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Novel diagnostic methods for rapid characterization of extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae

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NOVEL DIAGNOSTIC METHODS FOR RAPID CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE- AND CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE

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To my *teachers, friends and family*

ABSTRACT

Klebsiella pneumoniae and *Escherichia coli* are two of the most clinically important pathogens of the family Enterobacteriaceae. Both can cause severe infections including urinary tract infections (UTI), bloodstream infections (BSI) and pneumonia. Both are also involved in dissemination of antibiotic resistance. The prevalence of extended-spectrum β-lactamase- (ESBL) producing Enterobacteriaceae (EPE) and carbapenemase-producing Enterobacteriaceae (CPE) are increasing worldwide. This is a severe threat to public health, as antibiotic treatment becomes limited. The general aim of the thesis was to investigate the clonal diversity and prevalence of ESBL in invasive isolates of *Klebsiella pneumoniae* from the Stockholm area, the acquisition of EPE/CPE in healthy Swedish tourists travelling to regions with high prevalence of EPE/CPE, and finally to evaluate rapid detection and characterization methods for EPE and CPE.

In Paper I, we studied the clonal structure of *K. pneumoniae* from BSI patients in a well-defined geographical area, as well as the prevalence of ESBL in these strains. We also characterized the clinical isolates to investigate the association between phylogroups, bacterial virulence factors, 30-day mortality and comorbidity. A total of 139 samples were collected from the Department of clinical microbiology at Karolinska University Hospital, Stockholm, Sweden. Only five out of 139 isolates were detected as multidrug-resistant (MDR) and all five isolates were EPE. Only one isolate was detected as a CPE. Further, all isolates were tested for mucoid phenotypes, serotypes (K1, K2, K5, K20, K54 and K57) and screened for virulence genes using real-time PCR. To identify risk factors, we retrieved data from medical records including age, sex, time to adequate treatment, hospital acquired infections, comorbidity and mortality. The 30-day mortality was primary end-point. All isolates were further subjected to multilocus sequence typing (MLST) for phylogenetic analysis. We found that all isolates were divided into three distinct phylogroups; KpI (consisting of 96 isolates of *K. pneumoniae*) followed by phylogroup KpIII (consisting of 34 isolates of *K. variicola*) and phylogroup KpII (consisting of 9 isolates of *K. quasipneumoniae*). We observed 24/139 (17.3%) overall 30-day mortality. We also observed that *K. variicola* KpIII (29.4%) were highly associated with 30-day mortality compared to *K. pneumoniae* KpI isolates (13.5%). We also observed that several comorbidities; malignancy, diabetes mellitus, cirrhosis, biliary tract disorders and alcoholism among patients may influence the mortality.

In Paper II, we evaluated a commercial version of RAPIDEC® CARBA NP for rapid detection of carbapenemases against 276 Gram-negative bacilli. According to the manufacturer's protocol RAPIDEC® CARBA NP assay was performed on 138 carbapenemase-producers and 138 non-carbapenemase-producers. Carbapenemase detection was also performed by using conventional and real-time PCR. A total of 135 out of 138 carbapenemase-producers were detected successfully by the RAPIDEC® CARBA NP assay. The RAPIDEC® CARBA NP assay was unable to detect one OXA-48-producing *K. pneumoniae* and two *Acinetobacter baumannii*

producing OXA-23 and OXA-24 carbapenemases. The RAPIDEC® CARBA NP assay successfully detected 135 non-carbapenemase producers. A total of two false positives; one *Pseudomonas aeruginosa* with OprD loss and efflux, one *Enterobacter cloacae* with impermeability were detected by the RAPIDEC® CARBA NP assay. The overall sensitivity and specificity of the RAPIDEC® CARBA NP assay was 97.8% and 98.5%, respectively.

In Paper III, we investigated the acquisition of EPE and CPE among healthy Swedish travellers visiting regions with high prevalence of EPE and CPE; Southeast Asia, Indian subcontinent, North Africa and the Middle East. We studied 188 healthy adult travellers and excluded 13 pre-travel colonized tourists from the analysis. Molecular characterization was performed using real-time PCR and survey data were collected for analysis. We detected a total of 67/175 tourist were colonized by EPE strains and most dominant species was *E. coli* (n=65), followed by *K. pneumoniae* (n=1) and *Citrobacter freundii* (n=1). CTX-M type (CTX-M-1 n=49, CTX-M-9 n=12) was the most prevalent EPE type detected in 61 isolates but carbapenemases were absent. Only six travellers had strains with the virulence factor pyelonephritis-associated pili-encoding genes (*pap*) genes, and all of those had travelled to India. One traveller to Thailand was colonized with colistin resistance gene *mcr-1*. The highest colonization rate was detected among the travellers to the Indian subcontinent (49%) and northern Africa (44%) whereas lower rates were detected in travellers to Southeast Asia (19%) and Turkey (10%). EPE colonization was associated with diarrhea and antibiotic treatment during the trip but was not associated with sex, age, duration of trip, intake of proton-pump inhibitors, or use of oral cholera vaccine.

In paper IV, we assessed a CRISPR/Cas assisted optical DNA mapping method for rapid identification and characterization of plasmids during an EPE outbreak. We studied a total of 17 neonates initially colonized with ESBL-producing *K. pneumoniae* (ESBL-KP). In follow-up samples we observed that some of them were colonized with ESBL-producing *E. coli*. This method successfully detected two plasmids of different sizes; small size (80 kbp) and larger plasmid (162-222 kbp) in all ESBL-KP isolates. Using this method, we also observed that the $bla_{\text{CTX-M-15}}$ gene was located on the smaller size plasmid. In follow-up samples we also observed that the ESBL-KP clone was present and $bla_{\text{CTX-M-15}}$ was stable up to two years. This method demonstrated that plasmids can change over time: three deletions were observed in three plasmids of three isolates with different sizes (~5 kbp), (~55 kbp) and (~31 kbp) and an inversion of 31 kbp in another isolate. This method also confirmed that resistance plasmids were not transferred from one patient to another.

In conclusion, this thesis provides new knowledge on the prevalence of EPE and CPE among BSI patients and healthy Swedish tourists, along with describing the molecular features of ESBL- and carbapenemase-producing Enterobacteriaceae. This thesis also demonstrates the performance of a rapid phenotypic carbapenemase detection assay, as well as a novel CRISPR/Cas-assisted optical DNA mapping method for rapid plasmid characterization.

LIST OF SCIENTIFIC PAPERS

I. Makaoui Matallah, Malin Vading, **Muhammad Humaun Kabir**, Amina Bakhrouf, Mats Kalin, Pontus Naucler, Sylvain Brisse, Christian G Giske. *Klebsiella variicola* is a frequent cause of bloodstream infection in the Stockholm area, and associated with higher mortality compared to *K. pneumoniae.* PLoS One. 2014;9(11):e113539

II. **Muhammad Humaun Kabir,** Daniele Meubier, Katie L. Hopkins, Christian G. Giske and Neil Woodford. A two-centre evaluation of RAPIDEC® CARBA NP for carbapenemase detection in Enterobacteriaceae*, Pseudomonas aeruginosa* and *Acinetobacter* spp. J Antimicrob Chemother. 2015; 71(5):1213-6.

III. Malin Vading, **Muhammad Humaun Kabir**, Mats Kalin, Aina Iversen, Susanne Wiklund, Pontus Naucler, Christian G. Giske. Frequent acquisition of low-virulent strains of ESBL-producing *Escherichia coli* in travellers. J Antimicrob Chemother. 2016;71(12):3548-3555.

IV. Santosh K. Bikkarolla, Viveka Nordberg, Fredrika Rajer, Vilhelm Müller, **Muhammad Humaun Kabir**, Sriram Kesarimangalam, Albertas Dvirnas, Tobias Ambjörnsson, Christian G. Giske, Lars Navér, Linus Sandegren, Fredrik Westerlund. Optical DNA mapping combined with Cas9-targeted resistance gene identification for rapid tracking of resistance plasmids in a neonatal intensive care unit outbreak. MBio 2019;10(4). pii: e00347-19.

CONTENTS

LIST OF ABBREVIATIONS

AACs	N-acetyltransferases
ANTs	Aminoglycoside O-nucleotidetransferases
BSI	Bloodstream infections
EPE	ESBL-producing Enterobacteriaceae
ESBLs	Extended-spectrum β-lactamases
HAP	Hospital-acquired pneumonia
HUS	Hemolytic-uremic syndrome
HvKP	Hypervirulent *K. pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharides
mcr-1	Plasmid-mediated colistin resistance gene
MDR	Multi-drug resistance
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase-type β-lactamase
rmpA	Regulator of the mucoid phenotype
SSI	Surgical site infection
UTI	Urinary tract infections
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization

1 INTRODUCTION

1.1 Antibiotic resistance

In 1928, Sir Alexander Fleming serendipitously discovered a new drug with remarkable properties; penicillin. Subsequently, related compounds were developed with even broader antimicrobial spectrum – the cephalosporins, the carbapenems, and the monobactams. The Greek words *anti-* (means against) and *bios* (means life) are the source of the word antibiotic. Antibiotics are the substances that can kill or inhibit the growth of bacteria. Antibiotics can be chemical agents such as sulfa compounds or can be derived from natural origin with a chemical modification such as *Penicillium notatum*.

Micro-organisms capable of evading antibiotic action, commonly known as antibiotic resistance, is according to the World Health Organization (WHO) one of the greatest threats to human health. Estimates suggest that due to multidrug-resistant bacterial infections around 25,000 people are dying each year and the cost of treatment is about €1.5 billion annually in EU countries (1, 2). Over 2 million people are infected annually with antibiotic-resistant bacteria in the United States and 23,000 people are dying due to resistant bacterial infections (3) (4). It has been estimated that ten million people would die annually in 2050 if the development of resistance continues as now (5) (Figure 1).

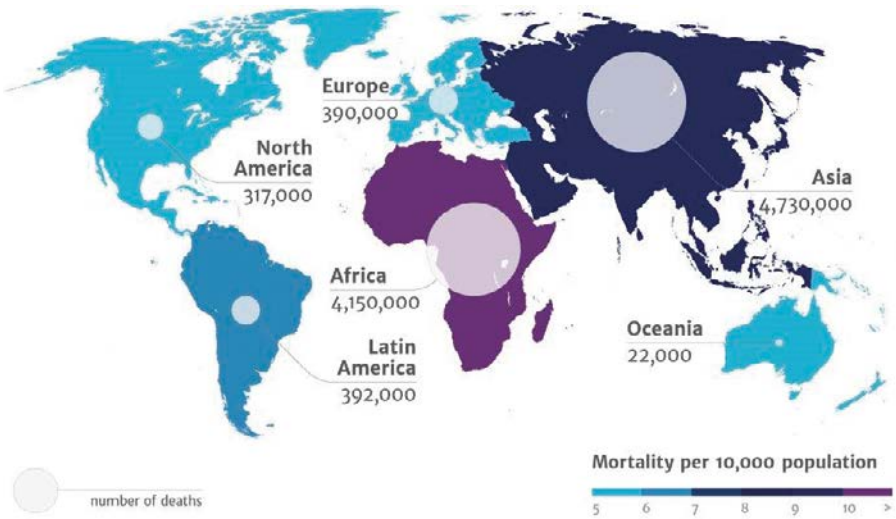

Figure 1. *Annual estimated deaths due to antimicrobial resistance by 2050. Rights to reprint from amr-review.org*[5].

1.2 Mechanisms of antibiotic resistance

Bacteria exhibit antibiotic resistance intrinsically or they can acquire it. Resistance mechanisms to some common antibiotics are listed in (Table 1) (6). Intrinsic resistance is the ability of a bacterium to resist antibiotic action by its inherent structural or functional characteristics. In comparison to Gram-positive bacteria, intrinsic resistance to a variety of compunds is rather common in Gram-negative due to i) differences in the composition of the cytoplasmic membrane; presence of anionic phospholipids is low in the cytoplasm of Gram-negative bacteria, ii) absence of susceptible targets and iii) inability of the antibiotics to cross outer membrane of Gram-negative bacteria. For example, vancomycin is active against Gram-positive bacteria by inhibiting peptidoglycan crosslinking by binding to D-Ala-D-Ala peptides, but it is not active against Gram-negative bacteria as it cannot reach to peptides in periplasm (7).

Gram-negative pathogens can also acquire resistance through gene mutations or horizontal gene transfer. The mechanisms of acquired resistance can be classified in four main classes; (i) antibiotic modification/inactivation, (ii) antibiotic target alteration, (iii) increased antibiotic efflux, and (iv) reduced antibiotic uptake (Figure 2) (8).

Antibiotic inactivation can occur by hydrolysis of β-lactam antibiotics. The four-membered β-lactam ring is hydrolyzed by β-lactamase enzymes (Figure 2A). Different groups of β-lactamases have been reported, which can inactivate different groups of antibiotics including, penicillin, cephalosporins and carbapenems (9). Some enzymes such as aminoglycoside *N*-acetyltransferases (AACs), aminoglycoside *O*-nucleotidetransferases (ANTs) and aminoglycoside *O*-phosphotransferases (APHs) can modify aminoglycoside antibiotics to exhibit resistance against aminoglycoside antibiotics (10).

Target alteration is another common mechanism of resistance against several classes of antibiotics where the target of antibiotic is changed. One example of such target alteration is DNA-topoisomerase complex. Mutation of the DNA gyrase encoding gene (*gyrA*) can alter the protein structure which reduces fluoroquinolone-binding affinity leading to resistance (11). Modification of specific nucleotides in the aminoglycoside binding site of 16S rRNA by 16S rRNA methylases can confer high-level aminoglycoside resistance (12).

Bacteria use efflux pumps to force out antibiotics from the cell so that they can lower the antibiotic concentration to sublethal levels (Figure 2B). This is a first line defence and it can force out different compounds, depending on the specificity of the efflux pump. Using efflux pumps, bacteria can develop multidrug resistance, as they can simultaneously export drugs of different drug classes (13).

Table 1. Mechanisms of resistance to different classes of antibiotics against Gram-negative bacteria. Modified from Kumar et al 2013 with permission from the publishers[6].

Antimicrobial class	Mechanism of Resistance	Specific means to achieve resistance	Examples
β-lactams	Degradation of antibiotics	Destruction of β-lactam ring by β-lactamases making it incapable to bind to penicillin binding protein (PBPs)	Resistance to penicillins, cephalosporins and carbapenems
	Decreased uptake	Decreased porin channel formation	Carbapenem resistance in *K. pneumoniae*, *Enterobacter* spp. and *P. aeruginosa*
Polymyxins	Altered target	Mutational changes in LPS. Modification of lipid A by enzyme MCR	Colistin resistance in *K. pneumoniae* and *E. coli*
Glycylcyclines	Efflux	Pump tigecycline and eravacycline out of the cell	Tigecycline resistance in *K. pneumoniae*
Trimethoprim-Sulfamethoxazole	DNA synthesis	Acquisition of *dfr* and *sul* genes that can circumvent the inhibitory effect of trimethoprim and sulfa in the synthesis pathway of tetrahydrofolate	Trimethoprim and sulfonamide resistance in *E. coli* and *K. pneumoniae*
	Permeability barrier and/or efflux	Permeability barrier and/or efflux act against both sulfonamides and trimethoprim	Trimethoprim and sulfonamide resistance in *E. coli* and *K. pneumoniae*
Aminoglycoside	Enzymatic modification	AACs, ANTs and APH enzymes modify aminoglycoside	Aminoglycoside resistance in Gram-negative bacilli
	Efflux	Downregulation or alteration of porin channels	Aminoglycoside resistance in Gram-negative bacilli
	Altered target	Modification of ribosomal proteins or 16S rRNA	16S ribosomal RNA (rRNA) methylases in Enterobacteriaceae and *P. aeruginosa* and *Acinetobacter* spp.
Fluoroquinolones	Decreased uptake and increased excretion	Alteration in the outer membrane diminishes uptake of drug. Activation of efflux pumps to excrete the drug	Fluoroquinolone resistant Gram-negative bacteria
	Altered target Enzymatic modification	Changes in DNA gyrase and topoisomerase or target protection (Qnr) AAC6´Ib-CR can confer FQ resistance through enzymatic modification	Fluoroquinolone resistant Gram-negative bacilli Fluoroquinolone resistant Gram-negative bacilli

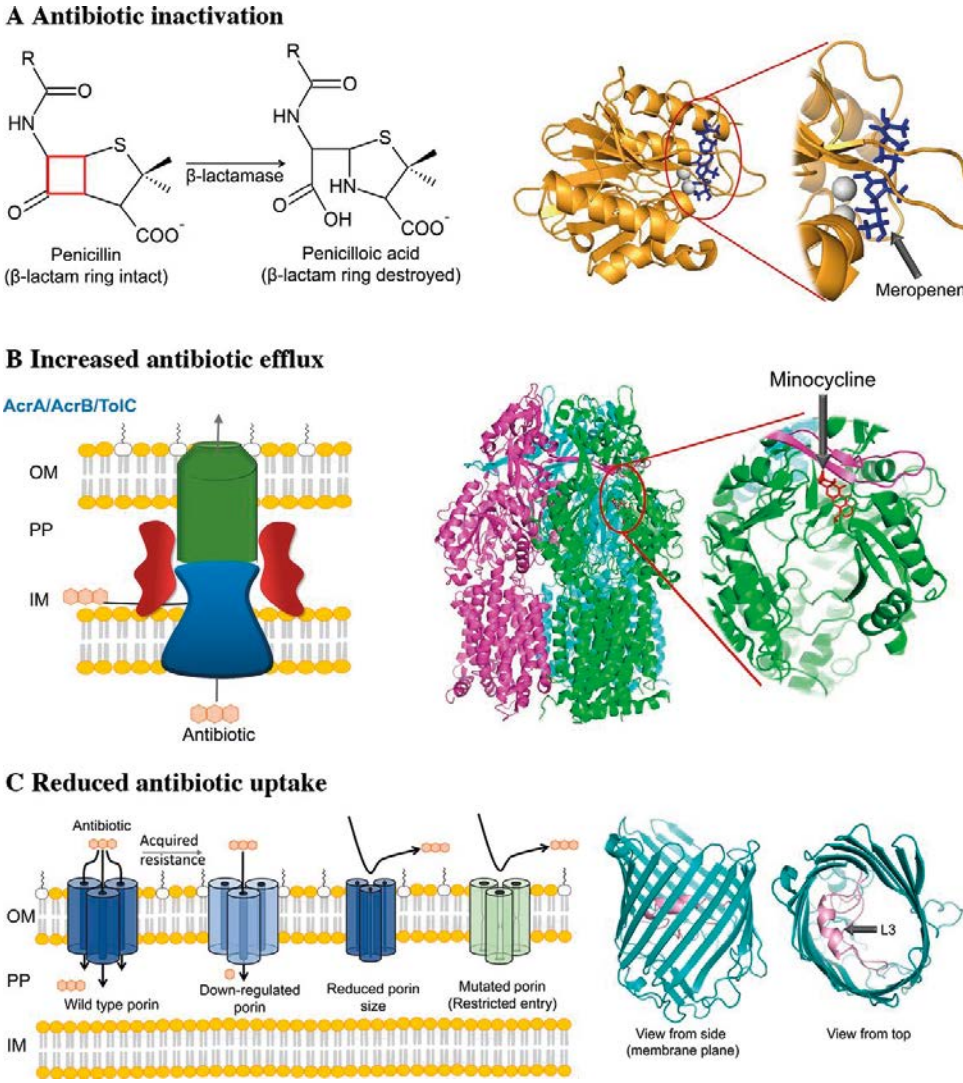

Figure 2. *Different mechanisms of antibiotic resistance (Rights to reprint from Essays in Biochemistry[8]).*

Gram-negative bacteria can reduce uptake of antibiotics by altering outer membrane permeability (Figure 2C). The outer membrane of Gram-negative bacteria is a barrier, but porins in the outer membrane facilitate the diffusion of small hydrophilic antibiotics such as β-lactam antibiotics. Gram-negative bacteria can acquire resistance through (i) down-regulation the expression of porins by mutations (e.g. loss of OmpF in *Escherichia coli* exhibits resistance to β-lactam antibiotics), (ii) substitution of smaller channel size porin with a large channel size porin (e.g.

Klebsiella. pneumoniae isolates exhibit resistance against β-lactam antibiotics by substitution of OmpK35 with a smaller channel size OmpK36), (iii) impaired porin function by mutation (e.g. permeation of benzylpenicillin is reduced in PenB porin of *Neisseria gonorrhoeae* by addition of two negatively charged amino acids in the channel-constricting loop 3) (14-16).

1.3 Enterobacteriaceae

Enterobacteriaceae is a family of rod-shaped Gram-negative bacteria, which are commensal inhabitants of the intestinal microbiota, but which are also a common source of both nosocomial and community-acquired infections. Enterobacteriaceae can cause a wide range of infections; cystitis, pyelonephritis, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections. *K. pneumoniae* and *E. coli* are the most common human pathogens within the Enterobacteriaceae family.

1.3.1 Klebsiella pneumoniae

In 1882, Carl Friedlander first isolated *Klebsiella pneumoniae* from the lungs of a patient with pneumonia (17). *Klebsiella* species are abundant in nature, they can be found in plants, animals and humans. They are one of the most important member of family Enterobacteriaceae of Gram-negative pathogenic bacteria. They are non-sporulating, lactose-fermenting, facultatively anaerobic, rod-shaped and non-motile bacteria. They can cause severe infections in humans, including respiratory tract infections, urinary tract infections, and bloodstream infections (18). They can cause both nosocomial and community-acquired infections. *Klebsiella* species are the third leading cause of hospital-acquired infections in United States (19) and they are among the leading causes of ventilator-associated pneumonia among intensive care units' patients (20). They are also considered as the second leading cause of bloodstream infections (BSI) among Gram-negative bacteria (18, 19).

Capsule, lipopolysaccharides (LPS), fimbriae, siderophores (enterobactin, aerobactin, salmochelin, yersiniabactin) and efflux pumps are considered as the main virulence factors of *K. pneumoniae* (18). Furthermore, some virulence factors; fimbriae, capsule and enterobactins are ubiquitous in all isolates of *K. pneumoniae* (21). Hypervirulent *K. pneumoniae* (HvKP) isolates are associated with a number of putative virulence factors; aerobactin and *rmpA* (regulator of the mucoid phenotype) both encoded by large virulence plasmids are present in hypervirulent HvKP isolates (22). Integrative and conjugative elements encoded iron acquisition system, such as yersiniabactin, and allantoin metabolism associated regions can also be present in HvKP isolates (23, 24). Capsules are composed of polysaccharide forming thick bundle of fibrillous structures that covers bacterium to protect from macrophage-mediated phagocytosis and bactericidal serum factors (25). There are 78 different capsular (K antigen) types, most importantly K1, K2, K5, K20, K54 and K57 are highly associated with invasive disease of *K. pneumoniae* (26).

1.3.2 *Escherichia coli*

Escherichia coli are non-sporulating, motile, lactose-fermenting, rod-shaped bacteria. They are also important member of the family Enterobacteriaceae of Gram-negative bacteria. They are found in the normal microbiota of the digestive tracts of humans and animals. *E. coli* have 171 somatic (O), 55 flagellar (H) and 80 capsular (K) antigens. Until today, there are over 160 serological types of *E. coli*. All these types are involved in causing different infections in human including, urinary tract infections (UTI), hospital-acquired pneumonia (HAP), sepsis, surgical site infection (SSI), gastrointestinal tract infections, hemolytic-uremic syndrome (HUS), meningitis and inflammation of the meninges, bloodstream infections (BSI), abdominal infections and pneumonia (27). Most *E. coli* are members of the commensal microbiota with low pathogenicity. In the mucus layer of the colon, *E. coli* are the most frequent facultative anaerobes with a quantity of 10^7-10^9 cfu/gram of feces (28).

Phylogenetic analysis of *E. coli* discloses eight phylogroups; A, B1, B2, C, D, E, F and clade I. Strains of the different phylogroups behave differently. Strains of A and B are mainly commensal bacteria, whereas strains of B and D are more virulent and pathogenic bacteria mostly associated with causing extra-intestinal infections (ExPEC) (29, 30). Virulence factors are important for a pathogen to attach with the mucosal surface of the host and to overcome immunologic response. The main virulence factors of uropathogenic *E. coli* include adhesins (e.g. pili associated with pyelonephritis (*pap*) encoding P-fimbriae and type 1 fimbriae encoded by *fimH*), toxins (e.g. alpha-hemolysin, cytotoxic necrotizing factor 1, autotransporter toxins), iron/heme acquisition systems and iron ion transporter, capsule production (K1/K2/K5) (31).

1.4 Antimicrobial resistance in Enterobacteriaceae

Infections caused by Enterobacteriaceae are usually treated with β-lactam antibiotics. These are broad spectrum antibiotics consisting of molecular ring-shaped structure, the β-lactam ring (Figure 2A). This group include penicillins, cephalosporins, monobactams and carbapenems.

Hydrolysis of β-lactam structure mediated by β-lactamases is the most common resistant mechanism for inactivation of β-lactam antibiotics. There are mainly four β-lactamases groups which can be identified based on substrate specificities: penicillinases, AmpC-type cephalosporinases, extended-spectrum β-lactamases (ESBLs), and carbapenemases (32). Decreased porin function or increased efflux are other types of β-lactam resistance mechanism exhibited by Enterobacteriaceae. Porin alterations and β-lactamase production can synergize in increasing antibiotic resistance in many pathogens of Enterobacteriaceae (7).

1.4.1 ESBL-producing Enterobacteriaceae (EPE)

Extended spectrum β-lactamases (ESBLs) are enzymes produced by Enterobacteriaceae that can hydrolyze β-lactam antibiotics mostly penicillins and cephalosporins but are susceptible to cephamycins and carbapenems (33). ESBLs were first described in Germany in 1983 (34), later a huge number of ESBLs have been detected. Several classification schemes are available, but the classification proposed by Giske et al in 2009 is most frequently used in Sweden and Norway (35). According to Giske et al classification, ESBL enzymes can be classified into three main groups; $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$ discussed in **Table 2**.

Table 2. ESBL classification according to Giske et al classification[35].

$ESBL_A$,	**$ESBL_M$**	**$ESBL_{CARBA}$**
CTX-M, SHV and TEM	AmpC, CMY and FOX	KPC, NDM, VIM and OXA-48
Degraded by clavulanic acid	Inhibited by cloxacillin	KPC is inhibited by boronic acid NDM and VIM are inhibited by DPA and EDTA OXA-48 is inhibited by avibactam

After the first discovery of CTX-Ms in the 1980s they have emerged in all continents. The $bla_{CTX\text{-}M}$ enzymes can be divided into five phylogroups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. The worldwide most dominant CTX-M-15 belongs to phylogroup CTX-M-1 (36-38).

The most well-known classification of β-lactamases is based on functional and molecular structure, Bush et al. classified β-lactamases into four groups in the 1995, "Group 1 cephalosporinases are not well inhibited by clavulanic acid, group 2 (molecular class A or D) β-lactamases are commonly inhibited by active site-directed β-lactamase inhibitors, group 3 metallo-β-lactamases are poorly inhibited by all classical β-lactamase inhibitors excluding EDTA and p-chloromercuribenzoate (pCMB) and group 4 penicillinases that are not inhibited by clavulanic acid" (33).

There is a homogenous distribution of ESBL genotypes observed in Nordic countries. In Sweden, $bla_{CTX\text{-}M\text{-}15}$ is found in 50-60% and $bla_{CTX\text{-}M\text{-}14}$ found in 10-15% of ESBL-producing *E. coli.* In Norway and Denmark, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ were found in 50% and 20% respectively (39, 40). In Finland, CTX-M-1 including $bla_{CTX\text{-}M\text{-}15}$ is the most prevalent ESBL genotype. Whereas, other parts of Europe have different ESBLs genotypes. In Spain, most prevalent ESBLs genotypes are $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}9}$ whereas $bla_{CTX\text{-}M\text{-}3}$ is the most prevalent ESBL genotype in Poland as well as other eastern European countries (41, 42). Again, the $bla_{CTX\text{-}M\text{-}15}$ is the most prevalent genotype in the African and Australian continents and also in

Canada. In US the most prevalent ESBL genotype used to be bla_{SHV} about a decade ago, but now $bla_{CTX\text{-}M\text{-}15}$ has become the most prevalent ESBL type also there (43-45). Heterogenous distribution of genotypes is observed in Asian continent. In Japan, CTX-M-9 is the most frequently encountered phylogroup whereas in China it is CTX-M-14 and in India CTX-M-15. CTX-M-8 is rare in other continents but it is common in South America along with CTX-M-2 (43, 46-49).

Transmission of EPE

ESBL can disseminate either by emerging bacterial clones or by horizontal gene transfer. Multiple factors are involved in dissemination of EPE; including humans, animals and environmental factors. Several studies have reported the transmission between household contacts (50, 51). Dissemination within households was observed in 32% (9/28) during a Norwegian outbreak of infants colonized by EPE at the neonatal intensive care unit (52). EPEs has been found in pets, poultry, cattle and birds (53-56). Dissemination of EPE in the community is very common in countries having high prevalence of intestinal EPE-colonization. Several other reservoirs for EPE are present, for example sewage water and the surface of fruits and vegetables (57, 58). NDM-1, ESBL and carbapenemase gene were reported from drinking water in New Delhi (59).

Prevalence of asymptomatic EPE carriage is not uniform throughout the world. In Sweden, during 2012-2013 the prevalence was 4.7% whereas it was much higher in Thailand 65.7% (60, 61). During 2015 EPE carriage was 19% in a tribal area in India but it was much higher 58.4% in outpatients, which has been reported by a study of rectal swabs in pediatric oncology unit (62, 63). With considerable variation, nearly 50% of the Enterobacteriaceae clinical isolates were reported to show resistance against extended-spectrum cephalosporins in all WHO region by 2014 (64).

In this era of globalization, drug resistant bacteria are spreading rapidly all over the world. EPE colonization is highly associated with international travel and the risk is higher while travelling to prevalent area. The risk of acquisition of EPE is higher while travelling to southeast Asia, especially India. Diarrhea and use of antibiotics during travel are both independent risk factors for acquiring EPE (65-69).

1.4.2 Carbapenemase-producing Enterobacteriaceae (CPE)

Carbapenemase production was detected in Enterobacteriaceae for the first time in 1993, in a clinical isolate of *Enterobacter cloacae*, in the form of a chromosomally encoded *NmcA* (70). Later, a wide variety of carbapenemases have been detected. Carbapenemases can be defined as the group of enzymes that can hydrolyze penicillins, in most cases cephalosporins and to varying degrees carbapenems and monobactams. The most clinically significant carbapenemases are;

Ambler class A, also known as functional group 2f, class B also known as group 3 and class D also known as group 2d. Class A carbapenemases are based on the presence of serine residues in the active site. Enzymes of this class can hydrolyze penicillins, cephalosporins, carbapenems and the monobactam aztreonam, with exception of Guiana extended spectrum (GES) which cannot hydrolyze aztreonam. Clavulanic acid, tazobactam and boronic acid can inhibit most of the members of this group. KPC, GES and IMI are the examples of class A carbapenemases. Class B carbapenemases also known as metallo-β-lactamases (MBL) are a group of carbapenemases who require a zinc ion in the active site for hydrolysis of β-lactams. Ethylenediaminetetraacetic acid (EDTA) can inhibit the activity of this group of enzymes by chelation. MBLs are not inhibited by clavulanic acid or tazobactam. Verona integron-encoded (VIM), German imipenemase (GIM), Seoul imipenemase (SIM) are the examples of this group. Class D carbapenemases are one of the most predominant and diverse group of carbapenemases and have weak hydrolytic activity against carbapenems. Currently, there is a lack of available specific inhibitors for OXA-48 like enzymes; OXA-23, OXA-24 and OXA-58 group. OXA-48 carbapenemases are identified in Enterobacteriaceae but OXA-48 like genes were identified only in *Acinetobacter baumannii* worldwide (71, 72)

Worldwide dissemination of CPE

Dissemination of carbapenemase-producing Enterobacteriaceae is still rare in Sweden but it is increasing rapidly. In total 115 CPE were identified in Sweden in 2015, among them 43 were identified in Stockholm. Most of the isolates were identified from fecal screening samples. In 2007-2012, out of the total of 94 isolates identified, 24 originated from clinical infections in Sweden (73). The Public Health Agency of Sweden reported a total of 144 cases of CPE in 2018 which is 28 cases more than in 2017 (74). In the United States in 1996, KPC enzymes were first detected from a *K. pneumoniae* isolate showing resistance to all β-lactams. Epidemic outbreaks of CPE were reported from Greece, the USA and Israel in the early 2000. Now, KPC is the most prevalent carbapenemase among Enterobacteriaceae in Europe and it has spread in South America and China also. KPC is also regarded as endemic in Greece (75, 76). European Antimicrobial Resistance Surveillance Network (EARS-Net) data reported that in Slovakia, Greece and Italy, proportion of carbapenem resistant *K. pneumoniae* were observed 63%, 57% and 44% respectively (77). KPC has been found both in Enterobacteriaceae and *Pseudomonas aeruginosa,* but is by far most commonly found in *K. pneumoniae* and is associated with the worldwide disseminated sequence type ST 258 (78). In the end of 1980s, IMP was first isolated from *P. aeruginosa* in Japan and VIM was first isolated from *P. aeruginosa* in 1997 in Verona, Italy. Later, both were disseminated to *K. pneumoniae* and other Enterobacteriaceae (79, 80). NDM-1 of Indian origin was detected in 2010 and the prevalence rate is very high in Indian subcontinent. The prevalence of NDM-producing Enterobacteriaceae in Indian and Pakistani

hospitals is ranging from 5 to 18.5%. NDM is also found in drinking water and seepage samples in New Delhi (59). As NDM is also found in *E. coli*, *Acinetobacter* spp and to some extent in *P. aeruginosa*, there is a high risk of dissemination of NDM within the community (81, 82). In 2001, the oxacillinase-type β-lactamase (OXA-48) enzyme was first isolated from carbapenem resistant *K. pneumoniae* in Turkey (83). OXA-48 like enzymes have caused outbreaks in UK, France, Germany, Belgium and the Netherlands and also disseminated in Northern Africa and the Middle East. Still, OXA-48 like enzymes are mostly isolated from *K. pneumoniae* and *E. coli* but can be found in other species as well (84).

1.4.3 Plasmid-mediated β-lactam resistance

Plasmids are circular DNA molecules serving as vectors for transmission of resistance genes and facilitating the spreading of ESBLs. Mobile genetic elements; insertion sequences and transposons are acquired by plasmids that mobilize the antimicrobial resistance genes. A plasmid can acquire one to several hundreds of genes encoding traits such as virulence, antibiotic resistance and metabolic functions. They are advantageous for bacteria but not vital for survival. Plasmids can acquire multiple physically linked genetic determinants that confer resistance to different classes of antibiotics commonly known as multi-drug resistance (MDR). ESBL-producing Enterobacteriaceae (EPE) show co-resistance most commonly to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (85, 86). MDR plasmids are self-conjugative, usually large (>50 kb) and encode sophisticated mechanisms that control their copy number and regulate the rate of replication (87). Plasmid replication is controlled by the replicon; is a DNA molecule, replicates from a single origin. Same replicon sharing plasmids cannot propagate stably within the same cell, which is the principle of the classification of incompatibility groups (Inc). IncFII, IncN and IncI are the predominant groups in Enterobacteriaceae (88).

1.4.4 Plasmid-mediated non β-lactam resistance

In EPE, plasmid-mediated co-resistance to other antibiotic groups are very common. Most clinically important plasmid-mediated non β-lactam resistance are described below.

Plasmid-mediated aminoglycoside resistance

Production of aminoglycoside modifying enzymes (AMEs) is the most common mechanism of aminoglycoside resistance. Aminoglycoside acetyl-transferase (*AAC* (6′)) is the most common AMEs in Enterobacteriaceae. In general, *AAC* (6′) expression confers resistance to both amikacin and gentamicin (89). Plasmid-mediated 16S rRNA methylase exhibits resistance to aminoglycoside antibiotics including gentamicin, amikacin and tobramycin. The most prevalent 16S rRNA methylase-encoding genes are *armA, rmtA, rmtC and rmtD* (90).

Plasmid-mediated quinolone resistance

Plasmid-mediated quinolone resistance gene was first identified from a urine sample with growth of *K. pneumoniae* in 1998 (91). The *qnr* gene encoding proteins bind with DNA-gyrase or topoisomerase and inhibit the mechanism of quinolones. *QepA* and *oqxAB* are plasmid-mediated genes that upregulate efflux pumps. The presence of *qnr* does not always confer clinical resistance, which has also been reported previously (92).

Plasmid-mediated colistin resistance

The first report of plasmid-mediated colistin resistance due to the *mcr-1* gene came from China in November 2015 (93). Chromosomally mediated colistin resistance gene has also been found in EPE. Changes of charge in lipid A due to modification by the enzyme MCR-1, encoded by gene *mcr-1* contributes to less interaction with polymyxin. The *mcr* gene was found in *E. coli* isolates from animals, food and patients. *E. coli* isolate harboring *mcr-1* has been reported for co-resistance of CTX-M-55 and an AmpC. Co-resistance was reported after one month of the first detection and the isolate was identified from blood samples in Denmark. In imported chicken meat, *mcr-1* was also reported (94). In our project III, we detected *mcr-1* for the first time in Sweden from a tourist travelling to Thailand. No gastrointestinal symptoms nor intake of antibiotic during the trip was reported. Later, another five cases of identification of *mcr-1* genes have been reported by the Public Health Agency, Sweden (95). It is too early to predict the future impact of *mcr-1* but the gene is able to transfer into rapidly disseminating epidemic EPE isolates such as *E. coli* ST131 and *K. pneumoniae* ST512 (96). Recently, *mcr-1* has been identified from a KPC producer from wound sample (97). Moreover, one clonal outbreak of *K. pneumoniae* ST45 carrying *mcr-1* and $bla_{\text{KPC-3}}$ was reported from Portugal with 16 cases (98). To date eight variants (*mcr-1-8*) have been reported (96, 99, 100). The clinical significance of CPE acquiring *mcr-1* could be life threatening due to the risk of total lack of effective antibiotic treatment against such infections.

1.5 Detection of EPE

ESBL-producing Enterobacteriaceae detection and characterization is mandatory for clinical laboratories for infection control purposes. According to CLSI and EUCAST, detection of ESBL mainly consists of two phases. At first, samples are subjected to screen for reduced sensitivity to certain antibiotics such as cefotaxime, ceftriaxone, ceftazidime and cefpodoxime. Then a confirmatory test is performed with the positive screening samples. According to the guidelines issued by EUCAST and CLSI, >1mg/L is a screening breakpoint for cefotaxime, ceftriaxone, ceftazidime and cefpodoxime, described in detail in Table 3 (101, 102). The most sensitive indicator for detection of ESBL is cefpodoxime, which

can be used for screening. Combination of cefotaxime and ceftazidime is more specific than cefpodoxime alone (103, 104). ESBL screening can be performed by using automated system such as VITEK 2 (bioMérieux), Microscan (Siemens), Phoenix (Becton-Dickinson) and classical disk diffusion methods.

Table 3. Screening methods for ESBL-producing Enterobacteriaceae from EUCAST[101].

Method	Antibiotic	Screening positive
Broth or agar dilution	Cefotaxime/ceftriaxone and ceftazidime	MIC >1 mg/L for either agent
	Cefpodoxime	MIC >1 mg/L
Disk diffusion	Cefotaxime (5 µg) or Ceftriaxone (30 µg) And Ceftazidime (10 µg)	Inhibition zone < 21mm Inhibition zone < 23mm Inhibition zone < 22mm
	Cefpodoxime (10 µg)	Inhibition zone < 21mm

1.5.1 Phenotypic confirmation methods

According to EUCAST guidelines, following phenotypic methods are recommended based on the *in vitro* inhibition of ESBL activity by clavulanic acid for ESBL confirmation such as the combination disk test (CDT), the double-disk synergy test (DDST), the ESBL gradient test, broth microdilution test and colorimetric test (101, 105, 106).

Combination disk testing (CDT) is performed by adding a disk or tablets containing cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid. The inhibition zone around the disk or tablet with cephalosporin alone and with clavulanic acid is measured. The test is interpreted as positive if the inhibition zone diameter around the cephalosporin with clavulanic acid is ≥5 mm larger than the cephalosporin alone (107).

Double-disk synergy test (DDST)

This test is performed by applying cephalosporins (cefotaxime, ceftazidime, cefepime) containing disks to plates next to a disk with clavulanic acid (amoxicillin-clavulanic acid). The results can be considered as positive if the inhibition zones around any of the cephalosporin disk are enhanced towards the disk containing clavulanic acid. The distance between the disks is important, for cephalosporin 30µg disk 20mm (centre-to-centre) is optimal.

Gradient test method

The Etest ESBL (bioMérieux) is a commercial kit available to perform ESBL confirmation test. The reading and interpretation can be performed according to manufacturer's instructions. The test is considered as positive if ≥ 8 fold reduction is found in the MIC of the cephalosporin plus clavulanic acid, compared with the MIC of the cephalosporin alone, or if a deformed ellipse or phantom zone is present. If the strip cannot be read due to growth exceeded to the MIC range of the strip, then the result is considered as indeterminate. The result is considered as negative in all other cases. The gradient test is not reliable for determining the MIC but can be used for confirmation of ESBL only.

Broth microdilution

Broth microdilution can be performed by using Mueller-Hinton broth containing two-fold serial dilution of cefotaxime, ceftazidime and cefepime at concentrations ranging from 0.25 to 512 mg/L with and without clavulanic acid at a concentration of 4 mg/L. Bacterial suspension is added to the each well of the microtitre plate and then incubated at 37°C for 18 to 24 hours. The test is considered as positive if a ≥ 8 fold reduction is found in the MIC of the cephalosporin plus clavulanic acid compared to the cephalosporin alone.

Biochemical (colorimetric) tests

In 2012, the ESBL NDP test was described for the first time. This test was performed by using cefotaxime as indicator antimicrobial and tazobactam as inhibitor (108). This test can be performed in 96-well plates or in separate tubes. The test will be considered as positive if the colour changes from red to yellow. It has been reported that the test was performed directly on patient samples (109).

Another, colorimetric test is β-LACTA test which can be carried out by using a chromogenic cephalosporin substrate (HMRZ-86). This test can be performed on isolates and also directly on clinical samples. An excellent sensitivity and specificity for *E. coli* and *K. pneumoniae* (96% and 100%, respectively) have been reported by a multicentre study conducted in Belgium and France (110).

1.5.2 Genotypic confirmation

Phenotypic confirmation test is not able to identify specific enzymes causing the production of ESBL. Genotypic confirmation is important and can be performed by using polymerase chain reaction (PCR), Eazyplex, ESBL gene sequencing or DNA microarray. Check-Direct ESBL Real-time PCR (Check-Points, Wageningen, The Netherlands) can be used for ESBL detection.

1.6 Carbapenemases detection

1.6.1 Phenotypic detection methods

Most clinical microbiology laboratories are using established phenotypic methods such as combination disk test, biochemical tests, spectrophotometry, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), and lateral flow assays for detection of carbapenemases. Phenotypic detection consists of two steps; screening and confirmation.

Screening of carbapenemases

Chromogenic media is used for screening of carbapenemase-producing Enterobacteriaceae from fecal samples. Chromogenic media contains chromogen that act as substrate for species-specific enzymes and changes colour due to degradation. Species can be identified by the colour of the colonies (111). CHROMagar KPC (bioMérieux, France) is a commercial chromogenic media that can detect VIM and KPC carbapenemases efficiently but OXA-48 with poor sensitivity (112). The highest sensitivity 76% and specificity 75% has been reported for CHROMagar KPC (113, 114). Another chromogenic media, Brilliance™ CRE contains carbapenem and two chromogens can detect carbapenemase-producing *K. pneumoniae* with higher sensitivity (98%) and specificity (79%) than other members of the Enterobacteriaceae. An overall sensitivity of 92% and specificity of 85% has been reported (111, 113-115). SUPERCARBA is a nonchromogenic media which can detect all class of carbapenemases with higher sensitivity and specificity than CHROMagar KPC. The sensitivity of detection of class A, B and D carbapenemases were 100, 90 and 100% respectively (113). Remel Spectra™ CRE (Thermo Fisher Scientific, Basingstoke, UK) is another type of chromogenic media having slightly higher sensitivity (97.8%) and specificity (86.4%) than CHROMagar KPC. One day of incubation time is required to obtain result, which is the main disadvantage of these media. In certain strains false negative results can be obtained due to absence of chromogen-specific enzymes (116). In these cases, disk diffusion cut-off for meropenem (<28 mm) and ertapenem (<25 mm) defined by EUCAST for screening of carbapenemaes in clinical isolates can be used (117). Screening of clinical isolates; blood isolates or urine isolates can be performed by disk diffusion method.

Confirmation of carbapenemases

Combination disk test

The commercial version of combination disk test is available from several manufacturers. MAST, in UK and Rosco, in Denmark were first to introduce the commercial combination disk test (118-120). Meropenem and various inhibitors are combined within the disk or tablets. Class A carbapenemases are inhibited by boronic acid, class B carbapenemases are inhibited by dipicolinic acid (DPA)

and ethylenediaminetetraacetic acid (EDTA). Avibactam can inhibit OXA-48 carbapenemase (121). Cloxacillin, AmpC β-lactamase inhibitor has also been added to the test. Cloxacillin, can differentiate between AmpC hyperproduction plus porin loss and carbapenemase-production.

Biochemical tests

Carba NP and CarbAcineto tests are commonly known as biochemical test having high sensitivity and specificity compared with culture-based methods. Turn-around time is also faster than with the culture-based methods. Nordmann and colleagues developed Carba NP and CarbAcineto tests for the first time in 2012 to identify carbapenemase producers in Enterobacteriaceae, *Pseudomonas* spp. And *A. baumannii.* The basic principle of these tests is the hydrolysis of imipenem, with a change of colour of a phenol indicator from red to yellow due to acid production from imipenem hydrolysis. High (100%) sensitivity and specificity of Carba NP test has been reported in many studies conducted on Enterobacteriaceae and *Pseudomonas* spp (122) (123, 124). A commercial version of Carba NP test RAPIDEC® CARBA NP is available (125).

The Blue-Carba test (BCT) is another type of biochemical test for rapid (<2 h) carbapenemase detection. The principle of the test is based on hydrolysis of imipenem by bacterial colonies which can be detected by changes in pH. Presence of a carbapenemase will change colour of the pH indicator bromothymol blue from blue to green/yellow or from green to yellow (126, 127). An excellent sensitivity for class A and B enzymes but suboptimal sensitivity for OXA-48 enzymes have been reported (128).

Another biochemical test is the β CARBA test™, which can be performed in < 2h. The test can be carried out by mixing 1 to 3 colonies in the reagents. After 30 min of incubation reading can be carried out. Test results will be considered as positive when colour changes from yellow to orange, red or purple. An excellent performance of β CARBA test™ for detection of CPE has been reported (127).

Spectrophotometry

Spectrophotometry is a well-known reference method for identification and differentiation of carbapenemases in Enterobacteriaceae using imipenem as substrate, but is used to a limited degree in clinical laboratories. Supernatant can be collected from sonication and centrifugation of 24 h-culture cell with imipenem. Absorbance per minute of imipenem and imipenem with bacterial extract were compared. The sensitivity 100% and specificity 98.5% has been reported for this test (129). With exception of class D carbapenemases, this test can detect efficiently other carbapenemases; VIM, IMP and SIM (129).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The MALDI-TOF MS can identify resistance genes, bacterial species and subspecies by using expressed proteins (130, 131). Ionized carbapenem and its degradation products can be detected after incubation of carbapenem with extracted bacterial proteins for 1-4 h which formed spectra of the carbapenem and its degradation products. MALDI-TOF MS can detect carbapenemases with 100% sensitivity and specificity (132-134). Incubation time can be reduced by adding sodium dodecyl sulphate (SDS) to the bacterial cell culture before incubation with the meropenem substrate. Using this modification, NDM-1, VIM-1, KPC, OXA-48 and OXA-162 in Enterobacteriaceae were detected with 100% sensitivity and specificity (130).

Lateral flow assays

Lateral flow assay, or immunochromatographic assay, has been described for detection of OXA-48 like carbapenemase. In this assay epitopes of OXA-48-like enzymes can be captured immunologically using colloidal gold nanoparticles which is bound to a nitrocellulose membrane within a lateral flow device. In this assay specific capture reagents based on monoclonal anti-OXA-48 antibodies are used to detect OXA-48 like enzymes directly (135). This is a rapid assay and it takes around 4 minutes to perform the assay. This assay can be performed on both clinical isolates and directly on positive blood cultures (136, 137). A recent modification triplex assay has been reported to detect OXA-48, KPC and NDM (138). Recently, one single centre performed a retrospective and prospective evaluation study and has reported highly accurate detection of all five major carbapenemase families (OXA-48, KPC, NDM, VIM, IMP). This study was based on multiplex lateral flow assay in which 296 enterobacterial isolates were examined. Overall, 100% sensitivity and 95.3 (retrospectively) to 100% (prospectively) specificity were reported (139).

1.6.2 Genotypic methods

Real-time PCR can detect and quantify resistance genes simultaneously by using fluorescent probes or dyes. Each gene has unique melting temperature which is used to identify specific resistance mechanisms (140). Multiple primers can be used to detect several genes simultaneously, commonly known as multiplex PCR. In multiplex real-time PCR, amplicon size of the target genes should be different for easy identification (141). The GeneXpert® (Cepheid, Sunnyvale, CA) is a commercial real-time PCR technique available to detect clinically relevant KPC, IMP, VIM, NDM and OXA-48 carbapenemases. Check-MDR Carba assay (Check-Points) is another commercial real-time multiplex PCR assay to detect KPC, NDM, OXA-48, VIM and IMP from rectal and throat swabs.

Eazyplex® SuperBug CRE assay (Amplex Biosystems, GieBen, Germany) is a commercially available assay that can detect the CTX-M-1-group and CTX-M-9-group of ESBLs and VIM (-1 to -37), NDM (-1 to -7), KPC (-2 to 15) and OXA-48-like (-48, -162, -204 and -244) carbapenemases directly from rectal swabs and bacterial culture (142). It is a loop mediated isothermal amplification based assay. One study evaluted this assay directly on urin samples and reported 100% sensitivity and 97.9% specificity (143).

1.7 CRISPR/Cas9 mediated optical DNA mapping

Optical mapping is a process of visualizing individual DNA molecules long range sequence information at kbp level. In this process, sequence specific labeled DNA can be imaged after stretching using a fluorescence microscope (Figure 3). Using optical DNA mapping we can study read lengths of DNA, it can be up to 1 Mbp, we can also extract information from single molecules and avoid averaging (144, 145). This method was first described by Schwartz et al in 1993, restriction enzymes were used to cut the DNA molecules at specific sites and the length of different fragments were visualized in an agarose gel (146). After that, a significant advancement has been observed in the field of optical mapping including different approaches for stretching and labeling were introduced. Most important advancements are stretching of DNA molecules on modified glass surfaces as well as nanofluidic devices (147).

Labeling of DNA molecules can be performed by enzyme based or affinity based techniques. Jo et al (148) introduced nick labeling, in this process a nicking enzyme was used to generate a single stranded break which is known as nick a 4-7 bp long recognition site. Then fluorescently labeled nucleotides were incorporated along the strand using DNA polymerase. DNA molecules were stained using YOYO to determine start and end of each molecule (148). Using CRISPR/Cas9 based approach, a highly adaptable 23 bp recognition site has been reported by McCaffery (149). Another way of labeling DNA is competitive binding that has been reported recently (150, 151). In this process AT specific non-fluorescent molecule netropsin and the fluorescent dye YOYO were used simultaneously. Both molecules will compete for binding site on DNA molecules. If we use accurate concentration, netropsin will bind to AT-site and prevent YOYO to bind to AT-site and YOYO will bind to GC-site. So, GC rich region will be bright and AT rich region will be dark. This process was used to characterize plasmids in the fourth project of this thesis.

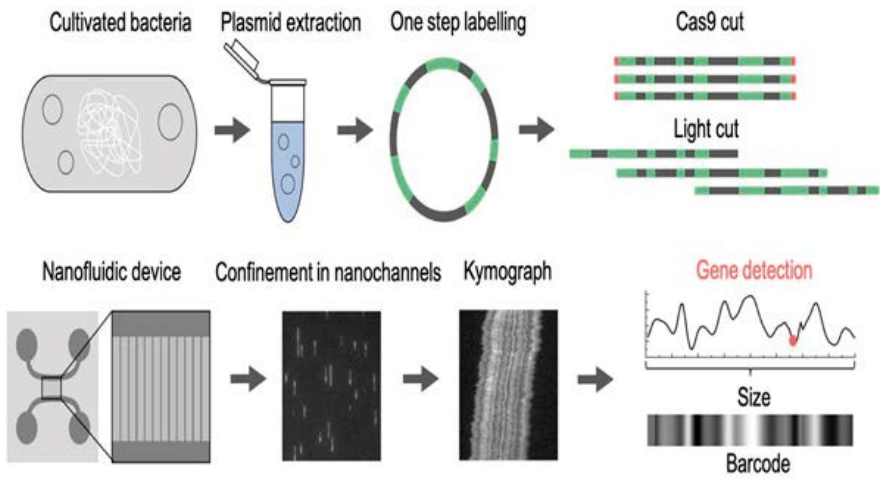

Figure 3. *Schematic diagram of optical mapping from paper IV. Rights to reprint from MBio.*

2 AIMS

The overall aim of this thesis was to investigate the clonality and proportion of ESBL-producers in *K. pneumoniae* from patients with bloodstream infections, and to study acquisition of EPE in healthy Swedish travellers travelling to high prevalence region of EPE. A second major aim was to evaluate rapid detection and characterization methods for EPE and CPE.

Specific aims

I) To characterize clinical isolates of *Klebsiella pneumoniae* and investigate the association with phylogroups, virulence factors and mortality during bloodstream infection (BSI) (Paper I)

II) To evaluate a commercial method the RAPIDEC CARBA NP test for detection of carbapenemase-producing Enterobacteriaceae, *Pseudomonas aeruginosa* and Acinetobacter spp (Paper II)

III) To identify bacterial colonizing characteristics that play key role for travellers to become carriers of ESBL-producing Enterobacteriaceae (EPE) (Paper III)

IV) To characterize risk factors for acquiring EPE in the intestinal microbiota during travel (Paper III)

V) To characterize resistance plasmids during an outbreak with an ESBL-producing strain of *K. pneumoniae* using optical DNA mapping, and further investigate whether the plasmid was transferred to ESBL-producing *E. coli* during outbreak (Paper IV)

3 MATERIALS AND METHODS

3.1 Sample collection

In **Paper I**, we studied a total of 139 isolates of *K. pneumoniae* (blood n=137, or cerebrospinal fluid n=1, or both n=2). All isolates were obtained from 139 adult (≥ 18 years old) patients admitted to Karolinska University Hospital, Solna during the years 2007 to 2009. In **Paper II**, we studied a total of 276 clinical isolates of Gram-negative bacilli. Detailed strain collection can be found in Table 4. Karolinska University Laboratory (KUL) and Publich Health England's (PHE) Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) reference Unit jointly performed this study. In **Paper III,** we studied a total of 188 healthy adults (≥ 18 years old) Swedish travellers travelling to highly EPE prevalent regions; Southeast Asia, Indian subcontinent, North Africa and the Middle East. We collected rectal swabs and survey data from 188 healthy Swedes travelling to the above-mentioned four high EPE prevalence regions. This study was performed at the Department of Clinical Microbiology, Karolinska University Hospital, Solna, Sweden. Rectal swabs were collected before and after the trip. Travellers, who did not provide both samples were excluded from the study. In **Paper IV,** we studied fecal samples from 17 neonates during an extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (EPE) outbreak at the NICU at Karolinska University Hospital in Stockholm, Sweden November 2008 and March 2009 which has been published previously (152). During follow-up, we collected fecal samples every second month from 14 surviving neonates from discharge to two years of age. We also conducted a five years' follow-up on 10 neonates due to drop-outs.

3.2 Ethical considerations

In this thesis, we used patient and human samples in project I, III and IV. We also used clinical data for analysis. We obtained ethical approval from the Karolinska Institutet Regional Ethics Committee of Stockholm for project I (**Paper I**) (recordal 2009/1985-31/4) and for project III (**Paper III**) (recordals 2013/1844-32 and 2015/1620-32). We also obtained ethical approval from regional ethical review board in Stockholm, Sweden (2009/734-31/4 and 2014/491-31/3) for project IV (**Paper IV**). No ethical approval was required for project II (**Paper II**) as the study was conducted on clinical strains. Moreover, no clinical data was used for analysis in project II.

The committee approved that we did not need to collect any written or verbal consent for project I from the study subjects as the study was conducted on large number of patients (n=139) and a large number of patients were died before the start of the project. It would not be possible to carry such a study where mortality has been analysed if written or verbal consents were required. Moreover, only

Table 4. Isolates tested by carbapenem resistance mechanism. Modified from paper II, rights to reprint from JAC.

	bla$_{KPC}$	*bla*$_{OXA-48}$	*bla*$_{NDM}$	*bla*$_{VIM}$	*bla*$_{IMP}$	*bla*$_{GES-5}$	*bla*$_{IMI}$	*bla*$_{OXA-23}$	*bla*$_{OXA-24}$	*bla*$_{OXA-51}$	*bla*$_{OXA-58}$	Non-carbapene-mases	Total
Klebsiella pneumoniae	28	15	18	1	1							20	83
Klebsiella oxytoca	2	1		1	1	2						1	8
Escherichia coli	3	5	3	1								62	74
Enterobacter complex	3	3	2	1	3	1	1					20	34
Serratia marcescens		1	2									3	6
Citrobacter freundii	2		1	1									4
Raoultella ornithinolytica		2											2
Raoultella planticola	2												2
Providencia complex			2										2
Proteus mirabilis			1										1
Morganella morganii			1										1
Acinetobacter baumannii			1		2			6	4	1[1]	1		15
Pseudomonas aeruginosa				11	2							31	44
Total	40	27	31	16	9	3	1	6	4	1	1	137	276

[1] Hyperproduction of OXA-51 was not detected.

limited clinical data were used anonymously in this study. We collected written consent from study subjects for project III. To pursue research project III and IV, most important ethical considerations is emotional impact of study participants. When study participants get to know that they are carrier of antibiotic resistant bacteria some may get psychological reactions and feel worried about being an asymptomatic carrier and which consequences this could have in the future. It is important to provide information to the participants about the implications of being a carrier, and to offer expert consultations to allow the individual to ask questions about fecal carriage and its implications.

3.3 Species identification and susceptibility testing

Species were identified by using VITEK2 (bioMérieux, Marcy-I'Etoile, France) in project I & IV and MALDI-TOF Biotyper (Bruker, Billerica, MA, USA) in project III.

In project I and III, antimicrobial susceptibility testing was carried out with the disk diffusion method on Isosensitest agar (Oxoid, Basingstoke, UK). The interpretation was performed following the guidelines of Swedish Reference Group for Antibiotics (SRGA) in project I and EUCAST guidelines were followed for interpretation in project III (117) (153). In project II, antimicrobial susceptibility was performed using meropenem and ertapenem disk by disk diffusion method and interpretation was performed following EUCAST guidelines at KUL. At AMRHAI, BSAC agar dilution with AMRHAI's standard Gram-negative antibiotic panel; ertapenem, imipenem and meropenem was used for susceptibility testing. BSAC breakpoints were used for interpretation.

3.4 ESBL Screening and subtyping

ESBL- and/or carbapenemase-producing isolates were investigated using Check-MDR (Checkpoints, Wangeningen, The Netherlands) in project I. In project III, screening of EPE was performed using ChromID ESBL agar (bioMérieux, Marcy-I'Etoile, France). Further, broth enrichment was performed using LB broth (Thermo Fisher Scientific, Waltham, USA) for all samples (n=188) and cultivated on an in-house agar on a chromogenic base (Liofilchem, Roseto degli Abruzzi, Italy) supplemented with 200 mg/L cloxacillin and 0.25 mg/L meropenem for an overnight incubation at 35°C. VITEK2 (bioMérieux, Marcy-I'Etoile, France) was also used for ESBL testing. Using Check-MDR (Checkpoints, Wageningen, The Netherlands) we performed ESBL subtyping and Diversilab, an automated rep-PCR system, was used for epidemiological typing.

3.5 Phenotypic and molecular detection of carbapenemase

In project II, RAPIDEC® CARBA NP (bioMérieux SA, Marcy l'Etoile, France) was used for phenotypic detection of carbapenemase producers. We followed the manufacturer's protocol, in brief: isolates were cultivated on Muller Hinton E agar for 18-24 hours. Incubation of RAPIDEC® CARBA NP tests were 30 minutes to two hours at 37°C. Reading was performed by visually comparing colours between test well and control well. Change of colour of the test well from red to yellow or orange was interpreted as positive. In case of positive result, incubation was stopped after 30 minutes otherwise continued to two hours before a negative result was interpreted.

In-house real-time or conventional PCR assays were used for molecular detection of carbapenemases. PCR primers were used to target the following groups of carbapenemases; KPC, NDM, VIM, OXA-23, OXA-24, OXA-48, OXA-58, IMP, GIM, SIM, SPM, GES, IMI and SME. We used antimicrobial susceptibility profile instead of PCR for rarely occurring carbapenemases for all isolates (154-162). False-positive isolates were further investigated using whole-genome sequencing (WGS) using a HiSeq sequencer (Illumina, Little Chesterford, UK). In-house bioinformatics pipeline was used for data analysis. We determined the presence of resistance genes according to previously described methods (163).

3.6 Serotyping and virulence and resistance gene detection

In project I, detection of serotypes and virulence genes were performed using real-time PCR according to previously described methods (164, 165). Community-acquired invasive disease has been associated with six major serotypes; K1, K2, K3, K4, K5, K6 and three virulence genes *allS*, *rmpA*, *wcaG* of *K. pneumoniae,* which were all investigated (166, 167). Six serotypes and three virulence genes specific primers were used for real-time PCR. In project III, we also investigated virulence factors and resistance genes using real-time PCR. PCR primers were specific for *fimAMT78*, *fimH*, *papAH*, *papC*, *papEF*, and *papG* allele I, II, III and *armA, rmtA, rmtB, rmtC, rmtD, qnrA, qnrB, qnrC, qnrD, qnrS*. We also investigated the presence of plasmid-mediated fluoroquinolones resistance gene aminoglycoside-(6)-N-acetyltransferase (*aac(6')-Ib*) and its point mutant *aac(6')-Ib-cr* using real-time PCR following previously published protocol (168, 169). We also investigated plasmid-mediated colistin resistance gene *mcr-1* against all isolates following previously published protocol (93). One isolate that carried the plasmid-mediated colistin resistance gene *mcr-1* was further analysed using Next-generation sequencing technology the Ion Torrent S5 XL system (Themo

Fisher Scientific, Waltham, MA, USA). The presence of plasmid-mediated colistin resistance gene *mcr-1* was confirmed by analyzing with ResFinder (170). We also confirmed the ST for this isolate following previously described method (171). Plasmid type was confirmed by using PlasmidFinder (172). All isolates of *E. coli* in project III were subjected to phylogrouping using real-time PCR following previously described protocol (29).

3.7 Phylogenetic analysis

In project I, we performed seven house-keeping genes based MLST for phylogenetic analysis of all isolates according to previously published methods (166, 173). Seven house-keeping genes were amplified and sequenced using primers and protocol, can be found on the web site (htt://bigsdb.web.pastur.fr). We determined nucleotide sequences for seven house-keeping genes by using ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). We obtained allelic and sequence type numbers by direct comparison with the *K. pneumoniae* MLST database (htt://bigsdb.web.pastur.fr). In case of new allele and ST, numbers were assigned by curator of the *K. pneumoniae* MLST database. All sequences were aligned by using Mega V5 (174). We identified phylogroups using Neighbor-joining trees based on concatenate sequences of all seven MLST loci for all isolates. We used three reference strains; ATCC13883 for phylogroup KpI: *K. pneumoniae*, SB59 for phylogroup KpII: *K. quasipneumoniae* and Kp342:ST146 for phylogroup KpIII: *K. variicola* to identify phylogroups (175). We confirmed phylogroups separately by using Neighbor-joining trees based on each allele. We also used Splits Tree v4 (176) for NeighborNet analysis to confirm phylogroups and recombination events. Nucleotide diversity of each gene for each phylogroup and for all isolates were calculated by using DnaSP (177). With the help of BioNumerics v7.0 (Applied Maths, Sint Maartens-Latem, Belgium), we produced minimal spanning tree (MST) (178). Isolates sharing six loci of their allelic profiles with one another member of the group were defined as clonal complexes. We identified clonal complexes by using eBURST V3 (179).

3.8 Clinical data

In project I, we retrieved medical data for finding association between phylogroups, 30-day mortality, patient risk factors, hospital versus community-acquired infections and antibiotic treatment. Assessment of comorbidity was performed by constructing Charlson comorbidity index (180). In case of more than one species were identified from specimens obtained within 24 hours from *K. pneumoniae* bloodstream infection were considered as polymicrobial infection. If the patient had been to hospital more than 30 days or if the sample was taken >48h after admittance, the episode was defined as hospital-acquired. In project III, study subjects

returned a filled questionnaire based on personal information and potential risk factors such as underlying chronic diseases, duration of travel, antibiotic treatment during travel, getting treatment from local healthcare before and after travel.

3.9 Plasmid characterization

In **paper IV**, we conducted bacterial plasmid characterization based on optical DNA mapping (Figure 3). We utilized the emission intensity variation along the DNA by adding fluorescent YOYO-1 and the non-fluorescent AT-selective netropsin, where AT-rich regions are dark and GC-rich region are bright. Visualization of the variation of emission intensity, the *DNA barcode* can be retrieved by stretching single intact plasmids in nanochannels and imaging them using fluorescence microscopy. Previously, it has been demonstrated that the method can measure the number and size of all large plasmids in a bacterial cell. It has also been demonstrated that it can produce a unique barcode for each plasmid which can later be used to identify conjugation of plasmids between different strains and species of bacteria. Latest improvement of the assay was done by adding the CRISPR/Cas9 system, now the method is able to demonstrate a specific (resistance) gene harbouring plasmid. Plasmids were extracted from 100 ml overnight bacterial cultures in LB broth by using the Macherey-Nagel NucleoBond Xtra Midi preparation kit according to the manufacturer's instructions. We precipitated eluted plasmid DNA into isopropanol and resuspended it in 1 × TE buffer. Then, we treated plasmid with Cas9 and guide-RNA (gRNA) targeting $bla_{CTX\text{-}M}$ group 1 gene according to our previously published protocol (181). To obtain DNA barcode, we stained plasmid and λ-DNA (New England Biolabs) at equal concentration with YOYO-1 (Invitrogen) at a molecular ratio of 1:2 (YOYO-1:bp) and with netropsin (Sigma-Aldrich) at a molar ratio of 100:1(netropsin:YOYO-1). Here, we used λ-DNA for internal size marker (48 500 bp). To obtain uniform staining, we incubated plasmid DNA (2μM) with YOYO-1 and netropsin in 10 μl of 0.5X TBE at 50°C for 30-60 min (182). We used Millipore water to dilute samples in order to obtain a final concentration of 0.05X TBE and 0.2 μM (bp) of DNA. We added 2.5% (v/v) β-mercaptoethanol (BME) to the solution to supress photo nicking and photo bleaching of DNA molecules. We fabricated nanofluidic channels in oxidized silicon, according to previously described methods (183). DNA molecules were stretched through nanochannels of dimensions 100 nm x 150 nm^2 and a length of 500 μm. We cleaned nanochannels with 5% w/w sodium hypochlorite solution and then washed a few times with loading buffer (0.05x TBE) before loading samples. This process enhanced the usage of nanofluidic chips more than 50 times. On average we used chips in each section at least 10 times. We placed 15μl of DNA solution to the reservoir and we used nitrogen gas to force DNA molecules into nanochannels. To image DNA molecules we used an inverted microscope (Zeiss AxioObserver.Z1) equipped with either a 63x (with 1.6 times optovar) or 100x

oil immersion objective (Zeiss, NA=1.46) and an EMCCD camera (Photometrics Evolve or Andor iXon). We imaged both circular or linear molecules for 200 frames with 100ms exposure by stretching into the nanochannels.

We analyzed data to identify the number of plasmids in each sample, their corresponding size, their sequence-specific barcode and possible location of a resistance gene ($bla_{\text{CTX-M}}$ group 1). In brief, we used the internal size marker λ-DNA to calculate the plasmid size. We also used conversion factor 1.8 to estimate whether directly from linearized plasmids, or from plasmids in their circular form (184). We compared linear fragments of similar size (+/- 10%), acquired either by Cas9 linearization, or via extensive light exposure (185). High degree of similar individual barcodes (typically Pearson Cross Correlation (CC) > 0.75) were merged into a consensus barcode to compare between different isolates, rendering a p-value (186). We studied the position of the double strand-breaks of the individual plasmid barcodes and the consensus barcode to detect the presence of a resistance gene (181).

Qiagen Genomic-tip 100 kit was used to extract complete genomic DNA (chromosomal DNA and plasmids) according to the manufacturer's instructions. Long read sequencing was performed using PacBio Sequencing technologies in collaboration with Science for Life Laboratory in Uppsala on a RSII system with one SMRT cell per genome. Sequencing library was prepared using Illumina MiSeq sequencing with two times 300-bp paired-end read lengths and Nextera XT DNA Library preparation kit (Illumina) following manufacturer's instructions. Overall generated chromosomal coverage was >25-fold for all isolates. The CLC Genomics workbench v.10 (CLC Bio, Aarhus, Denmark) including the CLC Microbial Genomics Module 3.0 was used to perform de novo assembly for each data set. Illumina reads were assembled using contig sequences from the PacBio sequencing as references. The resulting contigs were analyzed by mate-pair analysis and circularized by detection of redundant end-sequences. De novo assembly of non-matched reads from combined reference assemblies for each dataset was performed to verify that no plasmids were missed between isolates. Remaining contigs were analyzed for gene content by using BLASTn searches. Complete genome annotation was performed by using the NCBI Prokaryotic Genome Annotation Pipeline. ResFinder, PlasmidFinder, and VirulenceFinder, were used to detect resistance genes, plasmid replicons and virulence genes respectively (170, 172, 187).

4 RESULTS

4.1 Paper I

The aim of this study was to determine association between phylogroups, virulence factors and mortality in clinical isolates of *K. pneumoniae* causing BSI. Three distinct phylogroups were disclosed by phylogenetic analysis by multilocus sequence typing (MLST). The most predominant phylogroup was phylogroup KpI (corresponding to *K. pneumoniae* n = 96) followed by phylogroup KpIII (corresponding to *K. variicola* n = 34) and phylogroup KpII (corresponding to *K. quasipneumoniae* n = 9) (Figure 4). The highest mean genetic distance among phylogroups was 4.5% (KpII-KpIII), followed by 4.4% (KpI-III) and 4.0% (KpI-KpII). Thus, we observed that phylogroups KpI-KpII are more closely related compared to phylogroup KpIII. Neighbor-joining tree analysis also supported three phylogroups and suggested a high level of recombination within each group, as well as between the phylogroups. We also observed minor events of insertion/deletion (INDEL) in the *tonB* locus by aligning seven housekeeping genes. Considering all isolates, highest polymorphic sites were observed in *tonB* 19.6% and lowest polymorphic sites were observed in *gapA* 5.1% (Table 5) whereas only 10.7% polymorphic sites were observed in concatenate sequence. Average nucleotide diversity Π of 2.1% was observed for the whole population, whereas Π was much lower within the phylogroups; KpII (1.3%), KpI (0.37%) and KpIII (0.63%). The ratio of the number of non-synonymous substitution per non-synonymous site (Ka) to the number of synonymous substitution per synonymous site (Ks) was observed to be less than one, which indicated that housekeeping genes were evolved predominantly under purifying selection. An extensive haplotypic diversity with 116 distinct STs was observed by the MLST based population analysis. The clonal relatedness among sequence types (STs) were analysed using both MLST and eBURST method. A total of 48 isolates comprised 12 clonal complexes and remaining 91 isolates split into 79 singletons (STs that differed by two or more alleles from another ST).

Only 18 strains of 139 isolates were serotypable for tested capsular serotypes; K1, (1.4%; 2/139), K2 (5.0%; 7/139), K20 (1.4%; 2/139), K54 (2.2%; 3/139) and K57 (2.9%; 4/139). The predominant serotype was K2, whereas K5 was not found in any isolates. A total of 16 out of 18 capsular types were associated with phylogroup KpI and the remaining 2 were associated with KpIII. In this study virulence gene *wcaG* 8.6% (12/139) was the most frequently detected virulence gene followed by *allS* 4.3% (6/139) and *rmpA* 3.6% (5/139). A total of 8 (5.8% 8/139) isolates exhibited mucoid phenotype and among them two isolates were also positive for *rmpA*. The distribution of isolates harboring virulence genes were KpI (n=13), KpIII (n=5) and KpII (n=3).

A total of 17.3% (24/139) with 30-day mortality was observed. Isolates belonging to phylogroup KpIII were highly associated with the highest 30-day mortality of 29.4 % (10/34 cases), whereas isolates belonging to phylogroup KpI and KpII were associated with 30-day mortality of 13.5% (13/96 cases,) and 11.1% (1/9 cases), respectively. Out of the 24 events associated with 30-days mortality, only one was serotypable (K54) and one exhibited mucoid phenotype and/or (*wcaG* n=1 and *wcaG/allS* n=1). We observed low level of antibiotic resistance in general but the highest resistance rate 10.8% was observed against trimethoprim/sulfametoxazole followed by ciprofloxacin 8.6%, piperacillin/tazobactam 5.8%, gentamicin 3.6%, ceftazidime 4.3% and cefotaxime 3.6%. Isolates of KpI were more resistant against trimethoprim/sulfametoxazole compared to KpIII isolates. *K. variicola* (KpIII) isolates were found to be less resistant than isolates of *K. pneumoniae* (KpI) and *K. quasipneumoniae* (KpII) for several classes of antibiotics.

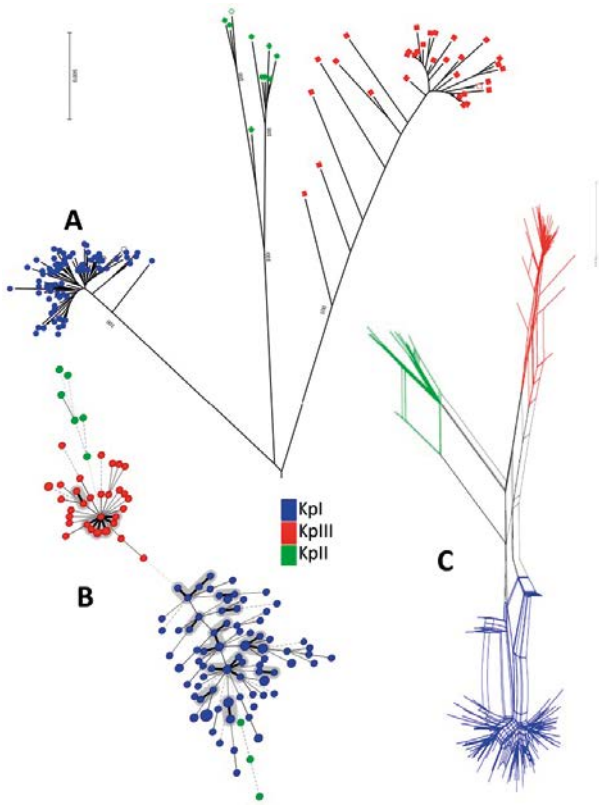

Figure 4. *Phylogenetic analysis based on Neighbor-joining trees (A), Minimal spanning tree (MST) (B), and NeighborNet analysis (C). Sequence type is represented by each circle. Black lines between STs indicate that they differ in one allele (thick lines), two and three alleles (thin), or four to seven alleles (dashed). Rights to reprint from PLoS One (Paper I).*

Table 5. Nucleotide diversity within seven housekeeping genes. N = number of samples subjected for analysis. Sites = Length of sequence after alignments. Net Site = Length of sequence subjected for analysis. S = polymorphic sites. Hap = haplotypes. Hd = haplotypic diversity. Π = nucleotide diversity. θ = average number of nucleotide difference per site. Ka/Ks = ratio of the number of non-synonymous substitution per non-synonymous site (Ka) to the number of synonymous substitution per synonymous site (Ks). Rights to reprint from PLoS One (Paper I).

Alleles	n	Sites	NetSites	S	Hap	Hd	Π	θ	Ka/Ks
gapA	139	450	450	23	18	0.692	0.00819	0.009683	0.0995
infB	139	318	318	28	19	0.756	0.01522	0.017127	0
mdH	139	477	477	59	26	0.805	0.02797	0.023598	0.0324
pgi	139	432	432	54	28	0.693	0.02405	0.023955	0.0144
phoE	139	420	420	41	41	0.939	0.01401	0.020316	0.0064
rpoB	139	501	501	41	27	0.815	0.01447	0.015582	0.023
tonB	139	438	402	79	47	0.952	0.04667	0.040646	0.0693
Concatenate, Total 139 strains	139	3 036	3 000	325	115	0.996	0.02138	0.021363	0.0344
Concatenate, KpI	96	3 024	3 006	115	75	0.993	0.00371	0.007448	0.0778
Concatenate, KpII	9	3 018	3 012	105	9	1	0.0132	0.012949	0.02
Concatenate, KpIII	34	3 018	3 006	155	31	0.995	0.00637	0.012936	0.0125

4.2 Paper II

We have evaluated a commercial version of RAPIDEC® CARBA NP against a total 276 Gram-negative bacilli consisting of (n=138) carbapenemase producers and (n=138) non-carbapenemase producers. A total of 135 isolates (97.8%) were correctly identified as carbapenemases producers using RAPIDEC® CARBA NP assay. One OXA-48-producing *K. pneumoniae*, one OXA-23 and one OXA-24-producing *Acinetobacter baumannii* were not detected in RAPIDEC® CARBA NP assay. Similarly, RAPIDEC® CARBA NP assay confirmed 135 (97.8%) negative isolates those were previously carbapenemases negative. One *Pseudomonas aeruginosa* with OprD loss and efflux, one ceftazidimase-producing *Klebsiella oxytoca* and one *Enterobacter cloacae* with impermeability were false positive in RAPIDEC® CARBA NP assay. Whole genome sequencing (WGS) was used to further analyse the three false positive isolates and known carbapenemases were absent in *P. aeruginosa* and *E. cloacae*. However, WGS analysis confirmed the

presence of the carbapenemase GES-5 in *K. oxytoca*, confirming that the positive result in the RAPIDEC® CARBA NP assay was in fact correct. After adjustment of GES-5 producer, overall sensitivity of the RAPIDEC® CARBA NP test was 97.8% and specificity was 98.5% (Table 6).

Table 6. Analytical sensitivity and specificity of RAPIDEC® CARBA NP test. Rights to reprint from JAC (Paper II).

	Sensitivity %	**Specificity %**	**True positive**	**True negative**	**False positive**	**False negative**	**Isolates tested**
Carbapenemase-producing isolates (overall)	97.8	-					139
Carbapenemase-producing Enterobacteriaceae (overall)	99.1	-	110	0	0	1	111
KPC	100	-	40	0	0	0	40
OXA-48-like	96.3	-	26	0	0	1	27
NDM[1]	100	-	30	0	0	0	30
VIM	100	-	5	0	0	0	5
IMP	100	-	5	0	0	0	5
IMI	100	-	1	0	0	0	1
GES	100	-	3	0	0	0	3
Carbapenemase-producing *Acinetobacter* (overall)	86.7	-	13	0	0	2	15
Carbapenemase-producing *Pseudomonas* (overall)	100	-	13	0	0	0	13
Carbapenem S or carbapenem R isolates without carbapenemase (overall)	-	98.5	0	135	2	0	137

[1] Including two isolates with NDM+OXA-48.

4.3 Paper III

We analysed a total 376 faecal samples from 188 healthy travellers. Thirteen of 188 (6.9%) were excluded from the analysis, as they were colonized with EPE before travel. We identified total 67 different EPE strains from 175 pre-travel negative participants. The predominant isolates were *E. coli* (n=65), followed by *K. pneumoniae* (n=1) and *Citrobacter freundii* (n=1). Carbapenemases-producing Enterobacteriaceae were not detected. Most EPE were of the the CTX-M type (61 strains) and most predominant type was CTX-M-1 (n = 49, 73.1%) followed by CTX-M-9 (n =12, 17.9%). A total 8 of 12 CTX-M-9-producing strains were acquired in South-East Asia (Figure 5).

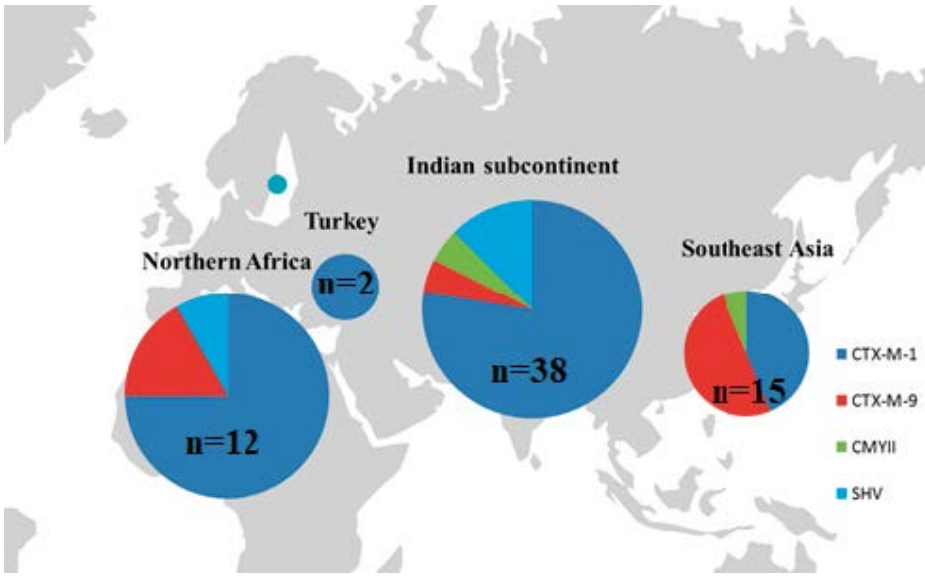

Figure 5. *Geographical representation of ESBL distribution. Circle size represents to colonization rate. Rights to reprint from JAC (Paper III).*

In 50 (76.9%) *E. coli* strains, type 1 fimbriae-encoding gene *fimH* was present, whereas *fimAMT78* was detected in 15 (23.1%) isolates. Pyelonephritis-associated pili-encoding genes were observed in six (9.2%) isolates and all these six isolates acquired *papEF* gene. Five isolates carried additional *papC* and *papGII* and two isolates harboured *papAH* gene. All *pap* harbouring strains were isolated from travellers travelling to India.

Phylogroup A (28/65, 43.1%) was the most commonly identified phylogroup, followed by phylogroup D (17/65, 26.2%), phylogroup B1 (12/65, 18.5%) and phylogroup B2 (8/65, 12.3%). Phylogroups B2 and D isolates were more commonly associated with multi-drug resistant (MDR) than the isolates of phylogroups A and B1. All isolates were identified as cefotaxime-resistant and 92.5% (62/67) isolates were resistant to ceftazidime. In other antibiotic classes, highest resistance 58.2% (39/67) was observed against trimethoprim/sulfamethoxazole, followed by ciprofloxacin 40.3% (27/67), gentamicin 29.9% (20/67), piperacillin/tazobactam 22.4% (15/67), amikacin 7.5% (5/67) and ertapenem 6.0% (4/67). A total 49.3% (33/67) isolates were detected as MDR strains. A total 46.3% (31/67) isolates harboured plasmid-mediated quinolone and fluoroquinolone resistance genes [*qnr* and/or *aac(6')-Ib*] and among them most commonly detected gene was *qnrS,* found in 20 isolates followed by *aac(6')-Ib* in 10 isolates, *qnrB* in 4 isolates and *qepA* in 1 strain. Ten out of 24 *qnr* harbouring isolates were resistant against ciprofloxacin. Two isolates harbouring 16S methylase gene *rmtB* were resistant to both gentamicin and amikacin. A CTX-M-55-producing *E. coli* isolate harbouring plasmid-mediated colistin resistance gene *mcr-1* was identified for the first time in Sweden from one traveler had travelled to Thailand in late 2013. The strain had a colistin MIC of 4 mg/L. ResFinder analysis also identified additional two aminoglycoside resistance genes [*aadA1A2* and *aac(3)-IId*], one $bla_{\text{TEM-1B}}$ and one fluoroquinolone resistance gene (*qnrS1*) in this isolate.

EPE colonization rates differed with different geographical region. Highest EPE colonization was observed in Indian subcontinent (49%) followed by northern Africa (44%) and lowest EPE colonization was observed in Turkey (10%) followed by Southeast Asia (19%). In agreement with previous studies, we found that diarrhea and antibiotic treatment during the trip were independent risk factors for EPE acquisition (OR 5.92), (65, 67, 68). EPE colonization was negatively associated with chronic diseases (OR 0.27). Sex, age, duration of trip, intake of proton-pump inhibitors, or use of oral cholera vaccine had no significant association with EPE colonization. The distribution of phylogroups differed between geographical regions. Strains belonging to phylogroup B2 (n=8) were only identified from travellers to Southeast Asia and the Indian subcontinent.

4.4 Paper IV

Initially, 17 neonates were colonized with $bla_{\text{CTX-M-15}}$ -producing *K. pneumoniae* (ESBL-KP), sequence type ST101. After storage, only 16 strains were viable and included in the molecular analysis. Optical mapping results of 16 ESBL-KP ST101 isolates confirmed that each isolate contained two different plasmids with different sizes. The sizes of the plasmids were approximately 80 kbp and 162–222 kbp.

Guide RNA was targeted to $bla_{\text{CTX-M-}}$ group1 genes which guide the Cas9 to cut at the same location of 80 kbp plasmid of all isolates. The location of $bla_{\text{CTX-M-}}$ group1 genes can be seen by using barcodes (Figure 6). Cas9 did not cut the larger plasmid which indicates that the larger plasmid did not acquire any $bla_{\text{CTX-M-}}$ group1 gene. Plasmids were linearized using light-induced DNA breaks to reveal the barcode. The size of the larger plasmid varied in different patient isolates, indicating that they all originated from same plasmid. All isolates had a large identical region (~160 kbp) but deletions were observed in three of the isolates; patient 3 (~5 kbp), patient 5 (~55 kbp) and patient 8 (~31 kbp). The deletion locations were clearly visible in the barcodes (Figure 6, right). This is how this method explains the variation in size of different plasmid. Considering these deletions, we confirmed that all of the larger plasmids had the same origin ($p<0.001$).

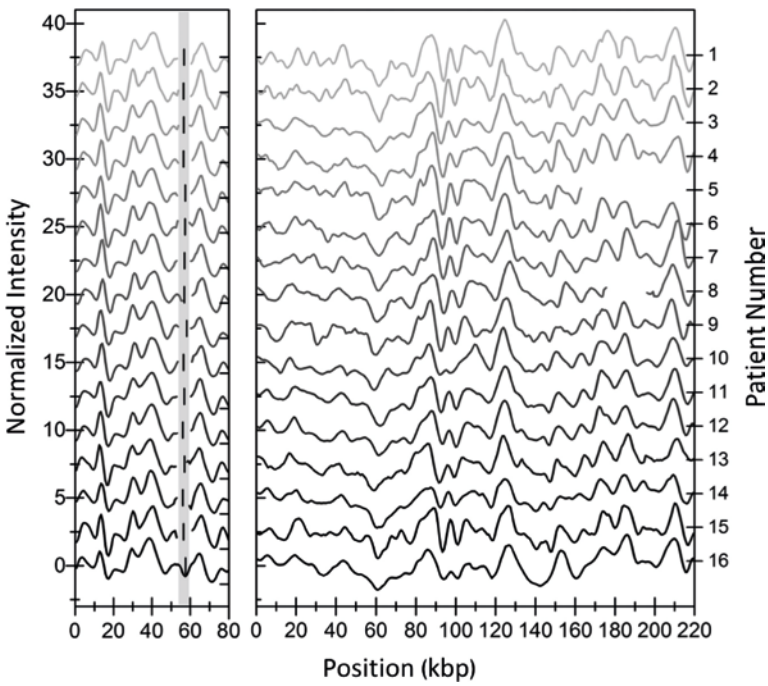

Figure 6. *Barcodes of different plasmids of ESBL-KP isolates from each of 16 patients. Rights to reprint from MBio (Paper IV).*

ESBL-KP isolates were collected from seven of the patients during the period of 6-24 months after initial isolation and were further investigated. In six isolates, 80 kbp plasmids harbouring $bla_{\text{CTX-M-15}}$ gene were present. The 80 kbp plasmids were identical with the initial five patients (p-< 0.001), whereas an inversion of about 31 kbp was present in the small plasmids of patient 6 (Figure 7, dashed region). In five out of six isolates, one larger plasmid was completely missing in an isolate from patient 4 (Figure 7 right). A region of 5-65 kbp deletions were observed in isolates from three patients. Three plasmids with size of 72±3.6, 122±6.3, and 204±10.2 kbp were observed in the isolate of patient 13 which was collected 9 months after initial isolate (Figure 7 bottom). No overlap was observed, and no $bla_{\text{CTX-M}}$ group 1 gene was detected in any of these three plasmids which indicated that the ESBL-KP isolate was unrelated to the outbreak.

Furthermore, genetic characterization of the plasmid content in all ESBL-KP samples were investigated using long-read sequencing on the PacBio platform to sequence the plasmid from the initial ESBL-KP from patient 1 (P1K0). We compared the barcodes obtained from optical mapping with the theoretical barcodes predicted from the PacBio sequencing and found excellent overlap (Figure 8). The size of smaller and larger plasmids were 80272 bp and 215872 bp, respectively, were determined by PacBio sequencing. Sequence analysis determined that the small plasmid 80 kbp contained IncR, and IncFIA origin. In addition to $bla_{\text{CTX-M-15}}$, it also contained $bla_{\text{TEM-1}}$, and genes encoding resistance to fluoroquinolones (*qnrB1*), sulphonamides (*sul2*), trimethoprim (*dfrA14*) and aminoglycosides (*aaac(3)-IId*), *aph(6)-Id*, *aph(3")-Ib*). The larger plasmid did not carry any resistance genes.

Additional ESBL-producing *E. coli* (ESBL-EC) were found in the follow up samples. The $bla_{\text{CTX-M-group-1}}$ gene was absent which indicates that ESBL phenotype of this isolates had a different genetic origin than ESBL-KP. Approximately, a size of 130 kbp identical plasmids were found in ten of the investigated ESBL-EC isolates. To validate the optical mapping data, we sequenced isolate P3E6 using PacBio and found a plasmid with size of 129 kbp, and a small size of 5,878 bp of cryptic plasmid. Resistance genes were absent in all of these plasmids, but the sequencing confirmed that $bla_{\text{CTX-M-15}}$ gene was located on the *E. coli* chromosome, which was in accordance with optical mapping results, as there was lack of Cas9 cleavage of any plasmids.

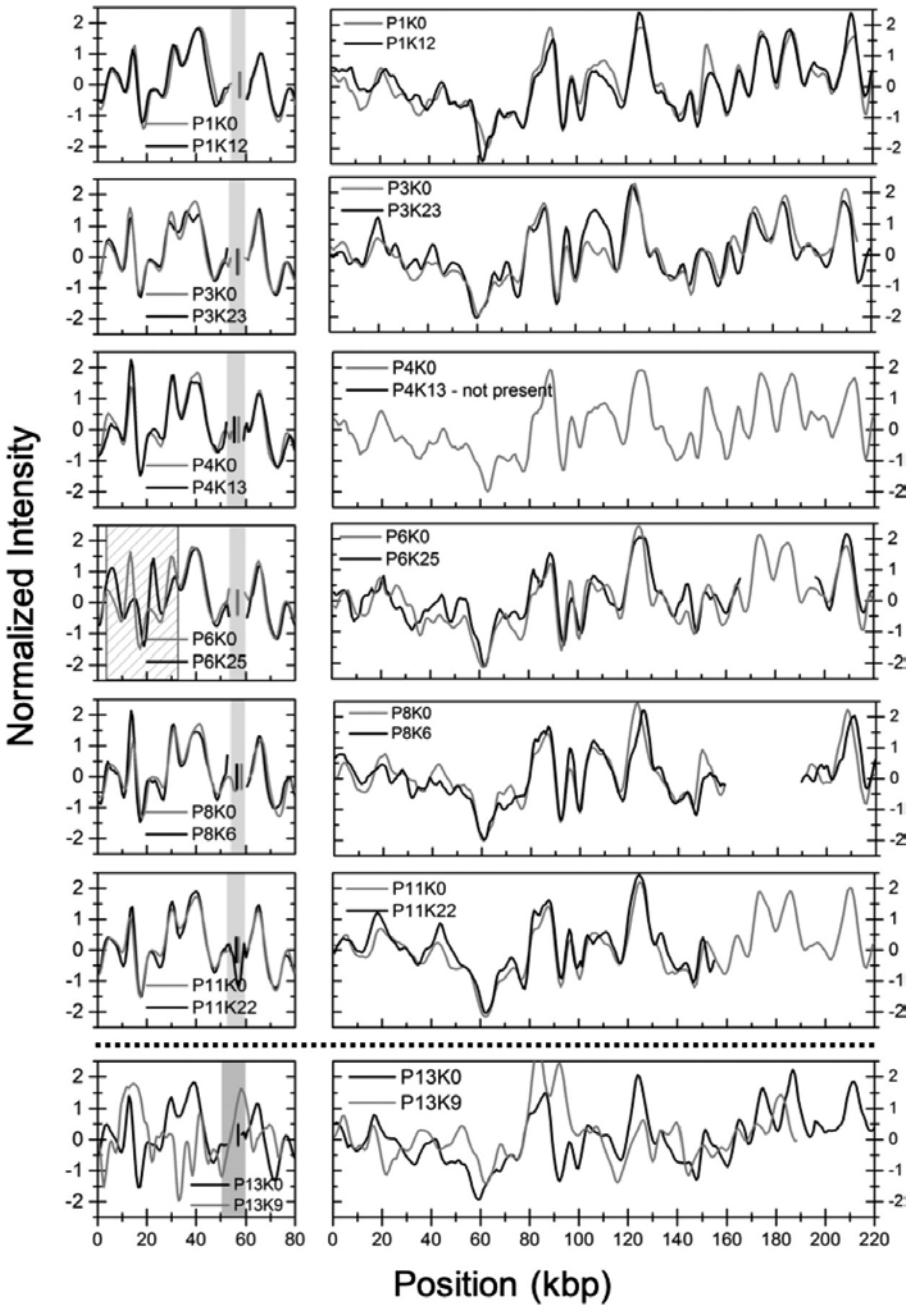

Figure 7. *Comparison between follow up ESBL-KP isolates (black lines) and corresponding initial ESBL-KP ST101 isolate (gray lines). Rights to reprint from MBio (Paper IV).*

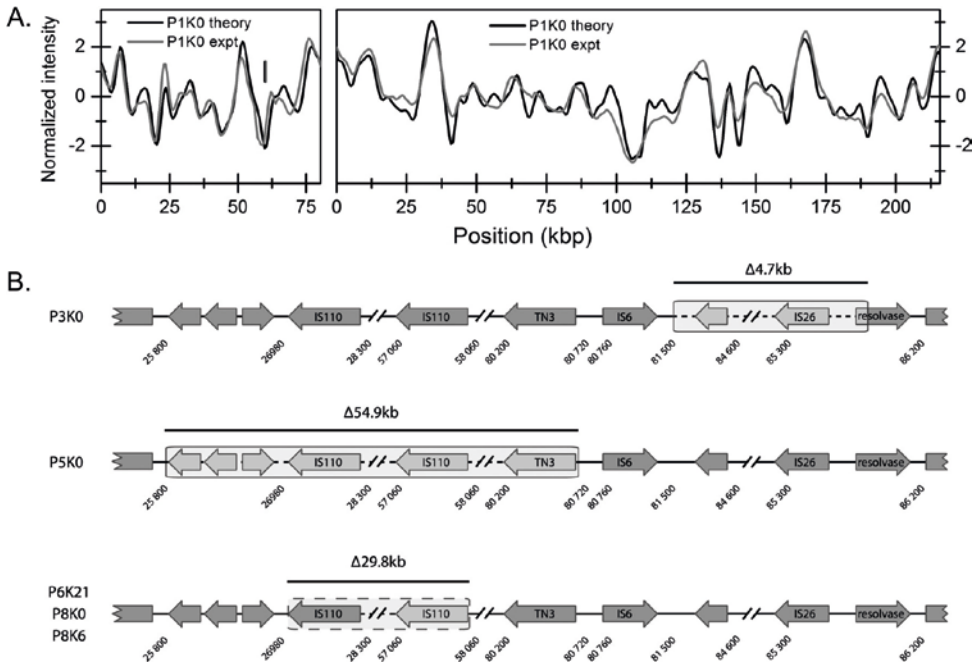

Figure 8. *Comparison between experimental optical map (gray) and theoretical optical map created from PacBio sequencing (black) for the two plasmids in P1K0. Rights to reprint from MBio (Paper IV).*

5 DISCUSSION

5.1 Major Findings

In the first project, we wanted to analyze clonal diversity and the prevalence of EPE and CPE in invasive isolates of *K. pneumoniae* isolated from BSI patients in a well-defined geographical area and we collected all samples from the department of clinical microbiology at Karolinska University Hospital, Stockholm. We observed very low prevalence of antimicrobial resistance among Swedish bloodstream infection (BSI) patients. Only five out of 139 isolates were detected as MDR (resistant to three or more than three antibiotic classes). All of these five isolates were EPE (two isolates CTX-M-1, one isolate CTX-M-1 and SHV, one isolate SHV) but only one isolate was a CPE (carbapenemase VIM), CTX-M-1 and CMYII. Then, we wanted to characterize *Klebsiella pneumoniae sensu lato* isolated from BSI patients to analyze their population structure. We also wanted to investigate potential linking between phylogroups, bacterial traits, mortality and comorbidity. We observed three distinct phylogroups, the largest phylogroup was KpI consisting of (n=96) isolates of *K. pneumoniae* followed by phylogroup KpIII consisting of (n=34) isolates of *K. variicola* and phylogroup KpII consisting of (n=9) isolates of *K. quasipneumoniae*. We also observed a high link between 30-day mortality and *K. variicola* (KpIII) (29.4%) isolates compared to *K. pneumoniae* (KpI) (13.5%) isolates. Although, *K. variicola* (KpIII) isolates had low virulence and resistance profile compared to *K. pneumoniae*. We observed several comorbidities; malignancy, diabetes mellitus, cirrhosis, biliary tract disorders and alcoholism among patients, which may have influenced the mortality rate.

Secondly, we wanted to investigate the acquisition of EPE and CPE among healthy people those were travelling to high prevalence of EPE and CPE region. We included 188 healthy adult Swedish tourists with planned travel to high EPE and CPE prevalent regions; Southeast Asia, Indian subcontinent, North Africa and the Middle East. We excluded 13 travellers from the analysis who were colonized with EPE before travelling. We found 67 out of 175 healthy Swedish tourists were colonized with different EPE strains; *E. coli* (n=65), *K. pneumoniae* (n=1) and *Citrobacter freundii* (n=1). We also observed that healthy Swedish tourist were not colonized with any carbapenemase producers. The most prevalent EPE type was CTX-M type detected in 61 isolates; 49 isolates were CTX-M-1 and 12 isolates were CTX-M-9. We also observed that Swedish healthy tourists were colonized with low virulence EPE isolates. Only six isolates acquired pyelonephritis-associated pili-encoding genes (*pap*) genes and travellers were colonized with these six isolates from India. Recently emerging colistin resistance gene *mcr-1* harboring *E. coli* was colonized with a traveler travelling to Thailand. We observed that only 8 out of 65 isolates were belonged to MDR associated phylogroups B2 and all of these 8 isolates were isolated from the travellers had travelled to South-East Asia

(n=2) and Indian subcontinent (n=6). As expected, highest colonization rates was observed in Indian subcontinent (49%) and northern Africa (44%) whereas lower rates was observed in Southeast Asia (19%) and Turkey (10%). We also observed that diarrhea and antibiotic treatment during the trip were associated with EPE colonization, but chronic diseases were negatively associated with EPE colonization. We did not find any significant association between sex, age, duration of trip, intake of proton-pump inhibitors, or use of oral cholera vaccine with EPE colonization.

Thirdly, we wanted to evaluate a rapid detection and characterization methods for CPE and EPE due to the urgent need of rapid, simple and reliable phenotypic detection method for low income settings. In low income settings, prevalence of EPE and CPE is high and can be migrated to low prevalence EPE and CPE area like Nordic countries (Showed in travellers study). We evaluated a commercial version of RAPIDEC® CARBA NP, colorimetric liquid-based assay for rapid detection of carbapenemases. The in-house Carba NP assay can be setup but will be challenging for routine laboratories due to high instability of carbapenems. To our knowledge, we reported for the first time two-centre evaluation of RAPIDEC® CARBA NP assay against 276 Gram-negative bacilli. We observed that RAPIDEC® CARBA NP assay can detect carbapenemases in less than 2.5 hours with high sensitivity (97.8%) and specificity (98.5%) without requiring any specialized equipment.

Finally, we evaluated a novel method based on optical DNA mapping assisted with CRISPR/Cas9 to characterize plasmids during EPE outbreak. This method should be equally useful to trace CPE outbreak, although this was not investigated in this paper. The basic of this method is, CTX-M group 1 gene was targeted by guide RNA to guide Cas9 to cut the same location of 80 kbp plasmid of all isolates. Using barcodes, location of CTX-M-1 gene (targeted gene) can be seen. We reported that this method successfully can detect different size of plasmids; small size 80 kbp and larger plasmid (162-222) kbp. We also reported that this method can track the changes of plasmid over time. We reported deletions in three plasmids of three isolates with different size (~5 kbp), (~55 kbp) and (~31 kbp) and inversion of 31 kbp in another isolate.

Despite not being part of the aims of the thesis, it is worth mentioning that rapid antimicrobial susceptibility testing (AST) is urgently needed. Although, it was not the goal of this thesis but to facilitate treatment decision and to prevent spreading of antimicrobial/antibiotic resistance resulting from abuse (misuse/overuse) of antibiotics, there is a major demand for development of rapid AST. AST used in this thesis (currently available techniques) require 16-18 hours to read out the results, but with novel methods this could drop to 30 minutes. This will be discussed in detail in the future perspectives section of this thesis.

5.2 Phylogroups of *K. pneumoniae*

Over the years, phylogenetic analysis of *K. pneumoniae* has resulted in the division into several phylogroups. Phylogroups KpI to KpIV were first reported in the year 2001 (175). Then, two more phylogroups KpV and KpVI were reported in 2017 (188). Another phylogroup KpVII has been reported recently (189). Phylogroups KpI to KpIV and KpVI have taxonomic status. Phylogroups KpI to KpIV and KpVI represent different taxa *K. pneumoniae (sensu stricto), K. quasipneumoniae* subsp. *quasipneumoniae, K. variicola* and *K. quasipneumoniae* subsp. *similipneumoniae* respectively. Phylogroups KpV and KpVII have no taxonomic status but Brisse and colleagues have recently proposed the names *K. variicola* subsp. *tropicalensis* for KpV and *K. africanensis* for KpVII (189). In this thesis, we tested three phylogroups; KpI, KpII and KpIII in project I. Phylogroups KpIV-KpVII were not tested. In fact, KpV and KpVII were not reported during the time we studied project I. We were able to identify all three phylogroups known at the time; KpI (69%), KpII (6%) and KpIII (24%). To our knowledge, this study was the first to find association between phylogroups, virulence factors and mortality data in a well-defined geographical area. In the year 2004, a study based on (n=420) isolates from 26 European hospitals also reported three phylogroups; KpI (82.1%), KpII (6.9%) and KpIII (11%) (190).

5.3 Misidentification of *K. variicola* and *K. quasipneumoniae*

The conventional clinical microbiology laboratory techniques are unable to distinguish *K. pneumoniae* (KpI)*, K. variicola* (KpIII) and *K. quasipneumoniae* (KpII) due to their closely overlapping biochemical properties (191). *K. pneumoniae* is adonitol positive but *K. variicola* and *K. quasipneumoniae* are adonitol negative. Adonitol fermentation and indole positivity are considered as the classifier but the phenotype is unstable and not consistent among all the isolates of these three species (192). The correct bacterial species identification using molecular diagnostics mainly depend on the availability of the databases. The database of common molecular diagnostics platforms; Verigene, Vitek or MALDI-TOF MS did not include *K. variicola* and *K. quasipneumoniae* until recently so either they were not identified or misidentified (193). Previously it has been reported that 2.5% to 10% of *K. pneumoniae* were misidentified and should have been reported as *K. variicola* (194-197). The current Bruker MALDI-TOF MS microorganism database included *K. variicola* in the year 2015 but still *K. quasipneumoniae* was not included, causing continuation of misidentifications (193, 194). Previous studies have showed overrepresentation of phylogroup KpI (*K. pneumoniae*) but in this study phylogroup KpIII (*K. variicola*) was also relatively common (190, 198). Previously, it has been reported that *K. variicola* causing bloodstream infection was incorrectly identified as *K. pneumoniae* using classical and automated identifications but later, correctly identified as *K. variicola* using next-generation sequencing (199).

5.4 Association of phylogroups, bacterial factors, mortality and comorbidity

In the first project of this thesis, to our knowledge we are the first to discuss the possible association with phylogroups, bacterial factors (virulence factors, serotypes), mortality and comorbidity. In accordance with previous studies we observed 17.3% overall 30-day mortality on BSI caused by *K. pneumoniae* (200, 201). High mortality ranges of 20% to 62% has been observed in *Klebsiella* bacteremia, reported previously (202). A recent study showed that the 30-day mortality rate was 40.7% in classical *K. pneumoniae* bacteremia compared with 37.1% hypervirulent *K. pneumoniae* bacteremia (203). One explanation could be that less virulent strains were acquired by severe sick patients and patients died due to underlying diseases. In our study, we also observed that a total of 10 isolates of *K. variicola* (Phylogroup KpIII), 13 isolates of *K. pneumoniae* (Phylogroup KpI) and one isolate of *K. quasipneumoniae* (Phylogroup KpII) were linked with 30-day mortality. Among them, only 3 isolates out of 24 cases of 30-day mortality were associated with investigated virulence factors; K54 (n=1), mucoid phenotype (n=1) and/or virulence genes (*wcaG* n=1 and *wcaG/allS* n=1). According to our knowledge, we reported for the first time that *K. variicola* bacteremia in adults were associated with higher 30-day mortality than bacteremia caused by *K. pneumoniae*. A recent study based on an outbreak of multidrug-resistant (MDR) *K. variicola* in a neonatal unit of Dhaka Medical College at Dhaka, Bangladesh in the year 2016 to 2017 reported that *K. variicola* is also highly associated with mortality (54.5%) due to BSI in neonates (204). In our study we observed a significant (P=0.037) difference in mortality among KpI and KpIII that has been shown both in univariate analysis and in multivariate analysis after adjustment of age, gender, time to get antibiotic treatment, comorbidity and polymicrobial infections. Clinical conditions of patients were more or less similar so that the reason for the difference in mortality between phylogroups remains inexplicable. In this type of study, it was complicated to measure time to appropriate antibiotic treatment and other recommended therapy due to the severity of disease. In this study, we also observed very low degree of antibiotic resistance but did not observe any significant changes among survivors and deaths, although 55.4% of the patients received adequate antibiotic treatment within 4 hours and 87.1% patients received adequate antibiotic treatment within 24 hours. Here, we studied BSI and mortality, but BSI may in many cases not be the main cause of death. Previously published reports suggested that several comorbidities such as malignancy, diabetes mellitus, cirrhosis, biliary tract disorders and alcoholism may suppress patient immune system and contributed to develop *K. pneumoniae* infections which may influence mortality rate (203, 205-207). We also found in our study that *K. pneumoniae* (phylogroup KpI) isolates were more resistant than *K. variicola* (phylogroup KpIII) isolates, which is in accordance to previously published report (190, 198). In our study we found only five isolates that were considered multidrug-resistant (MDR) belong-

ing to phylogroup KpI and all were ESBL-producers. This is in accordance with previously published report, they also found that most of the ESBL-producing isolates belonged to phylogroup KpI (208).

5.5 Phylogroups of *E. coli*

Six phylogroups; A, B1, B2, C, D and E of *E. coli* were first defined by a study based on multi-locus enzyme electrophoresis on 35 enzyme loci (209). Later, presence of all these above mentioned phylogroups were confirmed by DNA methods; rDNA restriction fragment length polymorphism, random amplified polymorphic DNA, MLST, whole genome sequencing (WGS)(210-212). Another new phylogroup F has been reported recently as a seventh phylogroup of *E. coli* (213). The most ancient phylogroups of *E. coli* are B2 and D, then emerged E, followed by the most recently diverged phylogroups C, B1 and A (213). The phylogenetic history of this species and the lifestyle of the strains are associated. The majority of extra-intestinal pathogenic strains belonged to phylogroups B2 and D whereas strains of recently diverged phylogroups C, B1 and A are associated with diarrhoeagenic strains (30). The latest phylogroup G has been reported in 2019 consisting of isolates being an intermediate between the phylogroup F and B2. Isolates of this phylogroup have been found to be associated with extra-intestinal virulence and acquired MDR (30). In this thesis we did not test all eight phylogroups of *E. coli*, since several were reported after the study was completed. We observed in this thesis that phylogroup A (43.1%) was more predominant than phylogroup B2 (12.3%) which is in accordance with the recently published report (2018), where they also found domination of phylogroup A (73%) than phylogroup B2 (33%) in travellers (214). Our findings and this recent study suggested that colonization of MDR strains were very few in healthy Swedish tourists. Strains of phylogroup A are responsible for causing dysentery, and we found that 28.7% of travellers suffered from travellers' diarrhoea. Based on the virulence profile of *E. coli* in this thesis we suggest that a majority of healthy travellers are colonized by less virulent strains of *E. coli*. In a previous study from our research group, we found B2 was the predominant phylogroup in stool samples which is in contrast of this study (215). It could be an explanation that previous study was performed on patients whereas the current study was performed on healthy populations.

5.6 Colonization and duration of EPE and CPE in travellers

To our knowledge, acquisition of CPE colonization is still rare in healthy travellers. In our study, we did not observe any CPE colonization in healthy tourists, but we observed that 34.9% of healthy tourists were colonized with CTX-M type EPE. The risk of developing clinical infections by EPE colonizing strains is not yet defined. It has been reported previously that 7.4% individual prior colonized

with third generation cephalosporin-resistant Enterobacteriaceae were at risk of acquiring bacteremia (216). In our study all isolates were likely less prone to cause clinical infections, as the majority of the isolates had limited number of virulence determinants and were community-acquired. During 26 months of follow-up of EPE positive travellers we did not observe any clinical infections due to EPE. It suggests that there is a risk of being colonized with EPE while travelling to high EPE regions, but the risk is very low for future clinical infections due to EPE colonization. However, there is still potential risk of dissemination of resistant strains from healthy travellers to patients, who may have a greater risk of developing disease.

A recently published report mentioned that 113 days and 60 days were the mean and median duration of carriage of CTX-M-EC, respectively. They found 31% of travellers being CTX-M-EC carrier at 6 months after travel. The longest duration of carriage of CTX-M-EC they observed was 11 months after travel (214). Another recent study reported that 11.3% travellers were persistently colonized at 12 months after travel (217) The limitation of our study was that we did not perform long term follow-up ESBL-EC carriage.

5.7 Rapid detection of CPE

In this thesis, we have evaluated a commercial version of RAPIDEC® CARBA NP test to detect carbapenemases and we found that this test was highly sensitive (97.8%) and specific (98.5%). We detected class A, including weak class A carbapenemase GES-5 and IMI, and class B carbapenemases using commercial version of RAPIDEC® CARBA NP assay. We observed that this commercial version was unable to detect two *A. baumannii* producing OXA-23 and OXA-24 and one *K. pneumoniae* producing OXA-48 but these enzymes were detected in other strains by RAPIDEC® CARBA NP thereby this issue was not consistent with this assay. A previous study also reported the failure of detection of some OXA-48 isolates with in-house CARBA NP test (218). The overall sensitivity 92.2% and specificity 100% were reported by a single-centre study (219). They also reported increased sensitivity to 100% by doubling the inoculum of bacteria but we did not follow this approach as in principle specificity could be affected negatively by this approach. However, the developer of original Carba NP test, Poirel and Nordmann also evaluated commercial version of RAPIDEC® CARBA NP assay against 176 isolates and reported 5 out 98 carbapenemase producers were detected as false negative; *P. aeruginosa* producing GES-2, *A. baumannii* producing OXA-23, *Acinetobacter pittii* producing OXA-40, and *Proteus mirabilis* producing chromosomally encoded OXA-23 (125). They also reported 3 out of 75 non-carbapenemase producers as false positive; two *A. baumannii* producing chromosomal AmpCs and one *Proteus vulgaris* producing CMY-2. We also observed two false positive results; one *Enterobacter cloacae* with impermeability and one *P. aeruginosa* with OprD loss in our study. Any other phenotypic carbapenemase detection methods were not tested in this thesis.

5.8 Plasmid characterization using CRISPR/Cas9 assisted optical DNA mapping

In this thesis, a novel method based on optical DNA mapping combined with CRISPR/Cas9 was developed for rapid and detailed characterization of plasmids from bacterial isolates. The purpose of this study was to characterize the ESBL plasmids from an NICU outbreak at Karolinska University Hospital Stockholm, Sweden and investigate whether several different ESBL-producing bacterial species were present due to horizontal spread of plasmid.

Using Cas9, we were able to identify the exact location $bla_{\text{CTX-M-1}}$ gene on one particular plasmid. Analyzing barcode from optical DNA mapping we were able to confirm the presence of particular plasmid in other isolates during outbreak (if any). We observed, at the beginning of outbreak $bla_{\text{CTX-M-1}}$ gene producing plasmid was identical in all isolates and it persisted within neonates more than a year after discharge from the NICU. DNA mapping also revealed that outbreak ESBL-KP plasmid was different from the ones observed in ESBL-EC and in one strain of ESBL-KP. This suggests that no horizontal transfer had occurred between these species. We also observed a small second chain of transmission in three patients as we found the same ESBL-EC plasmid in isolates from three patients. The $bla_{\text{CTX-M-1}}$ gene was located on the chromosome in these strains. We could conclude that additional plasmid-mediated dissemination of resistance was not taking place in this outbreak. The comparison between long-read PacBio sequencing and theoretical barcodes suggest that the prediction of the location of the $bla_{\text{CTX-M-15}}$ gene by Cas9 restriction was correct.

6 CONCLUSIONS

Paper I

1. Population structure of *K. pneumoniae* revealed three well-defined phylogroups; *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII) and *K. variicola* (KpIII). Phylogroup KpIII isolates were highly associated with 30-day mortality. High level of comorbidity was observed among the isolates of phylogroup KpI and KpIII.
2. Highly virulent *K. pneumoniae* clones are still rare in Stockholm area. Isolates belonging to phylogroup KpIII were more often associated with mortality than phylogroup KpI and KpII but were not associated with known virulence factors.

Paper II

3. The RAPIDEC® CARBA NP assay can detect carbapenemases within less than 2.5 hours with high sensitivity and specificity without requiring any specific equipment.
4. RAPIDEC® CARBA NP assay is a rapid, simple, and reliable tool for detection of carbapenemases.

Paper III

5. Isolates belonging to phylogroup B2 were only found in Southeast Asia and Indian-subcontinent but were absent in North Africa and Middle East.
6. Carbapenemases were absent, but we found the *mcr-1* gene encoding colistin resistance in an ESBL-producing strain from one of the travellers.
7. Antibiotic treatment during travel can increase the risk of EPE colonization.
8. The risk of colonization of EPE is higher than the risk of colonization of CPE while traveling to high-prevalence areas.

Paper IV

9. Optical DNA mapping combined with CRISPR/Cas9 method can readily demonstrate the number of plasmids, their size, and the location of resistance genes of interest
10. During an outbreak the plasmids change to a limited degree (e.g. deletions and inversions), and the plasmid typing can serve as an indirect tool for detecting strain outbreaks.
11. The findings with optical mapping were confirmed with long-read sequencing (PacBio), confirming the reliability of the novel technique
12. Optical DNA mapping could be a clinically useful novel molecular tool to characterize plasmids during hospital outbreaks with ESBL-producing Enterobacteriaceae.

7 FUTURE PERSPECTIVES

Antibiotic resistance is a severe threat to public health. It has been estimated that 10 million deaths and more than one trillion dollars in total economic impact would be the cause of antimicrobial resistance by 2050 if no action is taken. It is important for health care settings to determine antimicrobial susceptibility rapidly to be able to facilitate treatment decisions and avoid unnecessary broad-spectrum therapy. No phenotypic AST method has so far been available to obtain results within 30 minutes directly from clinical samples. An overnight culture is required to perform AST using currently available methods and all AST used in this thesis were performed following currently available methods. Depending on time and sample type (pure isolates/ direct samples) the following methods could be used in the near future to develop rapid phenotypic antibiotic susceptibility testing methods for clinical laboratories.

Atomic force microscopy (AFM) cantilever: The bacteria can accumulate on the surface of the cantilever. The movement of the bacteria can increase the amplitude of the cantilever fluctuations. These fluctuations can be sensed by using a sensing chamber. If bacteria are susceptible to antibiotics they will demonstrate decreased movement. Thus, the fluctuation of the cantilever will decrease and vise-versa. This can be used to identify MIC. It has been reported previously that MIC can be determined within 15 minutes after antibiotics treatment (220). Pure culture isolates may be required to perform the test, for which reason it may have to be combined with traditional culture before AST can be done from colonies.

Microfluidic agarose channel (MAC) system: In this method, AST can be determined by using immobilized bacteria in the agarose media. The mixture of bacteria and liquid agarose gel will be injected into the microfluidic channel and bacteria will be immobilized within the channel. Bacterial growth media and antibiotics will be introduced into the agarose by using a capillary valve. Antibiotic will diffuse into the agarose matrix that can be monitored in real time by using microscopy. Using the single bacterial time lapse images, growth of bacteria can be determined in different concentrations of antibiotics (221). This method may also require pure bacterial colonies and could be challenging to use directly on samples.

Isothermal micro calorimetry (IMC): In this method, bacterial growth can be determined by measuring the heat flowrate of a given bacterial suspension. In principle, produced heat is proportional to the reaction rate in the suspension. Using this data, MIC can be determined (222). This method can also distinguish between bacteriostatic and bactericidal effect of antibiotics. Using this method, antibiotic can be grouped into different mode of action; cell wall synthesis inhibitors, DNA/ protein synthesis inhibitors. This method can also be used for classification of new antibiotics. This method normally takes ~24 hours to obtain MIC results. Thus far, it has only been used on cultivated bacterial colonies.

Digital loop-mediated isothermal amplification (dLAMP): A recent published article reported that they have developed this ultrafast method for performing AST within 30 minutes from clinical urine samples, published 2017. It has been mentioned in this report that AST can be determined using digital nucleic acid quantification to measure the phenotypic response after antibiotic treatment to *E. coli* containing urine samples. In this report it has also been described that they used digital quantification of a DNA marker to lower the antibiotic exposure time to 15 minutes. Then, they showed digital AST (dAST) can distinguish the response of target pathogen and commensals. Finally, a rapid, high-resolution measurement method for quantifying NA targets was developed and optimized to reduce the measurement step to less than 10 minutes (222, 223). This method, like many other methods may work best on single colonies, and it is not clear whether it would work directly on complex samples.

Finally, advancement of development of rapid AST methods will provide health care providers with a powerful tool to tailor antimicrobial treatment, and thus to fight antimicrobial resistance.

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