The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug resistant Enterobacteriaceae

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Abstract

Escherichia coli ST131and *Klebsiella pneumoniae* ST258 emerged in the 2000s as important human pathogens; have spread extensively throughout the world and are responsible for the rapid increase in antimicrobial resistance among *E. coli* and *K. pneumoniae* respectively. *E. coli* ST131 causes extra-intestinal infections, is often fluoroquinolone resistant and associated with Extend-spectrum β-lactamase production especially CTX-M-15. *K. pneumoniae* ST258 causes urinary and respiratory tract infections and is associated with carbapenemases most often KPC-2 and KPC-3. The most prevalent lineage within ST131 is named *fimH*30 because it contains the

H30 variant of the type 1 fimbrial adhesin gene and recent molecular studies have demonstrated that this lineage emerged in early 2000's and was then followed by the rapid expansion of its sublineages H30-R and H30-Rx. *K. pneumoniae* ST258 comprises of 2 distinct lineages namely clade I and clade II. Moreover, it seems that ST258 is a hybrid clone that was created by a large recombination event between ST11 and ST442. Epidemic plasmids with $bla_{\text{CTX-M}}$ and bla_{KPC} belonging to the incompatiblity group F have contributed significantly to the success of these clones. *E. coli* ST131 and *K. pneumoniae* ST258 are the quintessential examples of international multidrug-resistant high risk clones.

Overview

1. Introduction
2. Expanded-spectrum β-lactamases
    a. Overview
    b. CTX-M
    c. Plasmid-mediated AmpC
    d. KPC
    e. NDM
    f. OXA-48-like
3. International multidrug resistant high risk clonal lineages
4. *Escherichia coli* Sequence Type 131
    a. Initial studies pertaining to *E. coli* ST131
    b. Recent developments pertaining to *E. coli* ST131

c. Does *E. coli* ST131 qualify as an international multidrug high risk clone?

5. *Klebsiella pneumoniae* Sequence Type 258

   a. Initial studies pertaining to *K. pneumoniae* ST258

   b. Recent developments pertaining to *K. pneumoniae* ST258

6. Laboratory Methods for the detection of *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST258

   a. Multilocus sequecenc typing

   b. Repetitive sequence based PCR typing

   c. Multilocus Variable Number of Tandem Repeats Analysis

   d. Polymerase Chain Reaction

   e. Matrix Assisted Laser Description/Ionization Time of Flight Mass Spectrometry

7. Role of plasmids in High Risk Clones

   a. Overview

   b. Plasmids associated with *E. coli* ST131

   c. Plasmids associated with *K. pneumoniae* ST258

   d. Broad host range plasmids (i.e. IncA/C, IncL/M, IncN)

6. Conclusion

Introduction

The Enterobacteriaceae, especially *Escherichia coli*, and *Klebsiella pneumoniae*, are common causes of nosocomial and community infections among humans (1). One of the most urgent areas of antimicrobial drug resistance is the rapid evolution of fluoroquinolone, cephalosporin and carbapenem resistance among Enterobacteriaceae which has spread globally during the last decade. Recently, the World Health Organization (WHO) released a report entitled: "Antimicrobial resistance: global report on surveillance 2014" (2). This report focused on antibiotic resistance among bacteria responsible for common, serious infections including bloodstream infections (BSIs), urinary tract infections (UTIs) and intra-abdominal infections (IAIs). It states that resistance to certain antibiotics among frequently isolated bacteria are rife in certain parts of the globe.

For *E. coli* the report states the following (2): "Resistance to one of the most widely used antibacterial medicines (i.e. the fluoroquinolones) for the treatment of UTIs caused by *E. coli* is very widespread. In the 1980s, when these drugs were first introduced, resistance was virtually zero. Today, there are countries in many parts of the world where this treatment is now ineffective in more than half of patients". Specifically for *K. pneumoniae* the WHO report states the following (2): "Resistance to the last resort agents (i.e. carbapenem antibiotics) for the treatment of life-threatening infections caused by a common intestinal bacteria, *K. pneumoniae* has spread to all regions of the world. *K. pneumoniae* is a major cause of hospital-acquired infections such as pneumonia, BSIs, infections in newborns and intensive-care unit patients. In some countries, because of resistance, carbapenem antibiotics would not work in more than half of people treated for *K. pneumoniae* infections".

The global dissemination of drug resistant organisms is troublesome for the medical practitioners because it decrease the available options for appropriate treatment. This contributes to increased patient mortality and morbidity (3). Therefore, it is not surprising that the global spread of drug resistance was recently recognized as a major threat to human health (4). Antimicrobial resistance endangers some of the accomplishments of modern medicine e.g. in a world without antibiotics it would be difficult to provide effective chemotherapy to cancer patients since most will die due to opportunistic multidrug resistant infections.

This pandemic comes at a time when Moore's law has enabled the analysis of large amounts of sequencing data enabling molecular epidemiologists to study antimicrobial resistance (5). The analysis of such data will enable bioinformaticians to determine the global dissemination of clones and plasmids responsible for antimicrobial resistance in ways that were not feasible ten years ago. Hopefully such new insights can yield urgently needed clues on how to prioritize tactics for limiting the global spread of multidrug resistant Enterobacteriaceae.

An 'eminent or successful' bacterial clone is a powerful source for the propagation of antimicrobial resistant genetic components (i.e. genes, integrons, transposons and plasmids) (6). They are able to provide stable platforms for the maintenance and propagation of genes responsible for antimicrobial resistance and had played an essential role in the recent global emergence of multidrug resistance among Gram negatives especially the Enterobacteriaceae.

Plasmids are extra chromosomal elements of circular DNA present in bacteria, which replicate independently of the host genome. The horizontal transfer of plasmids containing resistance genes is an essential mechanism for the dispersion of antimicrobial resistance (7). This free movement of plasmid encoded genes responsible for drug resistance has also been central to the recent and rapid global increase in antimicrobial resistance (8). Antimicrobial resistance

plasmids can broadly be divided into 2 main groups namely the narrow-host range group that most often belongs to the incompatibility (Inc) group F and the broad-host range group that belongs to the IncA/C, IncL/M and IncN. Broad-host range plasmids can easily be transferred between different species while narrow-host range plasmids tend to be restricted to species (9). Epidemic resistance plasmids belonging to IncF with divergent replicon types (e.g. FIA, FIB and FII) have the ability to acquire resistance genes and then rapidly disseminate among Enterobacteriaceae, especially among certain clones within species (9).

The focus of molecular epidemiologists when investigating antimicrobial resistant bacteria had been on analyzing chromosomal DNA, because the initial spread of successful clones did not necessarily require the in depth investigation of the role that plasmids had played in the dissemination of drug resistance genes. Molecular and bioinformatics tools for analyzing large amounts of plasmid DNA combined with refined techniques for large scale chromosomal analysis will likely provide a better understanding of the ceaseless movement of drug resistant genes throughout the microbial world (5).

The pandemics caused by multidrug resistant *E. coli* and *K. pneumoniae* (including fluoroquinolone, cephalosporin and carbapenem resistant isolates) are mostly due to the global dissemination of certain high risk clones namely *E. coli* ST131 and *K. pneumoniae* ST258. There is a strong relation of *E. coli* ST131 and *K. pneumoniae* ST258 with IncF epidemic plasmids containing FIA and FII replicon types. The reasons for the particular success of these high risk clones and their association with certain epidemic resistance plasmids are uncertain. However, their ability to spread swiftly is beyond dispute.

This review will provide insights on how antibiotic resistance evolves and spreads in bacterial populations and will attempt to highlight recent information about the continuous

interplay between bacterial clones and antimicrobial resistance plasmids. This article will expand the knowledge regarding the role, the importance and interdependence of high risk clones and epidemic plasmids in the global spread among multidrug resistant Enterobacteriaceae. It will also provide an overview on rapid laboratory methods that will aid clinicians and infection control measures in combating the spread of high risk clones.

Expanded-spectrum β-lactamases

Overview

Antibiotic therapy with cephalosporins that contain the oxyimino-side chain (i.e. cefotaxime, ceftazidime, ceftriaxone, and cefepime) is considered as one of the choice treatments for serious infections due to *Klebsiella* spp. and *E. coli*. Moreover, carbapenem resistant *E. coli* and *Klebsiella* spp is troublesome to medical physicians, since these drugs are often the last efficient treatment left for serious infections (1).

β-lactamases are the most common cause of resistance to various β-lactam agents. Several different schemes have traditionally been used for the classification of β-lactamases. These enzyme can be divided into either classes A, B, C and D (referred to as the molecular or Ambler classification based on amino acid sequences) or groups 1, 2 and 3 (referred to as the functional or Bush Jacoby classification based on substrate and inhibitor profiles). Classes A, C, and D β-lactamases are serine enzymes while the class B requires divalent zinc ions for its activity (10). Group 1 is referred to as serine cephalosporinases, group 2 as serine β-lactamases that includes penicillinases and broad-spectrum enzymes while group 3 is also called metallo-β-lactamases (MBLs) (10).

For the sake of simplicity we will use the term "expanded-spectrum" β-lactamases in this review article to stipulate those enzymes with activity against the cephalosporins with oxyimino side chain and/or the carbapenems. These enzymes consist of the class C plasmid-mediated or imported Amp C β-lactamases (e.g. CMY types), class A extended-spectrum β-lactamases [ESBLs] (e.g. CTX-M, SHV and TEM types), and carbapenemases (e.g. KPC types (class A), MBLs e.g. VIM, IPM, NDM types (class B), and the oxacilinases e.g. OXA-48-like enzymes) (class D). The production of expanded-spectrum β-lactamases causes non-susceptibility to various β-lactam agents and Enterobacteriaceae with these enzymes are often co-resistant to a variety of other classes of antibiotics. The most common global type of ESBL are the CTX-M-β-lactamases while the NDM, OXA-48 and KPC are the most frequent carbapenemases among nosocomial and community isolates of Enterobacteriaceae (11). Infections with ESBL and carbapemase-producing Enterobacteriaceae in the developed world are sometimes associated with travel to an endemic region (12). The features of the expanded-spectrum β-lactamases are outlined in the Table 1.

CTX-M β-lactamases

Extended-spectrum β-lactamases or ESBLs provide resistance to most of the β-lactam drugs, and are inhibited by certain β-lactamase inhibitors (13) (for a summary on which Enterobacteriaceae produce these enzymes, the classification, spectrum of activity, inhibition properties, types, endemic regions and molecular epidemiology of ESBLs, please refer to Table 1). The SHV or TEM types of ESBLs were common during the 1980s and 1990s (13), while the CTX-Ms became prominent since 2000 (14). Currently the CTX-M enzymes are present in various types of bacteria from all continents, being especially frequent in *E. coli* (15).

The CTX-Ms belong to the molecular class A or functional group 2be β-lactamases, and includes at least six lineages (i.e. CTX-M-1-like, CTX-M-2-like, CTX-M-8-like, CTX-M-9-like, CTX-M-25-like and KLUC-like) that differ from each other by ≥10% amino acid homology (14). The association of plasmids with $bla_{\text{CTX-M}}$ that belong to incompatibility groups IncF, IncN and IncK types, with certain insertion sequences (IS) (e.g. IS*Ecp1* or IS*CR1*) are able to capture and mobilize $bla_{\text{CTX-M}}$ genes effectively among members of the Enterobacteriaceae. IS elements can also act as strong promoters for the high-level expression of $bla_{\text{CTX-M}}$ (16) (Table 1). CTX-M-15 is the most universal type of ESBL among *E. coli* and has been associated with the presence of a clone named ST131 (17). It seems that this clone or sequence type was accountable for the global distribution and increase of *E. coli* with $bla_{\text{CTX-M-15}}$ during the early 2000s (more details are provided in the high risk clone section).

E. coli with CTX-Ms are responsible for nosocomial and community UTIs, BSIs and IAIs (14) and risk factors include the preceding use of antibiotics and visiting to certain endemic regions (18). The world-wide dissemination of *E. coli* with CTX-Ms has been very efficient and involved the health-care settings, community, livestock, companion animals, wildlife and the environment (19). Studies have also shown high transmission rates of *E. coli* with $bla_{\text{CTX-Ms}}$ within households (20).

The CTX-M pandemic had significantly contributed to the rapid global increase of cephalosporin resistance among Enterobacetriaceae with subsequent increased usage of the carbapenems for the medication of infections due to these MDR bacteria. Unfortunately, CTX-M-producing *E. coli* are often co-resistant to various antibiotic classes that includes cotrimoxazole, the aminoglycosides and the fluoroquinolones (15). This has important clinical implications because some of these drugs (e.g. cotrimoxazole) are popular oral treatment options

for community-acquired uncomplicated lower UTIs. Fortunately, fosfomycin, mecillinam and nitrofurantoin retain sufficient activity against a high percentage of *E. coli* with $bla_{\text{CTX-Ms}}$ (16).

CTX-M-producing *E. coli* is an important component among global multidrug resistant bacteria and should be regarded as a major target for surveillance, infection control and fundamental investigations in the field of antimicrobial drug resistance (21).

AmpC β-lactamases or cephalosporinases

Enterobacteriaceae with AmpC β-lactamases are important causes of cephalosporin and cephamycin resistance. For a summary on which Enterobacteriaceae produce imported or plasmid-mediated class C cephalosporinases, the classification, spectrum of activity, inhibition properties, types, endemic regions and molecular epidemiology of plasmid-mediated AmpC β-lactamases please, refer to Table 1. Since high risk clones and epidemic plasmids do not play important roles in the global dissemination of AmpC enzymes, we refer the readers to some excellent review articles on this topic (22-24).

KPC β-lactamases

The most clinical significant of the class A carbapenemases are the KPC-types (i.e. *Klebsiella pneumoniae* Carbapenemase) (25) (for a summary on which Enterobacteriaceae produce these enzymes, the classification, spectrum of activity, inhibition properties, types, endemic regions and molecular epidemiology of KPCs, please refer to Table 1). KPC-2 and -3 are the most prevalent isoenzymes among KPCs and bacteria with these β-lactamases are non-susceptible to a variety of β-lactam drugs including the majority of β-lactamase inhibitor combinations (26). KPC enzymes are especially prevalent in *Klebsiella* spp. and to a lesser extent in *Enterobacter spp.* (25).

Several hospital outbreaks most often due to *K. pneumoniae* with bla_{KPC-2} and $bla_{KPC-3,}$ have been reported from North America (especially the USA), South America (Colombia, Argentina), Europe (Greece, Italy, Poland), Asia (China) and Middle East (Israel) (26-28). Endemic regions of KPC-producing bacteria are shown in Table 1 (28). *K. pneumoniae* ST258 with bla_{KPC-2} and bla_{KPC-3} had significantly contributed to the world-wide distribution of this resistance trait (more details are provided in the high risk clone section) (28).

Plasmids with bla_{KPC} in association with the mobile element Tn*4401* are responsible for the effective spread of these genes among different types of Enterobacteriaceae (29) and with other antibiotic resistance determinants on the same plasmid provide an easy mechanism for carbapenemase genes to effectively spread as hitchhiker genes, even in the absence of carbapenem selection (30). Enterobacteriaceae with bla_{KPC} are often multidrug resistant that includes classes such as the aminoglycosides, fluoroquinolones and cotrimoxazole (31).

NDM β-lactamases

A different kind of metallo-β-lactamase (MBL), designated NDM, was described in *K. pneumoniae* and *E. coli* from Sweden during the late 2000s. This patient was previously admitted in a New Delhi hospital in India (32) (for a summary on which Enterobacteriaceae produce these enzymes, the classification, spectrum of activity, inhibition properties, types, endemic regions and molecular epidemiology of NDMs, please refer to Table 1). Patients visiting certain high risk regions and then returning to their respective home countries with NDM producing bacteria have been described and these enzymes are some of the most common carbapenemases identified in countries such as Canada, the UK and France (11). Since high risk clones and epidemic plasmids do not seem to play important roles in the global dissemination of NDMs, we refer the readers to some excellent review articles and recent reports on this topic (11, 33-36).

OXA-48-like β-lactamases

The molecular class D ß-lactamases is commonly referred to as OXAs and comprises over 400 enzymes with some variants that possess carbapenemase activity (also referred to as carbapenem-hydrolysing class D ß-lactamases or CHDLs) (37). For a summary on which Enterobacteriaceae produce these enzymes, the classification, spectrum of activity, inhibition properties, types, endemic regions and molecular epidemiology of CHDLs in Enterobacteriaceae please refer to Table 1. CHDLs are common in *Acinetobacter* spp., but the OXA-48-like types of CHDLs are most often encountered among the Enterobacteriaceae (37, 38). OXA-163 is different from other OXA-48-like enzymes, in that this enzyme has the ability to hydrolyse the oxyimino-cephalosporins. Enterobacteriaceae with OXA-163 are common in certain South American countries, especially Argentina (39). Since high risk clones and epidemic plasmids do not seem to play an important role in the global dissemination of OXA-48 derivatives, we refer the readers to some excellent review articles on this topic (37, 38).

International multidrug resistant high risk clonal lineages

Various definitions have been used to describe the resistance criterions among multidrug-resistant (MDR) Enterobacteriaceae. We will use the recent definition of MDR Enterobacteriaceae that was adopted by the European Center for Disease Prevention and Control (40). This states that a MDR Enterobacteriaceae isolate is non-susceptible to at least 1 drug in more than 3 antimicrobial categories that include the following: aminoglycosides, cephalosporins (divided into 3 groups), cephamycins, antipseudomonal penicillins with β-lactamase inhibitors, penicillins, penicillins with β-lactamase inhibitors, monobactams, carbapenems, folate pathway

Table 1. Characteristics of Enterobacteriaceae that produce Expanded-spectrum β-lactamases

Enzymes	Enterobacteriaceae	Classification	Examples	Spectrum of activity	Inhibition	Endemic areas	Molecular epidemiology
Extended-spectrum β-lactamases	*E. coli* *K. pneumoniae* Others (rare)	Class A	CTX-M-14, -15, others TEM, SHV	Penicillins Cephalosporins Monobactams	Clavulanic acid Tazobactam Sulbactam	Worldwide	ST131 IncF plasmids IS*Ecp1*
Plasmid-mediated or Imported AmpC β-lactamases	*K. pneumoniae* *E. coli* *Salmonella* spp Others (rare)	Class C	CMY, FOX, ACT, MOX, ACC, DHA	Penicillins Cephalosporins (not cefepime) Cephamycins Monobactams	Cloxacillin Boronic acid Avibactam	Worldwide	IncA/C, other plasmids
Metallo-β-lactacmases	*K. pneumoniae* *E. coli* Others (rare)	Class B	NDM-1 IMP, VIM,	Penicillins Cephalosporins Cephamycins Carbapenems	Metal chelators e.g. EDTA, dipicolinic acid	Japan (IMP), Taiwan (IMP), Indian subcontinent (NDM), Balkan states (NDM) Greece (VIM)	IncA/C, N , other plasmids Class I integrons
KPC carbapenemases	*K. pneumoniae*	Class A	KPC-2, 3	Penicillins	Clavulanic acid (weak)	USA , Greece, Italy, Israel, China, Brazil, Colombia,	Tn*4401* IncFII

	Enterobacter spp *E. coli* Others		others	Cephalosporins Cephamycins Carbapenems	Tazobactam (weak) Boronic acid Avibactam	Argentina	plasmids CC258
OXA-β-lactamases	*K. pneumoniae* *E. coli* Others (rare)	Class D	OXA-48, OXA-161 OXA-181 OXA-204 OXA-232	Penicillins Temocillin β-lactamase inhibitor combinations Carbapenems	NaCl	Turkey North Africa (Morocco, Tunisia)	Tn*1999* Inc L/M plasmids

inhibitors, glycylcyclines, fluoroquinolones, phenicols, phosphonic acids, polymyxins and tetracyclines.

A bacterial clone refers to the progeny of one bacterial cell through asexual reproduction, implying that the same clonal lineage consists of very closely related isolates that have recently diverged from a common ancestor (41). However, bacterial genomes are plastic and are subjected to genome re-arrangements (i.e. deletions, insertion sequences, etc.) and, to a variable extent, to localized recombinational events. Thus, bacterial isolates assigned to the same clone may not be identical, as the recent descendants of the same common ancestor may differ somewhat in genotype (42). Therefore, the strict definition of a clone tends to be loosened slightly in bacteriology, and clones are defined as isolates that are indistinguishable, or highly similar to each other as identified using a particular molecular typing procedure.

It is important to take into account that the identification of clones depends very much on the molecular typing technique used. The most common molecular techniques currently used by molecular epidemiologists to determine if isolates are clonally related, are multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), PCR typing (such as ERIC or RAPD) and multilocus variable number of tandem repeats analysis (MLVA) (43). PFGE is the technique with some of the highest discrimination when typing bacteria and in the mid 1990s, Tenover and colleagues provided guidelines to define clones or clusters (i.e. "possibly related with 4-6 bands difference") (44). MLST and MLVA are less discriminatory than PFGE and clones identified by these techniques will often have different pulsotypes e.g. an international collection of *K. pneumoniae* ST258 (as identified using MLST) consisted of 4 different pulsotypes that showed distinctive geographical distribution (45).

In this article the term *clone* will refer to any bacterium propagated from a single colony isolated at a specific time and place showing common phylogenetic origins. This implies that such isolates have similar traits as described by methods (e.g. biochemicals, molecular typing), indicating that they belong to the same group or lineage and possess a common ancestor. This implies that whole genome sequencing will provide the all of the information necessary to identify clones (43). The term clone has also become useful in molecular epidemiology, particularly in the study of possible relationships between isolates from different geographical areas. It has become well recognized that not all isolates of pathogenic species are necessarily equal, and that in a typical pathogenic species, a small number of clones, cluster or lineages are greatly over-represented among those isolates recovered from particular types of infection (46).

Short of whole genome sequencing, MLST have often been used to type several members that belong to the Enterobacteriaceae, especially *E. coli* and *K. pneumoniae*. This genotyping method assigns all isolates that are related to each other to a certain sequence or strain type (ST) using a numerical system (e.g. ST1, ST2 etc). Two standardized MLST schemes are widely used to type *E. coli* (e.g. Achtman and Pasteur), while the Pasteur MLST scheme is often utilized for *K. pneumoniae*. Please refer to the laboratory section on more details on MLST including the Achtman and Pasteur MLST schemes.

As described before, an ‘eminent or successful’ bacterial clone is a powerful source for the propagation of genetic antimicrobial resistant components (i.e. genes, integrons, transposons and plasmids) (6). Drug resistant determinants are provided to the offspring in a vertical fashion and such eminent or high risk clones increase the prevalence of antibiotic resistance by its enhanced ability to survive and reproduce efficiently. Moreover, due to the ability of high risk

clones to survive for long periods of time, they also play import roles in the horizontal transfer of dug resistance determinants to other bacteria, acting as efficient donors and recipients.

International multidrug resistant high risk clones have a global distribution and can remain viable for prolonged time periods in diverse areas (47). High risk clones have acquired certain adaptive traits that increase their pathogenicity and survival skills that is accompanied with the acquisition of antibiotic resistance determinants. They have the tenacity and flexibility to accumulate and then provide resistance and virulence genes to other isolates. High risk clones have contributed to the spread of global multidrug resistance through the transmission of different types of genetic platforms, including plasmids, and resistance genes among Gram negative bacteria (6). Examples of some multidrug resistant high risk clones among Enterobacteriaceae are *E. coli* ST38, ST69, ST131, ST155, ST393, ST405 and ST648; and *K. pneumoniae* ST14, ST37, ST147 and ST258 (47).

High risk clones most likely possess some types of biological factors that leads to increased 'fitness', providing these strains with a Darwinian edge over other isolates of the same specie (46). Such advantages will provide them with the abilities to out-contest other bacteria and become the principal part of the bacterial populace in that area. This will provide these clones with increased opportunities to spread as well as time to acquire antimicrobial drug resistance determinants from other bacteria. The spread of multidrug resistant high risk clones are especially facilitated by the selective pressures of antimicrobial drugs present in healthcare settings and used during food animal husbandry.

To qualify as an international multidrug resistant high risk clone, clones must possess the following characteristics (47): i) global distribution ii) association with various antimicrobial resistance determinants iii) colonizing and persisting in hosts for long time intervals (more than 6

months) iv) effective transmission among hosts vi) showing enhanced pathogenicity and fitness and v) cause severe and or recurrent infections.

Escherichia coli Sequence Type (ST) 131

Initial studies pertaining to *E. coli* ST131

Extraintestinal pathogenic *E. coli* or ExPEC is an important cause of nosocomial and community infections in humans (especially UTIs, BSIs and IAIs) (48). Resistance to certain antimicrobial classes (especially the fluoroquinolones and cephalosporins) among ExPEC was rare before 2000 but had increased exponentially since the mid to late 2000s (49).

During the mid-2000s, molecular typing of *E. coli* with $bla_{\text{CTX-M-15}}$ using PFGE in the UK and Canada identified different pulsotypes among these isolates. These pulsotypes were related but showed less than 80% similarity and did not fulfill the Tenover criteria for relatedness (44). The pulsotypes from the UK were named clones A-E while the Canadian pulsotypes were named clones 15A and 15AR (related to A) (50, 51). In 2008, investigators from Spain and France respectively collected *E. coli* with $bla_{\text{CTX-M-15}}$ from different countries that include Spain, France, Canada (including isolates from PFGE clusters 15A and 15AR), Portugal, Switzerland, Lebanon, India, Kuwait and Korea (52, 53). Both investigators performed MLST on this collection and ST131was present in all the countries that provided isolates the studies (52, 53). Clusters A – E from the UK were later identified as also belonging to ST131 (54). All the isolates of multidrug resistant *E. coli* ST131 typed with serotype O25b:H4, belonged to phylogenetic group B2 and harbored Inc F types of plasmids containing $bla_{\text{CTX-M-15}}$ (52, 53). These results of these two initial studies suggested that ST131 had appeared simultaneously in the community setting and seemingly unrelated in separate areas of the globe without any obvious link between the patients.

The results suggested that the appearance of ST131 was either due to contaminated common source type of epidemiology (e.g. water or food) and/or was being introduced into different regions via returning travelers to their respective home countries.

Subsequently, ST131 with CTX-M-15 was described in the United Kingdom (54), the rest of Canada (55), Italy (56), Turkey (57), Croatia (58), Japan (59), United States (60), South Africa (61) , Brazil (62) and Norway (63). Healthy Parisians in France were also found to be rectally colonized with *E. coli* ST131 without CTX-M-β-lactamases (64). Interestingly, ST131 showed high prevalence among fluoroquinolone resistant ESBL negative *E. coli* urinary isolates obtained from Canada (65). It quickly became apparent that *E. coli* ST131 with $bla_{\text{CTX-M-15}}$ were present among global isolates recovered from the community setting (66), hospitals (67), long term care settings including nursing homes (68) and interestingly, in companion animals (69).

Molecular epidemiologists were intrigued with this unexpected global appearance of *E. coli* ST131 mostly in the community setting, apparently in the same time frame but without any obvious connections between these patients. The first studies to provide insight into this fascinating issue occurred in Calgary, Canada and Auckland, New Zealand. The Auckland study reported on a series of patients that presented to local health care facility with community-onset UTIs due to CTX-M-15-producing *E. coli.* This was the author's first encounter with *E. coli* with $bla_{\text{CTX-M-15}}$ and of special interest was that all the patients had recently visited India as tourists or emigrated from India (70). The population-based surveillance study from Calgary showed that recent travel (i.e. within the previous 3 months) to the Indian Subcontinent, the African continent and Middle East was linked with notable high risks for developing community-onset UTIs (including upper UTIs) with CTX-M-producing *E. coli* (71). In a follow-up study from the same

Calgary investigators showed that these UTIs in returning travellers were mostly due to ST131 with *bla*$_{\text{CTX-M-15}}$ (72).

A retrospective molecular epidemiology investigation from Calgary, Canada during 2000-07 highlighted that *E. coli* ST131 with *bla*$_{\text{CTX-M-15}}$ had appeared during the mid-2000s as important etiology of community-acquired health care associated BSIs especially, especially during the closing period of the study (66). Community-onset cases were categorised into community-acquired or healthcare-associated infections (73). Healthcare-associated infections included patients attending community clinics, those from long term care facilities, or patients with infections that occurred within the first 48 hours of admittance to an acute care facility. *E. coli* ST131 was more probable to be multidrug resistant, to possess *aac(6')-Ib-cr*, and more probable to trigger community-onset BSIs, especially those secondary to upper UTIs.

It seems that the unexpected and sudden global appearance of *E. coli* with *bla*$_{\text{CTX-M-15}}$ in the community was related to the emergence of ST131 and was then followed by the subsequent expansion of this multidrug resistant clone. Global surveillance studies have shown that more than 50% of *E. coli* obtained in the Indian subcontinent from community specimens contain ESBLs (74). Therefore it is possible that the rectal colonization of returning travelers from certain endemic regions, could possibly have been critical for the early global spread of ST13 (12). However, it seems that international travel is not essential for the current *E. coli* ST131 global pandemic: A follow-up study during 2011 from the Calgary region, Canada, showed that international travelers to endemic regions such as India had a similar rectal colonization rates for *E. coli* ST131 than non-travelers (75).

The sudden world-wide appearance of *E. coli* with CTX-M-15 was most likely due to the acquisition of certain IncF epidemic plasmids encoding for *bla*$_{\text{CTX-M-15}}$ by a high risk clone such

as ST131. The combination of drug resistant epidemic plasmids, harboring multiple antibiotic resistance determinants, with the high risk clone's increased fitness due to several virulence factors, enabled ST131 to move effortlessly between the community, different hospitals and long-term care facilities. The horizontal transfer of plasmids with $bla_{\text{CTX-M-15}}$ between *E. coli* ST131 and non-ST131 isolates had also been described in certain areas such as France, Spain and Portugal (76).

Investigators from the USA and Canada characterized approximately 200 ExPEC isolates with varying levels of cotrimoxazole and fluoroquinolone resistance. These urine isolates originated from Manitoba (in Canada) acquired during the early 2000s (65). The overall prevalence of ST131 was 23 % and of special interest was that 99% of this clone was fluoroquinolone resistant, but only 2% was resistant to the cephalosporins (65). Results from this study provided some evidence that *E. coli* ST131 was initially a fluoroquinolone-resistant clone and later acquired plasmids harbouring $bla_{\text{CTX-Ms}}$.

One of the first *in vitro* studies that investigated the E. coli ST131 for presence of different virulence factors related to ExPEC, was performed by Johnson and colleagues (77). They investigated 127 ExPEC obtained in the USA during 2007 from the SENTRY and MYSTIC surveillance programs. The overall prevalence of ST131 was 17% and this clone was specifically associated with multidrug resistance and had a significantly higher overall virulence score when compared to non-ST131 *E. coli*. This was interesting to note that the presence of some virulence factors such as uropathogenic-specific protein (*usp)*; outer membrane protein (*ompT)*; secreted autotransporter toxin (*sat*), aerobactin receptor (*iutA*) and pathogenicity island marker (*malX*), corresponded specifically to ST131. Their results demonstrated that ST131 showed a typical virulence profile (when compared to other ExPEC) and it seemed that

multidrug resistance in association with some virulence factors may be essential for the ecological triumph of this clone. Subsequent studies from different investigators have shown similar virulence profiles among ST131 (19). Of special interest is the low prevalence of classical ExPEC-associated virulence factors (i.e. *pap* [P fimbriae], *cnF1* [cytoxic nectizing factor] and *hlyD* [α-hemolysin] among isolates than belong to ST131. The precise role of these virulence factor genes remain to be elucidated, however, it is possible that certain recognized virulence factors possibly play a part in the fitness and adaption of ST131 (78). Virulence factor genes such as *sat*, *iutA*, *malX*, *usp*, *iha*, *hra*, and *ompT* might increase the ability of *E. coli* ST131 to efficiently colonize human tissues rather than being responsible for virulence and causing infection (79).

As described before, PFGE analysis had shown that *E. coli* ST131 is not a single entity and can be separated into various pulsotypes. Molecular surveillance has demonstrated that ST131 with similar PFGE clusters are present in different regions or countries (53, 76, 80). A study that investigated a global collection of ST131 (isolates were obtained from 9 countries in North America, South America, Europe, Middle East, Asia and Oceania) identified 15 distinct pulsotypes; nearly half of ST131 belonged to four distinctive clusters showing a global distribution (81). However, ST131 isolates with less related pulsotypes can be present within the same location (80, 81). Johnson and colleagues genotyped with PFGE over 500 global ST131 ExPEC obtained during the late sixties until 2010, from multiple materials including humans, animals and the environment (82). This study proved that ST131 is highly divergent when using PFGE to analyse this clone. They also showed that a certain number of closely related pulsotypes preside internationally, and these so called "high-frequency" clusters only appeared during the last 10 year or so (82). Retrospective molecular surveillance from Calgary, Canada from 2000

until 2010 identified a prominent closely related pulsotype of ST131 causing a significant increase of ESBL-producing *E. coli* isolated from blood cultures (83). This cluster became especially dominant during 2009 and 2010.

Although ST131 first came to the attention of the medical community because of its particular association with ESBL-producing *E. coli* especially ExPEC with $bla_{\text{CTX-M-15}}$, it became apparent during the late 2000s that the most members of this clone were initially ESBL negative and resistant to the fluoroquinolones (19). Global surveillance studies have shown that ST131 was a fluoroquinolone resistant clone during the early 2000s and became strongly associated with $bla_{\text{CTX-M-15}}$ towards the end of 2000s (84).

Recent developments pertaining to *E. coli* ST131

The presence of ST131 among human clinical *E. coli* isolates varies by geographic region and host population. Recent surveillance studies have shown that the overall prevalence ranges from approximately 10% to nearly 30% of all *E. coli* clinical isolates (84).

Epidemiology and clinical issues

Like other ExPEC isolates, ST131 causes a variety of extraintestinal infections, including BSIs, pneumonia, UTIs, IAIs, and wound infections. ST131 is strongly associated with community-onset infections, especially in patients with regular contact with the health care settings. This was initially illustrated with infections due to ESBL-producing isolates (66, 85), and more recently with a population-based cohort study from the USA (86). Interestingly, *E. coli* ST131 has not been responsible for large nosocomial outbreaks in intensive care or high care units.

Infections with ST131 are the most common among the elderly and this sequence type has a high prevalence among residents in nursing homes and long-term-care-facilities (86). ST131 have also been detected in nonhuman sources, such as companion animals, other animals, food sources, and the environment (19). However, the prevalence of ST131 is substantially greater among humans than animals, food produce or the environment (87). It seems that the ST131 pandemic is primarily a human-based phenomenon and that this clone has somehow successfully adapted to human hosts. However, global studies regarding the prevalence of ST131 among non-human hosts are lacking.

There is a clear association between previous antibiotic consumption and colonization followed by infection due to ESBL-producing *E. coli* including ST131. Antimicrobial agents such as the fluoroquinolones and cephalosporins had most often been implicated in the selection for colonization and subsequent infections due to *E. coli* ST131 (86). The high prevalence of ST131 in community environments is associated with the extensive use of antimicrobial agents (i.e. the outpatient setting of health care settings, nursing homes and long term care centers). This indirectly supports the notion that antimicrobial usage played a role in the selection of ST131. However, the underlying basis for why *E. coli* ST131 has not been implicated in nosocomial outbreaks remains an enigma.

Population structure

The most prevalent lineage within ST131 is named *fimH*30 because it contains the H30 variant of the type 1 fimbrial adhesin gene *fimH* (88). The *fimH*30 lineage was first identified among ST131 obtained from different geographical regions and sources by means of subtyping over 1,000 historical and recent ExPEC isolates (both ST131 and non-ST131) using a combination of typing strategies, including sequencing of *fimH*, *gyrA*, *parC*, multilocus sequence

typing (MLST) and PFGE (88). Johnson and colleagues observed that the *fimH*30 ST131 lineage consisted of nearly seventy percent of recent fluoroquinolone resistant *E. coli* isolates, while it remained infrequent (i.e. less than one percent) among fluoroquinolone susceptible ST131 isolates. The ST131 *fimH*30 lineage first appeared in the early 2000s, became prominent in the mid-2000s and then expanded rapidly during the late 2000s. The majority of the *fimH*30 lineage has a very close genetic similarity, suggesting that they originated from a single *fimH*30-carrying ancestor (89). It seems that the dramatic global emergence of fluoroquinolone resistant ST131 ExPEC has been driven by the clonal expansion and dissemination of the *fimH*30 lineage. Additional support for a single ancestor was provided by the linkage between the ST131 *fimH*30 lineage with a specific *gyrA* and *parC* allele combination, despite the proof for the widespread generation of different *gyrA* and *parC* allele combinations among non-H30 lineages (88). A PCR-based assay that detects *fimH30*-specific single-nucleotide polymorphisms (SNPs) is available to rapidly and cost effectively detect the *H*30 lineage (90).

Price and colleagues, using whole-genome sequencing combined with phylogentic SNP analysis, recently identified two important sublineages within the ST131 *fimH*30 lineage, called H30-R and H30-Rx, because of their extensive antimicrobial resistance profiles (89). They initially analyzed just over 500 ST131 isolates using PFGE collected between 1967 and 2011 from different geographic regions and found that the *fimH*30 lineage showed significant different pulsotypes irrespective if the isolates were susceptible or resistant to the fluoroquinolone, or contained $bla_{\text{CTX-M}}$. These findings suggested that fluoroquinolone resistant mutations and CTX-M genes were horizontally acquired over a long period of time. However, when 105 of the isolates with significantly different pulsotypes underwent next generation sequencing combined with SNP analysis to reconstruct the phylogeny of ST131, the fluoroquinolone resistant *fimH*30

lineage and *fimH*30 with $bla_{\text{CTX-M-15}}$ formed a tight cluster irrespective if their pulsotypes were different. Moreover, when the authors performed additional sequencing with increased coverage, they found that within the fluoroquinolone resistant *fimH*30 lineage (named H30-R sublineage), isolates with $bla_{\text{CTX-M-15}}$ formed a distinct cluster named the H30-Rx sublineage that was separated from ESBL-negative fluoroquinolone resistant *fimH*30 isolates, by 3 core genome SNPs (89).

The phylogenetic analysis undertaken by Price and colleagues indicated that the ST131 *fimH*30 lineage comprised a series of nested sublineages that were most likely derived from a single common fluoroquinolone susceptible *fimH*30 ancestor (84). These investigators named these sublineages as follows: the H30-R sublineage or subclone was fluoroquinolone-resistant without $bla_{\text{CTX-M-15;}}$ the H30-Rx sublineage or subclone was fluoroquinolone resistant with $bla_{\text{CTX-M-15}}$ (89). This clonal structure results in a succession of antimicrobial resistance among the *fimH*30-associated lineages, from the most susceptible, H30 (fluoroquinolone susceptible, CTX-M negative), to the more resistant H30-R (fluoroquinolone resistant, CTX-M negative), to the most extensively resistant, H30-Rx (fluoroquinolone resistant, CTX-M positive). Whole genome sequencing combined with SNP analysis had indicated that the clonal expansion of the ST131 *fimH*30 lineage is the most dominant and important vehicle for the increasing global prevalence of fluoroquinolone resistance and $bla_{\text{CTX-M-15}}$ among ExPEC. These results were later independently confirmed by Petty and colleagues (91). The population structure of the *E. coli* ST131 *fimH*30 lineage, H30 sublineages and other ST131-associated lineages is illustrated in Figure 1.

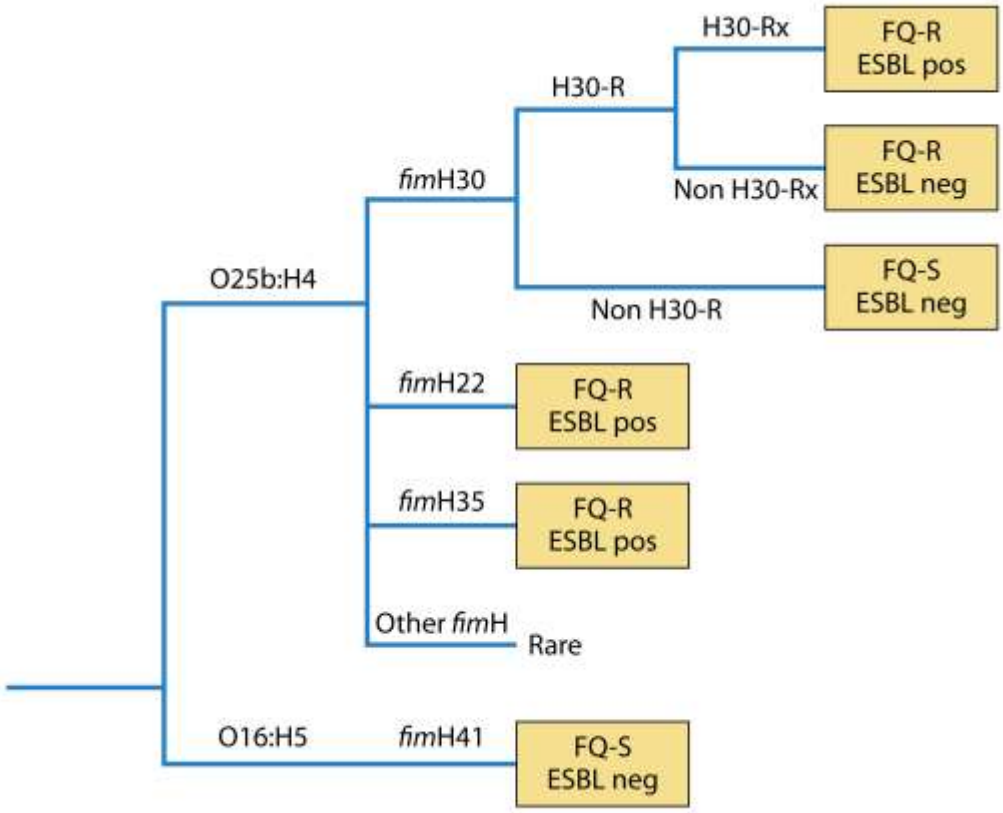

Figure 1. The population structure of the *Escherichia coli* ST131 *fimH*30 lineage, H30 sublineages and other ST131-associated lineages. FQ-R; fluoroquinolone-resistant, ESBL; extended-spectrum β-lactamases

The prevalence and distribution of the *fimH*30 lineage, H30-R and H30-Rx ST131 sublineages were initially described in populations within the USA. Among *E. coli* clinical isolates from various USA veterans centers obtained during 2011, showed the prevalence of ST131 was only 7% among fluoroquinolone-susceptible ExPEC, 78% among fluoroquinolone-resistant ExPEC and 64% among ESBL-producing *E. coli*; the ST131*fimH*30 lineage accounted for 12.5% of fluoroquinolone-susceptible ST131 isolates and increased up to 95% among fluoroquinolone-resistant and 98% of ESBL-producing ST131 isolates (90). Similarly, in a case-control study conducted in the Chicago region, over 50% of ESBL-producing ExPEC isolates

were identified as ST131; 98% were further characterized as *fimH*30 while 92% were characterized as H30-Rx (92). These studies supported the notion that there is a strong alliance linking the H30-Rx sublineage with $bla_{\text{CTX-M-15}}$. However, not all ST131 with CTX-M-15 are members of the H30-Rx sublineage.

In a population-based study of consecutively collected ExPEC isolates in Minnesota, the prevalence of the ST131 *fimH*30 lineage was high among elderly patients (i.e. older than 60 years), while infections due to non-H30 ST131 isolates was more common among younger patients (i.e. less than 2 years) (93). In a different multicenter USA study that analyzed >1,600 ExPEC isolates, CH clonotype 40-30 (that corresponded to the *fimH*30 lineage of ST131) was the most prevalent clonotype among all ExPEC and was statistically associated with recurrent or persistent UTIs and the presence of sepsis (94). The next generation sequencing study from Price and colleagues described earlier also observed that sepsis was significantly associated with infections due to the H30-Rx sublineage (89).

The first study outside the USA to determine the prevalence and distribution of the ST131 *fimH*30 lineage and its respective sub-lineages was a retrospective population-based surveillance study investigating BSIs due to flouroquinolone- resistant ExPEC from Calgary, Canada (2000-10) (95). This study demonstrated that the deluge of the H30-Rx sublineage towards the latter part of the study period was responsible for the significant increase in ST131 and fluoroquinolone resistant ExPEC (95) (Figure 2). The study recognized the relationship of H30-Rx with primary sepsis and BSIs due to prostate biopsies. This sublineage was also more likely to contain *aac(6')-lb-cr*.

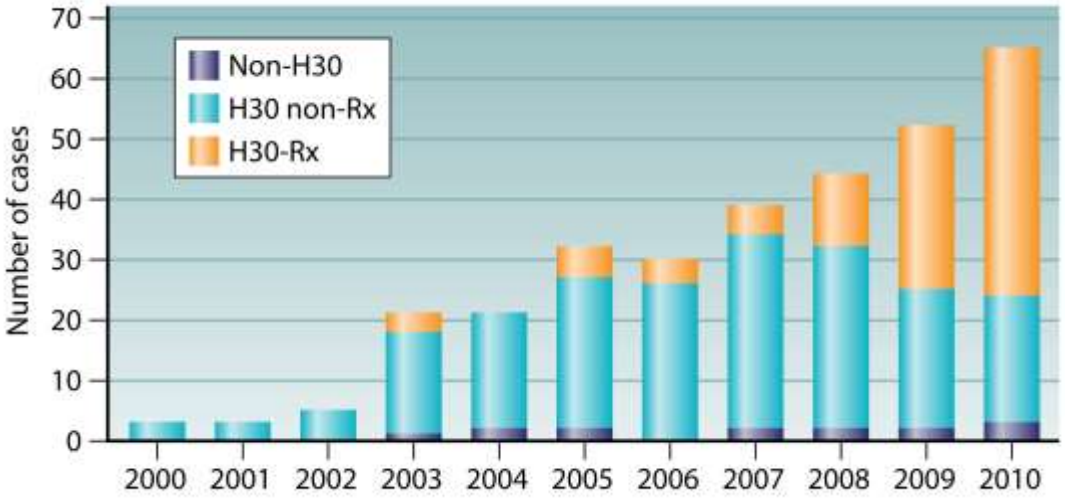

Figure 2. The number of *Escherichia coli* ST131, H30-R and H30-Rx sublineages isolated from blood in the Calgary Region from 2000 – 2010 (ref 95).

O16:H5 H41 and other ST131 lineages

As described before, several investigators noticed during the late 2000s that *E. coli* ST131 with ESBLs exhibited different pulsotypes (52, 55, 60, 61, 66). Interestingly, some ST131 isolates belonged to a well-defined cluster that was separated from the more prominent O25b:H4 clade and tested negative when using Clermont's ST131 PCR (81). (For more details on the Clermont PCR, please refer to the laboratory methods section). This cluster contained $bla_{\text{CTX-M-14}}$, was susceptible to the fluoroquinolones and with additional serotype and *fimH* allele characterization, it tested positive for O16:H5 and belonged to the *fimH*41 lineage (81). Of interest, the O16 and O25b serotypes within ST131 were identified as representing different STs when using the Pasteur Institute MLST scheme but isolates with these different serotypes were recognized as the same ST when using the Achtman MLST scheme (84).

The O16:H5 ST131 *fimH*41 lineage had distinct combinations of *gyrA* and *parC* alleles and surveillance data had shown that this lineage comprised of 1 – 5 % of *E. coli* ST131 (96). The *fimH*41 lineage are associated with resistance to trimethoprim-sulfamethoxazole and gentamicin (when compared to the *fimH*30 ST131 lineage) while ESBLs and fluoroquinolone resistance are rarely detected with this lineage. O16:H5 ST131 *fimH*41 isolates with $bla_{\text{OXA-48}}$ from the United Arab Emirates and Morocco have recently been described (97). A rapid PCR screening test was developed to detect the *fimH*41 lineage and combined the O16 *rfb* gene with the ST131-specific alleles of *mdh* and *gyrB* (96). (For more details on that PCR, please refer to the laboratory methods section).

Two additional ST131 lineages with the O25b:H4 serotype but showing significant different pulsotypes from the main *fimH*30 cluster have recently been reported (81). These isolates belonged to the *fimH*22 and *fimH*35 lineages and were also associated with $bla_{\text{CTX-M-15}}$, flouroquinolone resistance and presence of *aac(6')-lb-cr*. O25b:H4 ST131 *fimH*22 isolates with bla_{KPC} from Argentina and Colombia have recently been described (97). The population structure of the *E. coli* ST131 *fimH*30, *fimH*41, *fimH*22, and *fimH*35 lineages as well as the H30-R, H30-Rx sublineages, is illustrated in Figure 1.

Virulence

A Spanish study proposed a new classification system for ST131 into four virulence-associated groups (i.e. A, B, C and D) (also named virotypes) (98). These investigators performed *in-vitro* virulence characterization on a large global collection of ST131 and identified four virulence factors that showed a distinctive distribution among the different virotypes. These VFs include *afa* FM955459 (Afa/Dr adhesion), *iroN* (catecholate siderophore receptor), *ibeA* (invasion of brain endothelium) and *sat* (secreted autotransporter toxin) (98). Virotypes A and B

were related to antibiotic non-susceptibility, *bla*$_{\text{CTX-M-15}}$ and *aac(6')-lb-cr*. Virotype C was present in several countries and was linked to invasive infections when compared to other virotypes such as A and B (98).

An USA study described the ExPEC-associated virulence profiles of ST131 and its sublineages (H30-R and H30-Rx) among large *E. coli* collection from the Midwest near the Great Lakes region (92). They identified distinctive virulence profiles among these sublineages, with the H30-Rx sublineage having the highest aggregative virulence score (92). Colpan and colleagues had shown that the fluoroquinolone susceptible ST131 *fimH*30 lineage had a significantly different virulence profile than non-ST131 fluoroquinolone susceptible isolates. Moreover, this lineage gained resistance to the fluoroquinolones and acquired ESBLs, isolates tended to progressively accumulate additional virulence factors especially among the ST131 *fimH*30 lineage that acquired CTX-M-15 (90).

E. coli ST131 and carbapenemases

The development of resistance to the carbapenems in community pathogens such as *E. coli* is worrisome to the medical fraternity (49). The NDM and OXA-48 are the most prevalent carbapenemases present in nosocomial and community isolates of *E. coli*, while the VIM, IPM, and KPC β-lactamases are not yet commonly encountered in this species (11). Infections with carbapenemase-producing *E. coli* often occur in patients that had recently visited certain endemic regions such as the Indian subcontinent for NDMs and North Africa or Turkey for OXA-48 (12).

Due to the revolutionary world-wide triumph of ST131, carbapenemases among this clone has been carefully monitored by molecular epidemiologists. The carbapenemase gene *bla*$_{\text{NDM}}$ was first identified in ST131 during 2010 from patients in Chicago and Paris respectively (99, 100). Both patients had previously visited the Indian subcontinent. This was followed by

case reports of ST131 with *bla*$_{VIM}$ from Italy (101); ST131 with *bla*$_{KPC}$ from Ireland (102), France (103), USA (104), Italy (105), Taiwan (106), China (107); ST131 with *bla*$_{OXA-48}$ from the UK (108), Ireland (109), Algeria (110), Spain (111) and with *bla*$_{IMP}$ from Taiwan (112). The greatest cluster of ST131 with carbapenemases was recently described from Pittsburgh, USA (113). The authors characterized 20 isolates of KPC producing *E. coli*: 60% belonged to the ST131 *fimH*30 lineage while the *bla*$_{KPC}$ plasmid belonged to the IncF with FIIk replicons.

A recent study from the SMART and AstraZeneca global surveillance programs have shown that 35% of 116 carbapenemase-producing *E. coli* belonged to ST131 that was associated with fluoroquinolone resistance, the presence of *bla*$_{KPC}$, *fimH*30 lineage and virotype C (97). Genes for ESBLs such as *bla*$_{CTX-M-15}$ and *bla*$_{SHV-12}$ were also present in some of the ST131 with carbapenemases. ST131 was also isolated from Argentina, China, Colombia, Ecuador, India, Italy, Jordan, Morocco, Panama, Philippines, Puerto Rico, Thailand, Turkey, UAE, USA and Vietnam. *E. coli* ST131 with carbapenemases poses a significant new public health risk due to its worldwide distribution and relation with the dominant *fimH*30 lineage.

Does ST131 qualify as an international multidrug resistant high risk clone?

The pandemic emergence of *E. coli* ST131, and specifically its H30-R and H30-Rx sub-lineages, occurred over less than 10 years. It is a remarkable antimicrobial resistant success story rivalling the global pandemics caused by clones within methicillin resistant *Staphylococcus aureus* (i.e. ST5, ST8, ST36) and *Streptococcus pneumoniae* (i.e. ST236, ST320).

Global distribution and prevalence

E. coli ST131 was initially described among isolates with *bla*$_{CTX-M-15}$ from the following countries: Canada, France, Switzerland, Portugal, Spain, Kuwait, Lebanon, India, and Korea (52,

53). Subsequently, ST131 had been identified among ESBL, non-ESBL, fluoroquinolone resistant and fluoroquinolone susceptible *E. coli* from all corners of the world (19) . If investigators decide to determine the presence of ST131 among *E. coli* isolates collected from human sources, they will most likely detect ST131 among their collection. ST131 remains being over presented among antimicrobial resistant ExPEC; recent global surveillance have shown that ST131 consistently accounts for approximately 60% to 80% of fluoroquinolone-resistant isolates, and for 50-60% of ESBL-producing isolates, but only for 0% to 7% of fluoroquinolone - susceptible isolates (84). *E. coli* ST131 has a true global distribution and is present among ExPEC on all continents, except possibly Antarctica.

Association with antimicrobial resistance mechanisms

E. coli ST131 was initially described in ExPEC with CTX-M-15 and today is known to also be associated with fluoroquinolone resistance (83, 90, 92). Population genetics has indicated that the fluoroquinolone resistance in the ST131 *fimH*30 lineage is mostly due to *gyrA*1AB and *parC*1aAB mutations in gyrase and topoisomerase IV respectively (88). The *gyrA*1AB and *parC*1aAB mutations are present in 71% of fluoroquinolone resistant and in 62% of ESBL *E. coli* from various Veterans Administration hospitals in the USA (90). ST131 is also associated with the presence of the plasmid-mediated quinolone resistance determinant *aac(6')-lb-cr* (17). This resistance determinant causes decreased susceptibility to the fluroquinolones ciprofloxacin and norfloxacin, as well as resistance to the aminoglycosides tobramycin and amikacin.

Various expanded-spectrum β-lactamases (e.g. CTX-Ms, CMYs, SHV ESBLs, TEM ESBLs) have been detected in ST131 (19); CTX-M-15 in combination with TEM-1 and OXA-30 being by far the commonest. The presence of carbapenemases are still rare among ST131 although some enzymes had been reported from different parts of the world (19).

Other resistance determinants that have been characterized in ST131 including *mph*(A) (responsible for resistance to the macrolides), *cat*B4 (responsible for resistance to chloramphenicol), *tet*A (responsible for tetracycline resistance), *dfrA7* (responsible for trimethoprim resistance), *aadA5* (responsible for streptomycin resistance) and *sulI* (responsible for sulfonamide resistance). Therefore, isolates belonging to ST131 are most often non-susceptible to the cephalosporins, monobactams, fluroquinolones, trimethoprim-sulfamethoxazole, and the aminoglycosides (19).

Ability to colonize human hosts

Because intestinal colonization with ExPEC is believed to be a prerequisite for extraintestinal infection, it is possible that the enhanced ability to colonize the intestinal tract is partly responsible for the widespread dissemination of ST131. Several studies have shown that asymptomatic individuals can be rectally colonized with *E. coli* ST131 (19). In a mouse model of intestinal colonization, an ST131 isolate surpassed other commensal *E. coli* isolates mixed in a 1:1 ratio and administered enterally into streptomycin-pretreated mice. Although that study was limited by its evaluation of only a single ST131 isolate, it provided evidence that some ST131 isolates have the ability to efficiently colonize the intestine, bladder and kidney of mice (114). It is unclear whether the prevalence or duration of intestinal colonization in humans is different for ST131 when compared to other *E. coli* isolates. It is also unclear if the dominant *fimH*30 ST131 lineage, including the sublineages (i.e., H30-R and H30-Rx), has different intestinal colonization abilities when compared to other ST131 lineages (e.g., *fim*H41, *fimH*35 and *fimH*22).

The prevalence of *E. coli* ST131 among rectal isolates of *E. coli* varied considerably with the population studied, geographical regions, host characteristics, the presence of resistance

mechanisms as well as the time frame when the study was conducted: rectal colonization rates of ST131 that varied from 0 to 44% have been reported (19).

Effective transmission among hosts

The transmission of ST131 had previously been documented between different household family members (father to daughter; daughter to mother; sister to sister) and companion animals (dogs and cats in particular) (19, 84). A study from Switzerland had shown that ST131 were more likely to be transmitted between members of the same family than within patients in the hospital environment (20). A French day-care center recently reported that 7 children were rectally colonized with ST131 that produced CTX-M-15, suggesting that this clone was effectively transmitted within the day-care setting (115).

It seems that *E. coli* ST131 can effectively be transmitted between members of the same family and this might have played an important role in the emergence and dissemination of this sequence type within the community setting. However, whether ST131 is more efficiently transmitted than other *E. coli* isolates is uncertain and deserves additional studies.

Enhanced pathogenicity and fitness

In several molecular epidemiological studies, ST131, when comparing to non-ST131 ExPEC, consistently had a larger number of ExPEC-associated virulence factor genes and had significant higher aggregate virulence scores (77, 116). Moreover, within ST131, the *fimH*30 lineage and specifically the H30-Rx sublineage had characteristic virulence profiles with higher virulence scores when compared to non-H30 ST131 *E. coli*, most likely playing an important role in overall virulence and fitness of this sublineage (92).

In-vitro studies that investigated the maximal growth rate and the ability of ST131 to produce biofilms, had shown that this clone had a high metabolic potential, most likely increasing the fitness and ability of ST131 to establish intestinal colonization for long periods of time (117). *In vivo* studies that investigated the virulence potential of ST131 in animal models indicated that this sequence type kills mice (118), but are less virulent when compared to non-ST131 *E. coli* in the *Caenorhabditis elegans* and zebra fish embryo models (119).

Causing severe and or recurrent infections

It is unclear if ST131 cause more severe infections than other ExPEC *E. coli* but clinical epidemiological data suggests that ST131 are more likely to cause upper UTIs than lower UTIs (84). In a series of studies from Australia, ST131 accounted for 30% of pyelonephritis isolates among ExPEC isolates from women, versus only 13% of cystitis isolates and 4% of fecal isolates. Similar prevalence trends were seen among men and children (117). A study from the United Kingdom showed similar results where the prevalence of ST131 was 21% among bacteremia isolates as compared to only 7% among urinary isolates (120).

Whether ST131 is associated with worse clinical outcomes than other ExPEC isolates is unclear. Some studies suggest that ST131 is more likely to cause persistent or recurrent UTIs (86) while other investigators have found no significant difference in outcomes of infections with ST131 versus other ExPEC isolates (121). When investigators adjusted for host factors, no differences in cure or mortality were found between patients with infections due to ST131 and those with infections due to other ExPEC isolates (94). Of special interest, the H30-Rx sublineage of ST131, have demonstrated statistical and epidemiological association with sepsis (89).

To summarize; *E. coli* ST131 clearly have all of the essential characteristics that defines a high risk clone (Table 2). In fact this sequence type might be the quintessential example of an international multidrug-resistant high risk clone.

Table 2. Characteristics of *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST258 that defines them as high risk clones

Characteristic	***Escherichia coli* ST131**	***Klebsiella pneumoniae* ST258**
Global distribution	Endemic on all continents except Antarctica	ST258 is endemic in USA, Israel, Greece, Italy, Poland, Colombia; ST11 has been reported from China, Brazil; ST512 from Israel, Italy, Colombia, ST340 from Brazil, Greece
Association with various antimicrobial resistance determinants	Various but associated with fluoroquinolone resistance and CTX-M-15 (CTX-M-14 to a lesser extent)	Various but associated with KPC-2 and KPC-3
Ability to colonize and persist in hosts for long periods of time	Rectal colonization for up to 6 months	Rectal colonization for up to 12 months
Effective transmission among hosts	Transmission among family members	Successful nosocomial transmission for months after introduction
Showing enhanced pathogenicity and fitness and	Higher aggregate ExPEC-associated virulence scores, high metabolic potential and biofilm production	Unclear
Cause severe and or recurrent infections	More likely to cause upper UTIs, recurrent UTIs and H30-Rx sublineage is associated with sepsis	Mortality higher when compared to non-258 *K. pneumoniae* (most likely due to patient underlying conditions)

ExPEC (Extra-intestinal *E. coli*), UTIs (urinary tract infections)

Klebsiella pneumoniae Sequence Type (ST) 258

Initial studies pertaining to *K. pneumoniae* ST258

The rate and consequence with which carbapenem resistance had disseminated globally in *K. pneumoniae* had raised cause for alarm among the medical community at large. To date, bla_{KPC} has been found in more than 100 different sequence types (STs), but this pandemic is primarily driven by the spread of KPC-producing *K. pneumoniae* isolates that are members of clonal complex (CC) 258 (122). CC258 (founder member is ST292) consists of one predominant ST namely ST258, and to a lesser extent ST11, ST340 and ST512 that are single locus variants (SLVs) of ST258 (30, 122). *K. pneumoniae* ST258 is a prototype of a high risk clone and recent information about the epidemiology, genetic rearrangement and evolution of this successful clone had provided insights into the global spread of antimicrobial drug resistance.

K. pneumoniae with bla_{KPC} was first identified in a non-ST258 isolate during 1996 in the Southern United States (123). During the late 1990s to early 2000s there were sporadic reports of *K. pneumoniae* with bla_{KPC} from the North Eastern USA, however, large outbreaks due to related isolates were not described (124). In 2009, the Centers for Disease and Prevention from the USA in collaboration with investigators from Israel, performed MLST on *K. pneumoniae* with bla_{KPC} and they identified ST258 among isolates from the New York area collected during 2005 (125). As time progressed, ST258 was detected in geographically diverse regions of the USA and in 2009 it became apparent that ST258 was the predominant clone in this country being responsible for 70% of *K. pneumoniae* with bla_{KPC} obtained from different parts of this country (126). During the mid-2000s Israel experienced several nosocomial outbreaks of infections due to *K. pneumoniae* with bla_{KPC} that was caused by a clone (identified with PFGE) and named

clone Q (125). Interestingly, clone Q has a similar pulsotype than ST258 present in the USA. This was followed by global reports of ST258 among *K. pneumoniae* with bla_{KPC} from countries such Greece (127), Norway, Sweden (128), Italy (129), Poland (130), Canada (131), Brazil (132) and Korea (133) suggesting that this ST had characteristics of international multidrug resistant high risk clones. Recent reports from Israel (134) and Italy (135) demonstrated the endemicity and persistence of CC258 over time while remaining the predominant clone among *K. pneumoniae* with bla_{KPC}. Interestingly, Israel has seen an overall dramatic decrease in the incidence of KPCs among *K. pneumoniae* but ST258 still remain the most predominant clone (134).

The other SLVs from CC258 have the following global distribution: e.g. ST11 is the major ST among *K. pneumoniae* with bla_{KPC} from Asia (especially China) (136) and had also been described in Latin America (especially Brazil) (122). Other STs that belong to CC258 have been reported from Colombia (i.e. ST512), Italy (i.e. ST512), Israel (i.e. ST512), Brazil (i.e. ST340) and Greece (i.e. ST340) (122).

Recent developments pertaining to *K. pneumoniae* ST258

Population structure

The diversity in *K. pneumoniae* genomes is primarily due to mobile genes that move frequently by horizontal transfer between bacteria, including plasmids, phages, integrative conjugative elements (ICEs), and insertion elements (IEs) (30). Kreiswirth and colleagues recently performed whole genome sequencing on two *K. pneumoniae* ST258 urinary isolates from New Jersey and then did supplementary sequencing on a different global collection of just over eighty CC258 clinical isolates (137). The phylogenetic single nucleotide polymorphism

(SNP) analysis of the core genomes of these isolates showed that ST258 *K. pneumoniae* belonged to two well defined lineages named clade I and clade II. Clade I was associated with KPC-2 and clade II was associated with KPC-3. The genetic divergence between these two clades occurred in a 215-kb area that included the genetic material used for capsule polysaccharide biosynthesis (*cps*), an important virulence factor for *K. pneumoniae*.

The same group then compared the genetic structures of the *cp*s regions and disperse of SNPs in the core genomes of the ST258 clades I and II with other *K. pneumoniae* sequence types (i.e. ST11, ST442, and ST42) (138). Kreiswirth and colleagues found a 1.1-Mbp area in ST258 clade II that is identical to that of ST442, while the remainder part of the ST258 genome was homologous to that of ST11. This indicated that ST258 clade II is a hybrid or cross-breed clone that was created by a large recombination event between sequence types ST11 and ST442. The investigators then identified the same *cps* regions in ST42 and ST258 clade I. The likeness of the areas surrounding the *cps* regions from ST42, ST258 clade I and ST258 clade II indicated that the ST258 clade I evolved from ST258 clade II due to the replacement of the *cps* region from ST42. Recent developments regarding the population structure of *K. pneumoniae* ST258 is illustrated in Figure 3.

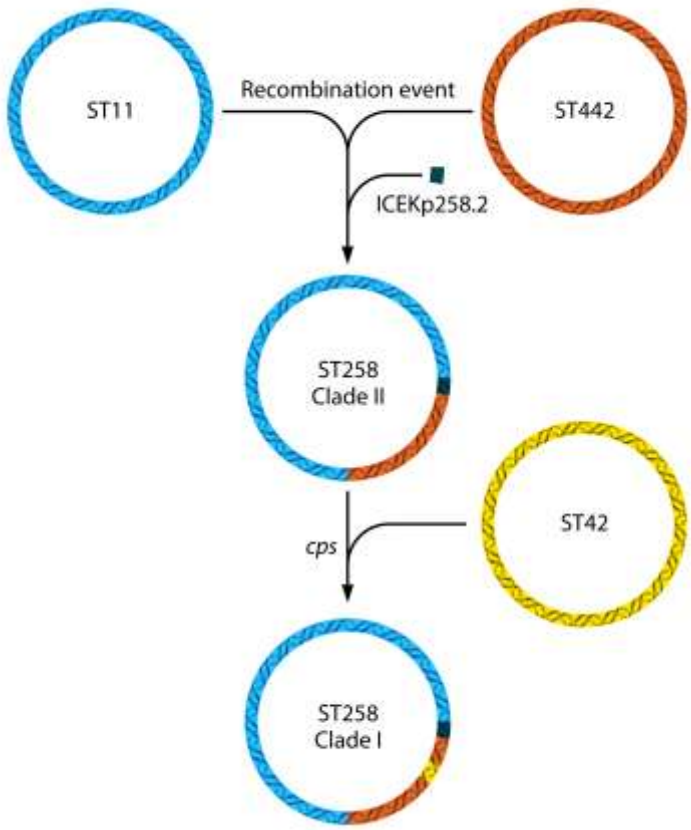

Figure 3. The population structure of *Klebsiella pneumoniae* ST258. ICE; integrative conjugative element *cps*; capsule polysaccharide biosynthesis gene region

The integrative conjugative element ICEKp258.2 contains gene clusters for a type IV pilus (i.e. *pilV*) and a type III restriction-modification system. A type IV pilus increases the exchange of plasmid DNA between bacteria and facilitates the attachment of bacteria to objects. Type IV pilus may therefore facilitate the movement of resistance genes (138). A type III restriction-modification system could also be responsible for "host specificity" i.e. preventing the exchange of certain mobile elements including plasmids among bacteria (138). A study from Israel investigated the specific association of ICEKp258.2 with *K. pneumoniae* ST258 and other sequence types by searching for the presence of *pilV* and showed that this gene cluster was

present only among ST258 isolates but absent in non-ST258 *K. pneumonaie* (including SLVs of ST258 such as ST11) (139). Their results shed some light on the different behaviours of ST258 and its close relative ST11: ST258 (that contains ICEKp258.2) is specifically associated with KPC and narrow-host range IncF plasmids (30) while ST11 (that lacks ICEKp258.2) is associated with various carbapenemases (KPC, VIM, IMP, NDM, and OXA-48) on a broad range of plasmids (e.g. IncF, Inc A/C, InL/M, IncN, not-typable) (140-142). Therefore, the restriction of plasmids and specific mobile elements by a type III restriction-modification system on ICEKp258.2, may explain some of the differences observed between these SLVs (i.e. ST258 and ST11). Moreover, *pilV* may also in part explain the high transmissibility and survival abilities of ST258 on living and nonliving surfaces. It is possible that ICEKp258.2 contribute significantly to the ecological success of *K. pneumonaie* ST258 (138).

Association with antimicrobial resistance mechanisms

High risk clones act as stable hosts that harbor resistance genes, transposons, integrons and plasmids which allow such elements to spread with such clones (6). One of the most consistent hallmarks of *K. pneumonaie* ST258 is its association with multidrug resistance determinants (133). The majority of antimicrobial resistant determinants present in ST258 are plasmid mediated and often this clone contained more than one plasmid, each with multiple individual resistant determinants (137). A recent study that used next generation sequencing with long reads examined the contents on plasmids in a single ST258 isolate. The investigators identified four plasmids containing 24 different resistance genes (143). Several investigators have shown that *K. pneumonaie* ST258 have multiple antimicrobial resistance determinants responsible for aminoglycoside resistance (i.e. *aac(6')-Ib*, *aadA2*, *aph(3')-Ia*), β-lactam resistance (i.e. bla_{KPCs}, $bla_{\text{OXA-9}}$, $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$), fluoroquinolone resistance (i.e. *oqxA*,

oqxB), macrolide-lincosamide-streptogramin B resistance (i.e. *mphA*), chloramphenicol resistance (i.e. *catA1*), trimethoprim resistance (i.e. *dfrA12*), and sulfonamide resistance (i.e. *sul1*) (143, 144). ST258 isolates can also decrease the number of porin channels that will lead to non-susceptibility to additional classes of antimicrobial drugs, leaving clinicians with very limited treatment options (145).

The emergence of colistin resistance among ST258 is a worrisome finding since this antimicrobial agent has remained as one of the last line salvation therapies for infections due to *K. pneumoniae* with bla_{KPC} (145). Colistin resistance among ST258 had been documented in several diverse geographical regions which likely indicated that colistin-resistant ST258 mutants can be selected over time after the introduction of this agent for the treatment of infections due to *K. pneumoniae* ST258 (145-147).

Ability to colonize human hosts

A recent study from Germany investigated the rectal colonization of patients following an outbreak due to *K. pneumonaie* ST258 and showed that 25% of patients remained colonized for up to one year after the outbreak was contained. Some patients were persistently colonized even beyond this time (148). A study from Greece found that 73% of the patients were rectally colonized with *K. pneumoniae* ST258 with bla_{KPC} on average within 9 days after exposure to this clone (149). Similar data had been reported from Italy that showed the effective colonization of neonatal patients by *K. pneumoniae* ST258 (150) as well as in long term acute care populations from the USA (151). However, in contrast to *E. coli* populations, ST258 has remained largely present among nosocomial patients with health care association, and are not readily identified among community dwellers.

Transmission within the health-care setting

In a detailed analysis of a recent USA ICU outbreak due to *K. pneumoniae* ST258, indicated that this clone can be very successfully transmitted between hosts for a long period of time (i.e. months) after the initial index patient was identified (152). In Italy successful spread of ST258 occurred rapidly within the hospital setting (129). The most notable and pronounced outbreaks due to ST258 was experienced in Israel after introduction of this clone during the early to mid-2000s (125). There was a sharp increase in cases and outbreaks in Israel during 2006-7 in acute care hospitals that was primarily driven by ST258. After the nationwide implementation of infection control guidelines during the late 2000s to limit the spread of *K. pneumoniae* with bla_{KPC}, there has been a steep decline in the incidence of infections due to ST258 throughout Israel (134).

Enhanced pathogenicity and fitness

Capsular polysaccharide is a recognized virulence factor enabling *K. pneumoniae* to evade phagocytosis (153). Recent papers have highlighted the likely importance of unique capsular polyscaccharides within ST258 and its probable importance in promoting global success of this clone (137). Characterization of the genome region from different clades of ST258 (i.e. clade I, clade II) showed that that capsule polysaccharide biosynthesis regions *cps*-1 and *cps*-2 (also referred to as $cps_{207\text{-}2}$ and $cps_{BO\text{-}4}$) are significantly different between clades I and II and are likely involved in the global distribution of these clades (138, 154). This *cps* region of diversification could be advantageous for *K. pneumoniae* ST258 clades I and II that manages to change the polysaccharide structure as a mechanism to evade host defenses. Capsule switching is used by certain bacteria to escape the host immune response (155). It is possible that the DNA interchange that occurs up and down stream of the *cps* regions may be an important method used

by *K. pneumoniae* to quickly expand and change over time (155). It would be interesting to determine what selection process is responsible for capsule switching among *K. pneumoniae* ST258 clades I and II.

As described before, *pilV* is present on ICEKp258.2 and may in part be responsible for the high transmissibility and survival abilities of *K. pneumoniae* ST258.This element is present in both clades of ST258 but it seems to have been acquired after the large recombination event between ST11 and ST442 to create ST258 clade II. It seems that the genetic material for KPC and the ICEKp258.2 element were independently acquired at two distinct points in time (137). A type-IV secretion system might have been beneficial to *K. pneumoniae* ST258 by increasing the fitness and survival abilities of this clone (156).

It is unclear if ST258 is more virulent when compared to other *K. pneumoniae* isolates. ST258 lacks well-characterized *K. pneumoniae* virulence factors, including K1, K2, and K5 capsular antigen genes, the aerobactin genes, and regulator of mucoid phenotype gene *rmpA* (157). A recent study demonstrated that ST258 is non-virulent in animal models, highly susceptible to serum killing and can rapidly undergo phagocytosis (157). A different group had shown that not all ST258 isolates produce the same results in a mouse lethality model but similar results were obtained in a moth (*Galleria mellonella*) virulence model (158). There has been evidence of virulence in a nematode model with the *Caenorhabditis elegans* killing and this effect was not diminished when the isolates were cured of bla_{KPC} plasmids (159). This study suggested that the bla_{KPC} plasmids contained additional factors which could result in persistence but not necessarily enhanced virulence. There was also some strain to strain variability in the nematode model.

To summarize; *K. pneumoniae* ST258 clearly has all of the essential characteristics that defines a high risk clone (Table 2).

Table 3. Laboratory methods for the detection of *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST258

Method	***Escherichia coli* ST131**	***Klebsiella pneumoniae* ST258**
Next generation sequencing	High-resolution, accurate, reproducible. Not yet routine	High-resolution, accurate, reproducible. Not yet routine
Multilocus sequence typing (MLST)	Gold standard, expensive, time consuming, 2 schemes (Achtman and Pasteur)	Gold standard, expensive, time consuming, Pasteur scheme
Pulsed field gel electrophoresis	Used during the late 2000s, poor method since ST131 consists of different pulsotypes	Used during the late 2000s, poor method since ST258 consists of different pulsotypes
Repetitive sequence-based PCR typing	Standardized fingerprinting kit, rapid, expensive	Standardized fingerprinting kit, rapid, expensive
Multilocus variable number of tandem repeats analysis	Rapid, cost effective, comparable to MLST	Not yet described
Polymerase chain reaction	Several techniques, rapid, inexpensive for screening large number of isolates	Several techniques, rapid, inexpensive for screening large number of isolates, multiplex for clade I and II
Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry	Rapid, inexpensive, not yet routine	Not yet described

Laboratory methods for the detection of *E. coli* ST131 and *K. pneumoniae* ST258

Due to the unprecedented success of *E. coli* ST131 and *K. pneumoniae* ST258, several investigators have designed rapid methods for the detection of these sequence types to aid molecular epidemiologists and surveillance studies (Table 3). Such techniques facilitated the enhanced detection of these sequence types as part of surveillance programs. These include PCR, PFGE, Diversilab repetitive PCR typing, abbreviated MLVA and more recently MALDI TOF mass spectrometry.

Next generation sequencing (NGS) is a promising new technology with substantial potential for clinical microbiology (160). This technique uses PCR to amplify individual DNA molecules that are immobilized on a solid surface, enabling molecules to be sequenced in parallel, leading to decreased costs and rapid turnaround times (161). NGS provides high-resolution DNA data in a rapid fashion that can be used for accurate typing of medically important organisms. Several *E. coli* ST131 and *K. pneumoniae* ST258 isolates have undergone NGS (89) and it is likely that this technique, combined with more user friendly and rapid bioinformatics, will most likely become the gold standard for the identification of these sequence types in the near future.

Multilocus sequence typing (MLST)

MLST is a sequence-based molecular typing method that determines the nucleotide sequences of some (i.e. 6-10) conserved housekeeping genes (162). This makes it suitable for continuous surveillance and is excellent for collating data obtained from several separate sources via web-based databases. MLST is often used to examine the evolutionary relationships between

bacteria that implies some form of common ancestry among isolates with the same ST. Relatedness between isolates when using MLST, is based on polymorphisms within conserved 'housekeeping' genes. MLST determine the population structure of different species and has led to the identification of certain global antimicrobial resistant STs or clones (162). MLST does not have the necessary discrimination to detect genetic changes among isolates involved in outbreak situations (43).

MLST is perfect for tracing multidrug resistant clones or sequence types (STs) across the globe (162). Unfortunately, MLST is expensive since it often uses Sanger sequencing, but this technique remains the benchmark for the identification of *E. coli* ST131 and *K. pneumoniae* ST258.

As described earlier, there are 2 MLST schemes that are often used to identify *E. coli* ST131. The Achtman scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) uses the gene sequences of seven housekeeping genes; *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), *recA* (ATP/GTP binding motif). The Pasteur institute *E. coli* scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html) uses eight housekeeping genes eight housekeeping genes: *dinB* (DNA polymerase), *icdA* (isocitrate dehydrogenase), *pabB*(p-aminobenzoate synthase), *polB* (polymerase PolII), *putP* (proline permease), *trpA* (tryptophan synthase subunit A), *trpB* (tryptophan synthase subunit B) and *uidA* (beta-glucuronidase). The scheme from the Pasteur institute tends to identify different sequence types among ST131 (as identified by the Achtman scheme). Most investigators use the Achtman MLST scheme to routinely identify ST131.

The Pasteur institute is the most popular MLST scheme used to identify *K. pneumoniae* ST258 (163). (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). It uses seven housekeeping genes that include the following: *rpoB* (beta-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor 2) and *tonB* (periplasmic energy transducer).

Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is the benchmark for molecular typing of medically important bacteria during outbreak investigations (43). PFGE involves the cutting of bacterial DNA into fragments of varying sizes, followed by the separation into fingerprints by gel electrophoresis and this technique is often used to track clones or clusters during outbreak investigations (164).Unfortunately, PFGE is arduous and it takes days to get results while personnel need to be technically skilled in this method to obtain reliable and reproducible results. Additionally, comparing various pulsotypes created in different locations remains a problem.

PFGE was extensively used during the late 2000's to recognize isolates that belonged to *E. coli* ST131 (66) and *K. pneumoniae* ST258 (126). However, PFGE is not a very good method to identify both sequence types because they consist of different pulsotypes that display high levels of intra-lineage genetic variations (45, 81). Both high risk clones often display less than 80% similarity and often do not satisfy the criteria for relatedness outlined by Tenover *et al* (82).

Repetitive sequence-based PCR typing

Three separate studies from Canada, UK and the USA evaluated the ability of the DiversiLab fingerprinting kit, a type of repetitive element PCR, to identify *E. coli* ST131 (165,

166) and *K. pneumoniae* ST258 (167). The DiversiLab system allowed the discrimination of ST131 isolates from other *E. coli* sequence types and ST258 from other *K. pneumoniae* sequence types. The Diversilab system offers kit-based standardized typing results in a rapid fashion but is unfortunately rather expensive and this prohibited its wide spread utilization and implementation of this technique for the recognition of *E. coli* ST131 and *K. pneumoniae* ST258.

Multilocus variable number of tandem repeats analysis (MLVA)

An abbreviated version of MLVA for the detection of *E. coli* ST131 that uses a benchtop capillary electrophoresis instrument was developed by Nielsen et al (168). The exclusion of two loci affected the discriminatory power of the abbreviated MLVA assay, making it less discriminatory than the original assay. However, this approach can be used as a high-throughput assay and did provide rapid typing results for *E. coli* ST131 that were comparable to MLST and PFGE.

Polymerase chain reaction (PCR)

PCR based techniques offer rapid and inexpensive methodologies (when compared to MLST and PFGE) to identify *E. coli* ST131 and *K. pneumoniae* ST258. These techniques are the most popular approaches used to screen for ST131 and ST258 among large numbers of *E. coli* and *K. pneumoniae* isolates. The PCR screening methods are based on the recognition of different SNPs within O serogroup and housekeeping genes specific for *E. coli* ST131 (19) and *K. pneumoniae* ST258 (169).

Several PCR screening methods are widely available to determine whether an *E. coli* isolate belongs to ST131. These techniques include a PCR for SNPs within the 5′ portion of the *rfb* locus specific for O25b (170) and SNPs within the *pabB* gene (171). Johnson and

colleagues designed a sequencing method based on SNPs within the *mdh* and *gyrB* genes (65). A novel technique and a rapid technique based on sequencing of the *fumC* and *fimH* loci (called CH or clono typing) identified the *fimH*30 ST131 lineage as clonotype CH40-30 (172). Blanco and colleagues designed a triplex PCR that was based on the detection of O25b *rfb* allele, $bla_{CTX\text{-}M\text{-}15}$ and the *afa/draBC* virulence factor (173). A recent PCR screening test combined the O16 *rfb* variant with the ST131-specific alleles of *mdh* and *gyrB* that can distinguish between the two serogroups of ST131 namely O25b (*fimH*30) and O16 (*fimH*41) (96). PCR methods are also available for the identification of the *fimH30* lineage and the H30-Rx sub-lineage of ST131 (88, 92, 96). The identification of the H30-Rx sub lineage requires an additional sequencing step to be performed. Just a caution: PCR-based screening of *E. coli* ST131 may infrequently identify isolates that belong to the clonal complex 131 isolates (which varies by one or two alleles from ST131), as ST131 and very rarely can misidentify non-ST131 *E. coli* as ST131.

The multiplex real time PCR from Chen and colleagues for the detection of *K. pneumoniae* ST258, targets 2 single-nucleotide polymorphisms (SNPs) in the *tonB* allele that are unique to ST258 and related STs (169). This PCR also included primers against the *gapA* allele (present in all *K. pneumoniae*) that serves as an internal control. The regular multiplex PCR from Adler and colleagues detects the presence or absence of 2 genes namely *pilv-l* and *prp* (139). Whole genome sequencing has shown that the *pilv-l* allele is specific for ST258 and related STs. Both studies included ST258 and ST512 as positive controls in their evaluation. Chen and colleagues have also developed a multiplex PCR for the identification of different lineages within ST258 i.e. Clade 1 and Clade II (174).

Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a high-throughput methodology based on the identification of the mass-to-charge ratio of peptides and small proteins, most of which are ribosomal (175). It has been increasingly adopted by clinical microbiology laboratories all over the world for quick and reliable microbial identification at the species level based on the comparison of mass spectral fingerprints (obtained from single colonies or crude extracts) with previously established reference databases. The use of MALDI-TOF MS for bacterial typing is based on the principle that sequence variations within a given taxa or subspecies level will be translated into corresponding protein or peptide sequences, and supported by a similar clustering obtained from either mass spectral data and genomic sequences, highlighting the usefulness of peak patterns as taxonomic markers (176). The simplicity, speed and low cost advantages associated with MALDI-TOF MS and its potential application on a large-scale basis might be useful to enable timely and appropriate diagnosis, infection control and individual patient management decisions.

Japanese and Portuguese investigators evaluated MALDI-TOF MS for the identification of *E. coli* ST131. The study from Japan used flexanalysis to successfully distinguish ST131 (O25b and O16 serotypes) from other sequence types with sensitivity of 97.0% and a specificity of 91.5% (177). The study from Portugal showed that MALDI-TOF MS fingerprinting analysis were able to discriminate between ST131, ST69, ST405, ST39 and between phylogenetic group B2 ST131 from other phylogenetic group B2 that did not belong to ST131 (178). MALDI-TOF is a promising tool for the detection of *E. coli* ST131 that might be available for routine use in the near future.

To our knowledge studies that evaluated MALDI-TOFF MS for the detection of *K. pneumoniae* ST258 has not yet been published.

Role of plasmids in high risk clones *E. coli* ST131 and *K. pneumoniae* ST258

Overview

Since plasmids are often maintained and exchanged independently of the bacterial host genome, they undergo evolutionary changes that are unrelated to the host. To understand the interdependence of plasmid acquisition and subsequent dissemination of them in high risk clones a brief description about the classification, similarities and differences among plasmids is required.

Plasmids consist of extra chromosomal DNA and require independent mechanisms for maintenance and coinheritance into daughter cells (9). Large portions of plasmid DNA show high degrees of plasticity that consist of several types of mobile elements such as insertion elements and transposons. This plasticity challenged studies investigating the evolutionary history and relatedness of different plasmids, even when investigators using the latest sequencing technologies. However, DNA that is required for replication of plasmids are conserved and can therefore be used as regions for the comparison and classification of different plasmids obtained over different time periods, geographical areas and from different bacteria. This is referred to as the "incompatibility group typing" scheme and is based on the characterization of unique or conserved replication areas on different plasmids and is primarily used to demonstrate relatedness and behavior of different plasmid groups (179, 180). It is important to remember that not all plasmids can be typed with this technique; plasmids that do not show results with incompatibility typing are often referred to as non-typable. Plasmids that belong to different

incompatibility groups exhibit variable behavior characteristics (e.g. narrow bacterial host range plasmids versus broad host range plasmids; high copy plasmids versus low copy plasmids).

The broad host range vs the narrow host range classification scheme of Proteobacteria plasmids is based on the diversity of the bacterial hosts into which plasmids can be transferred to and then be successfully maintained in a sustainably manner (181). The narrow-host range group of plasmids most often belongs to the incompatibility (Inc) group F that containes different replicons (e.g. FIA, FIB, FII) while the broad-host range group include the IncA/C and IncN types (among others). Broad-host range plasmids can easily be transferred between different species while narrow-host range plasmids tend to be restricted to certain species or even clones within species. This concept is important when evaluating plasmids in high risk clones as there is a predominance of narrow range plasmids (specifically IncF with certain β-lactamases [e.g. $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$]) in these clones. They have recently been termed "epidemic resistance plasmids" due to their propensity to acquire resistance genes and rapid dissemination among the Enterobacteriaceae (9). IncF plasmids used postsegregation killing and addiction systems to ensure their propagation among high risk clones. IncF plasmids are often present in *E. coli* and up to 70 % of plasmids characterized from human and avian *E. coli* belonged to IncF with different replicons (182).

Plasmids have co-evolved with bacteria providing them with rapid ways to evolve and adapt through the accumulation of point mutations, deletions and insertions. Plasmids can provide intact functional genes to various bacteria that will assist them with adapting to harsh conditions and unsuitable environments (183). Plasmids often contain various combinations of virulence, fitness and antimicrobial resistance genes and have contributed significantly to the success of antimicrobial resistant bacteria (184).

Once a resistance plasmid has been transferred to and replicate in a new bacterial host, the presence of antimicrobial agents will create artificial selective pressure that will select for a bacterial population with such resistance plasmids. However, in the absence of antimicrobial agents, the resistance plasmid may only be transiently retained by the bacterium if it is unstable within the host (185). The documentation of the long term stability and persistence of resistance plasmids in various bacterial populations during the absence of antibiotic selection pressures had received surprisingly little attention. Very little is known about the impact of plasmid stability on the long-term survival and host range of resistance plasmids especially in the absence of selection pressure created by antimicrobial agents.

As described before, there is a predominance of narrow range plasmids (i.e. IncF) among high risk clones such as *E. coli* ST131 and *K. pneumoniae* ST258 and it seems such clones with IncF plasmids provides a stable environment for antimicrobial resistance genes (186). Antimicrobial resistance determinants on epidemic plasmids provide a selective advantage to high risk clones and are likely central to their success (187, 188). IncF plasmids are diverse, complex, tend to be narrow range and are present in low copy number (189). The concept of incompatibility for this group is somewhat of a misnomer with several descriptions of multiple replicons being present in the same plasmid with one replicon remaining silent while another replicon is acting as the area of replication (189).

Plasmids associated with *E. coli* ST131

The initial description of *E. coli* ST131 in 2008 by Coque and colleagues included the characterization of eight plasmids harboring $bla_{\text{CTX-M-15}}$ in this ST (52). The sizes of the plasmids varied between 100kb – 150kb, with 3 different restriction fragment length polymorphism patterns and all belonged to IncF with FII and FIA replicons.

The first plasmids from ST131 to be entirely sequenced were obtained in the United Kingdom and included IncF plasmids (i.e. pEK499, pEK516) and the IncI1 plasmid pEK204 (190). Plasmid pEK499 turned out to be a fusion of FII and FIA replicons and contained genes for the following antimicrobial determinants: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *aac*(*6′*)*-Ib-cr*, *mph*(A), *cat*B4, *tet*A, *dfrA7*, *aadA5*, and *sulI*. pEK499 also encoded for the following postsegregation killing systems that are responsible for stable plasmid inheritance during vertical spread: *hok-mok* killing protein and modulator system, the *pemI-pemK* toxin-antitoxin system, the *vagC-vagD* virulence-associated genes, and the *ccdA-ccdB* toxin-antitoxin system. These postsegregation killing systems could be responsible for the persistence of pEK499-like plasmids among ST131 that can occur even in the absence of antibiotic selection pressure. The IncF pEK516 plasmid contained the FII replicon with the following antimicrobial resistance genes: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$ *aac*(*6′*)*-Ib-cr*, *aac* (*3*)*-II*, *cat*B4, and *tet*A. The pEK516 and pEK499 plasmids display 75% DNA sequence similarity, despite pEK516 being substantially smaller than pEK499. Plasmid pEK516 also encoded for the *pemI-pemK*, and *hok-mok* postsegregation killing systems, as well as the type I partitioning locus *parM* and *stbB*. Plasmid pEK204 belonged to a different incompatibility group IncI1 (i.e. a broad-host range type of plasmid) and contained *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{TEM-1}}$ accompanied by an IS*Ecp1*-like mobile element upstream from the *bla*$_{\text{CTX-M-3}}$. These initial plasmid studies were then followed by several global molecular epidemiology studies that characterized plasmids associated with CTX-Ms among ST131. The majority of the plasmids associated with *bla*$_{\text{CTX-M-15}}$ belonged to IncF and contained the fused FIA-FII or FII replicons (19). IncF plasmids present in *E. coli* ST131 can also encode for β-lactamases other than CTX-M-15, such as CTX-M-14, SHV-2, and SHV-12 while *bla*$_{\text{CTX-}}$

$_{M\text{-}15}$ had also been identified on plasmids that belong to other incompatibility groups such as IncI1, IncN, and IncA/C, as well as being on *pir*-type plasmids (19).

The narrow host range IncF and broad-host range plasmids (i.e. IncN, IncN2, IncI1, IncHI2, IncL/M, IncA/C, IncK, IncX4, IncU) as well as the RCR plasmids families had been associated with CTX-Ms enzymes but the IncF plasmids are by far the most common Inc type detected in *E. coli* ST131 harboring $bla_{\text{CTX-M}}$ (191). The $bla_{\text{CTX-M-15}}$ has mainly been found on IncF plasmids with fused FIA-FII or FII replicons in ST131, whereas Inc plasmids with different replicons (i.e. FIB) have been identified in non-ST131 ExPEC (192). Some IncF plasmids with $bla_{\text{CTX-M-15}}$ also encode for multiple virulence determinants (e.g. *iutA ompT*, *hlyF*, *iss* and *iroN*) (193). IncF plasmids provided various antimicrobial resistance determinants and virulence factors that confer additional selective and significant advantages for the ST131 host. IncF plasmids also used postsegregation killing and addiction systems to ensure their propagation and maintenance within *E. coli* ST131 and have played an essential role in the dissemination of the $bla_{\text{CTX-M-15}}$ among this high risk clone even in the absence of artificial selection pressure created by antimicrobial agents. The introduction of such IncF plasmids have created the H30-Rx sublineage and in some areas this sublineage is more prevalent than the H30-R sublineage (Figure 2) (95). Banerjee and colleagues have shown that the H30-Rx sublineage with $bla_{\text{CTX-M-15}}$ containing plasmids had significantly higher virulence scores when compared to H30-R sublineage and non-H30 ST131 implying greater virulence potential created by the presence of such IncF plasmids and possibly providing an explanation for the high prevalence of this sublineage (92). A different study showed that as ST131 gained resistance to the fluoroquinolones and acquired ESBLs, isolates (especially those that had acquired $bla_{\text{CTX-M-15}}$)

tend to accumulate additional virulence factors thus implying that the virulence factors on plasmids are contributed to the success of the H30-Rx sublineage (90).

Plasmids associated with *K. pneumoniae* ST258

Several different KPC-containing plasmids have been identified in ST258. They belong to IncF (with FIIk1, FIIk2, and FIA replicons), IncI2, IncX, IncA/C, IncR, and ColE1 and these plasmids often contain various genes encoding for non-susceptibility to different antimicrobial drugs (30). However, the most predominant *bla*$_{KPC}$ -plasmid types associated with *K. pneumoniae* ST258 is IncF with FIIk replicons (186). The first *bla*$_{KPC}$-plasmid identified in ST258 (named clone Q at that time) was obtained in 2006 from Israel and named pKpQIL (194). This was a 113-kb IncF plasmid with FII$_{K2}$ replicon containing Tn*4401a*, with a backbone very similar to the pKPN4 plasmid first characterized in 1994 from non-KPC antimicrobial resistant *K. pneumoniae* obtained in Massachusetts (194).

Retrospective plasmid analysis of *K. pneumoniae* with *bla*$_{KPC}$ from the New York and New Jersey areas isolated during the early 2000s showed that ST258 contained *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ on pKpQIL-like plasmids which were nearly identical to the Israeli pKpQIL plasmid described in 2006 (195). PKpQIL-like plasmids from the New York and New Jersey isolates were mostly associated with *bla*$_{KPC-2}$ and to a lesser extent with *bla*$_{KPC-3,}$ whereas the pKpQIL plasmids from Israel were mainly associated with *bla*$_{KPC-3}$ (195, 196). This suggests that ST258 with *bla*$_{KPC-3}$ on KpQIL plasmids were introduced during the mid-2000s from the USA into Israel (a Founder effect) followed by clonal expansion in Israel. The IncFIIk plasmids are also the most common plasmid identified among ST258 with *bla*$_{KPC}$ from several different geographically diverse areas including Canada, Poland, United States, Israel, Brazil, Italy, and Norway (132, 186, 197). There also appears to be an association between different plasmid Inc

groups and the ST258 clades I and II. The *bla*$_{KPC-3}$-associated IncI2 plasmids and *bla*$_{KPC-3}$-associated IncFIA plasmids were found exclusively in clade II while the pKpQIL- associated IncFIIk2 plasmids with *bla*$_{KPC-2}$ were detected in both clades I and II (137). pKpQIL plasmids were not only restricted to ST258 and were present in 33% of non-ST258 *K. pneumoniae* in the New York area (195).

The complete sequencing of plasmids associated with ST258 from large collections are revealing that they are evolving over time through large genetic rearrangements (186, 198, 199). This process is creating hybrid plasmids as was previously described in Italy with ST258 containing two different IncF plasmids namely pKpQIL-IT and pKPN-IT as well as a ColE-like plasmid with *bla*$_{KPC-2}$ (200). Both pKpQIL-IT and pKPN-IT have a very high degree of homology to the historic plasmids pKPN4 and pKPN3 from a non-KPC producing *K. pneumoniae* isolated in 1994 (200). This suggests that certain ancestral plasmids are particularly well suited to Enterobacteriaceae such as *K. pneumoniae* and are good candidates for sustaining the presence of *bla*$_{KPC}$ through multiple independent insertions and transposition events. This is further supported by a recent study from Korea that demonstrated that ancestral plasmids were present among ST258 isolates from various geographical regions and were obtained as early as 2002 (144).

Logic might dictate that when a single *bla*$_{KPC}$ plasmid entered into ST258 isolates at a single time point and it will develop diversity over time. However, it appears that *K. pneumoniae* ST258 isolates had acquired various types of *bla*$_{KPC}$ plasmids at multiple time points and different geographical locations around the globe, that was then followed notable local spread of ST258 with the specific KPC plasmid (30).

It seems that the presence of plasmids with *bla*$_{KPC}$ is central to the success of ST258. The loss of pKpQIL by ST258 has limited the ability of these isolates to successfully disseminate when compared to other *K. pneumoniae* without *bla*$_{KPC}$ and ST258 with pKpQIL (188). This would suggest that the *bla*$_{KPC}$ in combination with other virulence or persistence factors on the pKpQiL-like plasmids promoted the fitness and survival of ST258. This is further supported by the epidemiological observation that non-ST258 *K. pneumoniae* with *bla*$_{KPC}$ did not demonstrate the same global success as ST258 with *bla*$_{KPC}$. It appears that the successful global dissemination and survival of *K. pneumoniae* ST258 is in part dependent on the combination of *bla*$_{KPC}$ on IncF plasmids with factors inherently present on the chromosome of this high risk clone (187).

IncI2 with *bla*$_{KPC-3}$ can also successfully pair with ST258 and was recently detected in 23% of ST258 from the New York and New Jersey areas (201). Interestingly, this IncI2 plasmid also contained type IV pili which may contribute to successful dissemination of *K. pneumoniae* ST258.

Broad host range plasmids (i.e. IncA/C, IncL/M, IncN)

Broad host range plasmids are rare in *E. coli* ST131 and in *K. pneumoniae* ST258 (19, 30) . This is a curious finding and it seems that at this point in time, broad range plasmids do not play a meaningful role in the success and global dominance of *K. pneumoniae* ST258 and *E. coli* ST131.

Conclusion

There is no doubt that the high risk clones *E. coli* ST131 and *K. pneumoniae* ST258 are important human pathogens, have spread extensively throughout the world and are responsible for the sudden increase in multi-drug resistance among *E. coli* (17) and *K. pneumoniae* (122)

Table 4. Summary of *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST258

Characteristic	*Escherichia coli* ST131	*Klebsiella pneumoniae* ST258
Clonal complex	CC131 (ST131 being the dominant)	CC258 (ST258 being the dominant; also includes ST11, ST340, ST437, and ST512)
Multi-drug resistant High Risk clone	Yes	Yes
Endemic regions	Global	USA, Greece, Italy, Israel, Columbia
Resistance determinants	Various but associated with fluoroquinolone resistance and CTX-M-15 (CTX-M-14 to a lesser extent)	Various but associated with KPC-2 and KPC-3
Mobile elements	IS*Ecp*1	Tn*4401*
Plasmids	Various incompatibility groups but IncF with virulence factors are the most common	Various incompatibility groups but IncFIIk are the most common
Clades/Lineages	O25:*fimH*30 lineage that consists of the H30-R and H30-Rx sublineages O16:*fimH*41 lineage	Clade I associated with KPC-2 Clade II associated with KPC-3
Population biology	H30-R sublineage is associated with fluoroquinolone resistance and the H30-Rx with CTX-M-15	Clade II is a hybrid of ST11 and ST442 Clade I is a hybrid of Clade II and ST42
Acquisition	Community onset health care associated, LTCFs	Nosomial, LTCFs
Type of infections	UTIs, IAIs, BSIs	UTIs, RTIs, BSIs
Mortality	Unclear if higher than non-ST131 *E. coli*	Higher when compared to non-258 *K. pneumoniae* (most likely due to patient underlying conditions)

UTIs (urinary tract infections), IAIs (intra-abdominals infections), BSIs (blood stream associated infections), RTIs (respiratory tract infections), LTCFs (long term care facilities)

respectively. ST131 is responsible for extra-intestinal infections (especially UTIs), most often fluoroquinolone resistant and can also be linked with the production of CTX-M-15 (19) while ST258 is known to cause UTIs, respiratory tract infections, BSIs and is associated with carbapenemase production most often to KPC-2 and KPC-3 (202) (Table 4).

Biological evolution is defined as "descent with modification within a defined population" with the sole purpose for adaptation to environmental changes (203). This definition encompasses micro-evolution (i.e. changes in gene frequency within a population from one generation to the next) and macro-evolution (i.e. the descent of different species from a common ancestor over many generations). Evolution provides the means for members of a population to adapt, survive and reproduce in an effective manner. For evolution to progress in an orderly and efficient fashion, a selection process for desirable traits is required. Natural selection is due to the interaction of a population with the environment while artificial selection (also referred to as selective breeding) occurs due to human interaction. Antimicrobial therapy with the subsequent selection of antimicrobial resistant mutations is considered as being one of the most efficient artificial selection pressures introduced by humans (204).

E. coli ST131 has long been "flying under the antimicrobial resistance radar" and therefore did not generate the same awareness as other multidrug resistant bacteria. Retrospective molecular epidemiology surveys have demonstrated that ST131 was rare among *E. coli* from the 1990s and early 2000s (84, 95). However, during the mid to late 2000s, the numbers of fluoroquinolone resistant ST131 increased substantially that was followed by an explosive increase of isolates with $bla_{\text{CTX-M-15}}$ towards the end of the 2000s (19). The reasons for the sudden increase are not clear or well understood. Recent studies utilizing advanced technologies such as whole-genome sequencing combined with phylogenetic analysis have

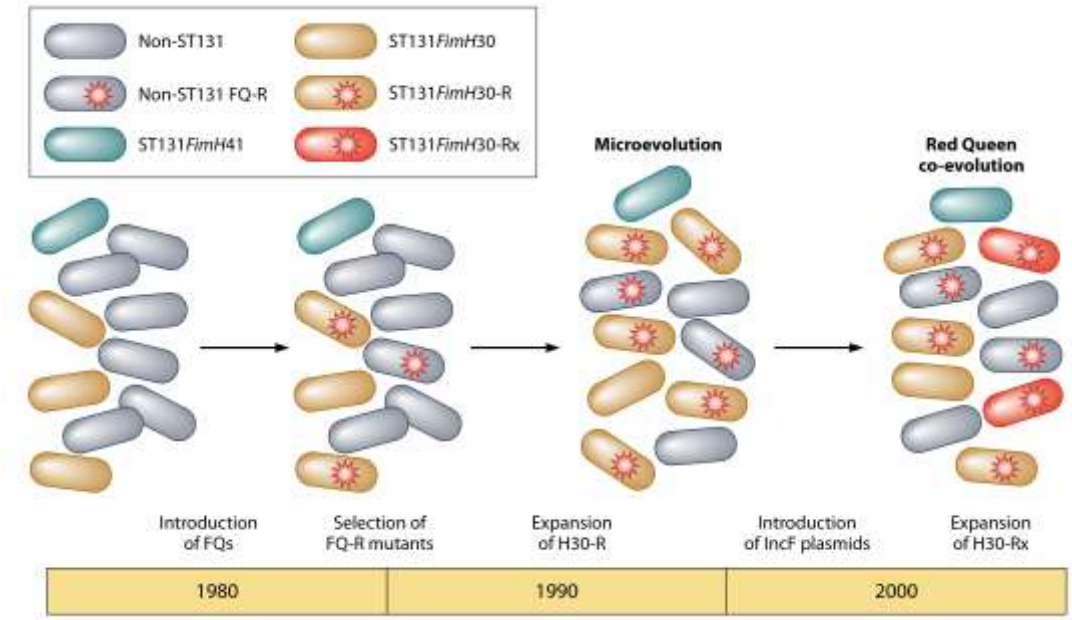

Figure 4. The change in the population structure of *Escherichia coli* due to selection pressures created by the fluoroquinolones and the oxyimino-cephalosporins. FQ; fluoroquinolone, FQ-R; fluoroquinolone-resistant

demonstrated that a lineage within ST131 named *fimH*30 emerged during the early 2000s. This was then followed by the rapid expansion of its sublineages H30-R and H30-Rx that had been responsible for the global emergence of fluoroquinolone resistance and CTX-M-producing ExPEC. The widespread use of antimicrobials had possibly selected for ST131 resulting in the emergence of ST131. The strong association between ST131 and fluoroquinolone resistance suggested that use of fluoroquinolones, has contributed to the expansion of the H30-R ST131 sublineage. Likewise, use of the oxyimino-cephalosporins may have driven the expansion of H30-Rx sublineage, which is strongly associated with CTX-M-15. The selection pressures created by the widespread use of the fluoroquinolones and the oxyimino-cephalosporins dramatically changed the population structure of *E. coli*, especially among ExPEC, and created one of the most prevalent and extensive global antimicrobial-resistant *E. coli* sublineages known

to the medical community (84).This is an excellent example of micro-evolution in action due to the artificial selection pressure created by the use of antimicrobial agents (Figure 4).

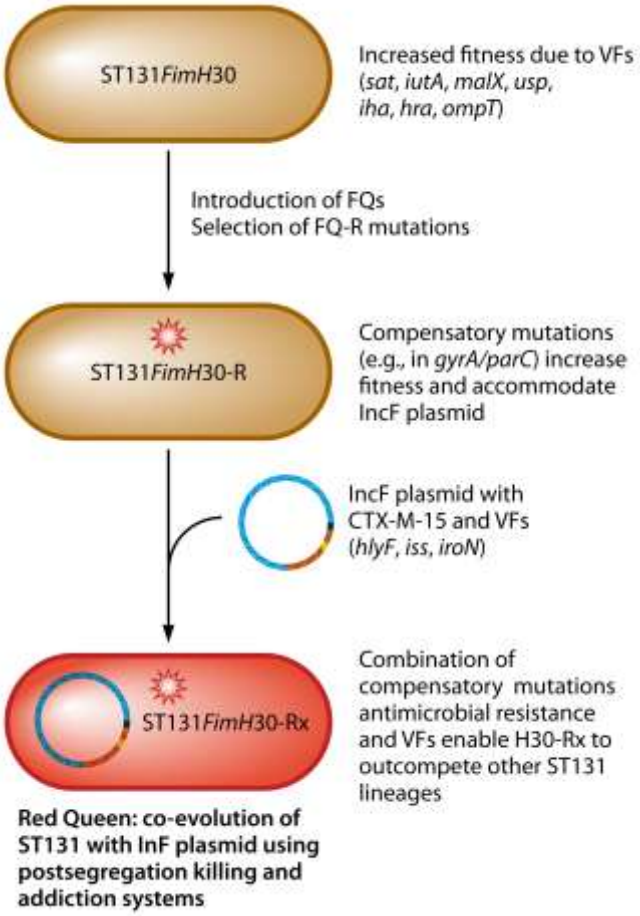

Figure 5. The possible factors that make *Escherichia coli* ST131 *fimH*30 lineage such a successful high-risk clone VFs; virulence factors, FQ; fluoroquinolone, FQ-R; fluoroquinolone-resistant

The prevalence of certain virulence genes such as *sat*, *iutA*, *malX*, *usp*, *iha*, *hra*, and *ompT* are consistently higher among ST131 (when compared to non-ST131 ExPEC) and these genes might increase the versatility, competitiveness and the capacity of ST131 to effectively infect the human body (Figure 5). The exact role of these virulence genes remain to be explained, however, it is possible that a combination of factors contribute to the fitness of ST131 rather than

being directly involved in the pathogenesis of infection (79). *In vivo* studies suggest that fluoroquinolone resistant *E. coli* undergo compensatory mutations and become as fit as fluoroquinolone susceptible isolates (46).

This brings up some intriguing issues: Is *E. coli* ST131 inherently more fit than other ExPEC clones and therefore, able to better survive in certain environments? Is this "fitness" due to certain virulence factors (e.g. *sat, iutA, malX, usp, iha, hra,* and *ompT*) that provided ST131 with opportunities to be exposed to and acquired certain narrow-host range IncF plasmids? And did the *gyrA*/*parC* compensatory mutations (i.e. *gyrA*1AB and *parC*1aAB) selected by fluoroquinolones, reduce the fitness costs associated with IncF plasmids? Is it perhaps possible that the mutations accommodating IncF plasmids increased the fitness of ST131, enabling them, with the aid of certain virulence factors and antimicrobial resistance determinants on the CTX-M-15 plasmids, to out compete other ExPEC clones? Colpan and colleagues have shown that fluoroquinolone susceptible *E. coli* ST131 have different virulence factor profiles than fluoroquinolone susceptible non-ST131 and as ST131 gained resistance to the fluoroquinolones and acquired ESBLs (especially CTX-M-15), they tended to accumulate additional virulence factors (90). Furthermore, *in-vitro* studies have shown that ST131 has high metabolic potential and enhanced ability to produce biofilms (19). These studies suggest that the inherent combination of different virulence factors, biofilm production and metabolic potential provide ST131 with means to be "more fit" that other ExPEC clones. Moreover, it seems that ST131 (especially the *fimH*30 lineage) has the ability to counteract for the fitness cost associated with antimicrobial resistance and also tends to accumulate virulence factors as this clone/lineage becomes progressively more antimicrobial resistant (90). With its combination of multidrug resistance and ecological success, the ST131 *fimH*30 lineage counters the hypothesis that

increased antimicrobial resistance entails a significant fitness cost. *E. coli* ST131 is most likely the best example of an international multi-resistant high risk clone (Figure 5).

The role of IncF plasmids with $bla_{\text{CTX-M-15}}$ in the success of ST131 is another intriguing issue. IncF plasmids provide various antimicrobial resistance determinants but some plasmids also encode for multiple virulence factors that confer additional selective and significant advantages for the ST131 host (193). IncF plasmids use postsegregation killing and addiction systems to ensure their propagation among high risk clones, and have played an essential role in the dispersion of $bla_{\text{CTX-M-15}}$ among *E. coli* ST131 by creating the H30-Rx sublineage. A population-based molecular surveillance study (2000-10) from Calgary showed that the H30-Rx sublineage with $bla_{\text{CTX-M-15}}$ became the most dominant sublineage towards the later part of the study period (95) (Figure 2). It is therefore possible that the co-evolution of certain sublineages within high risk clones (i.e. H30-Rx) and epidemic plasmids (e.g. IncF) provided rapid and continual adaptation for certain sublineages giving them the additional ability to outcompete other sublineages without such epidemic plasmids (Figure 5). This is consistent with both the macro- and micro-evolutionary versions of the Red Queen hypothesis as demonstrated recently using the microscopic roundworms *Caenorhabditis elegans* as hosts and the bacteria *Serratia marcescens* as parasites to create a host-parasite co-evolutionary system (205) (Figures 5 and 6).

Recent molecular studies have shown that *K. pneumoniae* ST258 consists of 2 distinct lineages namely clade I and clade II (137). Clade I was specifically associated with $bla_{\text{KPC-2}}$ and clade II with $bla_{\text{KPC-3}}$ (Table 4). The genetic differentiation between the two clades occurred in a 215-kb region that included genes responsible for capsule polysaccharide biosynthesis indicating that these 2 clades had distinct evolutionary pathways. Additional investigation showed that ST258 clade II is a cross-hybrid clone that was created by a large recombination event between

ST11 and ST442 (138). Moreover, it seems that ST258 clade I was created by the *cps* replacement in ST258 clade II that originated from ST42 (Figure 3). The integrative conjugative element ICEKp258.2 contains gene clusters for a type IV pilus (i.e. *pilV*) and a type III restriction-modification system. The *pilV* on ICEKp258.2 may in part be responsible the high transmissibility and durability of ST258 on foreign surfaces and it seems that this integrative conjugative element contribute significantly to the epidemiological success of *K. pneumonaie* ST258 (138).

The use of antimicrobial agents will continue to create selection pressure that gives high risk clones the opportunity to become effective intestinal colonizers and provide opportunities for them to cause infections. To prescribe more-effective empirical antimicrobial therapy, clinicians will require increased awareness about *E. coli* ST131 and *K. pneumoniae* ST258. More specifically the prevalence and extensive antimicrobial resistance capabilities of these high risk clones including which patients are at risk for infection. *E. coli* ST131 and *K. pneumoniae* ST258 cause similar infections to non-ST131 *E. coli* and non-ST258 *K. pneumoniae* respectively and clinical risk factors most likely will be insufficient to reliably identify patients with increased risk for infections with these clones. Therefore, the medical community needs cost effective diagnostic tests that have the ability to rapidly detect *E. coli* ST131 and *K. pneumonia* ST258. PCR and sequence based approaches have been developed and reliably detect both clones in a matter of hours (Table 3), but unfortunately due to cost and personnel issues, these methods are difficult to implement routinely in a diagnostic laboratory. Such approaches might provide timely and appropriate antimicrobial therapy that can help to improve clinical outcomes with infections due to *E. coli* ST131 and *K. pneumonia* ST258.

E. coli ST131 and *K. pneumoniae* ST258 and their linegaes/clades are now major threats to public health due to their world-wide distribution and association with multidrug resistance. The recent emergence of *E. coli* ST131 with carbapenemases poses a significant additional public health menace due to its relation with the very dominant *fimH*30 lineage.

Future studies should be undertaken to determine if antibiotic stewardship and which infection control bundles will decrease colonization and infections with *E. coli* ST131 and *K. pneumoniae* ST258. Additionally, vaccines that target *E. coli* ST131 serotypes O25b and O16, and those that target the *cps* region of *K. pneumoniae* ST258 clades I and II should also be developed. This might reduce the reservoir of asymptomatic individuals colonized with these high risk clones and will reduce the spread and infection due to *E. coli* ST131 and *K. pneumoniae* ST258.

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