virulent subclone from India

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This part is published as:

**Genomic and Functional Portrait of a Highly Virulent, CTX-M-15-Producing H30-Rx Subclone of *Escherichia coli* Sequence Type 131**

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**Antimicrobial agents and chemotherapy 59 (10), 6087-6095**



## Abstract

*Escherichia coli* sequence type 131 (ST131) is a pandemic clone associated with multi-drug resistant, extraintestinal infections and is attributed to the presence of CTX-M-15 extended-spectrum beta-lactamase gene and mutations entailing fluoroquinolone resistance. Studies on subclones within *E. coli* ST131 are critically required to direct and implement successful control efforts. Our study comprehensively analyzed the genomic and functional attributes of the two *H30-Rx* subclonal strains NA097 and NA114 belonging to ST131 lineage. We carried out whole-genome sequencing, comparative analysis, phenotypic virulence assays and profiling of antibacterial response of THP1 cells when infected with these subclones. Phylogenomic analysis suggested that the strains were clonal in nature and confined entirely to a single clade. Comparative genomics revealed that virulence and resistance repertoires were comparable among the *H30-Rx* ST131 strains except for the commensal ST131 strain, SE15. Similarly, seven phage specific regions were found to be strongly associated with the *H30-Rx* strains but were largely absent in the genome of SE15. Phenotypic analysis confirmed the virulence and resistance similarity between the two strains. However, the strain NA097 was found to be more robust than NA114 in terms of virulence gene carriage (*dra* operon), invasion ability ( $P < 0.05$ ) and antimicrobial resistance (streptomycin<sup>R</sup>). RT<sup>2</sup> gene expression profiling revealed a generic upregulation of key pro-inflammatory responses in THP1 cells, irrespective of ST131 lineage status. In conclusion, our study provides insights into the immunological basis of infection with ST131 clones and suggests clonal diversification of genomic and phenotypic features within the *H30-Rx* subclone of *E. coli* ST131.

## Introduction

Extraintestinal infections due to *Escherichia coli* (*E. coli*) cause significant morbidity, mortality and increased health care costs (1, 2). Management of such infections is problematic due to the increasing abundance and high spectrum of antimicrobial resistance ranging from fluoroquinolone to extended-spectrum cephalosporins and to the ‘last resort’ antimicrobials (eg, carbapenems) (3).

Many multidrug-resistant (MDR) bacterial strains are now being reported specially those belonging to the clones capable of rapid global dissemination (4, 5). The H30 ST131, is one such predominant *E. coli* clone or lineage that is responsible world over for a high proportion of community and hospital-acquired urinary tract and bloodstream infections (6-8). The *E. coli* H30-Rx subclone has emerged with a strong association to fluoroquinolone resistance and CTX-M-15 extended-spectrum  $\beta$ -lactamase (ESBL) production (9). The H30-Rx denotes presence of allele 30 for *fimH* (type 1 fimbrial adhesin gene), R denotes its association with fluoroquinolone resistance and x conveys its carriage of *bla*<sub>CTX-M-15</sub>. Clonal expansion has now been a dominant mechanism in the proliferation of antimicrobial resistance in ST131 (10). Alarming, ST131 strains harboring New Delhi metallo-beta lactamase (NDM-1) entailing carbapenem resistance have also been reported among *E. coli* in particular, and *Enterobacteriaceae* in general (11). The ST131 *E. coli* are not only prevalent in human populations, but also an interspecies transmission is being reported (12-14).

The epidemiological success of *E. coli* ST131 has been attributed to a wide variety of factors that include enhanced virulence and metabolic capabilities, ability to acquire extensive and newer resistance traits and better survivability in intestinal and extraintestinal sites (15, 16). Despite these, the exact reasons for the rapid emergence and successful spread of ST131 clones largely remain undefined. Whole genome sequencing has revolutionized the study of bacterial evolution. In order to understand recent evolutionary history of the most important

*E. coli* strains that are in circulation, it is necessary to analyze their diversity in terms of mutations, recombination, gene gain and loss in the population including their profile of bacteriophage content (17).

Although ST131 is being recognized as a pandemic clone involved in serious ExPEC infections posing grave public health threats (15), it received meager attention in countries such as India despite tremendous host diversity, poor sanitation, and widespread abuse of antibiotics amidst a non-existent antibiotic policy. In spite of an ongoing research on ST131's epidemiological significance, little is known regarding the host responses and immunological profile during infection of different niches by members of this lineage.

To gain insight into the pathogenicity and to identify distinct traits associated with the high risk *H30-Rx* subclone, we sought to investigate the whole genome sequence of one of the representative ST131 (NA097) strains and perform comparative genomics using several reference genomes; we characterized the ST131 strain NA097 for its functional and genomic attributes entailing antimicrobial resistance and pathogenicity. Additionally, we performed profiling of innate immune responses to *E. coli* infection in differentiated THP1 macrophages with an adherent and invasive ST131 *E. coli* (NA097). The results of this study extend support to the idea that the expansion of *H30-Rx* sublineage has contributed substantially to the dissemination of ST131 *E. coli* and provide a snapshot of the innate immune signatures of the host against ST131 infection.

## **Materials and methods**

### ***ST131 strains and genome sequencing***

The *E. coli* strain NA097 was isolated from the urine sample of a 29 year old male patient suffering from septicemia and reporting at the D.Y. Patil Hospital, Pune, India in 2009.

Another ST131 strain NA114 employed in this study was obtained during 2009-2011 and has been sequenced and reported previously by our group (18). The strain NA097 was characterized according to routine biochemical techniques and was stored in Luria-Bertani (LB) broth supplemented with 20% glycerol at -80°C. The high quality genomic DNA was obtained using Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany). Whole genome sequencing (WGS) was carried out on Illumina Miseq sequencer which generated 1.3 million paired-end reads of 251 bp with an insert size of 400-500 bp. After filtering and trimming the paired-end reads using NGS QC Toolkit, the high quality reads amounted to a genome coverage of approximately 34x. The reads were then assembled into contigs using Velvet (19) *de novo* assembler run with an optimum hash length of 77. The contigs were then screened for possible presence of plasmids based on their alignment to the online *E. coli* sequence databases with parameters such as 70% identity and query coverage using BLASTn (20). Only chromosomal contigs were ordered and scaffolded using in-house workbench, C-L-Authenticator, to avoid mis-scaffolding due to plasmid related contigs. The generated scaffolds along with plasmid contigs were used for further analysis. The draft genome was then submitted to RAST server (21) for annotation and the genome statistics from the resulting .gbk file was extracted using ARTEMIS (22). Further, gene prediction was carried out using GeneMarkS (23). The numbers of tRNAs/ rRNAs were identified using tRNAscan-SE (24) and RNAmmer (25) respectively.

### ***Genome data and comparative genomics***

A total of 107 whole genome sequences including those of ST131 strains NA097, NA114, JJ1886 and EC958 from NCBI, and four ST95 strains as described by Petty *et al* (4) were used to construct a core genome based phylogeny using Harvest (26) with the complete genome of SE15 strain taken as a reference. The resulting tree was visualized using Splits Tree program (<http://www.splitstree.org/>). BRIG (27) was used to visualize the genome

variations within clade C comprising NA097 along with other reference ST131 genomes from NCBI. In case of all the genomes, predicted protein sequences from GenemarkS were searched against the database of *E. coli* virulence genes downloaded from Virulence Factors Database (VFDB) (28) using BLASTp. Only hits with 80% identity and 70% query coverage were considered as positive hits. A comparative table was created based on the presence and absence of these virulence genes in all the compared genomes. A similar table was generated for putative resistance related genes after comparison with the Comprehensive Antibiotic Resistance Database (CARD) (29) using BLASTp (NCBI). PhiSpy (30) and PHAST (31) were used to identify the putative phage sequences in the genome. Insertion sequence (IS) elements and genomic islands were identified using IS finder (32) and Island Viewer (33), respectively. Predicted protein sequences were also searched against the CDD (34) database using Batch CD-Search at NCBI for functional categorization. In house scripts were used to extract the best hit and corresponding COG classes from the results.

### ***Molecular characterization and susceptibility testing***

*E. coli* phylogenetic groups (A, B1, B2, and D) were determined by a well-established PCR method as described earlier (35). The isolates were preliminarily assessed for ST131 status by an allele-specific PCR for *pabB* gene detection (36). Antimicrobial susceptibility was examined by standard Kirby Bauer method using ICOSA UTI (HiMedia Mumbai, India) antibiotic ring for 20 different antibiotics. ESBL production was detected using the double-disc synergy test. The results of both the tests were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) criteria. Metallo-beta-lactamase (MBL) production was screened by Meropenem and Meropenem-EDTA E-test strips (HiMedia, Mumbai, India) as per manufacturer's directions. Further, detection of ESBL (*bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>) and MBL genes (*bla*<sub>NDM-1</sub>) was carried out by PCR. Plasmids were determined using Kado lysis

and replicon types were identified using five multiplex and three single PCR reactions for the presence of total 18 plasmid types (37).

### ***Phenotypic characterization of strains***

Serum resistance assays were performed in 50% human serum (Pan Biotech) as described previously (38) with some modifications. Briefly, 5 µl of overnight bacterial culture was added to 495 µl of fresh LB media and grown for an hour. After centrifugation, the bacteria were resuspended in sterile 1 ml PBS, and 30 µl of culture with 270 µl of 50% human serum was added in triplicates to 96 well microtiter plates. An aliquot of 30 µl of each culture was serially diluted and plated from it (0 hr count) and the rest was incubated for 3 hr at 37°C. After incubation, the bacteria were serially diluted and plated (3hr count). Counting was done after overnight incubation and colony forming units (CFU) were calculated. The initial CFU was subtracted from the final count in order to obtain growth in serum and plotted into a graph. Hemagglutination assay was carried out in freshly isolated RBCs from human blood and results were interpreted as described elsewhere (39). Biotyping of strains for carbohydrate utilization of 35 sugar sources was performed using the Hi-Carbo kit (Himedia, Mumbai) as per manufacturer's protocol. Biofilm formation was examined three times in triplicates in 96 well microtiter plates with M63 media after 48 hr, as previously described (8).

### ***Epithelial cell adhesion and invasion assays***

Adhesion and invasion assay was done as described earlier (38) with some modifications. T24 and ACHN, the human bladder and kidney epithelial cell lines were grown in RPMI-1640 and Ham's F-12 medium (Invitrogen, USA) supplemented with 10% and 15% fetal bovine serum (Invitrogen, USA), respectively. Cells were grown in 5% CO<sub>2</sub> at 37°C. Cells were seeded into 24 well-plates 48 hr prior to cell infection assays. Infection was performed at multiplicity of infection (MOI) of 10. Following 3 hr of incubation at 37°C, the monolayer was washed thrice using 1X PBS (Gibco, USA) and subsequently lysed using 0.1% Triton X-

100 in 1X PBS for 10 min at 37°C. The serially diluted lysates were plated on LB agar plates for determination of CFUs of adherent bacteria. For invasion assay, the cells were incubated for 3 hr and the monolayer was washed thrice with 1X PBS and incubated additionally for 1.5 hr with fresh 1 ml Ham's F-12 media containing 100µg/ml gentamicin. After incubation, cells were lysed using 0.1% Triton X-100 in PBS for 10 minutes at 37°C. Lysates were serially diluted and CFUs were determined as in adhesion assay described above.

### ***Infection of cultured THP1 cells and RT<sup>2</sup> profiling of antibacterial response***

Monocyte cells were cultured and maintained in RPMI-1640 media (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C and 5% CO<sub>2</sub>. Prior to infection, about 1 million cells were seeded into 35mm petri dishes (Corning) and stimulated using phorbol myristate acetate (PMA) for 36 hr. Post stimulation, cells were incubated in antibiotic-free media for 12 hr. Subsequently, *E. coli* strains (NA097 and DH5alpha) were grown overnight followed by secondary growth to 0.95 OD at 600nm. *E. coli* was harvested and added to the differentiated THP1 cells at an MOI of 10. Similarly, another set of 1 million cells were treated with 1µg LPS. Infected cells were incubated for 3 hr. Total RNA was isolated using GenElute kit (Sigma, USA) and cDNA was synthesized from 1 µg of DNase (Sigma, USA) treated RNA, using a First Strand cDNA synthesis kit (Qiagen, Germany). Real time PCR was performed on cDNA using the RT<sup>2</sup> Profiler PCR Array system according to the manufacturer's instructions in an Eppendorf realplex 4 equipment. The experiments were done in triplicates and the data were analyzed using the online RT<sup>2</sup> Profiler Array Data Analysis Ver. 3.5 tool from Qiagen.

### ***Statistical Analysis***

Statistical analyses for biofilm assay, serum bactericidal assay, adhesion and invasion assay were carried out using non-parametric Mann-Whitney U test using GraphPad Prism 5.01 (<http://www.graphpad.com>). For calculations on qRT PCR, the custom online software was

used as directed in the PAHS-148Z RT<sup>2</sup> profiler manual (Qiagen) and calculations were obtained as per manufacturers conventions. p value <0.05 was considered to be significant.

### ***Accession Number***

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JSXJ000000000. The version described in this paper is version JSXJ010000000.

## **Results**

### ***Genome Statistics and comparative genomics***

The genome of NA097 comprised 187 contigs constituting an approximate size of 5.1 MB. The genome was predicted to encode ~ 5164 protein coding sequences with an average CDS length of 885 bp, amounting to a coding capacity of 88%. The genome revealed 51.83% G+C, 73 tRNAs and 3 rRNA genes. The functional annotation of NA097 genome using the COG database revealed a majority of genes related to metabolism (37%), followed by cellular processing and signaling (21%) and information storage and processing (17%). A fraction of the genes (14%) belonged to multiple classes while 10% remained to be poorly characterized (Fig S2). *In silico* multilocus sequence typing (MLST) analysis as per the MLST database (<http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli>) showed that this organism belongs to sequence type 131. We identified 13 families of Insertion Sequences (IS) elements within NA097 genome including at least one copy each of IS21, IS66, IS6, IS1, IS30, Tn3, IS1380, IS110, IS3, ISL3, IS4, IS200 and ISAs1.

Whole genome sequence based phylogeny of ST131 strains together with ST95 strains revealed three different clades (clade A, clade B and clade C) as described in Fig S1. The strains NA097 and NA114 clustered within clade C which predominantly included strains of

*H30* and *H30*-Rx subclones of ST131 *E. coli*. The outcome of UPEC (uropathogenic *E. coli*) specific virulence and resistance gene profiling as regards to five ST131 genomes are summarized in table S1 and table S2, respectively. The virulence and resistance repertoire was comparable among the ST131 strains except for the SE15 which had low virulence and fewer resistance genes. NA097 majorly revealed *dra* operon in contrast to pyelonephritis-associated pili (*pap*) operon in NA114. Both the strains shared similar resistance pattern except that the NA097 was additionally positive for *strA*, *strB*, *aac3'**IIC* genes. The genomes of EC958 and NA097 contained both *bla*<sub>TEM</sub> and *bla*<sub>CTX-M-15</sub> genes. SE15 was negative for both while the rest of the genomes had either of the two  $\beta$ -lactamase genes. Further, to understand the variations among genomes that clustered in clade C, we subjected four well characterized reference ST131 genomes including, EC958, jj1886, NA114 and SE15 with NA097 as a reference to comparative analysis using BRIG. The most common variation observed among SE15 - a commensal ST131 strain, and the four *H30*-Rx subclonal ST131 strains was the absence of seven phage associated regions in SE15 genome (Fig 1). Three major genomic islands, namely, GI-SelC, GI-pheV and GI-leuX were also found in the NA097 genome along with several other genomic islands of sizes ranging from 0.5 kb to 30 kb as predicted by Island viewer. The genome of NA097 exclusively contained the lysogenic phage P4 and other genomic regions encoding functions such as autoinducers, capsular polysaccharide synthesis, quorum sensing, aminoglycoside resistance and others that are labeled in blue in Fig 1. A detailed description of different genomic regions and phages is provided in a tabular form. The genome of NA097 shared similarity to the recently published EC958 *E. coli* ST131 genome sequence which contains several mobile genetic elements that would account for increased fitness or pathogenicity. Overall, the genomes of four *H30*-Rx strains were found to be highly clonal in nature despite their distinct geographical and temporal origins.

### ***Molecular characterization and susceptibility testing***

The strain NA097 was positive for typical characteristics of clone ST131: it was belonging to phylogroup B2, was PCR positive for *pabB* allele along with CTX-M-15 and TEM  $\beta$ -lactamase genes. Phenotypically, NA097 was tested to be an ESBL producer and was MBL negative. The strain was refractory to the following 13 of the 20 antimicrobial agents tested: ampicillin, ampicillin/sulbactam, carbapenem, belonging to class penicillin; cephalothin (1<sup>st</sup> gen), cefazolin (1<sup>st</sup> gen), cefotaxime (3<sup>rd</sup> gen), cefepime (3<sup>rd</sup> gen) of cephalosporins class, norfloxacin (1<sup>st</sup> gen), ciprofloxacin (2<sup>nd</sup> gen), moxifloxacin (4<sup>th</sup> gen), gatifloxacin (4<sup>th</sup> gen) of fluoroquinolone class, streptomycin and co-trimoxazole of aminoglycoside and trimethoprim/sulphamethazole class, respectively. The strain was completely susceptible only to the macrolide antibiotic class and to five other antibiotics belonging to the above described classes. Karyotyping revealed the presence of four plasmids but only two plasmids were able to be identified in PCR based replicon typing.

### ***Phenotypic characteristics of ST131 strains NA097 and NA114***

Biofilm formation is considered to be an important virulence property among pathogenic *E. coli* (40), especially those which cause urinary tract infections (UTIs) and sepsis. Therefore, we investigated the ability of two ST131 strains to form biofilms in M63 minimal medium. We observed significant specific biofilm formation (SBF) values for both NA097 and NA114 strains as compared to DH5alpha after 48 hr of incubation (Fig 2a). As serum resistance is reported to be a marker of virulence (41), we examined the survival of the two ST131 strains and DH5alpha in human serum after 3 hr. The mean CFU of growth for NA097, NA114 and DH5alpha were  $3.9 \times 10^8$ ,  $2.6 \times 10^8$ ,  $7.7 \times 10^7$ , respectively, while the blank showed no growth (Fig 2b). NA097 and NA114 were significantly resistant to serum as compared to DH5alpha. Moreover, the level of serum resistance was comparable between NA097 and NA114. Furthermore, NA097 showed mannose resistant hemagglutination (MRHA) in

human RBCs. To assess whether isolates of ST131 lineage share specific metabolic characteristics, we investigated the carbohydrate utilization ability of NA097 using 35 API test reagents (Himedia, Mumbai). Of the 35 sugars, NA097 was able to utilize 20 (57%) sugars. The metabolic capability of carbohydrate utilization of NA097 and NA114 was found comparable. However, they did not demonstrate an enhanced utilization of a panel of metabolic substrates compared to the data of metabolic utilization by *E. coli* belonging to other sequence types (15).

### ***Adhesion and invasion assay on human epithelial cells***

Considering the significance of human kidney and bladder epithelial cells in the development of acute UPEC related complications and to investigate whether the presence or absence of virulence genes impacts the pathogenic nature of the bacterium (42), we conducted *in vitro* cell adhesion and invasion assays using strains NA097 and NA114 along with the negative control (DH5alpha) on both the ACHN and T24 cells. We observed significant adhesion and invasion levels of NA097 in both the cell types as compared to DH5alpha (Fig 3). Conversely, NA114 was adherent on both cell types but was less invasive ( $1.3 \times 10^5$  CFU) in T24 cells and was non-invasive for the ACHN cells.

### ***RT<sup>2</sup> gene expression profiling of antibacterial response in THP1 cells***

In order to analyze the transcription of antibacterial response genes in differentiated human THP1 macrophages infected with ST131 *E. coli* strain NA097, DH5alpha strain and LPS, we performed quantitative PCR (qPCR) to determine the expression of 84 genes corresponding to inflammatory responses, pattern recognition receptors (PRRs), chemokines, cytokines, downstream signaling components, etc. The expression levels for 12 genes were unaltered in all the three groups (including control), while a total of 24 transcripts were identified to be significantly altered. In all the three categories, 12 genes were upregulated while five genes were downregulated. A majority of significantly upregulated genes comprised of

inflammation-coding transcripts entailing cytokines such as IL-8, IL-1 $\beta$  and TNF and chemokines such as CXCL1, CXCL2, CCL3 and the remaining significantly upregulated transcripts were encoding intracellular signaling factors such as BIRC3, MAP2K3, NFKB1, NFKBIA, NLRP3 and RIPK2. In contrast, transcripts corresponding to CARD6, TLR1, TLR6, PSTPIP1 and PYCARD were significantly downregulated (Fig 4). IL6 was upregulated more than 100 times in all the cases. Overall, the levels of transcriptionally altered genes were similar (with no significant difference) in all the three groups. The rest of the genes did not show any significant fold change compared to untreated control; nonetheless, few were upregulated differentially such as IFNB1, NOD1, SUGT1, NAIP etc. These data provide a comprehensive scenario of transcriptional regulation of macrophage responses to *E. coli* infection.

## Discussion

Previous work by our group showed that the ST131 *E. coli* in India have emerged as an important cause of community-acquired urinary tract infections (8, 15). Molecular epidemiology of these strains demonstrated that they are largely evolving clonally and are associated with several successful phenotypes that were likely involved in the worldwide dissemination of and infections due to ST131 *E. coli* (8, 15, 18, 43). This highlights the urgent need to understand this clonal pathogen in order to develop better control strategies. In this study, we investigated the comprehensive profile of antibacterial response of human macrophages upon ST131 infection. We further investigated the variations in phenotype of ST131 strains by analyzing the phenotypic traits against the whole genome sequence data of our two ST131 strains NA097 and NA114. Whole genome comparisons and phylogenetic comparisons were also performed with other sequenced genomes of ST131 clone.

Our data lend support to observations related to the adaptive diversification and clonal expansion of ST131 as the underlying mechanism for the evolution of fluoroquinolone resistant and ESBL producing ST131 *E. coli* (44, 45). Our data also reveal that the similarity in phenotypes observed between our two ST131 strains correlates with the observation of clonal genetic structure due to the evolution of a distinct subclone within ST131, namely the *H30-Rx* (10). The minor differences observed could be due to variations in virulence gene carriage as also observed by others (46).

ST131 *E. coli* is now the dominant extraintestinal pathogen world over and is a major etiological agent of bladder and kidney infections (47). This organism represents a classical example of evolution through both horizontal and vertical (clonal expansion) mechanisms. Although ST131 first caught attention because of its close association with ESBLs, especially the plasmid mediated CTX-M-15, recent studies on population genetic analysis of ST131 strains have identified that the global rise in extended-cephalosporin resistant ST131 *E. coli* infections is due to the expansion of a single, highly virulent subclone, *H30-Rx* (10). Phylogenomic analysis of two of our ST131 strains (NA114 and NA097) together with several reference genomes of ST131 (4) revealed that both the Indian strains NA097 and NA114 belonged to *H30-Rx* subclone (Fig S1). These results might represent only the tip of the ice berg and therefore warrant attention by clinicians and researchers as our previous studies have already documented that the ST131 *E. coli* are a major population among clinical ExPEC isolates in India (8, 15).

Earlier studies have reported the presence of mobile genetic elements (MGEs) in ST131 *E. coli* strains which are not found in other non-ST131 UPEC strains (4). Our study confirms this and newly documents the high conservation of seven prophage associated genomic regions among all the *H30-Rx* strains studied (Fig 1), and that these seven prophage elements were clearly absent in the commensal non *H30-Rx* ST131, SE15 genome (48). The genome

of our ST131 strain NA097 revealed higher genetic affinity to the recently published EC958 ST131 genome from England (49) followed by the genome of Indian ST131 strain NA114. This variation could possibly be due to the differences in arrangements of different genomic regions arising due to variable genome content encompassing mobile elements and other auxiliary genome components. However, such a degree of clonality between two geographically distinct isolates hints at the possibility of their potential to emerge and expand globally. Other studies have also observed phylogeographic patterns in spatially dispersed ST131 isolates (8).

Given the observed extensive genomic similarities among the strains within *H30-Rx* clade, we sought to determine if there were any phenotypic similarities between the two representative *H30-Rx* strains; NA114 and NA097. We compared the ability of our two *H30-Rx* strains to form biofilm *in vitro* for over 48 hr. There was no significant difference in the levels of specific biofilm formation between the two strains (Fig 2a). Further, we found that the two strains were equally resistant to serum bactericidal activity (Fig 2b). We also conducted *in vitro* cell adhesion and invasion assays on T24 - bladder epithelial cells and ACHN - kidney epithelial cells. In addition to the virulence associated phenotypes, an identical pattern was also observed for the adhesion capabilities of two strains on both the epithelial cells (Fig 3). However, the two strains differed significantly in their levels to invade the epithelial cells. The strain NA097 was highly invasive while NA114 showed insignificant invasion (Fig 3). This observed difference could be partly accounted to the presence of *dra* operon in NA097 strain, which was found to be absent in strain NA114. Goluszko *et al* have observed similar results wherein the insertional mutants of *dra* operon members were unable to invade the Hela cells (50). Finally, we compared the ability of our two ST131 strains to metabolize 35 carbon sources. We found a similar pattern of carbon utilization between the two strains. Overall, our data provide a correlation between genetic framework and the virulence phenotypes of two *H30-Rx* strains. In contrast, past studies have shown that

ST131 strains in general are genetically monomorphic but were phenotypically diverse (46). This could be possibly due to the fact that all such studies have analyzed ST131 strains without taking into account the recent subclones of ST131 namely the *H30-R* and *H30-Rx*. But when these subclones are studied separately they might give rise to clearer and distinct observations.

While several studies have investigated the pathogenesis of ST131 *E. coli* by *in vitro* and *in vivo* assays, the innate immune response involved and the molecular pathways governing these responses largely remain obscure. Deciphering the innate response against this pathogen is a key to understand the immunological basis of bacterial clearance from the host. Here, we performed transcriptome profiling of innate immune signatures in the context of host response to ST131 strains, the control strain DH5alpha and LPS alone. We detected that only the key inflammatory pathways were activated upon infection with either ST131 or DH5alpha or LPS. Hence, we could not identify major strain specific host responses, implying that in the post infected conditions, the capsular antigens would primarily associate with the immune responses and thus pathogenesis could not be correlated on the basis of the virulence genes or mobile genetic elements. Previous studies by Vimont *et al* (16) confirmed that the inflammatory responses of kidney cells upon infection with ST131 and non-ST131 were similar while Horn *et al* (47) showed similar histopathological outcomes in lung tissues after infection with pathogenic and faecal avian *E. coli*. These observations together with our results suggest that the fitness and epidemiological success of ST131 strains were more relevant in the upstream steps involved in onset of infection.

In conclusion, our study confirmed the presence of *H30-Rx* ST131 *E. coli* in Indian ST131 strains and provides more insights into the clonal diversification of genomic and phenotypic features of *H30-Rx* ST131 strains. The study also shows the immunological basis of the outcome of infection with ST131 strains. Such baseline observations could be of great

significance in understanding the emergence of virulent and globally disseminated *H30-Rx* strains. Further studies are needed to investigate if this clone undergoes evolutionary selection and whether that has any effect on its virulence and or resistance phenotypes.

## ACKNOWLEDGMENTS

We acknowledge funding received from Department of Biotechnology, Government of India [Ref.No.BT/HRD/NBA/34/01/2011(ix)]. We also acknowledge support received from University of Malaya High Impact Research Grant (reference no. UM.C/625/1HIR/MOHE/02 [A000002-5000 1]) for which NA is a lead collaborator and support from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673) - functional molecular infection epidemiology, an initiative of the German Research Foundation (DFG) and the University of Hyderabad (India), for which NA and LHW are the speakers. NA is an Adjunct Professor at the Academy of Scientific and Innovative Research (ACSIR), India. AR would like to acknowledge ICMR for the award of Junior Research Fellowship (JRF).

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## Figures legends

Figure 1: The BRIG figure represents comparison of NA097 with the four reference genomes of ST131 *E. coli* which were downloaded from NCBI. Blue regions (R1, R2, R3, R7 and R8) are present only in NA097, black region (R4) is present only in NA097 and EC958, green regions (R5 and R6) are absent in SE15 and red regions denote phage associated regions. The details of each region and their relevant sizes are tabulated.

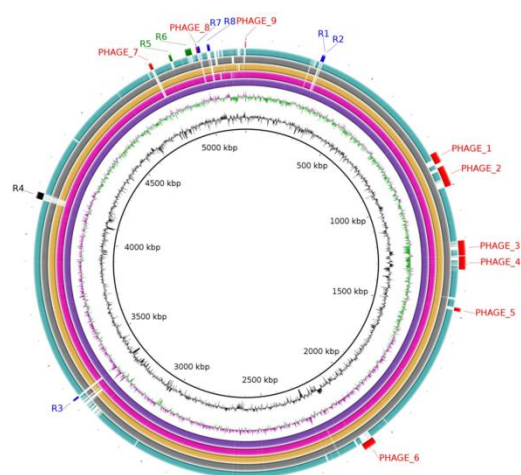
Figure 2: Biofilm formation capabilities and serum bactericidal resistance of NA114 and NA097. 2 (a) Specific biofilm formation (SBF) after 48 hr of incubation. 2 (b) Resistance towards human serum as determined by CFU count after 3 hr of incubation. For both the experiments strain DH5alpha was taken as a negative control and NA434 served as a positive control for the determination of SBF. The experiment was performed three times in triplicates and statistical values were obtained using non-parametric Mann-Whitney U test using GraphPad Prism 5.01 (<http://www.graphpad.com>); ns = non-significant, \*=  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ .

Figure 3: Adhesion and invasion capabilities of NA114 and NA097. 3 (a) and 3 (b) represent adhesion on human bladder epithelial cell line (T24) and human kidney epithelial cell line (ACHN) respectively, after 3 hr of incubation. 3 (c) and 3 (d) represents invasion capabilities in T24 and ACHN cell line after 3 hr of incubation and additional incubation for 1.5 hrs in media containing gentamicin (100µg/ml). DH5alpha was used as a negative control for adhesion and invasion assay and were performed three times in triplicates and statistical values were obtained using non-parametric Mann-Whitney U test using GraphPad Prism

5.01 (<http://www.graphpad.com>); ns= non-significant, \*= p<0.05, \*\*, p<0.01 and \*\*\*= p<0.001.

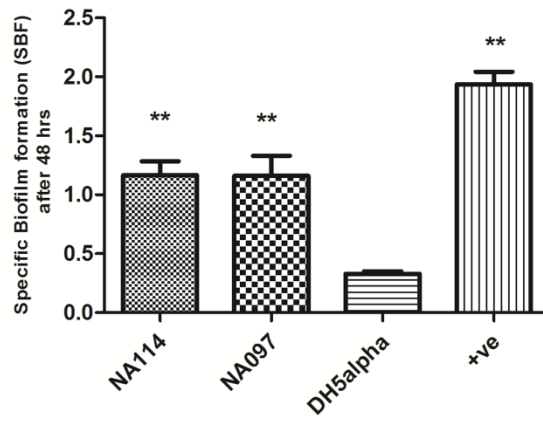
Figure 4: Clustered diagram indicating transcript levels of genes associated with bacterial infection in THP-1 cells. Group 1 = LPS treated cell sample, group 2 = cells infected with DH5alpha, and group 3 = cells infected with strain NA097. Untreated THP-1 cells were used as control. Online custom software RT<sup>2</sup> Profiler Array Data Analysis Ver. 3.5 from Qiagen (Qiagen, Germany) was used to obtain statistical values (student's t-test) as per RT<sup>2</sup> Profiler array instructions (\*= p<0.05, \*\* = p<0.01 and \*\*\*= p<0.001).

**Figure1:**

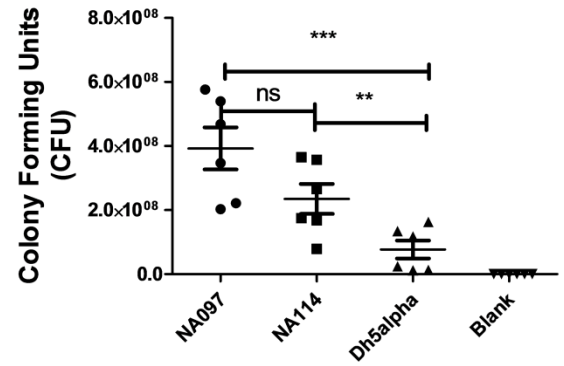


| S. No | Region | Size (kb) | Names of closest relative organism/ phage (if any) | Status/ coding proteins                                                                         |
|-------|--------|-----------|----------------------------------------------------|-------------------------------------------------------------------------------------------------|
| 1.    | Phage1 | 41.8      | P88                                                | incomplete                                                                                      |
| 2.    | Phage2 | 78.6      | BcepMu                                             | intact                                                                                          |
| 3.    | Phage3 | 56.3      | BP4795                                             | intact                                                                                          |
| 4.    | Phage4 | 50.4      | HK629                                              | intact                                                                                          |
| 5.    | Phage5 | 12.7      | mEp235                                             | incomplete                                                                                      |
| 6.    | Phage6 | 49.4      | ZF40                                               | intact                                                                                          |
| 7.    | Phage7 | 11.7      | P4                                                 | intact                                                                                          |
| 8.    | Phage8 | 5.9       | P88                                                | incomplete                                                                                      |
| 9.    | Phage9 | 3.1       | D108                                               | Incomplete                                                                                      |
| 10.   | R1     | 5.6       | -                                                  | site specific DNA methyltransferases, phage integrase etc.                                      |
| 11.   | R2     | 7         | -                                                  | Sam dependent methyltransferase, mobile element protein, Autoinducers LsrG, LsrF etc.           |
| 12.   | R3     | 7         | -                                                  | XcbB, CDP glycerol etc.                                                                         |
| 13.   | R4     | 25.2      | -                                                  | mobile element protein, type II restriction enzyme methylase, ATP dependent protease etc        |
| 14.   | R5     | 9.4       | -                                                  | Type I restriction modification system                                                          |
| 15.   | R6     | 23        | -                                                  | Flagellar synthesis apparatus                                                                   |
| 16.   | R7     | 9         | -                                                  | Autoinducers ABC transport system, mobile elements etc.                                         |
| 17.   | R8     | 10        | -                                                  | DNA recombinase protein, transposase, Aminoglycoside transacetylase, hypothetical proteins etc. |

Figure 2:

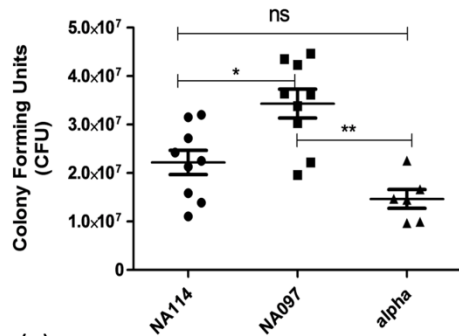


(a)

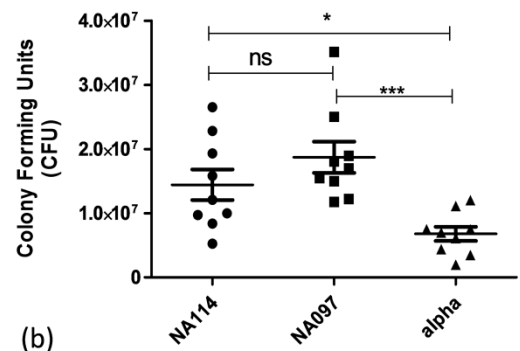


(b)

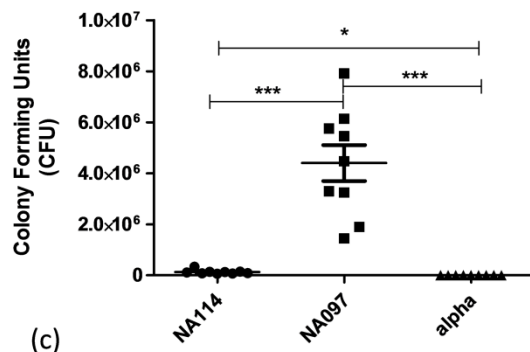
Figure 3:



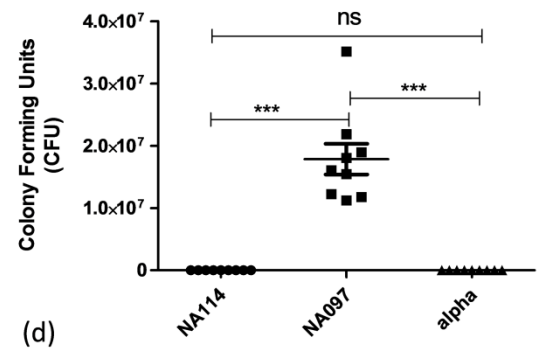
(a)



(b)

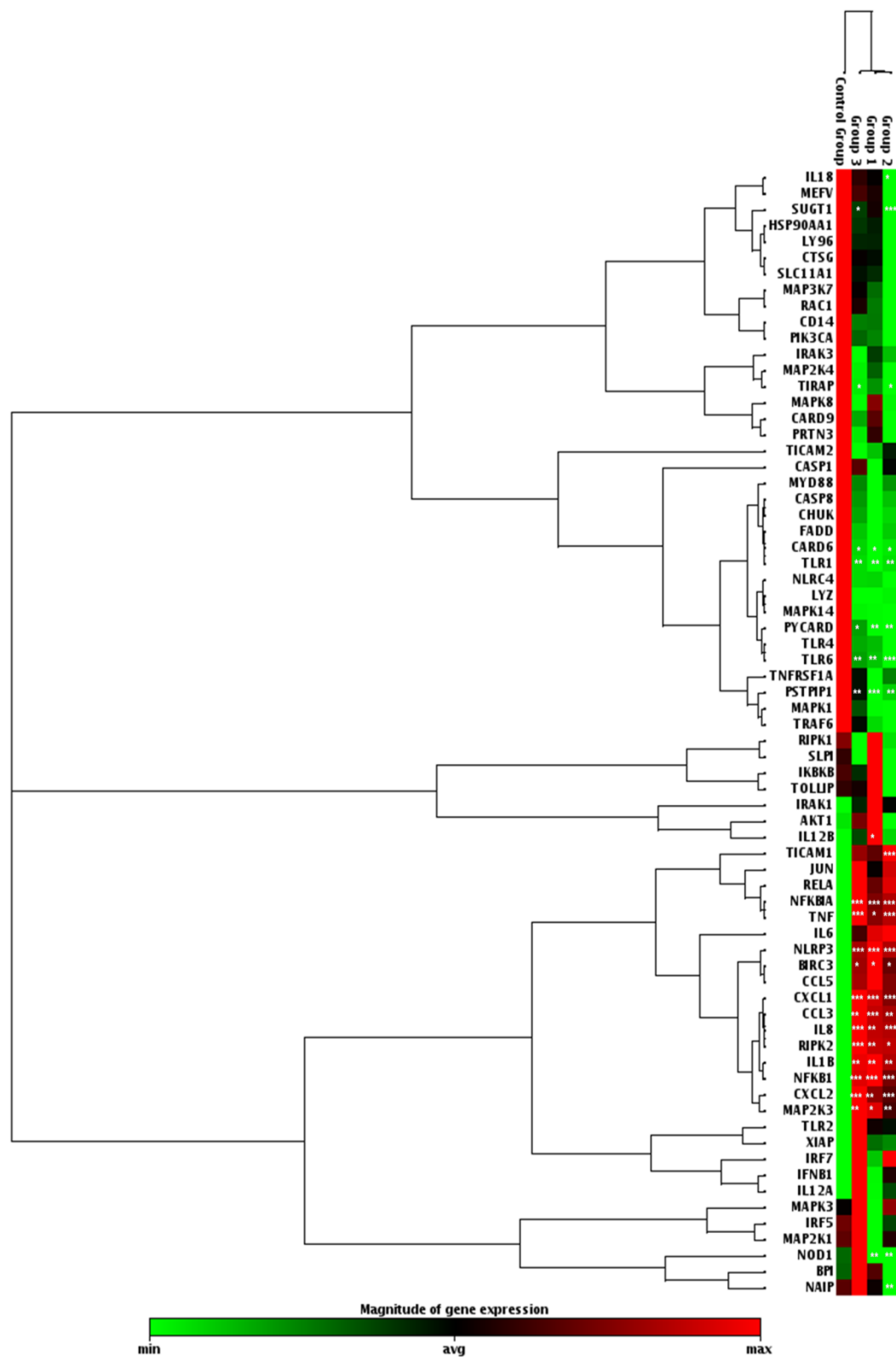


(c)



(d)

Figure 4:



Supplementary data

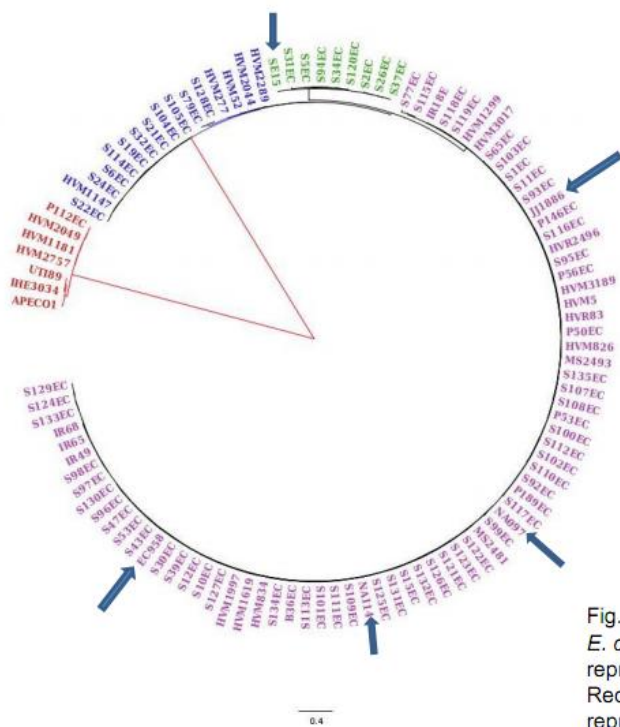


Fig. S1: Maximum Likelihood phylogenetic tree of 107 *E. coli* strains based on core genome SNPs. Green represents clade A, Blue clade B and Purple clade C. Red represents the outgroup members of ST95. Arrows represent the genomes that were used for BRIG analysis.

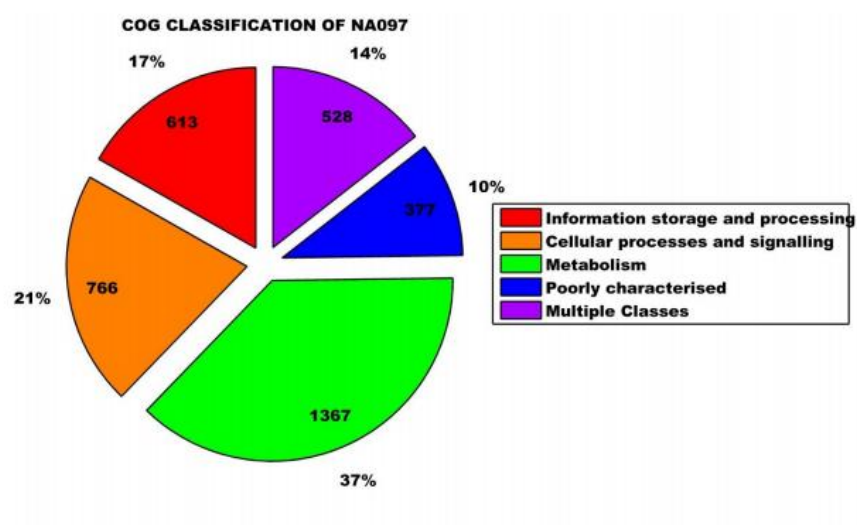


Fig. S2: COG functional classification of the annotated draft genome of NA097 from CDD database.

**Table S1:** Status table for UPEC specific virulence factors within the compared strains of ST131 *E. coli* as per the parameters described earlier.

| S. no | VF     | VFDB/ Gene bank ID | EC958 | SE15 | NA097 | NA114 | JJ1886 |
|-------|--------|--------------------|-------|------|-------|-------|--------|
| 1.    | fimB   | VFG0871            | -     | +    | -     | -     | -      |
| 2.    | draC   | VFG0944            | +     | -    | +     | -     | -      |
| 3.    | draB   | VFG0943            | +     | -    | +     | -     | -      |
| 4.    | draA   | VFG0942            | +     | -    | +     | -     | -      |
| 5.    | draP   | VFG0946            | +     | -    | +     | -     | -      |
| 6.    | draD   | VFG0945            | +     | -    | +     | -     | -      |
| 7.    | papF   | VFG0889            | -     | -    | -     | +     | -      |
| 8.    | papB   | VFG0881            | -     | -    | -     | +     | -      |
| 9.    | papD   | VFG0885            | -     | -    | -     | +     | -      |
| 10.   | papC   | VFG0884            | -     | -    | -     | +     | -      |
| 11.   | papG   | VFG0890            | -     | -    | -     | +     | -      |
| 12.   | papH   | VFG0883            | -     | -    | -     | +     | -      |
| 13.   | papK   | VFG0887            | -     | -    | -     | +     | -      |
| 14.   | papJ_2 | VFG0897            | -     | -    | -     | +     | -      |
| 15.   | TRAT1  | gi20095235         | +     | +    | +     | +     | +      |
| 16.   | entE   | VFG0932            | +     | +    | +     | +     | +      |
| 17.   | entB   | VFG0933            | +     | +    | +     | +     | +      |
| 18.   | chuT   | VFG0918            | +     | +    | +     | +     | +      |
| 19.   | entA   | VFG0934            | +     | +    | +     | +     | +      |
| 20.   | fimI   | VFG0874            | +     | +    | +     | +     | +      |
| 21.   | papI_2 | VFG0891            | +     | -    | +     | +     | +      |
| 22.   | chuS   | VFG0916            | +     | +    | +     | +     | +      |
| 23.   | entD   | VFG0929            | +     | +    | +     | +     | +      |
| 24.   | fepA   | VFG0923            | +     | +    | -     | +     | +      |
| 25.   | fimC   | VFG0875            | +     | +    | +     | +     | +      |
| 26.   | fepD   | VFG0926            | +     | +    | +     | +     | +      |
| 27.   | chuA   | VFG0917            | +     | +    | +     | +     | +      |
| 28.   | chuX   | VFG0920            | +     | +    | +     | +     | +      |
| 29.   | iutA   | VFG0936            | -     | -    | +     | +     | +      |
| 30.   | iucA   | VFG0940            | +     | -    | +     | +     | +      |
| 31.   | entC   | VFG0931            | +     | +    | +     | +     | +      |
| 32.   | fimA   | VFG0873            | +     | +    | +     | +     | +      |
| 33.   | ompA   | VFG1443            | +     | +    | +     | +     | +      |
| 34.   | fimE   | VFG0872            | +     | +    | +     | +     | +      |
| 35.   | kpsM   | VFG1450            | +     | +    | +     | +     | +      |
| 36.   | fimG   | VFG0878            | +     | +    | +     | +     | +      |
| 37.   | ibeB   | VFG1441            | +     | +    | +     | +     | +      |
| 38.   | sat    | VFG0902            | +     | -    | +     | +     | +      |
| 39.   | aslA   | VFG1444            | +     | +    | +     | +     | +      |

|     |      |           |   |   |   |   |   |
|-----|------|-----------|---|---|---|---|---|
| 40. | iucD | VFG0937   | + | - | + | + | + |
| 41. | kpsD | VFG1448   | + | + | + | + | + |
| 42. | fimF | VFG0877   | + | + | + | + | + |
| 43. | iucC | VFG0938   | + | - | + | + | + |
| 44. | ibeC | VFG1440   | + | + | + | + | + |
| 45. | fimD | VFG0876   | + | + | + | + | + |
| 46. | iucB | VFG0939   | + | - | + | + | + |
| 47. | Usp  | gi7416048 | + | + | + | + | + |
| 48. | traJ | VFG1445   | + | + | + | + | + |
| 49. | fimH | VFG0879   | + | + | + | + | + |
| 50. | chuY | VFG0921   | + | + | + | + | + |
| 51. | entF | VFG0930   | + | + | + | + | + |
| 52. | fepC | VFG0925   | + | + | + | + | + |
| 53. | chuU | VFG0922   | + | + | + | + | + |
| 54. | fepB | VFG0924   | + | + | + | + | + |
| 55. | chuW | VFG0919   | + | + | + | + | + |

**Table S2:** Status table of resistance associated genes as obtained from ARDB based on parameters as mentioned above within the compared strains

|     | Resistance    | Resistance class | EC958 | SE15 | NA097 | NA114 | JJ1886 |
|-----|---------------|------------------|-------|------|-------|-------|--------|
| 1.  | blaCTXM-15    | Beta lactam      | +     | -    | +     | +     | +      |
| 2.  | BlaTEM-1.     | Beta lactam      | +     | -    | +     | -     | +      |
| 3.  | OXA-1         | Beta lactam      | +     | -    | +     | +     | +      |
| 4.  | CMY-23        | Beta lactam      | +     | -    | -     | -     | -      |
| 5.  | AadA4         | Aminoglycoside   | +     | -    | +     | +     | -      |
| 6.  | AAC(6')-Ib-cr | Fluoroquinolones | +     | -    | +     | +     | +      |
| 7.  | AAC(3)-IIc    | Aminoglycoside   | -     | -    | +     | -     | -      |
| 8.  | acrD          | Efflux           | +     | +    | +     | +     | +      |
| 9.  | acrA          | Efflux           | +     | +    | +     | +     | +      |
| 10. | acrF          | Efflux           | +     | +    | +     | +     | +      |
| 11. | acrB          | Efflux           | +     | +    | +     | +     | +      |
| 12. | acrE          | Efflux           | +     | +    | +     | +     | +      |
| 13. | APH(6)-Id     | Aminoglycoside   | -     | -    | +     | -     | -      |
| 14. | APH(3'')-Ib   | Aminoglycoside   | +     | +    | +     | +     | +      |
| 15. | StrA          | Streptomycin     | -     | -    | +     | -     | -      |
| 16. | sul2          | Sulphonamides    | -     | -    | +     | -     | -      |
| 17. | Sul1          | Sulphonamides    | +     | -    | +     | +     | -      |
| 18. | dfrA17        | trimethoprim     | +     | -    | +     | +     | -      |
| 19. | catB2         | Phenolics        | -     | -    | +     | -     | -      |
| 20. | tetC*         | Tetracyclines    | +     | -    | +     | +     | -      |
| 21. | mdtK          | MDR efflux       | +     | +    | +     | +     | +      |
| 22. | mdtH          | MDR efflux       | +     | +    | +     | +     | +      |
| 23. | mdtF          | MDR efflux       | +     | +    | +     | +     | +      |
| 24. | mdtB          | MDR efflux       | +     | +    | +     | +     | +      |
| 25. | mdtD          | MDR efflux       | +     | +    | +     | +     | +      |
| 26. | mdtG          | MDR efflux       | +     | +    | +     | +     | +      |
| 27. | mdtL          | MDR efflux       | +     | +    | +     | +     | +      |
| 28. | mdtA          | MDR efflux       | +     | +    | +     | +     | +      |
| 29. | mdtE          | MDR efflux       | +     | +    | +     | +     | +      |
| 30. | mdtC          | MDR efflux       | +     | +    | +     | +     | +      |
| 31. | emrA          | MDR efflux       | +     | +    | +     | +     | +      |

|     |                                |                                  |   |   |   |   |   |
|-----|--------------------------------|----------------------------------|---|---|---|---|---|
| 32. | emrY                           | MDR efflux                       | + | + | + | - | + |
| 33. | emrK                           | MDR efflux                       | + | + | + | + | + |
| 34. | emrD                           | MDR efflux                       | + | + | + | + | + |
| 35. | asmA                           | Bile resistance                  | + | + | + | + | + |
| 36. | ompF                           | MDR                              | + | + | + | + | + |
| 37. | ECs3903                        | Fluoroquinolones                 | + | + | + | + | + |
| 38. | ECs2138                        | MAR regulator                    | + | + | + | + | + |
| 39. | ECs1739                        | Polymixin B/aminoglycoside/E DTA | + | + | + | + | + |
| 40. | ECs5045                        | Drug induced oxidative stress    | + | + | + | + | + |
| 41. | ECs5044                        | Drug induced oxidative stress    | + | + | + | + | + |
| 42. | parE                           | Fluoroquinolones                 | + | + | + | + | + |
| 43. | PhoQ                           | Antimicrobial Peptide resistance | + | + | + | + | + |
| 44. | EvgA                           | Acid                             | + | + | + | + | + |
| 45. | cpxA                           | Drug induced oxidative stress    | + | + | + | + | + |
| 46. | rpoB                           | Rifamycin                        | + | + | + | + | + |
| 47. | folP                           | Sulphonamides                    | + | + | + | + | + |
| 48. | tolC                           | MDR efflux                       | + | + | + | + | + |
| 49. | elongation   factor   Tu       | Kirromycin                       | - | + | + | + | + |
| 50. | gyrA                           | Fluoroquinolones                 | + | + | + | + | + |
| 51. | EnvR                           | acrAB repressor                  | + | + | + | + | + |
| 52. | envZ                           | MDR                              | + | + | + | + | + |
| 53. | uppP                           | Bacitracin                       | + | + | + | + | + |
| 54. | yjiO                           | MDR                              | + | + | + | + | + |
| 55. | rpoB                           | Rifamycin                        | + | + | + | + | + |
| 56. | yfbG                           | polymyxin                        | - | + | + | + | + |
| 57. | EvgA                           | Acid                             | + | + | + | + | + |
| 58. | gadX                           | Acid                             | + | + | + | + | + |
| 59. | mph(A)   protein               | Macrolide                        | + | - | + | + | - |
| 60. | CRP                            | MDR                              | + | + | + | + | + |
| 61. | Multidrug resistance protein B | Multi drug resistance            | + | + | + | + | + |
| 62. | RobA                           | Multi drug resistance            | + | + | + | + | + |
| 63. | MdfA                           | Multi drug resistance            | + | + | + | + | + |

|     |                       |                               |   |   |   |   |   |
|-----|-----------------------|-------------------------------|---|---|---|---|---|
| 64. | CpxR                  | Drug induced oxidative stress | + | + | + | + | + |
| 65. | mcrB                  | Bacteriophage resistance      | + | + | + | + | + |
| 66. | DLP12                 | MDR                           | + | + | + | + | + |
| 67. | baeR.   Sensor   BaeS | Multi drug exporter           | + | + | + | + | + |

# Genomic and Functional Portrait of a Highly Virulent, CTX-M-15-Producing *H30-Rx* Subclone of *Escherichia coli* Sequence Type 131

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*Escherichia coli* sequence type 131 (ST131) is a pandemic clone associated with multidrug-resistant, extraintestinal infections, attributable to the presence of the CTX-M-15 extended-spectrum  $\beta$ -lactamase gene and mutations entailing fluoroquinolone resistance. Studies on subclones within *E. coli* ST131 are critically required for targeting and implementation of successful control efforts. Our study comprehensively analyzed the genomic and functional attributes of the *H30-Rx* subclonal strains NA097 and NA114, belonging to the ST131 lineage. We carried out whole-genome sequencing, comparative analysis, phenotypic virulence assays, and profiling of the antibacterial responses of THP1 cells infected with these subclones. Phylogenomic analysis suggested that the strains were clonal in nature and confined entirely to a single clade. Comparative genomic analysis revealed that the virulence and resistance repertoires were comparable among the *H30-Rx* ST131 strains except for the commensal ST131 strain SE15. Similarly, seven phage-specific regions were found to be strongly associated with the *H30-Rx* strains but were largely absent in the genome of SE15. Phenotypic analysis confirmed the virulence and resistance similarities between the two strains. However, NA097 was found to be more robust than NA114 in terms of virulence gene carriage (*dra* operon), invasion ability ( $P < 0.05$ ), and antimicrobial resistance (streptomycin resistance). RT<sup>2</sup> gene expression profiling revealed generic upregulation of key proinflammatory responses in THP1 cells, irrespective of ST131 lineage status. In conclusion, our study provides comprehensive, genome-inferred insights into the biology and immunological properties of ST131 strains and suggests clonal diversification of genomic and phenotypic features within the *H30-Rx* subclone of *E. coli* ST131.

Extraintestinal infections due to *Escherichia coli* cause significant morbidity and death and increased health care costs (1, 2). Management of such infections is difficult due to the increasing abundance and wide spectrum of antimicrobial resistance, ranging from fluoroquinolones to extended-spectrum cephalosporins and to the “last resort” antimicrobials (e.g., carbapenems) (3).

Many multidrug-resistant (MDR) bacterial strains are now being reported, especially those belonging to clones capable of rapid global dissemination (4, 5). *H30* sequence type 131 (ST131) is one predominant *E. coli* clone or lineage that is responsible for large proportions of community- and hospital-acquired urinary tract infections (UTIs) and bloodstream infections throughout the world (6–8). The *E. coli* *H30-Rx* subclone has emerged with strong associations with fluoroquinolone resistance and CTX-M-15 extended-spectrum  $\beta$ -lactamase (ESBL) production (9); *H30* denotes the presence of allele 30 for *fimH* (type 1 fimbrial adhesin gene), R indicates its association with fluoroquinolone resistance, and x conveys its carriage of *bla*<sub>CTX-M-15</sub>. Clonal expansion has now become a dominant mechanism in the proliferation of antimicrobial resistance in ST131 (10). Alarming, ST131 strains harboring New Delhi metallo- $\beta$ -lactamase 1 (NDM-1), which confers carbapenem resistance, have also been reported among *E. coli* strains in particular and *Enterobacteriaceae* in general (11). ST131 *E. coli* strains not only are prevalent in human populations but also demonstrate interspecies transmission (12–14).

The epidemiological success of *E. coli* ST131 has been attributed to a wide variety of factors, including enhanced virulence and metabolic capabilities, the ability to acquire extensive and newer resistance traits, and better survivability in intestinal and extraintestinal sites (15, 16). However, the exact reasons for the rapid

emergence and successful spread of ST131 clones remain largely undefined. Whole-genome sequencing (WGS) has revolutionized the study of bacterial evolution. In order to understand the recent evolutionary history of the most important *E. coli* strains in circulation, it is necessary to analyze their diversity in terms of the mutations, recombinations, and gene gains and losses in the population, including their profiles of bacteriophage contents (17).

Although ST131 is recognized as a pandemic clone involved in serious extraintestinal pathogenic *Escherichia coli* (ExPEC) infections, posing grave public health risks (15), it has received meager attention in countries such as India, despite tremendous host diversity, poor sanitation, and widespread abuse of antibiotics with an unclear antibiotic policy. Despite ongoing research on the epidemiological significance of ST131, little is known regarding the host responses and immunological profiles during infection for members of this lineage.

Received 19 June 2015 Returned for modification 28 June 2015

Accepted 12 July 2015

Accepted manuscript posted online 20 July 2015

Citation Ranjan A, Shaik S, Hussain A, Nandanwar N, Semmler T, Jadhav S, Wieler LH, Ahmed N. 2015. Genomic and functional portrait of a highly virulent, CTX-M-15-producing *H30-Rx* subclone of *Escherichia coli* sequence type 131. *Antimicrob Agents Chemother* 59:6087–6095. doi:10.1128/AAC.01447-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01447-15>.

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To gain insight into its pathogenicity and to identify distinct traits associated with the high-risk H30-Rx subclone, we sought to investigate the whole-genome sequence of one of the representative ST131 strains (NA097) and to perform comparative genomic analysis using several reference genomes; we characterized the ST131 strain NA097 because of its functional and genomic attributes, including antimicrobial resistance and pathogenicity. Additionally, we performed profiling of innate immune responses to *E. coli* infections in differentiated THP1 macrophages with an adherent and invasive ST131 *E. coli* strain (NA097). The results of this study support the idea that expansion of the H30-Rx sublineage has contributed substantially to the dissemination of ST131 *E. coli*, and they provide a snapshot of the innate immune signatures of the host against ST131 infections.

## MATERIALS AND METHODS

**ST131 strains and genome sequencing.** The *E. coli* strain NA097 was isolated from a urine sample from a 29-year-old male patient with septicemia who presented to the D. Y. Patil Hospital (Pimpri, India) in 2009. Strain NA114 was isolated in 2009 and was sequenced and reported previously by our group (18). Strain NA097 was characterized with routine biochemical techniques and was stored at  $-80^{\circ}\text{C}$  in Luria-Bertani (LB) broth supplemented with 20% glycerol. High-quality genomic DNA was obtained using the Qiagen DNeasy blood and tissue kit (Qiagen, Germany). Whole-genome sequencing (WGS) was carried out with an Illumina MiSeq sequencer, which generated 1.3 million paired-end reads of 251 bp, with an insert size of 400 to 500 bp. After filtering and trimming of the paired-end reads using the NGS QC Toolkit (<http://www.nipgr.res.in/ngsseqtoolkit.html>), the high-quality reads amounted to genome coverage of approximately 34 times. The reads were then assembled into contigs using the Velvet *de novo* assembler (19), run with an optimum hash length of 77. The contigs were then screened for the possible presence of plasmids based on their alignment with the online *E. coli* sequence databases, with parameters such as 70% identity and query coverage, using BLASTn (20). Only chromosomal contigs were ordered and scaffolded using an in-house workbench, C-L-Authenticator, to avoid misscaffolding due to plasmid-related contigs. The generated scaffolds and plasmid contigs were used for further analysis. The draft genome was then submitted to the RAST server (21) for annotation, and the genome statistics from the resulting file were extracted using ARTEMIS (22). Gene prediction was carried out using GeneMarkS (23). The numbers of tRNAs and rRNAs were identified using tRNAscan-SE (24) and RNAmmer (25), respectively.

**Genomic data and comparative genomic analysis.** A total of 107 whole-genome sequences, including those of ST131 strains NA097, NA114, JJ1886, and EC958 from the NCBI and other genomes described by Petty et al. (4), were used to construct a core-genome-based phylogenetic tree using Harvest (26), with the complete genome of the SE15 strain taken as a reference. The resulting tree was visualized using the SplitsTree program (<http://www.splitstree.org>). BRIG (27) was used to visualize the genome variations within clade C, including NA097 along with other reference ST131 genomes from the NCBI. For all of the genomes, predicted protein sequences from GeneMarkS were searched against the database of *E. coli* virulence genes downloaded from the Virulence Factors Database (VFDB) (28) by using BLASTp. Only hits with 80% identity and 70% query coverage were considered positive hits. A comparative table was created based on the presence or absence of these virulence genes in all of the genomes compared. A similar table was generated for putative resistance-related genes after comparison with the Comprehensive Antibiotic Resistance Database (CARD) (29), using BLASTp. PhiSpy (30) and PHAST (31) were used to identify the putative phage sequences in the genome. Insertion sequence (IS) elements and genomic islands were identified using IS finder (32) and IslandViewer (33), respectively. Predicted protein sequences were also searched against the Conserved Domains

Database (CDD) (34) using NCBI Batch CD-Search, for functional categorization. In-house scripts were used to extract the best hits and corresponding cluster of orthologous groups (COG) classes from the results.

**Molecular characterization and susceptibility testing.** *E. coli* phylogenetic groups (A, B1, B2, and D) were determined with a well-established PCR method, as described previously (35). The isolates were preliminarily assessed for ST131 status with an allele-specific PCR assay for *pabB* gene detection (36). Antimicrobial susceptibility was examined with the standard Kirby-Bauer method, using an Icosa UTI-1 antibiotic ring (HiMedia, Mumbai, India), for 20 different antibiotics. ESBL production was detected using the double-disc synergy test. The results of both tests were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria. Metallo- $\beta$ -lactamase (MBL) production was screened with meropenem and meropenem-EDTA Etest strips (HiMedia, Mumbai, India), according to the manufacturer's directions. Detection of ESBL (*bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub>) and MBL (*bla*<sub>NDM-1</sub>) genes was carried out by PCR (15). Plasmids were determined using Kado lysis, and replicon types were identified using five multiplex and three single PCR assays for the presence of a total of 18 plasmid types (37).

**Phenotypic characterization of strains.** Serum resistance assays were performed in 50% human serum (Pan Biotech) as described previously (38), with some modifications. Briefly, 5  $\mu\text{l}$  of overnight bacterial culture was added to 495  $\mu\text{l}$  of fresh LB medium and grown for 1 h. After centrifugation, the bacteria were resuspended in 1 ml of sterile phosphate-buffered saline (PBS), and 30  $\mu\text{l}$  of culture with 270  $\mu\text{l}$  of 50% human serum was added in triplicate to 96-well microtiter plates. An aliquot of 30  $\mu\text{l}$  of each culture was serially diluted and plated (0-h count), and the rest was incubated for 3 h at  $37^{\circ}\text{C}$ . After incubation, the bacteria were serially diluted and plated (3-h count). Counting was performed after an overnight incubation, and CFU were calculated. The initial CFU value was subtracted from the final count in order to obtain growth in serum, and data were plotted on a graph. Hemagglutination assays were carried out with freshly isolated red blood cells (RBCs) from human blood, and results were interpreted as described elsewhere (39). Biotyping of strains for carbohydrate utilization of 35 sugar sources was performed using the Hi-Carbo kit (HiMedia, Mumbai, India), according to the manufacturer's protocol. Biofilm formation was examined in a total of nine replicates in 96-well microtiter plates with M63 medium, after 48 h, as described previously (8).

**Epithelial cell adhesion and invasion assays.** Adhesion and invasion assays were performed as described previously (38), with some modifications. T24 and ACHN cells (human bladder and kidney epithelial cell lines) were grown in RPMI 1640 medium and Ham's F-12 medium (Invitrogen) supplemented with 10% and 15% fetal bovine serum (FBS) (Invitrogen), respectively. Cells were grown in 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Cells were seeded into 24-well plates 48 h prior to cell infection assays. Infection was performed at a multiplicity of infection (MOI) of 10. Following 3 h of incubation at  $37^{\circ}\text{C}$ , the monolayer was washed three times using  $1\times$  PBS (Gibco) and subsequently lysed with 0.1% Triton X-100 in  $1\times$  PBS for 10 min at  $37^{\circ}\text{C}$ . The serially diluted lysates were plated on LB agar plates for determination of CFU of adherent bacteria. For invasion assays, the cells were incubated for 3 h and the monolayer was washed three times with  $1\times$  PBS and incubated for 1.5 h with 1 ml of fresh Ham's F-12 medium containing 100  $\mu\text{g}/\text{ml}$  gentamicin. After incubation, the cells were lysed with 0.1% Triton X-100 in PBS for 10 min at  $37^{\circ}\text{C}$ . Lysates were serially diluted and CFU were determined as in the adhesion assays described above.

**Infection of cultured THP1 cells and RT<sup>2</sup> profiling of antibacterial responses.** Monocyte cells were cultured and maintained at  $37^{\circ}\text{C}$  in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), in 5%  $\text{CO}_2$ . Prior to infection, about  $1\times 10^6$  cells were seeded into 35-mm-diameter petri dishes (Corning) and stimulated for 36 h using phorbol myristate acetate (PMA). After stimulation, cells were incubated for 12 h in antibiotic-free medium. Subsequently, *E. coli* strains (NA097 and DH5 $\alpha$ ) were grown overnight, followed by secondary growth to an optical

density at 600 nm of 0.95. *E. coli* was harvested and added to the differentiated THP1 cells at an MOI of 10. Similarly, another set of  $1 \times 10^6$  cells was treated with 1  $\mu$ g lipopolysaccharide (LPS). Infected cells were incubated for 3 h. Total RNA was isolated using a GenElute kit (Sigma), and cDNA was synthesized from 1  $\mu$ g of DNase (Sigma)-treated RNA, using a first-strand cDNA synthesis kit (Qiagen, Germany). Real-time PCR was performed on cDNA using the RT<sup>2</sup> Profiler PCR array system (Qiagen), according to the manufacturer's instructions, in an Eppendorf RealPlex 4 system. The experiments were performed in triplicate, and the data were analyzed using the online RT<sup>2</sup> Profiler array data analysis tool (version 3.5) from Qiagen.

**Statistical analysis.** Statistical analyses for biofilm assays, serum bactericidal assays, adhesion assays, and invasion assays were carried out using the nonparametric Mann-Whitney *U* test, using GraphPad Prism 5.01. For calculations with quantitative real-time (qRT)-PCR data, custom online software was used as directed in the PAHS-148Z RT<sup>2</sup> Profiler manual (Qiagen), and calculations were performed according to the manufacturer's instructions. *P* values of <0.05 were considered to be significant.

**Nucleotide sequence accession numbers.** This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under accession number JSXJ00000000. The version described in this paper is version JSXJ01000000.

## RESULTS

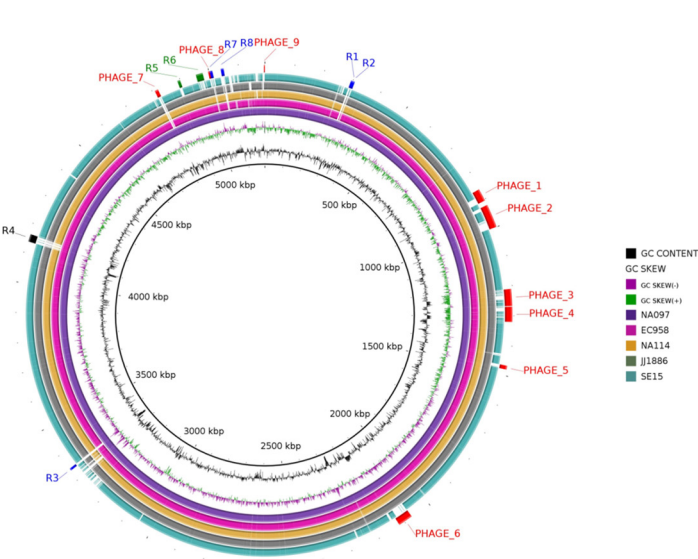
**Genome statistics and comparative genomic analysis.** The genome of NA097 comprised 187 contigs, constituting an approximate size of 5.1 Mb. The genome was predicted to encode ~5,164 protein coding sequences, with an average coding sequence length of 885 bp, amounting to a coding capacity of 88%. The genome revealed 51.83% G+C, 73 tRNAs, and 3 rRNA genes. The functional annotation of the NA097 genome using the COG database revealed a majority of genes related to metabolism (37%), followed by cellular processing and signaling (21%) and information storage and processing (17%). A fraction of the genes (14%) belonged to multiple classes, while 10% remained poorly characterized (see Fig. S2 in the supplemental material). *In silico* multilocus sequence typing (MLST) analysis using the MLST database (<http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli>) showed that this organism belongs to sequence type 131. We identified 13 families of insertion sequence (IS) elements within the NA097 genome, including at least one copy each of IS21, IS66, IS6, IS1, IS30, Tn3, IS1380, IS110, IS3, ISL3, IS4, IS200, and ISAs1.

Whole-genome sequence-based phylogenetic analysis of ST131 strains together with ST95 strains revealed three different clades (clade A, clade B, and clade C), as described in Fig. S1 in the supplemental material. The strains NA097 and NA114 clustered within clade C, which predominantly included strains of H30 and H30-Rx subclones of ST131 *E. coli*. The outcomes of uropathogenic *E. coli* (UPEC)-specific virulence and resistance gene profiling for five ST131 genomes are summarized in Tables S1 and S2 in the supplemental material. The virulence and resistance repertoires were comparable among the ST131 strains except for SE15, which had few virulence and fewer resistance genes. NA097 primarily revealed the *dra* operon, in contrast to the pyelonephritis-associated pili (*pap*) operon in NA114. The strains had similar resistance patterns except that NA097 was also positive for the *strA*, *strB*, and *aac3'* IIC genes. The genomes of EC958 and NA097 contained both *bla*<sub>TEM</sub> and *bla*<sub>CTX-M-15</sub> genes; SE15 was negative for both, and the rest of the genomes had one of the two  $\beta$ -lactamase genes. To elucidate the variations among genomes that clustered in clade C, we subjected four well-characterized reference

ST131 genomes, i.e., EC958, JJ1886, NA114, and SE15, with NA097 as a reference, to comparative analysis using BRIG. The most common variation observed for SE15 (a commensal ST131 strain) and the four H30-Rx subclonal ST131 strains was the absence of seven phage-associated regions in the SE15 genome (Fig. 1). Three major genomic islands, namely, GI-*selC*, GI-*pheV*, and GI-*leuX*, were also found in the NA097 genome, along with several other genomic islands with sizes ranging from 0.5 kb to 30 kb, as predicted by IslandViewer. The genome of NA097 exclusively contained the lysogenic phage P4 and other genomic regions encoding functions such as autoinduction, capsular polysaccharide synthesis, quorum sensing, aminoglycoside resistance, and others (labeled in blue in Fig. 1). A detailed description of different genomic regions and phages is also provided in Fig. 1. The genome of NA097 shared similarity with the recently published EC958 *E. coli* ST131 genome sequence, which contains several mobile genetic elements (MGEs) that would account for increased fitness or pathogenicity. Overall, the genomes of four H30-Rx strains were found to be highly clonal in nature, despite their distinct geographical and temporal origins.

**Molecular characterization and susceptibility testing.** The NA097 strain was positive for typical characteristics of clone ST131, i.e., it belonged to phylogroup B2 and was PCR positive for the *pabB* allele as well as CTX-M-15 and TEM  $\beta$ -lactamase genes. Phenotypically, NA097 was determined to be an ESBL producer and was MBL negative. The strain was refractory to the following 13 of 20 antimicrobial agents tested: ampicillin, ampicillin-sulbactam, and carbenicillin of the penicillin class; cephalothin (first generation), cefazolin (first generation), cefotaxime (third generation), and cefoperazone (third generation) of the cephalosporin class; norfloxacin, ciprofloxacin, moxifloxacin, and gatifloxacin of the fluoroquinolone class; and streptomycin and co-trimoxazole of the aminoglycoside and trimethoprim-sulfamethoxazole classes, respectively. The strain was completely susceptible only to the macrolide antibiotic class and to five other antibiotics belonging to the aforementioned classes. Kado lysis revealed the presence of four plasmids, but only two plasmids could be identified in PCR-based replicon typing.

**Phenotypic characteristics of ST131 strains NA097 and NA114.** Biofilm formation is considered to be an important virulence property among pathogenic *E. coli* strains (40), especially those that cause urinary tract infections (UTIs) and sepsis. Therefore, we investigated the ability of two ST131 strains to form biofilms in M63 minimal medium. We observed significant specific biofilm formation (SBF) values for both NA097 and NA114 strains, compared to DH5 $\alpha$ , after 48 h of incubation (Fig. 2a). Because serum resistance is reported to be a marker of virulence (41), we examined the survival of the two ST131 strains and DH5 $\alpha$  in human serum after 3 h. The mean CFU values for growth of NA097, NA114, and DH5 $\alpha$  were  $3.9 \times 10^8$ ,  $2.6 \times 10^8$ , and  $7.7 \times 10^7$  CFU, respectively, while the blank showed no growth (Fig. 2b). NA097 and NA114 were significantly resistant to serum, compared to DH5 $\alpha$ . Moreover, the levels of serum resistance for NA097 and NA114 were comparable. Furthermore, NA097 showed mannose-resistant hemagglutination (MRHA) in human RBCs. To assess whether isolates of the ST131 lineage shared specific metabolic characteristics, we investigated the carbohydrate utilization ability of NA097 using 35 API test reagents (HiMedia, Mumbai, India). Of the 35 sugars, NA097 was able to utilize 20 sugars (57%). The metabolic capabilities of NA097 and NA114 for



| S. No | Region | Size (kb) | Names of closest relative organism/ phage (if any) | Status/ coding proteins                                                                         |
|-------|--------|-----------|----------------------------------------------------|-------------------------------------------------------------------------------------------------|
| 1.    | Phage1 | 41.8      | P88                                                | incomplete                                                                                      |
| 2.    | Phage2 | 78.6      | BcepMu                                             | intact                                                                                          |
| 3.    | Phage3 | 56.3      | BP4795                                             | intact                                                                                          |
| 4.    | Phage4 | 50.4      | HK629                                              | intact                                                                                          |
| 5.    | Phage5 | 12.7      | mEp235                                             | incomplete                                                                                      |
| 6.    | Phage6 | 49.4      | ZF40                                               | intact                                                                                          |
| 7.    | Phage7 | 11.7      | P4                                                 | intact                                                                                          |
| 8.    | Phage8 | 5.9       | P88                                                | incomplete                                                                                      |
| 9.    | Phage9 | 3.1       | D108                                               | Incomplete                                                                                      |
| 10.   | R1     | 5.6       | -                                                  | site specific DNA methyltransferases, phage integrase etc.                                      |
| 11.   | R2     | 7         | -                                                  | Sam dependent methyltransferase, mobile element protein, Autoinducers LsrG, LsrF etc.           |
| 12.   | R3     | 7         | -                                                  | XcbB, CDP glycerol etc.                                                                         |
| 13.   | R4     | 25.2      | -                                                  | mobile element protein, type II restriction enzyme methylase, ATP dependent protease etc        |
| 14.   | R5     | 9.4       | -                                                  | Type I restriction modification system                                                          |
| 15.   | R6     | 23        | -                                                  | Flagellar synthesis apparatus                                                                   |
| 16.   | R7     | 9         | -                                                  | Autoinducers ABC transport system, mobile elements etc.                                         |
| 17.   | R8     | 10        | -                                                  | DNA recombinase protein, transposase, Aminoglycoside transacetylase, hypothetical proteins etc. |

FIG 1 Left, BRIG figure, representing a comparison of NA097 with four reference genomes of ST131 *E. coli* downloaded from the NCBI. Blue regions (R1, R2, R3, R7, and R8) are present only in NA097, the black region (R4) is present only in NA097 and EC958, green regions (R5 and R6) are absent in SE15, and red regions are phage-associated regions. Right, details of the regions and their relevant sizes.

carbohydrate utilization were found to be comparable. However, the strains did not demonstrate enhanced utilization of a panel of metabolic substrates, compared to the data on metabolic utilization by *E. coli* strains belonging to other sequence types (15).

**Adhesion and invasion assays with human epithelial cells.** Because of the significance of human kidney and bladder epithelial cells in the development of acute UPEC-related complications and because we wanted to investigate whether the presence or

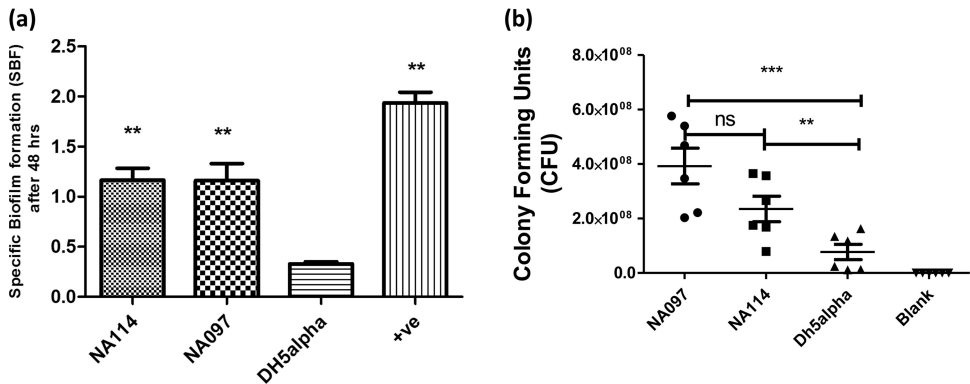


FIG 2 Biofilm formation capabilities and serum bactericidal resistance of *E. coli* strains NA114 and NA097. (a) Specific biofilm formation (SBF) after 48 h of incubation. (b) Resistance to human serum, as determined by CFU counting after 3 h of incubation. Strain DH5 $\alpha$  was used as a negative control for both experiments, and NA434 served as a positive control (+ve) for the determination of SBF. The experiment was performed in nine replicates for analysis of biofilm formation and six replicates for the serum assay, and statistical values were obtained with the nonparametric Mann-Whitney *U* test, using GraphPad Prism 5.01. ns, nonsignificant; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

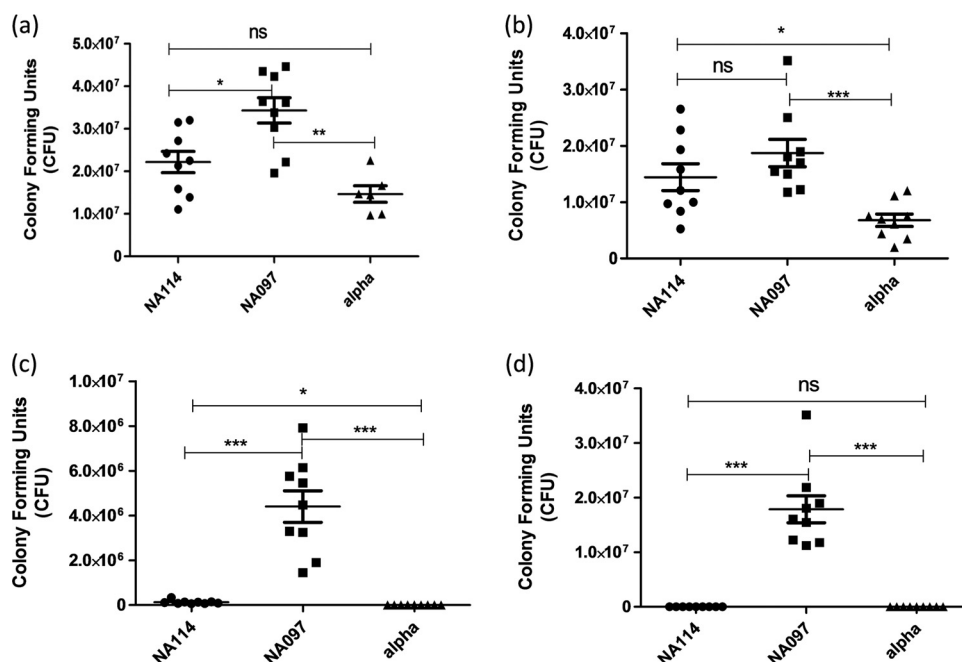


**FIG 3** Adhesion and invasion capabilities of *E. coli* strains NA114 and NA097. (a and b) Adhesion on the human bladder epithelial cell line T24 (a) and the human kidney epithelial cell line ACHN (b) after 3 h of incubation. (c and d) Invasion capabilities in the T24 cell line (c) and the ACHN cell line (d) after 3 h of incubation and additional incubation for 1.5 h in medium containing gentamicin (100  $\mu$ g/ml). DH5 $\alpha$  (alpha) was used as a negative control for the adhesion and invasion assays, experiments were performed three times in triplicate, and statistical values were obtained with the nonparametric Mann-Whitney *U* test, using GraphPad Prism 5.01. ns, nonsignificant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

absence of virulence genes affected the pathogenic nature of the bacteria (42), we conducted *in vitro* cell adhesion and invasion assays using strains NA097 and NA114, along with the negative control (DH5 $\alpha$ ), with both ACHN and T24 cells. We observed significant levels of adhesion and invasion of NA097 with both cell types, compared to DH5 $\alpha$  (Fig. 3). In contrast, NA114 was adherent with both cell types but was less invasive ( $1.3 \times 10^5$  CFU) with T24 cells and was noninvasive with ACHN cells.

**RT<sup>2</sup> gene expression profiling of antibacterial responses in THP1 cells.** In order to analyze the transcription of antibacterial response genes in differentiated human THP1 macrophages infected with the ST131 *E. coli* strain NA097, the DH5 $\alpha$  strain, and LPS, we performed quantitative PCR to determine the expression of 84 genes, corresponding to inflammatory response factors, pattern recognition receptors (PRRs), chemokines, cytokines, or downstream signaling components. The expression levels for 12 genes were unaltered in all three groups (including the control), while levels for a total of 24 transcripts were identified as being significantly altered. In all three categories, 12 genes were upregulated, while five genes were downregulated. A majority of significantly upregulated genes encoded cytokines such as interleukin 8 (IL-8), IL-1 $\beta$ , and tumor necrosis factor (TNF) and chemokines such as CXCL1, CXCL2, and CCL3; the remaining significantly upregulated transcripts encoded intracellular signaling factors such as BIRC3, MAP2K3, NFKB1, NFKBIA, NLRP3, and RIPK2. In contrast, transcripts corresponding to CARD6, TLR1, TLR6, PSTPIP1, and PYCARD were significantly downregulated (Fig. 4). IL-6 was upregulated more than 100-fold in all cases. Overall, the levels of transcriptionally altered genes were similar (with no significant differences) in all three groups. The rest of the genes did not show any significant fold changes, compared to the untreated

control; however, a few, such as IFNB1, NOD1, SUGT1, and NAIP, were upregulated differentially. These data provide a comprehensive picture of transcriptional regulation of macrophage responses to *E. coli* infections.

## DISCUSSION

Previous work by our group showed that ST131 *E. coli* strains in India have emerged as important causes of community-acquired urinary tract infections (8, 15). Molecular epidemiological analysis of these strains demonstrated that they are largely evolving clonally and are associated with several successful phenotypes that are likely involved in ST131 *E. coli* worldwide dissemination and infections (8, 15, 18, 43). This highlights the urgent need to understand this clonal pathogen, in order to develop better control strategies. In this study, we investigated the comprehensive profile of antibacterial responses of human macrophages upon ST131 *E. coli* infection. We further investigated the variations in the phenotypes of ST131 strains by analyzing the phenotypic traits versus the whole-genome sequence data for our two ST131 strains (NA097 and NA114). Whole-genome comparisons and phylogenetic comparisons were also performed with other sequenced genomes of ST131 clones.

Our data lend support to observations indicating adaptive diversification and clonal expansion of ST131 as the underlying mechanisms for the evolution of fluoroquinolone-resistant and ESBL-producing ST131 *E. coli* (44, 45). Our data also reveal that the similarity in phenotypes observed for our two ST131 strains correlates with the observation of the clonal genetic structure due to the evolution of a distinct subclone within ST131, namely, H30-Rx (10). The minor differences observed could be due to variations in virulence gene carriage, as observed by others (46).

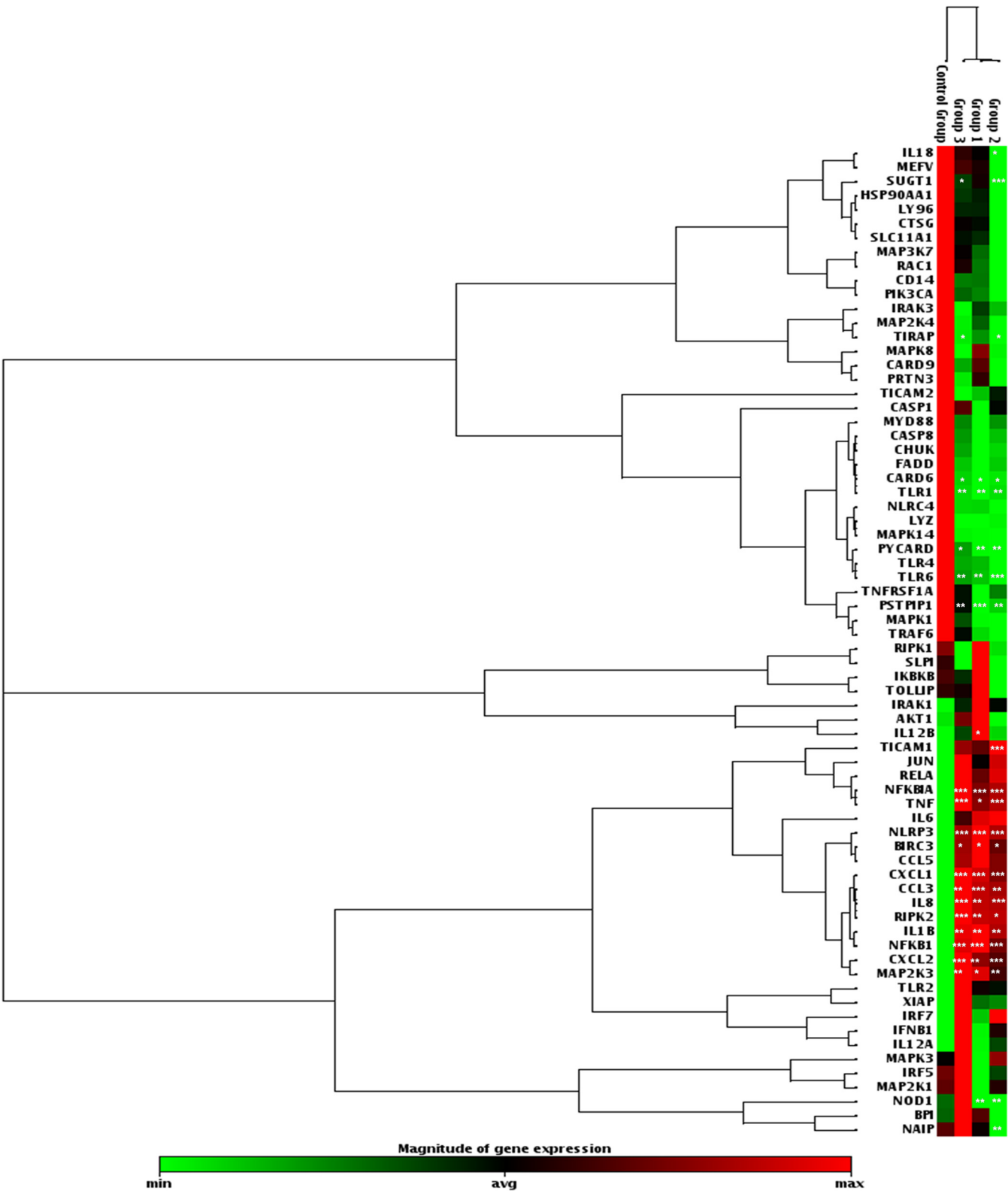


FIG 4 Cluster diagram indicating transcript levels of genes associated with bacterial infections in THP-1 cells. Group 1, LPS-treated cell sample; group 2, cells infected with *E. coli* strain DH5 $\alpha$ ; group 3, cells infected with strain NA097. Untreated THP-1 cells were used as the control. Online RT<sup>2</sup> Profiler array data analysis custom software (version 3.5) from Qiagen was used to obtain statistical values (Student's *t* test), according to the RT<sup>2</sup> Profiler instructions. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

ST131 *E. coli* is now the dominant extraintestinal pathogen throughout the world and is a major etiological agent of bladder and kidney infections (47). This organism represents a classic example of evolution through both horizontal and vertical (clonal expansion) mechanisms. Although ST131 first attracted attention because of its close association with ESBLs, especially the plasmid-mediated CTX-M-15, recent studies involving population genetic analysis of ST131 strains indicated that the global rise in extended-spectrum cephalosporin-resistant ST131 *E. coli* infections is due to the expansion of a single, highly virulent subclone, H30-Rx (10). Phylogenomic analysis of two of our ST131 strains (NA114 and NA097), together with several ST131 reference genomes (4), revealed that both of the Indian strains NA097 and NA114 belonged to the H30-Rx subclone (see Fig. S1 in the supplemental material). These results might represent only the first of many such findings and therefore warrant attention from clinicians and researchers, as our previous studies have already documented that ST131 *E. coli* strains represent a major population among clinical ExPEC isolates in India (8, 15).

Earlier studies reported the presence of mobile genetic elements (MGEs) in ST131 *E. coli* strains, which are not found in non-ST131 UPEC strains (4). Our study confirms this and documents the high level of conservation of seven prophage-associated genomic regions among all of the H30-Rx strains studied (Fig. 1); these seven prophage elements were clearly absent in the commensal non-H30-Rx ST131 SE15 genome (48). The genome of our ST131 strain NA097 revealed greater genetic affinity with the recently published EC958 ST131 genome from England (49), followed by the genome of the Indian ST131 strain NA114. This variation could possibly be due to differences in the arrangements of different genomic regions, arising from variable genomic contents encompassing mobile elements and other auxiliary genomic components. However, such a degree of clonality between two geographically distinct isolates hints at the possibility of their potential to emerge and to expand globally. Other studies have also observed phylogeographic patterns among spatially dispersed ST131 isolates (8).

Given the extensive genomic similarities observed among the strains within the H30-Rx clade, we sought to determine whether there were any phenotypic similarities between the two representative H30-Rx strains, i.e., NA114 and NA097. We compared the abilities of our two H30-Rx strains to form biofilms *in vitro* over 48 h. There was no significant difference in the levels of specific biofilm formation between the two strains (Fig. 2a). Furthermore, we found that the two strains were equally resistant to serum bactericidal activity (Fig. 2b). We also conducted *in vitro* cell adhesion and invasion assays with T24 bladder epithelial cells and ACHN kidney epithelial cells. In addition to the virulence-associated phenotypes, identical patterns were observed for the adhesion capabilities of the two strains on both types of epithelial cells (Fig. 3). However, the two strains differed significantly in their abilities to invade the epithelial cells. The NA097 strain was highly invasive, while NA114 showed insignificant invasion (Fig. 3). This observed difference could be partly attributable to the presence of the *dra* operon (which was found to be absent in the NA114 strain) in the NA097 strain. Goluszko et al. observed similar results, with insertional mutants of *dra* operon members being unable to invade HeLa cells (50). Finally, we compared the abilities of our two ST131 strains to metabolize 35 carbon sources. We found similar patterns of carbon utilization for the two strains. Overall, our data

provide a correlation between the genetic frameworks and the virulence phenotypes of two H30-Rx strains. In contrast, previous studies showed that ST131 strains in general were genetically monomorphic but phenotypically diverse (46). This might be due to the fact that all such studies analyzed ST131 strains without taking into account the recent subclones of ST131, namely, H30-R and H30-Rx. When these subclones are studied separately, they might yield clearer distinct observations.

While several studies have investigated the pathogenesis of ST131 *E. coli* using *in vitro* and *in vivo* assays, the innate immune responses involved and the molecular pathways governing these responses largely remain obscure. Deciphering the innate responses against this pathogen is key to understanding the immunological basis of bacterial clearance from the host. Here we performed transcriptome profiling of innate immune signatures in the context of host responses to ST131 strains, the control strain DH5 $\alpha$ , and LPS alone. We observed that only the key inflammatory pathways were activated upon infection with ST131, DH5 $\alpha$ , or LPS. Therefore, we could not identify major strain-specific host responses, which implies that, after infection, capsular antigens would be primarily associated with the immune responses and thus pathogenesis could not necessarily be correlated with repertoires of virulence genes or mobile genetic elements. A previous study by Vimont et al. (16) confirmed that the inflammatory responses of kidney cells after infection with ST131 versus non-ST131 were comparable. Also, Horn et al. (51) observed similar histopathological outcomes in lung tissues after infection with pathogenic versus fecal avian *E. coli*. These observations, together with our results, suggest that the fitness and epidemiological success of ST131 strains are more relevant in the upstream steps involved at the onset of infection.

In conclusion, our study confirmed the presence of H30-Rx ST131 *E. coli* among Indian ST131 strains and provided more insights into the clonal diversification of genomic and phenotypic features of H30-Rx ST131 strains. The study also shows the immunological basis of the outcomes of infection with ST131 strains. Such expository observations could be of great significance for understanding the emergence of globally disseminated, virulent H30-Rx strains. Further studies are needed to investigate whether this clone undergoes evolutionary selection and whether that has any effect on its virulence and resistance phenotypes.

## ACKNOWLEDGMENTS

We acknowledge funding received from the Department of Biotechnology, Government of India [grant BT/HRD/NBA/34/01/2011(ix)]. We also acknowledge support received from University of Malaya High Impact Research Grant UM.C/625/1HIR/MOHE/02 (A000002-5000 1) and support from Indo-German International Research Training Group GRK1673, an initiative of the German Research Foundation and the University of Hyderabad (India). A.R. acknowledges the Indian Council of Medical Research for the award of a junior research fellowship. N.A. is an adjunct professor at the Academy of Scientific and Innovative Research (AcSIR), New Delhi, India.

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## Chapter 4:

### Objective 3

**Molecular epidemiology of New Delhi metallo-beta-lactamase (NDM) positive, carbapenem resistant extraintestinal pathogenic *E. coli***

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This part is published as:

**Molecular Epidemiology and Genome Dynamics of New Delhi Metallo- $\beta$ -Lactamase-Producing Extraintestinal Pathogenic Escherichia coli Strains from India**

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**Antimicrobial agents and chemotherapy** 60 (11), 6795-6805



## Abstract

The global dissemination amidst increasing incidence of carbapenem-resistant, Gram-negative organisms has resulted in acute public health concerns. Here, we present a retrospective multicenter study on molecular characterization of metallo-beta-lactamase (MBL) producing clinical *E. coli* isolates recovered from extraintestinal infections in two hospitals in Pune, India. We screened a large sample size of 510 *E. coli* isolates for MBL production wherein we profiled their molecular determinants, antimicrobial resistance phenotypes, functional virulence properties, genomic features and transmission dynamics. Approximately ~8% of these isolates were MBL producers, the majority of which were NDM-1 (69%) type followed subsequently by NDM-5 (19%), NDM-4 (5.5%) and NDM-7 (5.5%). MBL producers were resistant to all antibiotics tested, except for colistin, fosfomycin and chloramphenicol which were effective up to a varying extent. Plasmids were found to be an effective means of dissemination of NDM genes and other resistance traits. All MBL producers adhered to and invaded bladder epithelial (T24) cells and demonstrated significant serum resistance. Genomic analysis of MBL producing *E. coli* revealed higher resistance but a moderate virulence gene repertoire. A subset of NDM-1 positive *E. coli* was identified as dominant sequence type 101 while two strains belonging to ST167 and ST405 harbored NDM-5. A majority of MBL producing *E. coli* strains revealed unique genotypes suggesting they were clonally unrelated. Overall, the co-existence of virulence and carbapenem resistance in clinical *E. coli* isolates is of serious concern. Moreover, the emergence of NDM-1 among the globally dominant *E. coli* ST101 warrants stringent surveillance and control measures.

## Introduction

Infections with Gram-negative multidrug-resistant (MDR) pathogens, particularly those caused by  $\beta$ -lactamase producing *E. coli* constitute a major reason for the 'global SOS' in public health (1). Carbapenems are often considered to be the last resort antibiotics available for treating infections caused by extended-spectrum- $\beta$ -lactamase (ESBL) or AMPc-producing bacteria (2) and are categorized in three major types; the KPC types, the metallo- $\beta$ -lactamases (MBLs) and the oxacillinases (3). The major geographically widespread MBLs comprise of imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), and New Delhi metallo-beta-lactamase (NDM), of which NDM has attracted significant worldwide attention as the NDM gene has been detected on different transferable plasmid types (both on typeable and untypeable) and has also shown to be chromosomally integrated in other bacterial species (4, 5).

NDM belongs to the amber class B  $\beta$ -lactamase. Unlike other classes of  $\beta$ -lactamases (A, C and D), NDM requires zinc-ions to catalyze the hydrolysis of  $\beta$ -lactam antibiotics and due to this, MBL activity can be inhibited in the presence of metal chelating agents such as ethylenediaminetetraacetic acid (EDTA) (6). NDM producing bacteria are mostly multidrug-resistant and show resistance towards sulfonamides, fluoroquinolones, aminoglycosides and macrolides but are susceptible to only a few antimicrobials, such as azetronam, tigecycline and colistin (7, 8). Recent reports suggest that this susceptibility is expected to be short lived (1). Moreover, NDM enzymes are generally not inhibited by any of the known and available  $\beta$ -lactamase inhibitors such as sulbactam, clavulanate and tazobactam (9).

NDM producing bacteria have been implicated in both hospital and community acquired infections and have been recovered from several infection sites including urinary tract infections, pneumonia, septicemia, wound infections, device-associated infections and companion animals (10-13). Although the exact geographical origin and the precise time for the emergence of the *bla*<sub>NDM</sub> gene are unknown, the first NDM-1 positive isolate was

recovered in 2009 from a Swedish patient who previously (2007) had an episode of hospitalization in New Delhi, India (14). Later in the year 2010, Kumarasamy *et al.* provided evidence that NDM-1 positive *Enterobacteriaceae* are widespread in India, Pakistan and the United Kingdom (15). Recent reports further indicate that several variants of NDM (NDM-1 to NDM-14) producing *Enterobacteriaceae* are currently spreading worldwide including United States, Netherlands, Australia, Canada, France, and Oman (16, 17) (<http://www.lahey.org/studies>). Most of these case reports/epidemiological studies provide evidence of connections of patients to the Indian subcontinent in the form of migration/travel visits or having received medical treatment. However, certain reports reflect on infection sources other than this region as patients without any obvious connection to these places have also been reported (18). Nevertheless, the Indian subcontinent particularly offers several risk factors for the emergence and spread of NDM-producing superbugs which include poor sanitation, easy access to broad spectrum antibiotics over the counter without proper prescription, increased medical tourism for health care and lack of stringent antibiotic policies.

Members of *Enterobacteriaceae*, particularly *E. coli* are often disseminating globally through distinct and successful lineages such as sequence types (ST) 131, 95, 10 etc. (19-22). Our previous studies have put forward strong evidences for the widespread occurrence of ST131 strains among extra-intestinal infections in India and have also reported existence of NDM-1 positive ST131 *E. coli*; thus representing a serious therapeutic challenge (23-27). This together with the implication of the Indian subcontinent in the current spread of NDM producing strains globally warrants serious attention of health care specialists and epidemiologists to surveillance and resistance tracking in India so as to monitor threats locally and globally. Also, there are very scarce data on the virulence traits associated with *E. coli* which produce NDM. With the advent of high-resolution, whole genome sequencing methods, it has now become easy to decipher the genomes of the NDM-producing isolates and understand the

underlying resistance and virulence mechanisms. The use of comparative genomics in infection tracking has enabled a comprehensive understanding of epidemiology, resistomes, virulomes and the genetic background of NDM-producing MDR *E. coli* strains.

In the present study, we sought to determine the prevalence of MBL producing phenotypes of strains from among a large number of *E. coli* isolates collected chronologically for six years (2009-2014) from a major tertiary hospital and to determine the antibiotic susceptibility patterns by taking into account the allelic diversity of *bla*<sub>NDM</sub> gene and the plasmid replicon types. Further, we determined the virulence traits of NDM producing *E. coli* by various phenotypic assays in conjunction with whole genome sequences of five MDR-NDM-producing *E. coli* strains and consequent analyses of resistomes, virulomes and genetic affinities as important parameters required to portray a correct epidemiological scenario. We believe that such exhaustive analyses potentially provide for a clear and solid epidemiological baseline to guide an effective antibiotic policy in an endemic country setting such as India.

## **Materials and methods**

### ***Bacterial isolates***

A total of 510 clinical isolates of *E. coli* were included in this study. The isolates were collected consecutively between January 2009 and December 2014 from in-patients and out-patients treated at two major tertiary care hospitals in Pune, India. The collection included clinical isolates from urine (*n*=393), pus (*n*=88), and other sources (*n*=29). All the isolates were obtained as pure cultures and were cryopreserved at -80°C in 20% glycerol. The isolates were revived on LB agar plates and LB broth prior to experiments.

### ***Detection of metallo-beta-lactamase production and its molecular characterization***

A total of 510 *E. coli* isolates were screened for MBL production using meropenem (with and without EDTA) with the help of Ezy MIC™ Strips (Himedia; India), as per the manufacturer's instructions. All positive *E. coli* strains were also screened for the presence of *bla*<sub>NDM-1</sub> by a gene specific PCR (28). *E. coli* strains confirmed as MBL producers were further analyzed by high fidelity PCR (with the use of Q5 DNA polymerase from NEB) using flanking primers of NDM gene as described earlier (29). Custom-sequencing of the resulting amplicons was performed and the nucleotide and translated protein-sequences were analyzed using MEGA 6 (30). NDM types were determined in accordance with the sequences available in NCBI database(s).

### ***Antimicrobial susceptibility testing and its molecular determinants***

Antimicrobial sensitivity of 39 strains against 23 different antibiotics was performed on Mueller-Hinton agar (MHA) plates by Kirby-Bauer disc diffusion method, using antibiotic discs (Hi Media) and ICOSA UTI rings (Hi Media), according to the manufacturer's protocols. Two in-house strains that were consistently resistant and susceptible to most of the antimicrobial agents were employed as positive and negative controls, respectively. Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute. For colistin, a zone of <11mm was considered as resistant. The minimum inhibitory concentration (MIC) values for four frontline antibiotics, namely tetracycline, cotrimoxazole, gentamicin and ciprofloxacin were determined by using HiComb MIC strip (Hi Media, India) as per the manufacturer's protocol. MIC values were determined and compared among a subset of 10 MBL strains, 10 non-ESBL producing strains and seven meropenem resistant transconjugants. All MBL-producing *E. coli* strains were analyzed for molecular determinants of antibiotic resistance by PCR. DNA template was prepared using a standard heat lysis method. Thereafter, gene specific primer sets were used to screen for the

following ESBL genes; *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>NDM-1</sub> (28, 31). Other antibiotic resistance genes, such as those conferring resistance to tetracycline [*tet* (A) and *tet* (C)], sulfonamides (*sul1*, *sul2*), streptomycin (*strA*), trimethoprim (*dfr*), including the quinolone resistance determinants (carried on plasmids) such as *aac* (6')-Ib, *qnrB*, and *qnrS* were amplified as per standard PCR protocols (32, 33). The amplicons were run on 1% agarose gel and the images were documented using a gel documentation system (Major sciences, USA). The correlation between the phenotypic and different genotypic determinants of resistance was calculated with the help of observed phenotypic resistance and the frequency of genotypic determinant probed.

### ***Conjugation, mating experiments and plasmid analysis***

Conjugal transfer of resistance genes was performed by broth mating or filter mating experiments for a subset of nine carbapenem resistant *E. coli* strains using sodium azide (NaI)-resistant recipient strain *E. coli* K12-J53 (34). Transconjugants were selected on selection plates supplemented with a combination of meropenem (4µg/ml) and NaI (50µg/ml). Besides this, other antibiotics and NaI combinations were used to select transconjugants. However, as meropenem containing transconjugants were the focus of this study the same were subjected to further analyses. Pure cultures of the transconjugants were preserved in 20% glycerol at -80°C. Antibiotic susceptibility and MICs were compared between the parental strains and their respective transconjugants. Plasmid classes were determined using PCR based replicon typing (35).

### ***Phenotypic assays***

A total of nine random isolates were analyzed for functional virulence traits as described below. Nine MBL *E. coli* strains were tested for their adhesion and invasion efficiency. Human bladder epithelial cell lines (T24) were cultured and maintained in RPMI1640 (Invitrogen, USA) medium with 10% fetal bovine serum (FBS). Cells were seeded in 24 well

plates and grown until monolayer was obtained. Infection was done at multiplicity of infection (MOI) 10. After 3 hr of incubation at 37°C with 5% CO<sub>2</sub>, cells were washed thrice with 1X PBS followed by lysis with 1ml of 0.1% Triton-X100. Cell lysates were serially diluted, plated on LB agar plates and incubated overnight at 37°C. For invasion assay, after 3 hr of incubation, the cells were washed and further incubated for 1.5 hr with 1 ml of RPMI medium containing 100µg/ml of fosfomycin. After incubation, cells were washed thrice with 1XPBS and processed in a similar way as described above. Assays were performed thrice with three technical replicates.

Serum resistance was determined for nine MBL *E. coli* isolates by *in vitro* time kill experiments as described earlier (22). Briefly, 5 µl of overnight grown culture was added to 495 µl of LB broth and incubated for one hr at 37°C in a shaker incubator. The culture was pelleted and resuspended in 1ml of 1XPBS and then 30 µl of this bacterial suspension was added to 96 well microtiter plate wells, each containing 270µl of 50% sterile human serum (PAN Biotech, Germany) in 1XPBS. 30µl serum was then collected (0 hour count), serially diluted and plated on LB agar plates for CFU enumeration. The plate was then incubated for 3 hr at 37°C. After the incubation time (3 hr count), 30µl sample was pipetted for serial dilution and plated as described above. Growth was measured in terms of CFU. Samples were considered serum resistant if the 3 hr CFU count equaled or exceeded the 0 hr CFU count of the same sample. Serum assay was performed twice in triplicates.

Total siderophore production was tested by streaking all 39 MBL-positive *E. coli* samples on chrome azurol S (CAS) agar plates (blue color media). Color change (haloes) from yellow to orange on the CAS agar media plates, after overnight incubation at 37°C, was recorded. No color change in media corresponded to non siderophore production by the culture.

### ***Enterobacterial intergenic repetitive element sequences (ERIC) PCR based strain typing***

ERIC-PCR based fingerprint analysis was performed as described previously (36). Strain specific DNA banding profiles obtained on 1.5% agarose gel images were compared using BioNumerics software (Version 7.1, Applied Maths, Belgium). After scoring the bands on dice similarity indices based on unweighted UPGMA (arithmetic mean), a dendrogram was generated in order to analyze similarity/diversity within carbapenem resistant *E. coli* strains. The definition of ERIC clusters was based on a Dice similarity index.

### ***Whole genome sequencing, assembly, annotation and genomic analysis***

Whole-genome sequencing (WGS) of five selected strains was carried on Illumina MiSeq with an insert size of 400 to 500 bp. The paired-end sequence reads were filtered and trimmed using NGS QC Toolkit (v2.3.3) (37). These high quality reads were assembled into contigs using SPAdes Genome Assembler (v3.6.1) (38). The contigs were then ordered and scaffolded using an in-house workbench, C-L-Authenticator (39) and the resulting ordered scaffolds were joined together by placing N's in between to construct draft genomes. These were then submitted to RAST (40) server for annotation, and the genome statistics were extracted using ARTEMIS (41). For all of the genomes, genes were predicted using GeneMarkS (42). Amino acid sequences of the predicted genes from all the strains were compared using BLASTp (43) against the database of *E. coli* virulence genes from the Virulence Factors Database (VFDB)(44). Only those with 60% identity and 85% query coverage were considered as present and in this way a presence-absence status of virulence genes in each of these strains was represented in the form of a heat map using R. A similar heat map was generated for putative resistance-related genes after comparison with the Comprehensive Antibiotic Resistance Database (CARD) (45), using BLASTp. Insertion sequence (IS) elements and putative phage sequences in the genome were identified using IS

finder(46) and PHAST (47) respectively. The Sequence Type (ST) of each of these strains was determined by submitting the contigs to the Centre for Genomic Epidemiology (CGE) server (<https://cge.cbs.dtu.dk/services/MLST/>).

### ***Accession numbers***

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions LXFJ000000000 (NA703), LXFK000000000 (NA724), LXEZ000000000 (NA086), LXFA000000000 (NA099) and LWTZ000000000 (NA084). The versions described in this paper are LXFJ010000000, LXFK010000000, LXEZ010000000, LXFA010000000 and LWTZ010000000, respectively.

### ***Statistical analysis***

All statistical analyses were performed using GraphPad prism (version 5.01). For adhesion and serum resistance non parametric Mann-Whitney U test was performed while for the invasion assay Wilcoxon matched pair tests were performed and p values were determined.

## **Results**

### ***Prevalence of MBL producers was low with high abundance of NDM-1 genotype***

Of the 510 clinical *E. coli* isolates, 39 (7.6%) were found to be MBL producers. These 39 MBL producing *E. coli* isolates were deemed to be of potential interest and taken further for in depth analyses, as described. All these 39 MBL *E. coli* isolates were also found to be ESBL producers, phenotypically. Most of the MBL producers (25/39) were from urine isolates, followed by pus (11/39) and other sources (3/39). The 39 MBL producers as identified at the phenotypic level were screened for the presence of major carbapenemase genes including, *bla*<sub>NDM</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>OXA-48</sub>. With the exception of the *bla*<sub>KPC</sub> gene, which was not

detected in any of the MBL producers, all the 36 (92%) MBL producers were *bla*<sub>NDM</sub> positive and 6 (15%) of them were also *bla*<sub>OXA-48</sub> positive. Sequence analysis of the full length NDM genes from 36 MBL producers revealed that majority of them belonged to *bla*<sub>NDM-1</sub> (25 isolates) followed by *bla*<sub>NDM-5</sub> (7 isolates). Further, the gene *bla*<sub>NDM-4</sub> was present in two isolates and the gene *bla*<sub>NDM-7</sub> was harbored by other two MBL producers.

***All MBL producing E. coli isolates were identified to be MDR and ‘pandrug resistant’***

Of the 23 antimicrobial agents representing 10 different classes, our MBL *E. coli* isolates showed an average resistance to nearly 18 antibiotics with maximum resistance to 21 and minimum to 10 antimicrobial agents. The frequencies of susceptibilities/resistance are indicated in figure 1. Among all MBL positive isolates, highest resistance was observed against beta-lactam antibiotic class while highest sensitivity (100%) was observed to the peptide antibiotic class, the colistin. Each of the MBL producing *E. coli* isolates was found to be at least 90% resistant to all the beta-lactam and beta-lactam/beta lactamase inhibitor combinations used, including ampicillin, carbenecillin, cefotaxime, cephalothin, cefparazone, cefepime, cefazolin, piperacillin /tazobactam and ampicillin/sulbactam. On the contrary, each of the MBL producing *E. coli* isolates was found to be sensitive to colistin. All MBL positive isolates were highly susceptible to fosfomycin and chloramphenicol (Fig 1). Other resistance patterns were as follows: tetracyclines and aminoglycosides class, 80% each and trimethiprime/sulphonamides class, 82%. Notably, all MBL positive isolates (100%) were identified to be MDR as they were resistant to three or more classes of antimicrobials. Moreover, all the MBL producing *E. coli* isolates were found to be ‘pandrug-resistant’ meaning that they were resistant to as many as 7 antimicrobial agents. The MIC values of MBL-ESBL producing *E. coli* and non-ESBL *E. coli* isolates varied widely for different antibiotics used in the study (Table 1). Overall, the MBL-ESBL producing *E. coli* isolates revealed higher MICs for all four frontline antimicrobial substances compared to non-ESBL

strains. The higher MIC values of the four antimicrobials revealed by the meropenem-transconjugants in comparison to the recipient strain J53 *E. coli* indicated horizontal transmission of respective resistance genes. All the 39 ESBL-MBL producers were negative for *bla*<sub>SHV</sub> and *bla*<sub>KPC</sub>. However, 31 isolates harbored *bla*<sub>CTX-M-15</sub> and 28 isolates harbored *bla*<sub>TEM</sub>. The correlation among different resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was found to be high for aminoglycosides (94% agreement) and sulfonamides (84% agreement) whereas the agreement between resistance to tetracycline and the presence of tet(A) and tet(C) was 69 % and that for fluoroquinolone was (67%). The overall prevalence of all the major resistance genes in 39 MBL producing *E. coli* isolates is presented in Fig 1.

#### ***Metallo-beta-lactamase resistance was naturally transferrable via plasmids***

NDM genes are basically located on plasmids. In order to determine the capability of these MBL positive *E. coli* strains to disseminate NDM genes horizontally, we carried out conjugation mating experiments with sodium azide-resistant recipient strain *E. coli* K12-J53. Out of the 9 representative MBL positive strains employed, we were able to obtain 7 meropenem containing transconjugants alongside several other transconjugants. However, only meropenem transconjugants were further analyzed for this study. The transconjugants were analyzed for their phenotypic drug resistance to four different classes of antibiotics, along with MBL and ESBL detection. All transconjugants were identified to be MBL and ESBL positive with co-resistance varying widely for different antibiotics used in the study. The detailed result is shown in Fig 2. Transconjugants were confirmed to be carrying NDM gene by gene specific PCR. PCR-based replicon typing revealed presence of majorly three plasmid types, FIA, FIB and F plasmids among transconjugants. These observations reflect the role of transmissible plasmids in dissemination of NDM based carbapenem resistance.

### ***MBL-producing E. coli isolates were moderately virulent***

Phenotypic virulence properties of the MBL positive *E. coli* isolates were assayed using adhesion, invasion, serum bactericidal and siderophore production assays. The two former assays were carried on nine randomly selected strains and the siderophore production was determined for all the 39 strains. Adhesive and invasive virulence properties are important features during intestinal as well as extraintestinal infections. In order to evaluate this, we quantitatively assayed the pathogen's ability to adhere and invade the urinary bladder epithelial cells. Compared to negative control strain DH5- $\alpha$  *E. coli*, all nine MBL-positive *E. coli* isolates showed significant adhesion to T24-cells with various efficiencies as illustrated in Fig 3a. Among the nine MBL positive isolates tested, NA084 isolate demonstrated highest adherence capability towards T24-cells. T24-cells were invaded by all investigated isolates in comparison to the negative control strain *E. coli* Dh5-alpha revealing weak invasion index. Numbers of intracellular bacteria are depicted in Fig 3b.

Resistance to serum is quite essential for the pathogens to survive and cause invasive infections; we found all the nine tested isolates to be resistant (100%) to serum bactericidal activity as compared to the control Dh5-alpha *E. coli*. Bacterial survival in serum over a period of three hours is depicted in Fig 3c. Though the virulence genes tested were not prevalent in these isolates, bacterial resistance towards bactericidal effect of serum could result from individual or combined effects of capsular polysaccharide, surface proteins and toxin secretions.

Siderophore production, as visualized by an iron mobilization zone around the bacterial colonies with orange haloes on the Chrome-Azurol S agar, was similarly prevalent among 36 (92%) out of the 39 MBL producing *E. coli* isolates. The role of iron in the virulence mechanism of human and animal pathogens is well established (48). Siderophore production has been shown to be more frequently associated with extra intestinal pathogenic *E. coli*.

### ***Whole genome analysis reveals high resistance, moderate virulence with MBL strains***

Out of the 39 MBL positive strains, whole genome sequencing of five representative strains (three NDM-1 and two NDM-5) was carried out. Their genome characteristics are represented in Table 2. The three NDM-1 strains belonged to ST101 while the two NDM-5 strains were characterized as ST167 and ST405. Whole genome based resistome reflected the high prevalence of resistance genes of all categories within all the strains similar to the phenotypic drug resistance (Fig 5). The virulome of these strains reflected moderate virulence wherein all the strains inherited genes belonging to adhesins, autotransporters, TTSS, T6SS. NA099 harbors least number of virulence genes while NA724 showed maximum genes. The details are shown in Fig 5. All the strains showed different phage content and variable IS elements. The upstream sequence analysis of these five NDM strains showed insertion elements such as *ISAba125* upstream to NDM-1 and *IS5* and truncated *ISAba125* upstream to NDM5 sequences.

### ***NDM strains reveal high genomic diversity***

The PCR based genotyping is used to analyse the genetic relationship of the organisms. ERIC PCR based dendrogram of these strains (Fig 4) revealed high diversity among these strains. However, when observed at lesser resolution (~65% identity) two diffusely clonal clades were observed. As expected, all the strains in this cluster harbored NDM-1 genes. Further, the *in silico* MLST analysis of whole genome sequences identified all the three strains to be ST101. Overall, this cluster might show clonally related ST101 strains which are strongly associated with the NDM genotype. No such clusters were obtained for any other NDM variants. Above observations also support that plasmids are the important means for NDM dissemination.

## Discussion

The emerging NDM, an acquired class B carbapenemase from among *Enterobacteriaceae*, has become a major public health concern worldwide (49) and infections with these pathogens pose a major healthcare problem particularly for developing countries such as India. Previous studies from our group have shown an endemic *E. coli* infection burden and the presence of clonal group ST131 isolates with NDM-1 (23-25, 50). This study was aimed at detection, comprehensive characterization and whole genome sequence analysis of MBL producing MDR organisms that can lead to successful infection. This study would likely be useful in guiding formulation of control strategies involving antimicrobial stewardship and public health interventions in order to control the escalation of life-threatening MDR bacterial infections involving ExPEC organisms.

In general, production of MBL in *Enterobacteriaceae* shows an increasing prevalence pattern. However, the prevalence of carbapenem resistance among 510 *E. coli* isolates analyzed throughout, 2009 to 2014 was found to be 7.6%, which was within the range of previously reported carbapenem resistance from India (51, 52); however, this might be variable in different geographical locations and different hospital settings. The dissemination of NDM carbapenemases involves several *bla*<sub>NDM</sub> gene variants that are associated with different plasmid types among several Gram-negative species. A total of 14 NDM types have been reported globally, which were due to single or more point mutations (<http://www.lahey.org/studies>). In our study, we identified four NDM variants responsible for MBL production; NDM-1 being the dominant variant, while other variants comprising NDM-4, NDM-5 and NDM-7 were observed with varying frequencies. Previous reports from India have shown the presence of NDM-6 type in addition to the above mentioned NDM types (52). As such, these four variants neither demonstrated any significant difference in MIC values for meropenem, nor did they show any specific association with different antimicrobial resistances. The carbapenems were referred to as the last resort antibiotics for

treatment of MDR bacterial infections; hence bacteria which are resistant to these agents are often termed as ‘superbugs’ as they possess resistant phenotype against different classes of broad spectrum antibiotics. The antibiogram of these 39 isolates (Fig 1) reflected similar scenario wherein all MBL strains (100%) revealed pandrug resistant phenotypes as they were completely resistant to almost all the empirically used antibiotics. Nevertheless, all the strains were susceptible to colistin and a majority of them were susceptible to fosfomycin. Polymyxins are the last resort antibiotics to treat infections caused by MBL producing *Enterobacteriaceae* (53), and as the combinational therapies are being advocated for treatment of MDR pathogens (54), the dual combination of colistin and fosfomycin could be the best option in effective treatment of carbapenem resistant infections which also minimizes the chances of resistance development.

The spread of antibiotic resistance genes is mediated by horizontal gene transfer (HGT) between bacteria (55). As multidrug-resistant plasmids serve as the most efficient means for dissemination of resistance traits *via* HGT, we also observed that the NDM genes and other resistances were transferred (*via* plasmids) to recipient strains as confirmed by us genotypically and phenotypically by conjugation experiments (Fig 2). ERIC PCR based clusters revealed the presence of NDM genes in *E. coli* isolates of mostly unique genotypes, suggesting that they are clonally unrelated and thereby have equal propensities to acquire the NDM genes (Fig 4). Recent reports have shown a greater association of NDM-1 genotype with sequence type (ST) 101, which is consistent with our findings as we also observed three NDM-1 bearing *E. coli* belonging to ST101 (56, 57). We identified NDM-5 to be present in *E. coli* isolates belonging to ST405 and ST167. These sequence types were also linked to NDM dissemination, worldwide (58-60). Further, we found that the NDM-1 sequences were preceded by *ISAba125* insertion element which belongs to IS30 family while NDM-5 were having IS5 insertion element upstream to their sequence followed by sequences of *ISAba125*. *ISAba125* are known to be located upstream to NDM in both *Acinetobacter* and *E. coli*, similar to

our observations. Krishnaraju *et al* have also described IS5 element to be followed by truncated *ISAbal25* sequence upstream of NDM gene (61, 62).

The virulence phenotypes such as adhesion to and invasion of epithelial cells play an important role in initiation and establishment of infection. The virulence gene complement as detected at whole genome level (Fig 5) and the adhesion/invasion (Fig 3a and Fig 3b) capabilities towards human urinary bladder epithelial cells demonstrated their pathogenic potential and associated risks during infection as these strains are resistant to all empirically used antibiotics. Peirano *et al.* have also shown the ability of CTX-M and NDM positive strains to adhere to Caco2 and HepG2 cell lines (63). The ability to resist the bactericidal effect of serum complement as determined by serum resistance assay (Fig 3c) points towards enhanced survival advantages of these strains in different extraintestinal infection sites.

In summary, our findings demonstrated that the human extraintestinal pathogenic–MBL producing *E. coli* were associated with promiscuous plasmid types, resulting in diverse range of clones harboring different variants of NDM. Furthermore, whole genome sequencing and genetic fingerprinting suggest that *bla*<sub>NDM-1</sub> may also disseminate *via* dominant clones such as ST101. The genomes newly sequenced herein would be an important addition to the existing UPEC/ExPEC genome archives and would be helpful in future studies involving comparative genomics and molecular epidemiology. Given that the emergence of newer variants of NDM1 in endemic countries and their association with different ExPEC infections is an alarming problem, large-scale surveillance and molecular genotyping studies are needed in order to develop strategies to prevent dominant clones potentially leading to an institutionalized outbreak.

## Acknowledgments

We acknowledge funding received from Department of Biotechnology, Government of India [Ref.No.BT/HRD/NBA/34/01/2011(ix)] and support from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673) - functional molecular infection epidemiology, an initiative of the German Research Foundation (DFG) and the University of Hyderabad (India), of which NA and LHW served as speakers. NA is an Adjunct Professor at the Academy of Scientific and Innovative Research (ACSIR), India. AR would like to acknowledge the Indian Council of Medical Research (ICMR) for the award of Junior Research Fellowship (JRF). We thank Ramani Baddam for her help in discussing the data and their interpretation and help with the analysis of plasmid sequences.

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**Table 1.** MIC values of 10 carbapenem resistant, 7 meropenem transconjuants, a recipient strain, and 10 non ESBL non carbapenem resistant *E. coli* (T represents transconjuants and ND = not determined)

| strain no                                        | Gentamicin (µg/ml) | Ciprofloxacin (µg/ml) | Co-trimoxazole (µg/ml) | Tetracycline (µg/ml) |
|--------------------------------------------------|--------------------|-----------------------|------------------------|----------------------|
| <b>Carbapenem resistant strains</b>              |                    |                       |                        |                      |
| NA084                                            | >240               | >240                  | >240                   | 3                    |
| NA644                                            | >240               | >240                  | >240                   | 5                    |
| NA708                                            | >240               | 60                    | >240                   | 3                    |
| NA086                                            | >240               | >240                  | >240                   | 2                    |
| NA086 T                                          | >240               | 0.01                  | 0.05                   | 0.1                  |
| NA099                                            | >240               | >240                  | >240                   | 0.1                  |
| NA099T                                           | 0.01               | 0.1                   | 0.01                   | 0.01                 |
| NA316                                            | >240               | >240                  | >240                   | 3                    |
| NA316T                                           | 0.1                | ND                    | 0.05                   | 0.1                  |
| NA635                                            | >240               | 120                   | >240                   | 5                    |
| NA635T                                           | 24                 | ND                    | >240                   | 3                    |
| NA703                                            | >240               | >240                  | >240                   | 0.001                |
| NA703T                                           | 16                 | ND                    | 0.05                   | 0.001                |
| NA724                                            | >240               | 120                   | >240                   | 3                    |
| NA724T                                           | 30                 | 0.01                  | 0.05                   | 0.1                  |
| NA805                                            | 60                 | 10                    | >240                   | 0.01                 |
| NA805T                                           | ND                 | 1                     | 0.05                   | 0.01                 |
| <b>Recipient strain</b>                          |                    |                       |                        |                      |
| K12-J53                                          | 0.001              | 0.008                 | 0.05                   | 0.01                 |
| <b>Non ESBL non carbapenem resistant strains</b> |                    |                       |                        |                      |
| NA433                                            | 0.1                | 60                    | >240                   | 10                   |
| NA010                                            | 0.1                | 0.008                 | 30                     | 0.1                  |
| NA295                                            | 0.01               | 0.01                  | 120                    | 0.1                  |
| NA053                                            | 0.25               | 1                     | >240                   | 2                    |
| NA057                                            | 0.1                | 30                    | >240                   | 0.1                  |
| NA120                                            | 0.01               | 0.1                   | >240                   | 0.5                  |
| NA140                                            | 0.1                | 0.01                  | >240                   | 30                   |
| NA150                                            | 0.1                | 0.25                  | 2                      | 0.01                 |
| NA203                                            | 0.01               | 0.01                  | 0.1                    | 0.01                 |
| NA274                                            | 0.1                | 10                    | >240                   | 30                   |

**Table2.** The genome statistics and sequence types of five whole genome sequenced NDM harboring strains comprising of three NDM-1 and two NDM-5 strains

| Strain | No. of raw reads | Genome coverage from filtered reads | No. of Contigs | ST  | Genome size in bases | G+C%  | CDS  | Coding <sup>o</sup> % | No. of rRNAs | NDM type | Source specimens type |
|--------|------------------|-------------------------------------|----------------|-----|----------------------|-------|------|-----------------------|--------------|----------|-----------------------|
| NA084  | 1741426          | 73.25                               | 109            | 101 | 5147001              | 50.59 | 5000 | 86.8                  | 13           | NDM1     | Urine                 |
| NA086  | 1643674          | 63.13                               | 115            | 101 | 5147281              | 50.59 | 4994 | 86.9                  | 13           | NDM1     | Urine                 |
| NA099  | 2406806          | 104.68                              | 155            | 101 | 5197495              | 50.57 | 5063 | 85.8                  | 17           | NDM1     | Urine                 |
| NA703  | 2401628          | 92.94                               | 211            | 167 | 5263381              | 50.66 | 5186 | 86.0                  | 16           | NDM5     | pus                   |
| NA724  | 2784910          | 118.36                              | 166            | 405 | 5424442              | 50.55 | 5273 | 87.0                  | 9            | NDM5     | pus                   |

## Figure Legends:

**Figure 1. Top panel:** Antibigram of MBL producing carbapenem resistant *E. coli* as tested by disc diffusion method for the following 23 different antibiotics: ampicillin (Amp), piperacillin/tazobactam (PIT), carbenicillin (CB), Ampicillin/Sulbactam (A/S), cefotaxime (CTX), cephalothin (CEP), cefaperazone (CPZ), cefepime (CPM), cefazolin (CZ), nalidixic acid (NX), ciprofloxacin (CIP), moxifloxacin (MO), gatifloxacin (GAT), tetracycline (TE), doxycycline HCL (DO), co-trimoxazole (COT), gentamicin (GEN), amikacin (AK), nitrofurantoin (NIT), clarithromycin (CLR), chloramphenicol (C), fosfomycin (FO) and colistin (CL). All MBL producing isolates were detected to be “pandrug-resistant”. **Bottom:** Identification of resistance determinants among 39 MBL producing *E. coli* isolates by gene specific PCRs.

**Figure 2.** *In-vitro* conjugation experiment and comparative analysis between clinical strains and their respective transconjugants revealed transfer of resistances via plasmids. Suffix T designates transconjugants.

**Figure 3.** A subset of 9 MBL producing *E. coli* isolates was tested for three virulence-associated phenotypes: **3a:** Adhesion to human bladder epithelial cells (T24) at MOI 10. **3b:** Invasion to human bladder epithelial cells (T24) and **3c:** Resistance to 50% human serum for 3 hr time period. Adhesion and invasion assays were repeated three times in triplicates while serum resistance assay was repeated twice in triplicates. All the nine MBL producing *E. coli* isolates demonstrated ExPEC-associated phenotypes. \*\* denotes p values <0.01 and \*\*\*denotes p values <0.001 as compared to control (Dh5-alpha).

**Figure 4.** Dendrogram based on phylogenetic analysis of 39 MBL producing carbapenem resistant clinical *E. coli* strains using ERIC PCR banding analysis by BioNumerics 7.5

(Applied Maths, Belgium). Dotted boxes indicate clusters into which majority of NDM-1 profiles segregated. ND = not determined.

**Figure 5.** Whole genome based identification of virulence (Left) and resistance (Right) determinants of five MBL producing *E. coli* isolates.

**Figure 1:**

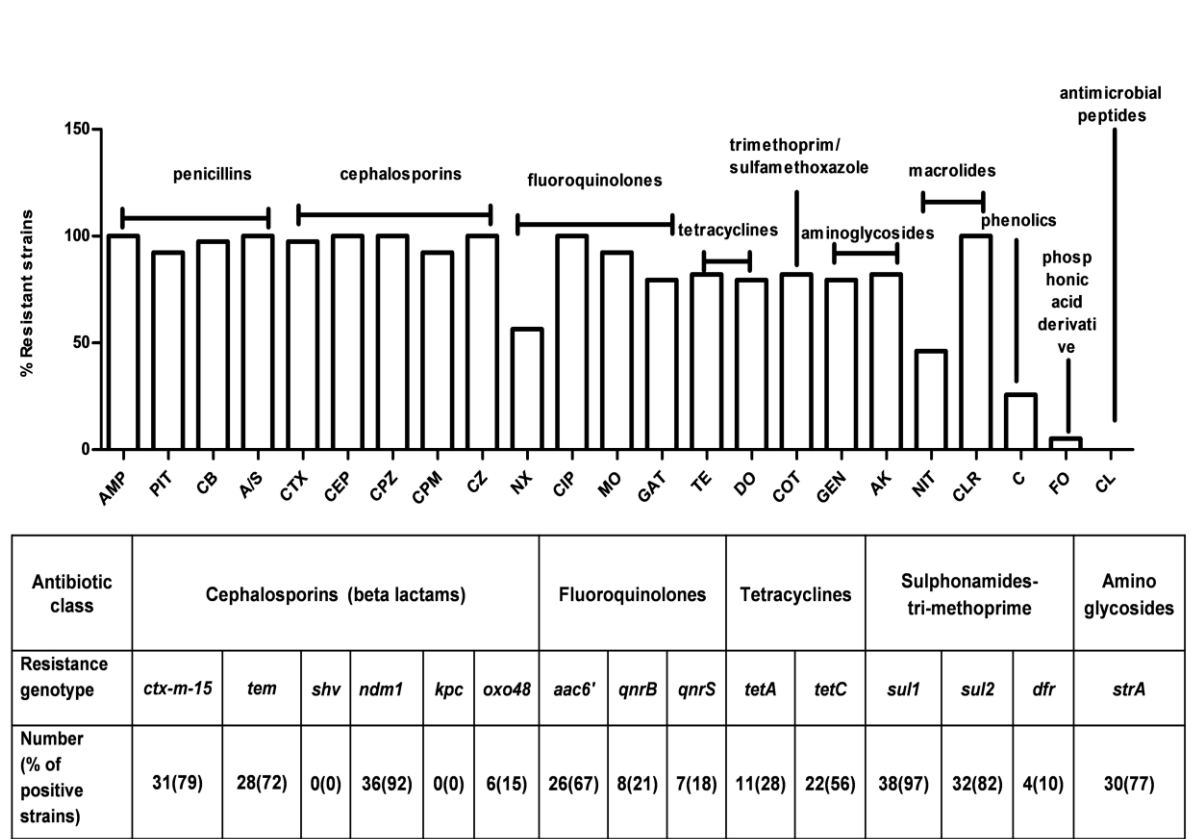


Figure 2:

|         | NA086 | NA086<br>T | NA099 | NA099<br>T | NA316 | NA316<br>T | NA635 | NA635<br>T | NA703 | NA703<br>T | NA724 | NA724<br>T | NA805 | NA805<br>T |
|---------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|
| MBL     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| ESBL    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Gen     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Cip     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Cot     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Tet     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| ndm     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| ctxm-15 |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| FIA     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| FIB     |       |            |       |            |       |            |       |            |       |            |       |            |       |            |

Figure 3:

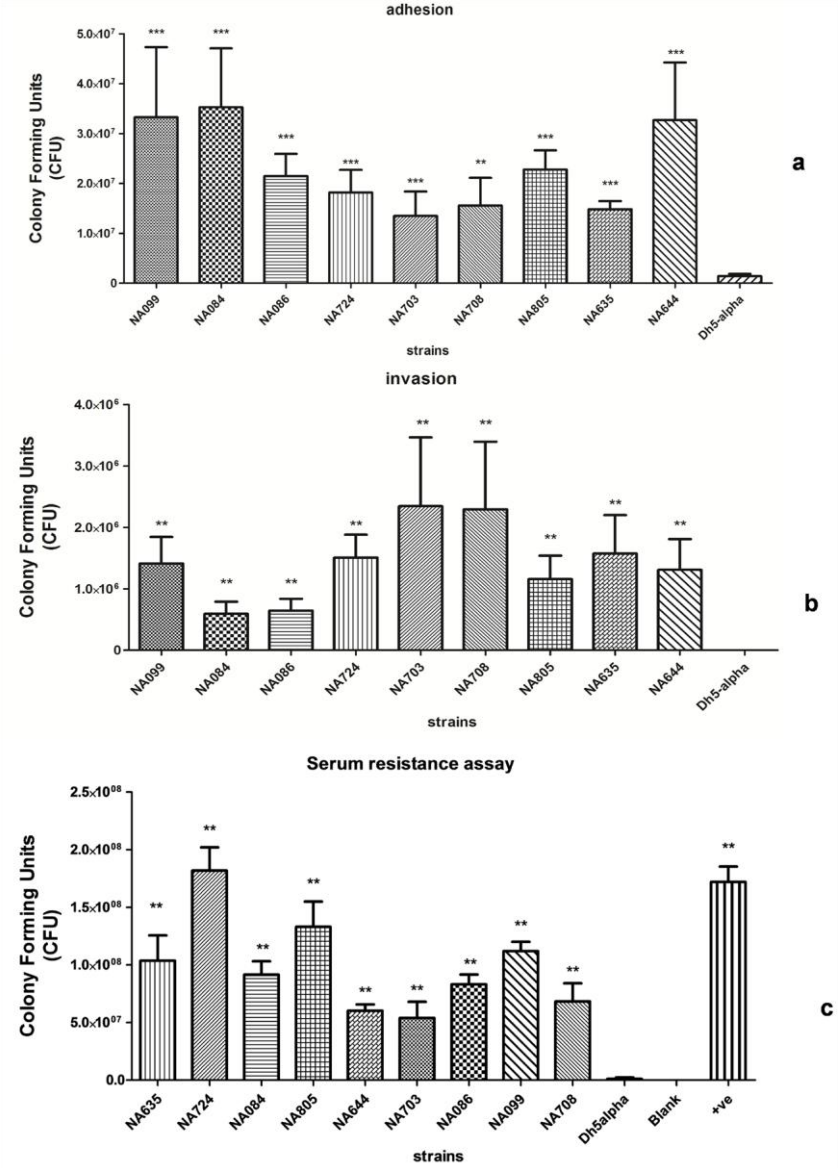


Figure 4:

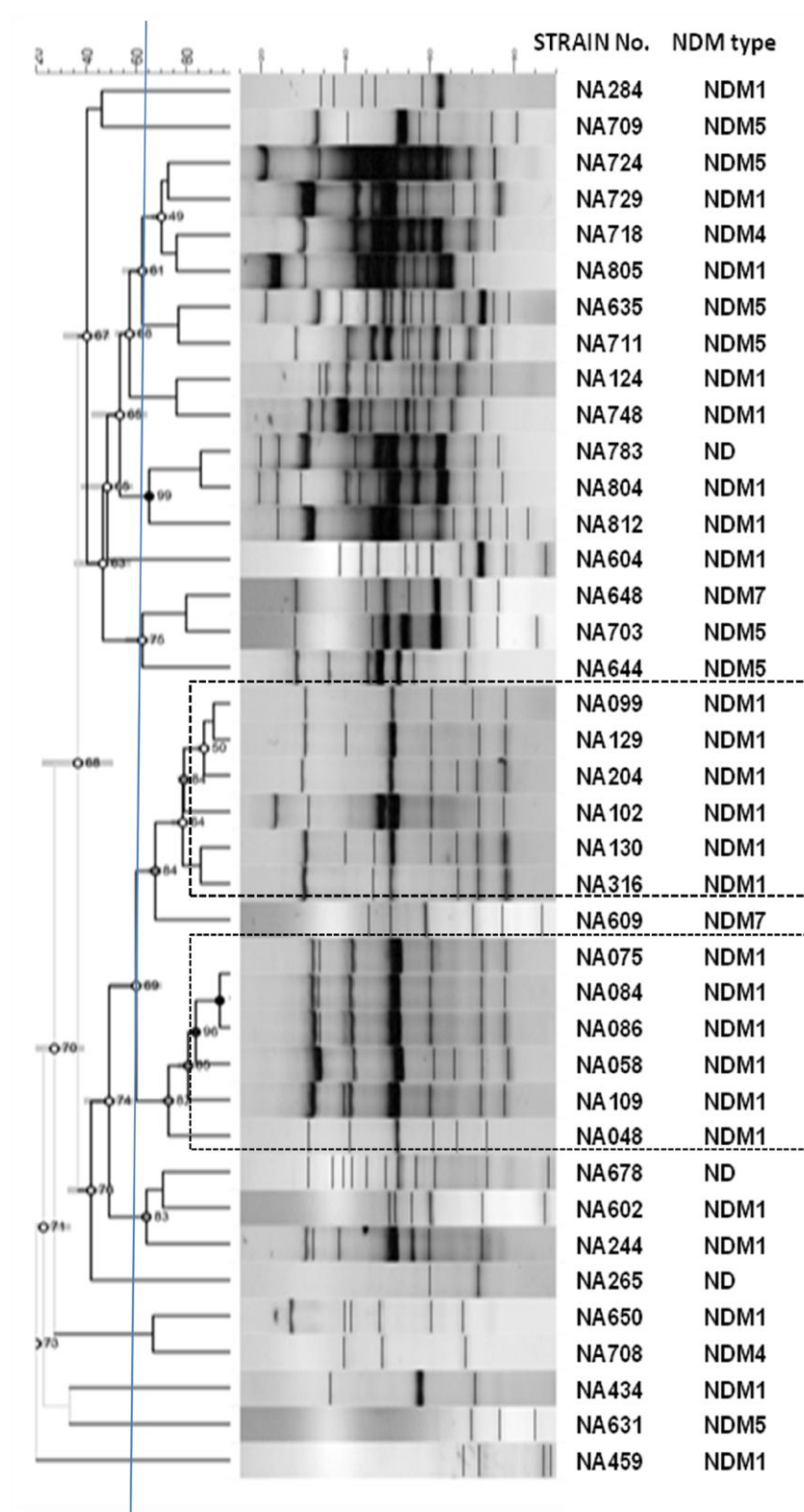
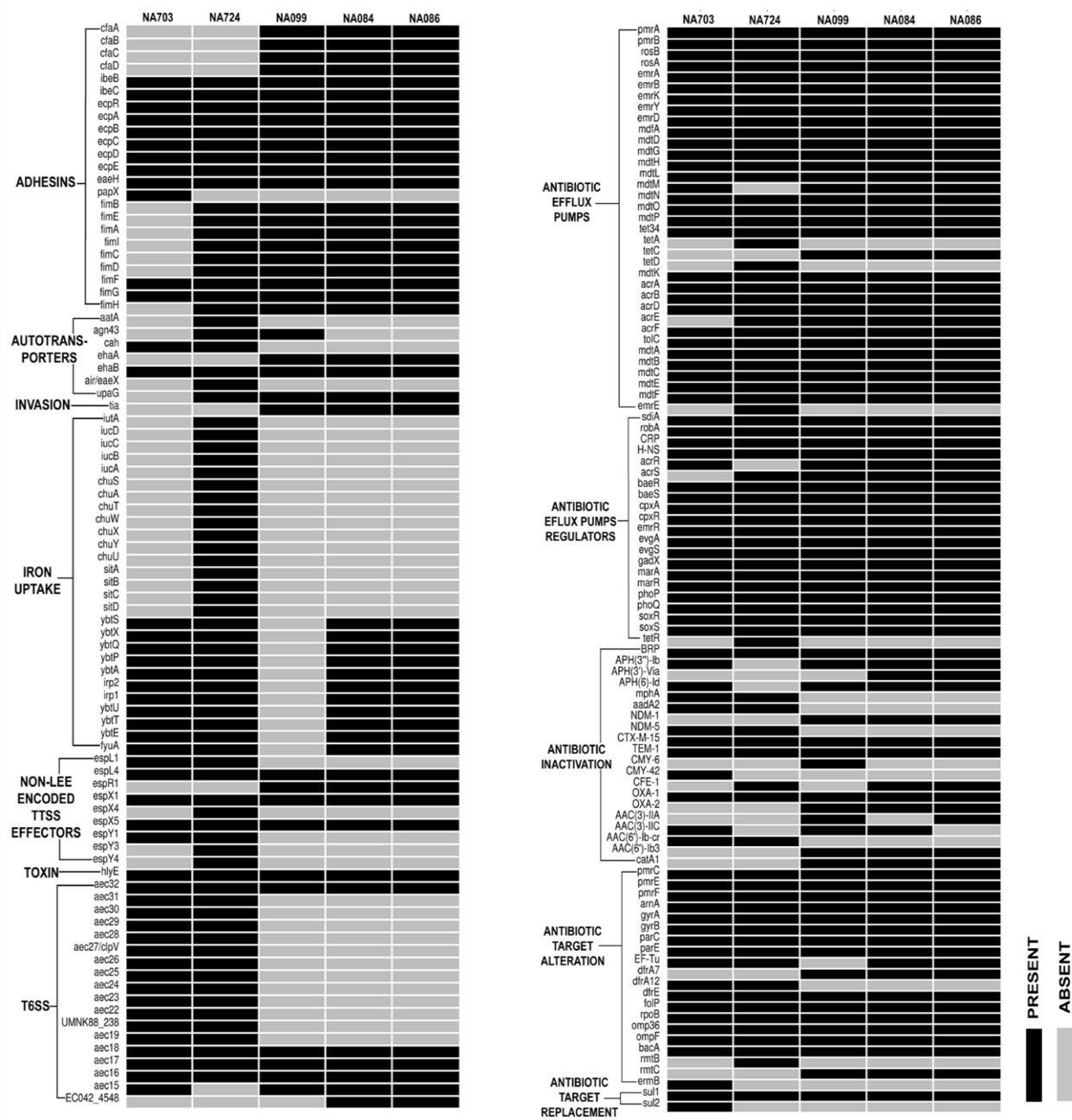


Figure 5:



# Molecular Epidemiology and Genome Dynamics of New Delhi Metallo- $\beta$ -Lactamase-Producing Extraintestinal Pathogenic *Escherichia coli* Strains from India

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The global dissemination and increasing incidence of carbapenem-resistant, Gram-negative organisms have resulted in acute public health concerns. Here, we present a retrospective multicenter study on molecular characterization of metallo- $\beta$ -lactamase (MBL)-producing clinical *Escherichia coli* isolates recovered from extraintestinal infections in two hospitals in Pune, India. We screened a large sample size of 510 *E. coli* isolates for MBL production wherein we profiled their molecular determinants, antimicrobial resistance phenotypes, functional virulence properties, genomic features, and transmission dynamics. Approximately 8% of these isolates were MBL producers, the majority of which were of the NDM-1 (69%) type, followed by NDM-5 (19%), NDM-4 (5.5%), and NDM-7 (5.5%). MBL producers were resistant to all antibiotics tested except for colistin, fosfomycin, and chloramphenicol, which were effective to various extents. Plasmids were found to be an effective means of dissemination of NDM genes and other resistance traits. All MBL producers adhered to and invaded bladder epithelial (T24) cells and demonstrated significant serum resistance. Genomic analysis of MBL-producing *E. coli* isolates revealed higher resistance but a moderate virulence gene repertoire. A subset of NDM-1-positive *E. coli* isolates was identified as dominant sequence type 101 (ST101) while two strains belonging to ST167 and ST405 harbored NDM-5. A majority of MBL-producing *E. coli* strains revealed unique genotypes, suggesting that they were clonally unrelated. Overall, the coexistence of virulence and carbapenem resistance in clinical *E. coli* isolates is of serious concern. Moreover, the emergence of NDM-1 among the globally dominant *E. coli* ST101 isolates warrants stringent surveillance and control measures.

Infections with Gram-negative multidrug-resistant (MDR) pathogens, particularly those caused by  $\beta$ -lactamase-producing *Escherichia coli*, constitute a major reason for the “global SOS” in public health (1). Carbapenems are often considered to be the last-resort antibiotics available for treating infections caused by extended-spectrum- $\beta$ -lactamase (ESBL)- or AmpC-producing bacteria (2) and are categorized in three major types: the KPC types, the metallo- $\beta$ -lactamases (MBLs), and the oxacillinases (3). The major geographically widespread MBLs consist of imipenemase (IMP), Verona integron-encoded metallo- $\beta$ -lactamase (VIM), and New Delhi metallo- $\beta$ -lactamase (NDM); NDM has attracted significant worldwide attention as the NDM gene has been detected on different transferable plasmid types (both on typeable and untypeable) and has also been shown to be chromosomally integrated in other bacterial species (4, 5).

NDM belongs to the amber class B  $\beta$ -lactamase. Unlike other classes of  $\beta$ -lactamases (A, C, and D), NDM requires zinc ions to catalyze the hydrolysis of  $\beta$ -lactam antibiotics, and due to this, MBL activity can be inhibited in the presence of metal chelating agents such as EDTA (6). NDM-producing bacteria are mostly multidrug resistant and show resistance toward sulfonamides, fluoroquinolones, aminoglycosides, and macrolides but are susceptible to only a few antimicrobials, such as aztreonam, tigecycline, and colistin (7, 8). Recent reports suggest that this susceptibility is expected to be short-lived (1). Moreover, NDM enzymes are generally not inhibited by any of the known and available  $\beta$ -lactamase inhibitors such as sulbactam, clavulanate, and tazobactam (9).

NDM-producing bacteria have been implicated in both hospi-

tal- and community-acquired infections and have been recovered from several infection sites, including those associated with urinary tract infections, pneumonia, septicemia, and wound infections as well as from device-associated infections and companion animals (10–13). Although the exact geographical origin and the precise time for the emergence of the *bla*<sub>NDM</sub> gene are unknown, the first NDM-1-positive isolate was recovered in 2009 from a Swedish patient who previously (2007) had an episode of hospitalization in New Delhi, India (14). Later, in the year 2010, Kumarasamy et al. provided evidence that NDM-1-positive *Enterobacteriaceae* are widespread in India, Pakistan, and the United Kingdom (15). Recent reports further indicate that several variants of NDM (NDM-1 to NDM-14)-producing *Enterobacteriaceae* are currently spreading worldwide, including in the United States, Netherlands, Australia, Canada, France, and Oman (16, 17) (<http://www.lahey.org/studies>). Most of these case reports/epidemiological studies provide evidence of connections of pa-

Received 23 June 2016 Returned for modification 21 July 2016

Accepted 25 August 2016

Accepted manuscript posted online 6 September 2016

Citation Ranjan A, Shaik S, Mondal A, Nandanwar N, Hussain A, Semmler T, Kumar N, Tiwari SK, Jadhav S, Wieler LH, Ahmed N. 2016. Molecular epidemiology and genome dynamics of New Delhi metallo- $\beta$ -lactamase-producing extraintestinal pathogenic *Escherichia coli* strains from India. Antimicrob Agents Chemother 60:6795–6805. doi:10.1128/AAC.01345-16.

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tients to the Indian subcontinent in the form of migration/travel visits or medical treatment received there. However, certain reports indicate infection sources outside this region with patients without any obvious connection to this area (18). Nevertheless, the Indian subcontinent particularly offers several risk factors for the emergence and spread of NDM-producing superbugs, which include poor sanitation, easy access to broad-spectrum antibiotics over the counter without proper prescriptions, increased medical tourism for health care, and lack of stringent antibiotic policies.

Members of *Enterobacteriaceae*, particularly *E. coli*, are often disseminated globally through distinct and successful lineages, such as sequence types (ST) 131, 95, 10, etc. (19–22). Our previous studies have put forward strong evidence for the widespread occurrence of ST131 strains among extraintestinal infections in India and have also reported the existence of NDM-1-positive ST131 *E. coli*, thus representing a serious therapeutic challenge (23–27). This, together with the implication of NDM-producing strains globally, warrants serious attention of health care specialists and epidemiologists with respect to surveillance and resistance tracking in India and elsewhere so as to monitor threats locally and globally. Also, the data on the virulence traits associated with NDM-producing *E. coli* strains are currently scarce. With the advent of high-resolution, whole-genome sequencing methods, it has now become easy to decipher the genomes of the NDM-producing isolates and understand the underlying resistance and virulence mechanisms. The use of comparative genomics in infection tracking has enabled a comprehensive understanding of epidemiology, resistomes, virulomes, and the genetic background of NDM-producing MDR *E. coli* strains.

In the present study, we sought to determine the prevalence and phenotypes of MBL-producing strains from among a large number of *E. coli* isolates collected chronologically for 6 years (2009 to 2014) from a major tertiary hospital and to determine the antibiotic susceptibility patterns by taking into account the allelic diversity of *bla*<sub>NDM</sub> genes and the plasmid replicon types. Further, we determined the virulence traits of NDM-producing *E. coli* by various phenotypic assays in conjunction with whole-genome sequences of five MDR NDM-producing *E. coli* strains and consequent analyses of resistomes, virulomes, and genetic affinities as important parameters required to portray a correct epidemiological scenario. We believe that such exhaustive analyses potentially provide for a clear and solid epidemiological baseline to guide an effective antibiotic policy in a country of endemicity such as India.

## MATERIALS AND METHODS

**Bacterial isolates.** A total of 510 clinical isolates of *E. coli* were included in this study. The isolates were collected consecutively between January 2009 and December 2014 from in-patients and outpatients treated at two major tertiary care hospitals in Pune, India. The collection included clinical isolates from urine ( $n = 393$ ), pus ( $n = 88$ ), and other sources ( $n = 29$ ). All the isolates were obtained as pure cultures and were cryopreserved at  $-80^{\circ}\text{C}$  in 20% glycerol. The isolates were revived on LB agar plates and in LB broth prior to experiments. Collection of isolates was in accordance with the guidelines/approvals of Institutional Biosafety Committees at participating institutes/centers, where appropriate.

**Detection of metallo- $\beta$ -lactamase production and its molecular characterization.** A total of 510 *E. coli* isolates were screened for MBL production using meropenem (with and without EDTA) with the help of Ezy MIC Strips (HiMedia; India), as per the manufacturer's instructions. All positive *E. coli* strains were also screened for the presence of *bla*<sub>NDM-1</sub> by a gene-specific PCR (28). *E. coli* strains confirmed as MBL producers

were further analyzed by high-fidelity PCR (with the use of Q5 DNA polymerase from NEB) using flanking primers of the NDM gene, as described earlier (29). Custom sequencing of the resulting amplicons was performed, and the nucleotide and translated protein sequences were analyzed using MEGA6 (30). NDM types were determined in accordance with the sequences available in the NCBI database(s).

**Testing for antimicrobial susceptibility and its molecular determinants.** Antimicrobial sensitivity of 39 strains against 23 different antibiotics was performed on Mueller-Hinton agar (MHA) plates by the Kirby-Bauer disc diffusion method using antibiotic discs (HiMedia) and Icosa urinary tract infection (UTI) rings (HiMedia), according to the manufacturer's protocols. Two in-house strains that were consistently resistant and susceptible to most of the antimicrobial agents were employed as positive and negative controls, respectively. Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute. For colistin, a zone of  $<11$  mm was considered resistant. The MIC values of four frontline antibiotics, namely, tetracycline, co-trimoxazole, gentamicin, and ciprofloxacin, were determined by using HiComb MIC strips (HiMedia, India) as per the manufacturer's protocol. MIC values were determined and compared among a subset of 10 MBL-producing strains, 10 non-ESBL-producing strains, and 7 meropenem-resistant transconjugants. All MBL-producing *E. coli* strains were analyzed for molecular determinants of antibiotic resistance by PCR. DNA template was prepared using a standard heat lysis method. Thereafter, gene-specific primer sets were used to screen for the following ESBL genes: *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>NDM-1</sub> (28, 31). Other antibiotic resistance genes, such as those conferring resistance to tetracycline [*tet*(A) and *tet*(C)], sulfonamides (*sul1* and *sul2*), streptomycin (*strA*), and trimethoprim (*dfr*) and including the quinolone resistance determinants (carried on plasmids) such as *aac*(6')-Ib, *qnrB*, and *qnrS*, were amplified as per standard PCR protocols (32, 33). The amplicons were run on 1% agarose gels, and the images were documented using a gel documentation system (Major Sciences, USA). The correlation between the phenotypic and different genotypic determinants of resistance was calculated with the help of observed phenotypic resistance and the frequency of the genotypic determinant probed.

**Conjugation, mating experiments, and plasmid analysis.** Conjugal transfer of resistance genes was performed by broth mating or filter mating experiments for a subset of nine carbapenem-resistant *E. coli* strains using the sodium azide-resistant recipient strain *E. coli* K-12 J53 (34). Transconjugants were selected on selection plates supplemented with a combination of meropenem (4  $\mu\text{g}/\text{ml}$ ) and sodium azide (50  $\mu\text{g}/\text{ml}$ ). In addition, other antibiotics and sodium azide combinations were used to select transconjugants. However, as meropenem-containing transconjugants were the focus of this study, the same were subjected to further analyses. Pure cultures of the transconjugants were preserved in 20% glycerol at  $-80^{\circ}\text{C}$ . Antibiotic susceptibility and MICs were compared between the parental strains and their respective transconjugants. Plasmid classes were determined using PCR-based replicon typing (35).

**Phenotypic assays.** A total of nine random isolates were analyzed for functional virulence traits as described below. Nine MBL-producing *E. coli* strains were tested for their adhesion and invasion efficiency. Human bladder epithelial cell lines (T24) were cultured and maintained in RPMI 1640 medium (Invitrogen, USA) with 10% fetal bovine serum (FBS). Cells were seeded in 24-well plates and grown until a monolayer was obtained. Infection was done at a multiplicity of infection (MOI) of 10. After 3 h of incubation at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ , cells were washed three times with  $1\times$  phosphate-buffered saline (PBS), followed by lysis with 1 ml of 0.1% Triton X-100. Cell lysates were serially diluted, plated on LB agar plates, and incubated overnight at  $37^{\circ}\text{C}$ . For invasion assays, after 3 h of incubation, the cells were washed and further incubated for 1.5 h with 1 ml of RPMI medium containing 100  $\mu\text{g}/\text{ml}$  of fosfomycin. After incubation, cells were washed three times with  $1\times$  PBS and processed in a way similar to that described above. Assays were performed three times with three technical replicates.

Serum resistance was determined for nine MBL-producing *E. coli* isolates by *in vitro* time-kill experiments as described earlier (22). Briefly, 5  $\mu$ l of overnight-grown culture was added to 495  $\mu$ l of LB broth and incubated for 1 h at 37°C in a shaker incubator. The culture was pelleted and resuspended in 1 ml of 1× PBS, and then 30  $\mu$ l of this bacterial suspension was added to 96-well microtiter plate wells, each containing 270  $\mu$ l of 50% sterile human serum (Pan Biotech, Germany) in 1× PBS. Thirty microliters of serum was then collected (0-h count), serially diluted, and plated on LB agar plates for CFU enumeration. The plate was then incubated for 3 h at 37°C. After the incubation time (3-h count), 30  $\mu$ l of sample was pipetted for serial dilution and plated as described above. Growth was measured in terms of CFU counts. Samples were considered serum resistant if the 3-h CFU count equaled or exceeded the 0-h CFU count of the same sample. The serum assay was performed twice in triplicates.

Total siderophore production was tested by streaking all 39 MBL-positive *E. coli* samples on chrome azurol S (CAS) agar plates (blue medium). Color changes (haloes) from yellow to orange on the CAS agar medium plates, after overnight incubation at 37°C, were recorded. No color change in the medium corresponded to no siderophore production by the culture.

**ERIC-PCR-based strain typing.** Enterobacterial intergenic repetitive element sequence (ERIC)-PCR-based fingerprint analysis was performed as described previously (36). Strain-specific DNA banding profiles obtained on 1.5% agarose gel images were compared using BioNumerics software (version 7.1; Applied Maths, Belgium). After the bands were scored on dice similarity indices based on the unweighted-pair group method using average linkages (UPGMA; arithmetic mean), a dendrogram was generated in order to analyze similarity/diversity within carbapenem-resistant *E. coli* strains. The definition of ERIC clusters was based on a Dice similarity index.

**WGS, assembly, annotation, and genomic analysis.** Whole-genome sequencing (WGS) of five selected strains was carried out on an Illumina MiSeq system with an insert size of 400 to 500 bp. The paired-end sequence reads were filtered and trimmed using the NGS QC Toolkit (version 2.3.3) (37). These high-quality reads were assembled into contigs using SPAdes Genome Assembler (version 3.6.1) (38). The contigs were then ordered and scaffolded using an in-house workbench, Contig-Layout-Authenticator (39), and the resulting ordered scaffolds were joined by placing Ns between them to construct draft genomes. These were then submitted to the RAST (40) server for annotation, and the genome statistics were extracted using ARTEMIS (41). For all of the genomes, genes were predicted using GeneMarkS (42). Amino acid sequences of the products of the predicted genes from all strains were compared using blastp (43) against the database of *E. coli* virulence genes from the Virulence Factors Database (VFDB) (44). Only genes with 60% identity and 85% query coverage were considered present, and in this way the presence-absence status of each virulence gene in each of these strains was represented in the form of a heat map using R. A similar heat map was generated for putative resistance-related genes after comparison with the Comprehensive Antibiotic Resistance Database (CARD) (45), using blastp. Insertion sequence (IS) elements and putative phage sequences in the genome were identified using IS Finder (46) and PHAST (47), respectively. The sequence type (ST) of each of these strains was determined by submitting the contigs to the server of the Centre for Genomic Epidemiology (CGE) (<https://cge.cbs.dtu.dk/services/MLST/>).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism (version 5.01). For adhesion and serum resistance, a nonparametric Mann-Whitney U test was performed while for the invasion assay Wilcoxon matched-pair tests were performed, and *P* values were determined.

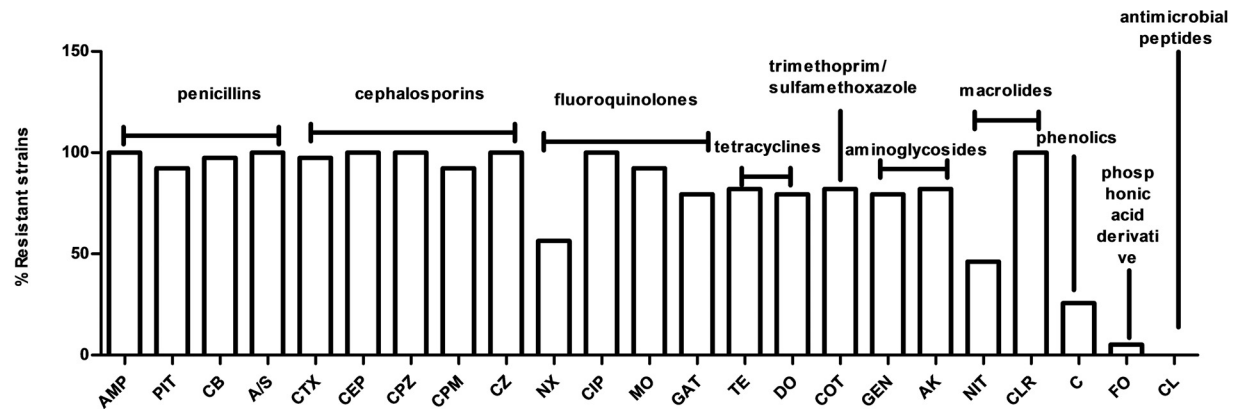
**Accession number(s).** The results of this whole-genome shotgun project have been deposited in the DDBJ/ENA/GenBank databases under the accession numbers [LXFJ00000000](#) (NA703), [LXFK00000000](#) (NA724), [LXEZ00000000](#) (NA086), [LXFA00000000](#) (NA099), and [LWTZ00000000](#) (NA084). The versions described in this paper are

[LXFJ01000000](#), [LXFK01000000](#), [LXEZ01000000](#), [LXFA01000000](#), and [LWTZ01000000](#), respectively.

## RESULTS

**Prevalence of MBL producers was low with a high abundance of the NDM-1 genotype.** Of the 510 clinical *E. coli* isolates, 39 (7.6%) were found to be MBL producers. These 39 MBL-producing *E. coli* isolates were deemed to be of potential interest and were used further for in depth analyses, as described above. All of the 39 MBL-producing *E. coli* isolates were also found to be ESBL producers phenotypically. Most of the MBL producers (25/39) were from urine isolates, followed by those from pus (11/39) and other sources (3/39). The 39 MBL producers as identified at the phenotypic level were screened for the presence of major carbapenemase genes, including *bla*<sub>NDM</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>OXA-48</sub>. With the exception of the *bla*<sub>KPC</sub> gene, which was not detected in any of the MBL producers, 36 (92%) MBL producers were *bla*<sub>NDM</sub> positive, and 6 (15%) of them were also *bla*<sub>OXA-48</sub> positive. Sequence analysis of the full-length NDM genes from 36 MBL producers revealed that the majority of them carried the *bla*<sub>NDM-1</sub> gene (25 isolates), followed by *bla*<sub>NDM-5</sub> (7 isolates). Further, the gene *bla*<sub>NDM-4</sub> was present in two isolates, and the gene *bla*<sub>NDM-7</sub> was harbored by two other MBL producers.

**All MBL-producing *E. coli* isolates were identified to be MDR and pan-drug resistant.** Of the 23 antimicrobial agents representing 10 different classes, our MBL-producing *E. coli* isolates showed an average resistance to nearly 18 antibiotics, with maximum resistance to 21 and minimum resistance to 10 antimicrobial agents. The frequencies of susceptibility/resistance are indicated in Fig. 1. Among all MBL-positive isolates, highest resistance was observed against the  $\beta$ -lactam antibiotic class while highest sensitivity (100%) was observed to the peptide antibiotic class, i.e., colistin. Each of the MBL-producing *E. coli* isolates was found to be at least 90% resistant to all the  $\beta$ -lactam and  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations used, including ampicillin, carbenicillin, cefotaxime, cephalothin, cefoperazone, cefepime, cefazolin, piperacillin-tazobactam, and ampicillin-sulbactam. In contrast, each of the MBL-producing *E. coli* isolates was found to be sensitive to colistin. All MBL-positive isolates were highly susceptible to fosfomycin and chloramphenicol (Fig. 1). Other resistance patterns were as follows: 80% of isolates were resistant to each of the tetracycline and aminoglycoside classes and 82% were resistant to the trimethoprim/sulfonamide class. Notably, all MBL-positive isolates (100%) were identified to be MDR as they were resistant to three or more classes of antimicrobials. Moreover, all the MBL-producing *E. coli* isolates were found to be pan-drug resistant, meaning that they were resistant to as many as seven antimicrobial agents. The MIC values of MBL-ESBL-producing *E. coli* and non-ESBL *E. coli* isolates varied widely for different antibiotics used in the study (Table 1). Overall, the MBL-ESBL-producing *E. coli* isolates revealed higher MICs of all four frontline antimicrobial substances than non-ESBL strains. That the MIC values of the four antimicrobials for the meropenem transconjugants were higher than those of the recipient strain J53 *E. coli* indicated horizontal transmission of respective resistance genes. All 39 ESBL-MBL producers were negative for *bla*<sub>SHV</sub> and *bla*<sub>KPC</sub>. However, 31 isolates harbored the *bla*<sub>CTX-M-15</sub> gene, and 28 isolates harbored *bla*<sub>TEM</sub>. The correlation among different resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was found to be high for aminoglycosides (94%



| Antibiotic class               | Cephalosporins (beta lactams) |        |      |        |      |       | Fluoroquinolones |       |       | Tetracyclines |        | Sulphonamides-tri-methopri-me |        |       | Amino glycosides |
|--------------------------------|-------------------------------|--------|------|--------|------|-------|------------------|-------|-------|---------------|--------|-------------------------------|--------|-------|------------------|
| Resistance genotype            | ctx-m-15                      | tem    | shv  | ndm1   | kpc  | oxo48 | aac6'            | qnrB  | qnrS  | tetA          | tetC   | sul1                          | sul2   | dfr   | strA             |
| Number (% of positive strains) | 31(79)                        | 28(72) | 0(0) | 36(92) | 0(0) | 6(15) | 26(67)           | 8(21) | 7(18) | 11(28)        | 22(56) | 38(97)                        | 32(82) | 4(10) | 30(77)           |

FIG 1 (Top) Antibiogram of MBL-producing carbapenem-resistant *E. coli* strains as tested by the disc diffusion method for the following 23 different antibiotics: ampicillin (Amp), piperacillin-tazobactam (PIT), carbenicillin (CB), ampicillin/sulbactam (A/S), cefotaxime (CTX), cephalothin (CEP), cefoperazone (CPZ), cefepime (CPM), cefazolin (CZ), nalidixic acid (NX), ciprofloxacin (CIP), moxifloxacin (MO), gatifloxacin (GAT), tetracycline (TE), doxycycline HCl (DO), co-trimoxazole (COT), gentamicin (GEN), amikacin (AK), nitrofurantoin (NIT), clarithromycin (CLR), chloramphenicol (C), fosfomycin (FO), and colistin (CL). All MBL-producing isolates were detected to be pan-drug resistant. (Bottom) Identification of resistance determinants among 39 MBL-producing *E. coli* isolates by gene-specific PCRs.

agreement) and sulfonamides (84% agreement), whereas the agreement between resistance to tetracycline and the presence of *tet(A)* and *tet(C)* was 69% and that for fluoroquinolone was (67%). The overall prevalences of all the major resistance genes in 39 MBL-producing *E. coli* isolates are presented in Fig. 1.

**Metallo-β-lactamase resistance was naturally transferable via plasmids.** NDM genes are basically located on plasmids. In order to determine the capability of these MBL-positive *E. coli* strains to disseminate NDM genes horizontally, we carried out conjugation mating experiments with the sodium azide-resistant recipient strain *E. coli* K-12 J53. Out of the nine representative MBL-positive strains employed, we were able to obtain seven meropenem-resistant transconjugants alongside several other transconjugants. However, only meropenem transconjugants were further analyzed for this study. The transconjugants were analyzed for their phenotypic drug resistance to four different classes of antibiotics, along with MBL and ESBL detection. All transconjugants were identified to be MBL and ESBL positive, with coresistance varying widely for different antibiotics used in the study. The detailed results are shown in Fig. 2. Transconjugants were confirmed to be carrying the NDM gene by gene-specific PCR. PCR-based replicon typing revealed the presence of primarily two plasmid types, the FIA and FIB plasmids, among transconjugants. These observations reflect the role of transmissible plasmids in dissemination of NDM-based carbapenem resistance.

**MBL-producing *E. coli* isolates were moderately virulent.** Phenotypic virulence properties of the MBL-positive *E. coli* iso-

lates were assayed using adhesion, invasion, serum bactericidal, and siderophore production assays. The two former assays were carried out on nine randomly selected strains, and siderophore production was determined for all 39 strains. Adhesive and invasive virulence properties are important features during intestinal as well as extraintestinal infections. In order to evaluate this, we quantitatively assayed the pathogen’s ability to adhere and invade the urinary bladder epithelial cells. Compared to the negative-control strain *E. coli* DH5α, all nine MBL-positive *E. coli* isolates showed significant adhesion to T24 cells with various efficiencies, as illustrated in Fig. 3a. Among the nine MBL-positive isolates tested, the NA084 isolate demonstrated highest adherence capability toward T24 cells. T24 cells were invaded by all investigated isolates, in contrast to the absence of invasion by the negative-control strain *E. coli* DH5α. Numbers of intracellular bacteria are depicted in Fig. 3b.

Resistance to serum is quite essential for the pathogens to survive and cause invasive infections; we found all nine tested isolates to be resistant (100%) to serum bactericidal activity compared to results with the control DH5α *E. coli*. Bacterial survival in serum over a period of 3 h is depicted in Fig. 3c. Though the virulence genes tested were not prevalent in these isolates, bacterial resistance toward the bactericidal effect of serum could result from individual or combined effects of capsular polysaccharide, surface proteins, and toxin secretions.

Siderophore production, as visualized by an iron mobilization zone around the bacterial colonies with orange haloes on the chrome azurol S agar, was similarly prevalent among 36 (92%) of

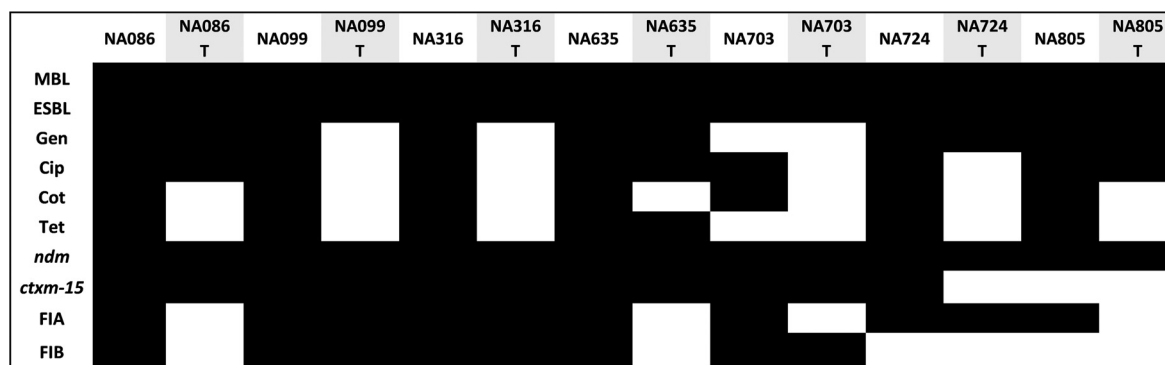
**TABLE 1** MIC values of carbapenem-resistant strains, meropenem transconjugants, a recipient strain, and non-ESBL non-carbapenem-resistant *E. coli* isolates from the present study

| Strain group and no.                                   | MIC ( $\mu\text{g/ml}$ ) <sup>b</sup> |               |                |              |
|--------------------------------------------------------|---------------------------------------|---------------|----------------|--------------|
|                                                        | Gentamicin                            | Ciprofloxacin | Co-trimoxazole | Tetracycline |
| Carbapenem-resistant strains ( $n = 17$ ) <sup>a</sup> |                                       |               |                |              |
| NA084                                                  | >240                                  | >240          | >240           | 3            |
| NA644                                                  | >240                                  | >240          | >240           | 5            |
| NA708                                                  | >240                                  | 60            | >240           | 3            |
| NA086                                                  | >240                                  | >240          | >240           | 2            |
| NA086 T                                                | >240                                  | 0.01          | 0.05           | 0.1          |
| NA099                                                  | >240                                  | >240          | >240           | 0.1          |
| NA099T                                                 | 0.01                                  | 0.1           | 0.01           | 0.01         |
| NA316                                                  | >240                                  | >240          | >240           | 3            |
| NA316T                                                 | 0.1                                   | ND            | 0.05           | 0.1          |
| NA635                                                  | >240                                  | 120           | >240           | 5            |
| NA635T                                                 | 24                                    | ND            | >240           | 3            |
| NA703                                                  | >240                                  | >240          | >240           | 0.001        |
| NA703T                                                 | 16                                    | ND            | 0.05           | 0.001        |
| NA724                                                  | >240                                  | 120           | >240           | 3            |
| NA724T                                                 | 30                                    | 0.01          | 0.05           | 0.1          |
| NA805                                                  | 60                                    | 10            | >240           | 0.01         |
| NA805T                                                 | ND                                    | 1             | 0.05           | 0.01         |
| Recipient strain ( $n = 1$ )                           |                                       |               |                |              |
| K-12 J53                                               | 0.001                                 | 0.008         | 0.05           | 0.01         |
| Non-ESBL non-carbapenem-resistant strains ( $n = 10$ ) |                                       |               |                |              |
| NA433                                                  | 0.1                                   | 60            | >240           | 10           |
| NA010                                                  | 0.1                                   | 0.008         | 30             | 0.1          |
| NA295                                                  | 0.01                                  | 0.01          | 120            | 0.1          |
| NA053                                                  | 0.25                                  | 1             | >240           | 2            |
| NA057                                                  | 0.1                                   | 30            | >240           | 0.1          |
| NA120                                                  | 0.01                                  | 0.1           | >240           | 0.5          |
| NA140                                                  | 0.1                                   | 0.01          | >240           | 30           |
| NA150                                                  | 0.1                                   | 0.25          | 2              | 0.01         |
| NA203                                                  | 0.01                                  | 0.01          | 0.1            | 0.01         |
| NA274                                                  | 0.1                                   | 10            | >240           | 30           |

<sup>a</sup> The total includes seven meropenem transconjugants (T suffix).<sup>b</sup> ND, not determined.

the 39 MBL-producing *E. coli* isolates. The role of iron in the virulence mechanism of human and animal pathogens is well established (48). Siderophore production has been shown to be more frequently associated with extraintestinal pathogenic *E. coli* (ExPEC).

**Whole-genome analysis reveals high resistance and moderate virulence of MBL strains.** Out of the 39 MBL-positive strains, whole-genome sequencing of five representative strains (three NDM-1 and two NDM-5) was carried out. Their genome charac-

**FIG 2** *In vitro* conjugation experiment and comparative analysis between clinical strains and their respective transconjugants revealed transfer of resistances via plasmids. The T suffix designates transconjugants. Gen, gentamicin; Cip, ciprofloxacin; Cot, co-trimoxazole; Tet, tetracycline. *ndm* and *ctxm-15* represent *bla*<sub>NDM</sub> and *bla*<sub>CTX-M-15</sub> genes, respectively. FIA and FIB are the plasmid replicon types. Black and white boxes indicate presence and absence of corresponding traits, respectively.

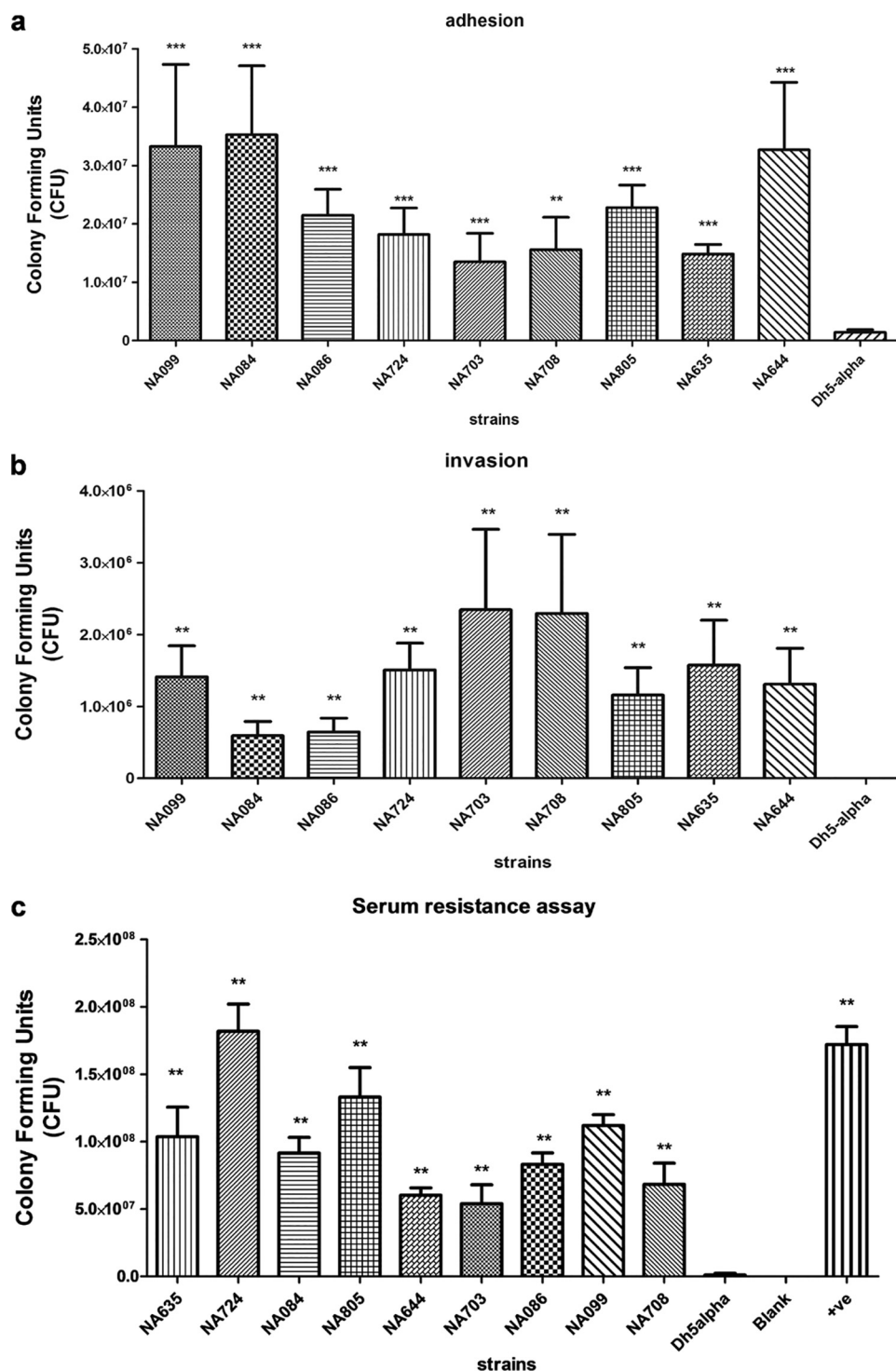


FIG 3 A subset of nine MBL-producing *E. coli* isolates was tested for three virulence-associated phenotypes: adhesion to human bladder epithelial cells (T24) at an MOI of 10 (a), invasion of human bladder epithelial cells (T24) (b), and resistance to 50% human serum for a 3-h time period (c). Adhesion and invasion assays were repeated three times in triplicates while the serum resistance assay was repeated twice in triplicates. All nine MBL-producing *E. coli* isolates demonstrated ExPEC-associated phenotypes. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , for results compared to those with the control (DH5 $\alpha$ ).

teristics are represented in Table 2. The three NDM-1 strains belonged to ST101 while the two NDM-5 strains were characterized as ST167 and ST405. The whole-genome-based resistome reflected the high prevalence of resistance genes of all categories

within all the strains, similar to the phenotypic drug resistance profile (Fig. 4). The virulome of these strains reflected moderate virulence wherein all the strains inherited genes belonging to adhesins, autotransporters, type three secretion systems

TABLE 2 The genome statistics and sequence types of five whole-genome-sequenced NDM-harboring strains comprising three NDM-1 and two NDM-5 strains

| Strain | No. of raw reads | Genome coverage from filtered reads (×) | No. of contigs | ST  | Genome size (no. of bases) | G+C content (%) | No. of CDS <sup>a</sup> | % coding capacity | No. of rRNAs | NDM type | Specimen source |
|--------|------------------|-----------------------------------------|----------------|-----|----------------------------|-----------------|-------------------------|-------------------|--------------|----------|-----------------|
| NA084  | 1,741,426        | 73.25                                   | 109            | 101 | 5,147,001                  | 50.59           | 5,000                   | 86.8              | 13           | NDM-1    | Urine           |
| NA086  | 1,643,674        | 63.13                                   | 115            | 101 | 5,147,281                  | 50.59           | 4,994                   | 86.9              | 13           | NDM-1    | Urine           |
| NA099  | 2,406,806        | 104.68                                  | 155            | 101 | 5,197,495                  | 50.57           | 5,063                   | 85.8              | 17           | NDM-1    | Urine           |
| NA703  | 2,401,628        | 92.94                                   | 211            | 167 | 5,263,381                  | 50.66           | 5,186                   | 86.0              | 16           | NDM-5    | Pus             |
| NA724  | 2,784,910        | 118.36                                  | 166            | 405 | 5,424,442                  | 50.55           | 5,273                   | 87.0              | 9            | NDM-5    | Pus             |

<sup>a</sup> CDS, coding sequences.

(TTSS), and type 6 secretion systems (T6SS). NA099 harbors the least number of virulence genes while NA724 carries the maximum number of genes. The details are shown in Fig. 4. All the strains showed different phage contents and variable IS elements.

The upstream sequence analysis of these five NDM strains showed insertion elements such as *ISAbal25* upstream of NDM-1 and *IS5* and truncated *ISAbal25* upstream of NDM-5 sequences.

NDM strains reveal high genomic diversity. PCR-based

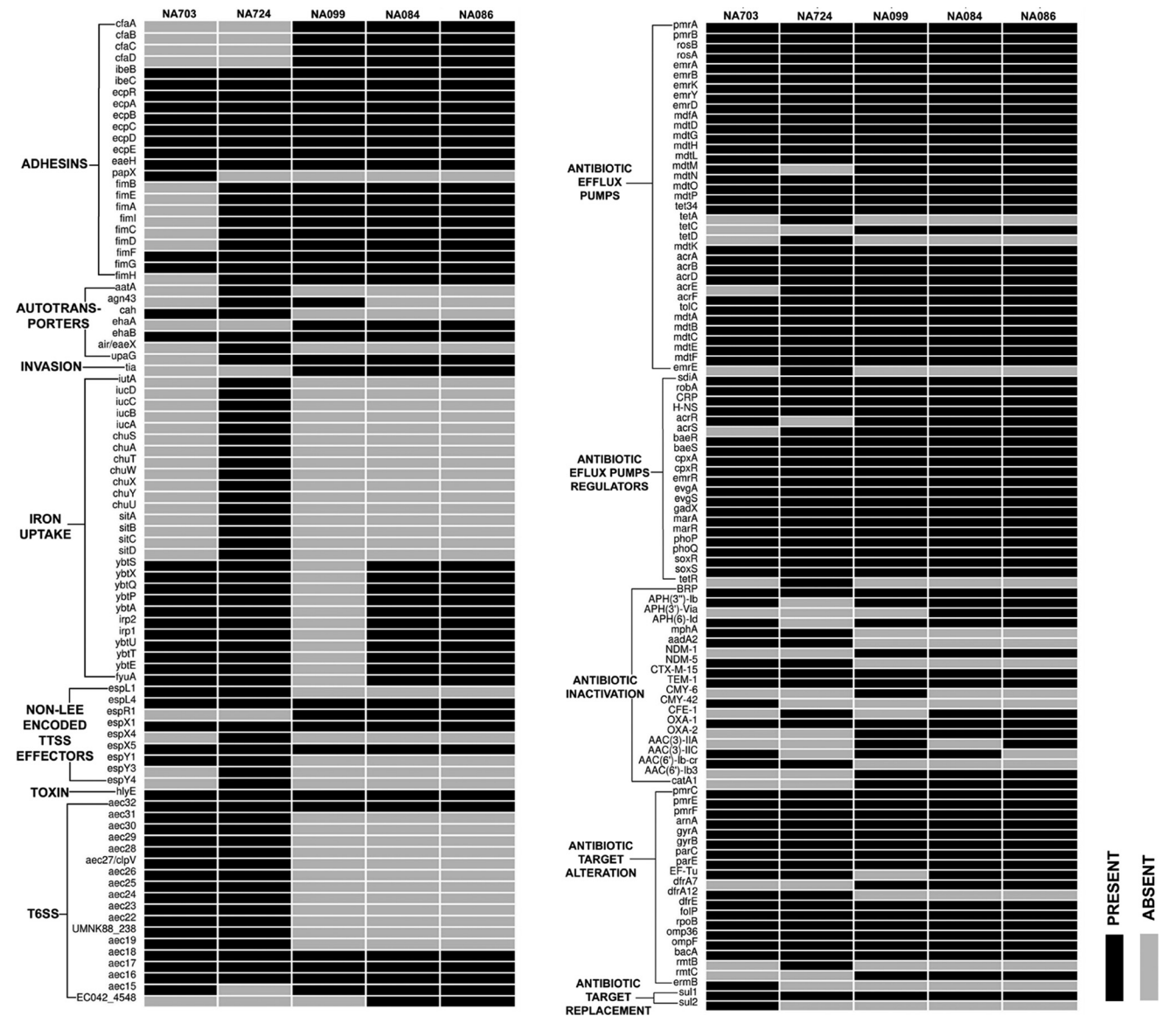


FIG 4 Whole-genome-based identification of virulence (left) and resistance (right) determinants of five MBL-producing *E. coli* isolates.

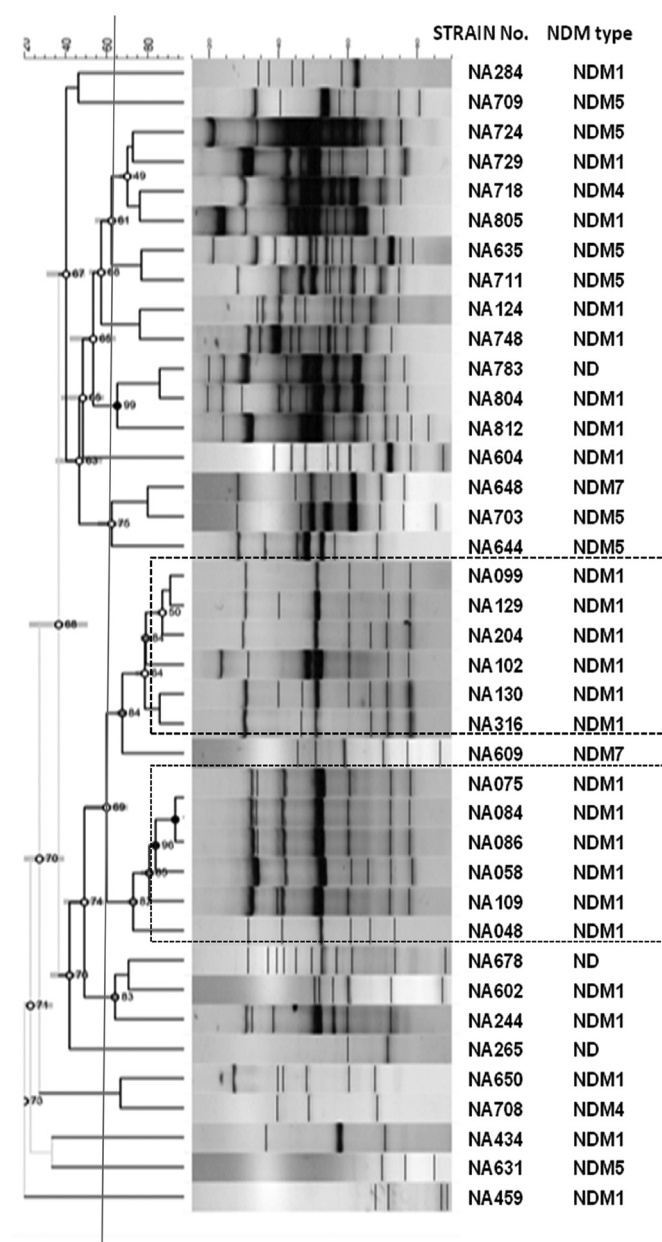


FIG 5 Dendrogram based on the phylogenetic analysis of 39 MBL-producing carbapenem-resistant clinical *E. coli* strains using ERIC-PCR banding analysis by BioNumerics, version 7.5 (Applied Maths, Belgium). Dotted boxes indicate clusters into which the majority of NDM-1 profiles segregated. ND, not determined.

genotyping was used to analyze the genetic relationships of the organisms. The ERIC-PCR-based dendrogram of these strains (Fig. 5) revealed high diversity among them. However, when strains were grouped at a lower threshold of ~65% identity, two diffusely clonal clades were observed. As expected, all the strains in this cluster harbored NDM-1 genes. Further, the *in silico* multilocus sequence typing (MLST) analysis of whole-genome sequences identified all three strains to be ST101. Overall, this cluster might include clonally related ST101 strains which are strongly associated with the NDM genotype. No such clusters were obtained for any other NDM variants. The above observations also support the

idea that plasmids are the important means for NDM dissemination.

## DISCUSSION

The emerging NDM, an acquired class B carbapenemase from among *Enterobacteriaceae*, has become a major public health concern worldwide also due to its medical and economic impact (49), and infections with NDM-positive pathogens pose a major health care problem, particularly for high-burden countries such as India. Previous studies from our group have shown an endemic *E. coli* infection burden and the presence of clonal group ST131 isolates with NDM-1 (23–25, 50). This study was aimed at detection, comprehensive characterization, and whole-genome sequence analysis of MBL-producing MDR organisms that can lead to successful infection. This study would likely be useful in guiding the formulation of control strategies involving antimicrobial stewardship and public health interventions in order to control the escalation of life-threatening MDR bacterial infections involving ExPEC organisms.

In general, production of MBL in *Enterobacteriaceae* shows an increasing prevalence pattern. However, the prevalence of carbapenem resistance among 510 *E. coli* isolates analyzed from 2009 to 2014 was found to be 7.6%, which was within the range of previously reported carbapenem resistance from India (51, 52); however, this prevalence might be variable in different geographical locations and different hospital settings. The dissemination of NDM carbapenemases involves several *bla*<sub>NDM</sub> gene variants that are associated with different plasmid types among several Gram-negative species. A total of 14 NDM types have been reported globally, which were due to one or more point mutations (<http://www.lahey.org/studies>). In our study, we identified four NDM variants responsible for MBL production, with NDM-1 being the dominant variant, while other variants comprising NDM-4, NDM-5, and NDM-7 were observed with various frequencies. Previous reports from India have shown the presence of the NDM-6 type in addition to the above-mentioned NDM types (52). These four variants did not demonstrate any significant difference in MIC values for meropenem, nor did they show any specific association with different antimicrobial resistances. The carbapenems are considered the antibiotics of last resort for treatment of MDR bacterial infections; hence, bacteria which are resistant to these agents are often termed superbugs as they possess a resistance phenotype against different classes of broad-spectrum antibiotics. The antibiogram of these 39 isolates (Fig. 1) reflected a similar scenario wherein all MBL-producing strains (100%) revealed pan-drug resistance phenotypes as they were completely resistant to almost all the empirically used antibiotics. Nevertheless, all the strains were susceptible to colistin, and a majority of them were susceptible to fosfomycin. Polymyxins are the antibiotics of last resort to treat infections caused by MBL-producing *Enterobacteriaceae* (53), and as combination therapies are being advocated for treatment of MDR pathogens (54), the dual combination of colistin and fosfomycin could be the best option for effective treatment of carbapenem-resistant infections, which also minimizes the chances of resistance development.

The spread of antibiotic resistance genes is mediated by horizontal gene transfer (HGT) between bacteria (55). As multidrug-resistant plasmids serve as the most efficient means for dissemination of resistance traits via HGT, we also observed that the NDM genes and other resistances were transferred (via plasmids)

to recipient strains, as confirmed genotypically and phenotypically by our conjugation experiments (Fig. 2). ERIC-PCR-based clusters revealed the presence of NDM genes in *E. coli* isolates of mostly unique genotypes, suggesting that they are clonally unrelated and thereby have similar propensities to acquire the NDM genes (Fig. 5). Recent reports have shown a greater association of the NDM-1 genotype with sequence type 101, which is consistent with our findings as we also observed three NDM-1-bearing *E. coli* isolates belonging to ST101 (56, 57). We identified NDM-5 to be present in *E. coli* isolates belonging to ST405 and ST167. These sequence types were also linked to NDM dissemination worldwide (58–60). Further, we found that the NDM-1 sequences were preceded by the IS*Aba125* insertion element which belongs to the IS30 family while NDM-5 has an IS5 insertion element upstream of its sequence, followed by sequences of IS*Aba125*. IS*Aba125* is known to be located upstream of NDM in both *Acinetobacter* and *E. coli*, similar to our observations. It has also been reported that the IS5 element is followed by a truncated IS*Aba125* sequence upstream of the NDM gene (61, 62).

The virulence phenotypes such as adhesion to and invasion of epithelial cells play an important role in initiation and establishment of infection. The virulence gene complement as detected at the whole-genome level (Fig. 4) and the adhesion/invasion (Fig. 3a and b) capabilities of the strains toward human urinary bladder epithelial cells demonstrate their pathogenic potentials and associated risks during infection as these strains are resistant to all empirically used antibiotics. Peirano et al. have also shown the ability of CTX-M- and NDM-positive strains to adhere to Caco2 and HepG2 cell lines (63). The ability to resist the bactericidal effect of serum complement as determined by serum resistance assays (Fig. 3c) points toward the enhanced survival advantages of these strains at different extraintestinal infection sites.

In summary, our findings demonstrated that the human extraintestinal pathogenic MBL-producing *E. coli* strains were associated with promiscuous plasmid types, resulting in a diverse range of clones harboring different variants of the NDM gene. Furthermore, whole-genome sequencing and genetic fingerprinting suggest that *bla*<sub>NDM-1</sub> may also disseminate via dominant clones such as ST101. The genomes newly sequenced herein would be an important addition to the existing uropathogenic *E. coli* (UPEC)/ExPEC genome archives and would be helpful in future studies involving comparative genomics and molecular epidemiology. Given that the emergence of newer variants of NDM-1 in countries of endemicity and their association with different ExPEC infections represent an alarming problem, large-scale surveillance and molecular genotyping studies are needed in order to develop strategies to circumvent the emergence of dominant clones potentially leading to institutional outbreaks and nosocomial epidemics of the future.

## ACKNOWLEDGMENTS

We acknowledge funding received from the Department of Biotechnology, Government of India [BT/HRD/NBA/34/01/2011(ix)], and support from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673), Functional Molecular Infection Epidemiology, an initiative of the German Research Foundation and the University of Hyderabad (India), for which N.A. and L.H.W. served as speakers. N.A. is an Adjunct Professor at the Academy of Scientific and Innovative Research, India. A.R. acknowledges the Indian Council of Medical Research for the award of a Junior Research Fellowship.

We thank Ramani Baddam for her help in discussing the data and their

interpretation and help with the analysis of plasmid sequences. The Department of Biotechnology and Bioinformatics is supported by DST-FIST and UGC-SAP-DRS (level 1) programs.

## FUNDING INFORMATION

This work, including the efforts of Lothar H. Wieler and Niyaz Ahmed, was funded by Deutsche Forschungsgemeinschaft (DFG) (GRK1673). This work, including the efforts of Niyaz Ahmed, was funded by Department of Biotechnology, Ministry of Science and Technology (DBT) (BT/HRD/NBA/34/01/2011(ix)).

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## Chapter 5:

### Summary and epilogue



ExPEC infections are increasingly being reported worldwide. Besides humans, ExPEC infections are reported in birds and other companion animals thus reflecting their zoonotic potential. The management of such infections is mediated with antibiotics that cost significant economic burden to public health budgets. The morbidity and mortality rates associated with these infections are significant. Particularly, in high disease burden, developing, populous countries such as India, it is quite important to have the epidemiological data of such scenario in order to track and monitor infection outbreaks. These data not only aid in policy decisions but also play prime role in formulating guidelines for clinical practices, in order to guide appropriate prescription of drugs during treatment. Previously it has been reported by our group that the multi drug resistant UPEC infections in India are of endemic proportions and need urgent attention before causing a catastrophic situation. *E. coli* being one of the predominant organisms in such infections needs to be monitored in appropriate ways. In the present situation, worldwide, the dissemination of MDR ST131 strains has been observed in case of all possible sources.

Of the various diseases associated with ExPEC pathotypes, SSTI are least studied in context of the involvement of *E. coli* in ExPEC infections. Thus, we compared isolates from SSTIs with other ExPEC pathotypes associated with pyelonephritis and septicemia to deduce genomic and functional relationships among the pathotypes. We observed that the SSTI *E. coli* are similar in virulence capabilities as those of other ExPEC pathotypes. A majority of isolates belonged to more virulent B2 and D phylogroups of *E. coli* classification. 67% of the isolates were multidrug resistant and 6% were MBL producers. Nearly 85% of the isolates were ESBL producers and CTX-M15 was the predominant genotype responsible for this resistance trait. Overall resistance between the isolates of pyelonephritis, septicemia and SSTI isolates was very much comparable. Eight genomes were sequenced from this collection and four genomes from our other studies

were together subjected to computational analyses to gain a comprehensive view of the broader picture. We observed similarities among the three groups of isolates in terms of virulence and resistance gene repertoires. Further, they harbored nearly similar genes of both resistance and virulence factors of *E. coli* with little strain specific variations. The *in silico* MLST analysis segregated these isolates into different sequence types such as ST38, ST68, ST167, ST405 and ST131. The two strains, namely NA633 and NA643 perhaps represented the first ST131 strains being reported from SSTI infections in India. The whole genome sequence based comparison further strengthened our observations and revealed ST specific commonality across different pathotypes and ruled out differences based on disease specific traits among these isolates. These studies also provided the functional molecular epidemiological insight of SSTI isolates in comparison to other ExPEC pathotypes demonstrating an indispensable aggregation of genomic as well as functional characteristics.

ST131 strains are globally disseminated and isolated from a variety of infections including all types of ExPEC infections and are presently the most studied sequence type within *E. coli* with global implications. ST131 presently can be designated into further subclones, of which three (*H30*, *H30-R* ST131 and *H30-Rx*) have been prominent and significant in terms of virulence potentials. We described genomic and functional characteristics of the most prevalent and virulent *H30Rx* subclone(s) from our collection as a representative of septicemic *E. coli* from India. The clone in conjunction with other reference genomes revealed high similarity within the *H30 Rx* clones and with seven phage specific regions found to be conserved within the *H30 Rx* strains. The commensal strains lacked the same. The *H30 Rx* subclone, in addition, also harbored certain strain specific regions. Their functional characterization in terms of biofilm formation and serum resistance revealed similarities while differences were observed in invasion capabilities between the two strains of the same *H30 Rx* subclone depicting the phenomenon of

clonal diversification within ST131 strains and survival advantages between the same clade strains.

Multidrug resistant strains, particularly those resistant to the newest IVth generation cephalosporins such as carbapenem are of global concern due to their invariable capability of resistance towards a wide variety of antibiotics belonging to nearly all classes. These organisms are often termed as *superbugs* as they leave very few therapeutic options for treatment. We characterized a large cohort of 510 clinical *E. coli* isolates to determine their resistance pattern towards meropenem (IV generation cephalosporin) and observed that nearly 8% of the isolates were metallo-beta-lactamase producers. Further, all the strains were pan drug resistant and showed an average resistance towards 18 antibiotics of 10 different classes. The only peptide antibiotic, colistin, was effective against all the carbapenem resistant strains while fosfomycin and chloramphenicol were also effective up to a certain extent. NDM was the predominant genotype responsible for MBL production (92%). Among the different variants, NDM1 was present in 69%, NDM5 in 19%, NDM4 in 5.5% and NDM7 in 5.5% of the isolates. Plasmid based replicon typing revealed FIA and FIB plasmids to be the predominant plasmid types whereas conjugation experiments revealed the transfer of NDM genotypes and phenotypes both; this possibly points at the role of plasmids in dissemination of this trait. ERIC PCR based genotyping revealed high diversity among the isolates but two small diffused clades of NDM-1 strains were observed at around 65% of identity which might represent the clonal strains of ST101 isolates as three strains sequenced under these clades belonged to ST101. The WGS of five selected strains comprised of the three NDM1 and two NDM5 isolates revealed three strains (NA084, NA086 and NA099) to belong to ST101 while the two strains (of NDM5 namely NA703 and NA724) belonged to ST167 and ST405. All these strains are associated with NDM and reported predominantly with high resistance traits globally. The virulome and

resistome based on WGS revealed high resistance while moderate prevalence of virulence genes. The functional virulence tests such as serum resistance assay and adhesion/invasion assays revealed the capability of these strains i) to survive under bactericidal effects of serum and ii) to adhere and invade the bladder epithelial cells, which is one of the most important steps in establishment of infection. Therefore, our study systematically deciphered the emerging multidrug resistance traits and the underlying genomic and functional characteristics of the strains we studied, in addition to determining their transmission dynamics at population level.

Overall, this study portrayed aspects of functional molecular infection epidemiology of ExPEC infections in India with a focus on SSTI infections and pan drug resistant phenotypes. This study also provides a comprehensive genomic snapshot of the underlying strains in India that would help clinicians understand mechanistic aspects of drug resistance phenotypes and their clonal or diverse origins relevant in clinical epidemiology. In addition to this, the study will help the healthcare authorities in preparation of suitable and effective antibiotic policies; the most important need of the hour. The study also provides essential baseline data for functional level infection studies needed to answer bigger questions such as i) what could be the spectrum of the functional characteristics of isolates during infection when tested in infection models? ii) what could be the factors responsible for higher acquisition as well as global dissemination of MDR strains? and iii) how the strains could be effectively differentiated into different lineages with respect of drug resistance traits?

## *Other publications*



## Genotypic and Phenotypic Profiles of Escherichia coli Isolates Belonging to Clinical Sequence Type 131 (ST131), Clinical Non-ST131, and Fecal Non-ST131 Lineages from India

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*Antimicrob. Agents Chemother.* 2014, 58(12):7240. DOI:  
10.1128/AAC.03320-14.  
Published Ahead of Print 22 September 2014.

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# Genotypic and Phenotypic Profiles of *Escherichia coli* Isolates Belonging to Clinical Sequence Type 131 (ST131), Clinical Non-ST131, and Fecal Non-ST131 Lineages from India

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In view of the epidemiological success of CTX-M-15-producing lineages of *Escherichia coli* and particularly of sequence type 131 (ST131), it is of significant interest to explore its prevalence in countries such as India and to determine if antibiotic resistance, virulence, metabolic potential, and/or the genetic architecture of the ST131 isolates differ from those of non-ST131 isolates. A collection of 126 *E. coli* isolates comprising 43 ST131 *E. coli*, 40 non-ST131 *E. coli*, and 43 fecal *E. coli* isolates collected from a tertiary care hospital in India was analyzed. These isolates were subjected to enterobacterial repetitive intergenic consensus (ERIC)-based fingerprinting, O typing, phylogenetic grouping, antibiotic sensitivity testing, and virulence and antimicrobial resistance gene (VAG) detection. Representative isolates from this collection were also analyzed by multilocus sequence typing (MLST), conjugation, metabolic profiling, biofilm production assay, and zebra fish lethality assay. All of the 43 ST131 *E. coli* isolates were exclusively associated with phylogenetic group B2 (100%), while most of the clinical non-ST131 and stool non-ST131 *E. coli* isolates were affiliated with the B2 (38%) and A (58%) phylogenetic groups, respectively. Significantly greater proportions of ST131 isolates (58%) than non-ST131 isolates (clinical and stool *E. coli* isolates, 5% each) were technically identified to be extraintestinal pathogenic *E. coli* (ExPEC). The clinical ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates exhibited high rates of multidrug resistance (95%, 91%, and 91%, respectively), extended-spectrum- $\beta$ -lactamase (ESBL) production (86%, 83%, and 91%, respectively), and metallo- $\beta$ -lactamase (MBL) production (28%, 33%, and 0%, respectively). CTX-M-15 was strongly linked with ESBL production in ST131 isolates (93%), whereas CTX-M-15 plus TEM were present in clinical and stool non-ST131 *E. coli* isolates. Using MLST, we confirmed the presence of two NDM-1-positive ST131 *E. coli* isolates. The aggregate bioscores (metabolite utilization) for ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates were 53%, 52%, and 49%, respectively. The ST131 isolates were moderate biofilm producers and were more highly virulent in zebra fish than non-ST131 isolates. According to ERIC-based fingerprinting, the ST131 strains were more genetically similar, and this was subsequently followed by the genetic similarity of clinical non-ST131 and stool non-ST131 *E. coli* strains. In conclusion, our data provide novel insights into aspects of the fitness advantage of *E. coli* lineage ST131 and suggest that a number of factors are likely involved in the worldwide dissemination of and infections due to ST131 *E. coli* isolates.

*Escherichia coli* is the most frequent causal agent of bacterial infections globally, and about 80% of urinary tract infections (UTIs) are caused by extraintestinal pathogenic *E. coli* (ExPEC) isolates (1). These ExPEC isolates are becoming increasingly resistant to frontline antibiotics, like ciprofloxacin and trimethoprim, resistance to which is frequently reported in Europe, America, and much of Asia (2). Moreover, the increasing prevalence of extended-spectrum  $\beta$ -lactamases (ESBLs) in ExPEC, mainly the non-TEM/SHV ESBLs, such as the CTX-M enzymes, has become a serious clinical problem globally and particularly during the last decade (3). Also, ever since the first report of CTX-M-producing *Enterobacteriaceae* (4), several surveys have reported on the presence of ESBLs in Indian clinical isolates (5–7). This has led to an understanding that an endemic incidence of CTX-M ESBL is imminent in many parts of India (8). This scenario could have motivated clinicians to resort to the widespread use of last-line antibiotics, such as carbapenems, to treat various life-threatening infections. Consequently, this could have selected bacteria with novel genes/enzymes that could degrade even the carbapenem group of antibiotics, with the best example being the NDM-1 gene (9, 10). Following the first description of NDM-1 in *Klebsiella pneumoniae* in 2008, a variety of bacterial species positive for NDM-1 carbapenemases was reported globally (5). The emer-

gence of antimicrobial-resistant superbugs is thus a global problem. However, the circumstances predominating in developing countries in particular pose serious concerns (11) despite the fact that there is no credible scientific evidence to indicate that the NDM-1 gene had its origins in India.

The increase in the prevalence of multidrug-resistant (MDR) bacteria in recent years has posed a significant risk to public health (12). Moreover, the dissemination of clonal organisms carrying a heavy antibiotic resistance background has aggravated the problem (13). The strains of sequence type 131 (ST131) form a pandemic clone that is rapidly and boundlessly disseminating in different countries across continents. These clonal pathogens are highly homogeneous in their virulence and antimicrobial resistance properties (14). Pulsed-field gel electrophoresis analysis re-

Received 12 May 2014 Returned for modification 28 June 2014

Accepted 16 September 2014

Published ahead of print 22 September 2014

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doi:10.1128/AAC.03320-14

vealed them to be 85% similar (15). Phylogenetic analysis based on whole-genome sequence data confirmed that the ST131 clones are genetically monomorphic in nature (16, 17). *E. coli* ST131 has emerged globally to become an important pathogen causing urinary tract and bloodstream infections within communities and hospitals (18). This clonal group is also responsible for the recent worldwide spread of CTX-M-15 ESBL types, which are known to frequently harbor fluoroquinolone resistance and for which limited treatment options exist (19). Clinical findings also suggest that the strains belonging to this group are highly virulent and have a fitness advantage (20). If this is the case, then the association of newer mechanisms of antibiotic resistance, such as NDM-1 with ST131 *E. coli* (21), could pose grave complications in the form of pandemics of life-threatening treatment failures.

The dissemination of resistance among bacteria is mainly thought to be due to mobile genes present on self-transmissible plasmids (22). However, a large cache of antibiotic-resistant bacterial clones goes undetected, as these clones often take refuge as normal microbiota in the gut of humans and animals. Moreover, the commensal bacteria are at risk of exposure to orally ingested antibiotics. In this way, they possibly acquire resistance genes and facilitate the spread of such genes to other bacteria of the gut and extant environment (23, 24).

Our previous study from India reported important data on clinical isolates of *E. coli* (7). However, a thorough comparative work describing ESBL-producing ST131 *E. coli* and other non-ST131 ESBL *E. coli* isolates has not been reported so far. Therefore, the objectives of this study were to assess the occurrence, diversity, and distribution of antimicrobial-resistant genotypes and ESBL phenotypes in order to deduce a molecular epidemiological scenario involving commensal bacteria, clinical ST131 types, and non-ST131 isolates of *E. coli*. We also determined 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. This comprehensive profiling of the Indian isolates of *E. coli* forms an essential molecular epidemiological perspective on the predominance and diversity/clonality of such isolates that will aid planning for mitigation with better control strategies.

## MATERIALS AND METHODS

**Sample collection and screening for ST131 strains.** A total of 350 *E. coli* isolates were collected from symptomatic UTI patients from a tertiary care hospital (D. Y. Patil Medical College and Hospital, Pimpri, Pune, India) in India between January 2009 and March 2011. Fifty *E. coli* isolates were also cultured from the feces of healthy individuals who had reported for routine health check-ups at the hospital. All isolates were tested for purity and were identified by routine laboratory methods. The O types of all *E. coli* isolates were initially determined with an allele-specific PCR targeting the *rfbO25* subgroup gene locus (25). ST131 strains were further confirmed by PCR-based detection of the *pabB* allele as described earlier (25). After this screening procedure, only 83 out of the 350 isolates and 43 fecal *E. coli* isolates were finally selected for further studies. Therefore, a total of 126 *E. coli* isolates were subjected to the thorough investigation described below. All microbiological study protocols for these *E. coli* isolates were approved by the Institutional Biosafety Committee of the University of Hyderabad.

**Antibiotic susceptibility testing.** Antibiotic susceptibility profiles were obtained for all *E. coli* isolates by using the standard disc diffusion method on Mueller-Hinton agar. The Clinical and Laboratory Standards Institute (CLSI) recommendations for antimicrobial susceptibility testing were followed (7). The antibiotic panel used was as follows: gentamicin (120 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), chloramphenicol

(30 µg), sulfamethoxazole-trimethoprim (25 µg), and tetracycline (30 µg). All strains were tested for the ESBL production phenotype using the CLSI method, as described previously (26). Resistance to carbapenems was detected by the Etest (HiMedia, India) method, with MICs being determined after a 24-h incubation at 37°C. Susceptibility was defined according to the breakpoints of the CLSI criteria. Additionally, the modified Hodge test was performed to detect carbapenemase production (27).

**Antimicrobial resistance gene testing.** The presence of genes encoding antimicrobial resistance was determined by PCR amplification of the respective genes. PCR amplification of the *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub> genes was carried out on all *E. coli* isolates using the PCR conditions and primers previously described (28). The presence of other antibiotic resistance genes, such as those conferring resistance to tetracycline [namely, *tet*(A), *tet*(B), and *tet*(C)], sulfonamides (*sul1*, *sul2*, *sul3*), streptomycin (*strA*, *strB*), aminoglycosides (*aadA1*-like, *aac4*), and trimethoprim (*dhfr*, *dfr*), and some other plasmid-mediated quinolone resistance determinants, such as *aac* (6')-Ib, was determined by PCR (29–34). Four well-known carbapenemase resistance genes, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>NDM-1</sub>, were also amplified as described previously (29, 35). All the *E. coli* isolates were screened by PCR for the presence of the integron-associated *int1* gene encoding the class 1 integrase with the help of primers described elsewhere (36).

**Conjugation and plasmid analysis.** Conjugative transfer of ESBL genes was tested by broth mating experiments using plasmid-free and sodium azide-resistant *E. coli* strains as the recipients (32). Putative transconjugants were selected on eosin-methylene blue agar plates containing sodium azide (100 µg/ml) and cefotaxime (4 µg/ml), and these were checked by PCRs for the presence of the relevant *bla* genes. Plasmids were classified into two major replicon types according to their incompatibility groups by using a PCR-based replicon-typing scheme (37).

**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. (38). All the *E. coli* isolates were screened for 6 major ExPEC-associated virulence genes by PCR, as described previously (28, 39). The virulence score for each isolate was determined on the basis of the number of virulence genes detected.

**Zebra fish lethality assay and biofilm formation.** Zebra fish were anesthetized by immersion in water containing 168 µg/ml of tricaine (Sigma) and then intraperitoneally injected with 10 µl of phosphate-buffered saline (PBS) containing *E. coli* ( $1 \times 10^6$  CFU/ml) using a 26.5-gauge syringe (Becton Dickinson, USA). After injection, the fish were returned to water tanks and closely monitored for mortality every 3 h for up to 1 week, as described earlier (40). Biofilm formation by *E. coli* isolates from the three groups was assayed by use of a 96-well polystyrene microtiter plate in the static biofilm model, as described in our previous study (7).

**Metabolic profiling.** The three categories of *E. coli* isolates were tested against 35 different substrates to check their metabolizing abilities by use of a KB009 Hi carbohydrate kit (HiMedia, Mumbai, India). Fifty microliters of inoculum was added into the wells of the strip. Upon incubation, the organisms underwent metabolic changes, which were indicated by a spontaneous color change in the medium due to a change of pH.

**Genotyping by ERIC-PCR.** *E. coli* strains were typed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). Crude DNA obtained from the *E. coli* isolates by direct cell lysis was analyzed by ERIC sequence-based PCR with the ERIC1R (5'-ATGTAAGCTCCTGGGGATT CAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers; PCR was performed as previously described (41). Amplicons were separated on a 1.5% agarose gel containing ethidium bromide (5 µg/ml) at 50 V for 3 h. The banding pattern of 126 *E. coli* isolates was analyzed using BioNumerics software (Applied Maths, Belgium). Bands were scored by the use of Dice similarity indices based on the unweighted-pair group method with arithmetic mean (UPGMA), and dendrograms were generated to draw relationships among the ERIC profiles.

TABLE 1 Contribution of ST131, non-ST131, and stool *E. coli* isolates to overall antimicrobial resistance rates

| Resistance trait                         | Overall prevalence of resistant phenotype in population (%) | Estimated fraction due to: |                             |                          |
|------------------------------------------|-------------------------------------------------------------|----------------------------|-----------------------------|--------------------------|
|                                          |                                                             | ST131 clinical isolates    | Non-ST131 clinical isolates | Stool non-ST131 isolates |
| Quinolone or fluoroquinolone             |                                                             |                            |                             |                          |
| Ciprofloxacin                            | 76                                                          | 0.40                       | 0.25                        | 0.34                     |
| Nalidixic acid                           | 95                                                          | 0.38                       | 0.23                        | 0.38                     |
| Sulfonamide-trimethoprim, co-trimoxazole | 68                                                          | 0.33                       | 0.23                        | 0.43                     |
| Aminoglycosides, gentamicin              | 45                                                          | 0.47                       | 0.21                        | 0.31                     |
| Phenicol, 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 <sup>a</sup>               | 20                                                          | 0.48                       | 0.52                        | 0.00                     |
| Multidrug resistance                     | 92                                                          | 0.33                       | 0.32                        | 0.33                     |

<sup>a</sup> Fisher's exact test did not identify any significant differences between the three groups of strains for the different traits analyzed, except for the MBL phenotype, in which the *P* value for ST131 and non-ST131 isolates was <0.05 in comparison with stool *E. coli* isolates.

## RESULTS

**ST131 status and patient demography.** A total of 350 *E. coli* isolates were screened by PCR with the help of a pair of primers directed at the *pabB* fragment, a specific signature of the ST131 lineage. Of these, 43 isolates (12.2%) were positive for ST131-specific amplicons. These ST131 strains were additionally tested for the *rfbO25b* locus by PCR, and their ST131 status was thus reconfirmed. None of the 43 stool isolates belonged to the ST131 lineage. Among the 43 ST131 *E. coli* isolates, 26 (60%) were from female patients with a mean age of 38 years and 17 (40%) were from male patients with a 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 ages of 35 and 45 years, respectively. Male patients (for both ST131 and non-ST131 isolates) were more likely to develop prostatitis than common UTI. Among the different wards/sections of the hospital, a majority of the ST131 isolates originated from surgical wards (30%) and the intensive care unit (33%), while 28% of the isolates were from different medical wards. Only 9% of the ST131 isolates came from the outpatient department. All the clinical non-ST131 isolates originated from nonsurgical medical wards.

**Phenotypic and molecular screening for antimicrobial resistance.** A total of 126 isolates (comprising three groups of 40 ± 3 randomly selected isolates each) representing ST131, non-ST131, and stool non-ST131 *E. coli* isolates underwent antimicrobial susceptibility testing. Disc diffusion test entailing 6 antibiotics of 5 different non-β-lactam classes showed high levels of resistance among all *E. coli* isolates regardless of their genetic lineage or clinical status (Table 1). The antimicrobial resistance rates for the three groups of strains, namely, ST131, non-ST131, and stool non-ST131 isolates, were as follows: for ciprofloxacin, 90%, 92%, and 76%, respectively; for tetracycline, 63%, 75%, and 86%, respectively; for gentamicin, 60%, 50%, and 42%, respectively; for co-trimoxazole, 63%, 75%, and 86%, respectively; and for chloramphenicol, 40%, 33%, and 25%, respectively. *E. coli* isolates within the three groups showed a nearly equal rate of resistance (95%) toward nalidixic acid. ST131 and non-ST131 isolates, including the fecal isolates, did not differ significantly in the prevalence of resistance to any of the particular antibiotics tested. However, aggregate resistance scores (resistance to all the 6 antibiotics) differed marginally among the three groups and were the highest

among the ST131 isolates (35%) and the lowest among the fecal isolates (14%). Among the three groups of strains, the proportions of multidrug-resistant (MDR) isolates were as follows: for ST131 and fecal isolates, 95% each; for non-ST131 isolates, 91%. With regard to ESBL production, 37 (86%) of the 43 ST131 isolates, 39 (91%) of the 43 fecal isolates, and 33 (83%) of the 40 non-ST131 isolates were ESBL producers. The rate of ESBL production among the three groups differed slightly, with higher rates of ESBL production being found in stool isolates, followed by ST131 isolates and then clinical non-ST131 isolates (Table 1).

The prevalences of the major genes for resistance to tetracycline, aminoglycosides, fluoroquinolones, sulfonamides, and trimethoprim in our isolates are reported in Table 2. All 126 isolates were negative for the *sul3*, *aac*, and *tet(C)* genes. The correlation between resistance genotypes (presence or absence of resistance genes) and phenotypes (resistance or susceptibility) was high for fluoroquinolones (94% agreement), co-trimoxazole (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%). The agreement between the ESBL genotypes and the ESBL-positive phenotype in the ST131 and stool isolates was 100%, and for non-ST131 isolates it was 84%. The main gene contributing to the ESBL phenotype among the ST131 strains was *bla*<sub>CTX-M-15</sub>, whereas for non-ST131 and fecal strains, it was *bla*<sub>CTX-M-15</sub> plus *bla*<sub>TEM</sub>. *bla*<sub>CTX-M-15</sub> was found to be strongly associated with ST131 status (93%). The possible combinations of ESBL production–CTX-M-15 production, ESBL production–fluoroquinolone resistance, and CTX-M-15 production–fluoroquinolone resistance gave rise to six subgroups with two subgroups per combination (Table 3). Among these, the dual-positive subgroup (ESBL positive, CTX-M-15 positive) was the most predominant (57%), followed successively by the CTX-M-15-positive–fluoroquinolone-resistant subgroup (55%), the ESBL-positive–CTX-M-15-negative subgroup (33%), the ESBL-negative–fluoroquinolone-resistant subgroup (8%), and the ESBL-negative–fluoroquinolone-susceptible and CTX-M-15-positive–fluoroquinolone-susceptible subgroup (with a low prevalence of 6% each). Within these subgroups, the ST131 strains contributed more or less equal numbers of representatives compared to the numbers contributed by the stool and clinical non-ST131 strains, except for subgroups 1 and 5. The prevalence of metallo-β-lacta-

TABLE 2 Molecular determination of antimicrobial resistance in 126 *E. coli* isolates

| Antibiotic class | Specific trait       | No. (%) of isolates                             |                                                     |                                                 | P value <sup>a</sup> |                    |
|------------------|----------------------|-------------------------------------------------|-----------------------------------------------------|-------------------------------------------------|----------------------|--------------------|
|                  |                      | ST131 <i>E. coli</i> isolates (group 1; n = 43) | Non-ST131 <i>E. coli</i> isolates (group 2; n = 40) | Fecal <i>E. coli</i> isolates (group 3; n = 43) | Group 1 vs group 2   | Group 1 vs group 3 |
| Tetracyclines    | <i>tet(A)</i>        | 24 (56)                                         | 17 (43)                                             | 7 (16)                                          | <0.001               | <0.001             |
|                  | <i>tet(B)</i>        | 5 (12)                                          | 22 (55)                                             | 10 (23)                                         |                      |                    |
| Aminoglycosides  | <i>strA</i>          | 33 (77)                                         | 19 (48)                                             | 24 (56)                                         | 0.006                | 0.040              |
|                  | <i>strB</i>          | 15 (35)                                         | 7 (18)                                              | 16 (37)                                         | 0.073                |                    |
| Fluoroquinolone  | <i>aac(6')-Ib-cr</i> | 36 (84)                                         | 17 (43)                                             | 36 (84)                                         | <0.001               |                    |
| Sulfonamides     | <i>sul1</i>          | 20 (47)                                         | 17 (43)                                             | 10 (23)                                         | 0.038                | 0.024              |
|                  | <i>sul2</i>          | 5 (12)                                          | 12 (30)                                             | 22 (51)                                         |                      | <0.001             |
| Trimethoprim     | <i>dfr</i>           | 18 (42)                                         | 7 (18)                                              | 21 (49)                                         | 0.016                | 0.011              |
|                  | <i>dhfr</i>          | 15 (35)                                         | 3 (8)                                               | 5 (12)                                          | 0.002                |                    |

<sup>a</sup> P values (determined by Fisher's exact test, two-tailed) are shown when P is <0.05.

mase (MBL)-positive strains was only 20% (25 out of a total 126 isolates), having a median resistance and resistance range higher than those of MBL-negative isolates (Table 3). Molecular detection revealed that two such MBL producers belonged to the ST131 group and, more importantly, harbored the *bla*<sub>NDM-1</sub> metallo-β-lactamase gene, in addition to the *bla*<sub>CTX-M-15</sub> ESBL gene. This suggests the emergence of metallo-β-lactamase NDM-1-producing ST131 *E. coli* isolates in India. Overall, resistance to antimicrobial agents other than those specified (Table 1) was common and differed insignificantly between the three groups.

**Conjugation and plasmid analysis.** Plasmid-based replicon typing of the two major incompatibility replicons, FIA and FIB, revealed that the ST131 strains were strongly associated with FIA and FIB at frequencies of 84% and 81%, respectively, and the non-ST131 strains were associated with FIA and FIB at frequencies of 15% and 20% respectively, whereas only 9% and 16% of stool *E. coli* strains were positive for the FIA and FIB replicons, respectively. For identification of the plasmids that were responsible for ESBL production, 10 representative strains from each group were tested by conjugation with the laboratory strain J53, which does not harbor plasmids of its own. We were successful in

getting 10 transconjugants out of the total of 30 representative strains tested. These comprised transconjugants derived from 2 non-ST131, 3 ST131, and 5 stool *E. coli* isolates. The transconjugants were subjected to an ESBL production test by the double-disc synergy method, and it was confirmed that all the 10 transconjugants contained an ESBL plasmid. They were also checked for their ability to confer coresistance to other classes of antibiotics, as described in Fig. 1. As observed, the conjugation propensity was higher among stool *E. coli* strains, followed by ST131 and non-ST131 strains. The transconjugants were found to confer resistance to none or a maximum of four antibiotics, but none of these transconjugants were coresistant to all the antibiotics to which their parent clinical strains were resistant.

**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 (groups A, B1, B2, and D), as shown in Table 4. Among the total population, group B2 was most prevalent phylogenetic group, corresponding to 49% of the isolates, followed in prevalence by the other groups: A (27%), D (16%), and B1 (8%). All the ST131 isolates were from group B2 (accounting for 43 [70%] of

TABLE 3 Antimicrobial resistance scores and range toward 6 non-β-lactam antibiotics among 6 groups of strains defined by the possible combination of ESBL and CTX-M-15 production, ESBL production and fluoroquinolone resistance, and CTX-M-15 production and fluoroquinolone resistance, including those for MBL-positive and MBL-negative strains

| Subgroup | Subset definition                              | All isolates    |                      | ST131 isolates  |                      | Non-ST131 isolates |                      | Fecal isolates  |                      |
|----------|------------------------------------------------|-----------------|----------------------|-----------------|----------------------|--------------------|----------------------|-----------------|----------------------|
|          |                                                | No. of isolates | Median (range) score | No. of isolates | Median (range) score | No. of isolates    | Median (range) score | No. of isolates | Median (range) score |
| 1        | ESBL positive, CTX-M-15 positive               | 72              | 4 (1–6)              | 35              | 5 (2–6)              | 21                 | 4 (2–6)              | 16              | 4 (2–5)              |
| 2        | ESBL positive, CTX-M-15 negative               | 41              | 4 (1–6)              | 15              | 4 (3–6)              | 2                  | 3 (3)                | 24              | 5 (1–6)              |
| 3        | ESBL negative, fluoroquinolone resistant       | 10              | 4 (3–6)              | 4               | 3 (3–4)              | 5                  | 4 (4–6)              | 1               | 0 (5)                |
| 4        | ESBL negative, fluoroquinolone susceptible     | 7               | 1 (0–3)              | 2               | 0 (0)                | 2                  | 2 (1–3)              | 3               | 1 (0–3)              |
| 5        | CTX-M-15 positive, fluoroquinolone susceptible | 69              | 4 (2–6)              | 38              | 5 (2–6)              | 20                 | 4 (2–6)              | 11              | 4 (2–5)              |
| 6        | CTX-M-15 positive, fluoroquinolone susceptible | 7               | 3 (0–4)              | 3               | 3 (0–3)              | 1                  | 0 (3)                | 3               | 3 (3–4)              |
| 7        | MBL positive                                   | 25              | 5 (2–6)              | 12              | 5 (2–6)              | 13                 | 4 (2–6)              | 0               | 0                    |
| 8        | MBL negative                                   | 58              | 4 (0–6)              | 31              | 4 (0–6)              | 27                 | 4 (1–6)              | 0               | 0                    |

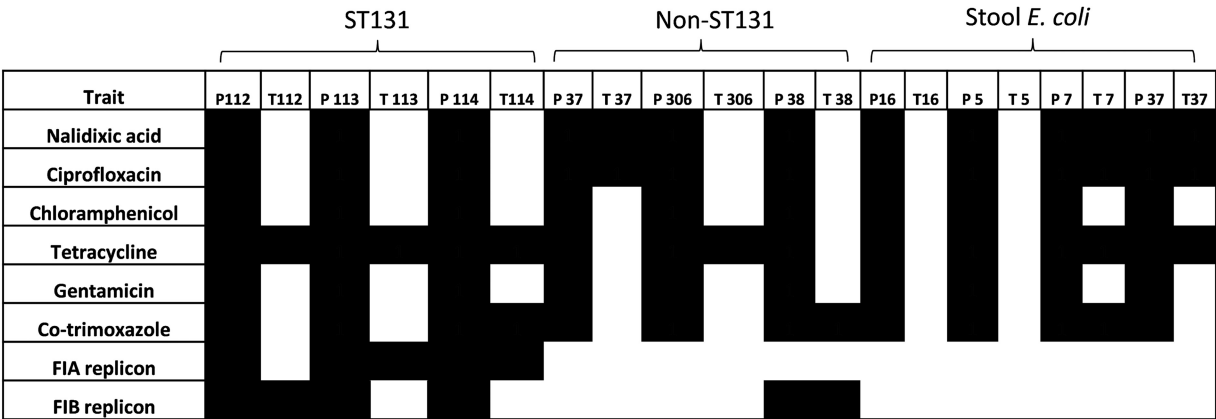


FIG 1 Agar diffusion test and screening of major replicon genes between clinical strains (designated P) and their respective transconjugants (designated T). Black squares, resistance to the indicated antimicrobial or the presence of the indicated replicon; white squares, susceptibility to the indicated antimicrobial or the absence of the indicated replicon.

the 62 isolates of the B2 type), whereas the non-ST131 and fecal isolates exhibited diverse phylogenetic groups, with B2 and A being predominant in the two groups, respectively. Although the non-ST131 isolates were likely to be from group B2 (38%), the phylogenetic group distribution was not significantly associated with CTX-M-15-positive and/or CTX-M-15-negative isolates. In contrast, the stool isolates were predominantly from group A, followed by groups D, B2, and B1. Overall, the distribution of isolates into phylogroups varied significantly among the three groups of *E. coli* isolates (except for group D, with which both non-ST131 and fecal isolates were affiliated in equal proportions).

The genotypes for 6 ExPEC-associated virulence markers were determined for all the 126 isolates comprising the three groups defined by ST131 type and clinical status. All the 6 virulence genes that we explored were detected once in at least 1 isolate. Among the three groups, the median virulence score and the virulence score range values increased progressively from a high of 2 (range, 0 to 5) for ST131 isolates to a low of 1 (range, 0 to 1) for stool isolates, with the intermediate score,

and a score of 1 (range, 0 to 3) for non-ST131 isolates. The ST131 isolates harbored a slightly higher and discrete virulence profile than the non-ST131 and stool isolates (Table 4). In the ST131 group, an aggregate of 81 isolates was positive for the 6 different virulence markers, whereas 25 and 29 isolates within non-ST131 and fecal groups, respectively, were positive for the 6 different virulence markers. Particularly, *sfa* and *foc* (encoding S fimbria and FIC fimbriae, respectively) and *papA* (encoding type 1 fimbrial adhesin) were predominant in ST131 strains. Coinciding with the greater prevalence of a few ExPEC-associated virulence markers among the ST131 isolates, a more significant proportion of ST131 isolates (58%) than non-ST131 and fecal isolates (5% each) technically fell in the ExPEC category. Moreover, among the ST131 isolates, the presence of CTX-M-15 was significantly associated with ExPEC status (98%).

**Biofilm formation.** We explored the biofilm-forming ability of the three categories of strains and found that the ST131 strains were indeed superior in their ability to form a biofilm, but their numbers were not significantly higher. This was followed by non-ST131 strains. The stool strains were weaker biofilm formers than the ST131 and non-ST131 strains (Fig. 2). This indicates that biofilm formation could possibly contribute to the resilience and predominance of ST131 strains, which offers them an advantage to

TABLE 4 Phylogenetic and virulence characteristics of 126 *E. coli* isolates

| Specific trait     | No. (%) of isolates with trait |                               |                                   |                               |
|--------------------|--------------------------------|-------------------------------|-----------------------------------|-------------------------------|
|                    | Total<br>(n = 126)             | ST131<br>isolates<br>(n = 43) | Non-ST131<br>isolates<br>(n = 40) | Fecal<br>isolates<br>(n = 43) |
| Phylogenetic group |                                |                               |                                   |                               |
| Group A            | 34 (27)                        | 0 (0)                         | 9 (22.5)                          | 25 (58)                       |
| Group B1           | 10 (8)                         | 0 (0)                         | 8 (20)                            | 2 (5)                         |
| Group B2           | 62 (49)                        | 43 (100)                      | 15 (37.5)                         | 4 (9)                         |
| Group D            | 20 (16)                        | 0 (0)                         | 8 (20)                            | 12 (28)                       |
| Virulence genes    |                                |                               |                                   |                               |
| <i>afa</i>         | 16 (13)                        | 7 (16)                        | 1 (3)                             | 8 (19)                        |
| <i>sfa-foc</i>     | 4 (3)                          | 4 (9)                         | 0 (0)                             | 0 (0)                         |
| <i>papA</i>        | 45 (36)                        | 29 (67)                       | 12 (30)                           | 4 (9)                         |
| <i>aer</i>         | 55 (44)                        | 32 (74)                       | 9 (23)                            | 14 (33)                       |
| <i>hlyD</i>        | 11 (9)                         | 6 (14)                        | 2 (5)                             | 3 (7)                         |
| <i>cnf</i>         | 4 (3)                          | 3 (7)                         | 1 (3)                             | 0 (0)                         |

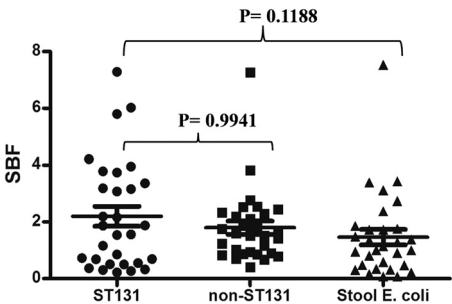


FIG 2 Comparison of the specific biofilm formation (SBF) capacities of ST131, non-ST131, and stool *E. coli* isolates. The mean value for each population is indicated by a dark line. The Mann-Whitney test shows that the differences between the three groups are statistically insignificant.

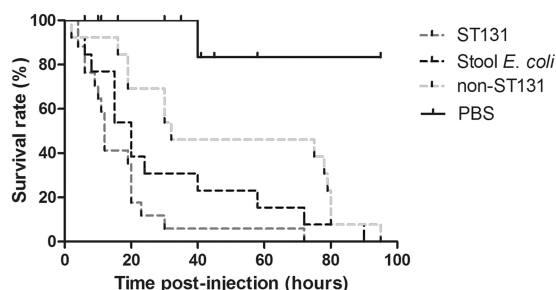


**FIG 3** Survival rates of adult zebra fish infected with *E. coli* isolates belonging to the three *E. coli* groups tested. Each experimental group contained 12 zebra fish, and each experiment was performed in duplicate. Using Kaplan-Meier analysis, we observed differences in survival rates. Although the virulence of ST131 isolates differed significantly ( $P = 0.0032$ ) from that of the clinical non-ST131 *E. coli* isolates, it did not differ significantly ( $P = 0.1820$ ) from that of stool non-ST131 *E. coli* isolates.

infect and cause chronic infections; a previous study has also shown that ST131 strains from global sources harbor virulence factors that potentially associate with *in vitro* biofilm formation (42).

**Zebra fish lethality assay.** The zebra fish infection model is considered a valuable tool to resolve the diverse virulence phenotypes of closely related ExPEC strains. The survival rate of the infected fish was determined to evaluate the virulence potential of ST131 strains and compare it with that of non-ST131 and stool *E. coli* isolates. In this study, three bacterial strains per category were tested; each bacterial strain was injected into four replicates of fishes. The control fish were injected only with PBS. The control fish were all healthy and survived even after 5 days postinjection. Bacterium-infected zebra fish exhibited apparent signs of discomfort, including an inflamed peritoneal area, improper swimming, and reduced gill movement. The ST131 strains were significantly more virulent than the non-ST131 and stool strains. Almost 90% of the zebra fish injected with ST131 strains died within 24 h postinjection. The lethality of the stool *E. coli* isolates was moderate, followed by the level of lethality of the non-ST131 clinical isolates. These results indicate that the ST131 strains were highly lethal for zebra fish (Fig. 3). 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 partly be accounted for by the presence of the *hlyA* and *aer* genes; in addition, the possibility that other unknown toxins or virulence factors are produced cannot be ruled out.

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

**Genotyping.** To illustrate the relationship of ST131 with other *E. coli* groups and to establish evidence for ST131's prominence,

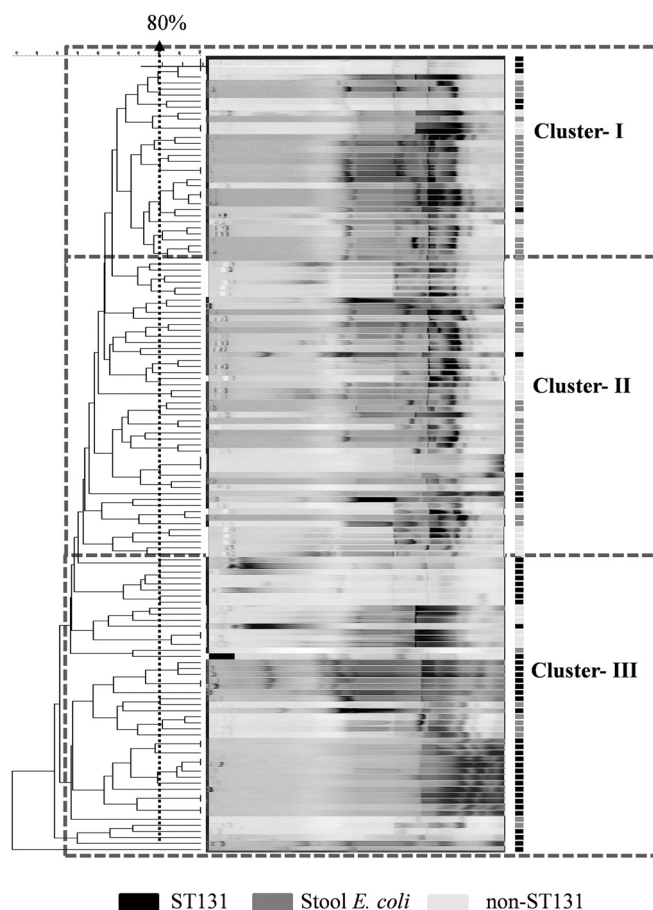


**FIG 4** ERIC-PCR-based dendrogram of 126 *E. coli* isolates comprising 43 ST131, 40 non-ST131, and 43 stool *E. coli* isolates produced by use of the UPGMA algorithm based on Dice similarity coefficients. The ST131 strains were genetically homogeneous (cluster III). The homogeneity of the ST131 strains was followed by that of non-ST131 (cluster II) and stool *E. coli* (cluster I) isolates.

ERIC-PCR-based fingerprinting was employed to assess the genomic diversity among the clinical ST131, clinical non-ST131, and stool non-ST131 *E. coli* isolates. The isolates in an ERIC-PCR-based dendrogram of 126 *E. coli* isolates could be broadly classified into three clusters, as defined by the ability of the 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 more genetically similar, followed by the non-ST131 strains and then the fecal strains (Fig. 4). Consistent with this, the ST131 isolates were more likely to exhibit similar ERIC-PCR-based fingerprinting profiles (30%) than the non-ST131 (18%) and stool *E. coli* (9%) isolates. The two NDM-1-positive isolates formed identical ERIC-PCR-based fingerprinting profiles, suggesting vertical transmission. However, several dissimilar isolates belonging to all the three categories harboring *bla*<sub>CTX-M-15</sub> were scattered throughout the dendrogram, suggesting possible horizontal gene transfer (regardless of ST131 and clinical status). These observations confirm our previous results (7) that the ST131 strains are evolving clonally, whereas the non-ST131 fecal isolates are genetically diverse, with non-ST131 clinical strains being intermediate in their genetic heterogeneity.

## DISCUSSION

Recent studies have shown a significant increase in the number of CTX-M-producing *E. coli* isolates possibly due to the dominant mechanisms of clonal expansion of *E. coli* ST131 (15, 16, 43). The association of ST131 with several ExPEC-associated virulence genes and broad-spectrum drug resistance, together with their propensities for rapid expansion and geographic dispersal, has led to the worldwide predominance of ST131 among extraintestinal pathogenic *E. coli* infections. However, the exact underlying mechanism conferring this behavior has not been identified yet. Having said this, it is very pertinent at this juncture to understand the epidemiology of the clone ST131. Past studies have analyzed selected factors for their association with ST131 and non-ST131 isolates (42, 44, 45). However, comprehensive analyses to effectively measure the association of several key factors with clinical ST131 isolates, non-ST131 isolates, and *E. coli* isolates from healthy adult subjects, particularly from developing countries like India, remain scarce. In this study of MDR *E. coli* isolates, obtained from clinical samples and stool specimens from India, we sought to define the associations of some of the key factors related to resistance profiles and transmissibility, virulence genotypes, and pathogenicity and also assessed the genetic affinities and metabolic capabilities of the three populations of *E. coli*. Such a study is also important since ST131 *E. coli* is presumed to be still evolving in terms of the acquisition of newer resistance and virulence genes, and the global expansion of such clones poses a significant public health threat. The current findings extend those from our previous study (7) and offer valuable insights into the basis of the epidemiology of CTX-M-15 dissemination in general and the success of ST131 *E. coli* in particular.

Our analysis of 126 systematically selected isolates comprised 43 (group B2) ST131 clinical *E. coli* isolates and 40 non-ST131 clinical isolates involved in nosocomial and community-acquired infections, along with 43 isolates from healthy individuals, including both ESBL producers and non-ESBL producers. We found that although *E. coli* clonal group ST131 was present in the wider uropathogenic *E. coli* (UPEC) population, it was particularly discerned among MDR ESBL producers. However, their prevalence was moderate; this is consistent with the idea that *E. coli* is a highly diverse species and the clinical isolates are usually represented by several small clonal groups. Remarkably, none of the stool *E. coli* isolates belonged to the ST131 clonal group. Moreover, we identified very few cases of ST131 *E. coli* infection in individuals in outpatient departments. We found a predominance (93%) of *bla*<sub>CTX-M-15</sub> among ESBL-producing ST131 *E. coli* isolates. Notably, over approximately 50% of non-ST131 and fecal isolates also harbored *bla*<sub>CTX-M-15</sub>, providing evidence of the widespread distribution of *bla*<sub>CTX-M-15</sub> and a strong association of ST131 with *bla*<sub>CTX-M-15</sub>, but not vice versa. The estimated overall comparable contributions of ST131, non-ST131, and stool *E. coli* isolates to non- $\beta$ -lactam antimicrobial resistance phenotypes suggest not only that it is ST131 that is responsible for the recent increase in antimicrobial resistance prevalence but also that the possibility that other factors contribute to the spread and maintenance of high resistance rates cannot be ruled out. Between the three *E. coli* groups, no single group demonstrated any unique resistance gene profile which was reflective of the observed phenotypes. Despite the similarity of our study to that of Kumarasamy et al. (5), our results were quite different: only two of the total clinical samples

analyzed by us were positive for NDM-1. Also, none of the ESBL-producing *E. coli* strains of fecal origin harbored NDM-1. Given this, the prevalence rates appear to be contrasting, although both the studies were performed on clinical samples from India. Plasmid-based replicon typing did not identify plasmid replicons common to the ST131, non-ST131, and stool *E. coli* isolates. Nevertheless, the ST131 isolates shared common plasmids, particularly plasmids positive for FIA-FIB replicons. Earlier studies also showed that ST131 strains are enriched with FIA-FIB replicons (14). Moreover, the transmissibility of these plasmids, together with their propensities to acquire new genes, implies an alarming potential to spread and diversify. The fluoroquinolones and extended-spectrum cephalosporins as well as aminoglycosides and  $\beta$ -lactam antibiotics are generally prescribed as empirical therapy for the treatment of hospital-acquired infections and urinary tract infections in particular. Having said this, the clinical and the public health implications of the observed high prevalence of multi-drug resistance and resistance to extended-spectrum cephalosporins and, in particular, the high rates of fluoroquinolone resistance among the clinical as well as the stool *E. coli* isolates are alarming, as it might narrow or deplete the treatment options.

Our results and the observation of a high prevalence of phylogroup B2 isolates among the ST131 isolates compared to its prevalence among the non-ST131 and stool *E. coli* isolates suggest the clonal expansion of ST131 from a common phylogenetic ancestor; this observation reinforces the explanations for ST131's remarkable epidemiological success, as phylogroup B2 is thought to be epidemiologically associated with extraintestinal virulence by means other than through the defined extraintestinal virulence traits.

Furthermore, the molecularly inferred virulence profiles as well as the pathogenicity of the ST131 isolates substantially exceeded those of the non-ST131 and stool *E. coli* isolates in zebra fish, and the ST131 isolates were slightly stronger biofilm formers. This profound virulence advantage observed for ST131 strains may compensate for their modest disadvantage for the development of resistance compared to the possibility of resistance development in the other non-ST131 and stool *E. coli* isolates.

The outcome of the zebra fish lethality assay added to our understanding that *E. coli* ST131 isolates possess an enhanced virulence potential and, thereby, an enhanced ability to cause invasive disease (20, 46, 47), although this may not consistently corroborate the findings of different studies performed in different settings and with different isolates (48–52). Nevertheless, the assay provided a much needed *in vivo* snapshot to establish the virulence attributes of our ST131 strains in adult zebra fish, even though the number of strains in each group was limited. However, scant numbers of strains have previously been used in some of the landmark studies conducted in this area (15, 53). Recently, two reports (15, 48) have shown that *E. coli* ST131 strains do not possess a higher virulence potential in causing invasive infections than other UPEC or other B2 group *E. coli* isolates. This disagreement with respect to virulence could possibly be due to several factors, such as differences in experimental conditions, the ST131 virulence reporter itself, and differences in study populations. Thus, these findings suggest that the ST131 isolates are not (universally) more virulent than the *E. coli* isolates of other sequence types. It is also possible that ST131 strains would act as efficient opportunistic pathogens in humans, given the fact that ST131 is currently the dominant extraintestinal human pathogen. How-

ever, the possibility that other host and environmental factors play a role cannot be ruled out. Furthermore, this discrepancy deserves an investigation by whole-genome comparative analysis of ST131 strains from different geographic origins, along with an experimental assessment of their role in pathogenesis. Overall, the ST131 strains were significantly more likely to qualify as ExPEC strains, and because the ST131 isolates were significantly more likely to be from group B2 than the non-ST131 and stool *E. coli* isolates, they may have enhanced fitness over other *E. coli* isolates. In addition to this, the ST131 strains were also found to be significantly more likely to be associated with the CTX-M-15 gene while also being resistant to a wide range of  $\beta$ -lactam and non- $\beta$ -lactam antibiotics. This supplemental property seems to provide a competitive advantage to ST131 *E. coli* isolates over other *E. coli* isolates.

Studies have reported variations in the virulence-associated gene profile (VAGF) among ST131 strains, which causes one to question the role of VAGF in the current success of ST131 (49, 50), and therefore, these studies have proposed other factors, such as an enhanced metabolic potential, that contribute to the fitness of this dominant clone. Also, it is well established that bacterial metabolic potential can enhance fitness, leading to increased pathogenesis. For example, a previous study has shown that carbohydrate metabolism in enterobacteria may increase bacterial virulence (51). In this connection, we tested and compared sugar source utilization and found that the ST131 strains were only moderately superior to the other *E. coli* strains tested in metabolizing the 35 sugars tested. Our data are not in total agreement with those of Gibreel et al. (52) or Alqasim et al. (49) because our study did not show either a significantly enhanced or a significantly reduced metabolic capacity of ST131 strains in comparison with the metabolic capacity of other non-ST131 *E. coli* strains. This discrepancy could be due to differences in the types of tests employed, differences in the study populations, and/or comparisons of biased samples. Given this, it will be necessary to profile a collection of strains that is as diverse as possible and as many strains from different geographic regions of the world as possible, and in particular, Indian isolates should be analyzed in a comparative genomics study and by use of phenotypic screens employing other well-known and completely sequenced isolates of the ST131 lineage.

Genotyping by ERIC-PCR showed that the ST131 isolates of this study formed a more or less distinct cluster representing the majority of ST131 strains or less these strains were compared to the other *E. coli* groups. This strongly suggests clonal expansion from a common ancestor. Even though it has been years since the emergence and dissemination of ST131 *E. coli* strains, they still maintain their clonal nature. This clonal nature, on the one hand, holds the danger of potentially causing disease outbreaks but, on the other hand, may facilitate effective control strategies involving vaccine development and transmission prevention.

In summary, we utilized comprehensive genotyping and phenotyping approaches to compare clinical ST131 *E. coli* isolates with clinical non-ST131 *E. coli* and stool non-ST131 *E. coli* isolates. Although our isolates displayed diversity in virulence, resistance, metabolic activity, and clonality, neither their affiliation with ST131 nor clinical status was significantly linked to the observed differences. Furthermore, our comprehensive data suggest that none of the key traits examined as described above *per se* contributed exclusively to the epidemiological success of ST131 *E.*

*coli* strains. Rather, the cumulative effect of some of the factors, such as their being of phylogenetic group B2, their sustained carriage of CTX-M-15 plasmids, and their slightly enhanced virulence, antimicrobial resistance, and metabolic activity, along with the high degree of clonal structure and some undefined evolutionary mechanisms, could provide a multilayered fitness advantage to ST131 strains. Hence, the biological basis for the success of ST131 could be a combination of some of the key traits explained above, but none of them could be the sole or exclusive characteristic responsible for the remarkable success of ST131 strains. This is also suggested by the fact that analysis of ST131 *E. coli* strains from different origins and comparison of these strains with well-characterized strains showed inconsistent results with respect to some of the key traits (42, 50). Further investigation based on the functional molecular infection epidemiology of ST131 *E. coli* isolates from different geographic origins and their comparison with other well-characterized strains from *E. coli* phylogroup B2 are needed to exactly determine the basis for the success of ST131 *E. coli*. Regardless of this, the broad multidrug resistance profile observed among all the three categories of *E. coli* strains and especially the continued acquisition of new antibiotic resistance determinants (NDM-1) by ST131 may limit therapeutic options in the future and warrant attention from the public health and clinical communities.

## ACKNOWLEDGMENTS

We acknowledge help from the Indo-German International Research Training Group, Internationales Graduiertenkolleg (GRK1673), on functional molecular infection epidemiology, an initiative of the German Research Foundation (DFG) and the University of Hyderabad (India), for which N.A. is a speaker. We thankfully acknowledge the support received from a University of Malaya High Impact Research Grant (reference no. UM.C/625/1HIR/MOHE/02 [A000002-5000 1]) in molecular genetics, for which N.A. is a lead collaborator. N.A. is an adjunct professor at the Academy of Scientific and Innovative Research (ACSIR), India.

We thank Lothar H. Wieler and Christa Ewers for stimulating discussions.

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# *Helicobacter pylori* cell translocating kinase (CtkA/JHP0940) is pro-apoptotic in mouse macrophages and acts as auto-phosphorylating tyrosine kinase



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## ARTICLE INFO

### Article history:

Received 30 April 2014

Received in revised form 26 June 2014

Accepted 28 July 2014

### Keywords:

*H. pylori*  
Plasticity region  
Inflammation  
JHP0940  
Apoptosis  
Kinases

## ABSTRACT

The *Helicobacter pylori* gene JHP0940 has been shown to encode a serine/threonine kinase which can induce cytokines in gastric epithelial cells relevant to chronic gastric inflammation. Here we demonstrate that JHP0940 can be secreted by the bacteria, triggers apoptosis in cultured mouse macrophages and acts as an auto-phosphorylating tyrosine kinase.

Recombinant JHP0940 protein was found to decrease the viability of RAW264.7 cells (a mouse macrophage cell line) up to 55% within 24 h of co-incubation. The decreased cellular viability was due to apoptosis, which was confirmed by TUNEL assay and Fas expression analysis by flow-cytometry. Further, we found that caspase-1 and IL-1 $\beta$  were activated upon treatment with JHP0940. These results point towards possible action through the host inflammasome. Our *in vitro* studies using tyrosine kinase assays further demonstrated that JHP0940 acts as auto-phosphorylating tyrosine kinase and induces pro-inflammatory cytokines in RAW264.7 cells. Upon exposure with JHP0940, these cells secreted IL-1 $\beta$ , TNF- $\alpha$  and IL-6, in a dose- and time-dependent manner, as detected by ELISA and transcript profiling by q-RT-PCR.

The pro-inflammatory, pro-apoptotic and other regulatory responses triggered by JHP0940 lead to the assumption of its possible role in inducing chronic inflammation for enhanced bacterial persistence and escape from host innate immune responses by apoptosis of macrophages.

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## Introduction

Even 30 years after the discovery of *Helicobacter pylori* (Marshall and Warren, 1984) and establishment of its role in gastric diseases, more than 80% of the population in developing countries (Hunt et al., 2011) as compared to 30–50% in developed countries (Covacci et al., 1999) is infected with the bacterium. However, the prevalence of *H. pylori* infection varies from geographic region to region throughout the world (Ferlay et al., 2010) which is more likely correlated with socioeconomic status of the population (Malaty and Graham, 1994) and sex; because in many populations males have 20–30% higher rates of infection than females (Replogle et al., 1995). Since *H. pylori* is the main cause of gastric cancer, WHO has

classified the bacterium as a class I carcinogen in 1994 and severity of the disease in the present scenario is so high that gastric cancer is the second leading cause of cancer related deaths worldwide and constitutes the fourth most common cancer type (Ferlay et al., 2010).

*H. pylori* causes infection of the gastric mucosa (Marshall and Warren, 1984) and is found to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcer, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997; Uemura et al., 2001). Although the severity of chronic inflammation depends on host genetic factors, environmental parameters and bacterial factors play an important role in the onset of gastric diseases. Among the bacterial factors encoded by various clinical *H. pylori* strains, one of the two most studied proteins is CagA which is encoded within the cytotoxin-associated genes (*cag*) pathogenicity island (PAI) and is about 40 kb in size (Covacci et al., 1993). It has been established that most of the genes from the *cagPAI* act as components of a type IV

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secretion system (T4SS) forming a syringe like structure that facilitates translocation of CagA and probably other macromolecular bacterial factors into the host cells (Backert et al., 2010). Moreover, the strains that harbour the *cagPAI* are associated with more severe gastric inflammation and overt outcomes as compared to *cagPAI*-negative strains. The other most studied *H. pylori* virulence factor is VacA (vacuolating cytotoxin A) which induces cytoplasmic vacuolation by forming membrane channels in epithelial membranes *in vitro* (Cover and Blaser, 1992). Besides this activity, it has a multifunctional role in colonization, virulence and immunomodulation (Cover and Blanke, 2005; Pachathundikandi et al., 2013). Apart from these two cardinal effectors, virulence genes such as BabA, SabA, SabB, OipA etc. (Basso et al., 2010; Odenbreit et al., 2009) code for the other important factors which facilitate adhesion of the bacterium to the gastric epithelium. In addition, IceA that carries a restriction endonuclease activity was also found to be associated with gastritis and duodenal ulcer (Xu et al., 2002). There is an on-going interest in another putative virulence factor, DupA, on account of its association and envisaged role in gastric diseases (Hussein et al., 2010; Shiota et al., 2010; Schmidt et al., 2009). Furthermore, findings on some other *H. pylori* gene products such as protease HtrA (Hoy et al., 2012), AnsB (asparaginase), GGT ( $\gamma$ -glutamyl transpeptidase) (McGovern et al., 2001), HP0986 (Alvi et al., 2011), HP-NAP (Satin et al., 2000) and HorB (Snelling et al., 2007) revealed their association with inflammation and pointed to their role in gastric diseases. Given this, the data available about these new generation bacterial factors do not seem to be adequate to fully understand pathogenesis of gastric inflammation and cancer. Therefore, it seems prudent to focus on the discovery of novel virulence factors and their biological roles relevant to the development and progression of gastric diseases. JHP0940 or CtkA is one such potential virulence factor that has been discovered and partly characterized (Rizwan et al., 2008) and its crystal structure has been solved (Kim do et al., 2010).

In the present study, we attempted to investigate the functional roles of JHP0940 which is located in a plasticity region gene cluster of the chromosome. The plasticity region is a highly variable zone that was identified by comparison of complete genome sequences of the two *H. pylori* strains (26695 and J99) that contain approximately 50% strain specific genes (Alm et al., 1999). Some of the genes in this region are thought to be acquired by *H. pylori* through horizontal gene transfer from some environmental bacteria (Datta et al., 2003). Thus, the plasticity zone genes provide an assumption that the proteins encoded by them may contribute to virulence or modulation of host immune responses. The association of *jhp940* gene with gastric cancer (Occhialini et al., 2000; Yakoob et al., 2010), expression of JHP940 protein in response to interaction of *H. pylori* with the gastric mucosa in Mongolian gerbils *in vivo* (Graham et al., 2002) and its antigenicity profile prompted us to look at its virulence properties in more detail. Previous observations indicated that JHP940 can induce pro-inflammatory cytokines IL-8 and TNF $\alpha$  and increases translocation of transcription factor NF- $\kappa$ B in the human macrophage cell line Thp1 (Rizwan et al., 2008). Consequently, it was reported that JHP0940 also acts as a cell-translocating Ser/Thr Kinase and could indirectly upregulate the phosphorylation of NF- $\kappa$ B at serine residue 276 in human gastric epithelial cells (Kim do et al., 2010).

In the present study, we further extend the functional acumen of the JHP0940 protein and report its interaction with the host immune apparatus as evident from serum antibody titres present in patients infected with JHP0940-positive *H. pylori*. We also found that JHP940 acts as an auto-phosphorylating tyrosine kinase. This is perhaps the first described tyrosine kinase in *H. pylori* and we could demonstrate that it induces strong pro-inflammatory responses as well as Fas-mediated apoptosis in murine macrophage cells.

## Materials and methods

### Bacterial strains

The *H. pylori* wild-type strains J99 (Alm et al., 1999), SJM180 (Kersulyte et al., 2003) and P1 (Mueller et al., 2012) were used in the study. *H. pylori* was grown in thin layers on horse serum GC agar plates supplemented with vancomycin (10  $\mu$ g/ml), nystatin (1  $\mu$ g/ml), and trimethoprim (5  $\mu$ g/ml) as described previously (Tegtmeyer et al., 2013). All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bacteria were grown at 37 °C for 2 days in an anaerobic jar containing a Campygen gas mix of 5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub> (Oxoid, Wesel, Germany) (Tegtmeyer et al., 2011).

### JHP0940 (Ctk) secretion assays

Bacterial wild-type strains were grown in BHI broth medium supplemented with 10% FCS for 12 h starting with an OD<sub>600nm</sub> ~ 0.2. The supernatant and the cell pellets were separated by centrifugation at 4000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21  $\mu$ m sterile filter (Sigma Aldrich). The resulting bacterial pellets and supernatants were analysed by immunoblotting (Boehm et al., 2012). Absence of live bacteria in the supernatant was also confirmed by incubation on agar plates showing no growth.

### Cell culture

Mouse macrophage RAW264.7 cell line was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The cells were trypsinized and seeded for the experiments.

### Expression and purification of recombinant JHP0940 protein

The *jhp0940* gene was cloned in pRSET-A expression vector and the protein was expressed in *Escherichia coli* BL21 (DE3) cells, induced with 0.1 mM IPTG. His-tagged JHP0940 was purified as described earlier (Rizwan et al., 2008) with minor modifications, using Cobalt based Talon<sup>®</sup> resin (Clontech, USA). The homogeneity of the protein was confirmed by SDS-PAGE (12%) and the amount of protein estimated by bicinchoninic acid assay (BCA). The purified protein JHP0940 was treated with polymyxin B beads for 4 h and separated by centrifugation at 10,000 rpm for 10 min. LPS contamination in polymyxin B treated protein was quantitated by *Limulus* amoebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific).

### Treatment of cells with JHP0940 and preparation of cell lysate

RAW264.7 cells were cultured in 60 mm culture dish at a density of  $4 \times 10^6$  per plate and treated with JHP0940 protein. Before harvesting, the cells were washed in cold PBS, scraped by cold plastic cell scraper, transferred to cold microfuge tube and lysed with ice cold lysis buffer (20 mM Tris HCl pH 7.4, 137 mM NaCl, 10% Glycerol, 1% NP-40, 2 mM EDTA, PI cocktail 1  $\mu$ l/ $1 \times 10^6$  cells, 1 mM PMSF, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>) by intermittent vortexing for 40–60 s and repeated 5 times after an interval of 10 min and followed by centrifugation at 14,000 rpm for 10 min at 4 °C. Supernatant was collected and quantified by BCA method for western blotting.

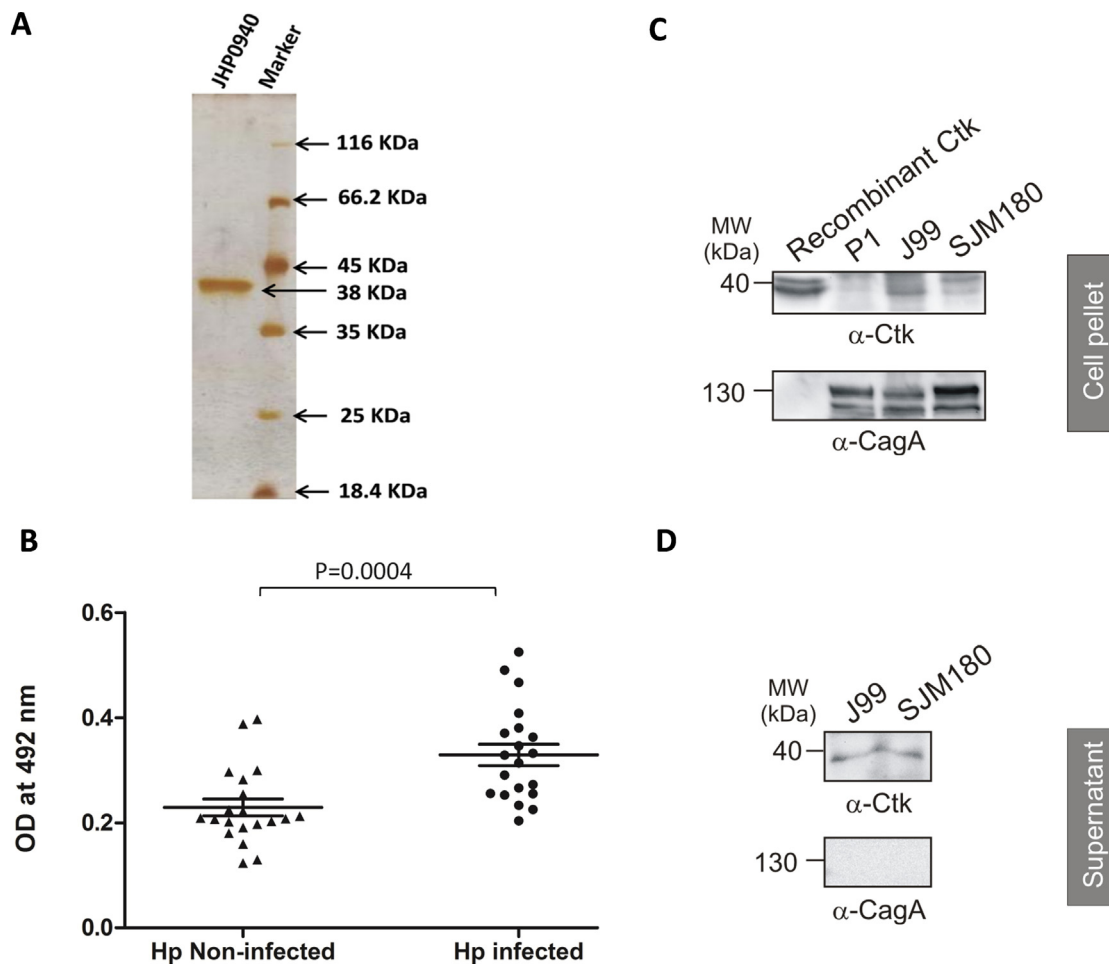


**Fig. 1.** (A) Purified JHP0940 in silver stained SDS gel. (B) ELISA of JHP0940 in two groups of human sera samples of 20 *H. pylori* infected and 20 healthy individuals. Statistically significant difference in *P* value (0.0004) was determined by two tailed Mann Whitney *U* test between healthy and *H. pylori* infected individuals. The data shown here represent mean  $\pm$  SEM of three independent experiments. (C) Total cell lysates of *H. pylori* wild-type strains J99, SJM180 and P1 were investigated for the expression of Ctk. Equal amounts of protein per sample was confirmed by immunoblotting using an  $\alpha$ -CagA antibody as control. (D) Ctk secretion assay in BHI medium with 10% FCS. The above *H. pylori* wild-type strains were grown for 12 h at 37 °C. Bacterial supernatants were filtered and prepared as described in the Materials and Methods section. The presence of secreted Ctk proteins in the supernatant was investigated by immunoblotting using the  $\alpha$ -Ctk antibody and as control with  $\alpha$ -CagA antibody. (OD: optical density; KDa: molecular weight in kilodalton).

#### Production of anti-JHP0940 polyclonal antibody in rabbit

A New Zealand white rabbit was given prime dose of 500  $\mu$ g of JHP0940 intramuscularly after acclimatization. First intra-dermal booster of JHP0940 (300  $\mu$ g) was given 21 days after the prime dose. Second intramuscular booster dose of the protein (300  $\mu$ g) was followed by third intramuscular dose of JHP0940 (300  $\mu$ g) 14 days after the second dose. Titre was checked 14 days after each booster dose. Serum was obtained by removing 20 ml blood from marginal ear vein of the rabbit. Serum was used in Western blot experiments to detect JHP0940.

#### Humoural response

Twenty human sera samples from each group of *H. pylori* infected patients and healthy subjects were obtained from the sera collection of Prof. Francis Megraud, INSERM-U853, Bordeaux (France) and used for performing ELISA. JHP0940 protein was coated in bicarbonate coating buffer @ 0.5  $\mu$ g/well in 96 well plates followed by overnight incubation at 4 °C; unbound surface was blocked with 1% BSA for 2 h at 37 °C. 1:50 dilution of sera (primary antibody) were used and incubated for 2 h at 37 °C. Anti-human HRP secondary antibody was used in 1:1500 dilution followed

by 1 h incubation at 37 °C. At each step, washing was done 3 times with PBST. This was followed by development of water soluble coloured reaction product with o-phenylenediamine dihydrochloride (OPD) substrate. The reaction was stopped by 3N H<sub>2</sub>SO<sub>4</sub> added @ 100  $\mu$ l/well and the plate was read by ELISA plate reader at 492 nm. *P* value was calculated by two tailed Mann Whitney *U* test between *H. pylori* infected and non-infected groups considering replicates of three independent experiments in each case.

#### ELISA for cytokine quantification

Murine macrophage RAW264.7 cells were cultured in the absence or presence of the endotoxin free JHP0940 protein for 24 h in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. Culture supernatants were harvested for the estimation of various cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) by two-site sandwich ELISA as described earlier (Khan et al., 2006; Kumar et al., 2013). In brief, the 96-well polyvinyl chloride microtitre plates were coated with purified anti-TNF- or anti-IL-6 or anti- IL-1 $\beta$  antibody at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated for overnight at 4 °C. The plates were washed with wash buffer (PBS with 0.05% Tween-20) and blocked with 1% BSA in PBS followed by incubation with the test samples for overnight at 4 °C. After

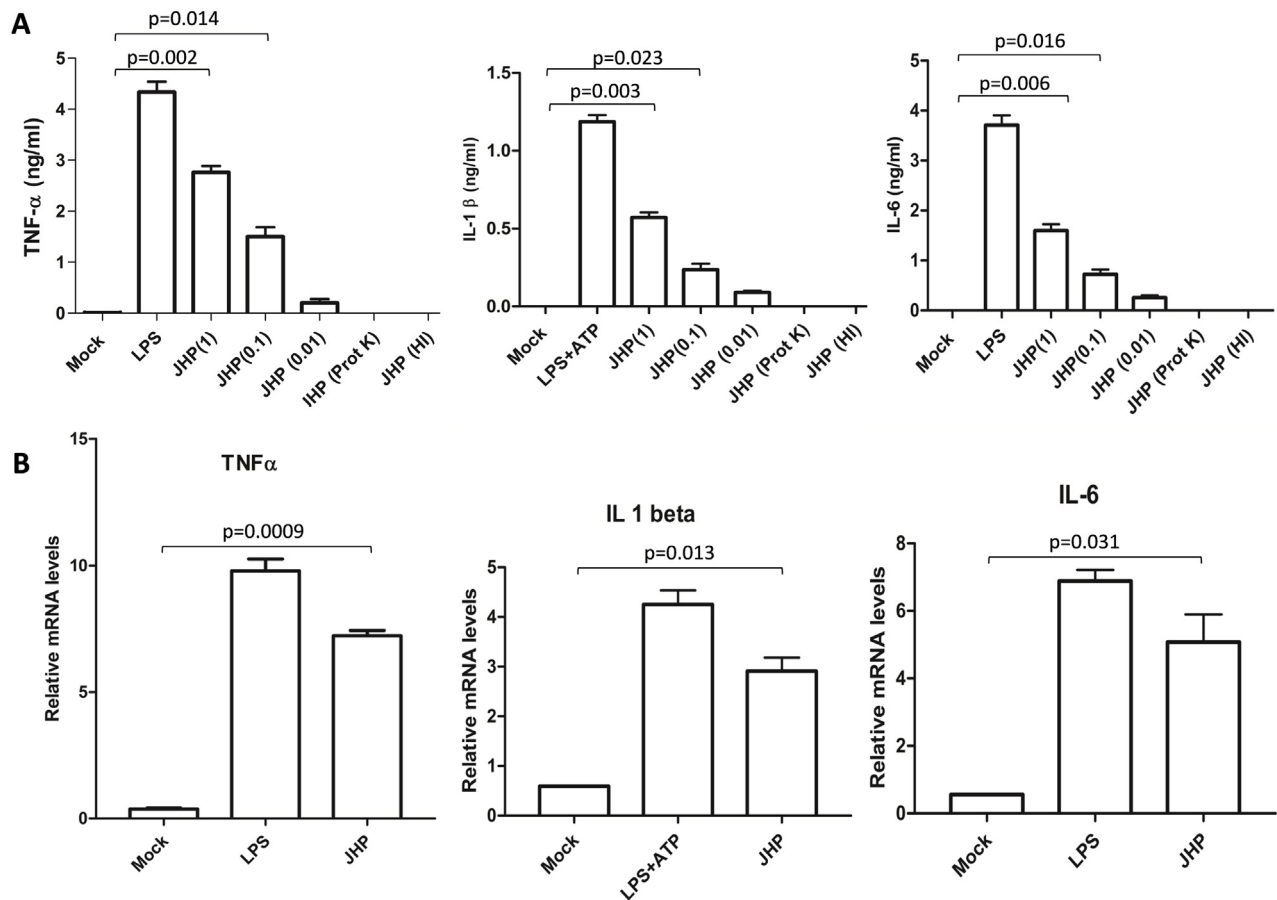


**Fig. 2.** (A) Treatment with JHP0940 protein in varying concentrations (0.01, 0.1 and 1  $\mu\text{g/ml}$ ) induced secretion of pro-inflammatory cytokines in mouse macrophage cell line (RAW264.7): TNF- $\alpha$  and IL-6 were measured at 24 h post treatment. For IL-1 $\beta$  analysis, the cells were treated (primed) with either JHP0940 (CtkA) or LPS (as positive control) for 3 h followed by 20 min of incubation with 5 mM ATP to activate the release of mature IL-1 $\beta$ . The cells treated with LPS (0.1  $\mu\text{g/ml}$ ), heat inactivated JHP0940 (100°C for 10 min) and Proteinase K were taken as controls. The data represent means  $\pm$  S.E. ( $n=3$ ) and  $P$  value for TNF- $\alpha$ , IL-6 and IL-1 $\beta$  was calculated separately by comparing with corresponding mock control. (B) Real time analysis of relative expression of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  after 6 h of JHP0940 protein treatment (1  $\mu\text{g/ml}$ ) of RAW264.7 cells indicated increased relative expression of above cytokines when compared to mock.  $\beta$ -2 microglobulin was used as endogenous control for normalization. The data represent mean  $\pm$  S.E. of duplicates ( $n=3$ ).  $P$  value for TNF- $\alpha$ , IL-6 and IL-1 $\beta$  was calculated separately by comparing with corresponding mock control.  $P < 0.05$  was considered as significant.

washing, plates were incubated with biotin conjugated anti-TNF- $\alpha$  or anti-IL-6 or anti-IL-1 $\beta$  antibody followed by incubation with streptavidin coupled to HRP. The HRP activity was detected using a chromogenic substance *o*-phenylenediamine tetrahydrochloride (Sigma) in citrate-phosphate buffer (pH 5.4) and  $\text{H}_2\text{O}_2$ . The reaction was terminated using 1 N  $\text{H}_2\text{SO}_4$ , and the absorbance values was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

#### Real time PCR

RAW264.7 cells were cultured in the absence or presence of the JHP0940 protein in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C for 6 h. Following incubation, cells were harvested. For RT-PCR of cytokines, mRNA was extracted from the stimulated cells by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's recommendation. Real-time PCR was performed on a MyiQ Icyler (BioRad), using SYBR Green SuperMix. Quantification of various cytokine levels were performed by the Ct method and normalized by  $\beta$ -2 microglobulin level.

#### Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out for checking cell viability of mouse macrophage RAW cells in 96 well culture plate using different concentration of JHP0940 protein. The cells were seeded at a density of  $5 \times 10^3$  cells per well in 200  $\mu\text{l}$  of complete medium followed by protein treatment for 24 h. 20  $\mu\text{l}$  of 5 mg/ml MTT per well was added followed by 3 h incubation in dark or until the colour of the formazan product developed. Culture medium was removed and 50  $\mu\text{l}$ /well DMSO was added followed by 5 min incubation in dark. The plate was measured at 570 nm.

#### Ladder assay

Ladder assay was performed according to method described by Gong et al. (1994) with minor modifications. Approximately one million RAW 264.7 cells were treated with JHP0940 protein (10  $\mu\text{g/ml}$ ) for 48 h. The cells were trypsinized and suspended in HBSS (Hank's buffer salt saline) solution and prefixed in ice cold 70% ethanol, stored in deep freezer for 24 h before the assay. After fixing, the cells were centrifuged at  $800 \times g$  for 5 min to remove ethanol and DNA was extracted with 0.2 M phosphate-citrate buffer

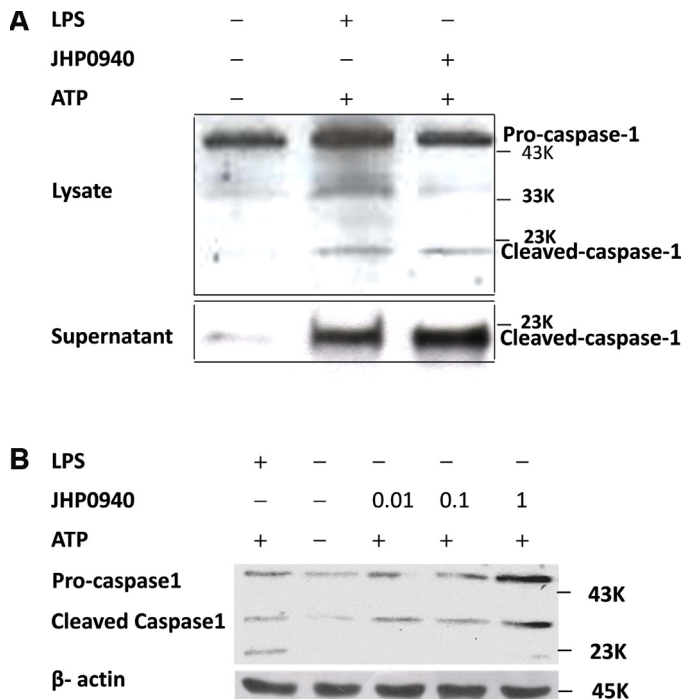


**Fig. 3.** Induction of caspase-1 in mouse macrophage RAW264.7 cell line on treatment with JHP0940: (A) Activation of caspase 1 was detected in whole cell lysate and in culture supernatants after priming either with JHP0940 (1  $\mu$ g/ml) or LPS (as positive control) for 3 h followed by activation with 5 mM ATP for 20 min. (B) Dose dependent activation of caspase-1 in the cells pre-treated with JHP0940 and LPS for 3 h followed by 5  $\mu$ M ATP treatment for 20 min. JHP0940 was used in varied doses of 0.01, 0.1 and 1  $\mu$ g/ml for 3 h. LPS (positive control) was used at 100 ng/ml concentration. (K: molecular weight in kilodaltons).

(PCB) at pH 7.8 by suspending the pellet in 40  $\mu$ l of PCB and incubating at room temperature for 30 min. Under these conditions, the partially degraded, oligonucleosomal DNA could be extracted selectively from the cells whereas the higher molecular weight DNA stayed associated with the nuclei. The extract (supernatant) was centrifuged at 1000  $\times$  g for 5 min and were treated with 1% Nonide NP40 (in water), 3  $\mu$ l RNase A (5 mg/ml in water) and incubated at 37  $^{\circ}$ C for 30 min. 6  $\mu$ l Proteinase K was added to it and incubated at 37  $^{\circ}$ C for 30 min. DNA loading dye was added to these samples and loaded to the 1.2% agarose gel with 0.5% EtBr. The gel was run at 4 V/cm and the bands were visualized.

#### Confocal microscopy

Confocal microscopy was used for visualization of DAPI stained nucleus of apoptotic cells. RAW264.7 cells were seeded at a density of  $0.1 \times 10^6$ /well on autoclaved coverslips in 24 well culture plate followed by protein treatment for 48 h. The cells were fixed in 4% paraformaldehyde and incubated for 15 min. After 3 washes with PBS the cells were permeabilized with pre-chilled 0.2% Triton-X followed by 15 min incubation at room temperature. DAPI with anti-fade reagent was added and the cells were mounted on slide followed by confocal microscopy for observing degraded nucleus.

For TUNEL assay,  $0.05 \times 10^6$  cells/well were seeded followed by protein treatment for 48 h. The cells were fixed and permeabilized as mentioned above. After washing, the cells were equilibrated for 10 min at room temperature with equilibration buffer provided with ApoAlert DNA Fragmentation Assay Kit (Clontech). This was followed by tailing reaction in TdT incubation buffer according to the manufacturer's protocol. Tailing reaction was terminated followed by washing with PBS. Cells were stained using DAPI with

anti-fade reagent followed by observation under confocal microscope.

#### Flow cytometry (expression of fas receptors on RAW264.7 cells)

To determine expression of Fas receptors on RAW264.7 cells, cells were seeded followed by treatment with JHP0940 for different time intervals and in different doses. Cells were harvested by trypsinization very carefully to avoid loss of receptors, washed in PBS and suspended in wash buffer (PBS + 0.5% BSA) followed by incubation with FITC-conjugated anti-mouse CD-95 monoclonal antibody for 30 min at 4  $^{\circ}$ C. For isotype control, cells incubated with FITC-conjugated mouseIgG1 were used. The incubation was followed by washing of the cells with the wash buffer. The cells expressing Fas receptor were detected by flow cytometry analysis on BD FACS Canto II (BD Biosciences) and analysed by FlowJo software (FlowJo).

#### Western blotting

Equivalent amount of proteins were separated on SDS-PAGE and transferred electrophoretically to PVDF membranes. The blots were blocked with 4% BSA followed by overnight incubation with primary antibodies such as anti-CagA (Conradi et al., 2012) and various commercial antibodies (NEB and Santa Cruz) at 4  $^{\circ}$ C in TBST (0.1% v/v) and 2% (w/v) BSA. The blots were washed and incubated with secondary HRP antibody in 2% (w/v) BSA for 1 h at room temperature. After washing with TBST the blots were developed with enhanced chemiluminescence reagent and exposed to X-ray film.

#### In vitro tyrosine kinase assay

Kinase reactions were carried out in 25  $\mu$ l of volume consisting of 5  $\mu$ l 2 $\times$  Assay buffer (2 mM DTT, 2 mM  $\text{MnCl}_2$ ,  $\text{Na}_3\text{VO}_4$ ), 5  $\mu$ l of 250  $\mu$ M ATP solution, 5  $\mu$ l of Src substrate (1 mg/ml), a synthetic peptide with sequence-KVEKIGEGTYGVVYK and 1  $\mu$ l Src or c-Abl enzymes (NEB) at 30  $^{\circ}$ C for 15 min as described (Lind et al., 2014). Both Src substrate and Src enzyme were replaced by JHP0940 in the test reactions for showing auto phosphorylation activity of the JHP0940. Then 25  $\mu$ l ADP-Glo reagent was added to deplete unused ATP, plate was shaken and allowed 40 min incubation at room temperature. 50  $\mu$ l kinase detection reagent was added and incubated for 30 min at room temperature followed by reading in luminometer (Promega).

#### Statistical analysis

Two tailed Mann-Whitney *U* test was performed for the humoral response in *H. pylori* infected patient v/s healthy control cohorts and *P* values were calculated at 95 per cent confidence level. Student's *t*-test was performed for the analysis of results wherever required. The data were expressed as the mean of triplicates  $\pm$  SEM unless or otherwise mentioned. *P* < 0.05 was considered as significant.

#### Results

##### *JHP0940 is likely presented to the immune system in H. pylori infected patients*

Earlier observations of our group showed that JHP0940 is associated with gastric disease in patients from different regions of the world and that enhanced activation of NF- $\kappa$ B occurs in cultured macrophages (Rizwan et al., 2008). This prompted us to investigate if the protein is expressed by *H. pylori* in gastric

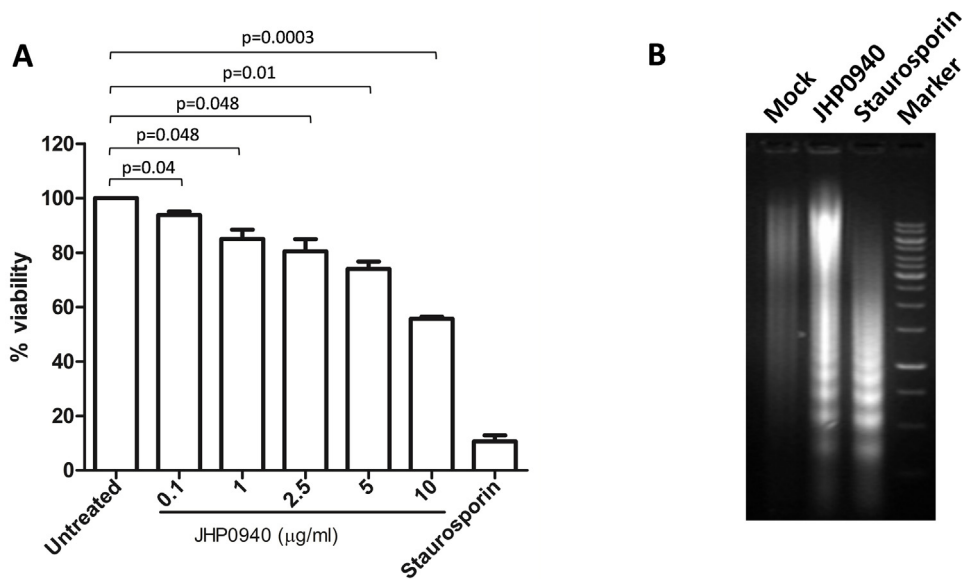


**Fig. 4.** (A) MTT assay of RAW264.7 cells after 24 h of treatment with different concentration of JHP0940 indicated dose dependent decrease in viability. The data are represented as means  $\pm$  S.E. ( $n = 3$ ).  $P$  value for different treatment experiments was calculated by comparing with untreated control.  $P < 0.05$  was considered as significant and  $P < 0.01$  as highly significant. (B) Ladder assay: The fragmentation of DNA of RAW264.7 cells on treatment with JHP0940 (10  $\mu$ g/ml) was found similar to RAW264.7 cells treated with staurosporin as positive control. In mock (untreated RAW264.7 cells), DNA fragmentation was not observed. (Marker: 1Kb DNA ladder).

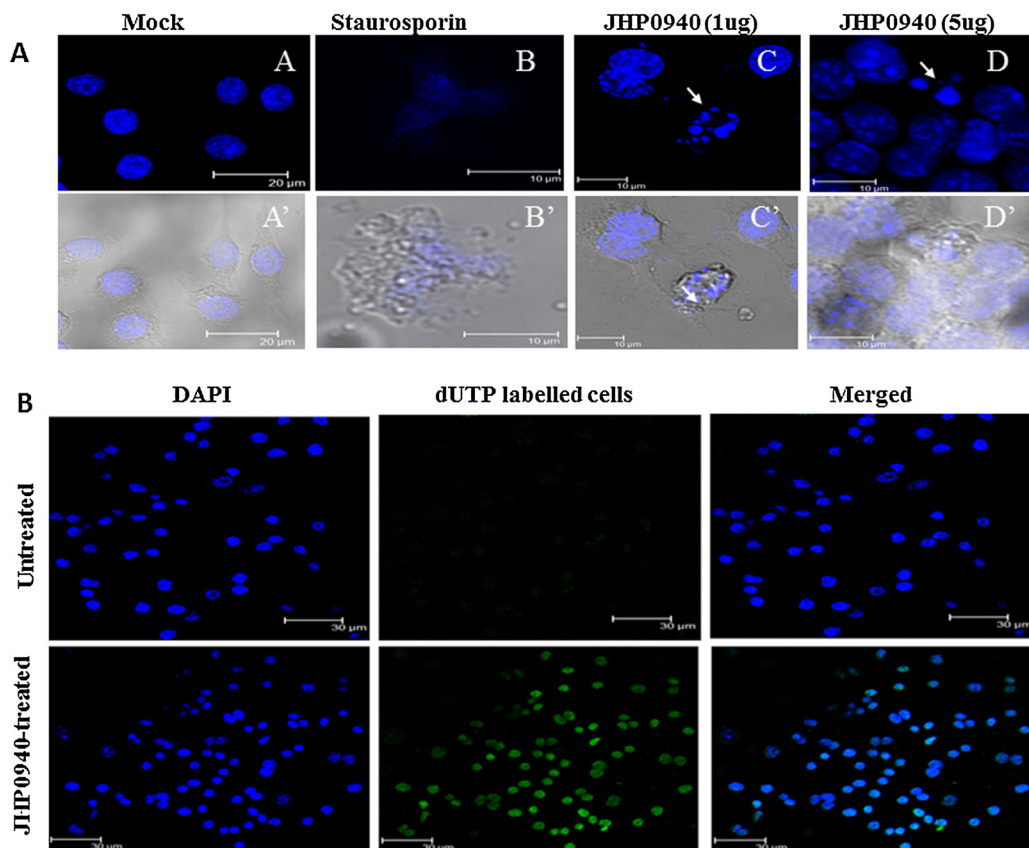


**Fig. 5.** (A) Confocal microscopy images indicating apoptosis of RAW264.7 cells on treatment with JHP0940 protein, after 48 h. Arrows in the images show apoptotic bodies. The RAW264.7 cells upon treatment with 1  $\mu$ g/ml and 5  $\mu$ g/ml of JHP0940 protein resulted in apoptosis as compared to the cells treated with staurosporin (0.1  $\mu$ g/ml) used as positive control. No apoptosis was observed in the mock experiment (untreated cells). (B) TUNEL assay showing apoptosis in RAW264.7 cells upon treatment with JHP0940 protein after 48 h. Fluorescence-labelled dUTP in the fragmented DNA of cells undergoing apoptosis was observed in cells treated with JHP0940 but such fluorescence was not observed in mock (untreated cells). The figures shown are representative of three independent experiments.

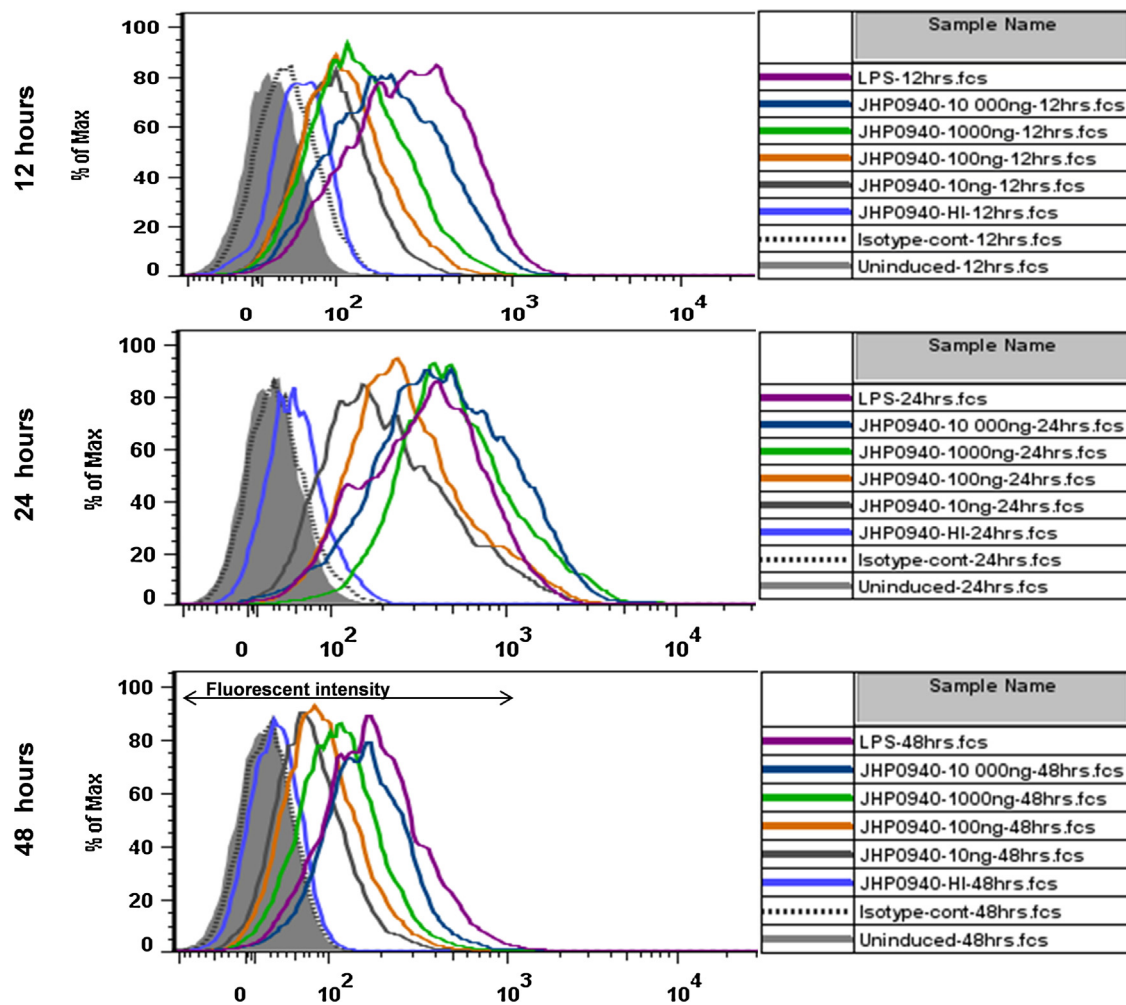


**Fig. 6.** Flow cytometry analysis showing expression of Fas receptors on RAW264.7 cells treated with JHP0940 in a dose dependent manner at 12, 24 and 48 h. The shift in the histogram peaks was observed in accordance with increase in concentration of JHP0940. RAW264.7 cells treated with LPS also indicated shift in histogram peak as compared to untreated. The shift in histogram peaks indicates increased expression of Fas receptors with respect to untreated.

environment and/or presented to the immune system. Since *H. pylori* colonizes the gastric epithelium, the bacterium should express some of its proteins/factors for gaining persistence while modulating the immune responses of the host. In this context, expression and exposure of factors to the immune system becomes important. To test this idea, we expressed and purified JHP0940 to homogeneity (Fig. 1A) and performed ELISA experiments with immobilized JHP0940 protein and human serum samples of *H. pylori* infected patients as compared to sera from non-infected healthy individuals. A significant difference in *P* value (0.0004) between *H. pylori* infected patients versus healthy group was found (Fig. 1B). This suggests that the protein expresses and presented to the immune system leading to the presence of anti-JHP0940 antibodies in the host.

#### *JHP0940 is expressed in a subset of H. pylori strains and is secreted into the culture supernatant*

Before its being presented to the immune system, we aimed to investigate if the Ctk protein is secreted into the extracellular environment. We tested this idea by fractionation studies of various wild-type *H. pylori* isolates including *ctk* gene-positive (J99 and SJM180) and *ctk*-negative (P1) strains, which were incubated for 12 h in liquid broth. Western blotting experiments showed that Ctk protein is expressed in strains J99 and SJM180, except P1 (Fig. 1C or

D). In addition, bands for Ctk were also found in the culture supernatant fraction. As a control, the type IV secretion effector protein CagA, used as loading control for the *H. pylori* cell pellets (Fig. 1C) was not found in the supernatant as expected (Fig. 1D), thereby excluding artificial lysis of bacteria during experiments. Given this, our results hint at the secretory nature of the protein, allowing us to study how JHP0940 might affect signal transduction events during interaction with macrophages.

#### *JHP0940 induces pro-inflammatory cytokines in RAW264.7 macrophage cells*

RAW264.7 cells were grown and treated with recombinant JHP0940 protein at a concentration of 10 ng to 1000 ng/ml for 24 h followed by ELISA-based quantitation of cytokines in the culture supernatant. We found that the cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were upregulated in a dose dependent manner (Fig. 2A). Furthermore, to confirm the pro-inflammatory properties of JHP0940, qRT-PCR was performed for mRNA profiling of the above cytokines (Fig. 2B). Our results from qRT-PCR corroborated with the ELISA results confirming the upregulation of the above cytokines (Fig. 2B). *H. pylori* infection is recognized by the presence of chronic gastric inflammation and there is increasing evidence that caspase-1 is involved in the activation of the two important pro-inflammatory cytokines (IL-1 $\beta$  and IL-18), which normally mediates bacterial

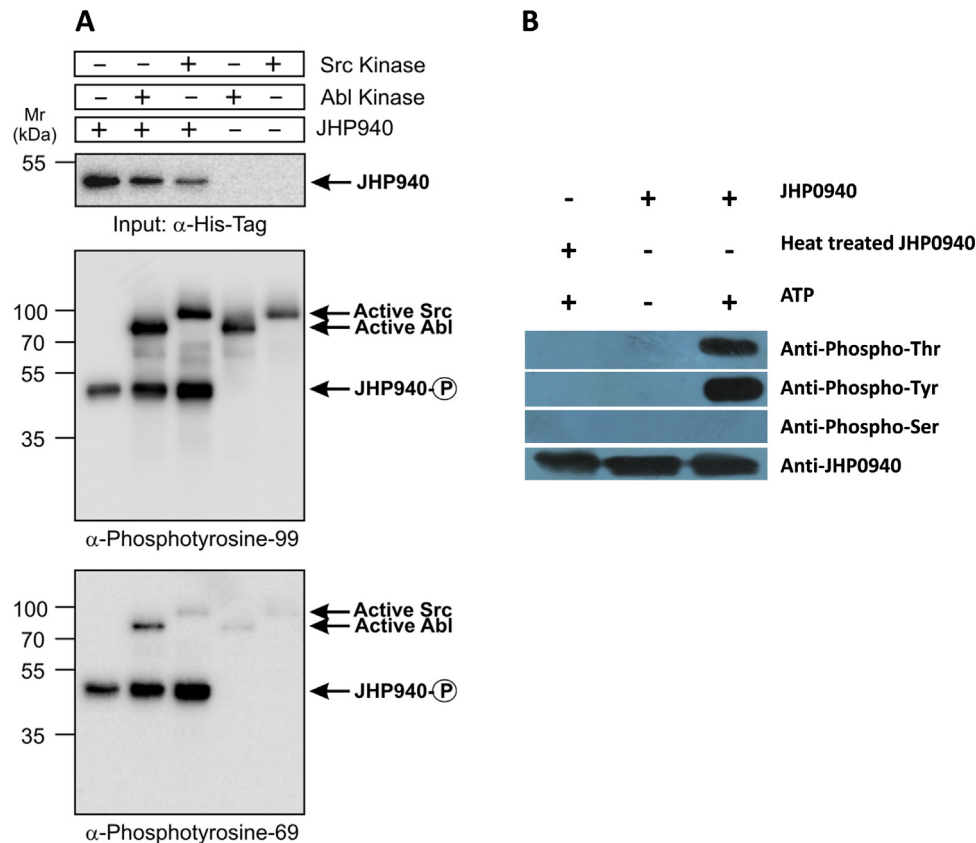


**Fig. 7.** (A) Western blot showing auto-tyrosine phosphorylation of JHP0940. Lane 1, from left represents kinase reaction with only JHP0940, lane 2 and 3 represent kinase reactions using JHP0940 along with Abl and Src kinase respectively. Lane 4 and 5 represent *in vitro* kinase reactions only with Abl and Src kinase respectively. Phosphorylation at tyrosine residues was probed using  $\alpha$ -phosphotyrosine-99 and  $\alpha$ -phosphotyrosine-69 antibodies. JHP0940 was probed with anti-His-tag antibody. (B). JHP0940 acts as auto phosphorylating kinase. *In vitro* kinase reactions were performed and phosphorylation at tyrosine and threonine residues was determined by respective anti-tyrosine and anti-threonine antibodies.

clearance after infection (Hitzler et al., 2012; van de Veerdonk et al., 2011). In this regard, we investigated the involvement of JHP0940 in caspase-1 induction. We found that caspase-1 expression was induced upon treatment of RAW264.7 cells with recombinant JHP0940. We observed that JHP0940 induces caspase-1 processing and activation in a dose dependent manner (Fig. 3B). We also observed that active caspase-1 is secreted into the cell culture supernatant (Fig. 3A). This observation partly corroborates with the above observation related to IL-1 $\beta$  secretion by murine macrophage cells that were treated with JHP040. Given these findings, we espouse the hypothesis that JHP0940 could most likely contribute to *H. pylori* pathogenesis by producing chronic gastric inflammatory responses and consequently ensuring *H. pylori* colonization. In our experiments, every time before treating the cells, JHP0940 protein was incubated with polymyxin B for 4 h to remove putative endotoxin contamination and to rule out the effect of bacterial endotoxin. Furthermore, we used proteinase K treated JHP0940 as control to ensure that the effect was not due to bacterial endotoxins.

#### JHP0940 decreases viability of RAW264.7 cells by inducing apoptosis through Fas receptors

Persistence of *H. pylori* in the gastric environment is the hallmark of this pathogen, not allowing the immune system to clear infection. The role of putative bacterial proteins/factors in immune evasion and related processes, however, is not yet fully clear. We therefore hypothesized that JHP0940 could induce caspase-1 activation to trigger macrophage apoptosis, thus facilitating

enhanced bacterial survival. We therefore investigated the viability of RAW264.7 cells in response to exposure with JHP0940 protein. We observed that the protein induced cell death in these cells in a dose dependent manner leaving 56% of viable cells at 24 h upon treatment with 10  $\mu$ g/ml of JHP0940. The cell viability was accessed by MTT assay for 24 h in a dose dependent manner (Fig. 4A).

Cell viability assays performed with RAW264.7 cells intrigued us to identify the cause of cell death and therefore, we performed the ladder assay. We observed that JHP0940 induced DNA fragmentation similar to the staurosporin control, explaining its role in apoptosis (Fig. 4B). To confirm these observations, we investigated the nuclear morphology of RAW264.7 cells by staining with DAPI using confocal microscopy. Our results revealed that JHP0940 induced degradation of chromosomal DNA in RAW264.7 cells (Fig. 5A). Furthermore, these observations were corroborated by TUNEL assay results (Fig. 5B). TUNEL assay revealed that most of the cells undergo apoptosis. These results were further confirmed by expression of Fas receptor on the surface of JHP0940-treated RAW264.7 cells in a time- and dose-dependent manner (Fig. 6). The expression of Fas receptors on murine macrophages intrigued us to test if JHP0940 acts as non-receptor tyrosine kinase. There are evidences where c-Abl non-receptor tyrosine kinases are involved in expression of Fas receptors (Gu et al., 2012).

#### JHP0940 acts as an auto-phosphorylating tyrosine kinase

In a previous study, it was shown that JHP0940 acts as cell-translocating ser/thr kinase (CtkA) and leads to phosphorylation

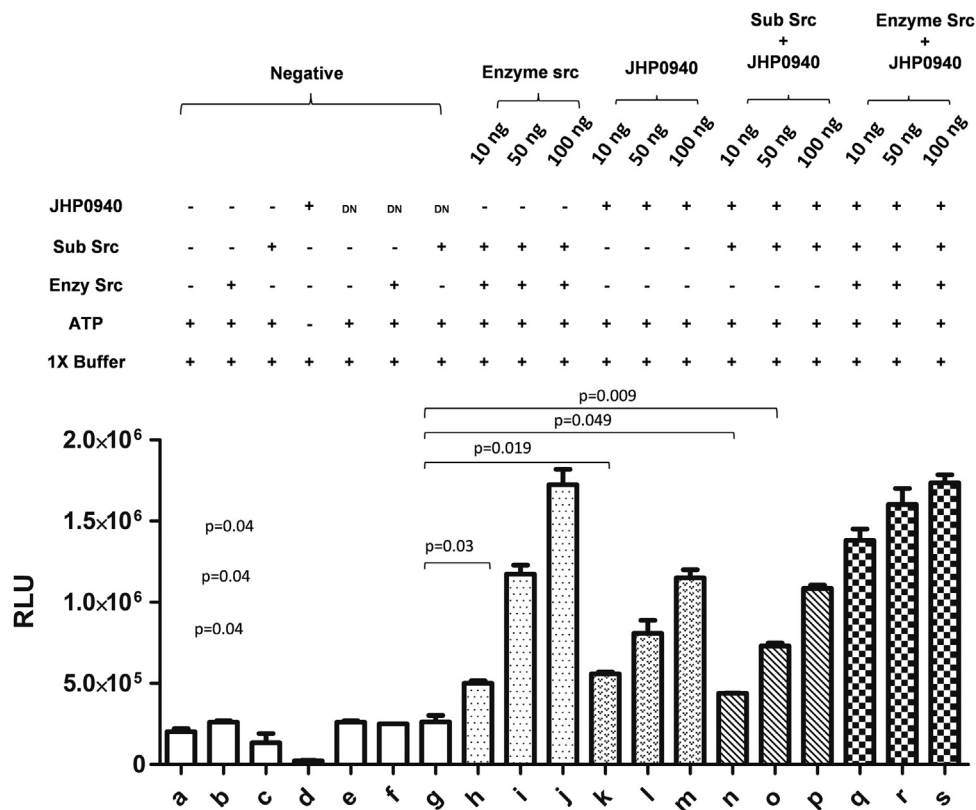


**Fig. 8.** *In vitro* tyrosine kinase assay. On X-axis, a to g are negative controls while h, i and j are reactions with increasing concentrations of Src enzyme (10, 50 and 100 ng) serving as positive controls. k, l and m are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) only. n, o and p are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src substrate (5 µg/reaction, the same concentration was maintained throughout the experiment). q, r and s are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src enzyme (50 ng/reaction). Y-axis represents relative light units (RLU). The data are represented as mean ± S.E. of three independent experiments. DN represents denatured protein JHP0940. P values have been indicated in comparison with test and control samples.

at serine residue 276 residue of p65 subunit of NF-κB in cultured AGS gastric epithelial cells (Kim do et al., 2010). But, the association of the gene jhp0940 with gastric cancer (Santos et al., 2003) intrigued us to check if it can also act as a tyrosine kinase and hence we performed *in vitro* tyrosine kinase assay. In our results, JHP0940 was found to be an auto phosphorylating tyrosine kinase apart from its described serine/threonine kinase activity on western blot analysis (Fig. 7). In an *in vitro* tyrosine kinase assay, JHP0940 was used in enzyme range concentrations in the kinase reaction and when it was used along with the enzyme provided with the kit, it revealed cumulative enzymatic activity (Fig. 8).

Discussion

Putative virulence-associated genes of *H. pylori* have been categorized into three groups: strain specific genes, phase variable genes and genes with different structures and phenotypes (Yamaoka, 2008). The strain specific genes are further sub-classified into those encoded by two pathogenicity islands, the cagPAI, which encodes various virulence factors along with its T4SS and the effector protein CagA and plasticity region clusters (called plasticity zones) encoding nearly half of the strain-specific genes including another T4SS, called tfs3 (Kersulyte et al., 2003; Fernandez-Gonzalez et al., 2014). These genes within the plasticity region are seemingly acquired extraneously and are of utmost interest because of their association with the gastric disease (Yamaoka, 2008). In previous studies, various genes of the plasticity region i.e., dupA, jhp0940, jhp0945, jhp0947 and jhp0949 have been shown to be associated with various stages of gastric disease (Yamaoka,

2008). The first characterized member from this region, JHP0940 or CtkA, intrigued us to explore it in detail due to pre-existing observations such as its association with gastritis and gastric cancer in different geographical regions, high antigenicity index and *in vivo* expression in gastric mucosa of Mongolian gerbils (Graham et al., 2002). Furthermore, the mechanism of secretion of JHP0940 from *H. pylori* and its transfer to the gastric epithelial cells is not fully clear. It has also not been confirmed if JHP0940 uses T4SS-mediated injection mechanism like CagA or it harnesses some other secretory mechanism similar to other Gram-negative bacteria. However, our ELISA results portray it as a secretory protein (Fig. 1). The cell-translocating activity of the protein has been shown using GFP-fused full length recombinant protein in HeLa cells (Kim do et al., 2010). Regarding the pro-inflammatory nature of the protein, we do not rule out the involvement of kinase activity of JHP940 in IL-1β secretion since there are reports where death associated protein kinase, a type of serine/threonine kinases is required for IL-1β production (Chuang et al., 2011). But, at the same time, non-receptor tyrosine kinase members of the c-Abl family are also shown to involve in T-cell mediated inflammation (Gu et al., 2012). It is well established that IL-1β plays a crucial role in inflammation and maintenance of infection (Dinarello, 2009). Subsequent to NF-κB activation, pro-IL-1β is synthesized in its pro-form, which is then cleaved by the action of caspase-1 to produce mature IL-1β. It is reported that the activation of caspase-1 is dependent on formation of multi-protein complexes, called inflammasome (Martinon et al., 2009; Dinarello, 2009; von Moltke et al., 2013). Recent studies identified the bacterial cag pathogenicity island and the cooperative interaction among host innate receptors TLR2, NOD2, and NLRP3

as important regulators of IL-1 $\beta$  production in *H. pylori* infected murine dendritic cells (Kim et al., 2013). Here, our study by Western blot analysis of caspase-1 revealed its involvement in secretion of IL-1 $\beta$  (Fig. 3) and therefore, we propose a strong pro-inflammatory role for Ctk, with the involvement of inflammasome components, at least in murine macrophage cells.

In order to examine the effects of recombinant JHP940 on mouse macrophage cells in more detail, we observed decreased viability of host cells by MTT assay. This was followed by the visualization of apoptotic bodies in the DAPI-stained nucleus. Furthermore, the TUNEL assay confirmed occurrence of apoptosis in response to treatment of the cells with JHP940 protein. In a previous *in vitro* study, translocation of GFP-tagged JHP940 into HeLa cells was documented, indicating its cell-translocating behaviour (Kim et al., 2010). The authors also found that transiently expressed protein translocates into the nucleus in a time dependent manner in AGS cells, although the protein doesn't contain a nuclear localization signal (NLS). These observations together with our findings based on an *in vitro* non receptor tyrosine kinase assay and its apoptotic effect on mouse macrophage cell line through Fas expression (death receptor) revealed that the activity of JHP940 mimics the function of a non-receptor tyrosine kinase such as c-Abl. Upon caspase activation, c-Abl retained tyrosine kinase activity and triggered Fas induced apoptosis as previously shown (Barila et al., 2003). The Fas receptor activated by specific ligands, triggers assembly of a complex called as Death Inducing Signalling Complex (DISC) leading to the cleavage and activation of more downstream executive caspases and thus apoptosis (Raina et al., 2005). Moreover, epithelial and endothelial non-receptor tyrosine kinases (Etk) mediate apoptosis in small cell lung cancer cells (Cheng et al., 2010). We also found enhanced expression of Fas receptors in the mouse macrophage cell line in a time and dose dependent manner (Fig. 6).

Somehow, JHP940 does not share any sequence homology with c-Abl tyrosine kinases in any of the databases but it is predicted as cytoplasmic protein (score of 8.96 out of 10) by PSORTb v3.0.2 prediction tool (Gardy et al., 2003). The protein was found to have one SH2 domain when searched for domains in Scansite Motif Scanner database (<http://scansite.mit.edu/motifscan.seq.phtml>). Also, it revealed homology with one of the SH2 domains of CrkII. This SH2 domain of CrkII serves as binding site for SH3 domain of c-Abl mediated by Crk phosphopeptide and thus regulates biological functions (Donaldson et al., 2002). Despite homology with SH2 domain of CrkII, there is no experimental evidence to show that JHP940 acts as an adaptor molecule like CrkII.

Other encoded protein from the plasticity region of *H. pylori* strain 26695, HP0986 (TieA), showed the same pro-inflammatory and pro-apoptotic behaviour but tested negative for kinase activity. However, HP0986 interacted with TNFR1, also having an endonuclease activity hinting at the potential pathobiological role of proteins/factors encoded by plasticity region genes (Alvi et al., 2011; Devi et al., 2014).

It will not be surprising that JHP940, acting as an autophosphorylating tyrosine kinase and by translocating into the nucleus activates transcription factor(s) apart from NF- $\kappa$ B to modulate signalling in the host cells. It hints that JHP940 may be a secretory protein, though the secretory mechanism of the protein is not established, which bacteria could use in modifying the signalling responses of the host cells. Thus, on the basis of our above observations and results, we assume that on one hand JHP940 helps *H. pylori* to orchestrate inflammation as a survival mechanism and on the other hand it averts immune recognition by limiting macrophages through Fas mediated apoptosis. Further, it will be pertinent to investigate if JHP940 acts as dual specific kinase or it acts as serine/threonine or tyrosine kinase in certain specific

conditions and or cellular environment to help *H. pylori* in progression of gastric disease.

## Acknowledgements

We would like to thank P. Sandhya Rani for inputs during manuscript editing. We acknowledge funding support from the Department of Biotechnology, Government of India (BT/PR6921/MED/29/699/2013). ST would like to acknowledge University Grants Commission, India for the award of Senior Research Fellowship. SD would like to acknowledge partial support from the HIR project of the University of Malaya (UM.C/625/1/HIR/MOHE/CHAN-02-Molecular Genetics). NA is an Academy Professor of the Academy of Scientific and Innovative Research, India and Visiting Professor at the Institute of Biological Sciences, University of Malaya, Kuala Lumpur. The work of Steffen Backert, Judith Lind and Nicole Tegtmeyer is supported through a German Research Foundation grant (project B10 of CRC-796).

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