

Burden of Antibiotic Resistance in Neonates from Developing Societies
The role of *Klebsiella pneumoniae* in neonatal sepsis

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by

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Summary

The Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) study was a multi-site international study in 12 sites across seven different low- and middle-income countries (LMICs) in Africa and South Asia. The primary aim of BARNARDS was to assess the burden of antibiotic resistance in neonates looking at neonatal morbidity and mortality. In BARNARDS, *K. pneumoniae* was the leading Gram-negative bacterial cause of neonatal sepsis and was frequently isolated from rectal samples from mothers (MR), neonates (BR) and samples from the clinical environment (ENV).

I found that *K. pneumoniae* was a particularly dominant species recovered from samples in Ethiopia and Pakistan, and for different reasons. Firstly, multiple outbreaks, or epidemiological clusters (that are still being investigated with the local clinical sites) of isolates recovered from blood cultures were isolated over a period of 12 months (2016-2017) from Addis Ababa, Ethiopia. Secondly, in Islamabad Pakistan, the rectal carriage of carbapenemase positive Gram-negative bacteria (GNB) was high, particularly in neonates, and the most frequently identified species was *K. pneumoniae*. In sampling the clinical environment to ascertain the frequency of carbapenemase colonisation within the wards and surrounding hospital environment, I also found that *K. pneumoniae* was often recovered in multiple countries.

With this in mind this PhD study characterised BB, MR, BR and ENV *K. pneumoniae* from Pakistan and Ethiopia. The population structure and phylogeny of *K. pneumoniae* was studied with focus on their resistome and virulome to characterise the genomic traits, and antibiotic susceptibility testing for a phenotypic characterisation. The results showed that *K. pneumoniae* were resistant to many antibiotics used in treatment including carbapenems and cephalosporins and harboured high numbers of ARG belonging to different classes, including those encoding carbapenemases and extended-spectrum β -lactamases. Furthermore, the *K. pneumoniae* population in Ethiopia and Pakistan varied, for example, in Pakistan the most common ST was ST15 whereas in Ethiopia ST35 and ST37 were the most common. Additionally, carbapenemase ARG were frequently found among isolates from Pakistan, with ST15 isolates carrying *bla*_{NDM-1} and *bla*_{OXA-181}, but carbapenemase ARG were not frequently found among isolates from Ethiopia. Patterns of virulence determinants also varied, for example, Ethiopian *K. pneumoniae* showed capsule loci

KL15, as the most common whereas Pakistani isolates displayed KL112, also, *ybt* was the most common siderophore loci among both PP and ES isolates but *iuc* and *iro* loci were rare in Pakistani isolates.

Lastly, *K. pneumoniae* belonging to the same were found among different sample types indicating the possible transmission of bacteria capable of causing sepsis between mother, neonate and the environment. Of note, this thesis detected cases whereby transmission of the same of *K. pneumoniae* strain between the mother, neonate and clinical environment is likely to have occurred.

Poster and Oral presentations at major international conferences

27th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Vienna, Austria, 22 – 25 April, 2017.

High levels of AMR *Enterobacteriaceae* present in the maternal faecal flora in low middle income countries

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Burden of antibiotic resistance in neonates from developing societies (BARNARDS): *Klebsiella pneumoniae* in neonatal sepsis

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List of abbreviations

Agly	Aminoglycoside antibiotic resistance gene
AMC	Amoxicillin/clavulanate
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
ARG	Antibiotic resistance gene
ATM	Aztreonam
AZM	Azithromycin
BARNARDS	Burden of Antibiotic Resistance in neonates from Developing Societies
BB	Neonate blood sample
BHK	Bhara Kahu
<i>bla</i>	β -lactamase antibiotic resistance gene
BR	Neonate rectal sample
BS	Biological sepsis
BSI	Blood stream infection
CAZ	Ceftazidime
CG	Clonal group

CHUK	University Central Hospital of Kigali
CIP	Ciprofloxacin
cKp	Classic <i>Klebsiella pneumoniae</i>
<i>clb</i>	Colibactin
CMOSH	Chittagong Maa-O-Shishu Hospital
CNK	Carbapenemase negative <i>Klebsiella pneumoniae</i>
CPE	Carbapenemase producing <i>Enterobacteriaceae</i>
CPK	Carbapenemase positive <i>Klebsiella pneumoniae</i>
cps	Capsular polysaccharide synthesis locus
CPS	Capsule polysaccharide
CRE	Carbapenemase resistant <i>Enterobacteriaceae</i>
CRKp	Carbapenemase producing <i>K. pneumoniae</i>
CRO	Ceftriaxone
CSE	Carbapenemase susceptible <i>Enterobacteriaceae</i>
CST	colistin
CTX	cefotaxime
ENV	Clinical environment swab sample
EOS	Early onset sepsis
ES	Ethiopia
ESBL	Extended-spectrum b-lactamase
ETP	ertapenem
Fcyn	fosfomycin antibiotic resistance gene
FEP	cefepime
Flq	fluoroquinolones antibiotic resistance gene
Flq SNP	fluoroquinolones SNP
FOF	Fosfomycin
gDNA	genomic DNA
GEN	Gentamicin
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
HCCA	α -Cyano-4-hydroxycinnamic acid
HGT	Horizontal gene transfer
HiR	High risk clone
hvKp	Hypervirulent <i>Klebsiella pneumoniae</i>
IA	Infants admission pathway
IMP	Imipenemase
IPM	Imipenem
iro	salmochelins
iuc	aerobactin
KH	Kabgayi Hospital
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
KpSC	<i>Klebsiella pneumoniae</i> species complex
KWMCH	Kumudini Women's Medical College Hospital
LMIC	Low-middle income country
LOS	Late onset sepsis
LPS	Lipopolisaccharide
LVX	Levofloxacin
LZD	Linezolid

MBL	Metallo-b-lactamase
MDR	Multidrug resistant
MEM	Meropenem
MfBS	Mortality following sepsis
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MIN	Minocycline
MLS	macrolides antibiotic resistance gene
MLST	Multilocus sequence type
MMSH	Murtala Mohammad Specialist Hospital
MR	Mother rectal sample
ND	Not determined
NHA	National Hospital Abuja
NICED	National Institute of Cholera and Enteric Diseases
OXA	Oxacillinase
Phe	phenicol antibiotic resistance gene
PIMS	The Pakistan Institute of Medical Sciences
PLA	Pyogenic liver abscess
PP	Pakistan
PTA	Potential target attainment
RIF	Rifampicin
Rif	rifampin antibiotic resistance gene
ST	sequence type
StPHMMC	St. Paul's Hospital Millennium Medical College
Sul	sulfonamides antibiotic resistance gene
<i>tet</i>	tetracyclines antibiotic resistance gene
TGC	Tigecycline
Tmt	thrimetrprim antibiotic resistance gene
TOB	Tobramycin
TZP	Piperacillin/tazobactam
UTI	Urinary tract infection
V	Vancomycin agar plates
VAN	Vancomycin
VE	Vancomycin and ertapenem agar plates
VIM	Verona integron encoded metallo-b-lactamase
WDH	Wuse District Hospital
WHO	World Health Organisation
<i>ybt</i>	yersiniabactin

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Declaration

This thesis is the result of my own independent work, except where otherwise stated, and the views expressed are my own. This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is it being submitted concurrently for any other degree or award.

Ana Daniela Sanches Ferreira

31 March 2021

1. General Introduction

1.1 Antibiotics and antibiotic resistance

Antimicrobial drugs are substances that are capable of either killing or controlling the growth of microorganisms. These are classified based on their molecular structure, mechanism of action and spectrum of activity. In addition, these can be synthetic drugs or naturally occurring compounds when produced by microorganisms such as *Bacteria* and fungi. Antibiotics are natural antimicrobials and synthetic antibacterial drugs include quinolones and sulfonamides (Fig. 1). Antimicrobials display several different mechanisms of action. Quinolones prevent the supercoiling of DNA in bacteria whereas treatments such as sulfamethoxazole, a sulfonamide, in combination with trimethoprim block the synthesis of folic acid, thus, inhibiting nucleic acid synthesis (1). Antibiotics, such as tetracyclines, aminoglycosides and macrolides, disrupt translation by interacting with ribosome subunits ultimately inhibiting protein translation. Other antibiotics inhibit transcription by inhibiting RNA synthesis. β -lactams inhibit cell wall synthesis by inhibiting the transpeptidation step of cell wall synthesis and include penicillins and cephalosporins (1). Lastly, polymixins such as colistin, target the outer membrane of GNB by interacting with cations from the phosphate groups of the membrane lipids, destabilising the lipopolysaccharide (LPS) which in turn increases the permeability of the membrane, leading to leakage of the cytoplasmic content consequently causing cell death (2). In addition, fosfomycin, initially reported as phosphonomycin, inhibits cell wall synthesis through a different mechanism than β -lactams, by inactivating the first step in the peptidoglycan chain formation of the cell wall (3).

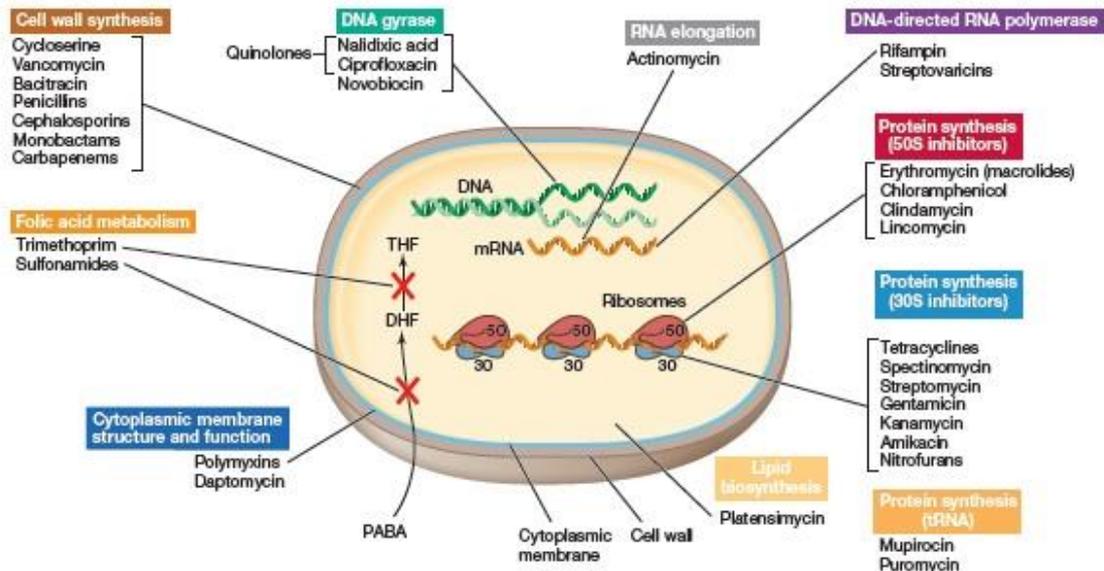


Fig. 1: Targets of antibacterial drugs- Agents are classified according to their target structures in the bacterial cell. THF, tetrahydrofolate; DHF, dihydrofolate; mRNA, messenger RNA. Reproduced from (1)

Antibiotic resistance is the ability bacteria have to withstand antibiotics effects. It happens when antibiotics lose their ability to either inhibit bacterial growth or kill microorganisms (1, 4). As antibiotics display different mechanisms of action bacteria display different mechanisms of resistance. Bacteria are not only naturally (i.e. intrinsically) resistant to certain antibiotics but also capable of acquiring resistance to antibiotics through mutation or by horizontal gene transfer (HGT). An example of intrinsic resistance is the inability of many compounds, such as vancomycin a glycopeptide antibiotic, to cross the outer membrane of GNB. In addition, many genes responsible for resistance to antibiotics belonging to different classes have been identified (5).

Moreover, bacteria can acquire resistance through several mechanisms including reducing permeability and increasing efflux as well as mutation and modification of antibiotic targets. In addition to the direct modification of antibiotics by hydrolysis or the transfer of a chemical group. Gram-negative bacteria (GNB) are intrinsically less permeable than Gram-positive (GPB) to antibiotics because their outer membrane forms a permeability barrier. Also, in most *Enterobacteriaceae*, outer-membrane proteins such as porins function as non-specific channels. Therefore, antibiotic resistance is achieved by down regulation of porins or by replacing porins with selective channels. Furthermore, reducing porin expression significantly

contributes to resistance to carbapenems and cephalosporins. Likewise, efflux pumps are major contributors to intrinsic GNB resistance to antibiotics used to treat GNB infection. The hydrolysis or modification of antibiotics is a major mechanism of antibiotic resistance. Numerous enzymes are capable of degrading or modifying different classes of antibiotics including β -lactams, aminoglycosides, phenicols and macrolides. Aminoglycoside antibiotics, for example, are particularly susceptible to modification because these are large molecules with exposed hydroxyl and amide groups. In fact, there are three main classes of enzymes that modify aminoglycosides and confer high levels of resistance, the acetyltransferases, phosphotransferases and nucleotidyltransferases (5).

More importantly, a diverse range of β -lactamases are capable of hydrolysing different β -lactams including penicillins, cephalosporins, carbapenems and monobactams. These enzymes include extended-spectrum β -lactamases (ESBLs) and carbapenemases as IMP (imipenemase), VIM (Verona integron encoded metallo β -lactamase), *K. pneumoniae* carbapenemase (KPC), OXA (oxacillinase) and NDM (New Delhi metallo- β -lactamase) enzymes found in GNB such as *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii*. In addition, the presence of these enzymes has underpinned the emergence of isolates resistant to all β -lactams antibiotics which in turn has serious implications for treatment of serious infections.

In summary, bacteria can display numerous mechanisms of resistance to different classes of antibiotics which can affect the treatment of infections as antibiotics play an important medical role (5).

1.1.1 β -lactamase producing *Enterobacteriaceae*

β -lactams antibiotics are commonly used treatment for bacterial infections and play an important role in promoting antibiotic resistance among GNB globally. There is an alarming increase of antibiotic resistance among community and hospital acquired infections as among GNB β -lactamases are widely considered the most important mechanism of β -lactam resistance in GNB, though in the extent of resistance seen in these pathogens in practice involves an interplay between membrane permeability, efflux and the rate of hydrolysis by any β -lactamases produced (6). Moreover, multidrug drug resistance bacteria, such as GNB *E. coli*, *K. pneumoniae* and *A. baumannii* are of particular interest. In some regions of the globe bacteria

belonging to these species are frequently resistant to most penicillin, cephalosporins, carbapenems, penems and monobactams due to the common presence of carbapenemases and ESBLs in *Enterobacteriaceae* (6).

β -lactamase differ from each other in relation to their substrate (type of β -lactam inactivated), inhibitor profile, and amino acid sequence homology. Based on amino acid sequence homology β -lactamases are divided into different classes according to the Ambler system. The different classes include class A, C and D, serine based β -lactamases and class B metallo- β -lactamases (MBLs) which are zinc based (6-8). In a review by Bush and Bradford multiple carbapenemases in the same GNB, excluding *Acinetobacter* spp. was summarised in the Table 1 shown below (9).

Table. 1: Multiple carbapenemase produced by the same GNB, excluding *Acinetobacter* spp Reproduced from (9).

Carbapenemase class			Producing organism	No. of Isolates	Yr of Isolation	Location
Class A	Class B	Class D				
KPC-2	IMP-8 or IMP-10	ND ^a	<i>S. marcescens</i> , <i>P. aeruginosa</i>	7	2009–2011	Brazil, Puerto Rico
KPC-2 or KPC-3 or KPC-17	NDM-1	ND	<i>C. freundii</i> , <i>E. cloacae</i> , <i>Enterobacter hormaechei</i> , <i>K. pneumoniae</i>	9	2010–2016	Brazil, China, Colombia, India, USA
KPC-2 or KPC-3 or KPC-18	VIM-1 or VIM-type	ND	<i>C. freundii</i> , <i>E. cloacae</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i>	36	2008–2017	Greece, Spain, USA
KPC-type	VIM-type	ND	<i>K. pneumoniae</i>	50	2013–2018	Greece
KPC-3, SME-1	ND	ND	<i>S. marcescens</i>	3	2015	USA
KPC-2 or KPC-3, NMC-1	ND	ND	<i>K. pneumoniae</i>	4	2013–2017	Malaysia, USA
KPC-2, NMC-1	ND	OXA-48	<i>K. pneumoniae</i>	1	2013	Malaysia, USA
KPC-type	ND	OXA-48	<i>K. pneumoniae</i>	7	2013–2014	Malaysia, Taiwan
ND	IMP-4, VIM-2	ND	<i>S. marcescens</i>	15	2015	Egypt
ND	IMP-4 or IMP-8, NDM-1	ND	<i>K. pneumoniae</i>	6	2013	China, Malaysia
ND	NDM-1 or NDM-type	OXA-48 or OXA-48-like	<i>C. freundii</i> , <i>E. cloacae</i> , <i>Enterobacter ludwigii</i> , <i>E. coli</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>Enterobacteriaceae</i>	91	2007–2018	Belgium, England, France, Romania, India, Iran, Saudi Arabia, Spain, Tunisia, Turkey, USA
ND	NDM-type, VIM-type	OXA-48	<i>K. pneumoniae</i> , <i>Enterobacter</i> spp.	2	2011, 2018	USA, Vietnam
ND	NDM-1 or NDM-5 or NDM-7	OXA-181	<i>E. coli</i> , <i>K. pneumoniae</i>	15	2010–2017	Belgium, Canada, Egypt, India, Norway, South Korea, Sri Lanka
ND	NDM-1	OXA-232	<i>K. pneumoniae</i>	4	2012–2018	India, South Korea, USA
ND	VIM-type	OXA-48-type	<i>C. freundii</i> , <i>Enterobacter</i> spp., <i>E. coli</i> , <i>K. pneumoniae</i> , <i>Enterobacteriaceae</i>	22	2006–2016	Egypt, France, India, Kuwait, Spain, Turkey

^aND, not detected.

Numerous β -lactamases harboured by GNB include AmpC β -lactamases, which belong to Ambler class C, conferring resistance to penicillins and oximino-cephalosporins, monobactams and cephamycins. Also, most class C enzymes are not inhibited by classical inhibitors such as clavulanate, sulfabactam and tazobactam, nevertheless, newer inhibitors are effective including avibactam, relebactam and vaborbactam. AmpC enzymes can be found in clinical isolates of *C. freundii*, *E. aerogenes*, *E. cloacae* complex, and *Serratia marcescens*, that harbour chromosomal inducible AmpC. Even though, these enzymes are more frequently found in the chromosome AmpC has been mobilised and therefore widely found in plasmids being described in a plasmid in 1989 from a *K. pneumoniae* isolate for the first time. Plasmid-based AmpC variants CMY originated from *Aeromonas hydrophila* and *C. freundii* but have been found in various genera of *Enterobacteriaceae* including for example, *K. pneumoniae*, *E. aerogenes* and *E. coli* (9).

1.1.2 Extended - spectrum β -lactamases among *Enterobacteriaceae*

ESBLs are often plasmid-encoded β -lactamases. These confer resistance to all penicillins, third generation cephalosporins (i.e. ceftazidime, cefotaxime and ceftriaxone) and monobactams (i.e. aztreonam) but not to cephamycins (i.e. cefoxitin, cefotetan) and carbapenems (i.e. imipenem, meropenem and ertapenem). In addition, these enzymes can be inhibited by clavulanic acid, sulfabactam and tazobactam as well as by avibactam, relebactam and vaborbactam (9).

Several types of ESBLs, belonging to class A, have been described including CTX-M, TEM and SHV type. TEM type β -lactamases evolved from parent enzymes TEM-1, TEM-2 and was first reported in *E. coli* and is capable of hydrolysing penicillins and first generation cephalosporins. SHV type β -lactamase are derived from *Klebsiella* spp. and have evolved from parent SHV-1 generally found in *K. pneumoniae*. SHV-1 confers resistance to ampicillin, and piperacillin but not oximino-cephalosporins (i.e. ceftazidime, cefotaxime and ceftriaxone) (10).

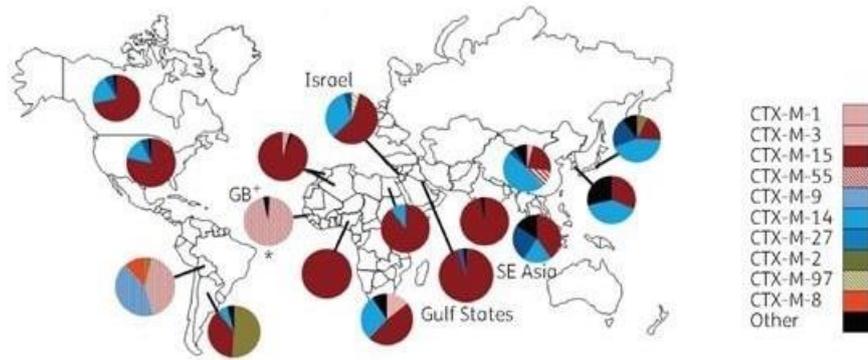


Fig. 2: Global distribution of CTX-M variants from 2009 to 2017 in which CTX-M-15 is the most frequently found ESBL. Reproduced from (11)

Moreover, CTX-M type β -lactamases are the most widely distributed ESBLs with more than 220 enzymes clustered into five subfamilies including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (Fig. 2). In addition, CTX-M enzymes are usually more effective against cefotaxime and ceftriaxone than ceftazidime but those belonging to the CTX-M-1 and CTX-M-9 subfamilies have the ability to hydrolyse ceftazidime successfully but can be inhibited by commercially available β -lactamase inhibitors (7).

CTX-M-15 belongs to the CTX-M-1 subfamily and is the most widely spread globally being frequently isolated from humans, animals, and the environment. More importantly, the prevalence of CTX-M-15 has increased markedly during the mid-late 2000s and early 2010s being frequently described in most regions across the globe.

Furthermore, *bla*_{CTX-M} antibiotic resistance genes (ARG) are associated with specific conjugative genetic elements including *ISEcp1*-like insertion sequences (IS), class 1 integrons and transposons. In addition, a variety of plasmids have been described to harbour *bla*_{CTX-M} including plasmids belonging to the FII group as well as plasmids and replicons belonging to IncN, Inc11, and IncL/M. Mobile genetic elements (MGE), such as plasmids, contribute to the mobilisation of ARG acting as vehicles HGT. As a result, CTX-M β -lactamases are not only commonly found in *E. coli*, *K. pneumoniae*, but also, in other *Enterobacteriaceae* including *Salmonella* spp., *C. freundii*, *Enterobacter* spp. and *S. marcescens* (11, 12). Thus, the movement of plasmids and IS are contributing to the global dissemination of ARGs such as ESBLs. However, the spread of ARG worldwide can also be associated with several disseminated sequence types (ST) which have played an important role in the

emergence of antibiotic resistance bacteria. Among these are *E. coli* ST131 associated to *bla*_{CTX-M} ARGs, particularly *bla*_{CTX-M-15}, and *K. pneumoniae* ST258 associated with *bla*_{KPC} ARGs (7, 11, 12). Also, several studies have shown that *bla*_{CTX-M-15} is predominantly carried by IncF plasmids whereas *bla*_{CTX-M-14} is, for example, carried within among numerous types of plasmids including IncF plasmids in the Far East and IncK in Western Europe. Furthermore, as HGT of plasmids occurs in the human gut, animals, and in the environment, and because IncF plasmids are mainly restricted to *Enterobacteriaceae* (11). These plasmids are able to be stably maintained in commensal *E. coli* which offers a possible explanation for the global spread of community carriage of ESBLs (11). Thus, the worldwide dissemination of *E. coli* ST131 likely occurred due to the acquisition of IncF plasmids harbouring *bla*_{CTX-M-15} by a high risk clone (HiR) such as ST131 that has the ability to move between the community, different hospitals, and long-term-care facilities (13).

In overall, there was a rise in the prevalence of *bla*_{CTX-M-15} in most countries this being a dominant ARG in most regions with the exception of China, South-East Asia, South Korea, Japan and Spain, where *bla*_{CTX-M-14} is more prevalent, and South America, where *bla*_{CTX-M-2} is still significant (11). In a tertiary hospital in Daejeon, Korea among 471 *E. coli* isolates causing UTI, recovered between 2011 and 2014, 80 carried *bla*_{CTX-M} ARG and among those 31 carried *bla*_{CTX-M-15} and 46 carried *bla*_{CTX-M-14} (14).

Different carbapenemases and carbapenemase producing *Enterobacteriaceae* have been identified globally as well (15). Also, infections caused by carbapenemase producing bacteria, especially carbapenemase producing *Enterobacteriaceae* (CPE) have become a global health concern. These bacteria have the ability to spread quickly and colonise patients and healthcare environments (16).

1.1.3 Carbapenemase among *Enterobacteriaceae*

More importantly, bacterial isolates carrying carbapenemases are often resistant to several antibiotics' classes ultimately limiting treatments. Also, globally distributed carbapenemase are usually associated with certain regions and countries (Fig. 3). Globally disseminated carbapenemases include class A KPC, class B or MBLs NDM, VIM and IMP and class D carbapenemases which commonly found in *A. baumannii*, mainly OXA carbapenemases. However, OXA-48 and variants such as

OXA-181 and OXA-232 have been found in *Enterobacteriaceae*. In addition, these enzymes are usually encoded in MGEs which facilitates their dissemination (16).

Carbapenemase genes found in the chromosome include SME and IMI/NMC found mainly in *S. marcescens* and *E. cloacae* complex. These confer resistance carbapenems but not extended-spectrum cephalosporins (9).

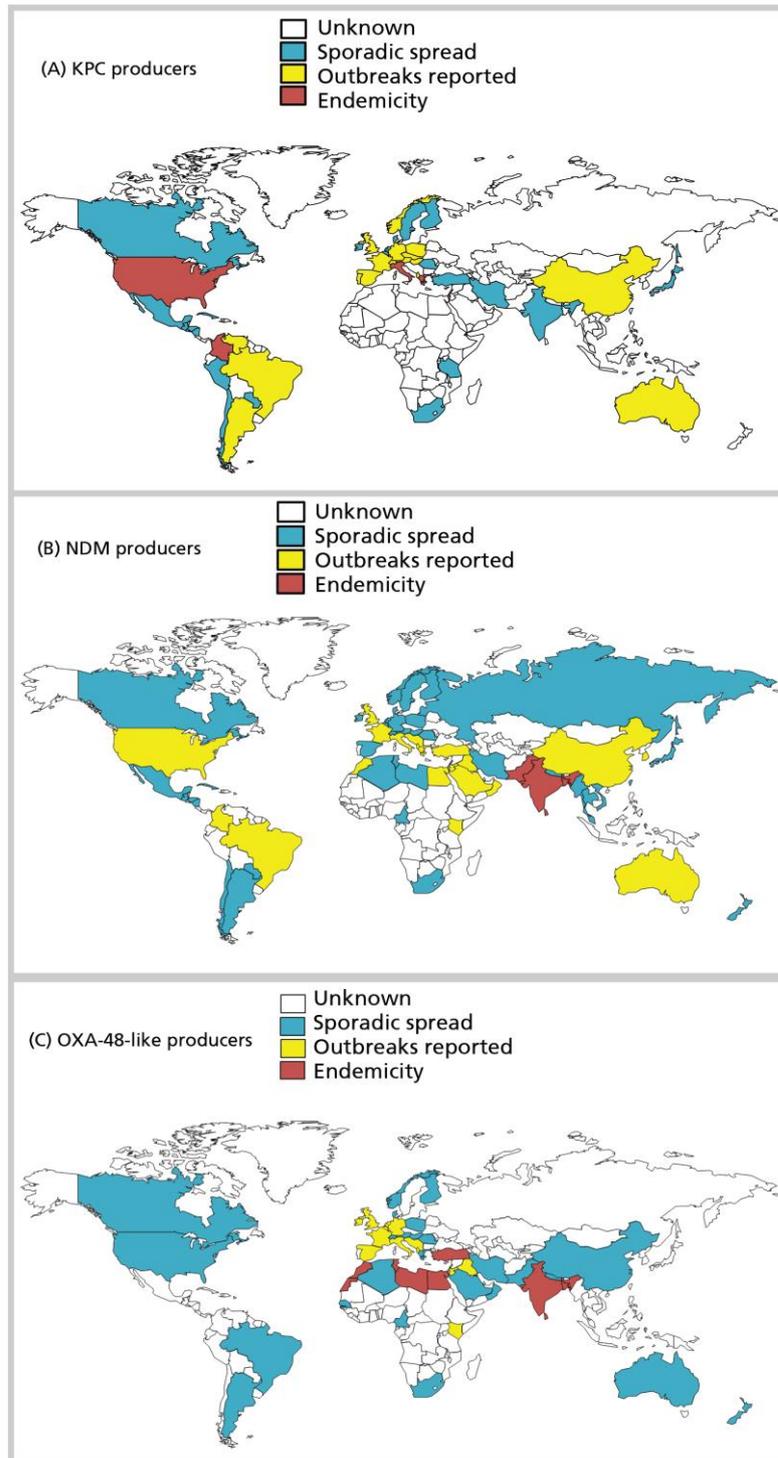


Fig. 3: Global spread of carbapenemase producers (a) KPC producers; (b) NDM producers and (c) OXA-48-like producers (16).

However, most frequently described carbapenemases worldwide are those acquired by bacteria. These include KPC, OXA and IMP, VIM and NDM, carbapenemases (9). KPC β -lactamases were first identified in the United States in the late 1990s and have now become the most common carbapenemase spread globally. It is widely spread among *K. pneumoniae* isolates in the United States but also in Europe in, for example, Italy and Greece where it has become endemic. KPC has been extensively found among *K. pneumoniae* but also in other *Enterobacteriaceae* (16). These β -lactamases confer resistance to cephalosporins, monobactams and carbapenems and are only inhibited by the commercially available avibactam, relebactam and vaborbactam (9). KPC β -lactamases are found among *Enterobacteriaceae* and *P. aeruginosa*. *bla*_{KPC} is usually found within the *Tn4401* transposon, however, the success of KPC dissemination can be attributed primarily to the spread of *K. pneumoniae* isolates belonging to the CC258, specifically ST258, found in both hospitals and the community. Also, *bla*_{KPC-2} and *bla*_{KPC-3} genes have been located on IncFII_{K2}-like plasmids. Additionally, it is important to note that often plasmids carrying *bla*_{KPC} also harbour other ARG such as aminoglycoside ARGs and other β -lactamases (17, 18). Outbreaks of KPC producing *K. pneumoniae* belonging to ST258 have been described for example in Brazil (19), Ecuador (20), Poland (21), United States (22) and Spain (23). Also, an extensive genomic study of 684 *K. pneumoniae* isolates from 244 hospitals in 32 countries showed that these isolates carried one or more carbapenemase, with 311 carrying *bla*_{KPC-like}. Additionally, in this study, the most frequently found STs were ST258 and ST512, which are single locus variants (24). Other STs such as ST11 are mainly found in China and Taiwan and ST25, ST147 and ST512 in the Americas and Europe have been reported as well.

OXA β -lactamases are a diverse group and those that have carbapenemase activity include OXA-23, OXA-40, and the increasingly prevalent OXA-48 group with variants OXA-162, OXA-181 and OXA-232. These confer resistance to penicillins and broad-spectrum carbapenems and are poorly inhibited by β -lactamase inhibitors except for avibactam. Also, OXA-48 hydrolyses clinically important penicillins and imipenem and meropenem (25). It was first described in a *K. pneumoniae* isolate in Turkey and subsequently spread in Europe and the Mediterranean region (26). In Spain, different species of *Enterobacteriaceae* have been reported to carry OXA-48 including *E. coli*, *K. pneumoniae* and *Citrobacter* spp. In addition, a survey study in 2015 of 11 559 *Enterobacteriaceae* isolates from 30 countries on five continents

reported 40 OXA-48 producing strains among these the majority were *K. pneumoniae* from Europe and most of these from Turkey (9). Besides, Europe, OXA-48-like enzymes have been reported in Africa, Asia, and South America (15, 27). In South Africa a study in twelve academic tertiary hospitals from four provinces revealed OXA-48 as the most common carbapenemase followed by NDM (28). In Taiwan, the transmission of an ST11 *K. pneumoniae* carrying OXA-48 in a single hospital was described (29). Also, in North Africa and the Middle East OXA-48 is endemic associated with MGEs as for example *Tn1999* variants on IncL plasmids whereas variants OXA-162 and OXA-244 are found in Europe. Variants OXA-181 and OXA-232 are endemic in the Indian subcontinent often found in countries including India, Bangladesh, Pakistan and Sri Lanka as well as being found in African countries as for example Angola and Nigeria. In addition, unlike OXA-48 which is associated with *Tn1999* within a IncL plasmids these variants are associated with *Tn2013* often on ColE2, and IncX3 types of plasmids. The presence of OXA-48-like carbapenemases on MGEs contributes to dissemination of these enzymes nevertheless, the global spread is largely associated with HiR clones. These include *K. pneumoniae* ST147, ST307, ST15, and ST14 and *E. coli* ST38 and ST410 which have been associated the global dissemination of OXA-48, OXA-181, OXA-232, and OXA-204 (25).

In relation to MBLs, that are capable to hydrolyse most β -lactams including carbapenems with the exception of monobactams. VIM β -lactamases were first described in Italy and France in 1997 and in majority found in *K. pneumoniae* and *E. cloacae* complex. Alongside with IMP carbapenemases these are less frequently found in carbapenemase producing bacteria. IMP carbapenemase are in majority found in Japan and in other regions of Asia, nevertheless, reports have increased in Middle Eastern countries (9, 30).

More importantly, the NDM MBL has spread worldwide and there are 24 NDM variants that have been identified in 11 bacterial species. However, *bla*_{NDM} has been more frequently found in *K. pneumoniae* and *E. coli* isolates with certain STs being the most prevalent such as ST11, ST14, ST15 and ST147 in relation to *K. pneumoniae*, and as ST167, ST410 and ST617 regarding *E. coli*. In addition, *bla*_{NDM} has been found within plasmid replicons such as IncX3, IncFII or IncC. Also, despite being identified globally NDM producing strains have the highest prevalence in Indian subcontinent, middle East, and the Balkans. Furthermore, in a global surveillance program *bla*_{NDM} was the third most common carbapenemase gene after *bla*_{KPC} and *bla*_{OXA-48-like}. with

heterogenous prevalence the highest being shown, for example, in Egypt, India and Serbia (30, 31). In addition, NDM positive isolates have been reported in China as well (32). Among the carbapenemase producing *K. pneumoniae* and *E. coli* isolates the majority of isolates produced KPC (50%) or NDM-type carbapenemases (33.5%) (32). Additionally, in a survey across Europe around 8% of carbapenem-resistant *K. pneumoniae* and 10% of carbapenem-resistant *E. coli* were NDM positive (24). Moreover, international travel has been associated with the spread of NDM as following its first report its incidence increased in the Mediterranean and Europe with the large majority of initial cases being associated with previous hospitalisation in the Indian subcontinent or Balkans region (33, 34). For example, *bla*_{NDM} was detected in *Klebsiella* and *E. coli* isolated from clinical infections in Italy and in *K. pneumoniae* causing hospital associated infections in Greece. These isolates were believed to be first introduced via travel from India and the Balkans region. Although, *bla*_{NDM} are largely isolated from *Enterobacteriaceae*, *Acinetobacter* spp. also frequently carry *bla*_{NDM} and have been described in Africa, the Americas, Asia and Europe. Lastly, the dissemination of NDM producers in South Asia, especially India, Pakistan and Bangladesh, represents the greatest clinical and epidemiological burden of MBL carbapenemases globally (30, 35).

1.2 Ecology of *Klebsiella pneumoniae*

K. pneumoniae is a GNB belonging to the *Enterobacteriaceae* family. It was first described by Carl Friedlander in 1882 after it was isolated from a pneumonia patient (36, 37).

K. pneumoniae exhibits vast genetic and phenotypic diversity. These bacteria can colonise a variety of hosts and be associated with a variety of environments. It can be found in plants, water, soil and animal hosts, and in the healthcare environment including medical devices as well as colonising the respiratory tract, gut, nasopharynx, oropharynx, and skin without causing pathology. Additionally, these bacteria have been found in dogs, dairy cattle and carried by birds and insects and is a member of the human gut microbiota *K. pneumoniae* (37-39).

Thus, the environment is a probable reservoir for human acquisition either as colonisation or infection. It has been shown that environmental and clinical *K. pneumoniae* are similar in terms of biochemical, virulence and pathogenicity.

However, capsule types may differ between clinical and environmental sources and clinical *K. pneumoniae* are commonly more resistant to antibiotics than environmental isolates (37).

K. pneumoniae is considered an opportunistic pathogen because it can cause severe infections. *K. pneumoniae* remains as one of the most common nosocomial pathogens and is among the top three causative agents of neonatal sepsis. In addition, ESBL producing and carbapenem resistant *K. pneumoniae* (CRKp) are recognized by the World Health Organization (WHO) as a significant public health threat accounting, in Europe alone, for >90 000 and >7 000 deaths infections and deaths, respectively (39).

Most *K. pneumoniae* infections affect neonates, the elderly, the immunocompromised and patients with implanted medical devices. The most common infections caused by *K. pneumoniae* include urinary tract infections (UTIs), pneumonia and wound or soft-tissue infections. All these having the possibility of leading to bacteraemia and/or systemic sepsis (39-41). Additionally, *K. pneumoniae* employs several mechanisms to evade host defences and suppress innate immune responses. For example, *K. pneumoniae* is able to limit the activation of inflammatory responses and nullify phagocytosis by neutrophils and macrophages as well as being able to prevent the TLR-dependent activation of host defences (39).

1.3 *K. pneumoniae* and the gut microbiota

K. pneumoniae is also a common commensal in the gut and respiratory tract though its prevalence varies according to health care exposure, location, and age increasing in diversity with frequent dynamic turnover. The gut microbial community is extremely diverse and individual (39, 42, 43). As the development of the microbiota in infants is dynamic and several factors play an important role in shaping it, including the environment, diet, genetics and host physiology. In addition, the age at delivery and the mode delivery, also, affect the composition of the gut microbiota of infants. For example, term infants born vaginally, initially acquire a microbiota similar to the mother's vaginal microbiota (mostly composed by *Lactobacillus* and *Prevotella* spp.) and the initial gut microbiota of preterm infants resembles that of the hospital surfaces and intubation tubing bacterial communities, in which *K. pneumoniae*, *S. epidermidis* and *E. coli* are often found (43). Furthermore, infections thought to result from the lack

of control of commensal *K. pneumoniae* present the most risk for vulnerable patients which include neonates, the elderly, the immunocompromised and patients inserted with medical devices. Thus, *K. pneumoniae* carried in the gut is deemed to be a risk factor for acquired hospital infection caused by *K. pneumoniae* (39). The microbiota plays an important role in health and disease as it offers protection against colonization by pathogenic bacteria, but antibiotics enable changes in the microbiota that might promote infection (42). Moreover, preterm infants were described to carry diverse *Klebsiella* populations encoding and producing several antimicrobial and virulence associated determinants. These populations included *K. pneumoniae*, *K. quasipneumoniae*, *K. grimontii* and *K. michiganensis* and these were described to encode several β -lactamases whether they were recovered from infants suffering from necrotizing enterocolitis, sepsis, or were healthy. Also, few differences were identified between *Klebsiella* isolates from healthy and sick infants regarding their phenotype and genotype, and because β -lactamases were found among all isolates it indicated that healthy infants also contributed to the resistome (44).

Furthermore, antibiotic treatment was found drive the enrichment of ARG among the microbiota and MDR *E. coli*, *Klebsiella* and *Enterobacter* can also dominate the preterm infant gut microbiota. In addition, following a specific antibiotic treatment the ARG enriched are usually unique to said treatment and correlated with the predominance of a certain bacteria. For example, meropenem, cefotaxime, and ticarcillin/clavulanate can substantially reduce species richness, contrarily, antibiotics commonly administered such as gentamicin and vancomycin had non-uniform effects. Additionally, treatment with ticarcillin/clavulanate and ampicillin was found to enrich for a numerous ARG that were correlated with *K. pneumoniae* (45).

1.4 Population structure of *K. pneumoniae*

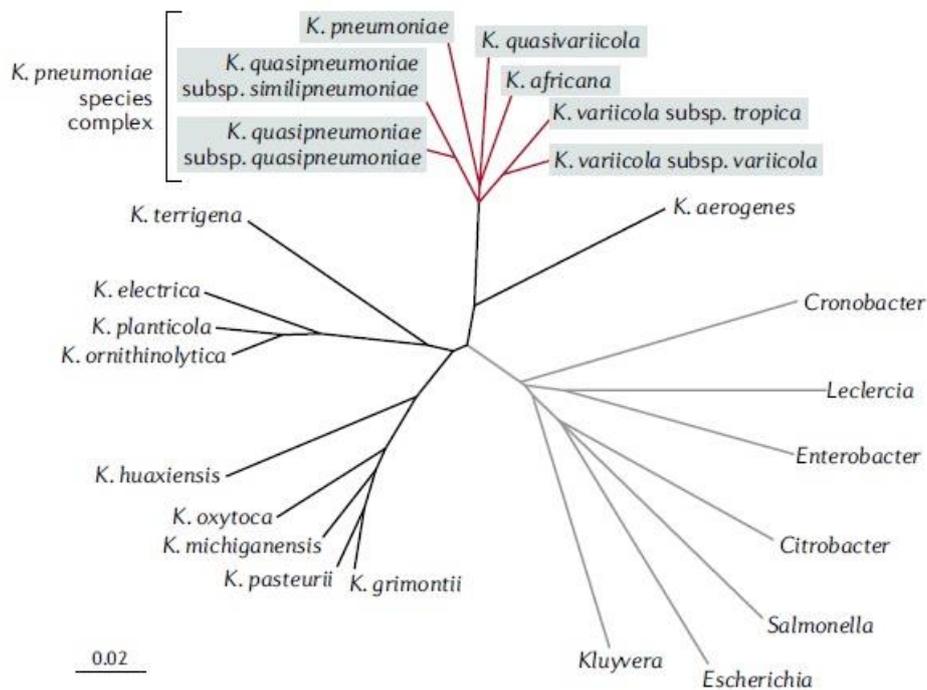


Fig. 4: *K. pneumoniae* species complex taxonomic position. Close relatives of *K. pneumoniae* (red branches) and other *Klebsiella* species (black branches) in addition to other *Enterobacteriaceae* are shown in a whole genome-based tree Reproduced from (39)

WGS studies showed that a significant part of isolates identified by biochemical and proteomics assays as *K. pneumoniae* actually belong to closely related species sharing 95-96% average nucleotide identity with *K. pneumoniae*. In fact, *K. pneumoniae* comprises several phylogenetics groups that are part of the *K. pneumoniae* species complex (KpSC). The KpSC included 7 phylogroups (Kp1 to Kp7) that correspond to the different described species that share 90% average nucleotide identity (Table 2; Fig. 4). The different member species of the KpSC were defined by $\geq 3\%$ genome wide average nucleotide identity which in turn has enabled the identification of new members. Furthermore, *K. pneumoniae sensu stricto* is highly prevalent among clinical isolates accounting for $\sim 85\%$ of the isolates whereas *K. variicola* seems to be associated with plants because it carries a nitrogen-fixing operon and cellulases that are absent in other species, in addition, *K. quasipneumoniae* is mostly associated with carriage. However, these two species are capable of causing infection in humans as well (39, 46).

Table 2: Species of the *K. pneumoniae* complex and respective phylogroups

Species	Phylogroup	References
<i>K. pneumoniae</i>	Kp1	(47)
<i>K. quasipneumoniae</i> subsp. <i>quasipneumoniae</i>	Kp2	(48)
<i>K. quasipneumoniae</i> subsp. <i>similipneumoniae</i>	Kp4	(48)
<i>K. variicola</i> subsp. <i>variicola</i>	Kp3	(49)
<i>K. variicola</i> subsp. <i>tropica</i>	Kp5	(50) (described as subsp. <i>tropicalensis</i> in this paper)
<i>K. quasivariicola</i>	Kp6	(51)
<i>K. africana</i>	Kp7	(50)(described as <i>africanensis</i> in this paper)

In relation to the species genome *K. pneumoniae* usually has a size of ~5-6 Mbp with approximately ~5000 to 6000 encoding genes, 1743 genes comprising the core-genome (genes conserved among the members of the species). Accessory genes content, present in less 10% of genomes, widely varies as individual *K. pneumoniae* genomes were shown to carry thousands of additional accessory genes some likely carried in plasmids and acquired from other bacteria, not only from other *Klebsiella* species but also *Enterobacteriaceae*, *Vibrio* sp. and *Acinetobacter* sp. Thus, the total pan-genome of *K. pneumoniae* is markedly diverse which highlights the genomic plasticity within this species (39, 46, 52).

Nevertheless, *K. pneumoniae* demonstrated a highly structured population defined by numerous different lineages that correspond closely to defined clonal groups (CG). These CG are usually identified based on the seven gene multilocus sequence type (MLST) in to order to allow comparison with original MLST scheme which covers the KpSC. Also, it is possible to distinguish *K. pneumoniae* clones based on their accessory genomes and overlap between multidrug resistant (MDR) and virulent *K. pneumoniae* is rare. For example, antimicrobial resistance (AMR) has only been occasionally reported in CG23 *K. pneumoniae* hypervirulent clone. Also, the acquisition of AMR plasmids by this clone is rare as well as long term plasmid maintenance and transmission among human isolates. In contrast, other clonal groups such as CG258 and CG15 are often associated with carriage of ESBL and/or carbapenemase ARG, in particular *bla*_{KPC} and *bla*_{CTX-M-15}, respectively (39, 46, 52, 53).

Despite the capability of numerous *K. pneumoniae* clones to cause infection and spread widely there are certain CG that contribute more markedly to infection. These include MDR clones which can be responsible for localised outbreaks in a single hospital or in health-care networks or can spread globally. The most highly resistant lineages include CG258, CG15, CG20 (CG17), CG29, CG37, CG147, CG101 (CG43) and CG307. These lineages are not related but are frequently responsible for MDR nosocomial infections and/or outbreaks (39) *K. pneumoniae* MDR isolates are able to spread due to clonal expansion and HGT of antibiotic resistant elements to susceptible strains which occur simultaneously but the spread of *K. pneumoniae* HiR plays the main role of dissemination. These originate either as a result of a local transmission of a specific clone or due to the import of a new clone from an endemic area. Among the HiR is the MDR CG258 comprising three different STs (ST258, ST11 and ST512) responsible for the majority of outbreaks. ST258 is the most widely spread member of the CG usually being associated with *bla*_{KPC-2} or *bla*_{KPC-3} and carriage of multiple ARG. In addition to sometimes harbouring OmpK35 and OmpK36 truncations and mgrB colistin resistance conferring mutations. There are HiR MDR clones that have successfully disseminated including ST14, ST147, ST37 and ST101, which usually carry carbapenemase ARG, and ST15 and ST17, which usually harbour ESBL ARG (53). Other lineages comprise hypervirulent *K. pneumoniae* among which the most common is CG23 followed by CG65 and CG86 (39). Also, cases of hypervirulent *K. pneumoniae* are mainly reported in Asian countries as Taiwan, China, South Korea and Iran, however, these have been reported in Europe and the Americas as well (54).

Moreover, hypervirulence and MDR rarely overlap in the *K. pneumoniae* population and MDR *K. pneumoniae* are more highly diverse than hypervirulent *K. pneumoniae*. ARG are uncommon among hypervirulent clones and virulence determinants such as aerobactin (*iuc*), salmochelin (*iro*) and *rmpA/rmpA2* are rare among MDR *K. pneumoniae*. Nevertheless, yersianiabactin (*ybt*) siderophore locus are frequently identified across both MDR and hypervirulent *K. pneumoniae* clones (1.7 *K. pneumoniae* virulence determinants and 1.8 Infections caused by virulent *K. pneumoniae*). The distribution of plasmids is also asymmetric across the *K. pneumoniae* population. The presence of plasmids (harbouring MGE ICEKp or not) associated with virulence determinants is associated with low number of ARG. In contrast, among *K. pneumoniae* genomes without virulence plasmids the distribution

of ARG is slightly higher in genomes where ICE K_p was found. Moreover, although, the influence of recombination on nucleotide diversity is marked among all *K. pneumoniae* it is greater in MDR clones when compared with hypervirulent *K. pneumoniae*. In addition, recombination events have a strong influence on the high diversity of capsule and LPS loci found among MDR clones. However, there is low diversity of capsule and LPS loci among hypervirulent *K. pneumoniae* which either carry KL1 and KL2 capsule loci plus O1/O2v1 or O1/O2v2 LPS loci. Furthermore, the pan-genomes of MDR *K. pneumoniae* are more diverse than hypervirulent clones and display more variation (55).

Lastly, MDR *K. pneumoniae* were associated with higher plasmid diversity than hypervirulent clones. MDR clones often acquire/lose plasmids and transfer is frequent between these clones, whereas hypervirulent *K. pneumoniae* seldom acquire plasmids but stably maintain them (55).

1.5 Antibiotic resistance in *K. pneumoniae*

K. pneumoniae is one of the most concerning bacteria in relation to antibiotic resistance. It is highly prevalent, a major source of antibiotic resistance and resistance to all clinically used treatments has been observed among *K. pneumoniae*. These bacteria are intrinsically resistance to ampicillin through the production of the SHV enzyme encoded by the core ARG *bla*_{SHV}. Also, *K. pneumoniae* has been found not only to harbour acquired ARG, as β -lactamases including ESBLs and carbapenemases, but also, aminoglycoside, quinolone, polymyxins, tigecycline and fosfomicin ARG as well. Furthermore, despite *K. pneumoniae* carrying multiple resistance mechanisms fitness is not necessarily reduced which allows growth and transmission between patients (39, 41, 53).

Antibiotic resistance among *K. pneumoniae* is often caused by horizontally acquired ARG, mainly through large conjugative plasmids, but chromosomal mutation happens as well (41). Therefore, it is not uncommon for resistance to almost all antibiotics to occur in *K. pneumoniae* as a result of carriage of distinct sets of ARGs. Also, transfer of ARG between *K. pneumoniae* strains isolated from hospital patients and the hospital environment has been detected as well as between *K. pneumoniae* and other *Enterobacteriaceae*. Additionally, mutational resistance in *K. pneumoniae* includes intrinsic expression of efflux pumps (*acrAB* and *oqxAB*) enabling for example,

resistance to fluoroquinolones and tigecycline. Other mutational resistance includes the reduced permeability of the outer membrane due to the truncation of *OmpK35* and *OmpK36* porins that allow for resistance to extended spectrum cephalosporins and diminished susceptibility to carbapenems and fluoroquinolones. Furthermore, in *K. pneumoniae* fluoroquinolone resistance is mediated by combined substitutions in the *gyrA* and *parC* topoisomerases (41). Also, regarding quinolone resistance, *K. pneumoniae* carries plasmid mediated quinolone resistance ARG. Among these *qnr*, including *qnrA* and *qnrB* genes, and *aac6'-Ib-cr* ARG, responsible for inhibiting quinolone activity and for resistance to fluoroquinolones and aminoglycosides, respectively, have been found. In addition, *K. pneumoniae* ST11, ST15 and ST147 often display both resistance mechanisms (39, 53).

Colistin resistance is conferred through inactivation, mutation, or deletion of the *mcrB* gene, or by the acquisition of plasmid acquired *mcr* ARG. As well as by the increased activity of LPS-modifying gene regulators such as *phoPQ*, *pmrA* and *pmrD* which in turn leads to overexpression of *pmrB* that also enables resistance to colistin (41, 53). In *K. pneumoniae* tigecycline resistance is mediated by the overexpression of efflux pumps such as AcrAB-TolC and oqxAB and their regulators. Additionally, efflux pump *tetA* ARG responsible for tetracycline resistance were described in tigecycline resistant *K. pneumoniae*. *K. pneumoniae* aminoglycoside resistance is mainly mediated by drug modification as acetylation, adenylation or phosphorylation by ARG carried on plasmids. These ARG include *aac*, *ant* plus *aad* and *aph* gene families which comprise all classes of aminoglycoside resistance genes (53, 56).

The dissemination of carbapenemase and ESBL ARG are of concern within the clinical environment. These include the carbapenemase ARG *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{NDM-1} and the ESBL *bla*_{CTX-M-15}. As mentioned before, these are associated with MGEs such as transposons mobilised across different plasmids allowing their spread to other strains and species and have been reported in different lineages of *K. pneumoniae*. Among *K. pneumoniae*, carbapenemase KPC is mainly linked to ST258 and with transposon *Tn4401*, OXA-48 is mobilised by *Tn1999* frequently associated with IncL/M and NDM-1 has been associated with diverse plasmid types. ESBL CTX-M-15 is often harboured in IncFII plasmids that harbour other ARG as well (41).

Also, hypervirulent *K. pneumoniae* (hvKp) are generally susceptible to commonly used antimicrobials, however, reports of convergence of antibiotic resistance and hypervirulence among *K. pneumoniae* have emerged, particularly in

Asia where hvKp is more prevalent and MDR *K. pneumoniae* prevalent as well (57). In China, reports of ESBL-producing and carbapenem resistant hvKp showed strains carrying mainly *bla*_{CTX-M} and *bla*_{KPC} (54, 58-60). Fewer cases were identified in Europe and America these usually carrying ESBLs and *bla*_{KPC} as well (54, 61, 62).

Moreover, *K. pneumoniae* shows incredible ability to incorporate ARG in which HGT plays an important role enabling the rapid spread of antibiotic resistance (Fig. 5). Also, the emergence ESBL producing *K. pneumoniae* led to the increased use of carbapenems in turn leading to the emergence of plasmid mediated carbapenemases.(53).

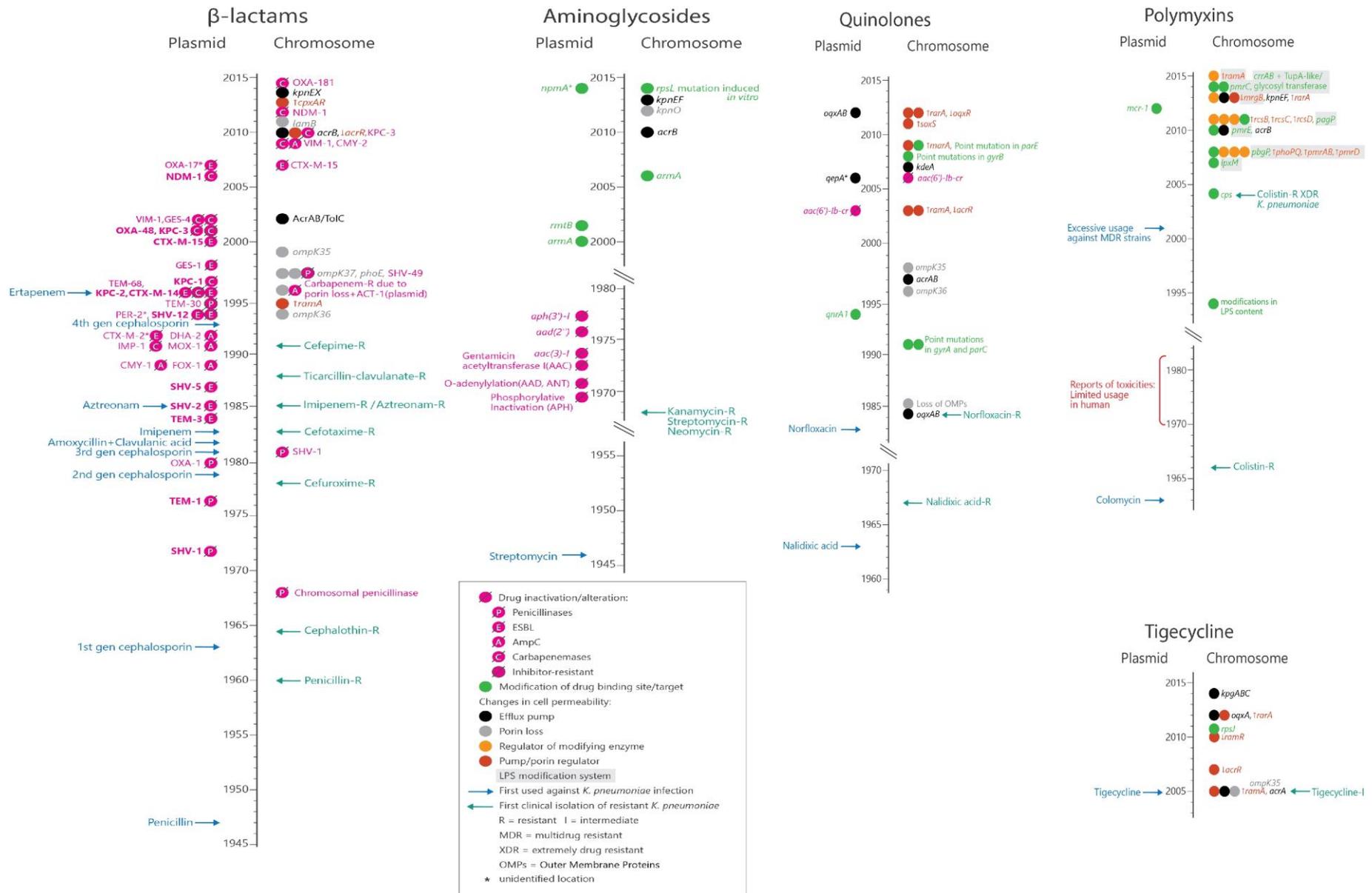


Fig. 5 :Timeline displaying the development of the resistome of *K. pneumoniae* showing different ARG against five antibacterial classes. Arrows on the left to the timeline indicate the first clinical use of the specified antibiotics. Arrows to the right of the timeline indicate the first appearance of resistance in a clinical *K. pneumoniae* isolate. Reproduced from (53).

Furthermore, globally spread MDR *K. pneumoniae* frequently harbour a larger load of ARG than hypervirulent isolates, usually carrying ARG to ≥ 6 classes of ARG (38). These MDR clones are often responsible for infections in different environments despite no particular virulence determinant being associated to these clones. As mentioned before, the most widely disseminated clones belong to CG258 and predominantly belonging to ST258 and ST11. These are common carriers of KPC-2 and KPC-3. Other members of the group harbour a diverse carbapenemases as NDM-1 and OXA-48 and ESBLs such as CTX-M-15. *K. pneumoniae* belonging to CG14/15 are globally spread MDR clones as well. These clones are frequently associated with ESBLs namely CTX-M-15 and carbapenemases such as KPC, NDM-1, OXA-181 and VIM-1(39).

1.6 Burden of antibiotic resistant *K. pneumoniae* in infection

In relation to antibiotic resistance *K. pneumoniae* has a marked ability to spread and acquire ARG allowing resistance to most antibiotics in addition to successful global dissemination of HiRs. Additionally, *K. pneumoniae* is an important nosocomial pathogen able to cause serious infections as, for example, UTIs and BSIs. However, resistance to carbapenems among *K. pneumoniae* has spread globally, namely in hospital acquired infections, for which treatment has become increasingly difficult (18). A recent survey in Europe described frequent occurrence of carbapenemase producing *K. pneumoniae* showing resistance to last-line antibiotics such as colistin, fosfomicin and tigecycline. Furthermore, *K. pneumoniae* was shown to be a driver of carbapenem resistance in the hospital environment in Europe due to the ability that these bacteria have to spread in this environment (24, 63).

However, the burden of antibiotic resistant infection is mainly felt in low- and middle-income countries (LMICs), where the rate of antimicrobial resistance in sepsis is alarming in Asia and Africa. *K. pneumoniae* being among the resistant GNB described to cause healthcare related sepsis. For example, *K. pneumoniae* causing infection was reported in China, India, Pakistan and Nepal, but also, in Nigeria, Egypt, Angola and Ethiopia (64-69).

A systematic review of carbapenem resistant *Enterobacteriaceae* causing neonatal sepsis in China showed that *K. pneumoniae* mainly carrying NDM-1 was isolated in different studies and one of the leading causes of neonatal sepsis. These

isolates were resistant to carbapenems and cephalosporins but not to colistin or fosfomycin. High levels of resistance to third generation cephalosporins among *K. pneumoniae* causing neonatal late onset sepsis (LOS) were reported in China, as well (70, 71). Another study in India revealed *K. pneumoniae* as the most frequent neonatal sepsis causing agent. In addition, these isolates were in majority resistant to carbapenems, cephalosporins and aminoglycosides (72). Furthermore, NDM-1 producing *K. pneumoniae* were found among bacteria responsible for neonatal sepsis in a hospital in north-east India. In this study the *K. pneumoniae* isolated also displayed resistance ARG to not only carbapenems (*bla*_{NDM-1}) and ESBLs (*bla*_{CTX-M-15}) but also quinolones (*qnrS1*; *qnrB1*) and aminoglycosides (*aac6'-Ib*) (73). In Pakistan, *K. pneumoniae* frequently carrying NDM-1 were recovered from three children's hospitals in neonatal and paediatric patients. In addition, these isolates displayed high percentages of resistance to commonly used antibiotics such as ceftazidime, cefepime, imipenem, meropenem, amikacin and ciprofloxacin (68). Other studies have described neonatal sepsis caused by antibiotic resistant *K. pneumoniae*. Neonates in Karachi, Pakistan developed *K. pneumoniae* LOS where an increase of antibiotic resistance to many antibiotics was seen between 2006 and 2011. Most of the isolates (80%) were resistant to ampicillin/clavulanic acid, gentamicin, aztreonam, and cephalosporins, in addition to the increasing rise in resistance to amikacin, fluoroquinolones, piperacillin/tazobactam, and imipenem (74). Moreover, in Lahore, Pakistan, *K. pneumoniae* was found as the most frequent bacteria causing neonatal septicaemia as well as displaying resistance to β -lactams, amoxicillin/clavulanate and amikacin (75). And a single strain of NDM producing *K. pneumoniae* was the cause of three temporally separated clusters of neonatal infection in Nepal. All these outbreak *K. pneumoniae* belonged to ST15 and harboured plasmids carrying multiple ARG. The plasmids were highly conserved among the isolates and carried ARG responsible for resistance to aminoglycosides (*armA*; *aac6'-Ib-cr*), β -lactams (*bla*_{OXA-1}), phenicols (*catB3*), trimethoprim (*dfpA12*) and tetracyclines (*tetA*, *tetR*) in addition to *bla*_{NDM-1} (76)

Despite the sparse available data on carbapenem resistant *Enterobacteriaceae* causing infection in children and infants in the WHO African region reports including carbapenem resistant *Klebsiella* spp. have emerged (77). In Nigeria, one study *K. pneumoniae* showed that was among the bacteria responsible for early onset sepsis (EOS) being the second most frequent isolated (78). In another study, *K. pneumoniae* was among the responsible agents for sepsis in new-borns, where these isolates

displayed increased resistance to antibiotics to gentamicin and ampicillin (79). Furthermore, in Egypt, carbapenem-resistant *K. pneumoniae* harbouring OXA-48 and NDM-1 were the causative agents of neonatal sepsis in an intensive care unit in Cairo. Another study in Egypt reported *K. pneumoniae* was isolated from blood and endotracheal samples from neonates diagnosed with sepsis. *K. pneumoniae* was the most frequent bacteria isolated among the samples studied and displayed marked resistance to cephalosporins, piperacillin/tazobactam but lower resistance to amikacin (80, 81). Moreover, *K. pneumoniae* frequently harbouring carbapenemase OXA-181, ESBL CTX-M-15, quinolone *qnrS* ARG were the second most frequently isolated bacteria among hospitalised children in a study in Angola. These isolates were mostly resistant to ceftazidime, ciprofloxacin, gentamicin, and tobramycin (64). Moreover, sepsis caused by *K. pneumoniae* has been described in Ethiopia as well. *K. pneumoniae* was reported as one of the main causative agents of community acquired sepsis and half of the *K. pneumoniae*, in this study, were resistant to gentamicin and ceftriaxone. In another study in Northwest Ethiopia, *K. pneumoniae* mainly resistant to ceftriaxone was among bacterial isolates causing neonatal sepsis as well. Furthermore, in a cross-sectional study conducted in an intensive care unit in a specialized hospital in North Ethiopia found *K. pneumoniae* to be the most frequent cause of neonatal sepsis. These isolates were resistant to common antibiotics such as ceftazidime, ceftriaxone, gentamicin and amoxicillin/clavulanate (65, 82, 83).

More importantly, infection is one of the leading causes of under-5 years old child mortality and resistance to β -lactams has made an important contribution to the world's child mortality. Furthermore, the rise in use of antibiotics has increased in LMICs mainly due to both the rising of incomes and high rates of hospitalisation and subsequently hospital related infections. In LMICs the ability to acquire second-line antibiotics is limited, and resistance to the WHO recommended regimen of ampicillin and gentamicin in neonatal sepsis pathogens has emerged (84, 85). Therefore, neonatal infections are often exacerbated by MDR GNB that frequently include ESBL and carbapenemase producing *K. pneumoniae* isolates (84).

1.7 *K. pneumoniae* virulence determinants

In order to cause infection *K. pneumoniae* displays virulence determinants that enable it to do so. Several virulence determinants have been well described to have an important role in *K. pneumoniae* infection (Fig. 6).

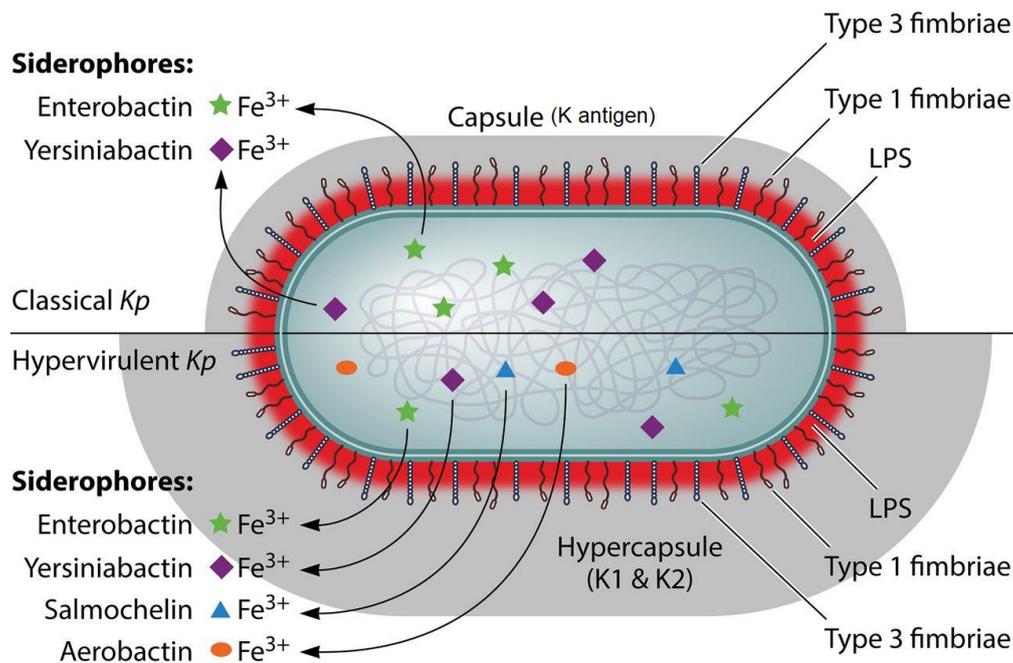


Fig. 6: Well characterised *K. pneumoniae* virulence determinants. Reproduced from (86)

These consist of capsule, lipopolysaccharide (LPS), acquired siderophores, colibactin, and fimbriae (86, 87).

The capsule is a matrix that coats the cell comprised of acidic polysaccharides composed by repeating units of three or six sugars (86, 88). The capsular polysaccharide (K antigen) is a pathogenicity factor important for *K. pneumoniae* to establish infection (39). Also, 77 capsular types were identified by serological testing however, sequencing of the capsular polysaccharide synthesis (*cps*) locus has allowed further discrimination based on *wzi* gene typing. Initially these allowed for the identification of 135 different *wzi* types that correspond with serological K types. Many *cps* genes, such as *wzi*, are conserved but others are present or absent depending on the capsule types. The combination of these genes defines a K locus that encodes a distinct capsule type with >138 different combinations described so far (57, 89, 90). The capsule prevents phagocytosis, opsonophagocytosis and serum killing. It also hinders bactericidal action of antimicrobial peptides such as human β defensins 1 and

3 and it blocks complement mediated lysis (86-88) In addition, it shields *K. pneumoniae* LPS from recognition by immune cell receptors protecting *K. pneumoniae* from epithelial cells, macrophages, neutrophils, and dendritic cells by preventing binding and internalization (86, 88). Furthermore, the production of increased amounts of capsular polysaccharides contributes to the hvKp phenotype which is mediated by the *rmpA* and/or *rmpA2* hvKp specific factors (86, 88).

The LPS is a major component of the outer leaflet of all GNB membrane and consists of the highly conserved lipid A anchored in the outer membrane, the variable O-antigen the outermost component and the core polysaccharide that connects the other two components (37, 88). Its role in *K. pneumoniae* infection consists of protection against the complement and humoral defences. In addition, the LPS is a significant mediator of septic shock by promoting an inflammatory cascade (37).

K. pneumoniae isolates have historically been classified into serotypes based on the recognition by antibodies of variations of surface polysaccharides, particularly O-antigens and K-antigens. These result in different O and K serotypes. K serotypes are more diverse than O serotypes. At least 79 different K antigens have been identified whereas only 9 O antigens have been identified. Also, the study of a large collection of *K. pneumoniae* isolates from different infection types demonstrated the genetic diversity of O and K antigens. Among the studied dataset the most common serotype O1 was associated with 43 different K serotypes. Other common O serotypes identified were O3, O2 and O5 these were associated with a large number of different K serotypes as well, 29, 30, and 20 K serotypes, respectively. The most common serotype was K2, and it was associated with two O serotypes, O1 and O2. K1 and K64 were common among the studied set of isolates and were associated with two and four O serotypes. Furthermore, K2 and K1 serotypes were correlated with invasive, and community acquired infection, respectively. In addition, K1 and K2 were notably associated with the presence of siderophore, colibactin clusters and *rmpA* factors and determinants have previously been associated with hvKp (89, 90).

Other virulence determinants important for *K. pneumoniae* infection are the siderophores. These are iron-chelating or iron scavenging molecules that allow *K. pneumoniae* to acquire iron from the host in order to survive and propagate during infection (86, 87). *K. pneumoniae* expresses several siderophores including enterobactin, yersiniabactin, salmochelin and aerobactin. Also, *K. pneumoniae* can produce more than one siderophore simultaneously which aids successful

colonisation of different tissues and/or avoid deactivation of one by the host. Different siderophores exhibit different levels of affinity for iron the highest being attributed to enterobactin and lowest to aerobactin. In addition, the contribution to virulence of each siderophore varies. Enterobactin is considered the primary iron uptake system used by *K. pneumoniae* because it is ubiquitous among both hvKp and classical *K. pneumoniae* (cKp). During, *K. pneumoniae* respiratory infection enterobactin has been shown to have an important role in both colonisation and dissemination. Nevertheless, it can be neutralized by the antimicrobial protein lipocalin-2, which has high affinity for enterobactin inducing inflammatory response upon binding (37, 86).

However, yersiniabactin possesses an entirely different structure than enterobactin and it avoids lipocalin-2 binding, therefore enabling bacterial colonisation and growth within the host. Also, yersiniabactin is the most common high virulence determinant. It has been associated with *K. pneumoniae* isolated from bacteraemia and invasive infections, as for example, liver abscesses, being observed among ~18% of cKp and 90% of hvKp clinical isolates. Furthermore, whereas enterobactin is harboured on the chromosome, the yersiniabactin locus (*ybt*) that encodes the siderophore and its receptor is mobilised by the integrative conjugative element (ICE) *ICEKp*. *ICEKp* is the most common MGE associated with virulence that facilitates the spread of these virulence determinants. In addition, *ICEKp* elements can encode other virulence determinants as salmochelin and colibactin. Furthermore, the *ybt* locus display considerable diversity, 11 *ybt* loci and 17 distinct lineages were identified and associated with 14 different *ICEKp* structural variants. The presence of *ybt* was significantly associated with, not only, liver abscess isolates, but, also, with blood isolates. It is frequently present in *K. pneumoniae* carbapenemase associated from the CG258 and in hypervirulent CG23. However, it is also present in the wider population of *K. pneumoniae*. Among the population of *ICEKp* several sub lineages are each associated with a unique complement set of genes in addition to the yersiniabactin synthesis locus (86, 91, 92).

Siderophores aerobactin (*iuc*) and salmochelin (*iro*) are less prevalent than *ybt*. These siderophores contribute to *K. pneumoniae* virulence by avoiding lipocalin-2 as well. *iuc* has a completely different structure than enterobactin and *iro* is a glycosylated form of enterobactin. Additionally, *iuc* and *iro* lineages are genetically diverse as well. There are five *iro* and six *iuc* lineages described among *K. pneumoniae*. These have been shown to be mobilised through the population by plasmids. Among analysed

genomes most lineages found included *iuc1/iro1* or *iuc2/iro2*. Each lineage carried by different plasmids KpVP-1 and KpVP-2, respectively. These plasmids can also carry *rmpA* factors that were associated with the presence of *iuc* and *iro*. More importantly, *iro* and *iuc* MGEs are stably maintained among hvKp strains and have been detected in MDR strains. *iro* and *iuc* were only found among *K. pneumoniae* and not in other members of the KpSC (91, 92). Additionally, colibactin is a genotoxic polyketide that promotes DNA damage in eukaryotic cells, mucosal and gut colonisation and dissemination to the blood and other organs (39).

Lastly, fimbriae allow *K. pneumoniae* to adhere to host surfaces. Type 1 and type 3 fimbriae are commonly found in *K. pneumoniae*. Type 1 and type 3 fimbriae promote infection by allowing *K. pneumoniae* to adhere to human mucosal or epithelial surfaces and type 3 fimbriae are important mediators of biofilm formation (37, 87).

1.8 Infections caused by virulent *K. pneumoniae*

K. pneumoniae can be responsible for diverse and life-threatening infections. These include pneumonia, sepsis, urinary tract infection (UTI), bacteraemia, meningitis, and pyogenic liver abscesses. However, *K. pneumoniae* strains capable of causing invasive infection in healthy people have emerged, these being referred as hvKp (93). hvKp are associated with pyogenic liver abscess (PLA) but they can also cause pneumonia, for example. PLA caused by *K. pneumoniae* are more commonly reported in South Asia, for example in Taiwan and Korea, whereas *E. coli*, *Streptococcus* sp. or *Staphylococcus* sp. usually cause PLA in Central Europe *Klebsiella* spp. being less frequent (94), though, cases have been reported in Spain, in Madrid (95) and Barcelona (96), and in France (97) and Ireland (98).

In contrast to the cKp *K. pneumoniae* strains that are usually responsible for pneumonias and UTIs and bacteraemia and immunocompromised individuals. hvKp infection is usually community acquired and cKp infection is nosocomial and frequently multidrug resistant. These *K. pneumoniae* strains are often responsible for serious bacteraemia which can be either primary or secondary arising from spread from lung or bladder infection. In addition, pneumonia caused by these bacteria can be hospital acquired or community acquired however, the former is more prevalent than the latter. It presents as other nosocomial pneumonias with symptoms that may include, for example, cough, pulmonary infiltrates, and fever. Hospital acquired pneumonia

caused by *K. pneumoniae* affects 8 to 12% of ventilated patients and 7% of non-ventilated patients. In relation to community acquired pneumonia *K. pneumoniae* is rarely the cause in North America, Europe and Australia estimated to cause 3 to 5% of cases. Nevertheless, it is a more common agent across Asia and Africa, likely due to the prevalence of hvKp in these regions (86). Furthermore, although *K. pneumoniae* is an important causative agent of community acquired pneumonia in western countries, *K. pneumoniae* is more prevalent in several Asia and Pacific countries, such as Malaysia and Singapore (99). Also, a retrospective study in Taiwan concluded that *K. pneumoniae* pneumonia was associated with high mortality and hvKp was prevalent among patients (100). In addition, another study in Japan reported that *rmpA*-positive *K. pneumoniae* isolates were frequently responsible for not only pneumonia but also UTIs (101). In addition, hospital acquired pneumonia caused by hvKp *K. pneumoniae* has been reported in China (102). The presence of *K. pneumoniae* isolates responsible for pneumonia with occasional fatalities was described as well. During a study *K. pneumoniae* isolates from bloodstream infections, hospital-acquired pneumonia, and intra-abdominal infections were collected from 10 cities in China during February to July 2013. hvKP being usually found and associated with virulence determinants common among these strains. These virulence determinants included K1, K2, K20 antigens, and *rmpA* genes. In addition, these strains were also more prevalent among liver abscess, sepsis, and invasive infections (102). Finally, hvKp was reported as responsible for a fatal outbreak among patients suffering from ventilator associated pneumonia (103). Regarding *K. pneumoniae* causing UTIs these have been reported to commonly express K1/K2 serotypes as well as hypermucoid phenotype associated with *rmpA* factors (104). Also, overlap between antibiotic resistance and virulence determinants among UTI causing *K. pneumoniae* has been reported among hospital and community acquired UTIs. These *K. pneumoniae* isolates were shown to harbour aerobactin siderophore related genes among both hospital and community pathogens and K2 antigens were found as well. Additionally, antimicrobial resistance was more common among hospital pathogens with isolates having unusual K64 and K62 antigens (105). Furthermore, *K. pneumoniae* is responsible for UTIs in children as well as in patients with chronic kidney disease (106, 107).

The association between *K. pneumoniae* and liver abscesses is reported more frequently in Asia, for example, in countries like China and Taiwan. These isolates

frequently belonged to CG23 and CG65 and serotypes K1 and K2 in addition siderophore loci were detected as well (108). Nevertheless, non-K1/K2 isolates are capable to cause primary liver abscess have been described in Taiwan (109). Even though, liver abscess is usually caused by K1 or K2 serotype and *rmpA* carrying *K. pneumoniae* (110).

Furthermore, *K. pneumoniae* is among the agents responsible for blood stream infections (BSI) among critically ill patients. In addition, BSI causing hvKp have been reported to be responsible for high mortality rates in a study from India (111, 112). *K. pneumoniae* also causes BSI among paediatric patients. A study in Japan described BSI caused by *Klebsiella* spp. which showed K1 and K2 serotypes as well as siderophore production and *rmpA* factors (113, 114). hvKp can also cause other infectious syndromes including musculoskeletal and soft tissue infection, central nervous system disease, abdominal disease, thoracic disease and endophthalmitis (93).

Type 1 and type 3 fimbriae have been described as playing a role in *K. pneumoniae* infection as the mediators of adhesion. Type 1 and type 3 fimbriae are expressed by environmental and clinical *K. pneumoniae* isolates. The most clinically important function of fimbriae is biofilm formation and binding to abiotic surfaces. Type 3 fimbriae were shown to be strongly associated with biofilm formation and an important virulence determinant associated with catheter associated infections. Therefore, the ability of *K. pneumoniae* to bind to endotracheal tubes of ventilated patients contributes for colonisation and persistence and lung infection caused by these bacteria. Type 1 fimbriae were described as an important determinant in UTIs caused by *K. pneumoniae* but do not affect the ability of these bacteria to infect the lung or colonise the intestine. Lastly, an outbreak, in neonatal care unit, of *ybt* producing *K. pneumoniae* strain has been described (115). This strain showed high pathogenicity among extremely premature infants infection leading to fulminant disease including necrotizing enterocolitis and sepsis. Virulence determinants including *rmpA*, *iuc* and *clb* were detected in *K. pneumoniae* isolates causing neonatal infections including UTI and sepsis (115, 116).

1.9 Treatment options for infection caused by *K. pneumoniae*

The increasing prevalence of carbapenemase producing *Enterobacteriaceae* has led to a decrease in treatment options. However, there are treatments that can be used these include colistin, tigecycline and fosfomycin or different β -lactam/ β -lactamase inhibitor combinations as, for example, ceftazidime/avibactam, aztreonam/avibactam. Although, colistin was used for treatment of GNB infection before its use was replaced by the safer drugs, because of adverse side effects including nephrotoxicity and neurotoxicity. However, the use of colistin has recently increased due to the rise in carbapenemase resistance. It is also active against most carbapenemase bacteria but colistin resistant KPC, NDM, OXA-48 producing *K. pneumoniae* have been reported (8).

Tigecycline has a broad spectrum of action against GPB and GNB such as carbapenemase producing *Enterobacteriaceae*. Treatments using tigecycline are usually administered in combination therapy, for example, with gentamicin. Nevertheless, reports of increasing resistance to tigecycline in KPC producing *K. pneumoniae* have emerged as well. Regarding fosfomycin, it can be used for several types of infections when in intravenous formulation and for systemic infections it is usually administered in combination with for example colistin and tigecycline. Carbapenemase producing *Enterobacteriaceae* including KPC producing *K. pneumoniae* remain mostly susceptible. Also, aminoglycoside gentamicin cannot be used to treat infections caused by NDM producing *Enterobacteriaceae* because these are often resistant to aminoglycosides. For treatment gentamicin is usually used in combination with other antibiotics such as colistin or with a carbapenem (8). Moreover, *K. pneumoniae* infections can be treated using triple combinations of antibiotics belonging to different classes these can include a polymyxin or tigecycline, and an aminoglycoside or carbapenem. In addition, triple combinations based on a polymyxin are important for the treatment of carbapenemase producing *K. pneumoniae* because few antimicrobials are effective against these strains and because polymyxin resistance is increasing (117).

Nevertheless, combinations as ceftazidime/avibactam, aztreonam/avibactam have been used to treat infections caused by *K. pneumoniae*. The ceftazidime/avibactam combination demonstrated excellent in vitro activity against GNB including *K. pneumoniae* producing ESBLs and AmpC and it was associated with

good response in patients with infections caused by non-susceptible to ceftazidime GNB (118, 119).

Furthermore, a retrospective study into infections caused by KPC-producing *K. pneumoniae* saw that the thirty-day mortality was significantly lower among patients treated with ceftazidime/avibactam than that of the patients treated with other drugs (120). Also, in another study evaluating the use colistin versus the use of ceftazidime/avibactam among patients suffering from infection caused by carbapenemase producing *Enterobacteriaceae* including *K. pneumoniae* ceftazidime/avibactam was concluded to be a reasonable option for treatment (121). Moreover, although, ceftazidime/avibactam still maintains high efficacy, against treatment of carbapenemase producing bacteria resistance to ceftazidime/avibactam has been reported in several studies. These mechanism of resistance among *K. pneumoniae* include the mutation of KPC-3 and CTX-M-14 (122).

Despite, the efficacy of ceftazidime/avibactam against KPC producing *K. pneumoniae* ceftazidime/avibactam is not effective against MBL producing bacteria, but the aztreonam/ avibactam combination has shown *in vitro* activity against carbapenemase producing GNB as well (118). Aztreonam/avibactam has been shown to be effective against MBL producing *Enterobacteriaceae* including *K. pneumoniae* lowering minimum inhibitory concentrations (MICs) of aztreonam resistant isolates by 8-fold in a study from China. In addition, another study in China also indicated that aztreonam was highly effective against NDM producing *K. pneumoniae* (123, 124). Additionally, other studies have indicated the efficacy of aztreonam/avibactam among *Enterobacteriaceae* including carbapenemase producing *K. pneumoniae* and NDM producing *K. pneumoniae* (31, 125, 126). Moreover, Chew et al. proposed that aztreonam/avibactam should be a more effective treatment where MBLs and carbapenemase *Enterobacteriaceae* predominate after testing the combination against a panel of *K. pneumoniae* and other *Enterobacteriaceae* coharbouring carbapenemase ARG including *bla*_{NDM} and *bla*_{OXA-48}-like ARG (127). In addition, other combinations as for example meropenem/vaborbactam can be an option for treatment of carbapenem producing *Enterobacteriaceae* and cefiderocol a new siderophore cephalosporin was shown to be effective against KPC and NDM producing *K. pneumoniae* in a respiratory tract infection in a rat model (128).

Additionally, there is clinical interest in using ceftazidime/avibactam and aztreonam combinations in view of the limited therapeutic options available for treating

some infection with MBL producing *Enterobacteriaceae*. Case reports, case series and animal models suggest the combination could be safe and effective. No controlled clinical trials exist to confirm these hypotheses (129-131).

Furthermore, other therapy options are being studied for treatment of infections caused by *K. pneumoniae* as vaccines and antibody-based treatments. These strategies can target the capsule (CPS) or LPS surface polysaccharides of *K. pneumoniae*. In relation to vaccines targeting the capsular polysaccharides the most successful vaccine attempt was a 24 valent CPS vaccine that reached phase I clinical trial, but this vaccine provided incomplete coverage and proved too costly. Nevertheless, recently a candidate vaccine for carbapenem-resistant *K. pneumoniae* has been developed providing a lead in the development of a vaccine. This is a glycoconjugate vaccine, that provided immunization through a synthetic hexasaccharide glycoconjugate and has resulted in high antibody production in mice and rabbits. These antibodies were active against carbapenem resistant *K. pneumoniae* CPS and promoted phagocytosis (132). Another vaccine study focused on the K1 and K2 serotypes of hypervirulent *K. pneumoniae* because these two serotypes cause the majority of hypervirulent infections reporting a bioconjugate vaccine that protected mice against *K. pneumoniae* hypervirulent infection (133). The LPS was studied as an alternative for a vaccine as well. It demonstrated that immunization with purified LPS was able to offer protection against a lethal dose of *K. pneumoniae* in a mouse model (134). Moreover, another study reported the development of a recombinant protein vaccine using the YidR protein because this protein is highly conserved among *K. pneumoniae* irrespective of serotype. The vaccine produced strong induction of antibodies in a mouse model after infection with *K. pneumoniae* (135). Also, other therapies are based on monoclonal antibodies use K serotypes as targets. These included anticapsular antibodies against carbapenem resistant ST258 *K. pneumoniae* that were capable of agglutinating strains and promoted bacteria killing processes by inhibiting biofilm formation, activating the complement and promoting the deployment of neutrophils. It was also shown to promote opsonophagocytosis and intracellular killing of carbapenem resistant *K. pneumoniae* (136). Another study of an antibody-based therapy focused on the hypervirulent *K. pneumoniae* K1 serotype with the therapy shown to decrease the dissemination of hypervirulent *K. pneumoniae* in sepsis and pulmonary infection in a

mouse model. It was also shown to enhance clearance of bacteria from the liver and opsonophagocytosis (137).

Overall, treatment of multidrug resistant GNB infections is increasingly challenging because fewer drugs are active and not available in many countries. With the rise of carbapenem resistance potential treatments include newer β -lactamase inhibitors and older drugs. Successful treatment strategies require good knowledge of the patient's clinical profile and knowledge of the local microbiologic epidemiology, including resistance rates and predominant resistance determinants (128, 138).

1.10 The BARNARDS project and this PhD dataset

The Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) study was a multi-site international project funded by the Bill and Melinda Gates Foundation. The study ran from March 2015 to December 2017 the project. It was active in 12 different sites across seven different low and middle-income countries (LMICs) in Africa (Ethiopia, Nigeria, Rwanda and South Africa) and South Asia (Bangladesh, India, Pakistan).

BARNARDS aimed to assess the burden of antibiotic resistance in neonates, particularly focusing on the prevalence of neonatal sepsis, and mortality following neonatal sepsis. Through establishing a consistent and standardised protocol ensuring all clinical sites used the same laboratory equipment and reagents, BARNARDS aimed to determine the prevalence of MDR GNB bacteria carried within normal microbiota by of mothers, their infants and that present within the clinical environment. An additional aim was to assess the proportion of neonatal sepsis cases caused by MDR GNB bacteria. Through a questionnaire, BARNARDS also captured socio-demographic traits such as education, access to clean water, sanitation conditions, living conditions, socioeconomic status, and antibiotic usage to explore potential risk factors for the development of neonatal sepsis. The background and a main result synopsis of the BARNARDS project are included in Appendix 1 and the clinical sites enrolled are described in Appendix 2.

1.10.1 Prevalence of neonatal sepsis caused by *K. pneumoniae*, and the carriage of carbapenemase positive among *K. pneumoniae* throughout the BARNARDS study

As part of the BARNARDS project *Klebsiella* spp. carbapenemase positive isolates were found among the different sample types on study and across several countries. In total 1345 bacterial isolates were identified during BARNARDS among these 275 (20%) belonged to *Klebsiella* spp. Furthermore, 258 infections, in total, were caused by *K. pneumoniae*. The outcome of infection for these cases were alive for 138 (53.5%) infants, deceased for 53 (20.5%) infants and for 67 (26%) infants the outcome of infection was untraceable. Also, the onset of sepsis for infections which the outcome was alive was 65 (47%) EOS, 68 (49%) LOS and for five (4%) cases the onset was not determined. Infection cases that the outcome was deceased included 25 (47%) EOS and 25 (47%) LOS cases and for three (6%) cases the onset of infection

was not determined. Additionally, overall, the onset of *K. pneumoniae* infection included 125 (48%) EOS and 118 (46%) LOS cases and for 15 (6%) cases the onset was not determined.

Sepsis cases in Pakistan caused by *K. pneumoniae* (n=42) included 23 (55%) EOS, 7 (17%) LOS and for 12 (28.5%) cases the onset was not determined. The outcome of these infections was alive for eight (19%) cases, deceased for three (7%) and for 31 (74%) cases the outcome of infection was untraceable. Among the infection cases that the outcome was alive four (50%) were EOS cases, one (12.5%) LOS and for three (37.5%) the onset of infection was not determined. Infections with an outcome of deceased included one (33%) EOS case and for two (67%) cases the onset was not determined (Fig. 7a).

K. pneumoniae was responsible for 95 sepsis cases in Ethiopia among these the onset of infection was for 66 (69.5%) cases EOS and for 28 (29.5%) cases LOS and for one case the onset was untraceable. In relation to, the outcome of these infections 63 (66%) were alive. The onset of infection among these cases was EOS for 43 (68%) infections and LOS for 20 (32%) cases. For 14 (15%) infections the outcome was deceased and the onset among these was EOS for 12 (86%) cases, one (7%) LOS case and for one (7%) case the onset of infection was not determined. Additionally, for 18 (19%) infection cases the outcome was untraceable among these the onset was for 11 (61%) cases EOS and for seven (39%) cases LOS (Fig. 7b).

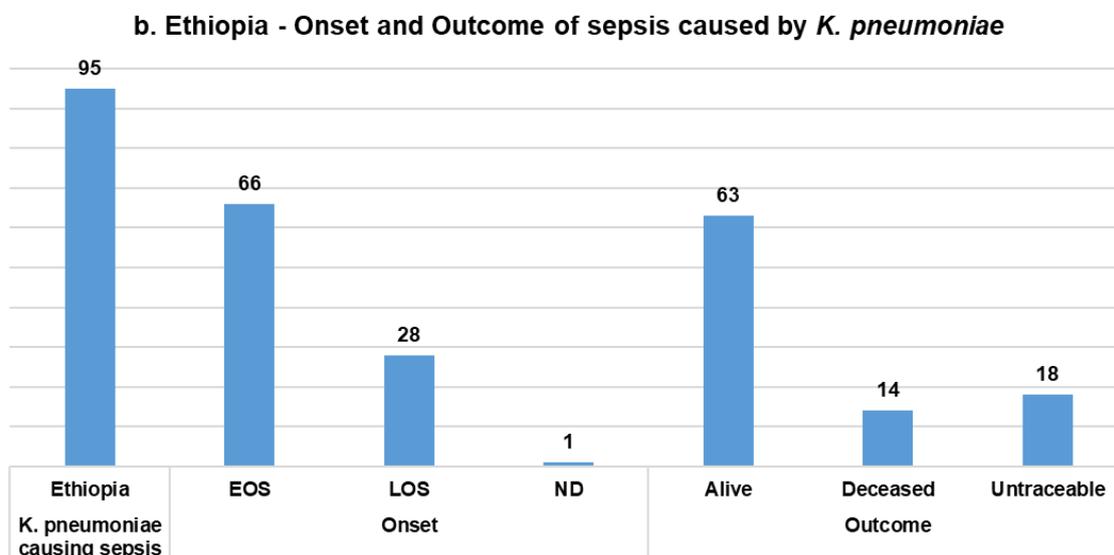
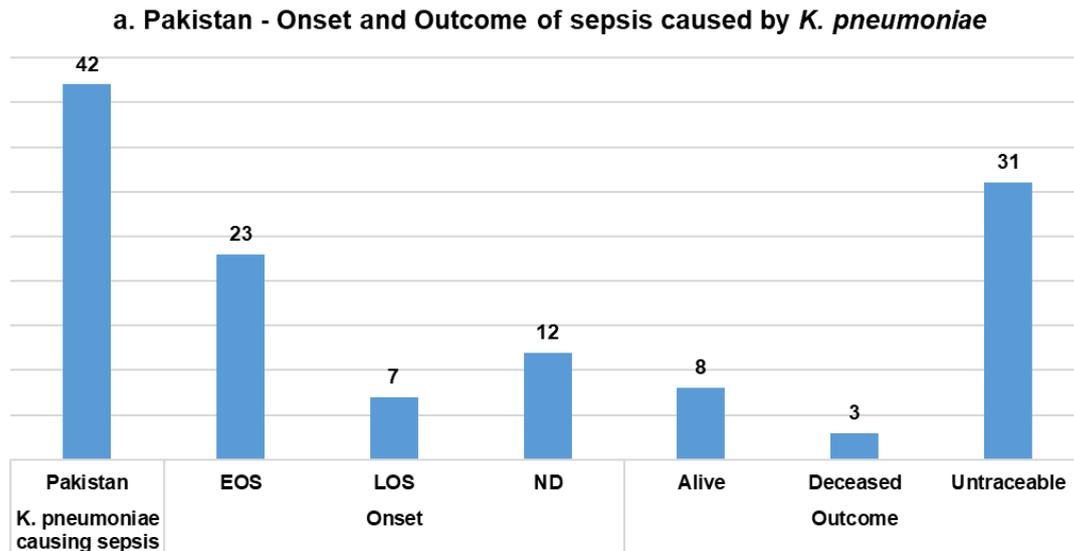


Fig. 7: Onset and outcome of sepsis among *K. pneumoniae* from Pakistan (a.) and Ethiopia (b.). **a.** In Pakistan 42 sepsis cases were caused by *K. pneumoniae*. The onset was EOS (n=23), LOS (n=7) and not determined (ND; n=12). For most cases, the outcome was untraceable (n=31) and the outcome for eight and three cases was alive and deceased, respectively. **b.** In Ethiopia 95 sepsis cases were caused by *K. pneumoniae*. The onset was EOS (n=66), LOS (n=28) and not determined (ND; n=1). The outcome was in majority alive (n=63) and the outcome was deceased (n=14) and was untraceable for 18 sepsis cases. The number of *K. pneumoniae* isolates causing sepsis is displayed in addition to the number of isolates causing early (EOS) and late (LOS). The number cases where the outcome of sepsis was alive, and deceased is shown too. ND means the onset was not determined.

Results, also, showed that the most commonly carried carbapenemase ARG among *Klebsiella* spp. isolates was *bla*_{NDM} (97%) followed by *bla*_{OXA-48-like} (17%) and *bla*_{KPC} (2%). Fig. 8 displays the percentages of carbapenemase ARG across the sites/countries from which *K. pneumoniae* were recovered.

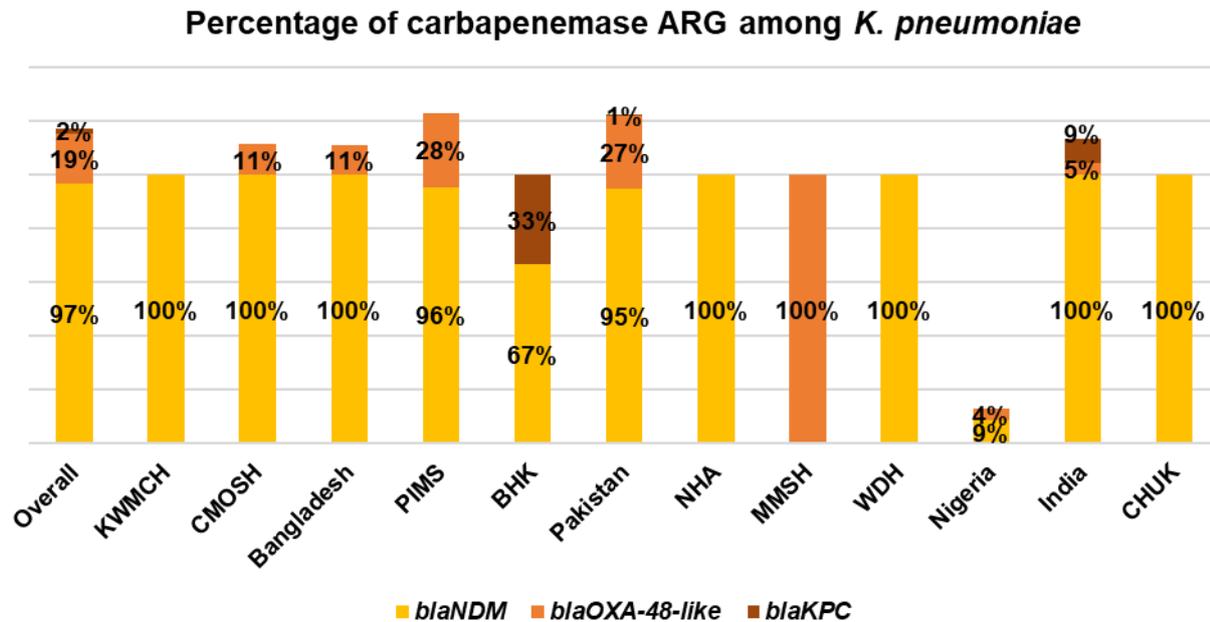


Fig. 8: Percentage of *bla*_{NDM}, *bla*_{OXA-48-like} and *bla*_{KPC} carbapenemase ARG among *K. pneumoniae* found in all countries/sites in BARNARDS. Among *K. pneumoniae*, *bla*_{NDM} (97%) showed the highest percentage followed by *bla*_{OXA-48-like} (19%) and *bla*_{KPC} (2%). With *bla*_{NDM} ARG percentages of 100% showed in KWMCH and CMOSH (Bangladesh), NHA and WDH (Nigeria), India and CHUK and *bla*_{OXA-48-like} was 100% in MMSH (Nigeria). *bla*_{KPC} were mostly found in Pakistan in BHK (33%).

In addition, *K. pneumoniae* in majority carried *bla*_{NDM} followed by *bla*_{OXA-48}-like and *bla*_{KPC}.

Among isolates recovered from MR 54 (9%) were identified as *Klebsiella* spp. from a total of 625 isolates. Isolates belonging to *Klebsiella* spp. (n=176; 31%) were among a total of 561 isolates recovered from BR samples. *Klebsiella* sp. isolates were among ENV isolates were 44 (28%) among 159 isolates in total recovered from ENV samples.

Moreover, *Klebsiella* spp. isolates were mostly identified as *K. pneumoniae* (n=508; 22%) including isolates recovered from MR, BR, ENV and BB (n=2394;100%) samples. MR isolates identified as *K. pneumoniae* (n=50;2%) were from Pakistan (n=29;1%), Bangladesh (n=16;0.7%), India (n=8;0.3%) and Nigeria (n=3;0.1%). PCR screening revealed that 49 (2%) MR *K. pneumoniae* carried *bla*_{NDM}, *bla*_{OXA-48}-like (n=7;14%) and *bla*_{KPC} (n=2;4%).

K. pneumoniae were found in BR from Pakistan (n=80;3%), Bangladesh (n=47;2%), Nigeria (n=18;0.75%) and India (n=14;0.6%). Among BR samples in overall, *K. pneumoniae* (n=161;7%) was the most frequently identified species. After PCR screening these isolates commonly tested positive *bla*_{NDM} (n=156;97%). In addition, 32 isolates tested positive for *bla*_{OXA-48}-like, and two isolates tested positive for *bla*_{KPC} ARGs.

Regarding ENV *K. pneumoniae* (n=39;2%) these were recovered from Pakistani (n=36;1.5%), Nigerian (n=2;0.08%), Rwandan (n=1;0.4%) samples. ENV *K. pneumoniae* tested positive for *bla*_{NDM} (n=37;47%) and for *bla*_{OXA-48}-like (n=8;10%).

BB *K. pneumoniae* (n=62;3%) carried *bla*_{NDM} ARG as well. These were found among isolates from Bangladesh (n=6;9.7%), Ethiopia (n=16;26%), India (n=1;2%), Nigeria (n=24;3%), Pakistan (n=8;13%), Rwanda (n=2;3%) and South Africa (n=5;8%). *bla*_{CTX-M-15} was carried by BB *K. pneumoniae* (n=220;9%). Fig. 9 displays the distribution of *K. pneumoniae* across sample types and countries in BARNARDS.

Sample type	BB n=258	Ethiopia n=95 Pakistan n=44 Nigeria n=57 Bangladesh n=19 India n=15
	BR n=161	Pakistan n=80 Bangladesh n=47 Nigeria n=18 India n=14
	MR n=50	Pakistan n=29 Bangladesh n=16 Nigeria n=8 India n=3
	ENV n=39	Pakistan n=36 Nigeria n=2 Rwanda n=1

Fig. 9: Number of carbapenemase positive *K. pneumoniae* isolates recovered from the different sample types (BR, MR and ENV) and BB isolates from countries enrolled in BARNARDS.

2.2 Defining the dataset for this PhD

K. pneumoniae isolates in Pakistan and Ethiopia were responsible for neonatal sepsis (including outbreaks) and recovered were frequently isolated from rectal samples from mothers (MR), infants (BR) and samples from the clinical environment (ENV). Thus, I chose to study the *K. pneumoniae* population recovered in these clinical sites.

<i>K. pneumoniae</i> n=465	BARNARDS n=274	MR n=22	Pakistan n=22
		BR n=87	Pakistan n=87
		ENV n=37	Pakistan n=37
		BB n=128	Pakistan n=42 Ethiopia n=86
	This PhD n=191	MR n=90	Pakistan n=70 Ethiopia n=30
		BR n=87	Pakistan n=19 Ethiopia n=68
		ENV n=14	Pakistan n=7 Ethiopia n=7

Fig. 10: Number of *K. pneumoniae* isolates from Pakistan and Ethiopia recovered for this PhD (orange) and for BARNARDS (blue). *K. pneumoniae* isolates from different sample types (BB, BR, MR and ENV) were included in this PhD study. *K. pneumoniae* from BARNARDS were carbapenemase positive and *K. pneumoniae* from Pakistan recovered in this study were all carbapenemase negative and *K. pneumoniae* from Ethiopia were in majority carbapenemase negative with two BR being carbapenemase positive.

However, due to the BARNARDS initial screening focusing on the recovery of carbapenemase producers, to evaluate the wider *K. pneumoniae* population for my PhD additional isolates were recovered, to include carbapenemase sensitive strains (Fig. 10). Retrospective culture of MR, BR and ENV was performed to phenotypically identify for *K. pneumoniae* based on their agar colour differentiation. Following isolation of *K. pneumoniae*, PCR screening was performed to select for isolates that tested negative for carbapenemase ARG (*bla*_{NDM/OXA-48-like/KPC})

In total 103 carbapenemase negative *K. pneumoniae* (CNK) were recovered from Ethiopia and 96 CNK from Pakistan. During additional screening two carbapenemase positive *K. pneumoniae* (CPK) from Ethiopia were recovered as part of this PhD (Fig. 10). The entire dataset of this PhD is listed in Appendix 3 detailing STs, ARG and virulence determinants carried by each isolate.

Below Table 3 and Table 4 show the number and percentage of CPK and CNK from Pakistan and Ethiopia, respectively.

Table 3: Number of *K. pneumoniae* isolates from Pakistan and percentages of CPK and CNK in total and per sample type characterised in this PhD.

<i>K. pneumoniae</i> from Pakistan characterised in this PhD				
Total number of isolates = 284				
	BB 42 (15%)	BR 106 (37%)	MR 92 (32%)	ENV 44 (15.5%)
Percentages of CPK and CNK (n;%)				
In Total				
	CPK =177 (62%)			
	CNK = 107 (38%)			
Among isolates from different sample types (n;%)				
	BB	BR	MR	ENV
CPK	31 (70.5%)	87 (82%)	22 (24%)	37 (84%)
CNK	11 (25%)	19 (18%)	70 (76%)	7 (16%)

Table 4: Number of *K. pneumoniae* isolates from Ethiopia and percentages of CPK and CNK in total and per sample type characterised in this PhD.

<i>K. pneumoniae</i> from Ethiopia characterised in this PhD				
Total number of isolates = 191				
	BB	BR	MR	ENV
	86 (45%)	68 (36%)	30 (16%)	7 (4%)
Proportion of carbapenemase positive and negative (n;%)				
In Total				
CPK = 2 (1%)				
CNK = 189 (99%)				
Among isolates from different sample types				
	BB	BR	MR	ENV
CPK	0 (0%)	2 (3%)	0 (0%)	0 (0%)
CNK	86 (100%)	66 (97%)	30 (100%)	7 (100%)

In order to study the genomic traits of *K. pneumoniae* in light of their role in neonatal sepsis, clinical data collected as part of BARNARDS was included for subsequent analysis. The population structure of the *K. pneumoniae* isolates included in my PhD included in Appendix 4 and clinical data in Appendix 5.

3. Aims of this PhD

My PhD characterises the population of *K. pneumoniae* from two BARNARDS clinical sites PIMS (Pakistan) and from St Paul's Hospital (Ethiopia). This thesis characterises two distinct populations of *K. pneumoniae* isolates from the two clinical sites to build upon the foundations of BARNARDS to provide further information that could guide clinical treatments for neonatal sepsis, infection control practices in neonatal wards, and contribute to the development of *K. pneumoniae* vaccines.

Aim 1

To identify whether transmission of *K. pneumoniae* has occurred between mother and neonate (vertical transmission) and/or between mother/neonate and the clinical environment (horizontal transmission)

Objectives

- a. To determine whether *K. pneumoniae* isolates can be recovered from the rectal microbiota of the mothers' (MR) and neonates (BB, BR) and the clinical environment (ENV).
- b. To perform whole genome sequencing (WGS), determine the multi locus sequence types (MLST) and generate core genome phylogenetic trees to summarise the *K. pneumoniae* population from the mother, neonatal and clinical environmental samples collected in Pakistan and Ethiopia.
- c. To perform core genome SNP analysis on the dominant ST groups/clades across the four sample types (BB, MR, BR, ENV) and ascertain whether strong or weak genomic and epidemiological data exists to evidence transmission.

The results that meet this aim are within chapter 5 and 6 , sections 5.1 and 6.1 and chapter 7.

Aim 2

To determine the extent of antimicrobial resistance in neonatal sepsis in the two clinical sites (Ethiopia and Pakistan).

Objectives

- a. To establish whether *K. pneumoniae* sepsis isolates are susceptible to empirical therapy used for neonatal sepsis at the study sites.
- b. To establish if key resistance markers used for screening colonising the mothers' and neonates' rectal microbiota are associated (*K. pneumoniae* identified within carriage) with antimicrobial resistance to commonly used antibiotics and other acquired antibiotic resistance genes (ARGs).
- c. To establish if dominant strains associated with antibiotic resistant phenotypes are also identified as causes of neonatal sepsis.
- d. To identify the different plasmid types carrying antibiotic resistance genes and explore the similarity of plasmid sequences within the dominant *K. pneumoniae* ST groups.

The results that meet this aim are within chapters 5 and 6, sections 5.2 to 5.4 and 5.6 and 6.2 to 6.4 and 6.6 and chapter 7.

Aim 3

To contextualise the *K. pneumoniae* isolates from Pakistan and Ethiopia in this study within a published to a global collection.

Objectives

- a. To understand how the dominant STs from Pakistan and Ethiopia in this study fit among other STs globally.
 - i. Performing a literature search to collate representative *K. pneumoniae* sequence and meta data.
 - ii. Performing a core genome phylogenetic analysis
- b. To understand how genomic surveillance can be helpful regarding therapeutics options such as vaccines and antibodies.

The results that meet this aim are within chapter 7, section 7.6.

Aim 4

To characterise the virulome of *K. pneumoniae* isolates collected across all four sample types (BB, BR, MR and ENV)

- a. To establish whether common virulence determinants were only associated to *K. pneumoniae* causing sepsis or were associated with the mothers' and neonates' microbiota and the clinical environment.
- b. To establish if dominant clones were associated with common virulence determinants found in this study phenotypes are also identified as causes of neonatal sepsis.
- c. To determine if these virulence determinants influence outcome and onset of sepsis.

The results that meet this aim are within chapters 5 and 6, sections 5.5 and 6.5 and chapter 7.

Aim 5

To determine whether relationships exist between particular STs, antimicrobial resistance and virulent traits to the clinical outcome of neonatal sepsis.

Objectives

- a. To collate available clinical epidemiological data from within the larger BARNARDS dataset.
- b. To perform statistical analyses to determine whether associations exist between carriage of particular ARG and virulence determinants and the clinical outcome of sepsis among the dominant STs in this study.

The results that meet this aim are within chapters 5 and 6, sections 5.7 and 6.7 and chapter 7.

4. Materials and Methods

4.1 Microbiological analysis

4.1.1 Study design and Sampling

The two clinical partners included from BARNARDS for extensive *Klebsiella* characterisation during this PhD study were Ethiopia (ES), St Paul's Millennium Medical College, Addis Ababa (a.), and Pakistan (PP), Pakistan Institute of Medical Sciences (b.) (all BARNARDS clinical sites are described in Appendix 2 and the methods described in Appendix 6).

a. [St Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia](#)

Saint Paul's Hospital is a teaching and tertiary referral hospital administered directly under the federal Ministry of Health of Ethiopia located on the country's capital with a population of 3.3 million (2007 census). This site was co-managed by the Boston Children's Hospital / Harvard University and BARNARDS worked directly with them. St Paul's hospital is one of the largest hospitals in Ethiopia covering a study catchment population of 250,000 people with 8000 births per year.

b. [The Pakistan Institute of Medical Sciences \(PIMS\), Islamabad, Pakistan](#)

BARNARDS was managed in Pakistan by Quaid-i-Azam with PIMS acting as the main clinical centre. Both institutions are based in Islamabad federal territory of Pakistan with an estimated population of 2 million that include Islamabad the country's capital. PIMS is a region leading tertiary level hospital established in 1985 as research-oriented health sciences institute. The institute includes 22 medical and surgical specialist centres including three semi-autonomous hospitals participating in the BARNARDS project. These include the Islamabad Hospital with 592 beds, the Children's Hospital, with 230 beds specialising in paediatric care and the Maternal and Child Health Care Centre with 125 beds specialising in obstetrics, gynaecology and neonatology.

Sample types collected during BARNARDS and analysed in this PhD

To understand the epidemiology of MDR bacteria carried as normal microbiota and their impact in the development of neonatal sepsis different sample types were collected to be studied during BARNARDS. Between November 2015 and November 2017, women in labour or immediately post-partum and were recruited prospectively following consent. Neonates who presented to clinical sites with suspected sepsis in the first 60 days of life were recruited (with their mothers) upon consent and followed up for the first 60 days of life or until study withdrawal/neonatal death. For this PhD, samples were screened for the presence of *K. pneumoniae* isolates. Isolates and clinical data of mothers and neonates that withdrew from the BARNARDS study was excluded from the study and from my PhD.

- Mother (MR) and Baby Rectal (BR) samples were collected from mothers that consented to participate and from their neonates who presented with signs of sepsis within > 7 to 60 days of life. Rectal samples from mothers that consented to participate whose neonate did not present with signs of sepsis were also collected. All samples were stored on charcoal swabs for transport under UN3373 regulations and processed at Cardiff University.

- Baby Blood samples (BB) were collected from neonates presenting with signs of sepsis within <7 days of life. Between 0.5-2 mL of blood was collected and processed using BactAlert (at the local sites). All positive blood culture bottles were plated onto blood agar (at the local sites) and bacterial isolates were stored on charcoal swabs. All isolates were shipped to Cardiff University under UN3373 regulations. All BB isolates were phenotypically identified on site. Biological sepsis (BS) was assigned to neonates with blood culture positive sample(s).

- Clinical Environmental Sample (ENV), for example, swabs were taken from a delivery table in the delivery room, the baby matrix in a NICU and a gown used in a labour room.

Standard Operating Procedures (SOPs) were created and followed in all BARNARDS enrolled sites included PIMS, Pakistan and St Paul's Hospital Ethiopia and ethical approval was obtained from local ethics committees before the study

began (<https://barnards-group.com/publications/>). Ethical approval letters obtained for St Paul's Hospital, Ethiopia and PIMS, Pakistan are included as Appendix 7.

4.1.2 Processing of sepsis *Klebsiella* isolates

BB isolates were shipped to the UK with a species identification performed at the local clinical sites. *Klebsiella* isolates were plated onto V media and incubated overnight at 37°C. All recovered isolates were stored in cryogenic TS/72 beads (Technical Service Consultants, UK) at -80°C and the original swabs stored at 4°C.

4.1.3 Processing of rectal swabs and environmental swabs

MR, BR and ENV samples that were shipped to the UK were streaked onto chromogenic agar UTI media (Liofilchem®, Italy) supplemented with vancomycin (V, 10mg/L) and incubated overnight at 37°C. Possible *K. pneumoniae* isolate colonies selected from V plates were streaked onto the same media for purity based on their blue phenotypic presentation provided by the chromogenic agar. V media agar plates were used in order to promote the growth of carbapenemase negative isolates. Carbapenemase positive (CRE+) isolates were isolated from MR, BR, and ENV samples stored and recovered during BARNARDS (as part of the primary aim of the study). CRE+ isolates were streaked onto vancomycin and ertapenem agar plates (VE, 10mg/L and 2mg/L, respectively). Colonies from MR, BR and ENV were similarly selected based on their blue phenotypic presentation. All recovered isolates were stored in cryogenic TS/72 beads (Technical Service Consultants, UK) at -80°C and the original swabs stored at 4°C.

4.1.4 Carbapenemase genes screening and isolates identification

Pure colonies recovered from V were resuspended in 150 µL molecular grade water and used as the genomic DNA (gDNA) template for multiplex PCR analysis to assess the presence of *bla*_{NDM}, *bla*_{OXA-48-like} and *bla*_{KPC}. using the Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, USA). PCR amplification was carried out for 30 cycles consisting of denaturation at 95°C for 30s, annealing at 61°C for 30s and extension at 72°C for 30s, after an initial denaturation at 95°C for 5 minutes. Final extension was performed at 72°C for 10 min. Amplicons were subjected to electrophoresis in a 1.5% agarose gel at 200 V for 45 min in 1xTBE buffer containing 2% of ethidium bromide. Primers used are shown in Table 5. Gels were visualised and

photographs recorded under UV light with the UV light with the Syngene G: Box XX6 instrument.

Preliminary identification of candidate carbapenemase negative *K. pneumoniae* isolates was done using Microflex LT MALDI-TOF MS (Bruker Daltonik, GmbH, UK) with α -Cyano-4-hydroxycinnamic acid (HCCA) matrix (Sigma Aldrich), following the manufacturers' guidelines.

Table 5: Primers used for single and multiplex PCR screening.

Primers	Target	5'-3' Sequence	Reference
NDM-M-F NDM-M-R	<i>bla_{NDM}</i>	AGCTGAGCACCGCATT CTCAGTGTCGGCATCAC	Walsh group (139)
KPC-M-F KPC-M-R	<i>bla_{KPC}</i>	TAGTTCTGCTGTCTTGTCTC CCGTCATGCCTGTTGTC	Walsh group (139)
OXA-48-M-F OXA-48-M-R	<i>bla_{OXA-48-like}</i>	GGCGTAGTTGTGCTCTG AAGACTTGGTGTTTCATCCTT	Walsh group (139)

4.1.5 Minimum Inhibitory Concentration Determination (MIC)

Antibiotic susceptibility testing was performed for both carbapenemase positive and carbapenemase negative (CRE-). *K. pneumoniae* isolates. CRE- isolates were tested for 18 antibiotics and two antibiotic/inhibitor combinations whereas CRE+ isolates were tested for 18 antibiotics in addition to four antibiotic/inhibitor combinations. For CRE+ isolates the antibiotic/inhibitor combinations used were amoxicillin/clavulanate, piperacillin/tazobactam, ceftazidime/avibactam and aztreonam/avibactam. For CRE- isolates amoxicillin/clavulanate and piperacillin/tazobactam were the only combinations tested. Agar dilution was performed to determine the minimum inhibitory concentrations (MICs) for cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides, tetracyclines, colistin and fosfomycin for all isolates in study. The complete list of antibiotics and concentrations tested are listed in Table 6. All results were interpreted following the EUCAST v.9 2019 guidelines (140) with CLSI M-100 Ed29:2019 clinical break points (141) being used to interpret aztreonam/avibactam MIC results. *E. coli* ATCC25922 (n=1), *P. aeruginosa* ATCC27853 (n=1), *K. pneumoniae* 700603 (n=1) and *S. aureus* ATCC29213 (n=1) were used for routine quality and performance test control. Muller-Hinton II (MHII) (BD™ BBL™ Mueller Hinton II Agar) agar square

plates (35 mL) were prepared for each antibiotic concentration to be tested after the corresponding antibiotic dilutions were made. For the 35 mL square plates a maximum of 80 isolates were tested, hence 75 isolates and the five controls were tested at the same time. To prepare the antibiotic solutions serial dilutions were performed to obtain the concentrations tested (Table 6). This was done by weighing the antibiotic powder to prepare a stock solution for each antibiotic and antibiotic/inhibitors using the appropriate solvent. All antibiotics were dissolved in sterilised water. With the exceptions of amoxycillin, piperacillin, ceftazidime and aztreonam being in a saturated bicarbonate sodium solution, ciprofloxacin being dissolved in a hydrochloric acid solution (0.1M) and minocycline that was dissolved in dimethylsulphoxide (DMSO). All solutions were prepared in universal containers including antibiotic stock solutions and the antibiotic solutions added to the MHAll agar. To prepare antibiotic stock solutions the amount of antibiotic needed in milligrams was calculated using the formula below:

$$\text{Weight of antibiotic (mg)} = \frac{1000}{\text{Potency given by the manufacturer } (\mu\text{g/mg})} \times$$

$$\times \text{Volume required (mL)} \times \text{Final concentration of solution (mg/L)}$$

Three stock solutions were prepared for each antibiotic and antibiotic/inhibitor combinations. These stock solutions were prepared to allow easier preparation of the agar plates corresponding to each concentration of the tested antibiotic and antibiotic/inhibitor combinations because these were prepared by serial dilution.

The three stock solutions included:

Stock A 2560mg/L = Weight of antibiotic in mg + 2 mL solvent,

Stock B: 80mg/L = 500 μ L of stock A + 15.5 mL of solvent;

Stock C: 2.5mg/L = 500 μ L of stock B + 15.5ml of solvent.

The appropriate volume of each solution was then added to the appropriate universal container corresponding to the tested concentrations plus 35 mL of molten MHAll agar. The preparation of the antibiotic dilutions range was performed as follows:

From stock A

1.75 mL of stock A in 35 mL agar = 128 μ g/mL

875 μ L of stock A in 35 mL agar = 64 μ g/mL

437.5 μL of stock A in 35 mL agar = 32 $\mu\text{g}/\text{mL}$

218.7 μL of stock A in 35 mL agar = 16 $\mu\text{g}/\text{mL}$

109.4 μL of stock A in 35 mL agar = 8 $\mu\text{g}/\text{mL}$

From stock B

1.75 mL of stock B in 35 mL agar = 4 $\mu\text{g}/\text{mL}$

875 μL of stock B in 35 mL agar = 2 $\mu\text{g}/\text{mL}$

437.5 μL of stock B in 35 mL agar = 1 $\mu\text{g}/\text{mL}$

218.7 μL of stock B in 35 mL agar = 0.5 $\mu\text{g}/\text{mL}$

109.4 μL of stock B in 35 mL agar = 0.25 $\mu\text{g}/\text{mL}$

From stock C

1.75 mL of stock C in 35 mL agar = 0.125 $\mu\text{g}/\text{mL}$

Clavulanate (2mg/mL), tazobactam (4mg/mL) and avibactam (4mg/mL) were added after preparing a solution of each using sterile water and then adding 175 μL to amoxicillin, piperacillin and aztreonam solutions (just before pouring the MHAII agar into the corresponding plates). Glucose-6-phosphate was added the agar media complemented with fosfomycin (2.5 mg/mL Glucose-6-phosphate in 500 mL MHAII molten agar, just before pouring the MHAII agar into the corresponding plates). The MHAII agar plus antibiotic solution and antibiotic/combinations were poured into the corresponding plate and left to set for 20 min. Afterwards, the set plates were dried in a drying cabinet (maximum temperature) for 20 min.

K. pneumoniae isolates were plated onto V media and incubated overnight at 37°C. After confirming the purity of the isolates overnight plate growth, a colony from the overnight growth was suspended in sterile saline (0.85% NaCl w/v) to the density of a MacFarland 0.5 turbidity standard (approximately corresponding to $1-2 \times 10^8$ CFU/mL for *E. coli*). After confirming the purity of the isolates overnight V plate growth. The bacterial suspensions were diluted 10x in 48 sterile well-plate prior to inoculation onto each antibiotic agar plate using a multipoint inoculator attaching a sterilised pinhead. 180 μL of sterile saline (0.85% NaCl w/v) plus 20 μL of the 0.5 McFarland bacterial suspension was added into each well of the 48 sterile well-plate used as the multipoint inoculator template. Each well corresponded to a single isolate. Using a multipoint inoculator allowed to test the wide panel of antibiotics and antibiotic/combinations and a higher number of isolates each time an experiment was

performed enabling faster testing of the isolates in study. After inoculation plates were left facing upwards on the bench for 30 min to allow the inoculation spots to dry then the plates were inverted and incubated overnight at 37°C.

Table 6: Antibiotics used for MIC testing. All antibiotics are grouped by classes and all concentrations used are listed. MIC tests were performed as per EUCAST v.9 2019 guidelines (140) and breakpoints. CLSI M100 Ed29:2019 breakpoints (141) were used to interpret aztreonam/avibactam MIC results.

Antibiotic class	Antibiotics tested	Concentrations tested (µg/mL)
Penicillins	Ampicillin	0, 4, 8, 16, 32
	Amoxicillin/clavulanate	
	Piperacillin/Tazobactam	
Cephalosporins	Ceftriaxone	0, 0.5, 1, 2, 4
	Cefotaxime	
	Ceftazidime	
	Ceftazidime/Avibactam	
	Cefepime	
Carbapenems	Imipenem	0, 1, 2, 4, 8
	Meropenem	
	Ertapenem	0, 0.25, 0.5, 1, 2
Monobactams	Aztreonam	0, 0.5, 1, 2, 4
	Aztreonam/Avibactam	
Aminoglycosides	Gentamicin	0, 1, 2, 4, 8
	Amikacin	0, 4, 8, 16, 32
	Tobramycin	0, 1, 2, 4, 8
Tetracyclines	Tigecycline	0, 0.5, 1, 2, 4
	Minocycline	
Fluoroquinolones	Levofloxacin	0, 0.5, 1, 2, 4
	Ciprofloxacin	0, 0.125, 0.5, 1, 2
Other agents	Colistin	0, 1, 2, 4, 8
	Fosfomycin	0, 16, 32, 64, 128

4.2 Whole Genome Sequencing

4.2.1 DNA extraction and quantification

For gDNA extraction a colony of each isolate was grown in 1.8 mL of LB broth and incubated at 37°C, 140 rpm for 18h. DNA extraction was performed using the Qiagen QIAmp DNA mini kit (Qiagen, Germany), with an additional RNase (10 mg/L) treatment, on the automated QIAcube platform (Qiagen, Germany). DNA quantification was performed using the Qubit fluorometer 3.0 with the dsDNA HS assay kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions.

4.2.3 Preparation of genomic libraries and Sequencing

Genomic libraries were prepared using the Nextera® XT V2 DNA Library Prep Kit, (Illumina,USA).

Firstly, gDNA concentration of all samples were normalised to 0.2 ng/µg. Once the gDNA was normalised it was 'tagmented' before PCR amplification. Amplification was performed using a program of 12 cycles consisting of denaturation at 95°C for 10s, annealing at 55°C for 30s and extension at 72°C for 30s, after an initial denaturation at 95°C for 30s minutes. Final extension was performed at 72°C for 5 min. Subsequently, the amplified library gDNA was purified through bead-based normalisation using AMPure XP beads (Beckman Coulter, UK) in a ratio of 3:2 to remove shorter than 250 bp library fragments and longer than 1500 bp using the bead based normalisation protocol within the Nextera XT v2 kit, to ensure equal representation. Following quantity normalisation, DNA was eluted into a single stranded library using 0.1 NaOH, as per manufacturer's instructions.

Lastly, diluted pooled libraries were loaded into the MiSeq cartridge for pair-ended WGS on an Illumina MiSeq platform (Illumina,USA). For each sequencing run 48 isolates were multiplexed to provide depth coverage of >15x. V3 chemistry was used to generate fragment lengths up to 300bp (600 cycles).

4.3 Bioinformatic analysis

Bioinformatics analysis was performed using a high-performance computing cluster at Cardiff University (ARRCA). The pair-ended fastq files generated by the MiSeq System were analysed using several software programs. This included software used for quality control checks before proceeding to further analysis focusing on phylogeny, resistome and virulome. A schematic of the bioinformatic analysis described in this chapter is included in Appendix 8. All parameters used for each software program used in this study are listed in Appendix 9 and only parameters that deviated from the default settings are described here. All phylogenetic trees were annotated and visualised using iTOL (142), and heatmaps were generated using Morpheus (Morpheus, <https://software.broadinstitute.org/morpheus>). These tools were used because they provided a user friendly and online accessible platform. iTOL allows the display, annotation directly from Microsoft Excel spreadsheets and management of phylogenetic trees. Morpheus also allows the upload of Microsoft Excel spreadsheets to enable viewing of the studied dataset as a matrix.

4.3.1 Sequence quality assessment and processing

For quality control, reports were generated using fastqc (v0.11.8) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) before and after removing the Nextera adapter sequences and low-quality bases by applying Trimgalore (v0.5.0) ([https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ to the pair-ended fastq files](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/to_the_pair-ended_fastq_files)). Per base sequence content GC (scores of over 20), per base sequence quality (showing uniform distribution) and the mean GC percentage distribution (mean of 57%) were used to assess sequence quality of each sequenced isolate. SPAdes (v3.12)(143) was used to assemble the trimmed pair-ended fastq files into contigs fasta files because this was designed with small single cell genomes in mind. To apply SPAdes the -k command was used to set k-mers lengths for read lengths of 250 bp because V3 chemistry was used during sequencing to allow generation of fragment lengths up to 300bp and the -careful option was used to minimize the number of mismatches. Then, the trimmed fastq files were mapped to the assembled contigs fasta files using BWA (v0.7.15)(144), using the bwa-index and bwa-mem commands in order to index and align the input files. Samtools (v1.3.1)(144) was used by applying the sort and index options to sort the alignments by the leftmost

coordinates and index the generated BAM files. BWA was chosen as the aligner since it allows for the mapping of low divergent genomes to a reference having in mind that the isolates studied in this PhD all belong to the same species. Using Samtools sorted BAM files enabled the extraction of alignments overlapping genomic regions, and indexing enabled the use of IGV genome viewer. Pilon (v1.2.3)(145) was used to identify inconsistencies between the assembled contigs files and the evidence in the alignment BAM files to improve and generate the final genome assemblies in fasta format specifying mindepth 0.5 in order to call variants if there was coverage at this depth. Both the assembled contigs and the final genome assemblies' fasta files generated for each isolate were assessed for quality using quast (v5.0.2)(146). To pass quality control assembled contigs/genomes had to display typical *K. pneumoniae* CG content of 57%, a total genome size in the range of 5 to 6.5Mb. The resulting alignment BAM files for each sequencing run were used to assess mean coverage of over 15x using Qualimap (v2.2.1)(147).

4.3.2 Genome annotation and typing

All final genome assemblies were annotated using Prokka (v1.14.0)(148) specifying the genus *Klebsiella*. Prokka was used for annotation because it allows rapid annotation of genomes using a set of different prediction tools to identify genomics features when provided with sequences generated by *de novo* assembly software such as SPAdes (143). Kleborate (v0.2.0)(91, 92) was used for species identification (using the `-species` command), MLST and for characterisation of key acquired ARG, including acquired ARG, SNPs and gene truncations (specifying the `-resistance` command which also turns on screening for chromosomal mutations associated with clinical resistance in KpSC) and virulence determinants such as the *iro*, *iuc* and *rmpA/rmpA2* loci. O:K locus profiles were determined using Kaptive via *wzc* and *wzi* alleles typing (90, 149) (<https://github.com/katholt/Kaptive>). Kleborate and Kaptive were chosen to characterise the genomes studied in my PhD because these are tools developed to specifically screen *K. pneumoniae* genomes and KpSC species. Plasmid replicon content was characterised using Abricate (Seemann T, Abricate, Github <https://github.com/tseemann/abricate>) to search the associated Plasmidfinder database (150). By using Abricate it was possible to screen the large number of contigs from the total genomes studied because this program allows to search its associated databases as well as the users' own database.

4.3.3 Phylogenetic analysis

To assess the relatedness between isolates Roary (v3.12.0)(151) was used to create a core genome alignment. The annotated gff3 files of each isolate genome (output from Prokka) were used as input to create a pangenome and the option `-e` was activated to create a multifasta alignment of the core genes. Roary rapidly builds large-scale pan genomes of large datasets of prokaryotic genomes from the same species identifying core and accessory genomes thus being chosen to analyse this PhD dataset (151). Raxml (v8.2.9) (152) was used to generate a preliminary maximum likelihood phylogenetic trees because it is capable to produce trees with good likelihood scores when analysing large datasets. The phylogenetic trees obtained through Raxml (v8.2.9)(152) were used to generate final maximum likelihood phylogenetic trees using ClonalFrameML (v1.11)(153) to account for recombination during phylogenetic reconstruction seeing that this is an important feature for diversity

in bacteria. Thus, providing a more accurate phylogeny of the isolates studied in my PhD. For global context phylogenetic analysis of *K. pneumoniae* a strain collection was built by downloading *K. pneumoniae* sequences available online from other studies including Lam et al. 2018 (92) and David et al. 2019 (24) and from other BARNARDS clinical sites (Appendix 4). These strains were analysed as described above before phylogenetic analysis.

SNP analysis

SNP analysis was performed using *ska* (v1.0)(154), *snippy* (v4.4.5)(155) and *Gubbins* (v2.3.4)(156). *ska* was used in order to choose a reference genome to generate a core SNP phylogeny for major ST groups. The *ska* fasta command was used to obtain the split kmer files needed as input in order to determine a reference for each SNP phylogeny corresponding each ST. The mash-like distance between each *K. pneumoniae* isolate belonging to each major ST was calculated, applying the *ska* distance command inputting the split kmer files. The reference genome was chosen based on having on average the lowest mash-like distance between itself and the other isolates. An internal reference from the PhD dataset was chosen because these genomes would have more similarities and would be a better representation of the *K. pneumoniae* population found in PIMS and St Paul's hospital (157). With this in mind *ska* (v1.0)(154) was used because it is able to analyse genome sequence data from closely related prokaryotic genomes through split kmers allowing for the comparison of the studied genomes.

Using *snippy*(v4.4.5)(155) a SNP phylogeny was generated by obtaining a whole genome SNP alignment by applying the *snippy-core* command after calling the SNPs of each *K. pneumoniae* genome sequence using the corresponding reference. The *snippy-clean_full_aln* command was applied to the whole genome SNP alignment obtained to remove low coverage characters, before it was passed through *Gubbins* to remove recombination. For SNP analysis *Gubbins* was chosen instead of *ClonalFrameML* because it only uses SNPs outside the regions of recombination for phylogenetic reconstruction. Then, *snp-sites* was applied to the multi-fasta core SNP alignment generated by *Gubbins* to extract the SNPs. This core SNP alignment was then used to generate the SNP phylogenetic trees using *Raxml* and *pairsnp*

(v0.0.7;<https://github.com/gtonkinhill/pairsnp>) was used to generate pairwise SNP matrixes applying default settings.

4.3.4 Plasmid content analysis and plasmid scaffold analysis

To analyse the dissemination of plasmids carrying *bla*_{NDM-1}, *bla*_{OXA-181} and *bla*_{CTX-M-15} plasmid scaffolds sequences harbouring these genes were obtained from long read sequences of ST37 ES-BB30 and ST15 PP-BB8. These were obtained by Oxford Nanopore Technology sequencing and assembly against the short reads using Unicycler (v0.4.7)(158). This work was performed by Dr Kirsty Sands at Cardiff University for the BARNARDS project. From each representative genome a complete plasmid sequence was obtained and analysed during this PhD study. To select the plasmid sequences only the long read sequences of ST37 ES-BB30 and ST15 PP-BB8 gfa files were visualised using Bandage (159) and contigs sequences corresponding to plasmids were saved as individual fasta files using the output menu option of save selected node sequences to FASTA. Afterwards, all reference plasmids were typed for ARG and annotated as described above using Abricate (Seemann T, Abricate, Github <https://github.com/tseemann/abricate>) to search the plasmidfinder (150) and resfinder associated databases. Reference plasmids were annotated using SnapGene software (from Insightful Science; available at snapgene.com) using transfer of annotated reference sequences downloaded from NCBI.

Screening *K. pneumoniae* genomes for this PhD reference plasmids

Plasmid sequences used as references within this PhD were formatted as databases. The databases were generated using BLAST+ (v.2.7.1)(160) applying the `makeblastdb` command, and Abricate was used to screen the short sequences of all ES *K. pneumoniae* and PP *K. pneumoniae* specifying these plasmid databases. BLAST+ (v.2.7.1)(160) was used to generate the reference plasmid databases because the database format was appropriate to use with Abricate (Seemann T, Abricate, Github <https://github.com/tseemann/abricate>). By using the `-db` command Abricate (Seemann T, Abricate, Github <https://github.com/tseemann/abricate>) allows to search a specific associated database.

Mapping reference plasmids to ES and PP *K. pneumoniae* putative plasmid scaffolds

Putative plasmid scaffolds were generated from the short read sequences of all ST35 ST37 and ST218 ES *K. pneumoniae* and ST15 *K. pneumoniae* using SPAdes plasmidSpades.py script using as input the trimmed pair-ended fastq files corresponding to each isolate in study (161). The reference plasmids were used as plasmid scaffolds for mapping short reads of ST35 ST37, ST218 ES *K. pneumoniae* and ST15 PP *K. pneumoniae*. Alignments were generated using BWA (v.0.7.15)(144) and samtools (v1.3.1)(144) as described in 4.3.1, using the reference plasmid scaffolds as the reference. Resulting alignments were visualised using IGV (v2.8.13)(162), a tool for viewing of genomic data including alignments and variants. The alignments were generated for ES and PP *K. pneumoniae* according to sample type of origin to determine whether plasmid similarity across the clusters was present and then visualised in IGV (v2.8.13)(162). To view the generated alignments the BAM/SAM files obtained from BWA (v.0.7.15)(144) and samtools (v1.3.1)(144) mapping were used as input for IGV(v2.8.13)(162). The default parameters of allow to visualise alignments at low coverage depth and allowed to view the alignments obtained. Also the displayed coverage track by IGV (v2.8.13)(162) enabled the assessment of greater than 20% nucleotide differences between the reference plasmid and the isolates' putative plasmid scaffolds.

4.4 Statistical analysis

Microsoft Excel and IBM SPSS Statistics 25 were used for statistical analysis of results from this PhD study. Microsoft Excel was used to calculate all frequencies in percentages and correspondent graphs as seen in 2. The Pearson's chi-square statistical tests and Kaplan-Meier statistics were applied using IBM SPSS Statistics 25. For the Pearson's chi-square statistical tests were considered statistically significant with a $p < 0.05$ result and for Kaplan-Meier statistics a 95% confidence interval was considered, $p < 0.05$.

5. Results – *K. pneumoniae* from St Paul's Hospital, Ethiopia

5.1 Phylogeny and population of ES *K. pneumoniae*

The relatedness of *K. pneumoniae* recovered from different sample types collected at St Paul's Hospital (n=191;100%) was studied in relation to their phylogeny and population (Appendix 4 includes a description of the ES *K. pneumoniae* population, Appendix 11 includes Supplementary Fig. 1 displaying the STs found in total). Studying the total ES *K. pneumoniae* population allowed for greater understanding of the relatedness of ES-BB isolates in relation to the other *K. pneumoniae* (MR, BR and ENV) (Fig. 11). The pangenome of the ES *K. pneumoniae* population was constituted by 1895 core genes (found in 99% to 100% of the isolates) and 57 119 accessory genes in a total of 59 014 genes. Whilst the *K. pneumoniae* population collected from samples from St Paul's hospital are diverse with 39 STs identified in total (Appendix 11 includes Supplementary Fig. 1 displaying the STs found in total). Within this section, I will focus on assessing genomic relatedness within the four most dominant STs; ST35 (n=35;18%), ST37 (n=35;18%), ST218 (n=22;11.5%) and ST45 (n=14;7%).

ST35 (total n=35;100%, BB n=33;94% ENV n=2;6%) and ST37 (total n=35;100%, BB n=27; 77%, BR n=5;14%, MR n=2;6%, ENV n=1;3%) were the two largest STs found from Ethiopia, followed by two smaller clusters composed of ST218 (n=22;11.5%) and ST45 (n=14;7%) *K. pneumoniae*. ST35, ST37 and ST218 *K. pneumoniae* were recovered from patients and hospital surfaces from St Paul's Hospital between May and December 2017. In beginning of May 2017, the first ST35 ES-BB *K. pneumoniae* was isolated and in the end of May the first ST218 ES-BB isolate was recovered (Fig. 13). The first ST37 ES-BB was isolated in the end of July 2017. However, most *K. pneumoniae* isolates of these three STs (n=72) were obtained between the 24th of September and the 1st of November 2017 (Fig. 13). During these months, 15 (43%) ST35 ES-BB and two (6%) ST35 ES-ENV were taken in October 2017. ST37 ES-BB (n=12;34%), three (8.5%) ES-MR and five (14%) ST37 ES-BR samples were also taken in October 2017 with three (8.5%) ES-BB samples recovered in the beginning of November. However, ST218 samples (n=19;86%) were recovered between May (one;4.5% ES-BB), June (four;18% ES-BR and one;4.5% ES-MR), August (five;23% ES-BB and two;9% ES-ENV) and September 2017 (two;9% ES-BB, one;4.5% ES-BR and two;9% ES-MR). The sampling dates of one (3%) ST37 ES-BR

sample one (4.5%) ST218 ES-BB and two (9%) ST218 ES-BR samples were not determined. Fig. 11 summarises the most common virulence and resistance ARG carried by all ES *K. pneumoniae* isolates highlighting ST35, ST37, ST218 and ST45. Additionally, Fig. 12 displays core genome phylogenies for ST35, ST37, ST218 and ST45 *K. pneumoniae*. *K. pneumoniae* belonging to different clusters showed differences in virulence markers for example ST218 *K. pneumoniae* all harboured *ybt*, *iuc* and *iro* loci and ST45 carried *ybt* loci, while only one ST37 (ES-BR42 found in 10/10/2017) carried *ybt* and ST35 did not carry these loci. In relation to surface polysaccharides loci ST35 showed in majority O1v2 and all displayed KL108 while all ST37 *K. pneumoniae* displayed O4 and KL15 where ST218 distinctly harboured O2v2 and KL57. When applying a chi-square statistical test to understand if the KL and OL were associated with the dominant STs, KL108 ($p < 0.001$), KL15 ($p < 0.001$) and KL57 ($p < 0.001$) were significantly associated with ST35, ST37 and ST218 respectively. Likewise, the OL showed by ST35 (O1v2, $p < 0.01$), ST37 (O4, $p < 0.01$) and ST218 (O2v2, $p < 0.01$) were significantly associated.

Considering aminoglycosides (Agly) ARG most ES *K. pneumoniae* carried *aac* ARG but also carried *aad* and *aph* including ES ST35, ST37 and ST218. However, ST35 and ST218 isolates did not carry *aph* ARG. In addition, isolates belonging to ST35, ST37 and ST218 also carried *tet* and fluoroquinolone (Flq) ARG. ST35, ST37 and ST218 carried *tet* ARG, however, ST35 and ST218 isolates did not carry Flq ARG. The resistome of ES *K. pneumoniae* is characterised in 5.2 and 5.2.1.

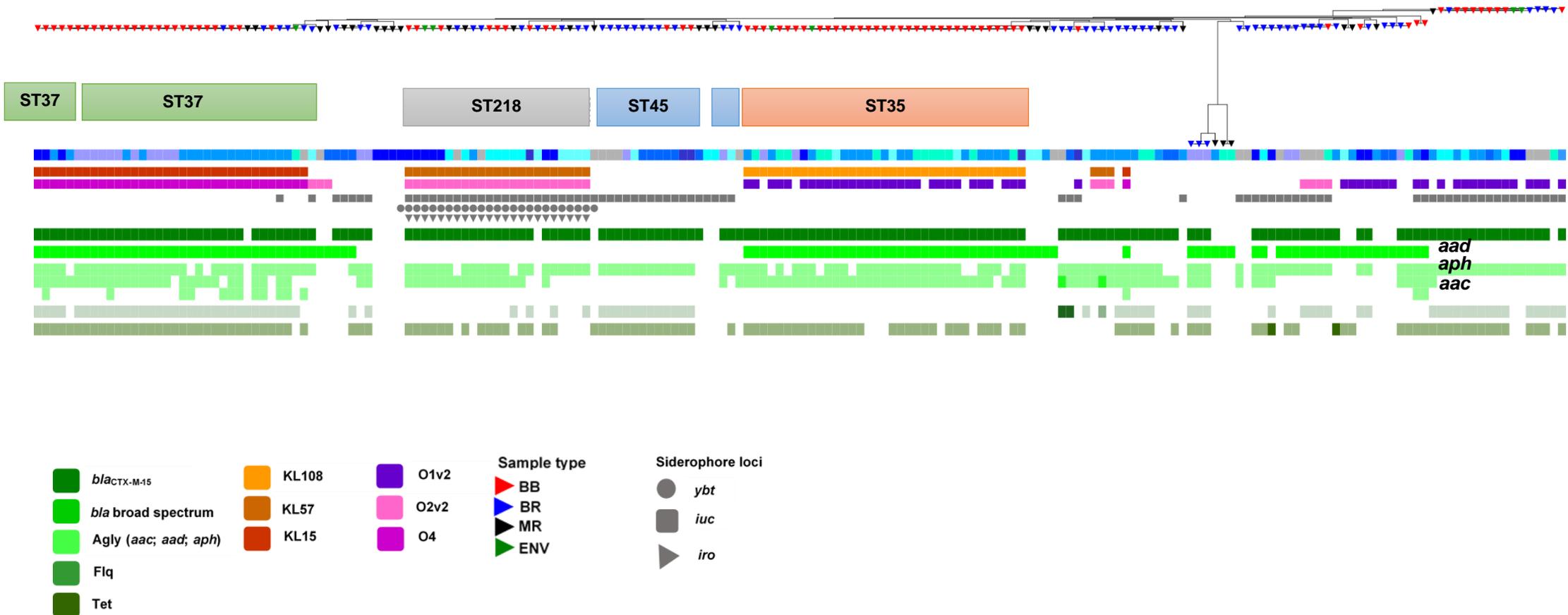


Fig. 11: Core genome maximum likelihood phylogeny of the population of *K. pneumoniae* from Ethiopia. Phylogeny and distribution the most common ARG, KL, and OL and presence of siderophore loci. ST35, ST37 and ST218 formed the largest clusters being the more frequent STs. ES ST37 (n=35), ES ST37 (n=35) and ES ST218 (n=22 followed by ES ST45 (n=14). ES ST35, ST37 and ST218 carried KL and OL detected among the most commonly found. Only ES ST218 *K. pneumoniae* carried *ybt*, *iuc* and *iro* concurrently. ST37 *K. pneumoniae* were found among BB, BR, MR and ENV and ES ST35 isolates among BB and ENV samples. However, BB *K. pneumoniae* were found among clades composed mostly of ST37 and ST35 BB isolates. ST35 BB *K. pneumoniae* belong the same clade, while MR, BR and ENV ST37 isolates belong a different clade within the same cluster. ST218 and ST45 *K. pneumoniae* composed other two clusters. ST218 ES *K. pneumoniae* were recovered from BB, BR, MR and ENV. ST45 *K. pneumoniae* formed the smaller cluster comprised in majority of BR isolates. Sample type of the isolates is also shown as circles BB (red), BR (blue), MR (orange) and ENV (green). Tet indicates the presence of *tet* ARG, Flq the presence of fluoroquinolone ARG, Agly the presence of aminoglycoside ARG. *bla* broad spectrum indicates the presence of broad spectrum β -lactamases ARG, and *bla*_{CTX-M-15} the presence of *bla*_{CTX-M-15} ARG

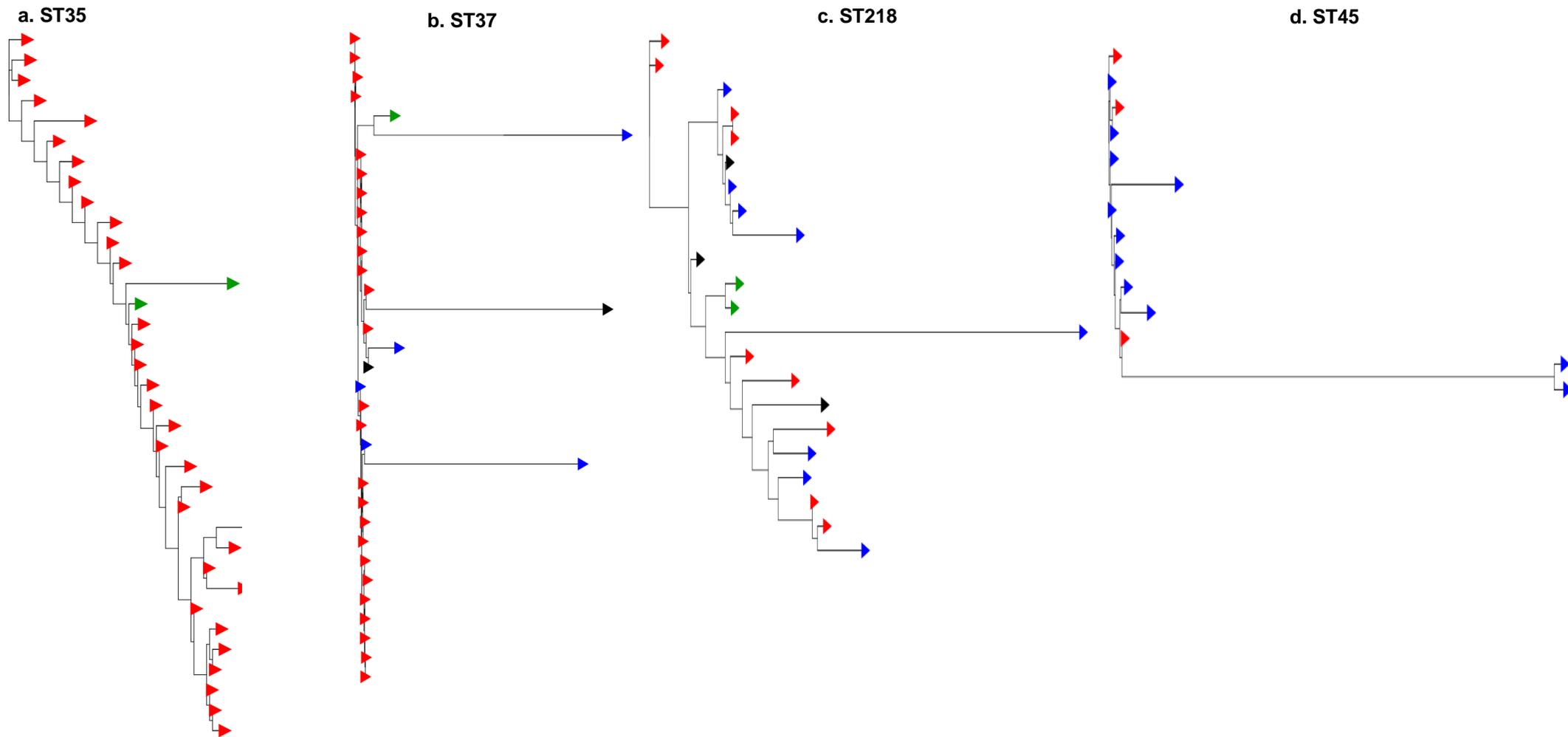


Fig. 12: Core genome maximum likelihood phylogeny of (a.) ST35 ES *K. pneumoniae* were phylogenetically similar when considering ES-BB isolates and one ST35 ES-ENV indicative that these isolates spread as outbreak among primarily neonates but could have disseminate through the clinical environment too **(b.)** Among ES ST37 *K. pneumoniae* ES-BB were markedly phylogenetically similar among ES-BB but ES-BR and ES-MR *K. pneumoniae* could have affected the spread of these isolates as these were among phylogenetically similar isolates to ST37 ES-BB as well. Despite that other ST37 ES-BR, ES-ENV and ES-MR were also found to diverge. **(c.)** ST218 were shown not to be as phylogenetically similar as ST35 and ST37 ES *K. pneumoniae* clusters. Particularly ST218 ES *K. pneumoniae* which of sample type of origin were more varied than ST35 **(d.)** ST45 ES *K. pneumoniae* were in majority phylogenetically similar with the exception of two BR isolates that were shown to diverge from the majority of ES ST45 isolates. *K. pneumoniae* isolates sample type of origin is indicated by the circles red (BB), blue (BR), orange (MR) and green (ENV).

SNP analysis of dominant ES ST groups

SNP analysis was performed for ST37, ST35 and ST218 *K. pneumoniae* isolates to evaluate the relatedness of these isolates and to determine whether transmission events occurred between the mother, neonate and the clinical environment (Fig. 15, Fig. 14 and Fig. 17 respectively).

By constructing a timeline of BARNARDS sampling (Fig. 13) it was possible to assess whether transmission events were likely between the clinical environment and mother/neonate, and whether clinical epidemiology data supports the hypothesis for vertical transmission between the mother and her neonate. As shown in Fig. 13 it is noticeable that *K. pneumoniae* belonging to ST37, ST35 and ST218 were recovered predominantly during the months of August and September 2017. Among the most common STs, ST218 isolates were often found during August and September and from the beginning of October until the end of December 2017. ST35 and ST37 were the lineages more commonly found (Fig. 13). Specifically, ST37 was most frequently found between October and November 2017 with ST35 *K. pneumoniae* being the most common during December 2017 (Fig. 13). To perform the SNP analysis of ES ST35, ST37 and ST218 as well as of PP ST15 *K. pneumoniae* appropriate reference genomes from each ST group were selected within ES and PP *K. pneumoniae* isolates sequenced within my PhD. To choose each reference genome the pairwise distance between each *K. pneumoniae* isolate belonging to each major ST was calculated and each reference genome was chosen by having in average the lowest mash-like distance between itself and the other isolates.

SNP analysis of ST35

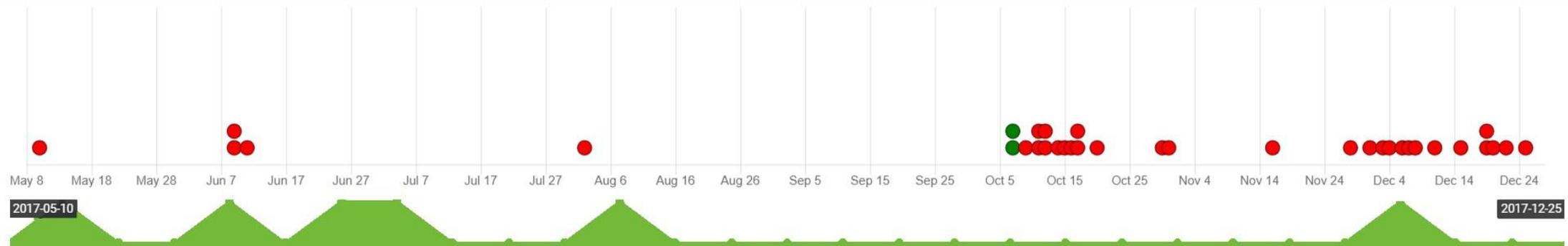
Among ST35 ES isolates (n=35) the lowest number of pairwise SNPs was zero, displayed by six (17%) ST35 ES-BB isolates recovered on December 2017 and the highest number of pairwise SNPs was 10, displayed by ES-ENV3 recovered on 7/10/2017 (Fig. 14)(Fig. 13b). ST35 ES-BB isolates showing a difference of one SNP (n=9;26%) included eight (23%) BB isolates and one (3%) ENV isolate (ES-ENV4) recovered on October, November and December 2017. Eight (23%) ST35 ES-BB showed two pairwise SNP difference in relation to the reference (ES-BB31 recovered in October 2017) and these recovered November and December 2017. With five (14%) and four (11%) ST35 ES-BB isolates showing three (recovered on June and October

2017) and four pairwise SNPs difference (recovered in May, June and October 2017), respectively. These results therefore suggested that most ST35 isolates (n=33;94%, 32 (91%) ES-BB and one (3%) ES-ENV) showed between zero and four pairwise SNPs in relation to the internal reference suggesting that these isolates were very closely related and responsible for an outbreak in St Paul's Hospital. Particularly between the months of October and December 2017 when ST35 ES-BB isolates showing zero (n=6;17%), one (n=9;26%), two (n=7;20%), three (n=3;8.5%) and four (n=2;6%) pairwise SNPs difference were more commonly recovered. Furthermore, having in mind that closely related ST35 ES-BB isolates were recovered in May and July 2017 suggested that ST35 *K. pneumoniae* isolates that later on caused an outbreak likely emerged in earlier in the year. Due to sporadic sampling however, it was not possible to ascertain if these isolates could have spread from the clinical environment seeing that only ST35 ES-ENV4 (recovered in October 2017) was shown to be phylogenetically similar to ST35 ES-BB isolates. ST35 ($p < 0.001$) was found to be significantly associated with BB sample type when applying the chi-square statistical test further supporting ST35 as one of the main agents of sepsis caused by *K. pneumoniae* at St Paul's Hospital.

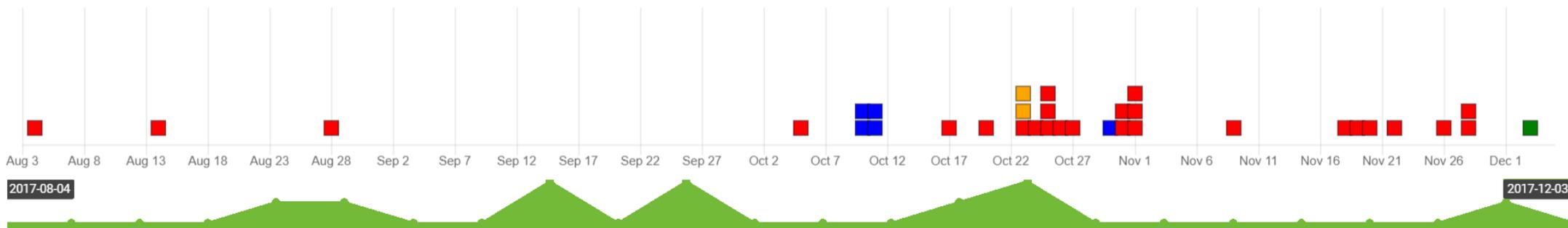
a. *ES K. pneumoniae* ST35 (circle), ST37 (square) and ST218 (triangle) Timeline including BB (red), MR (orange) and ENV (green)



b. *ES K. pneumoniae* ST35 (circle) Timeline including BB (red) and ENV (green)



c. ES *K. pneumoniae* ST37 (square) Timeline including BB (red), MR (orange) and ENV (blue)



d. ES *K. pneumoniae* ST218 (triangle) Timeline including BB (red), MR (orange) and ENV (green)



Fig. 13: Timeline of ES *K. pneumoniae* belonging to ST35, ST37 and ST218 across the different sample types of origin from May 2017 to December 2017 at St Paul’s Hospital, Ethiopia. **a.** Timeline of ES *K. pneumoniae* ST35, ST37 and ST218 isolates recovered between May and December 2017. These isolates belonged BB, BR, MR and ENV samples types of origin. In May 2017, the first BB *K. pneumoniae* were isolated these belonged to ST35 and ST218. ST35 ES-BB *K. pneumoniae* and ST218 ES-BR and MR isolates were then recovered in June 2017. *K. pneumoniae* isolates were then only recovered from the end of July 2017 with ST35 ES-BB, ST37 ES-BB and ST218 ES-BB and ENV being isolated. In September 2017 *K. pneumoniae* isolates were recovered again. From the end of September, these were ST218 isolates from BB, BR and MR samples, in October 2017 *K. pneumoniae* isolates recovered included ES *K. pneumoniae* belonging to ST35 (BB and ENV), ST37 (BB, BR and MR) and ST218 (BR). In November 2017 only ES-BB ST35 and ES-BB and ENV ST37 *K. pneumoniae* were recovered. **b.** displays the timeline of ST35 *K. pneumoniae* only. **c.** displays the timeline of ST37 *K. pneumoniae*. **d.** displays the timeline of ST218 *K. pneumoniae*.

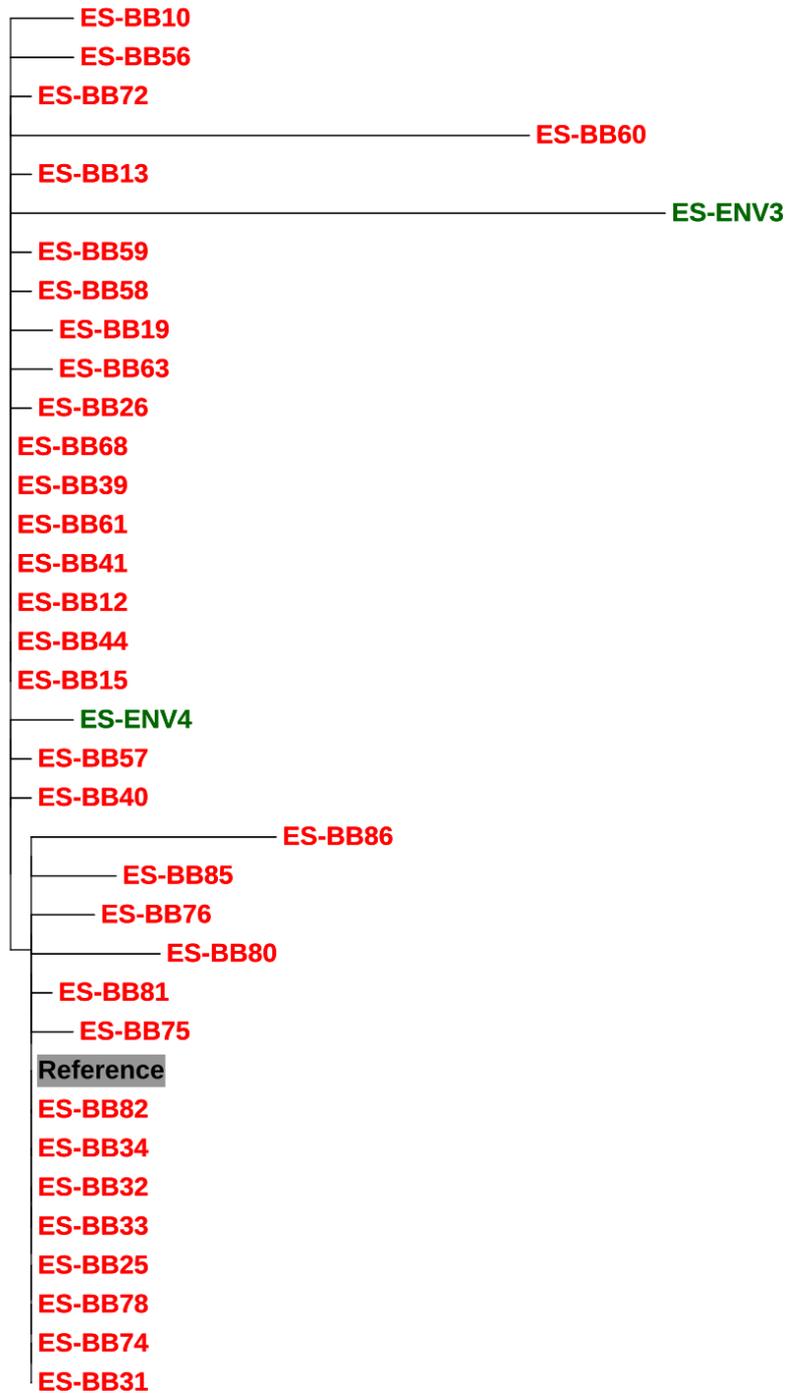


Fig. 14: SNP analysis of ST35 *K. pneumoniae* from Ethiopia. ES ST35 *K. pneumoniae* formed two clusters. ES-BB ST35 belonging to both clusters were phylogenetically similar. One cluster included ES-BB31 that was used as reference to which most ES-BB were phylogenetically similar. Also, One ST35 ES-ENV was phylogenetically similar to ES-BB isolates belonged a different cluster. Two ST35 ES *K. pneumoniae* diverged being less similar to other isolates these were ES-BB60 and ES-ENV3.

SNP analysis of ST37

ST37 ES isolates showed between 0 and 42 pairwise SNPs difference in relation to the reference ES-BB22 recovered in November 2017 (Fig. 15). The lowest number of pairwise SNPs was showed by ST37 ES-BB24, ES-BB29, ES-BB73 that were also recovered in November 2017. The highest number was shown by three (8.5%) isolates ES-MR3 (SNPs=42) recovered on the 23/10/2017, ES-BR2 (SNPs=42) recovered in 11/10/ 2017 and ES-BR57 (SNPs=14) showed the highest differences in relation to the reference (Fig. 15)(Fig. 13c). However, among ST37 ES-BB only, ES-BB16, ES-BB20, ES-BB54, ES-BB71 showed one pairwise SNP in relation to the reference, with the other ST37 ES-BB (n=22;100%) isolates displaying between two and five pairwise SNPs. Among these most ST37 ES-BB (n=7;32%) isolates, recovered in October (n=5;23%) and November 2017 (n=2;9%), showed two pairwise SNPs difference (Fig. 13c). Likewise, seven (32%) ES-BB showed three pairwise SNPs difference and were recovered in August (n=1;4.5%), October (n=3;14%) and November (n=3,14%) 2017. Two (9%) ST37 ES-BB isolates, collected in October 2017, had four pairwise SNPs difference and two (9%) ST37 ES-BB isolates recovered in August 2017 showed five pairwise SNPs difference (Fig. 13c). Regarding ST37 ES-BR isolates ES-BR1 and ES-BR43 recovered in October 2017 showed nine and two pairwise SNPs difference in relation to the reference. Together this data suggested that ST37 ES-BB (n=26;74%) and ES-BR (n=2;6%) *K. pneumoniae* were phylogenetically similar and that ST37 *K. pneumoniae* was another source of an outbreak in St Paul's Hospital, specifically during October and November 2017 (Fig. 13). ST37 was significantly associated with BB ($p<0.001$) and ST37 isolates were also significantly associated with BR samples ($p=0.012$), further indicating that this ST was one of the main agents of sepsis among ES *K. pneumoniae* and that the neonates' microbiota could be an important reservoir of these isolates.

With SNP analysis indicating that two (6%) ST37 ES-BR were very closely related to the ES-BB isolates it would be plausible to hypothesise that ST37 *K. pneumoniae* are colonising the neonates' gut microbiota prior to causing to infection. ST37 ES-MR isolates, ES-MR3 and ES-MR30 were recovered during October 2017 with 32 and four pairwise SNPs difference in relation to the

reference, respectively. These results indicated that ES-MR30 was closely related to ES-BB22, the reference. In fact, ST37 ES-MR30 and ES-MR3 and ES-BB17 *K. pneumoniae* isolates correspond to a mother and her neonate who developed sepsis. Analysing the ES-BB17, ES-MR3 and ES-MR30 isolates pairwise SNPs in relation to ES-BB17 showed that ES-MR30 (SNPs=2) was closely related to ES-BB17 but ES-MR3 (SNPs=596) was not. Furthermore, the timeline of recovery of ES-BB17 and ES-MR30 pair samples shown in Fig. 16 indicated that these isolates were recovered from samples collected two days apart. Hence, transmission between mother and the neonate could have been possible when considering ES-BB17 and ES-MR30.

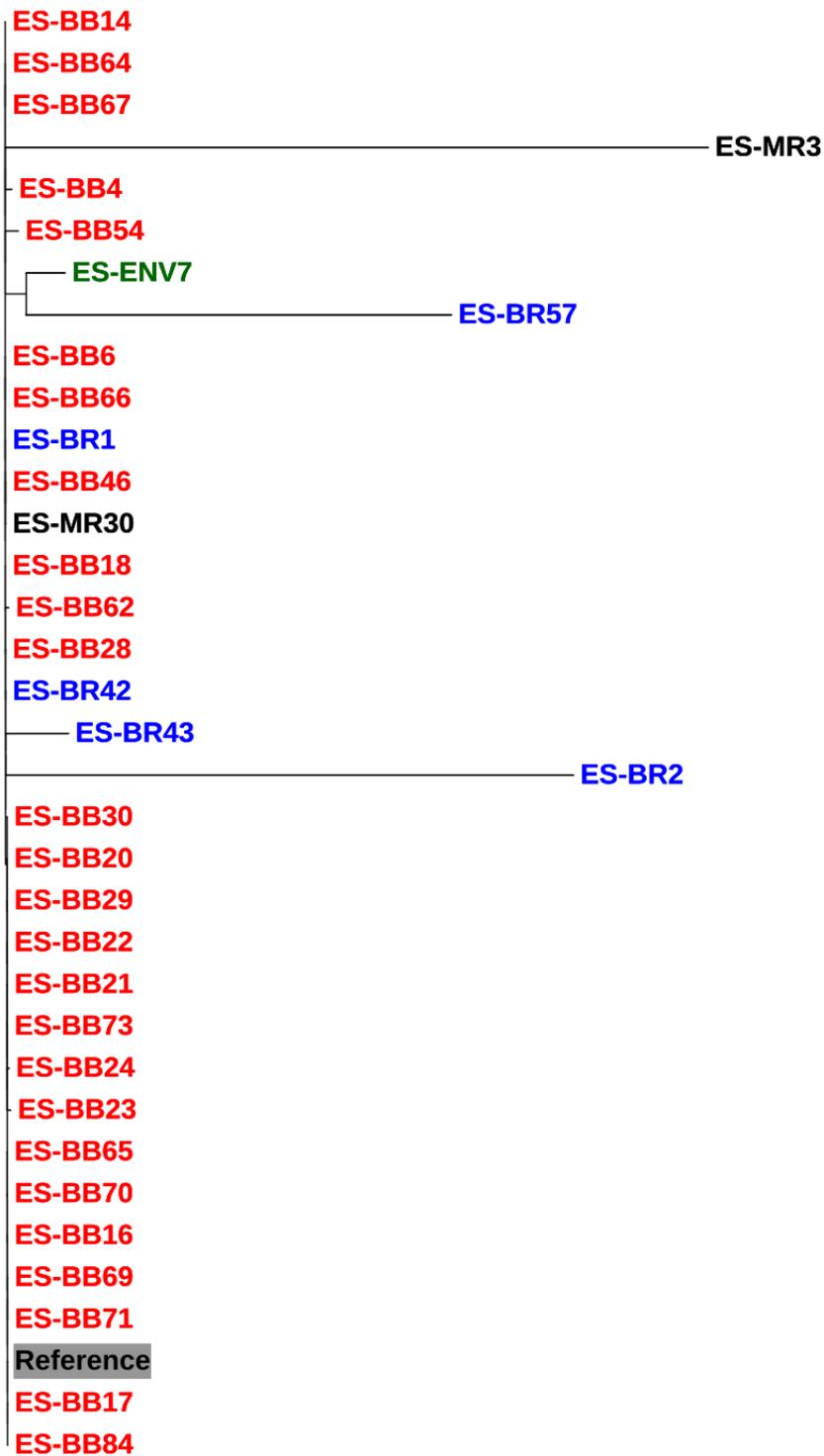


Fig. 15: SNP analysis of ST37 *K. pneumoniae* from Ethiopia. ES ST37 *K. pneumoniae* formed one cluster in which most isolates were phylogenetically similar among ES-BB, ES-BR, ES-MR isolates with four isolates (ES-MR3, ES-BR57, ES-BR2 and ES-ENV7) diverging. ES-BB22 was used as reference.

SNP analysis of ST218

Regarding ST218 *K. pneumoniae* (n=22;100%) the lowest number of pairwise SNPs in relation to the reference (ES-BB27) was 20 showed by ES-BB5 and the highest number was shown by ES-BR20 (SNPs=138) (Fig. 17). All isolates were recovered in August or October 2017 (Fig. 13d). Among ST218 ES-BB isolates ES-BB5 (SNPs=20) and ES-BB45 (SNPs=21) showed the lowest pairwise SNP differences and were recovered in August 2017. Other ST218 ES-BB isolates displayed between 26,30,33 and 36 SNPs. With ES-BB49 (SNPs=42) and ES-BB51 (SNPs=48) SNPs difference indicating that these were less similar in comparison to the other ES-BB. Considering ST218 ES-BR (n=8;100%), four (50%) isolates recovered in June 2017 these included ES-BR65 (SNPs=30), ES-BR66 (SNPs=40), ES-BR67 (SNPs=37), ES-BR68 (SNPs=47) that were recovered from the same BR sample and the corresponding ES-MR sample was also collected. From the MR sample it was possible to recover the ST218 ES-MR16 *K. pneumoniae* isolate, that showed 29 pairwise SNPs difference. Similarly to the ST37 case of mother-neonate (ES-MR30 and ES-BB17), ST218 ES-MR16 and the corresponding ES-BR isolates from the same neonate (ES-BR65, ES-BR66, ES-BR67 and ES-BR68) were analysed together. In relation to ES-MR16 the pairwise SNPs difference of ES-BR65 (SNPs=2), ES-BR66 (SNPs=16), ES-BR67 (SNPs=6), ES-BR68 (SNPs=27) were low in numbers suggesting that transmission between the mother and the neonate could have occurred. Specially, in the case of ES-BR65 and ES-BR67 which showed the lowest number of SNPs and were recovered on the same day Fig. 16 further evidencing possible transmission between mother and neonate. Two (9%) ST218 ES-ENV isolates showed 27 and 33 SNPs and were recovered in August 2017. Considering Fig. 13 these closely related ST218 ENV isolates indicating that the clinical environment could be another source of ST218 *K. pneumoniae* that could cause infection or colonise the neonates' gut microbiota.



Fig. 16: Timeline of the ES ST37 and ES ST218 *K. pneumoniae* pairs cases. In June 2017 ST218 ES-BR66 and ES-MR16 were collected and later in October 2017 ES ST37 ES-BB17 and ES-MR30 were collected.

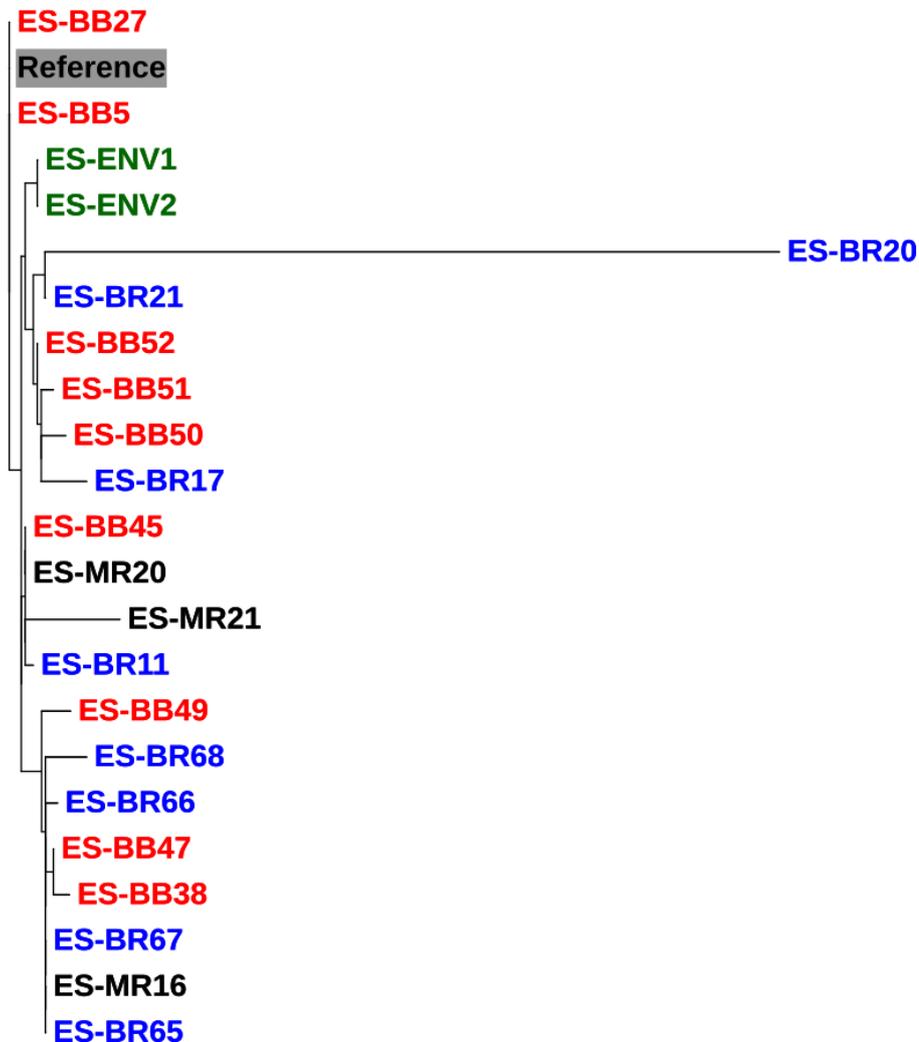


Fig. 17: SNP analysis of ES ST218 *K. pneumoniae* from Ethiopia. ST218 ES *K. pneumoniae* formed three clusters within each isolate were phylogenetically similar though these were diverging. With divergence being seen particularly with ES-BR20 that was less similar to the other ST218 isolates. ES-BB27 was used as a reference.

Phylogeny of *K. pneumoniae* from Ethiopia – discussion

Despite the high rates of sepsis and antibiotic resistance in Africa there is a lack of data regarding the population characterisation of the bacteria causing sepsis in this region of the world (163). *K. pneumoniae* has a marked ability to spread and acquire ARG (38) and is also responsible for causing infections such as sepsis. Furthermore, there are reports of sepsis caused by cephalosporin resistant *K. pneumoniae* from Ethiopia (83, 164) (1.5 Antibiotic resistance in *K. pneumoniae* and 1.6 Burden of antibiotic resistant *K. pneumoniae* in infection). Thus, these results allowed some insight into the population of *K. pneumoniae* recovered from St. Paul's Hospital, Ethiopia in the context of neonatal sepsis. Initial data from the Ethiopian study site revealed *K. pneumoniae* to be the dominant species causing sepsis and therefore during this study I retrospectively analysed corresponding mother/neonatal and clinical environmental samples. Through microbial culture and WGS analysis to match STs, I incorporated isolates from the other sample types (mother/neonatal rectal microbiota and clinical environment) in these core genome SNP analyses.

Isolates belonging to ST35 (n=35) and ST37 (n=35) were responsible for major epidemiological clusters that caused *K. pneumoniae* sepsis in Ethiopia, particularly, between August and September 2017, but also through October 2017. Following discussion with the Ethiopian team it was known that deep cleaning was undertaken at St Paul's Hospital in September 2017, and this could have influenced the recovery of isolates and arguably explains the gap in the timeline (Fig. 13). Clusters of ST35, ST37 and ST218 were not only composed of sepsis causing *K. pneumoniae* (ES-BB) but also from neonatal rectal samples (ES-BR), maternal (ES-MR) and ES-ENV in some cases there is evidence to support the theory of transmission of *K. pneumoniae* between the mother and her neonate or from the clinical environment to the neonate. This was particularly evident in the case of the possible transmission of (a.) a ST37 MR sample (ES-MR30) and ES-BB17 (sepsis isolate) between mother and neonate and (b.) a ST218 MR sample (ES-MR16) and four ES-BR (neonatal rectal sample).

More importantly, seeing that among ST37 and ST218 ES *K. pneumoniae* vertical transmission might have occurred reinforces the importance of not only screening blood isolates but also isolates from other sources such as the mother

and neonate microbiota. Furthermore, ST37 ES-BB17 and ES-MR30 carried *bla*_{CTX-M-15} and ST218 ES-MR16 and the four ST218 ES-BR carried not only *bla*_{CTX-M-15} but also carried *ybt*, *iuc*, *iro* and *rmpA* virulence determinants. This is worrying because it indicated that *K. pneumoniae* can drive both antibiotic resistance and virulence which in turn could cause more difficult to treat infections (18, 86). In contrast most ST35 and ST37 *K. pneumoniae* did not carry siderophore loci but were responsible for most neonatal sepsis cases. The fact that these *K. pneumoniae* carried not only *bla* ARG but also other ARG (5.2 Resistome of ES *K. pneumoniae*) could have contributed to the presence of these isolates during August 2017 until December 2017 and their ability to cause sepsis.

ES *K. pneumoniae* were shown to harbour ARG such as *bla*_{CTX-M-15} that is responsible for resistance for penicillins and cephalosporins (9) which would make these *K. pneumoniae* isolates resistant to treatments including cefotaxime which of concern because such treatments were commonly prescribed in St. Paul's Hospital between August 2017 and December 2017 to treat *K pneumoniae* sepsis. Likewise, because these *K. pneumoniae* harbour *aac*, *aph* and *aad* ARG responsible for resistance to aminoglycosides commonly used for treatment such as gentamicin would make these isolates resistant to treatments that included gentamicin which were prescribed in St Paul's Hospital too (165, 166). These isolates displayed resistance to penicillins and cephalosporins (Fig.18) when tested for antibiotic susceptibility which is in line with these isolates carrying broad spectrum β -lactamases such as *bla*_{SHV-11} and *bla*_{SHV-33} (5.4. Antibiotic susceptibility and ARG among *K. pneumoniae* belonging to ST35; ST37 and ST218). Similarly, these isolates were resistant to gentamicin and tobramycin but susceptible to amikacin possibly due the absence of, for example, *rmt* Agly ARG (5.2 Resistome of ES *K. pneumoniae*). Nevertheless, sepsis caused by ST35 and ST37 *K. pneumoniae* was in majority alive (73% and 65% respectively)

Lastly, knowledge about the capsule polysaccharides, typed here as KL, and liposaccharides, typed here as OL, could provide information for the development of an alternative therapeutic to antibiotics as a vaccine or antibodies, respectively. Vaccines targeting the capsule polysaccharides have been studied before however the high variability of the KL (89) has made development difficult (167). Nevertheless, the characterisation of the KL and OL

profiles could provide information about the most prevalent loci among a *K. pneumoniae* population within a clinical setting and provide a more targeted approach. For example, in this study the most prevalent KL were KL108, KL15 and KL57 displayed and significantly associated to ST35, ST37 and ST218, respectively, could be used as targets for a vaccine (168). On the other hand, OL such as O1v2, O4 and O2v2 were showed by ST35, ST37 and ST218 and could be used as a target for antibody therapeutics (168).

As seen in Fig. 13a less ST37 *K. pneumoniae* isolates diminished by the end of November 2017 indicating that surveillance of KL and OL serotypes would have to be done regularly in order to develop an appropriate vaccine or antibody because the dominant ST seemed to be changing during the year, but, in LMICs such as Ethiopia, performing such studies might be difficult (8.1.2 What was the contribution of plasmids to the spread of ARG among PP and ES).

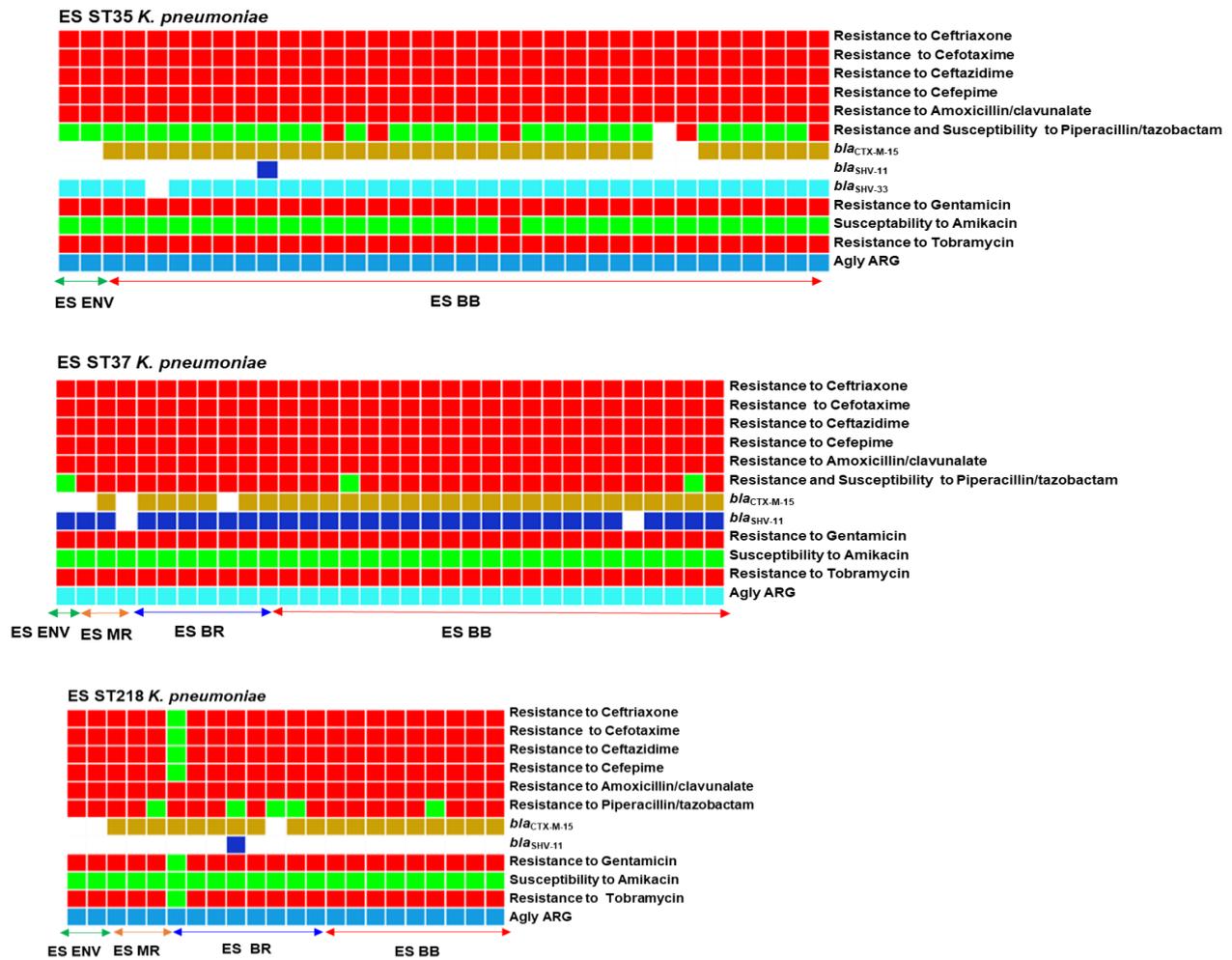


Fig. 18: Antibiotic resistance (red) and susceptibility (green) to cephalosporins (ceftriaxone, cefotaxime, ceftazidime and cefepime), penicillin treatments (amoxicillin/clavulanate and piperacillin/tazobactam) and aminoglycosides (gentamicin, amikacin and tobramycin) along with presence of ARG responsible for resistance to such antibiotic treatments. Broad spectrum β -lactamases such as *bla*_{SHV-11} and *bla*_{SHV-33}, ESBL *bla*_{CTX-M-15} and aminoglycoside (Agly) ARG among ES ST35 *K. pneumoniae* (first heatmap), ES ST37 *K. pneumoniae* (second heatmap) and ES ST218 *K. pneumoniae* (third heatmap).

5.2 Resistome of ES *K. pneumoniae*

The total ES *K. pneumoniae* isolate population (n=191;100%) carried several acquired ARG that confer resistance to β -lactam, aminoglycosides, tetracyclines and fluoroquinolones. SNPs that confer resistance to fluoroquinolones and truncations that confer resistance to β -lactams and to colistin were also detected. Fig. 19 summarises the percentages of the most frequent ARG and resistance conferring mutations.

All ES *K. pneumoniae* carried β -lactam ARG these included *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{OXA}, and *bla*_{TEM} ARG. (Appendix 3; Appendix 10) The most frequent *bla*_{CTX-M} ARG being *bla*_{CTX-M-15} harboured by 163 (85%) ES isolates with one ES *K. pneumoniae* carrying *bla*_{CTX-M-14} and two *bla*_{CTX-M-24}. In relation, to *bla*_{SHV} ARG these included *bla*_{SHV-11} (n=61; 32%) as the most common, followed by *bla*_{SHV-187} harboured by 49 (26%) ES *K. pneumoniae* and, *bla*_{SHV-33} (n=41; 21.5%), *bla*_{SHV-26} (n=17; 9%) and *bla*_{SHV-28} (n=14; 7%). The *bla*_{OXA} ARG found included *bla*_{OXA-1} and *bla*_{OXA-10} with 88 (46%). *bla*_{OXA-1} and *bla*_{OXA-10} were harboured by 44 (23%) of ES *K. pneumoniae*. *bla*_{TEM} ARG included *bla*_{TEM-1D} (n=133; 70%), *bla*_{TEM-30} (n=12; 6%) were carried by ES isolates. Two ES *K. pneumoniae* harboured carbapenemase ARG *bla*_{NDM-1} and six (3%) ES isolates displayed *OmpK* truncations including *OmpK35* (n=4;2%) and *OmpK36* (n=3;1.5%) truncations.

Agly ARG were harboured by all ES *K. pneumoniae* among these ARG *aac*, *aad*, and *aph* ARG were found. Different *aac*, *aad* variants were harboured by ES *K. pneumoniae*. In relation to *aac* ARG the most common was *aac3-Ila* (n=116; 61%) followed by *aac3-IId* (n=31; 16%) with only one ES isolate carrying *aac6Ib-cr* and most ES *K. pneumoniae* carried *aadA1* (n=68, 36 %) and *aadA16* (n=40; 21%). In contrast only one variant of *aph* was found among 15 (8%) ES *K. pneumoniae* which carried *aph3-Ia*.

Fluoroquinolone resistance (Flq) ARG *qnrB1* (n=44; 23%) was the most frequently carried by ES *K. pneumoniae* harbouring Flq ARG only, followed by *qnrS1* (n=36; 19%). One ES isolate carrying *qnrB38* only and another ES isolate carrying *qnrB6* only. Mutations associated with Flq resistance identified were *gyrA-83T* in one (0.5%) isolate and *gyrA-83Y*, *gyrA-87A* and *parC-80I* in two (1%) isolates. These isolates simultaneously harboured *qnrB38* and *qnrS1*, respectively. ES *K. pneumoniae* carried *tetA* ARG and 36 (19%) carried *tetD*.

Additionally, two (1%) ES isolates harboured *tetA* and *tetD* and other two (1%) ES isolates carried *tetB*. Also, two (1%) ES *K. pneumoniae* displayed *pmrB* truncations that confer resistance to colistin and one (0.5%) ES isolate harboured *fosA2* that confers resistance to fosfomycin.

The following sections detail the ARG in the context of the different sample types of origin of the ES *K. pneumoniae* isolates because these could influence the distribution of ARG harboured by the different isolates because isolates would be subjected to different selective pressures. Also, allowing to identify key markers of resistance differences and/or similarities between ES-BB which caused sepsis and ES-BR, MR and ENV.

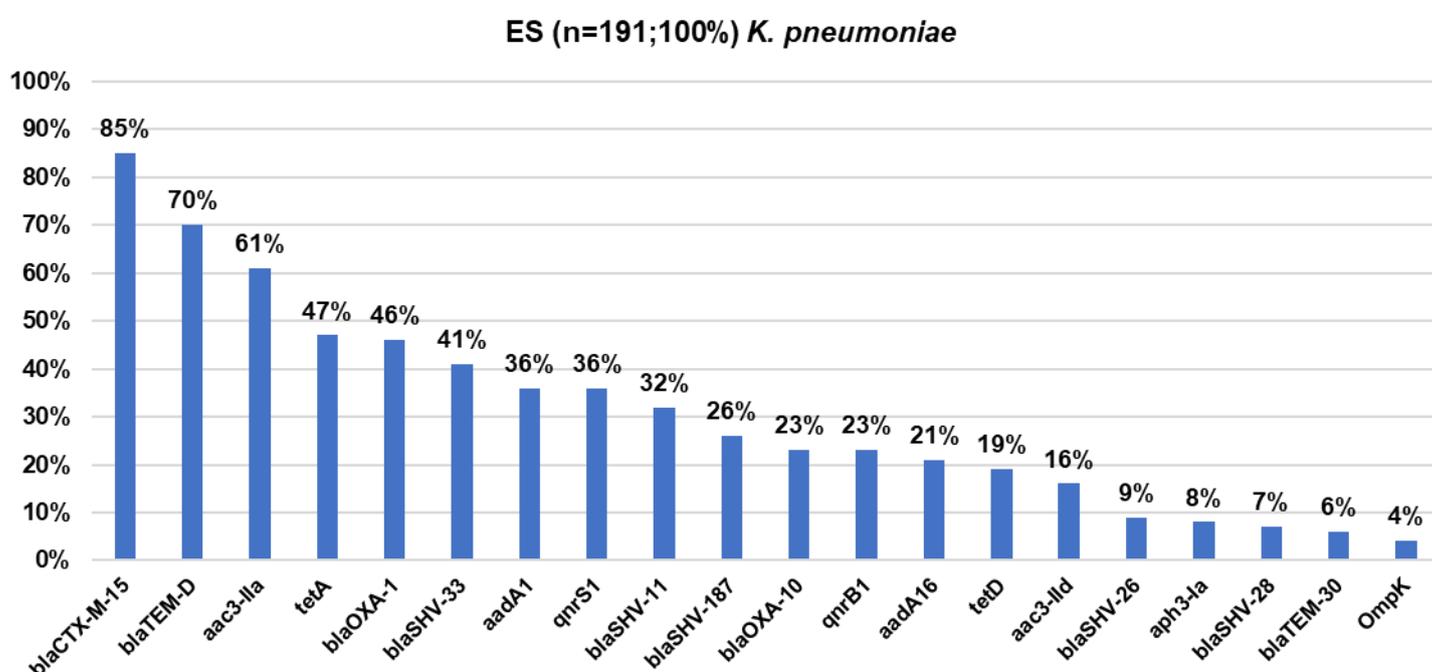


Fig. 19: Percentages of frequent of ARG and antibiotic resistance conferring mutations carried by *K. pneumoniae* from Ethiopia (ES). The most common ARG was *bla*_{CTX-M-15} (85%) followed by *bla*_{TEM-1D} (70%) and *aac3-Ila* (61%). With *tetA* and *bla*_{OXA-1} being carried by 46% and 47%. In contrast, 9% of ES isolates carried *bla*_{SHV-26}, and *aph3-Ia*, *bla*_{SHV-28} and *bla*_{TEM-30} were carried by 8%, 7% and 6%, respectively. Additionally, 4% of ES *K. pneumoniae* carried *OmpK* truncations.

5.2.1 Resistome of ES *K. pneumoniae* across ES-BB, BR, MR and ENV samples

ES-BB (n=86;100%) all carried β -lactamase ARG and Agly, ARG, 41 (48%) ES-BB *K. pneumoniae* carried Flq ARG and 77 (89.5%) carried *tet* ARG. Fig. 20 summarises the percentages of the described ARG and other ARG found. In Appendix 3 the ARG carried by each ES *K. pneumoniae* are listed.

All ES-BB (Fig. 20a) harboured *bla*_{CTX-M-15} and no other *bla*_{CTX-M} ARG was found among ES-BB *K. pneumoniae*. Other *bla* ARG carried by ES-BB included *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} ARG. Among which the most frequently found were *bla*_{OXA-1} (n=42; 49%), *bla*_{SHV-33} (n=37; 43%) and *bla*_{TEM-1D} (n=71; 83%). The most commonly found Agly ARG variants were *aac3-IIa* (n=50;58%), *aadA1* (n=34; 39,5%) and *aadA16* (n=33; 38%) whereas *aph3-la* was carried by eight (9%) ES-BB isolates. Other ARG that ES-BB *K. pneumoniae* carried included Flq and *tet* ARG. The Flq ARG found included *qnrB1* (n=16; 19%) and *qnrS1* (n=25; 29%) and the most common *tet* ARG carried was *tetA* (n=50; 58%). However, no Flq SNPs and colistin conferring truncations were displayed by ES-BB *K. pneumoniae* and only one (1%) ES-BB displayed an *OmpK* truncation.

Likewise, all ES-BR (n=68;100%) (Fig. 20b) carried *bla* and Agly ARG. Among, ES-BR the most harboured *bla* ARG were *bla*_{CTX-M-15} (n=60; 88%), *bla*_{TEM-1D} (n=48; 70%), *bla*_{OXA-10} (n=57;85%). Agly ARG commonly carried included *aac3-IIa* (n=54; 79%), *aadA1* (n=27; 40%), and *aph3-la* was harboured by six (9%) ES-BR *K. pneumoniae*. ES-BR isolates carried Flq and *tet* ARG including *qnrB1* (n=26; 38%) which was the most frequent but, in contrast to ES-BB, other *qnr* ARG were harboured by ES-BR *K. pneumoniae*. These included *qnrS1* was carried by seven (10%) ES-BR and *qnrB38* and *qnrB6* by one (1.5%) ES-BR isolate each. Three (4%) ES-BR harboured Flq SNPs simultaneously with *qnr* ARG, two (3%) ES-BR harboured *gyrA-83Y*, *gyr-87A*, *parC-80I* and *qnrS1* and one (1.5%) ES-BR isolate carried *gyrA-83T* and *qnrB38*. Also, as ES-BB the most frequently found *tet* ARG among ES-BR was *tetA* (n=31; 46%) with two (3%) more ES-BR *K. pneumoniae* carrying *tetA* together with *tetD*, other ES-BR isolates carried *tetD* (n=7; 10%). The *pmrB* resistance colistin conferring truncation was displayed by one (1.5%) ES-BR isolate, and similarly to, ES-BB *K. pneumoniae* , three (4%) ES-BR isolates showed *OmpK35* truncations.

When considering *bla* ARG harboured by ES-MR (n=30;100%) (Fig. 20c), WGS revealed that only ten (33%) ES-MR *K. pneumoniae* harboured these ARG in contrast to ES-BB (100%) and ES-BR (60%) *K. pneumoniae* that in majority harboured *bla* ARG. *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} ARG. Among ES-MR these were again harboured by small percentages of isolates in comparison to ES-BB and ES-BR (*bla*_{OXA-1} n=5, 17%; *bla*_{OXA-10} n=7, 23%; *bla*_{SHV-187} n=10; 33% and *bla*_{TEM-1D} n=8; 27%). Regarding, Agly ARG *aac3-IIa* (n=7; 27%) was the most common ARG, followed by *aadA1* carried by four (13%) ES isolates. Similarly, Flq ARG were less frequent among ES-MR (*qnrS1* n=3;10%; *qnrB1* n=2;7%) than among ES-BB and ES-BR and no Flq SNPs were shown by ES-MR *K. pneumoniae*. Additionally, *tetA* and *tetB* were harboured by seven (23%) and two (7%) ES-MR isolates. *OmpK35* (n=1;3%) and *OmpK36* (n=2;7%) and *pmrB* (n=1;3%) truncation were displayed by ES-MR *K. pneumoniae*. Lastly, ES-ENV (n=7;100%) (Fig. 20d) all harboured *bla*_{CTX-M-15}, *aph3-Ia* and most harboured *bla*_{TEM-D} (n=6;86%) and *aac3-IIId* (n=5; 71%).

More importantly, the comparison of the percentages of the ARG carried by ES *K. pneumoniae* showed that these percentages were frequently higher among ES-BB compared to ES-BR, followed by ES-ENV and ES-MR (Fig. 20). For example, the percentage of *bla*_{CTX-M-15}, which is a widely spread ESBL and responsible for resistance to cephalosporins but not to carbapenems, was higher among ES-BB *K. pneumoniae* (100%), followed by ES-BR (80%) and ES-MR (33%). The same was displayed in relation to Agly ARG which were harboured by all ES-BB and ES-BR, but most ES-MR did not carry Agly ARG (Fig. 20). Furthermore, these results were in line the percentages of resistance of ES *K. pneumoniae* regarding cephalosporins, carbapenems and other β -lactam antibiotics where the lowest percentages of resistance were shown by ES-MR *K. pneumoniae*. So, when comparing the percentages of ARGs among the different sample types of origin of ES *K. pneumoniae* the results showed that there were differences in the percentages of ARGs carried possibly reflecting the different stresses these were subjected to. Also, the high percentage of ARG among ES-ENV could suggest that ES-ENV could possibly be acting as a reservoir of ARG despite the possibility of mother-neonate transmission of ES-BB being indicated among ES *K. pneumoniae* (5.1 Phylogeny and population of ES *K. pneumoniae*). Nevertheless, it is important to notice that only seven ES-ENV isolates were

recovered, thus, not providing an appropriate indication of the percentage of *K. pneumoniae* harbouring ARG in the clinical environment in St Paul's hospital.

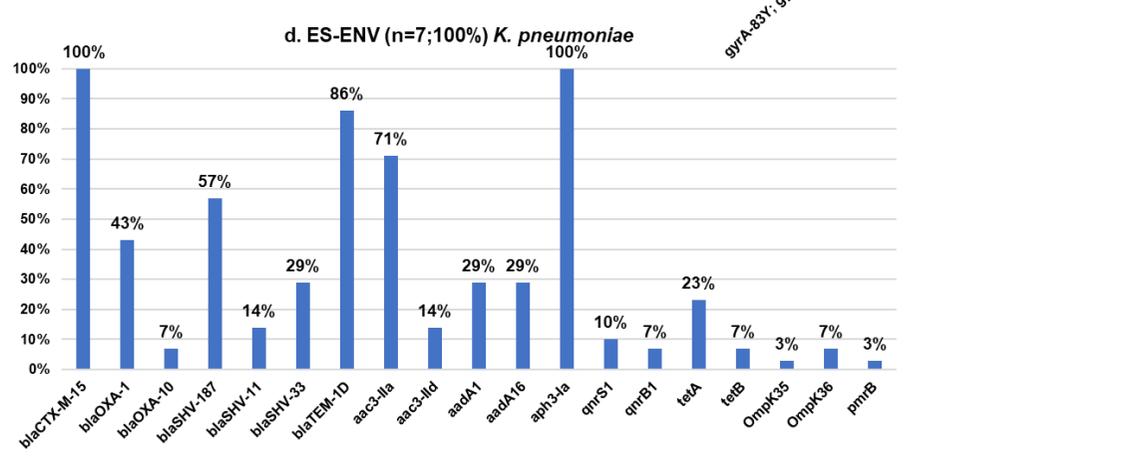
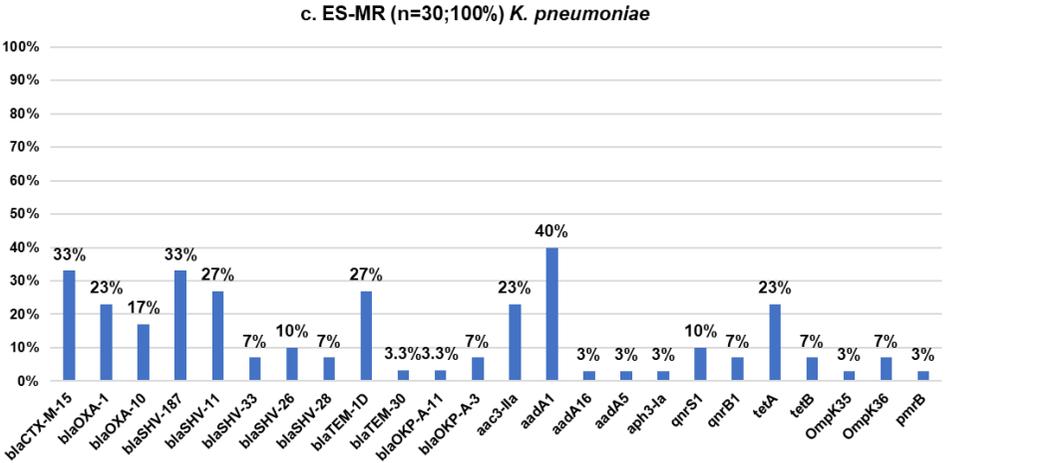
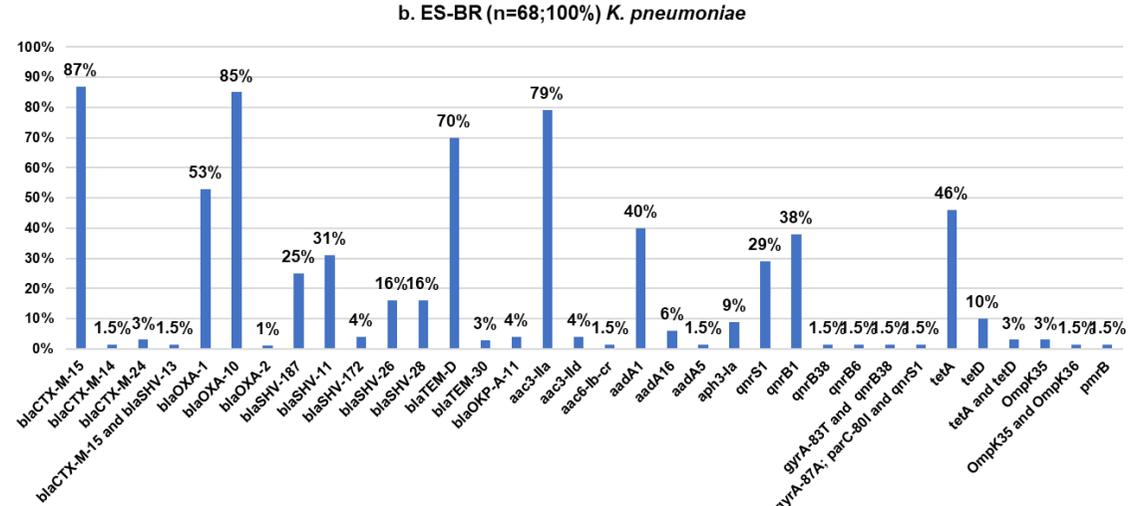
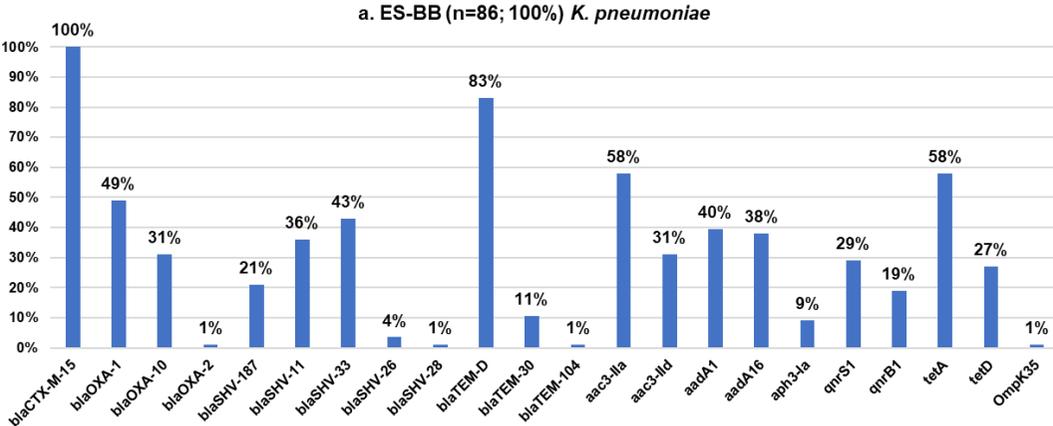


Fig. 20: Percentages of frequent of ARG and antibiotic resistance conferring mutations among *K. pneumoniae* from Ethiopia (ES) across the different sample types of origin.
a. Among ES-BB *K. pneumoniae* all isolates carried *bla*_{CTX-M-15} followed by *bla*_{TEM-1D} (83%) and *aac3-IId* (58%) and *tetA* (58%), in contrast, *aph3-Ia* and *bla*_{SHV-26} was carried by 9% and 4% of the ES-BB isolates, respectively. **b.** ES-BR *K. pneumoniae* frequently carried *bla*_{CTX-M-15} (87%) followed by *bla*_{OXA-10} (85%) and *aac3-Ila* (79%) with 70% of ES-BR isolates carrying *bla*_{TEM-1D}. Contrarily, *tetD* and *aph3-Ia* were harboured by 10% and 9% of ES-BR isolates, respectively. **c.** ES-MR *K. pneumoniae* frequently carried *aadA1* (40%) followed by *bla*_{CTX-M-15} (33%) and *bla*_{SHV-187} (33%), in contrast, 10% of ES-MR isolates carried *qnrS1* with 7% of ES-MR isolates carrying *qnrB1*, *tetB* and *OmpK36*. **d.** Among ES-ENV all *K. pneumoniae* carried *bla*_{CTX-M-15} and *aph3-Ia*, 86% carried *bla*_{TEM-1D} and 71% carried *aac3-Ila* ARG. In contrast, *bla*_{OXA-10}, *qnrB1*, *tetB* and *OmpK36* were carried by 7% of the ES-ENV isolates.

5.3 Antibiotic susceptibility profiles of ES *K. pneumoniae*

The antibiotic susceptibility profile of 191 (100%) ES *K. pneumoniae* were determined (Fig. 21) across the different sample types with ES-BB *K. pneumoniae* tested as part of the BARNARDS project.

Among the total ES *K. pneumoniae* in total the percentage of resistance to cephalosporins ranged from 85% to 87% (ceftriaxone (n=166;86%), cefotaxime (n=167;87%), ceftazidime (n=164;85%) and cefepime (n=163;85%)). Also, 162 (84%) ES isolates were resistant to amoxicillin/clavulanate combination and aztreonam (n=166;86%). However, carbapenem percentage of susceptibility of ES *K. pneumoniae* was 99% (n=190) to imipenem and 98% to both ertapenem (n=188) and meropenem (n=189). Likewise, among aminoglycosides the percentage of susceptibility to amikacin was high (n=190;99%) but, in contrast, ES *K. pneumoniae* showed high percentage of resistance to gentamicin (n=157;82%) and tobramycin (n=155;81%).

Furthermore, the percentage of susceptibility of ES *K. pneumoniae* to fosfomycin and colistin was both high at 91% (n=174) and 100% (n=191), respectively. Nevertheless, the majority of ES *K. pneumoniae* displayed high percentage of resistance to minocycline (n=190;99%) and to tigecycline (n=166;86%). Also, ES *K. pneumoniae* displayed 59% (n=113) and 47% (n=91) of susceptibility to the tested tetracyclines levofloxacin and ciprofloxacin, respectively. However, these *K. pneumoniae* also showed increased exposure of 39% (n=74) for levofloxacin and 15% (n=29) to ciprofloxacin and displayed 38% (n=72) of resistance to ciprofloxacin.

Generally, ES *K. pneumoniae* were frequently resistant to cephalosporins and in contrast in majority susceptible to carbapenems amikacin (99%), colistin (100%) and fosfomycin (91%). Carbapenemase *bla_{NDM}* positive isolates (n=2,100%) were susceptible to aztreonam/avibactam. In the following sections I will detail the antibiotic susceptibility profiles according to the origin sample type to study whether different stresses these isolates could be subjected to influence the antibiotic susceptibility profile phenotype of these isolates and if it is in accordance the ARG found through WGS.

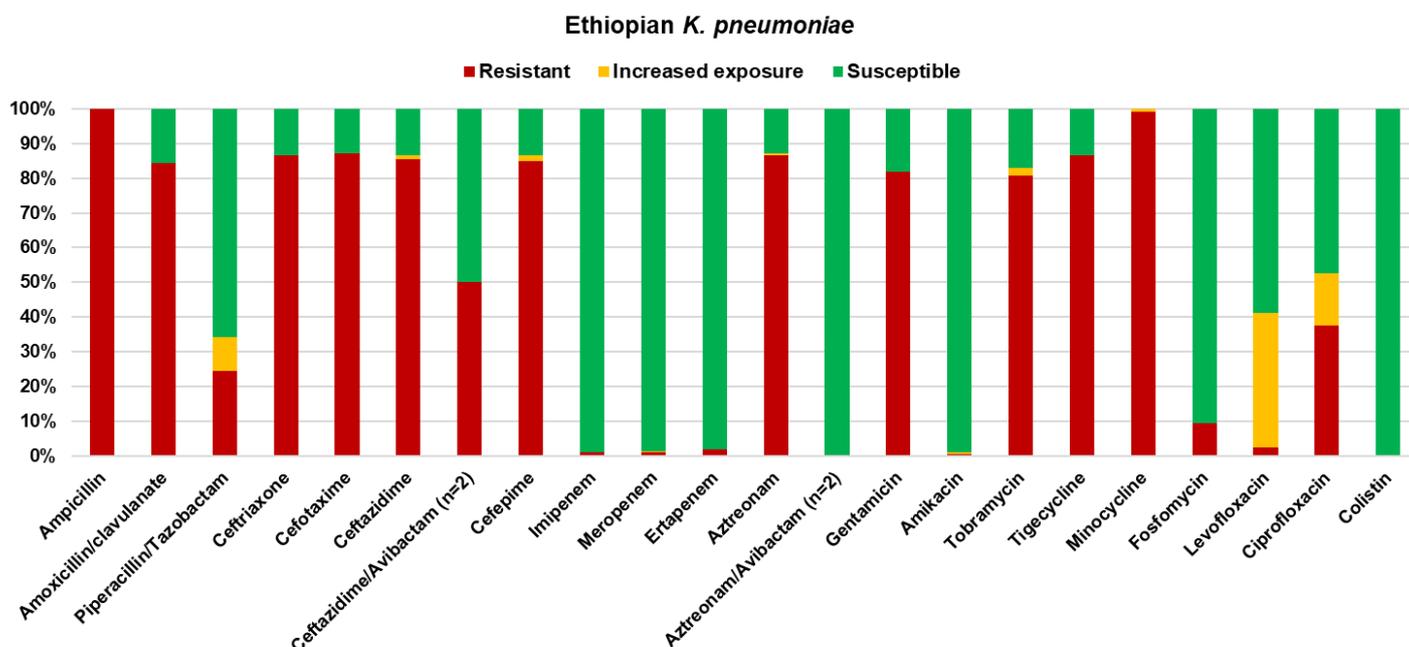


Fig. 21: Antibiotic susceptibility profile of *K. pneumoniae* isolates from Ethiopia. Isolates Ethiopia show high percentage of susceptibility to imipenem (99%), meropenem (98%), ertapenem (98%), amikacin (99%) and colistin (100%) in contrast to the high percentage of resistant to ceftriaxone (86%), cefotaxime (87%), ceftazidime (85%), cefepime (85%) and minocycline (99%). ES *K. pneumoniae* displayed increased exposure to levofloxacin (39%) with most isolates being susceptible (59%), likewise, most ES isolates were susceptible to ciprofloxacin (47%) despite the increased exposure (15%) and resistance (38%) displayed. The colour of the bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green).

5.3.1 ES *K. pneumoniae* antibiotic susceptibility profiles of BB, BR, MR and ENV isolates

ES-BB (n=87%) isolates showed high percentages of resistance to most of the β -lactams tested (Fig. 22a). These included cephalosporins. ES-BB isolates displayed high percentages of resistance to cefotaxime (n=86;99%), ceftazidime (99%), ceftriaxone (n=85;98%) and cefepime (n=85;98%). However, these resistance percentages were lower among ES-BR (n=68;100%) (Fig. 22b) ceftriaxone (n=62;91%), cefotaxime (n=62;91%), cefepime (n=61;90%) and ceftazidime (n=60,88%) and ES-ENV (n=7;100%) (Fig. 22d) that were all resistant to the cephalosporins tested. ES-MR isolates (n=30;100%) (Fig. 22c) were susceptible to ceftriaxone (n=18;60%), cefotaxime (n=18;60%), cefepime (n=18;60%) and aztreonam (n=18;60%) and 63% of the ES-MR isolates (n=63) were susceptible to ceftazidime.

ES-BB (Fig. 22a) were mostly susceptible to the carbapenems imipenem (n=86;99%), meropenem (n=85;98%) and ertapenem (n=84;97%). Likewise, ES-BR (Fig. 22b) isolates were in majority susceptible to imipenem (n=86;99%), meropenem (n=86;99%) and ertapenem (n=86;99%) and all ES-MR (Fig. 22c)

and ES-ENV (Fig. 22d) isolates were susceptible to imipenem, meropenem and ertapenem. In addition, ES-BB isolates showed 99% (n=86) and 44% (n=38) of resistance to amoxicillin/clavulanate and piperacillin/tazobactam, respectively. However, resistance percentages to amoxicillin/clavulanate and piperacillin/tazobactam were lower among ES-BR (88%; n=60 and 9%; n=6, respectively) with ES-MR isolates being susceptible to amoxicillin/clavulanate (n=21;70%) and piperacillin/tazobactam (n=26;87%).

Regarding the fluoroquinolones tested ES-BB displayed 53% (n=46) and 49% (n=43) susceptibility to levofloxacin and ciprofloxacin (Fig.22a), respectively. When considering ES-BR (n=35;51%) (Fig. 22b), ES-MR (n=27;90%) (Fig. 22c) and ES-ENV (n=5;71%) (Fig. 22d) isolates these were also in majority susceptible to levofloxacin, with ES-MR and ES-ENV showing higher percentages of susceptibility than ES-BB isolates. However, the percentage of susceptible ES-BR isolates (n=21;31%) to ciprofloxacin was lower in comparison to ES-BB (n=43;49%) isolates. The ES-MR isolates percentage of susceptibility (n=24;80%) to ciprofloxacin was higher in comparison to ES-BB (49%), ES-BR (29%) and ES-ENV (43%).

Furthermore, percentage of resistance shown by ES-BB *K. pneumoniae* (n=86), ES-BR (n=68) and ES-ENV (n=7)(Fig. 22a,b and d) to the tested aminoglycosides was high. ES-BB (n=85) displaying 98% resistance to gentamicin and tobramycin. In comparison, ES-BR isolates percentages of resistance to gentamicin (n=58;85%) and tobramycin (n=55;81%) were lower but all ES-ENV were resistant to gentamicin and tobramycin. Contrarily, ES-MR isolates were in majority susceptible to gentamicin (n=23;77%) and tobramycin (n=22;73%). However, most ES-BB isolates (n=85) were susceptible to amikacin displaying 98% of susceptibility and all ES-BR, ES-MR and ES-ENV were susceptible to amikacin.

Regarding, the tested tetracyclines ES-BB showed a percentage of resistance to minocycline of 99% (n=86) whereas 56% (n=49) were susceptible to tigecycline (Fig. 22a). In comparison ES-BR percentage of susceptibility to tigecycline (n=17;25%) was lower than among ES-BB isolates, likewise, ES-MR (n=18;60%) and ES-ENV (n=4;57%) were in majority susceptible to tigecycline. Also, as ES-BB isolates ES-BR (n=68;100%), ES-MR (n=30;100%) and ES-ENV (n=6;86%) were in majority resistant to minocycline (Fig. 22).

Lastly, ES-BB, showed a high percentage of susceptibility to fosfomycin and colistin, 97% (n=84) and 100% (n=86), respectively. Similarly, ES-BR (n=59;87% and n=68; 100%, respectively), ES-MR (n=24;80% and n=30;100%, respectively) and ES-ENV (n=7;100% to both) were in majority susceptible to colistin and fosfomycin, with the ES-BR showing higher percentages of susceptibility to fosfomycin (Fig. 22).

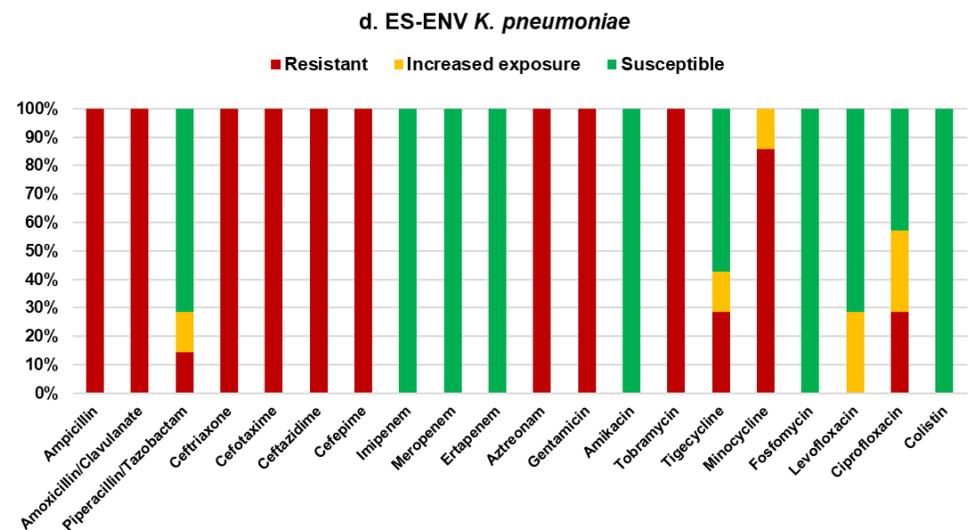
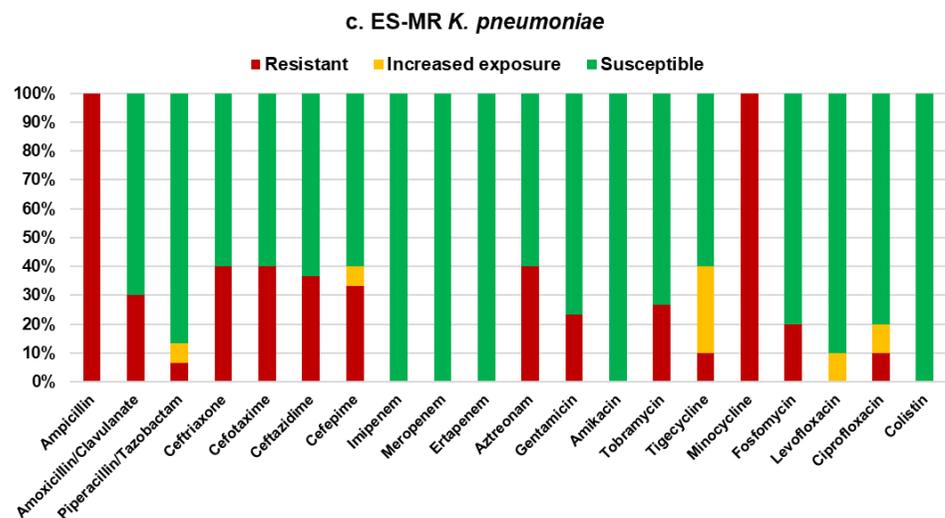
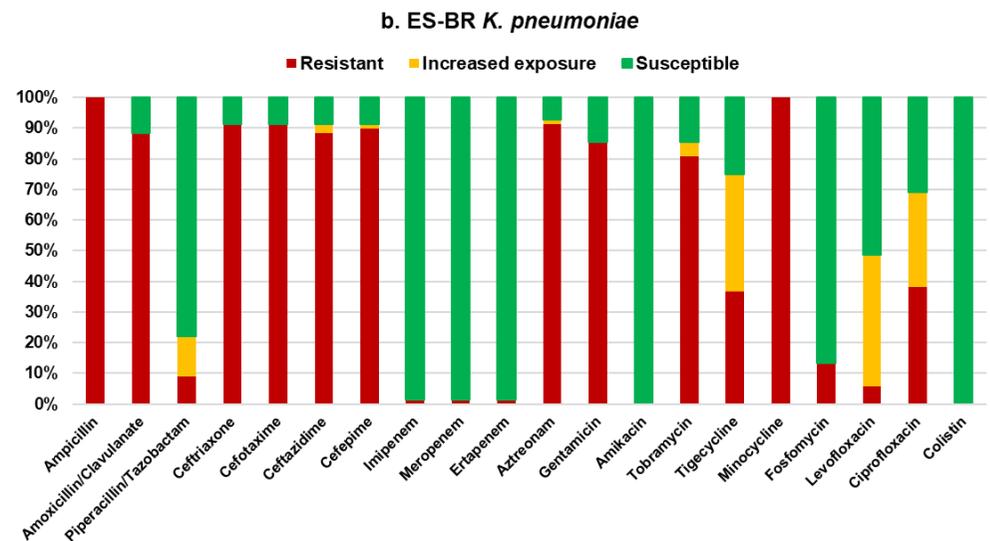
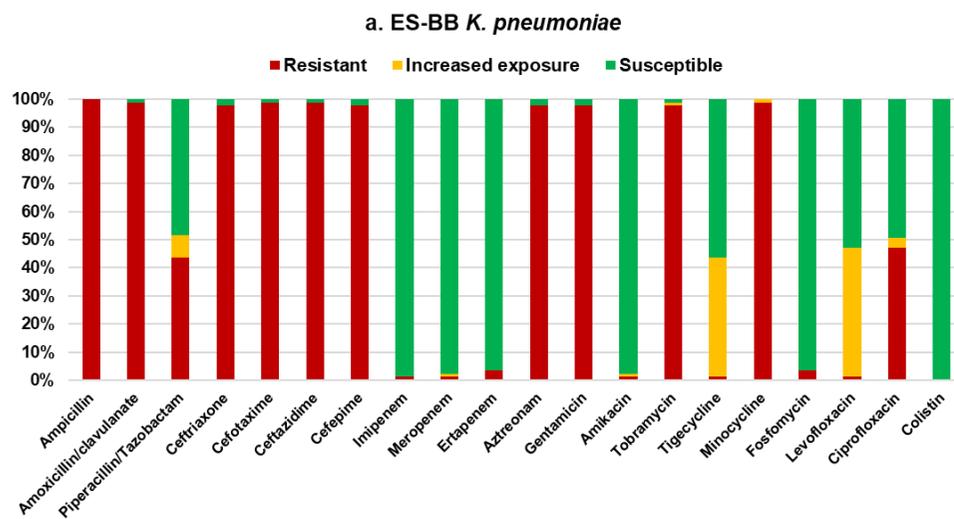


Fig. 22: Antibiotic susceptibility profile of ES-BB, BR, MR and ENV *K. pneumoniae*. **a.** ES-BB *K. pneumoniae* displayed high percentages of susceptibility to imipenem (99%), meropenem (98%) and ertapenem (97%) in contrast to resistance to ceftriaxone (98%), cefotaxime (99%), ceftazidime (99%), cefepime (98%). ES-BB *K. pneumoniae* were susceptible to colistin (100%) followed by amikacin (98%) and fosfomycin (97%). **b.** Most ES-BR were susceptible to imipenem (99%), meropenem (99%) and ertapenem (99%). Amikacin and colistin being the most effective antibiotics whereas ES BR displayed high percentage of resistance to ceftriaxone (91%), cefotaxime (91%), ceftazidime (88%) and cefepime (90%). **c.** ES-MR were in majority susceptible to the antibiotics tested including carbapenems imipenem (100%), meropenem (100%) and meropenem (100%) and aminoglycoside amikacin (100%). In contrast ES-MR *K. pneumoniae* were resistant to minocycline (100%). **d.** All ES-ENV resistant to cephalosporins but susceptible to carbapenems. Amikacin, colistin and fosfomycin being effective as well. All ES-ENV were susceptible to these antibiotics. The colour of the bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green)

In summary, ES *K. pneumoniae* recovered from BR, MR and ENV displayed high rates of resistance to gentamicin, but ES-MR isolates displayed lower percentages of resistance to cephalosporins than isolates from the other sample types including ES-BB *K. pneumoniae*. With the same applying to other antibiotics as for example tobramycin. Nevertheless, despite sample type of origin all ES *K. pneumoniae* were susceptible to colistin and amikacin.

5.3.2 Antibiotic susceptibility profile of ES *K. pneumoniae* – discussion

When considering ES *K. pneumoniae* in total these were shown to be mostly susceptible to carbapenems and all were susceptible to colistin. However, ES *K. pneumoniae* displayed high percentages of resistance to cephalosporins and to tetracyclines. Although being reasonably susceptible to piperacillin/tazobactam thus this treatment would be viable for some sepsis cases in St Paul's Hospital in Ethiopia.

More importantly, in majority ES-BB *K. pneumoniae* were resistant to cephalosporins, therefore, contributing to the big burden of cephalosporin resistance among ES isolates studied seeing these were recovered in higher numbers. In addition, the high percentage of resistance to gentamicin was also of concern among ES-BB. This is important because gentamicin, cefotaxime and ceftazidime were included in the antibiotics prescribed to treat *K. pneumoniae* sepsis in St Paul's hospital and gentamicin and cefotaxime were among empirically described antibiotics (169). Considering the high percentages of resistance to these antibiotics these can become unfeasible treatments for *K. pneumoniae* sepsis. Even though, MIC results presented amikacin as the best option for treatment. However, as mentioned before amikacin is not available in Ethiopia. Additionally, ES-BR *K. pneumoniae* were in majority resistant to these antibiotics as well. Further showing that *K. pneumoniae* resistant to antibiotics used in treatment are among the neonate's microbiota which of concern if these *K. pneumoniae* later cause sepsis. Seeing that the high percentages of resistance and lack of availability of other antibiotics that could be used reduce the choice of antibiotics for treatment (169). Other treatments could include colistin, tigecycline or fosfomycin considering that ES-BB were in majority susceptible to these antibiotics however these like amikacin are not available in Ethiopia. Leaving as options for treatment fosfomycin and carbapenems that place a heavy burden on the average daily wage in Ethiopia. For example, meropenem places a burden of 234%,

which again reduces the availability of treatments to patients (169) and these would not be viable treatments for *K. pneumoniae* sepsis seeing that *K. pneumoniae* is intrinsically resistant to ampicillin (46) (1.5 Antibiotic resistance in *K. pneumoniae*).

Also, regarding ES-MR *K. pneumoniae* MIC results showed higher percentages of susceptibility, however, considering the results in 5.1 possible transmission between mother and neonate of sepsis causing *K. pneumoniae* is possible. In this case, ES-MR30 and ES-BB17 were resistant cephalosporins and gentamicin and susceptible to carbapenems, colistin and amikacin. Also, WGS results showed that these isolates carried ARG responsible for resistance these antibiotics as *bla_{OXA}*, *bla_{TEM}* and *bla_{SHV}* ARG.

Hence it is important to carefully consider prescribing antibiotics to mothers prior to delivery if *K. pneumoniae* capable of sepsis could be spread between mother and neonate. Considering that in BARNARDS neonates of mothers that reported the use of antibiotics three months prior to enrolment had higher odds of BS (170). Regarding, ES-ENV *K. pneumoniae* even though MIC results were shown these were only related to seven isolates which only allowed for small insight of the probable resistance pattern of *K. pneumoniae* in the clinical environment in St Paul's Hospital.

5.4 Antibiotic susceptibility and ARG among ES *K. pneumoniae* belonging to ST35, ST37 and ST218

ES-BB (n=33) and ENV (n=2) belonged to ST35 and ES-BB (n=26), ES-BR (n=6) and ES-MR (n=2) and ES-ENV (n=1) belonged to ST37. Fig. 23 shows the antibiotic susceptibility profile for both ES ST35 and ST37. Also, MIC₅₀ and MIC₉₀ values were determined for ST35 and ST37 ES isolates. A summary of the determined MIC₅₀ and MIC₉₀ is shown in Table 7.

ES ST35 (n=35) and ES ST37 (n=35) displayed high percentage of resistance (100%) to β -lactams namely to cephalosporins (cefepime, ceftazidime, cefotaxime, and ceftriaxone), amoxicillin/clavulanate and aztreonam. MIC₅₀ and MIC₉₀ of >4 mg/L were determined for the cephalosporins tested and aztreonam. ST37 isolates piperacillin/tazobactam MIC₅₀ and MIC₉₀ was of >32 mg/L. Furthermore, WGS typing of ARG carried by ES ST35 and ES ST37 isolates revealed that several β -lactamase ARG associated with resistance to the tested antibiotics were harboured by the isolates. These included ESBL ARG such as *bla*_{CTX-M-15} often responsible for high percentages of resistance to cephalosporins. The presence of *bla*_{CTX-M-15} among ST35, ST37 and ST218 *K. pneumoniae* was significantly associated (p<0.001) with resistance to the tested cephalosporins (ceftriaxone, cefotaxime, ceftazidime and cefepime) in this PhD. Also, ST35 (p=0.006 and p=0.008, respectively) and ST37 (p=0.005 and p<0.001, respectively) were significantly associated with resistance to amoxicillin/clavulanate and piperacillin/tazobactam, but ST218 was only associated (p<0.001) with resistance to piperacillin/tazobactam. Likewise, for resistance to ceftriaxone, cefotaxime, ceftazidime and cefepime ST35 (p=0.010, p=0.012, p=0.010 and p=0.010, respectively) and ST37 (p=0.009, p=0.011, p=0.009 and p=0.009, respectively) were significantly associated.

Overall, ES-BB ST35 (n=33) carried ESBL ARG in addition to other β -lactamase ARG. Also, the percentage of resistance of ES ST35 isolates to amoxicillin/clavulanate was 100% (n=35; MIC₅₀ and MIC₉₀ of >32 mg/L) whereas the percentage of resistance to piperacillin/tazobactam was 6% (n=2). In this case piperacillin/tazobactam MIC₅₀ determined were of 4 mg/mL and MIC₉₀ of 16mg/L for

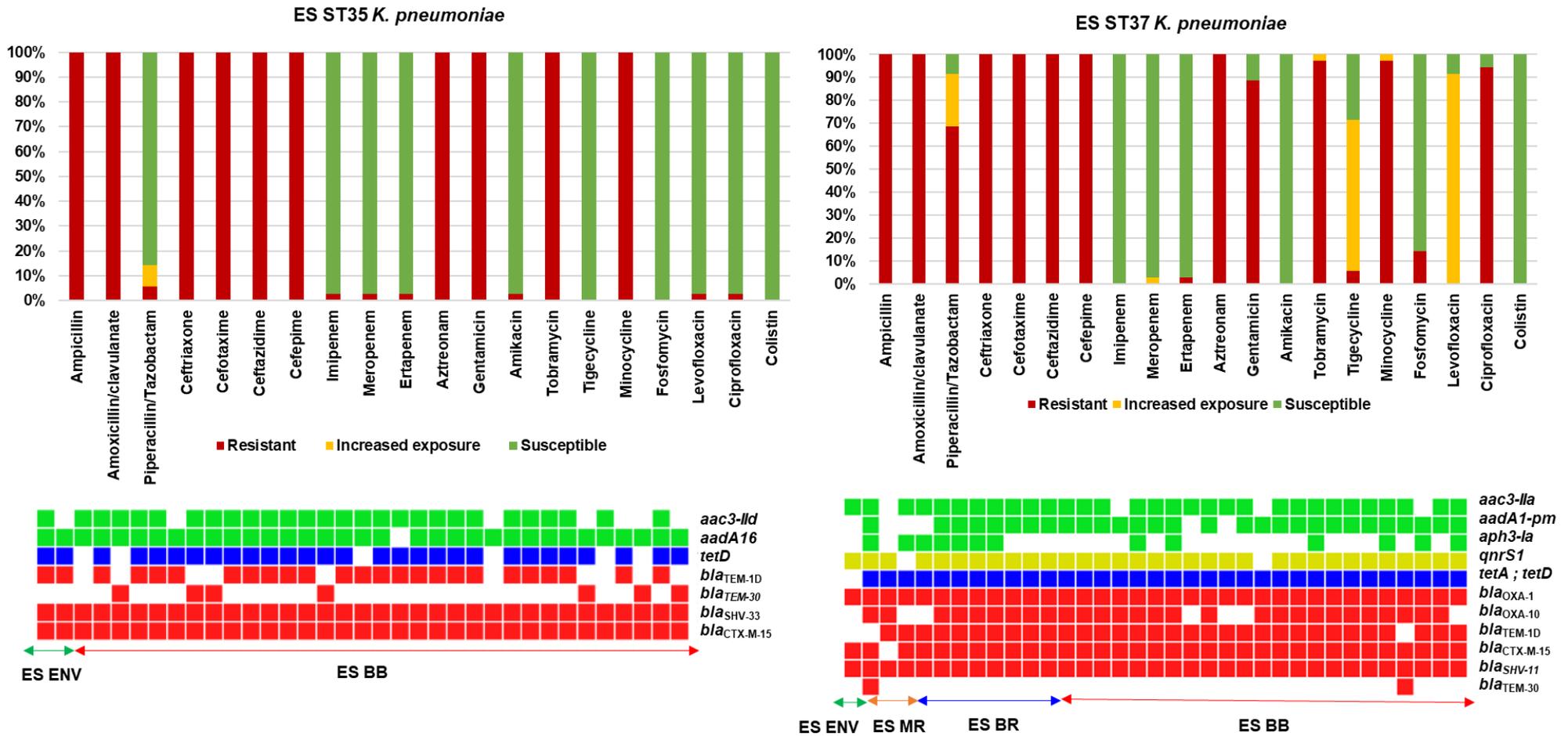
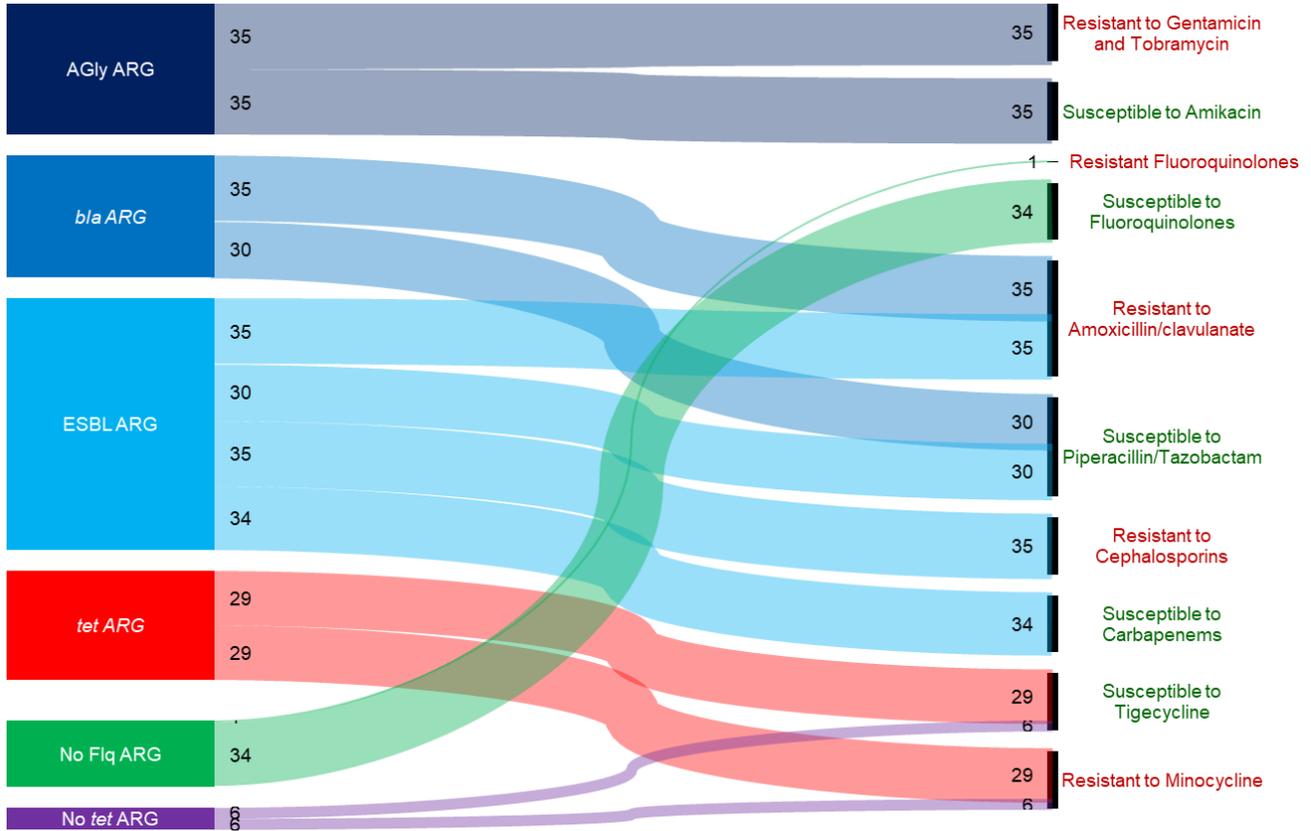


Fig. 23: Antibiotic susceptibility and ARG profile of ES ST35 and ST37 *K. pneumoniae*. ES ST35 and ES ST35 isolates were resistant (100%) to cephalosporins including cefepime, ceftazidime, cefotaxime and ceftriaxone. In majority ES ST35 and ST37 were susceptible (97% to 100%) to carbapenems including imipenem, meropenem and ertapenem. Both ES ST37 and ST35 were susceptible to amikacin (97% and 100%, respectively) and resistant to gentamicin (100% and 89%, respectively) and tobramycin (100% and 97%, respectively). Most ES ST35 were susceptible to ciprofloxacin (97%) and levofloxacin (97%) whereas most ES ST37 were resistant and showed increased exposure (94% and 91%, respectively). The colour of the graphs bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green). Heatmaps show the presence of aminoglycoside ARG (green), tetracycline ARG (blue), fluoroquinolones (yellow) and β -lactamase ARG (red) carried by ES ST35 from ES-BB and ES-ENV and ES ST37 *K. pneumoniae* from ES-BB, ES-BR, ES-MR and ES-ENV.

ST35 ES isolates. ES-BB ST37 (n=26;100%) carried ESBL and broad-spectrum β -lactamase ARG. Furthermore, ES ST37 isolates were among BR (n=6) and MR (n=2) as well. In relation to BR isolates these carried different ARG simultaneously and MR isolates did as well. Likewise, ST35 ES isolates, ST37 *K. pneumoniae* were all resistant to amoxicillin/clavulanate but were mostly resistant (n=24;69%) to piperacillin/tazobactam.

In contrast, the majority of ES *K. pneumoniae* ST35 and ST37 were susceptible to carbapenems. ES ST35 isolates displayed a high percentage of susceptibility to imipenem (n=34;97%), meropenem (n=34;97%) and ertapenem (n=34;97%). The determined MIC₅₀ and MIC₉₀ were 1 mg/mL for imipenem and meropenem and 0.25 mg/L for ertapenem. ES ST37 showed high percentage of susceptibility to carbapenems as well including to imipenem (n=35;100%), meropenem (n=34;97%) and ertapenem (n=4;97%). For these isolates MIC₅₀ and MIC₉₀ for imipenem and meropenem were both of 1 mg/L. Additionally, MIC₅₀ and MIC₉₀ for ertapenem were 0.25 mg/mL and 1 mg/L, respectively. These phenotypic results were consistent with WGS data indicating that carbapenemase ARG were not carried by ES isolates (Fig. 23 and Fig.24) (5.2 Resistome of ES *K. pneumoniae*).

ST35 ES *K. pneumoniae*



ST37 ES *K. pneumoniae*

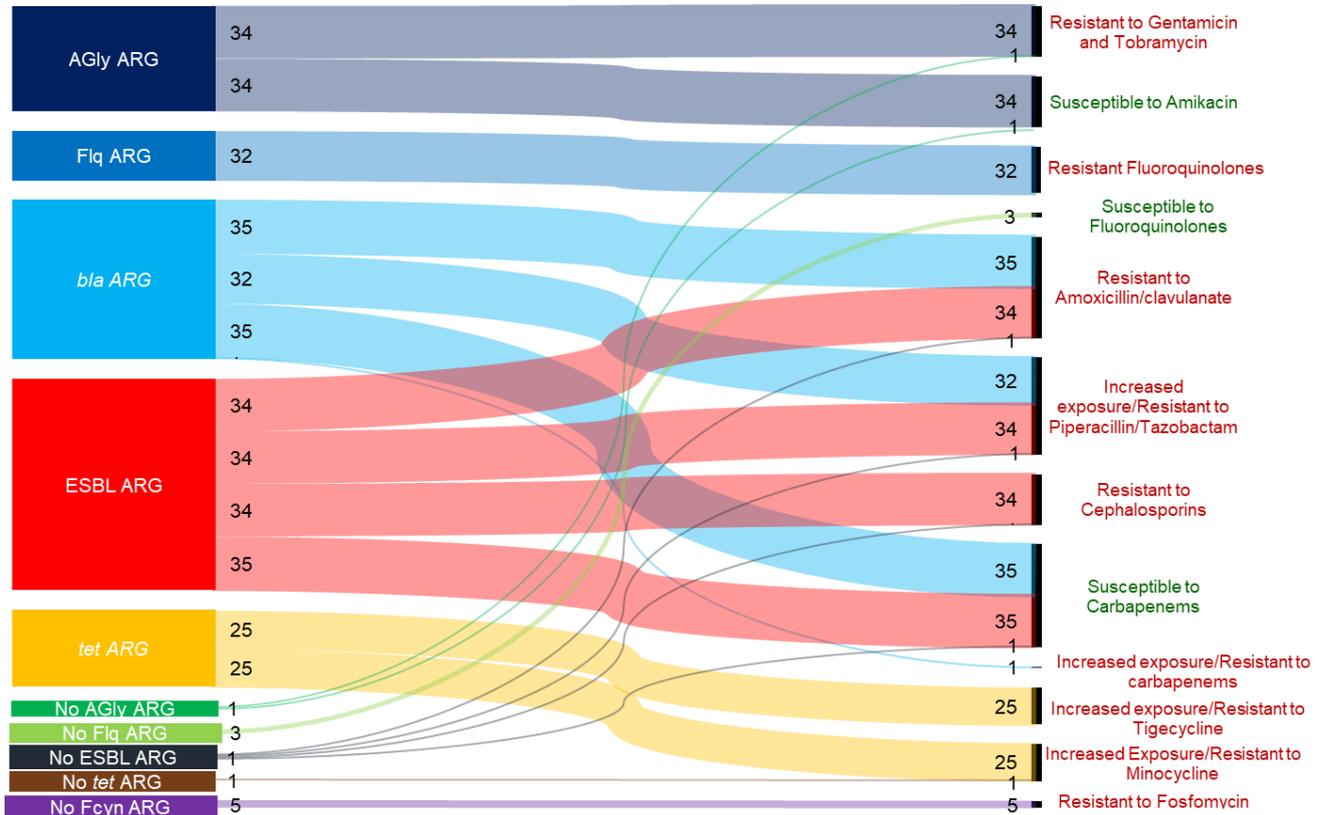


Fig. 24: Sankey diagrams linking ST35 and ST37 *K. pneumoniae* isolates that carry or not carry ARG with their antibiotic susceptibility profile. The number of isolates that carried ARG (left) are linked to the antibiotic susceptibility profile displayed by these isolates to which each ARG could confer resistance to (right). The number of isolates is indicated in each coloured band, the number of isolates carrying that type of ARG (left) and the number of isolates displaying the respective antibiotic susceptibility (right). The isolates that carried ARG displayed either resistance or increased exposure to the antibiotics that they would confer resistance to and ST35 and ST37 PP isolates that did not carry ARG showed susceptibility. However, when no Flq ARG were carried one ST35 ES isolates was resistant to the tested fluoroquinolones and six ST35 ES isolates were susceptible to tigecycline but resistant to minocycline without carrying *tet* ARG. Five ST37 ES isolates did not carry Fcyn ARG but were resistant to fosfomycin and one ST37 ES isolate did not carry *tet* ARG but displayed increased

Table 7: ES ST35 and ST37 *K. pneumoniae* MIC₅₀ and MIC₉₀ for the antibiotics tested. Among ES ST35 and ST37 *K. pneumoniae* most MIC₅₀ and MIC₉₀ determined were over the clinical break point with imipenem, meropenem, ertapenem, amikacin, fosfomycin and colistin being under the clinical break point.

Antibiotic	ES ST35		ES ST37	
	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)
Ampicillin	>32	>128	>32	>128
Amoxicillin/clavulanate	>32	>32	>32	>32
Piperacillin/tazobactam	4	16	>32	>32
Ceftriaxone	>4	>4	>4	>4
Cefotaxime	>4	>4	>4	>4
Ceftazidime	>4	>4	>4	>4
Cefepime	>4	>4	>4	>4
Imipenem	1	1	1	1
Meropenem	1	1	1	1
Ertapenem	0.25	0.25	0.25	1
Aztreonam	>4	>4	>4	>4
Gentamicin	>8	>8	>8	>8
Amikacin	4	4	4	4
Tobramycin	>8	>8	>8	>8
Tigecycline	0.5	1	2	2
Minocycline	>4	>4	>4	>4
Fosfomycin	16	16	16	>128
Levofloxacin	0.5	0.5	1	1
Ciprofloxacin	0.25	0.25	2	>2
Colistin	1	1	1	1

Regarding the aminoglycosides tested ES ST35 and ES ST37 showed high percentage of resistance to gentamicin (n=35;100% and n=31;89%, respectively) and tobramycin (n=35;100% and n=34;97%). Gentamicin and tobramycin MIC₅₀ and MIC₉₀ values determined were of > 8 mg/L for both ST35 and ST37 ES isolates, suggesting the presence of ARG responsible for resistance to aminoglycoside antibiotics and this was confirmed by WGS. Most ES-BB and ES-ENV ST35 and ES ST37 isolates carried more than one aminoglycoside ARG. In contrast, both ES ST35 and ES ST37 displayed high percentage of susceptibility to amikacin (n=34;97% and n=35;100%, respectively, Fig. 23; MIC₅₀ and MIC₉₀ 4 mg/mL). Also, these isolates displayed high percentage of resistance to the tetracycline minocycline (100% for ST35 and 97% for ST37). Both ES ST35 and ST37 isolates carrying *tet* ARG associated with resistance to tetracyclines. *tet* ARG were harboured by 27 ES-BB and by 2 ES-ENV ST35 isolates. All ES-BB ST37 and ES-BR (n=5) and ES- MR (n=2) ST37 harboured *tet* ARG as well. ST35 and ST37 ES isolates displayed MIC₅₀ and MIC₉₀ of >4 mg/L for

minocycline. Susceptibility to tigecycline was high for ST35 isolates (n=35;100%; MIC₅₀ 0.5 mg/L and MIC₉₀ 1 mg/L) whereas ST37 isolates resistance percentages of 6%(n=2) (MIC₅₀ and MIC₉₀ 2 mg/L).

Percentage of resistance to fluoroquinolones showed by ST37 isolates was high with ciprofloxacin at 94% (n=34) and an MIC₅₀ of 2 mg/L and MIC₉₀ of >2mg/L. Levofloxacin displayed increased exposure (n=32;92%) with both an MIC₅₀ and MIC₉₀ at 1 mg/L. Furthermore, ST37 ES-BB (n=32) isolates WGS revealed that fluoroquinolone ARG *qnrS1* was harboured by these isolates but no fluoroquinolone resistance conferring SNPs were harboured. In contrast, ST35 ES isolates (n=35) did not carry fluoroquinolone ARGs (Fig. 23). Additionally, MIC₅₀ and MIC₉₀ ciprofloxacin and levofloxacin determined for these isolates were of 0.25 mg/L and 0.5 mg/L, respectively.

All ES ST35 and ST37 isolates were susceptible to colistin (MIC₅₀ and MIC₉₀ 1 mg/L). In addition, ST35 isolates (n=35) showed 100% susceptibility to fosfomycin and ST37 showed 86% (n=30). MIC₅₀ and MIC₉₀ values determined for fosfomycin were of 16 mg/L. For ST37 ES isolates the MIC₅₀ was 16 mg/L and MIC₉₀ >128 mg/L.

ES ST218 *K. pneumoniae*

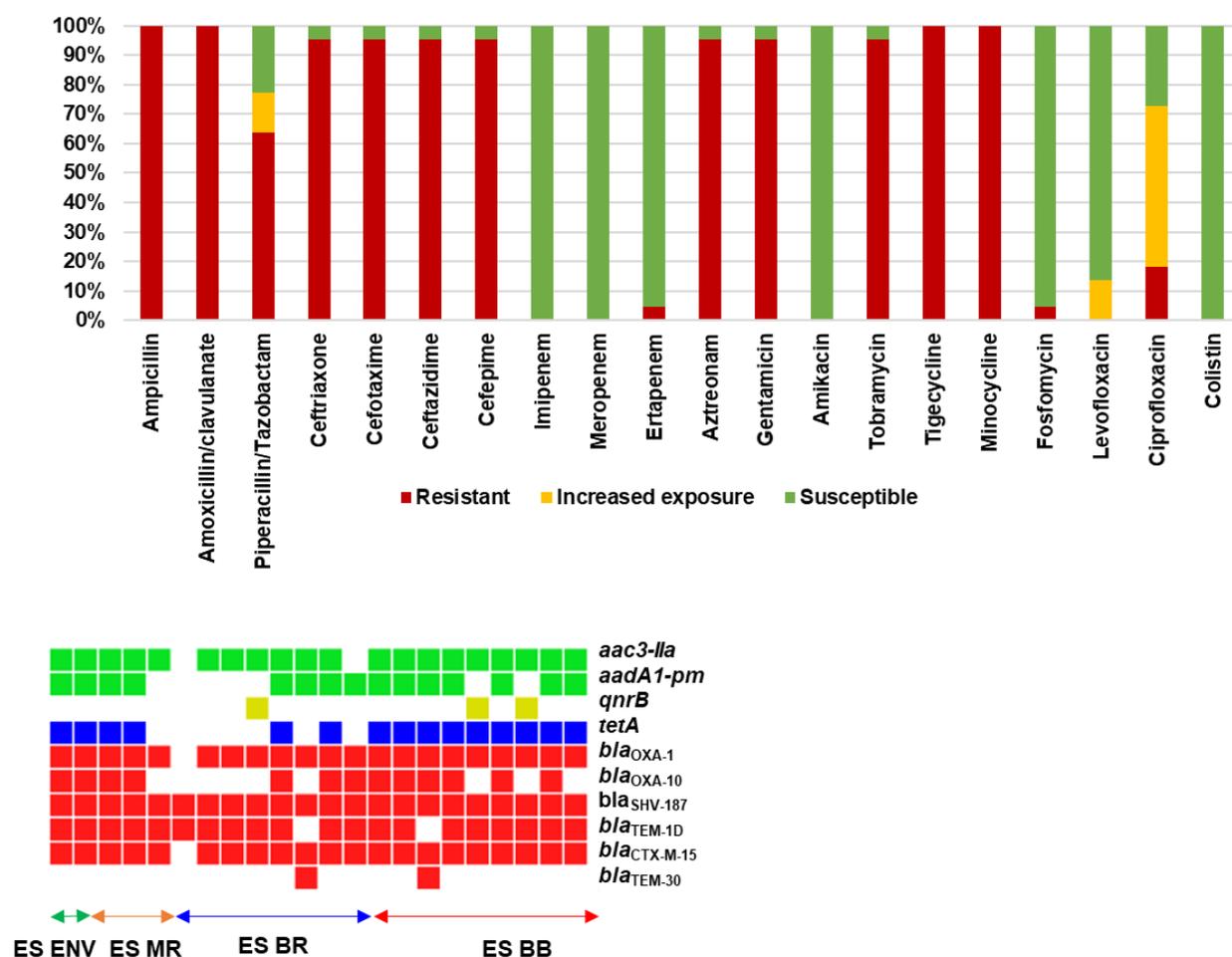


Fig. 25 Antibiotic susceptibility and ARG profile of ST218 *K. pneumoniae*. Most ES ST218 *K. pneumoniae* were resistant to ceftriaxone (95%), cefotaxime (95%), ceftazidime (95%) and cefepime (95%), in contrast, being in majority susceptible to imipenem (100%), meropenem (100%) and ertapenem (95%). These isolates were resistant to amoxicillin/clavulanate (100%). In majority resistant to piperacillin/tazobactam (64%) and showing increased exposure (14%). Most ES ST218 *K. pneumoniae* were resistant to aztreonam (95%), gentamicin (100%), tobramycin (95%), tigecycline (100%), minocycline (100%). These isolates showed increased exposure to ciprofloxacin (55%) but were susceptible to levofloxacin (86%). ES ST218 *K. pneumoniae* were mostly susceptible to colistin (100%) and fosfomycin (95%). The colour of the graphs bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green). Heatmaps show the presence of aminoglycoside ARG (green), tetracycline ARG (blue), fluoroquinolones (yellow) and β -lactamase ARG (red) carried by ST218 *K. pneumoniae* from ES-BB, ES-BR, ES-MR and ES-ENV.

Likewise, ST218 *K. pneumoniae* were resistant to ceftriaxone (n=21;95%), cefotaxime (n=21;95%), ceftazidime (n=21;95%) and (n=21;95%) (MIC₅₀ and MIC₉₀ > 4 mg/mL) which is line with the presence of *bla_{CTX-M-15}* among these isolates. In addition, resistance to other β -lactam is supported by the presence of other *bla* ARG such as *bla_{OXA-1}*, *bla_{OXA-10}*, *bla_{TEM-30}* and *bla_{TEM-1D}*. A summary of the determined MIC₅₀ and MIC₉₀ for ST218 *K. pneumoniae* is shown Table 8. For example, isolates carrying *bla_{TEM-30}* ARG could explain the resistance to piperacillin/tazobactam because this ARG encodes an enzyme that confers resistance to β -lactam/inhibitors combinations (171). However, these isolates were mostly susceptible to imipenem (n=22;100%)

(MIC₅₀ and MIC₉₀ 1 mg/mL), ertapenem (n=22;100%) (MIC₅₀ 0.25 mg/mL and MIC₉₀ 0.5 mg/mL) and meropenem (n=21;95%) (MIC₅₀ and MIC₉₀ 1 mg/mL) and showed no carbapenemase ARG. ST218 *K. pneumoniae* were also susceptible to amikacin (n=22;100%) but not to gentamicin (n=21;95%) and tobramycin (n=21;95%), with MIC₅₀ and MIC₉₀ of 4 and >8 being determined, which can be explained by the presence of *aac3-lla* and *aadA1-pm* Agly ARG. Among other antibiotics these isolates were mainly susceptible to levofloxacin (n=19;86%) (MIC₅₀ and MIC₉₀ 0.5 mg/mL) seeing that only three ST218 isolates carried *qnrB* ARG, but showed increased exposure to ciprofloxacin (n=12;55%, MIC₅₀ and MIC₉₀ of 0.5 mg/mL and 2 mg/mL, respectively). In contrast, most ST218 isolates were resistant to tigecycline (n=22;100%, MIC₅₀ 4 mg/mL and MIC₉₀ >8 mg/mL) and minocycline (n=22;100%) and carried *tetA* ARG. Regarding colistin and fosfomycin the majority of ST218 isolates were susceptible to fosfomycin (n=21;95%, MIC₅₀ and MIC₉₀ of 16 mg/mL and 32 mg/mL, respectively), and all ST218 *K. pneumoniae* were susceptible to colistin (MIC₅₀ and MIC₉₀ of 1 mg/mL, respectively). The antibiotic susceptibility profiles and ARG carried by ST218 *K. pneumoniae* are shown in Fig. 25. And the link between ARG and antibiotic susceptibility profile is shown in Fig. 26.

Overall, most ST35, ST37 and ST218 ES isolates were resistant to cephalosporins (100%), aminoglycosides gentamicin (89% to 100%) and tetracycline minocycline (97% to 100%). Lastly, most isolates were susceptible to colistin (100%), amikacin (100%) in addition to carbapenems.

Table 8: ES ST218 *K. pneumoniae* MIC₅₀ and MIC₉₀ for the antibiotics tested. Among ES ST218 *K. pneumoniae* most MIC₅₀ and MIC₉₀ determined were over the clinical break point with imipenem, meropenem, ertapenem, amikacin, fosfomycin and colistin being under the clinical break point.

Antibiotic	ST218		Antibiotic	ST218	
	MIC ₅₀	MIC ₉₀		MIC ₅₀	MIC ₉₀
Ampicillin	>32	>32	Amikacin	4	4
Amoxicillin/clavulanate	>32	>32	Tobramycin	>8	>8
Piperacillin/Tazobactam	8	>32	Tigecycline	4	>8
Ceftriaxone	>4	>4	Minocycline	4	>4
Cefotaxime	>4	>4	Fosfomycin	16	32
Ceftazidime	>4	>4	Levofloxacin	0.5	0.5
Cefepime	>4	>4	Ciprofloxacin	0.5	2
Imipenem	1	1	Colistin	1	1
Meropenem	1	1	Gentamicin	>8	>8
Ertapenem	0.25	0.5	Aztreonam	>4	>4

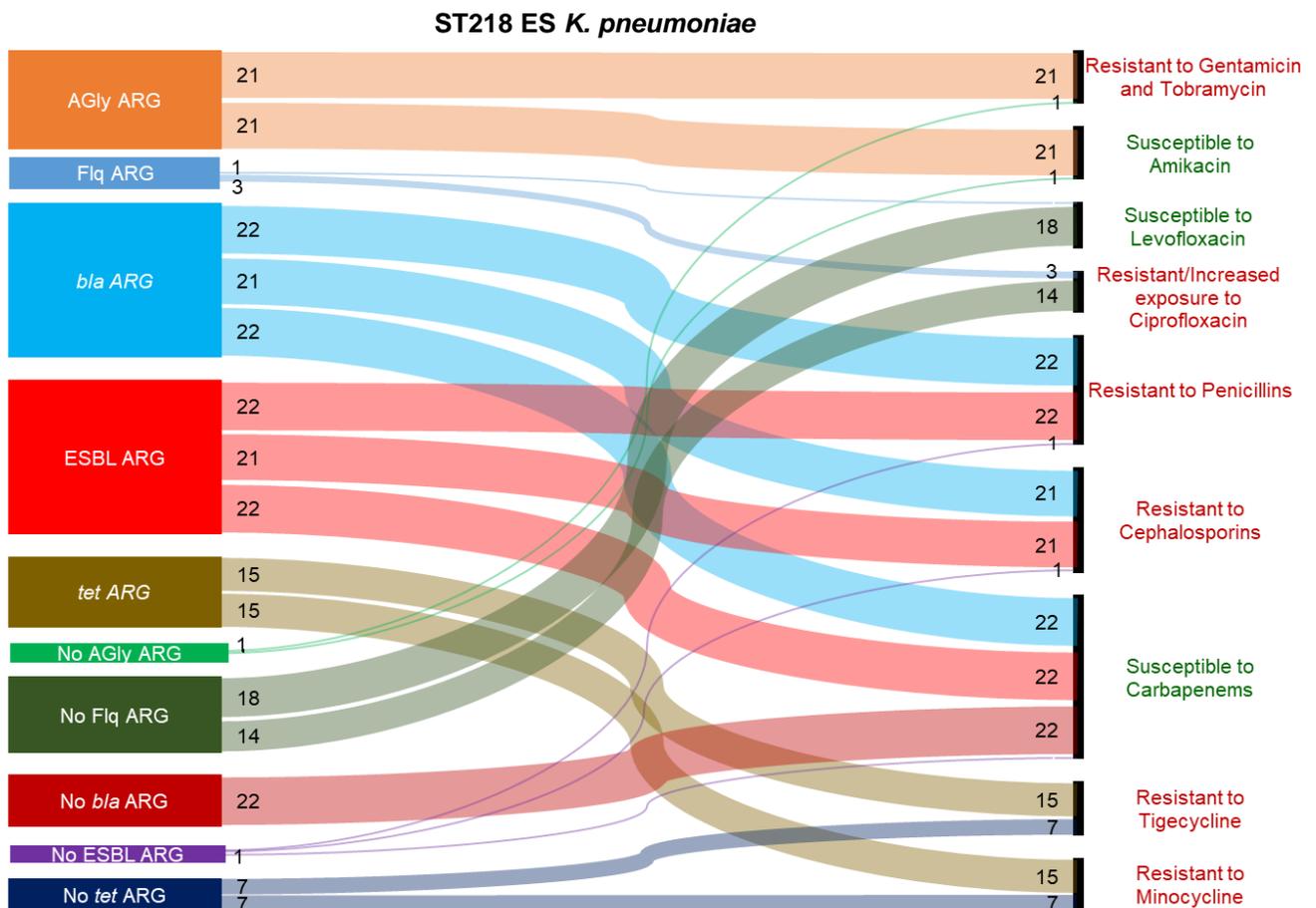


Fig. 26: Sankey diagrams linking ST218 *K. pneumoniae* isolates that carry or not carry ARG with their antibiotic susceptibility profile. The number of isolates that carried ARG (left) are linked to the antibiotic susceptibility profile displayed by these isolates to which each ARG could confer resistance to (right). The number of isolates is indicated in each coloured band, the number of isolates carrying that type of ARG (left) and the number of isolates displaying the respective antibiotic susceptibility (right). The isolates that carried ARG displayed either resistance or increased exposure to the antibiotics that they would confer resistance to and ST218 PP isolates that did not carry ARG showed susceptibility. However, when no Flq ARG were carried 14 ST218 ES isolates resistant/increased exposure to ciprofloxacin but 18 ST218 ES isolates were susceptible to levofloxacin. One ST218 ES isolate was resistant to gentamicin and tobramycin but it did not carry Agly ARG. In addition, ST218 ES isolates either carrying Agly ARG or not were susceptible to amikacin. Seven ST218 ES isolates did

5.4.1 Antibiotic susceptibility and ARG among ST35, ST37 and ST218 ES *K. pneumoniae* – discussion

Taken together, the results above showed that among ES *K. pneumoniae* belonging to the most frequently identified STs displayed differences in relation to antibiotic susceptibility and as expected in ARG harboured as well. As these *K. pneumoniae* were shown to carry ARG associated with resistance to the antibiotics tested. More importantly, looking at antibiotics commonly used for treatment ES *K. pneumoniae* only showed susceptibility to carbapenems, amikacin, fosfomycin and colistin. This is of concern as it seriously reduces available options for treatment. However, ST35 *K. pneumoniae* displayed susceptibility to ciprofloxacin, levofloxacin and tigecycline, and ST218 isolates showed susceptibility to levofloxacin. Conversely, ST37 *K. pneumoniae* showed resistance to ciprofloxacin, levofloxacin and tigecycline. Antibiotic susceptibility profiles could be explained by the ARG harboured by these isolates as revealed through WGS. For example, *rmt* Agly ARG were not found which is in line with the susceptibility to amikacin (165, 166) and cephalosporins resistance was in line with the presence of *bla*_{CTX-M-15}. Also, resistance to antibiotics such as aminoglycosides and cephalosporins could also have been driven by its regular use to treat neonatal sepsis at St Paul's hospital. In fact, gentamicin and cefotaxime were among to the antibiotics usually prescribed to treat *K. pneumoniae* infections (169) between August and December 2017 which was when these ES *K. pneumoniae* were recovered (Fig. 13). Furthermore, despite most ST35 and ST37 isolates being responsible for sepsis and the spread of ARG, such as carbapenemase and ESBL but also Agly and Flq ARG, HGT could have contributed too. (5.6 Plasmid replicon content types harboured by ES *K. pneumoniae*.) This is indicated by the ARG found among ES *K. pneumoniae* from the different sample types of origin, the evidence of transmission seen among ES isolates in a previous section and HGT that can contribute to the spread of ARG among these isolates (5.6 Plasmid replicon content types harboured by ES *K. pneumoniae*). Thus, it is possible for *K. pneumoniae* isolates from the rectal microbiota (MR and BR) and from the clinical environment (ENV) to contribute to the increase of resistance to the antibiotics that showed MIC₅₀ and MIC₉₀ values below the clinical breakpoint. These include antibiotics such as amikacin, ciprofloxacin and levofloxacin which could be used for treatment of infection caused by ST35, ST37 and ST218 *K. pneumoniae*. Amikacin particularly would be the best option as its MIC₅₀ and MIC₉₀ were below the clinical breakpoint. However, these

results raise concern because options for treatment are seriously reduced in St Paul's hospital because carbapenems can be difficult to obtain in LMICs, and amikacin is not available in Ethiopia (169). In addition, not only availability of antibiotics would have to be considered when choosing a treatment but also its safety when treating neonates, for example, fluoroquinolones are not usually prescribed for children due to the possible risk of adverse effects such as gastrointestinal disturbances and should only be used in the case of serious infection (172, 173).

5.5 *In-silico* serotyping of ES *K. pneumoniae*

ES *K. pneumoniae* recovered from ES-BB, ES-BR, ES-MR and ES-ENV were characterised separately to assess differences in virulence determinants among the ES *K. pneumoniae*. This analysis was also performed to understand the genomic virulent traits of ES-BB isolates because virulent *K. pneumoniae* often cause serious infection. Particularly *K. pneumoniae* carrying virulent determinants as hypermucoidy genes, *rmpA* and/or siderophore loci (86).

In relation to the siderophore loci displayed by ES-BB *K. pneumoniae* the most frequently identified was *ybt* carried by 25 (29%) of 86 ES-BB isolates (Fig. 27). Among ES-BB *K. pneumoniae* most commonly identified lineage was *ybt* 9 associated with ICEKp3 (n=10;40%) followed by *ybt* 16 associated with ICEKp12 (n=8;32%) and *ybt* 13 associated with ICEKp2 (n=3;12%). *iro* and *iuc* loci were carried concurrently by nine (36%) ES-BB and identified as *iro* 1 and *iuc* 1 lineages. Likewise, among ES-BR (n=64) and ES-MR (n=30) the most common siderophore loci was *ybt* found in 28 (44%) and 10 (16%) isolates, respectively (Fig. 27). However, ES-BR *K. pneumoniae* more frequently harboured an unknown *ybt* (n=10;36%) than *ybt* 13 associated with ICEKp2 (n=9;32%) and *ybt* 9 associated with ICEKp3 (n=9;32%), which were commonly found as well among ES-BB isolates. Nevertheless, *iro* 1 (n=8;29%) and *iuc* 1 (n=8;29%) loci lineages were identified among ES-BR isolates as in ES-BB *K. pneumoniae*. In relation to ES-MR the most frequent siderophore loci was *ybt* (n=10) similarly to ES-BB and ES-BR *K. pneumoniae* but in contrast the displayed *ybt* loci most frequently by ES-MR isolates was an unknown *ybt* locus (n=4;40%), followed by *ybt* 9 associated with ICEKp3 (n=3;30%), also commonly identified among ES-BB isolates, and *ybt* 10 associated with ICEKp4 (n=3;30%) which was harboured by one ES-BB isolate too. *iro* 1 (n=4;21%) and *iuc* 1 (n=4;21%) loci lineages were identified among ES-MR isolates as well. Additionally, one ES-MR isolates carried an unknown *iuc* loci lineage. The siderophore loci harboured by 4 (57%) ES-ENV *K. pneumoniae* (Fig. 27) included *ybt* 16 associated with ICEKp3 (n=2;50%) and *ybt* 9 associated with ICEKp3 (n=2;50%). And *iro* 1 and *iuc* 1 loci lineages were carried simultaneously by two (50%) ES-ENV isolates that also harboured *ybt* 9. These being the same lineages found among ES-BB *K. pneumoniae* suggesting that ES-ENV *K. pneumoniae* could have contributed to the spread from the clinical environment of these virulence determinants to ES-BB *K. pneumoniae*.

Lastly, ES-BB *K. pneumoniae* harboured the *rmpA* associated with KpVP-1 and *rmpA2* genes these were carried by three (3%) and six (3.5%) ES BB isolates, respectively. ES-BB harboured *rmpA_2* (n=3) and *rmpA_1* (n=1). In comparison, ES-BR harboured *rmpA_2* (n=6;9%) and *rmpA_1* (n=1;1.5%) and *rmpA2_2* was carried by seven (10%) ES-BR isolates, thus, among ES-BR these virulence determinants were found more commonly. Additionally, the *rmpA_2* associated with KpVP-1 and *rmpA2_8* were carried by two (7%) ES-MR isolates, simultaneously and two (28.5%) ES-ENV *K. pneumoniae* carried *rmpA_1* and *rmpA_2* associated with KpVP-1, respectively, both carried *rmpA2_8*.

In relation to the KLs (Fig. 28) and OLs (Fig. 28) identified 11 different KL and five different OL were identified among ES-BB isolates (n=86). Among the KL showed by ES-BB the most frequently identified was KL108 (n=33;38%) followed by KL15 (n=25;37%) and KL57 (n=10;15%). In comparison, there was more diversity in the KL types in the ES-BR (n=68) isolates with 18 different KLs and 10 different OLs. Additionally, KL25 (n=7;10%) and KL15 (n=6;9%) were displayed by ES-BR isolates as well. In relation to ES-MR *K. pneumoniae* there was also large diversity with 15 different KLs detected. The most frequently identified KLs among ES-MR isolates included KL64 (n=5;17%), followed by KL57 (n=4;13%), which was shown by ES-BB isolates as well, and KL20 (n=3;10%). Also, ES-ENV (n=7) commonly showed KL108 (n=2;28.5%), KL57 (n=2;28.5%) and KL15 (n=1;14%), that were displayed among ES-BB *K. pneumoniae* too, in addition, ES-ENV displayed KL39 (n=2;28.5%) as well.

Regarding OLs displayed by ES-BB *K. pneumoniae* the most commonly shown were O1v2 (n=41;48%) followed by O4 (n=26;30%) and O2v2 (n=10;12%). ES-BR (n=15;22%) and ES-MR (n=6;20%) also displayed O2v2 among the most frequent OL of these sample types. However, the most frequent OL among ES-BR included other OL such as O1v1 (n=11;16%) and O2v1(n=10;15%) and ES-MR frequently displayed O2v1 (n=9;30%) and O1v1 (n=4;13%). In addition, ES-BR and ES-MR showed a higher number of different OLs showing 10t and seven different OLs, respectively.

ES *K. pneumoniae* siderophore loci distribution per sample type

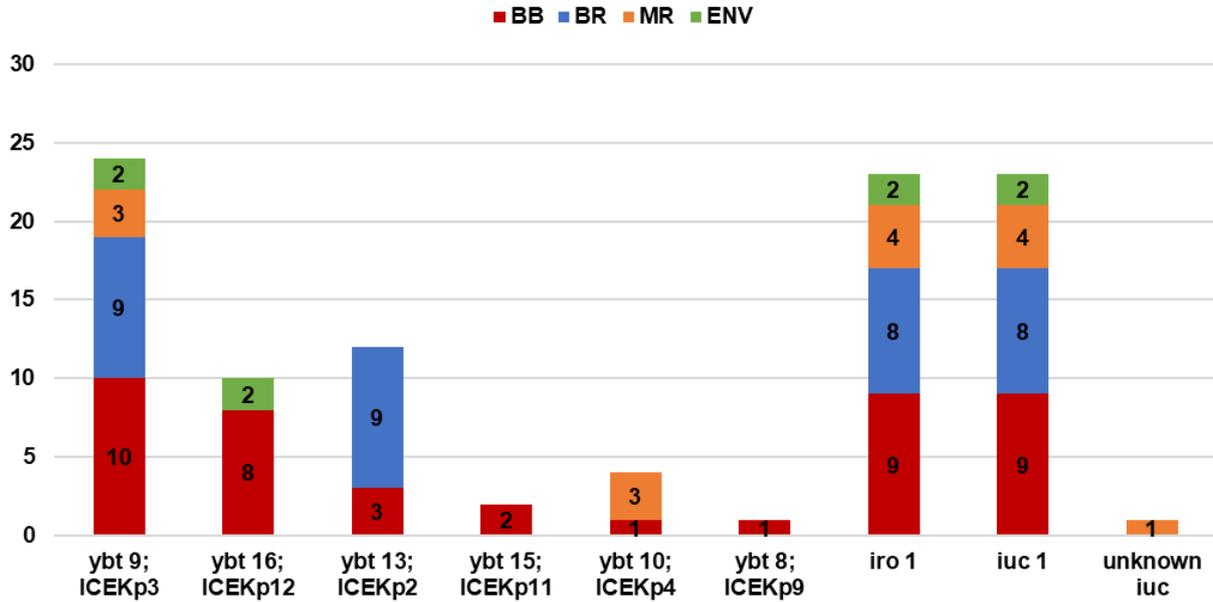


Fig. 27: Distribution of acquired siderophore loci lineages carried by ES-BB, BR, MR and ENV *K. pneumoniae*. *ybt9; ICEKp3* was the most common *ybt* lineage (n=24) among ES isolates being carried by ES-BB (n=10), ES-BR (n=9), ES-MR (n=3) and ES-ENV (n=2). *iro 1* and *iuc 1* were carried by ES-BB (n=9), ES-BR (n=8), ES-MR (n=4) and ES-ENV (n=2).

5.5.1 *In silico* serotyping of ES-BB, BR, MR and ENV *K. pneumoniae* - discussion

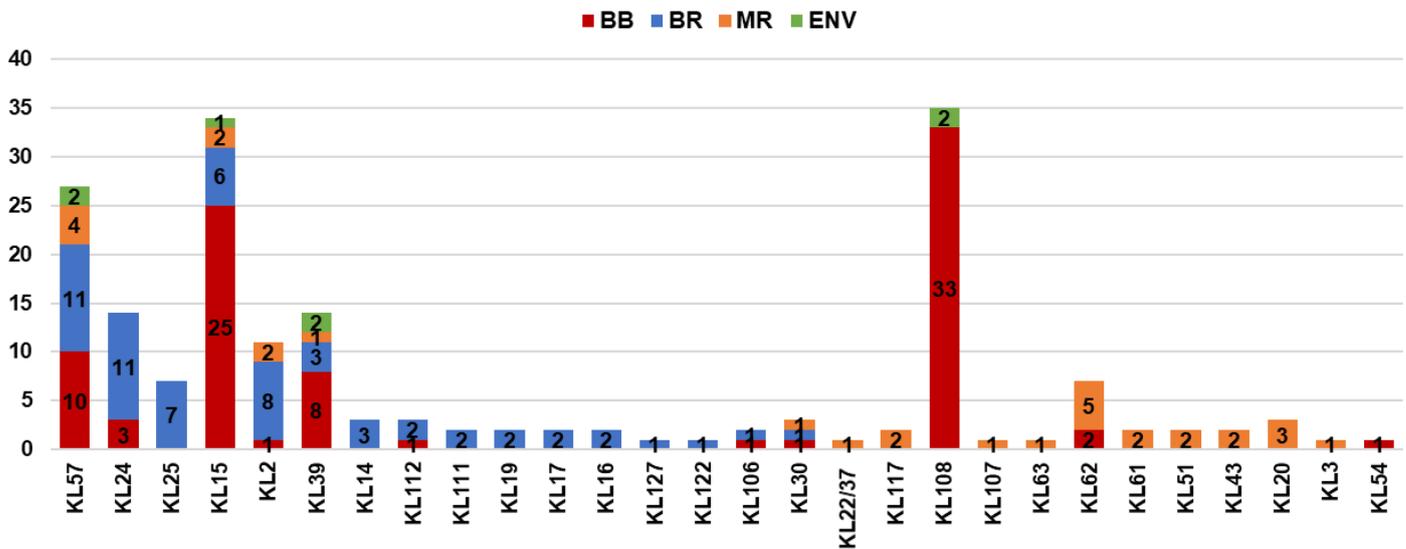
In summary, ES *K. pneumoniae* displayed 30 different KLs among which the most common were KL15, KL108 and KL57, and these were displayed by ES *K. pneumoniae* recovered from all different sample types in study (Fig. 28). However, considering the most common KL among the different sample types of origin was distinct since ES-BB showed KL108, ES-BR KL24 and ES-MR KL62 with only ES-ENV (n=2) showing KL108 the same as ES-BB *K. pneumoniae*. Different distribution of OL was also displayed among ES isolates (Fig. 28). ES isolates carried 11 different loci that included O1v1, O2v2 and O1/O2v1 as the most common, and, likewise, among the different sample type of origin the most frequent OL was different. Because ES-BB *K. pneumoniae* showed O1v1, ES-BR O2v2, ES-MR O2v1 with only ES-ENV (n=3) showing O1v1 the same as ES-BB *K. pneumoniae*. KL loci play an important role in helping *K. pneumoniae* establishing infection and OL loci in protecting *K. pneumoniae* from the immune system (86) (1.7 *K. pneumoniae* virulence determinants).

Moreover, among the common KL, shown by ES *K. pneumoniae* (n=191;100%), KL1 and KL2 associated with hvKP strains (59) were not commonly found. Nevertheless, KL2 was shown by one (0.5%) ES-BB, eight ES-BR (4%) and two (1%) ES-MR indicating that *K. pneumoniae* are present in the microbiota of mothers and neonates. This is important because ES *K. pneumoniae* found in this study are community recovered isolates, seeing that the samples were taken at admission to hospital. Moreover, because hvKP often cause community acquired infection the results indicated that *K. pneumoniae* with the potential to cause severe infection was circulating in the community (93) (1.8 Infections caused by virulent *K. pneumoniae*).

Furthermore, among, ES-BB, BR, MR and ENV *ybt*, *iro* and *iuc* siderophore loci were found too *ybt* being the most commonly found, in addition, *rmpA* and *rmpA2* were found among these isolates too. Nevertheless, *ybt*, *iuc* and *iro* loci were more commonly harboured by ES-BB and ES-BR this of concern because these were *K. pneumoniae* that caused sepsis and found among the microbiota of neonates. Virulence determinants such as siderophore loci and *rmpA* have been associated with hypervirulent infection caused by *K. pneumoniae* (93, 174). Moreover, these virulence determinants allow *K. pneumoniae* to better survive within the host thus improving

ability to cause serious infection which is namely of concern among ES-BB belonging to ST218 (n=22) (5.1 Phylogeny and population of ES *K. pneumoniae*) that carried *ybt*, *iuc* and *iro* in addition to *rmpA* hypermucoidy determinants indicating that these could be hypervirulent (86, 88). More importantly when considering the outcome of sepsis caused by ST218 ES-BB (n=9) only in three (33%) cases the outcome was alive with four (44%) deceased and two (22%) untraceable. Taken together these results possibly indicate that sepsis caused by these ES isolates can be severe and possibly exacerbated by the presence of ARG such as *bla*_{CTX-M-15} and Agly ARG harboured by ST218 *K. pneumoniae* reducing the treatment options. In addition, less common KL, such as KL64 and KL62, have been described among antimicrobial resistant *K. pneumoniae* causing infection (175, 176), and in this PhD study KL62 was found within *K. pneumoniae* from ES-MR.

ES *K. pneumoniae* KL distribution per sample type



ES *K. pneumoniae* OL distribution per sample type

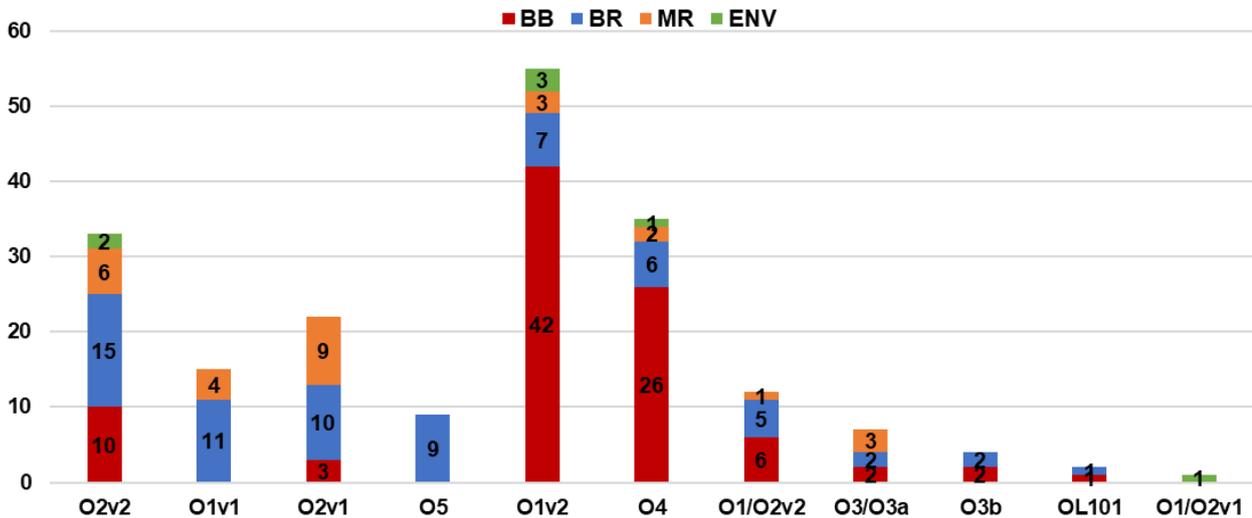


Fig. 28: Distribution of common KL and OL among ES *K. pneumoniae* from different sample types of origin. The most frequently detected KL was KL108 (n=35) followed by KL15 (n=34) and KL57 (n=27). Among ES-BB *K. pneumoniae* the most frequent KL was KL108 (n=33) followed KL15 (n=25) and KL57 (n=10). ES-BR *K. pneumoniae* frequently carried KL57 (n=11) and KL24(n=11) followed by KL2 (n=8). Among ES-MR the most common KL was KL62 (n=5) followed KL57(n=5) and KL20 (n=3). ES-ENV *K. pneumoniae* frequently carried KL57 (n=6), KL39 (n=2) and KL108 (n=2) followed by KL15 (n=1). The most frequently detected OL was O1v2 (n=55) followed by O4 (n=35) and O2v2 (n=33). In relation to OL among the distinct sample types of origin, ES-BB *K. pneumoniae* most commonly displayed O1v2 (n=42), followed by O4 (n=26) and O2v2 (n=10); ES-BR isolates frequently displayed O2v2 (n=15) followed by O1v1 (n=11) and O2v1 (n=10). Among ES-MR *K. pneumoniae* commonly carried O2v1 (n=9) followed by O2v2 (n=6) and O1v1 (n=4) and among ES-ENV isolates the most common OL was O1v2 (n=3) followed O2v2 (n=2) and O4 (n=1).

5.6 Plasmid replicon types harboured by ES *K. pneumoniae*

In total, 93 ES *K. pneumoniae* carried Col type plasmids and 187 carried Inc type plasmid replicons. In addition, two ES harboured trfA replicons and one ES isolate carried rep7_1_repC cassette. Fig. 29 summarises common plasmid replicon types harboured by ES *K. pneumoniae* across the different sample types.

ES-BB (n=84)	ES-BR (n=66)	ES-MR (n=30)	ES-ENV (n=7)
Col	Col	Col	Col
IncF	IncF	IncF	IncF
IncH	IncH	IncH	IncH
IncR	IncR	IncR	IncR
IncQ	IncQ	IncQ	IncQ
IncN	IncN		

Fig. 29: Types of common plasmid replicons harboured by *K. pneumoniae* from Ethiopia among the different sample types of origin ES-BB, ES-BR, ES-MR and ES-ENV-

Col type plasmids harboured by ES *K. pneumoniae*

Seven different Col type plasmid replicons found among the total ES *K. pneumoniae* included replicons ColRNAI (n=115;53%), Col(MGD29) (n=44;20%), Col(Ye4449) (n=41;19%), Col(BS512) (n=10;5%), ColpVC (n=3;1%), Col156 (n=1;0.4%) and Col8282 (n=1;0.4%) . Also, these replicons were found among 93 ES *K. pneumoniae* recovered from different sample types of origin being harboured by ES-BB (n=44;47%), ES-BR (n=34;36.5%), ES-MR (n=12;13%) and ES-ENV (n=3;3%). Also, the distribution of different Col type replicons was distinct across sample types of origin among ES *K. pneumoniae* (Fig. 30).

Inc type plasmids harboured by ES *K. pneumoniae*

Inc type replicons carried by ES *K. pneumoniae* included IncF, IncH, IncN, IncQ and IncR replicons. In total 187 ES *K. pneumoniae* (n=84;45% ES-BB, n=66;35% ES-BR, n=30;16% ES-MR and n=7;4% ES-ENV) carried IncF (n=542) replicons (Fig. 30). Regarding, IncFIA(HI1) replicons (n=69;37%) were more often found in BB and BR *K. pneumoniae* isolates (n=34;18% ES-BB, n=23;12% ES-BR, n=9;5% MR and n=2;0.5% ES-ENV). IncH, IncR, IncQ and IncN replicons were also detected within ES *K. pneumoniae*, however, these were found in lower numbers comparatively.

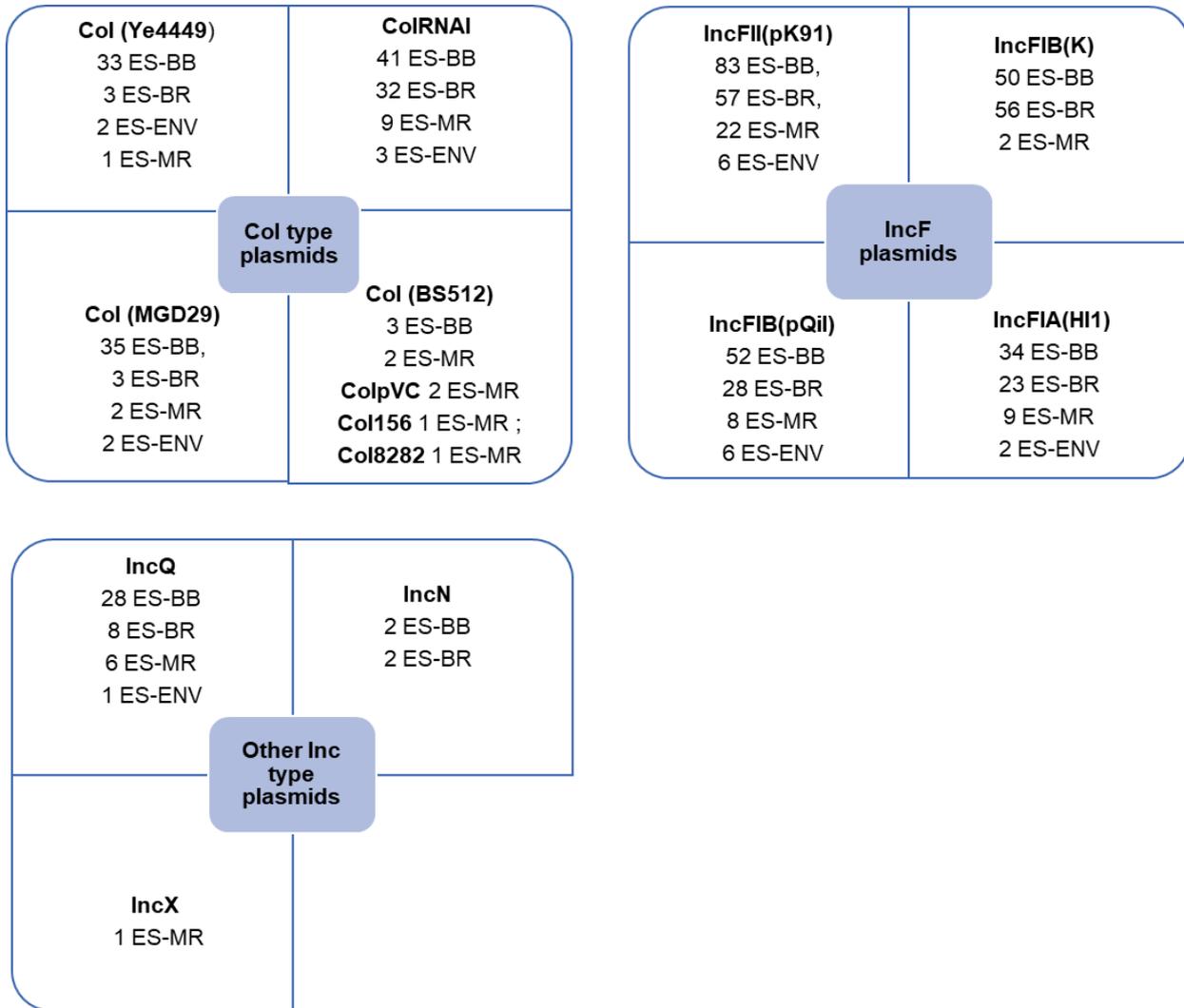


Fig. 30: Col and Inc type plasmid replicons distribution among the ES *K. pneumoniae* across the different sample types of origin. Col type replicons were found among ES-BB, ES-BR and ES-ENV *K. pneumoniae* but were mostly found among ES-BB isolates, with ColRNAI being the most frequently found among ES-BB (n=41), ES-BR (n=32, ES-MR (n=9) and ES-ENV (n=3). **IncF plasmids** were the most commonly found among ES *K. pneumoniae* with IncFII(pK91) being found among 83 ES-BB, 57 ES-BR, 22 ES-MR and 6 ES-ENV. **Other Inc type** plasmids included IncQ, IncN and IncX plasmids, with the most frequently found being IncQ harboured by 28 ES-BB, 8 ES-BR, 6 ES-MR and one ES-ENV.

5.6.1 Inc type plasmids carrying *bla*_{CTX-M-15} dissemination among ES *K. pneumoniae*

Within ES-BB *K. pneumoniae* it was noticeable that most blood culture isolates (BB) carried *bla*_{CTX-M-15}. Plasmids often carry ARG which implies they are important in the dissemination and spread of AMR via mechanisms including HGT. Thus, I studied if similar plasmids harbouring *bla*_{CTX-M-15} were carried by the ES *K. pneumoniae* belonging to the dominant ES STs.

This analysis used the complete plasmid sequences carrying *bla*_{CTX-M-15} harboured by ST37 ES-BB30 to understand if these were among ST37, ST35 and ST218 ES *K. pneumoniae* and among ES *K. pneumoniae* belonging to other STs (5.1 Phylogeny and population of ES *K. pneumoniae*). Initial analysis of the genome assembly (short read and long read sequencing hybrid) through Bandage showed that ES-BB30 harboured two plasmids carrying *bla*_{CTX-M-15}. These will be referred to in these section as pKpnST37_ESBB_1 (187kb) and pKpnST37_ESBB_2 (128kb) (Fig. 31). In addition, pKpnST37_ESBB_1 and pKpnST37_ESBB_2 plasmids ARG and replicon are listed in Appendix 11. Although, these plasmids were selected for a preliminary assessment of the dissemination of *bla*_{CTX-M-15}, these plasmids also carried other ARG. pKpnST37_ESBB_1 harboured ARG including, for example, *bla*_{TEM-1} (Fig. 31; Appendix 12). Agly ARG such as *aph3-1a*, *aac3-11a* and Flq ARG *qnrS1*. In contrast, pKpnST37_ESBB_2 despite carrying *bla*_{CTX-M-15} as well, it harboured other ARG such as *bla*_{OXA-1}, *bla*_{OXA-10} and *tetA* ARG which were not carried by pKpnST37_ESBB_1 (Fig. 31). Thus, although both plasmids were harbouring *bla*_{CTX-M-15} the ARG screening on the plasmid sequences also indicated that these could be responsible for the spread of resistance to β -lactams and other antibiotics simultaneously such as aminoglycosides. When screening the plasmid sequences against plasmidfinder, pKpnST37_ESBB_1 showed IncFIB, IncQ and IncH replication systems whereas pKpnST37_ESBB_2 showed IncR and IncFII replication systems. This is important because it suggested that these plasmids had different conjugative abilities (189) that could influence the spread of *bla*_{CTX-M-15} not only among ES-BB *K. pneumoniae* but also across ES-BR, ES-MR and ES-ENV isolates.

In order to perform a preliminary and exploratory assessment of whether the same plasmids were present in the ES *K. pneumoniae* population, both pKpnST37_ESBB_1 and pKpnST37_ESBB_2 sequences were used as to build a blast database with. Then, these were used to screen for the presence of the plasmids

(pKpnST37_ESBB_1 and pKpnST37_ESBB_2) by assessing the coverage and identity of hits produced (from the database) against the short read contigs of all ES *K. pneumoniae* using Abricate. However, seeing that the ES *K. pneumoniae* isolates sequences are short read sequences and the plasmids used as reference are long read sequences there would be numerous hits to numerous contigs of the ES *K. pneumoniae* isolates short read sequences. Thus, only contigs matches to pKpnST37_ESBB_1 and pKpnST37_ESBB_2 with over 10% of coverage and >95% identity were considered for these results. Considering the total ES *K. pneumoniae* and for pKpnST37_ESBB_1 in relation the total ES *K. pneumoniae* were found among ES-BB (n=16) sequences contigs, and results showed matches among ES-BR (n=15), ES-MR (n=3) and ES-ENV pKpnST37_ESBB_2 contigs matches were found among a total of 95 *K. pneumoniae* among ES-BB (n=54), among ES-BR (n=34), ES-MR (n=5) and ES-ENV (n=2) *K. pneumoniae* sequences contigs as well showing average percentage of coverage of 19%. Results also suggesting that pKpnST37_ESBB_2 probably spread among ES *K. pneumoniae* from different sample types of origin.

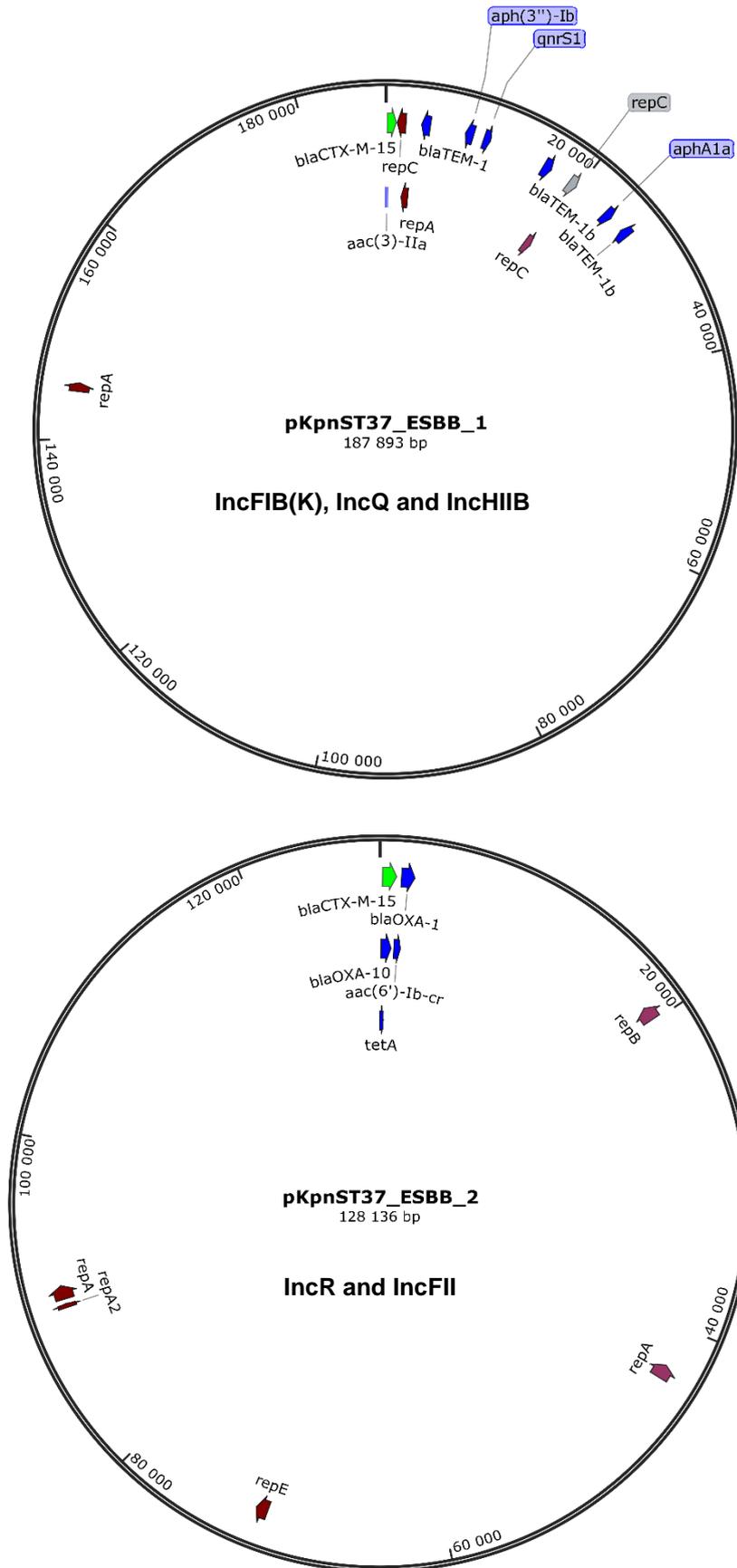


Fig. 31: ST37 ES-BB30 pKpnST37_ESBB_1 and pKpnST37_ESBB_2. pKpnST37_ESBB_1 had a size 187 893 bp with IncFIB(K),IncQ and IncHIIB. The ARG harboured included *bla*_{CTX-M-15}, *bla*_{TEM-1}, *aph3-^{''}Ib*, *aphA1a*, *aac(3)-IIa* and *qnrS1*. pKpnST37_ESBB_2 had 128 136 bp with IncR and IncFII. The ARG harboured included *bla*_{CTX-M-15}, *bla*_{OXA-1}, *bla*_{OXA-10} and *tetA*.

Inc type plasmids carrying *bla*_{CTX-M-15} dissemination among ES ST35, ST37 and ST218 *K. pneumoniae*

The presence of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 was studied among ST35, ST37 and ST218 ES *K. pneumoniae* separately as these formed major clusters within the ES *K. pneumoniae* population. Amongst ST37 *K. pneumoniae* the average coverage among contigs matches to pKpnST37_ESBB_1 sequence was 13%. These contigs were found among ES-BB (n=13;37%), ES-BR (n=4;11%), ES-MR (n=1;3%) and ES-ENV (n=1;3%) in a total of 19 (54%) ES ST37 *K. pneumoniae* with an average of coverage of 14%. In contrast, contigs hits to pKpnST37_ESBB_1 among ST35 and ST218 ES *K. pneumoniae* coverage was lower than 10% in overall with the highest percentage of coverage found was 2% and 4%, respectively suggesting that pKpnST37_ESBB_1 was probably not harboured by ES *K. pneumoniae* belonging to these STs. The highest percentage of coverage was seen among ST37 ES *K. pneumoniae* (24%) with coverage of contigs showing percentages of coverage between 73% and 10%. Nevertheless, these percentages of coverage were found mostly found among ST37 ES-BB (n=25;67.5%) *K. pneumoniae*, followed by ES-BR (n=6;17%) and ES-MR (n=1;3%) *K. pneumoniae* isolates with the highest percentages of coverage being found among ES-BB *K. pneumoniae*.

Also, when screening for pKpnST37_ESBB_2 among ES ST218 *K. pneumoniae* contigs the percentage of average coverage (12%) was lower than among ST35 and ST37 *K. pneumoniae* contigs, however, contig hits to pKpnST37_ESBB_2 were found among ES-BB (n=6;27%), ES-BR (n=6;27%), ES-MR (n=1;4.5%) and ES-ENV (n=1;4.5%). Likewise, pKpnST37_ESBB_1 contigs harboured by ES *K. pneumoniae* belonging to other STs commonly belonged to ST45 including seven ES-BR and two ES-BB.

Thus, considering these results both plasmids carrying *bla*_{CTX-M-15} were disseminated among ES *K. pneumoniae* belonging to the different sample types of origin, despite being more common among ES-BB *K. pneumoniae*. However, distribution of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 was different regarding, ES *K. pneumoniae* belonging to ST35 and ST218 because results suggested that these probably did not harbour pKpnST37_ESBB_1 whereas pKpnST37_ESBB_2 was probably carried by ES *K. pneumoniae* belonging to ST35, ST37 and ST218.

A blast type screening approach of the ES *K. pneumoniae* to find hits of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 using contigs generated from short-

read sequences did produce hits, however, these are likely to include low coverage and identity matches and may not evidence the presence of the sample plasmid.

Therefore, in addition, to the first approach, I used the long-read sequences of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 plasmids and mapped these against the putative plasmid scaffolds generated from short-read sequences of ST35, ST37 and ST218 ES *K. pneumoniae* using plasmidSPAdes. This analysis was performed to better understand if the same plasmids were harboured among the ST35, ST37 and ST218 ES *K. pneumoniae* and was restricted to these STs because these represent the major ST groups responsible for the majority of sepsis cases in St Paul's Hospital.

Mapping pKpnST37_ESBB_1 and pKpnST37_ESBB_2 to ES ST35, ST37 and ST218 *K. pneumoniae*

By mapping the plasmid sequences/reads of *K. pneumoniae* isolates against the reference plasmids, I was able to understand if the same plasmids are present in isolates of the same ST group, and if those plasmids were conserved/similar across isolates of different STs (i.e. ST37, ST35 and ST218).

Concerning ES ST37 *K. pneumoniae* (n=34;100%) from ES-BB (n=27;79%), BR (n=5;15%) and MR (n=2;6%) samples of origin. The visualization of the alignment to pKpnST37_ESBB_1 (Fig. 32) showed that this plasmid was harboured by ST37 from different sample types from BB (n=23;68%), BR (n=5;15%), and MR (n=2;6%) samples but not from ENV samples. There was low variation in relation to pKpnST37_ESBB_1 as shown in Fig. 32. When pKpnST37_ESBB_2 (Fig. 33) was aligned to ST37 ES-BB *K. pneumoniae* short-reads, I found that ST37 BB isolates (n=26;76%), the plasmid was recovered from the read mapping. However, alignment to ES-MR ST37 plasmid putative scaffolds was not possible because no reads were mapped. The alignment to ST37 ES-BR *K. pneumoniae* (n=5;15%) showed high variation (read bases that did not match the reference) in relation to pKpnST37_ESBB_2 indicating that either this plasmid was not harboured by these isolates, or another similar plasmid was present. However, this was not possible to ascertain through this analysis.

In summary it is also important to consider that 2166 reads among ES ST37 *K. pneumoniae* were mapped to pKpnST37_ESBB_1 (Fig. 32) with mean coverage of 0.34 and in relation to pKpnST37_ESBB_2 (Fig. 33) the number of reads aligned to this plasmid was of 1416 with a mean coverage of 0.4.

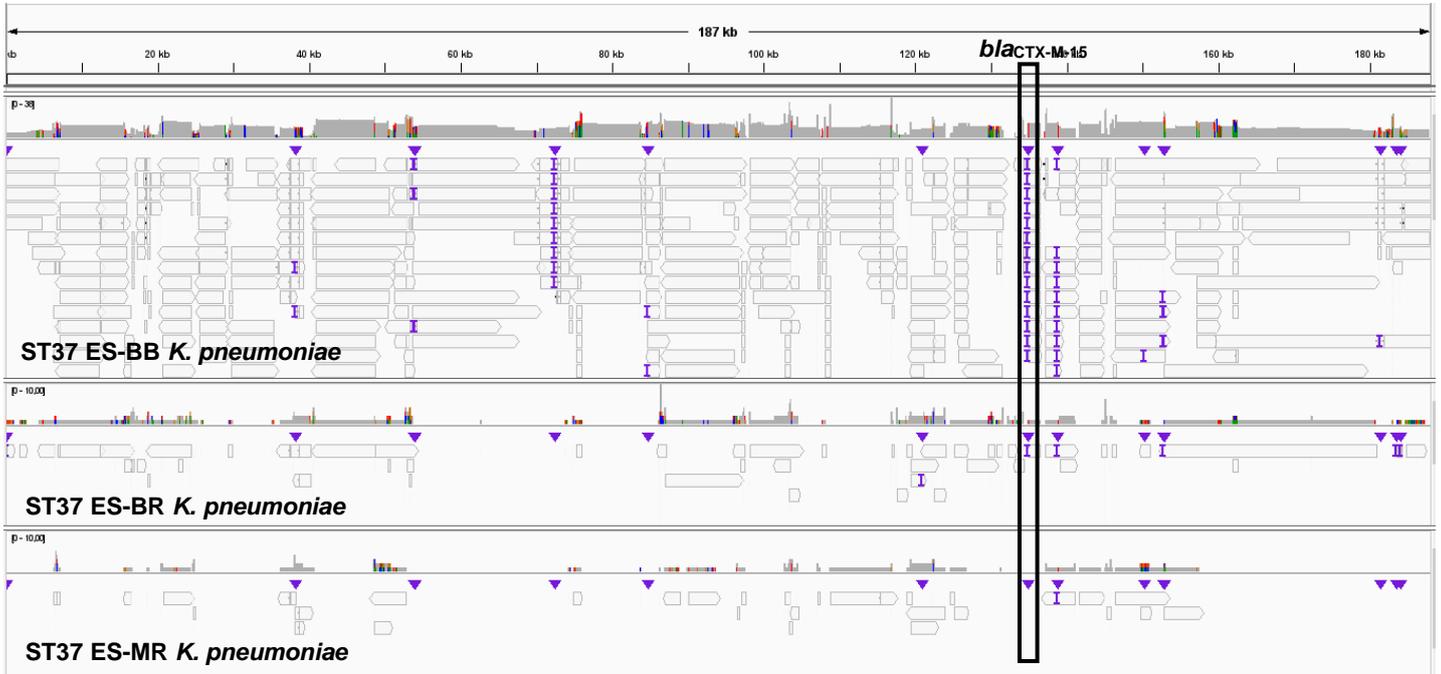


Fig. 32: ST37 *K. pneumoniae* putative plasmid scaffolds alignment using pKpnST37_ESBB_1 (187 kb) as reference. ST37 ES-BB, ST37 ES-BR and ST37 ES-MR *K. pneumoniae* alignment to pKpnST37_ESBB_1 showed that high number of reads were aligned of pKpnST37_ESBB_1 and across its length, particularly among ST37 ES-BB. Among ST37 ES-MR and ES-ENV isolates reads aligned were in lower number but these were found also across the reference plasmid. The alignment indicates that pKpnST37_ESBB_1 was present among ST37 *K. pneumoniae* from ES-BB, ES-BR and ES-MR samples. Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). The black rectangle indicates the region of pKpnST37_ESBB_1 (137504 bp to 138379bp) in which *bla*_{CTX-M-15} was found. The purple arrows and I indicate insertions. Dark grey represents the alignment coverage.

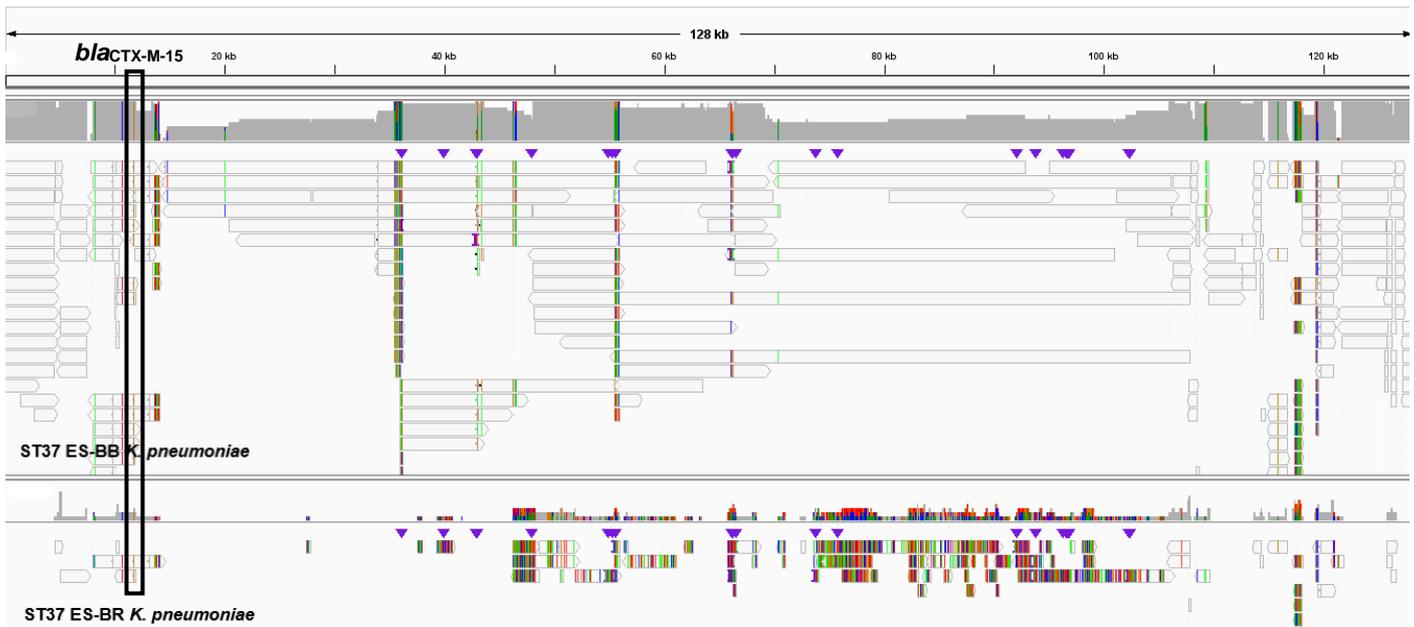


Fig. 33: ST37 *K. pneumoniae* putative plasmids scaffolds alignment using pKpnST37_ESBB_2 (128 kb) as reference. ST37 ES-BB and ST37 ES-MR *K. pneumoniae* alignment to pKpnST37_ESBB_2 showed that high number of large reads were aligned of pKpnST37_ESBB_2 and across its length, particularly among ST37 ES-BB. Among ST37 ES-BR isolates reads aligned were in lower number and showed higher variation than the reads aligned among ES-BB *K. pneumoniae*. The alignment indicates that pKpnST37_ESBB_2 was present among ST37 *K. pneumoniae* from ES-BB, ES-BR but not among ES-MR samples (read bases that did not match the reference). The black rectangle indicates the region of pKpnST37_ESBB_2 (10815bp to 11691bp) in which bla_{CTX-M-15} was found. Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation. The purple arrow indicates insertions. Dark grey represents the alignment coverage.

Among ST35 ES-BB short read putative plasmid scaffolds alignment to pKpnST37_ESBB_1 (Fig. 34) and pKpnST37_ESBB_2 (Fig. 35) the number of mapped reads was of 1069 with mean coverage of 0.05 and 1527 with mean coverage of 0.25. By visualising the alignment of ST35 ES *K. pneumoniae* putative plasmids scaffolds to pKpnST37_ESBB_1 and pKpnST37_ESBB_2 it was possible to further indicate that pKpnST37_ESBB_1 was not carried by ES ST35 *K. pneumoniae* contrary to pKpnST37_ESBB_2 that was harboured by ES ST35 isolates considering that larger reads were aligned to pKpnST37_ESBB_2 among ES ST35 showing regions without variation being indicated. Additionally, the alignment of putative plasmid scaffolds found among ST35 ES-ENV showed that these were not aligned to pKpnST37_ESBB_1 and pKpnST37_ESBB_2 sequences suggesting that these were not present in the clinical environment of St Paul's Hospital. Nevertheless, these were only two ES-ENV isolates belonging to ST35 and these plasmids could still be harboured by other ES-ENV isolates, as was seen among ST218 ES-ENV *K. pneumoniae*. In contrast to, ES-ENV ST37 isolates to which pKpnST37_ESBB_1 and pKpnST37_ESBB_2 were not aligned.

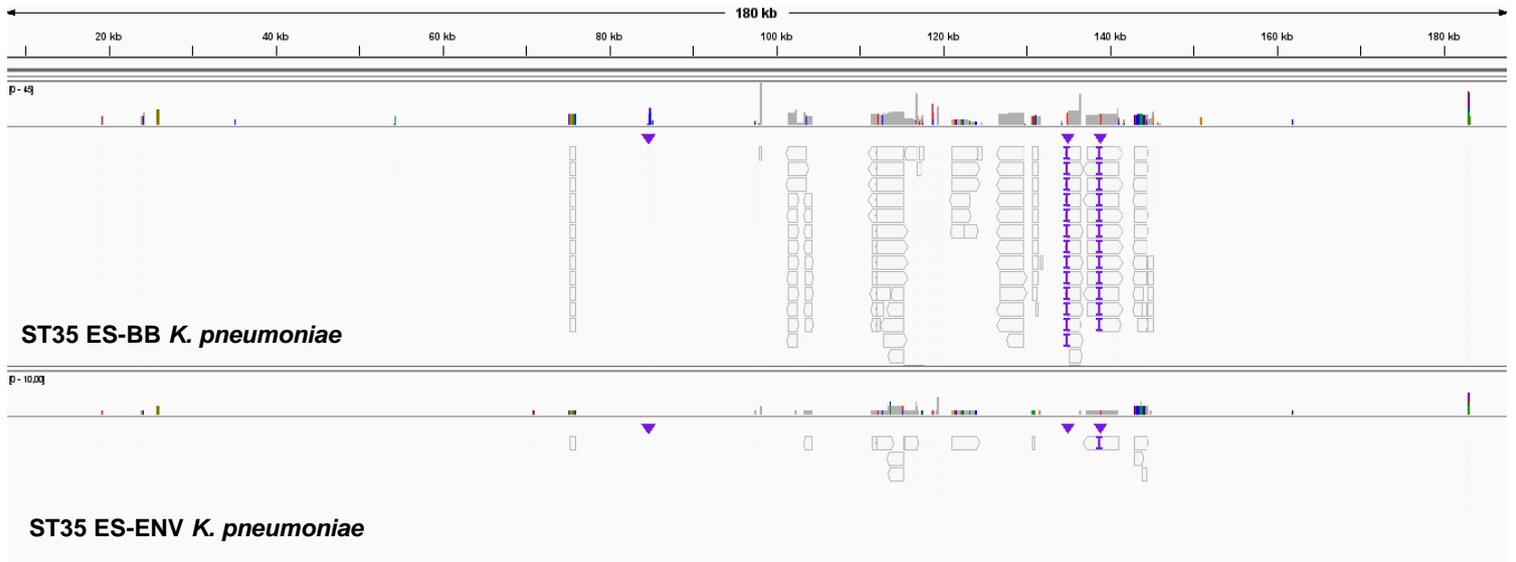


Fig. 34: ST35 ES *K. pneumoniae* putative plasmid scaffolds alignment using pKpnST37_ESBB_1 (187kb) as reference. ST35 ES-BB and ST35 ES-ENV *K. pneumoniae* alignment to pKpnST37_ESBB_1 showed that small reads were aligned to pKpnST37_ESBB_1 indicating this plasmid was not present among ST35 ES isolates. The small reads aligned were found in higher number among ST35 ES-BB than ES-ENV. Several regions with variation in relation to pKpnST37_ESBB_1 sequence was present (highlighted by different colours). Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). The purple arrows and | indicate insertions. Dark grey represents the alignment coverage.



Fig. 35: ST35 *K. pneumoniae* putative plasmids scaffolds alignment using pKpnST37_ESBB_2 (128kb) as reference. Alignment to pKpnST37_ESBB_2 showed that high number of reads were aligned to pKpnST37_ESBB_2 and across its length, particularly among ST35 ES-BB. Among ST35 ES-ENV isolates reads aligned were in lower number but these were found also across the reference plasmid. The alignment indicates that pKpnST37_ESBB_2 was present among ST37 *K. pneumoniae* from ES-BB, ES-ENV samples despite displaying areas of high variation alongside areas few variations. The black rectangle indicates the region of pKpnST37_ESBB_2 (10815bp to 11691bp) in which *bla*_{CTX-M-15} was found Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). The purple arrows and | indicate insertions. Dark grey represents the alignment coverage.

Regarding, ES ST218, despite the alignment to pKpnST37_ESBB_1 (Fig. 36) of putative plasmids scaffolds being obtained only small contigs were mapped among ES-BB, ES-BR and ES-ENV belonging to this ST. Alignment of pKpnST37_ESBB_2

(Fig. 37) showed frequent variation among putative plasmids scaffolds, however, similar plasmids scaffolds could be harboured by these isolates. So, these results suggest that pKpnST37_ESBB_1 was not found among ST218 ES-ENV *K. pneumoniae*. Also, the number of aligned reads to pKpnST37_ESBB_1 and pKpnST37_ESBB_2 was 1005 mean coverage of 0.12 among ES ST218 *K. pneumoniae*.

As mentioned previously, screening of the ST35, ST37 putative plasmids scaffolds using the resfinder database available through Abricate revealed that *bla*_{CTX-M-15} was harboured among these so considering the alignments in Figs. 32-37 together these suggest that pKpnST37_ESBB_1 and pKpnST37_ESBB_2 played a role in spreading *bla*_{CTX-M-15} particularly among ES ST37 *K. pneumoniae* when considering the regions of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 where *bla*_{CTX-M-15} was not mapped among all ES ST35, ST37 and ST218 plasmid scaffolds contigs. In summary, pKpnST37_ESBB_1 was indicated not to be harboured by ES ST35 and ST218 putative plasmid scaffolds through screening of these scaffolds using pKpnST37_ESBB_1 as the reference database. Contrarily, pKpnST37_ESBB_2 was found among ST35 putative plasmids scaffolds of ES-BB and ES-ENV isolates. Also, regarding ST218 ES *K. pneumoniae* pKpnST37_ESBB_1 and screening of ST218 putative plasmids scaffolds indicated that both pKpnST37_ESBB_1 was carried by ST218 *K. pneumoniae*.

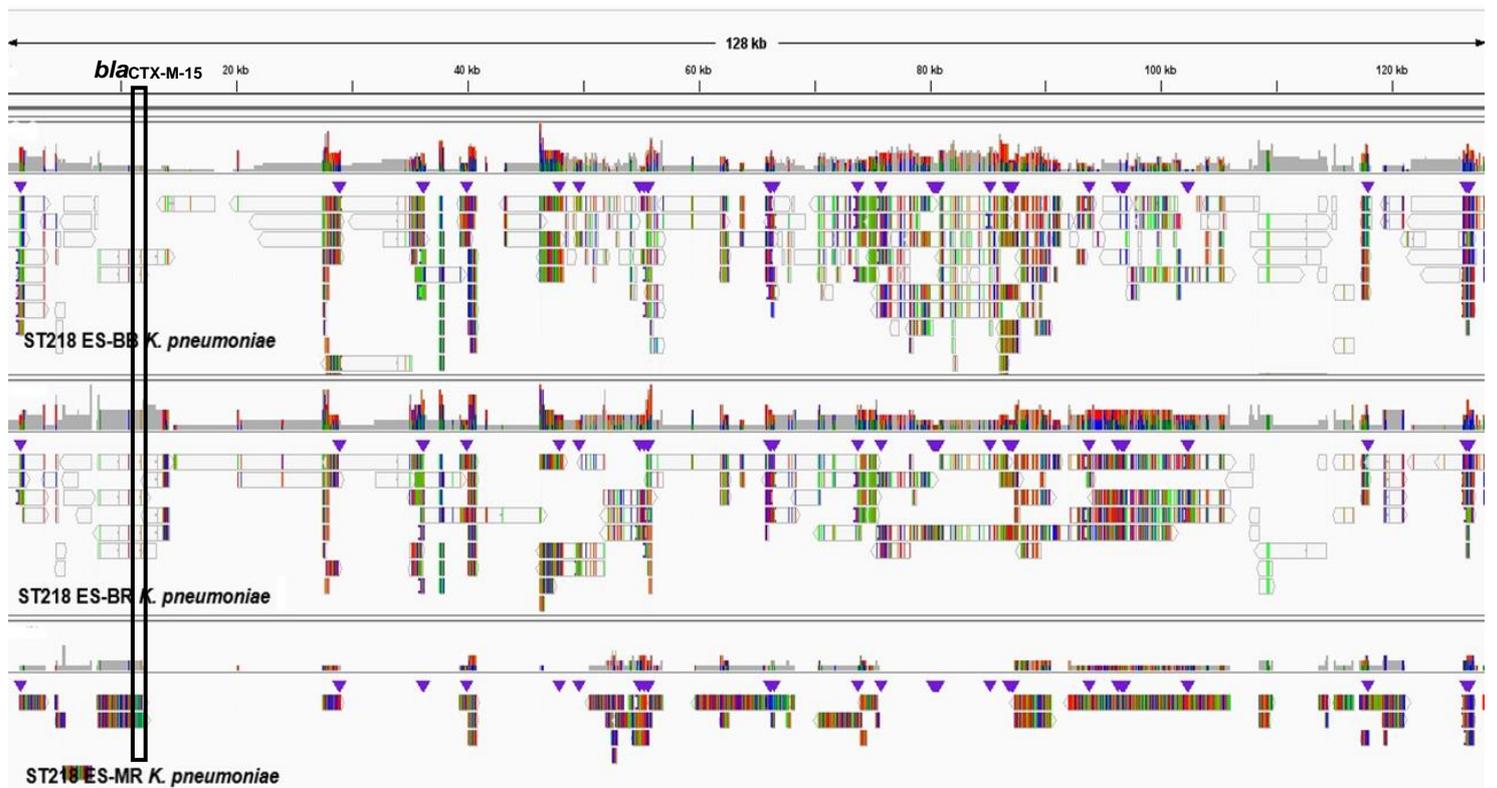


Fig. 36 ST218 *K. pneumoniae* putative plasmids scaffolds alignment using pKpnST37_ESBB_2 (128kb) as reference. Alignment of ST218 isolates from the different sample types of origin was displayed across the reference plasmid indicating that pKpnST37_ESBB_2 was found among ST218 *K. pneumoniae* despite variation being displayed. Alignment to reference plasmid is more frequent among ST218 ES-BB *K. pneumoniae* than among ST218 ES-BR and ES-MR. The black rectangle indicates the region of pKpnST37_ESBB_2 (10815bp to 11691bp) in which *bla*_{CTX-M-15} was found Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). Dark grey represents the alignment coverage.

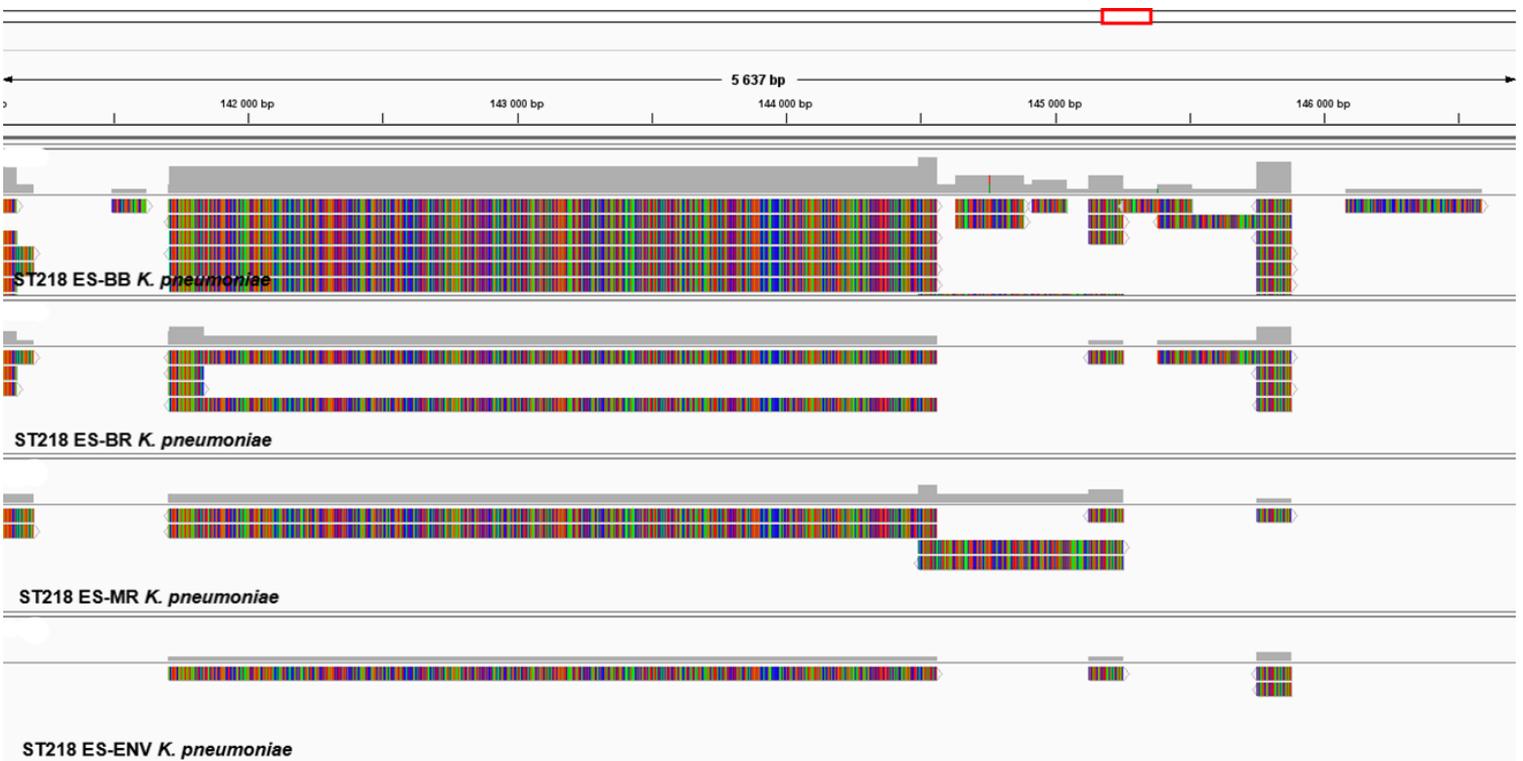


Fig. 37: ST218 *K. pneumoniae* putative plasmids scaffolds alignment using pKpnST37_ESBB_1 (187kb) as reference. Alignment of ST218 from the different sample types of origin was displayed in small regions of the reference plasmid and with high variation indicating that pKpnST37_ESBB_1 was not found among ST218 *K. pneumoniae*. Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). Dark grey represents the alignment coverage.

5.7 Assessing relationships between ARG, virulence determinants and clinical data among ES *K. pneumoniae*

The impact of ARG and acquired siderophore loci was studied among the dominant STs responsible for sepsis in St Paul's Hospital. Among ES *K. pneumoniae* resistance to β -lactams including cephalosporins in this PhD was high and ES *K. pneumoniae* belonging to major STs all carried *bla*_{CTX-M-15} (5.4 Antibiotic susceptibility and ARG among ES *K. pneumoniae* belonging to ST35, ST37 and ST218). So, the presence of this ARG was studied to understand if it affected the outcome of sepsis caused by ST35, ST37 and ST218 ES-BB *K. pneumoniae* as these were responsible for most sepsis cases at St Paul's Hospital. Likewise, the presence of siderophore loci influence on the outcome of sepsis was studied too.

The outcome of sepsis among ES-BB (n=68;100%) belonging to ST35, ST37 and ST218 was in majority alive (n=45;66%) and the onset of sepsis EOS (n=47;69%) (Fig. 38).

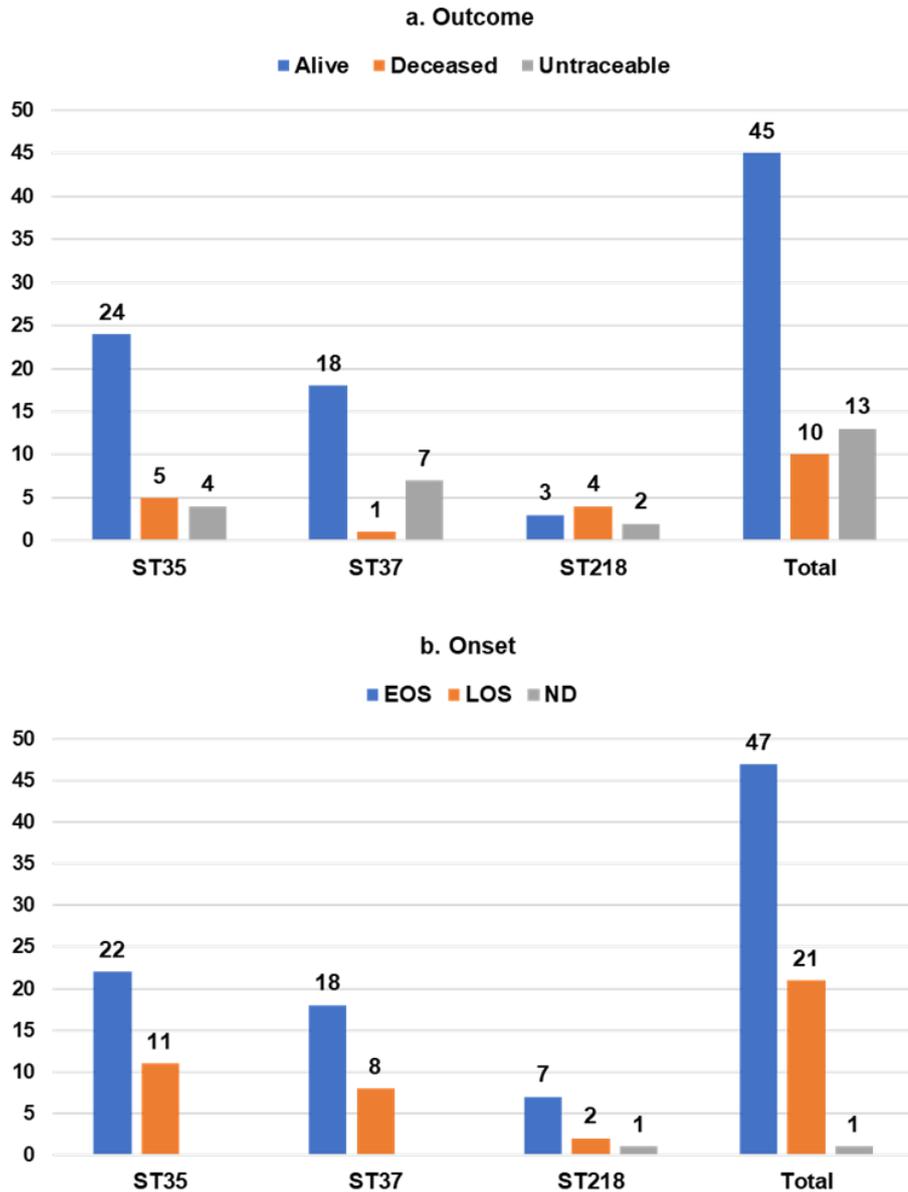


Fig. 38: Outcome and onset of sepsis caused by ES-BB *K. pneumoniae* belonging to ST35, ST37 and ST218 and in total. a. In total the outcome was in majority alive for 45 cases followed by deceased for 10 cases and for 13 cases the outcome was untraceable for 13 cases. Among sepsis cases caused by ST35 ES-BB *K. pneumoniae* the outcome of most cases was alive (n=24) and cases with outcome deceased and untraceable were five and four, respectively. Among sepsis caused by ST37 ES-BB isolates the outcome was in majority alive (n=18) and deceased (n=1) and untraceable (n=7). In relation to sepsis caused by ST218 ES-BB the outcome was deceased (n=4), alive (n=3) and untraceable (n=2). **b.** The onset of sepsis among ES-BB *K. pneumoniae* was in majority EOS (n=47) followed by LOS (n=21) and not determined (ND; n=1). Among sepsis caused ST35 ES-BB *K. pneumoniae* the onset in majority EOS (n=22) and LOS (n=11) and sepsis caused by ST37 ES-BB isolates the onset was EOS (n=18) and LOS (n=8). Among sepsis caused by ST218 the onset of infection was EOS (n=7), LOS (n=2) and not determined (ND; n=1)

ST35, ST37 and ST218 *K. pneumoniae* were studied using Kaplan-Meier statistics to assess the cumulative survival of neonates that were diagnosed with sepsis caused by *K. pneumoniae* belonging to these STs. Fig. 41 shows that neonates suffering from sepsis caused by ST35 (97%, 94%, 90% and 80%) and ST37 (96%) showed higher percentages of survival than neonates that suffered from sepsis caused by ST218 *K. pneumoniae* (86%, 51% and 26%). Nevertheless, the high

percentages of cumulative survival among these neonates were expected seeing that most neonates survived sepsis caused by these isolates. With most deaths occurring among neonates suffering from sepsis caused by ST35 (n=5) and ST218 (n=4) which is in line with the cumulative survival decrease among sepsis caused by ST35 and ST218.

In addition, the presence of *bla*_{CTX-M-15} carried by ST35, ST37 and ST218 ES *K. pneumoniae* was studied in relation to the outcome of sepsis. The survival plot in (Fig. 39) shows the percentage of cumulative survival was high in the presence of *bla*_{CTX-M-15}, which is not surprising seeing that all ST35, ST37 and ST218 isolates carried this ARG, and most neonates survived sepsis caused by these isolates (Fig. 38). However, when studying if the presence of siderophore loci influenced the outcome of sepsis, it was possible to see that the percentages of cumulative survival were lower when siderophore loci were carried by isolates causing sepsis (Fig. 39). Probably reflecting the neonates that died because of sepsis caused by ST218 *K. pneumoniae* which all carried siderophore loci. In contrast to ST35 and ST37 that in majority did not carry siderophore loci (5.1 Phylogeny and population of ES *K. pneumoniae*). This is also in line with lower percentage of survival shown when sepsis was caused by ST218 isolates. Hence, these results suggested that the siderophore loci could have affected the outcome of sepsis.

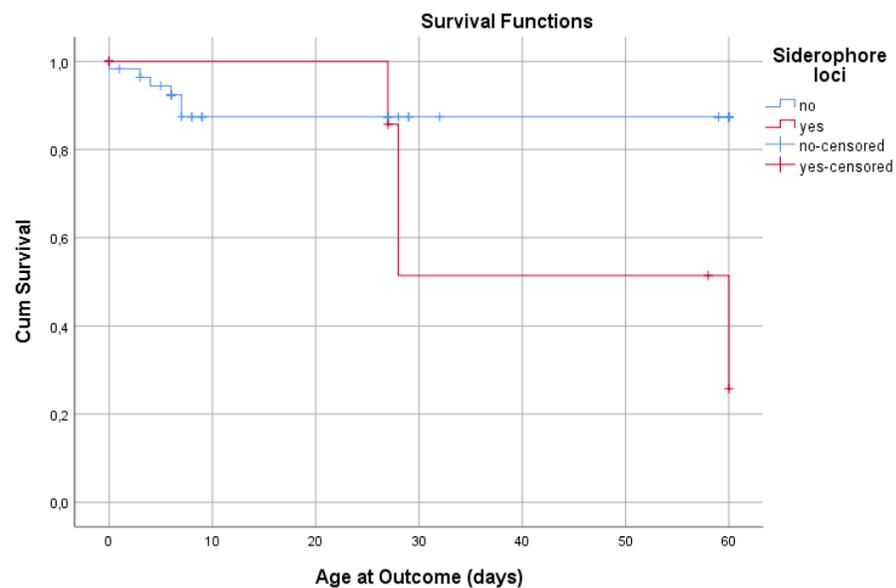
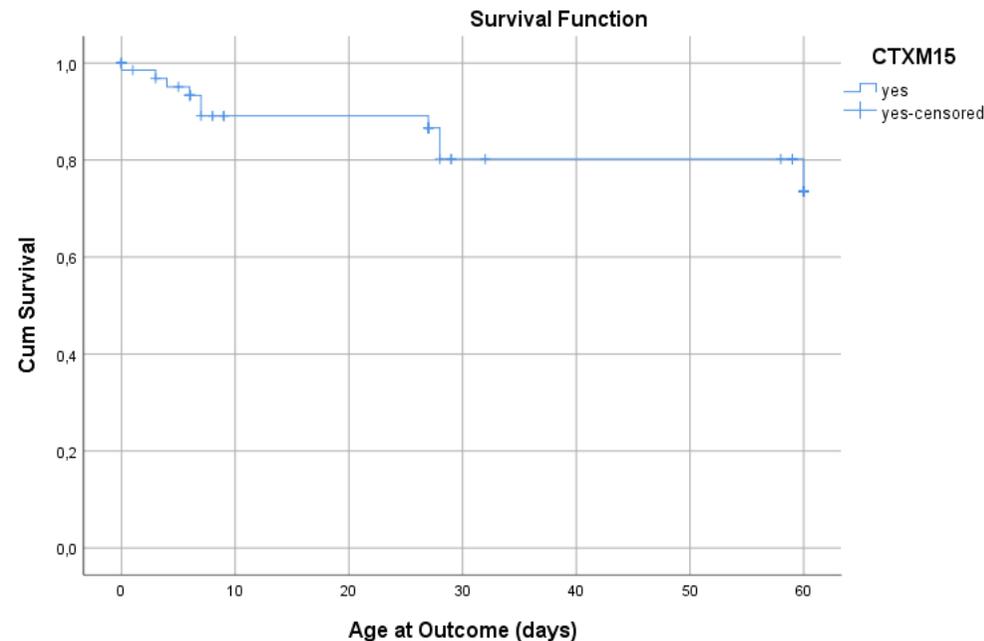
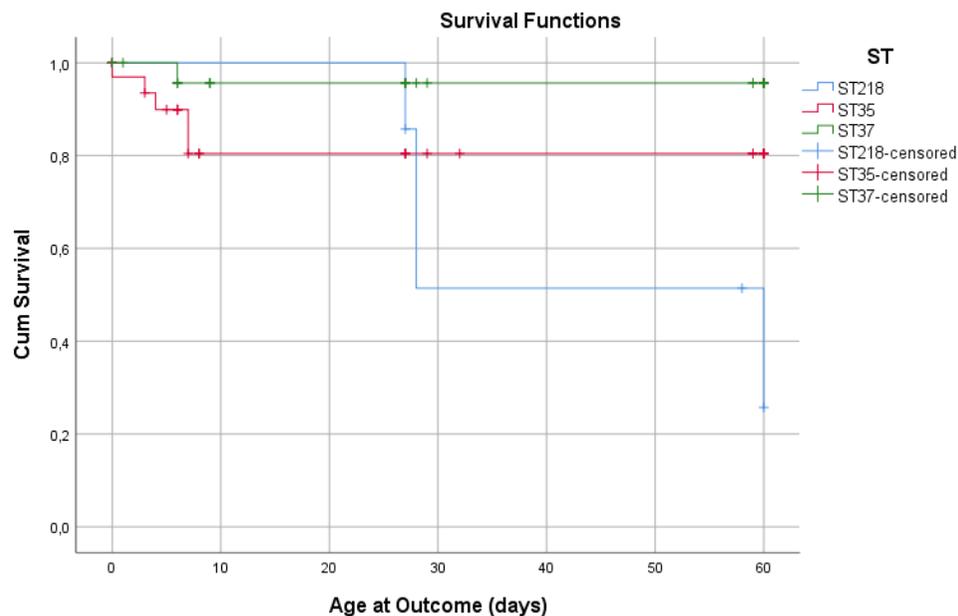


Fig. 39: Cumulative survival plots for sepsis caused by ST35, ST37 and ST218 *K. pneumoniae* and for the presence of $bla_{CTX-M-15}$ (CTXM15) and for the presence of siderophore loci at the age of outcome days. For the ST survival plot the cumulative survival percentage for ST218 (blue line) was 86%, 51%, 26%; for ST35 (red line) was 97%, 94%, 90 and 80% and for ST37 (green line) was 96%. High percentages of cumulative survival among these neonates were expected seeing that most neonates survived sepsis caused by these isolates. With most deaths occurring among neonates suffering from sepsis caused by ST35 and ST218. For the presence of $bla_{CTX-M-15}$ the percentages of cumulative survival were of 99%, 97%, 95%, 93%, 89%, 86%, 80% and 74%. The high percentages of survival while carrying of $bla_{CTX-M-15}$ (yes; blue line) , were in line with the fact that most neonates survived sepsis caused by these isolates, despite carrying this ARG. For the presence of siderophore loci (yes; line) was 86%, 51% and 26% and when siderophore loci were not present (no; red line) 96%, 94%, 92% and 87%. This percentages were lower when siderophore loci were carried by isolates possibly because of neonates that died due to sepsis caused by ST218 *K. pneumoniae* which all carried siderophore loci. Cross in blue (no-censored) and red line (yes-censored) indicate where data relating to the sepsis age at outcome was not available, this is indicated in the lines corresponding to the presence or absence of the studied ARG and siderophore loci. For the ST plot cross in blue (ST218-censored), red line (ST35-censored) and green line (ST37-censored) indicate where age at outcome data relating to sepsis caused by isolates belonging to these STs was not available

6 Results - *K. pneumoniae* from PIMS, Pakistan

6.1 Phylogeny and population of PP *K. pneumoniae*

The phylogeny of *K. pneumoniae* from Pakistan (n=284;100%) is depicted in Fig. 40. The pangenome of PP *K. pneumoniae* population was constituted by 2297 core genes, found in 99% to 100% of the isolates, and 68, 449 accessory genes, in a total of 70, 746 genes.

One major cluster was formed by ST15 *K. pneumoniae* (n=87;31%) and two other smaller clusters were formed by ST29 (n=16;6%) and ST20 (n=15;5%) *K. pneumoniae* from Pakistan. The other isolates belonged to 85 different STs (Appendix 3, 4 and 11). ST15, ST20 and ST29 were the most frequent STs found among these isolates.

The ST15 *K. pneumoniae* (n=87;100%) cluster was composed by closely related isolates from BB (n=22;25%), BR (n=41;47%), MR (n=9,10%) and ENV(n=15;17%) isolates (Fig. 42). ST20 isolates among BR and ENV *K. pneumoniae* were shown to be closely related and MR isolates were also present within the ST20 cluster. The same is shown for ST29 *K. pneumoniae* though ST20 and ST29 *K. pneumoniae* did not include BB isolates. *K. pneumoniae* included in the phylogeny clustered according to their ST regardless of sample types. As it shown by the phylogenetic tree (Fig. 40) where closely related STs are shown together in close clades and branches of the phylogeny. Fig. 40 also shows the common virulence determinants and ARG harboured among these isolates. For example ST15, ST20 and ST29 *K. pneumoniae* harboured *ybt* loci whereas most the other isolates did not carry these determinants. In addition, KL112 (n=90;32%) was the most frequent KL and often displayed by ST15 isolates (n=87;31%). Also, KL112 was significantly associated with ST15 ($p < 0.001$) in this PhD. The most common OL O1v1 (n=109;38%) carried by 63 (22%) ST15 PP isolates significantly associated with this ST as well. ST15 *K. pneumoniae* in majority harboured *bla*_{NDM-1} and *bla*_{OXA-181} in contrast ST20 and ST29 carried *bla*_{NDM-1} only. In addition, all ST15 *K. pneumoniae* (n=87;100%) carried *OmpK* truncations whereas ST20 (n=15;100%) and ST29 (n=16;100%) did not. *tet* ARG were carried by all ST20 and ST29 *K. pneumoniae* but was rarely seen in. However, Agly ARG were harboured by the majority of PP *K. pneumoniae* (n=197;69%) despite of ST the same being shown for *bla*_{CTX-M-15} (n=191;67%). As well for other ARG such as *bla* ARG with broad spectrums (*bla*_{SHV-11}, *bla*_{SHV-32} and *bla*_{OKP})

and Flq ARG (*qnrS1* and *qnrB1*) and Flq SNPs (*gyrA-83F*, *gyrA-87A*, *parC-80I*). (6.2
Resistome of PP *K. pneumoniae*)



Fig. 40: Core genome phylogeny of the population of *K. pneumoniae* from Pakistan and distribution of the most common ARG, and virulence determinants KL, OL and siderophore loci. The most common STs clustered together despite sample type of origin. A major cluster of PP ST15 isolates was noted and was comprised by phylogenetically similar isolates. PP ST20 and ST29 *K. pneumoniae* comprised other clusters of phylogenetically similar isolates. ST15 PP *K. pneumoniae* in majority carried *bla*_{CTX-M-15}, *bla*_{NDM-1} and *bla*_{OXA-181}; Agly ARG, and, Flq ARG and SNPs and *OmpK* truncations. These isolates all displayed KL112, O1v1 and O1/O2v1 and harboured *ybt* loci in majority. Sample type of the isolates is also shown as circles BB (red), BR (blue), MR (orange) and ENV (green). *tet* indicates the presence of *tet* ARG, Flq the presence of fluoroquinolone ARG and SNPs, Agly the presence of aminoglycoside ARG. *bla* broad spectrum indicates the presence of broad spectrum β -lactamases ARG, *bla*_{CTX-M-15} the presence of *bla*_{CTX-M-15} ARG, *bla*_{NDM-1} the presence of *bla*_{NDM-1} ARG, *bla*_{NDM-1} and *bla*_{OXA-181} the presence of *bla*_{NDM-1} and *bla*_{OXA-181} ARG

SNP analysis of PP ST15

ST15 *K. pneumoniae* (n=87;100%), as the dominant ST group were studied in relation to SNPs to further characterise their relatedness (Fig. 43) and identify possible events of transmission between mother and neonate. To facilitate this analysis a timeline for ST15 PP *K. pneumoniae* (Fig. 42) was created. From February 2016 to November 2017 (across the duration of sampling) ST15 *K. pneumoniae* were recovered PIMS including BB, BR, MR and ENV. BB ST15 *K. pneumoniae* were mostly recovered between February 2016 and June 2016 with three BB isolates being recovered between February and June 2017. Also, most ST15 PP isolates were recovered between June and August 2016. With, ST15 PP-BB were recovered between May and August 2016, PP-BR between February and November 2016 and PP-MR were recovered in March, July and December 2016, indicating that ST15 *K. pneumoniae* were present in the microbiota of neonates and responsible for sepsis during those months (Fig. 42). PP-ENV ST15 *K. pneumoniae* were recovered in June and August 2016 indicating that these were possibly already present in the clinical environment.

Fig. 43 shows that PP ST15 *K. pneumoniae* from the BB, BR, MR and ENV sample type of origin were phylogenetically similar with two (2%) MR isolates, three (3%) PP-BR and two (2%) ST15 PP-BB isolates being less similar. These included PP-MR79 (SNPs=539) and PP-MR87 (SNPs=524) recovered in July and September 2017; PP-BR24 (SNPs=356), recovered in April 2017 and PP-BR13 (SNPs=241) and PP-BR101 (SNPs=356) recovered in June and September 2017, respectively. In addition, PP-BB30 and PP-BB2 recovered in March 2017 and May 2017, respectively, were also less similar showing 180 and 181 pairwise SNPs difference in relation to PP-BR89 which was the reference. Also, among ST15 PP-BB (n=22;25%) the lowest number of pairwise SNPs was shown by PP-BB38 (SNPs=7), PP-BB40 (SNPs=9) and PP-BB41 (SNPs=9). Most ST15 PP-BB isolates showed between 12 and 23 pairwise SNPs difference these isolates being recovered in August 2016 (n=3;3%), July 2016 (n=2;2%) and February 2017 (n=1;1%)(Fig. 41)(Fig. 43). Regarding, ST15 PP-BR (n=41;47%) isolates 36 (41%) PP-BR isolates showed between 5 and 33 pairwise SNPs in relation to the reference. These isolates were recovered between March and November 2016 (n=16;18%) but were also recovered in April and May 2017 (n=3;3%) (Fig. 44)(Fig. 45).

In relation, to ST15 PP-MR (n=9;10%) were recovered in July and August 2016 these included PP-MR31 (SNPs=11), PP-MR82 (SNPs=19), PP-MR30 (SNPs=14), PP-MR18 (SNPs=30) and PP-MR81 (SNPs=192). In 2017, four (n=4.5%) ST15 PP-MR were recovered including PP-MR75 (SNPs=11) recovered in March and PP-MR86 (SNPs=4) and PP-MR87 (SNPs=524) recovered in September 2017. Lastly, ST15 PP-ENV were recovered in May (n=2;2%) and July (n=2;2%) 2016 included PP-ENV11 (SNPs=11) and PP-ENV10 (SNPs=258)(Fig. 42)(Fig. 43).

Phylogenetically similar PP ST15 *K. pneumoniae* were recovered from BB (n=22;25%) , BR (n=35;40%), MR (n=7;8%) and ENV (n=10;11.5%) in PIMS between 2016 and 2017 and these isolates showed between four and 37 pairwise SNPs (n=68;78%). Nevertheless, 16 (18%) ST15 *K. pneumoniae* that were not phylogenetically similar showing between 100 and 539 and also were from the different sample types of origin (BB n=4;4.5%, BR n=5;6%, MR n=3;3%, ENV n=4;4.5%). Nevertheless, suggesting that ST15 phylogenetically similar isolates formed an epidemiological cluster in PIMS during the sampling period.

Also, seeing that no mother-neonate *K. pneumoniae* pairs were found among PP isolates but three ST15 PP-BB and PP-BR isolates pairs related to the same neonate were found. Thus, suggesting the possibility that PP-BR *K. pneumoniae* found among the microbiota of neonates could spread among the neonates' microbiota and/or cause sepsis. Studying these pairs individually it was possible to understand that the PP-BB31/PP-BR61 were the most similar because it showed one pairwise SNP when comparing these two isolates. This pair was followed by the PP-BB42/PP-BR91 pair which showed 29 pairwise SNPs difference between these two isolates. In contrast, the pair of PP-BB6 and PP-BR15 displayed a high number of pairwise SNPs, 84 when comparing both isolates. These results indicate that the ST15 PP-BB *K. pneumoniae* could have been present in neonates' microbiota prior to cause infection.

When considering Fig. 41 showing the timeline of the ST15 PP-BB/PP-BR isolates further suggested that the *K. pneumoniae* causing sepsis were carried by the neonates microbiota and caused sepsis later on. Regarding, the PP-BB31/PP-BR61 pair case, the PP-BB isolate was recovered on the 23/4/2017 and the PP-BR sample collected on 21/4/2017. Thus, the sepsis causing isolate was recovered two days after the PP-BR sample was collected indicating that the PP-BB isolate could have been present in the neonates' microbiota prior to infection which is supported by the low number of pairwise SNPs that these isolates displayed. Likewise, PP-BB6 (23/6/2016) and PP-BR15 (19/6/2016) were recovered in June 2016 with four days difference which suggested that the PP-BB isolate was present in the neonates microbiota prior to causing infection but these were the less similar isolates among the studied pairs. It was not possible to ascertain if the sepsis causing isolate was harboured in neonates' microbiota prior to infection in this case. In the case of PP-BB42 and PP-BR91 pair only the date of recovery of the sepsis causing isolate was available (22/11/2017) therefore it was not possible to know if the PP-BB isolate was among the neonates' microbiota before infection.

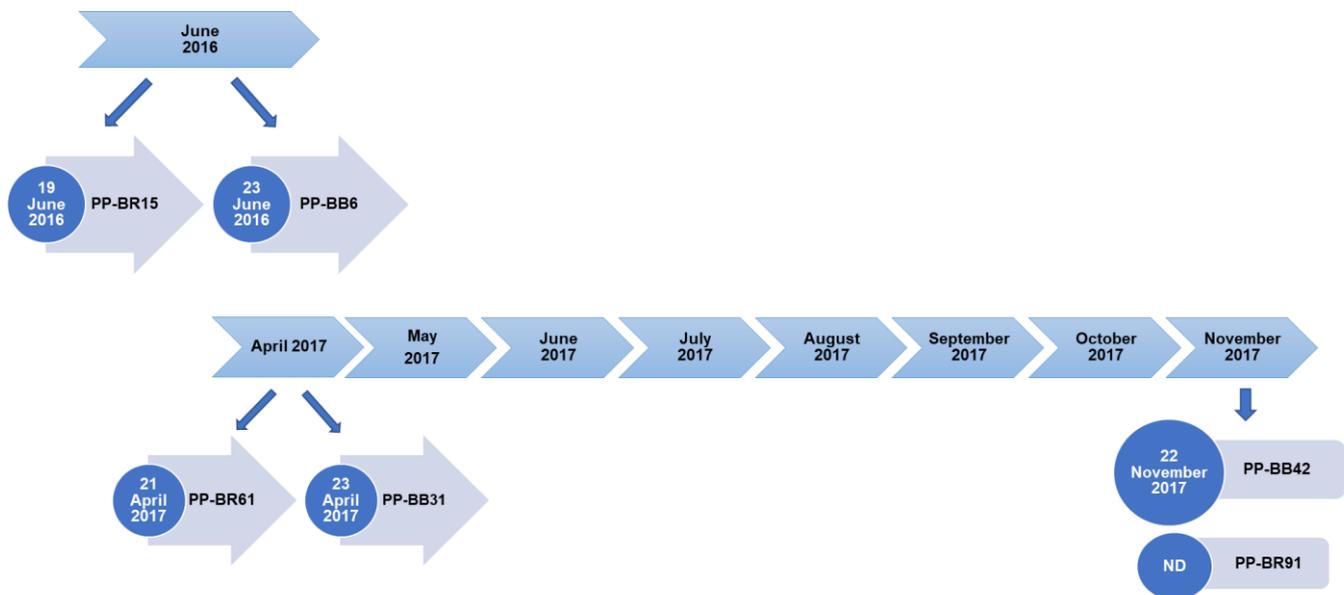


Fig. 41 :Timeline of the ST15 *K. pneumoniae* PP-BB/BR case pairs. The first case samples PP-BB6 and PP-BR15 taken in June 2016. The other case samples being taken in 2017. In April 2017 PP-BB31 and PP-BR61 were taken and in November 2017 PP-BB42 and PP-BR91.

ST15 PP *K. pneumoniae* Timeline including BB (red), BR (blue), MR (orange) and ENV (green)

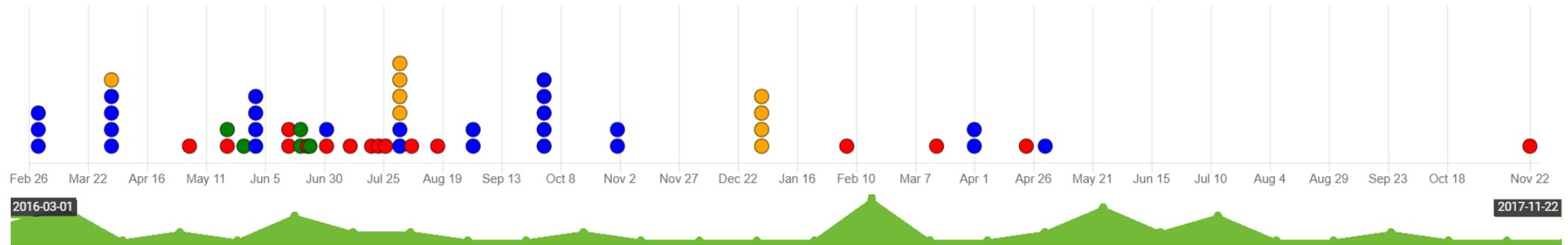


Fig. 42: Timeline of ST15 *K. pneumoniae* from different sample types of origin from January 2016 to November 2017 at PIMS, Pakistan. *K. pneumoniae* belonging to ST15 were firstly isolated from MR and BR samples in February 2016 with ST15 PP-BB *K. pneumoniae* recovered in June 2016 through August 2016 and as well as individual isolates recovered in February, April, and December 2017. In October 2016 only ST15 PP-BR *K. pneumoniae* were isolates and in July and December 2016 ST PP-MR were recovered. In April and May 2017 both ST15 PP-BR and PP-BB were isolated. ST15 PP-ENV *K. pneumoniae* were only isolated in May and June 2016.

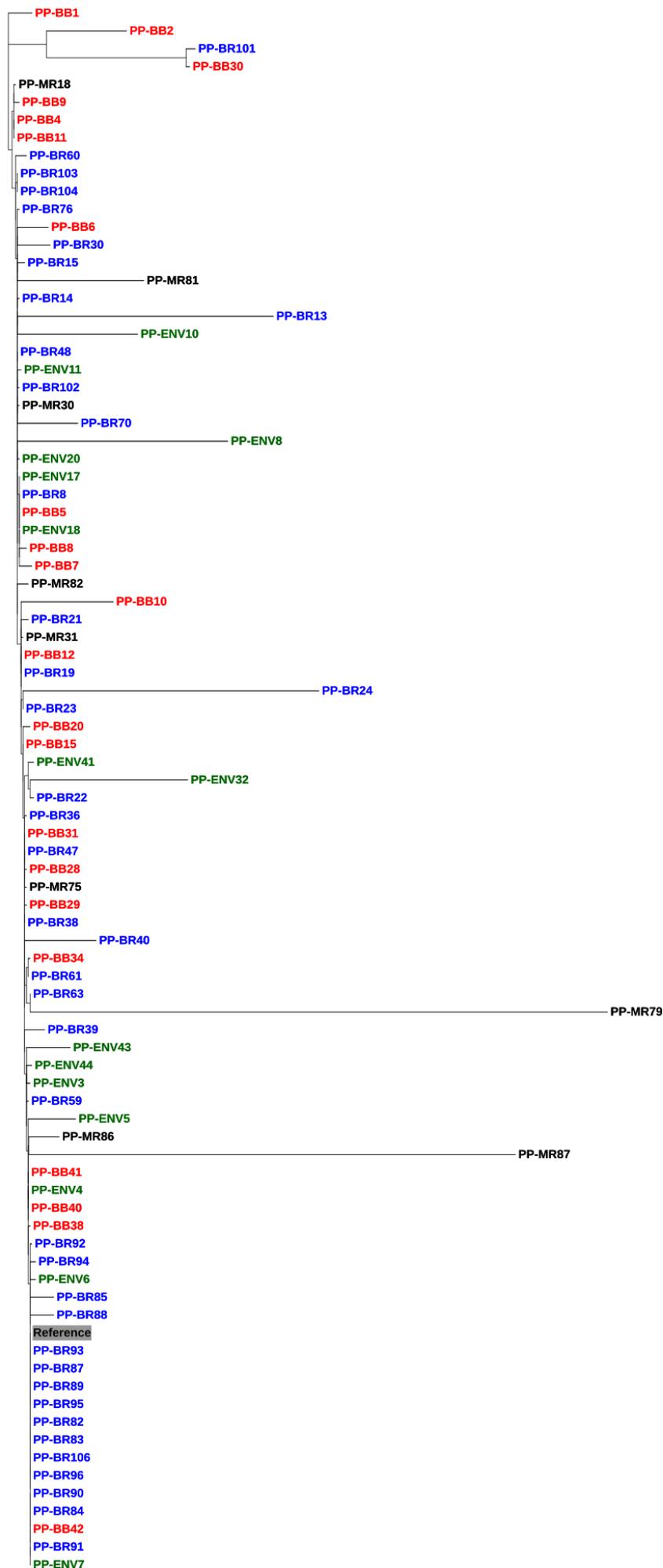


Fig. 43: SNP analysis of ST15 *K. pneumoniae* from Pakistan. ST15 PP *K. pneumoniae* were in overall phylogenetically similar but different clades were formed. With PP-BB, BR and MR and ENV shown to diverge markedly within these clades. PP-BR89 was used as the reference.

6.1.1 Phylogeny of PP *K. pneumoniae* – discussion

Likewise, as for the ES *K. pneumoniae* the genomic relatedness analysis of PP *K. pneumoniae* aimed to provide insight into the *K. pneumoniae* population in PIMS, Pakistan in context of neonatal sepsis looking into the possible transmission between the mothers, neonates, and the clinical environment. In total, 87 of the 284 *K. pneumoniae* recovered across all sample types from Pakistan belonged to ST15. As ST15 was also responsible for the majority of sepsis caused by *K. pneumoniae* in Pakistan, I studied the phylogeny of these ST15 isolates further. PP ST15 *K. pneumoniae* were found across at least an 18-month period between February 2016 and November 2017 with the majority isolated between June to August 2016, and this was the same time period the PP-BR and PP-ENV were found too. This finding was an initial indication that *K. pneumoniae* responsible for infection could have spread amongst the neonates or from the clinical environment.

SNP analysis revealed ST15 PP-BB *K. pneumoniae* encompassed isolates that were closely related indicating ST15 PP-BB *K. pneumoniae* was circulating in the neonates' microbiota seeing since PP-BR89 was used as the reference. The same was suggested among PP-MR and PP-ENV though among these isolates pairwise SNPs number was higher. Thus, *K. pneumoniae* capable to cause sepsis were likely to spread between mothers, neonates, and the clinical environment suggesting that the clinical environment and the mother rectal microbiota might have been a reservoir for ST15 *K. pneumoniae* capable to cause infection. SNP analysis also suggested that ST15 was established in PIMS because isolates belonging to this ST were recovered throughout the sampling period in addition the wide pairwise SNP range could multiple introductions of ST15 isolates and divergence among these isolates. In addition, ST15 was significantly associated with BB ($p=0.001$), BR ($p=0.023$) and with MR ($p<0.001$) further supporting that ST15 was established among the mother and neonates' microbiota and as a sepsis agent. Even though, ST15 isolates were found from the clinical environment with low number of SNPs PP-ENV sample type was not associated with ST15 ($p=0.588$), Therefore, this was probably not the main reservoir of ST15.

Despite not being able to match a specific ST15 PP-ENV isolate with a specific ST15 sepsis case it was possible to match ST15 PP-BB and PP-BR isolates. PP ST15 *K. pneumoniae* recovered from BB and BR samples from the same neonate were studied in more detail to evaluate whether *K. pneumoniae* causing sepsis were among the neonates' gut microbiota. Considering both the number of pairwise SNPs as well as the timeline of PP-BB isolates culture and when the BR sample was taken, especially in the case of pair PP-BB31/ PP-BR61. It was indicated that *K. pneumoniae* in the neonate's microbiota could be responsible for causing sepsis (44). As such, the SNPs phylogeny results showed that closely related *K. pneumoniae* were found, not only, among ENV, MR, BR, but also, BB isolates, indicating that *K. pneumoniae* that caused sepsis could be able to spread between the mothers, neonate, and clinical environment.

Furthermore, all PP *K. pneumoniae* were shown to harbour a wide range of ARG including ARG as ESBLs and carbapenemases and other β -lactamases as *bla*_{CMY}, *bla*_{SHV-28} and *bla*_{TEM-1D} as well as Agly ARG, among others (Fig. 46, section 6.2). The mentioned ARGs are of importance because these confer resistance to antibiotic treatments such as piperacillin/tazobactam/amikacin, cefotaxime and imipenem that were empirically prescribed in PIMS when sepsis was suspected (9, 10, 169). Also, PP *K. pneumoniae* were in majority resistant to these antibiotics when tested in this PhD indicating that these ARG were the drivers of resistance to these antibiotics (6.3 Antibiotic susceptibility profiles of PP *K. pneumoniae*). More importantly, ARG and resistance to antibiotics prescribed to treat *K. pneumoniae* sepsis were displayed by PP ST15 *K. pneumoniae* including carbapenems and cephalosporins, aminoglycosides and fluoroquinolones as well. It is also important to notice that ST15 *K. pneumoniae*, in contrast with the other PP isolates, displayed *OmpK* truncations that increase antibiotic resistance as well, by impairing the entrance of the antibiotic into the bacteria (177) Furthermore, PP *K. pneumoniae* including ST15 isolates carried *rmt* Agly ARG whereas ES *K. pneumoniae* did not carry it. This could explain why PP *K. pneumoniae* were resistant and ES *K. pneumoniae* were susceptible to amikacin because these ARG confer resistance to this antibiotic (165, 166). In relation to virulence determinants KL112 was the most common capsule polysaccharide found among all ST15 PP *K. pneumoniae* and O1v1 was the most common LPS found. Additionally, *ybt* was the most found siderophore loci among

PP isolates including ST15 isolates. KL2 often associated with hypervirulence was found among PP *K. pneumoniae* which are usually displayed by hypervirulent *K. pneumoniae* (93) indicating that hypervirulence might be appearing among *K. pneumoniae* recovered in PIMS which could lead to serious infection. Moreover, seeing that KL112 was displayed and associated to ST15 *K. pneumoniae* and was also the most common KL among PP isolates it could be a target for a possible vaccine. Likewise, O1v1 could be a possible target for antibody treatment in order to help prevent possible sepsis caused by *K. pneumoniae* in PIMS as these would target a high number of isolates (168) (1.9 Treatment options for infection caused by *K. pneumoniae*). These therapies could aid the treatment of *K. pneumoniae* sepsis in PIMS seeing that most isolates were displayed resistance to antibiotics used in treatment. Therefore, options for treatment become increasingly reduced not only by resistance to most of the antibiotics tested and prescribed but also by the availability of these antibiotics in Pakistan.

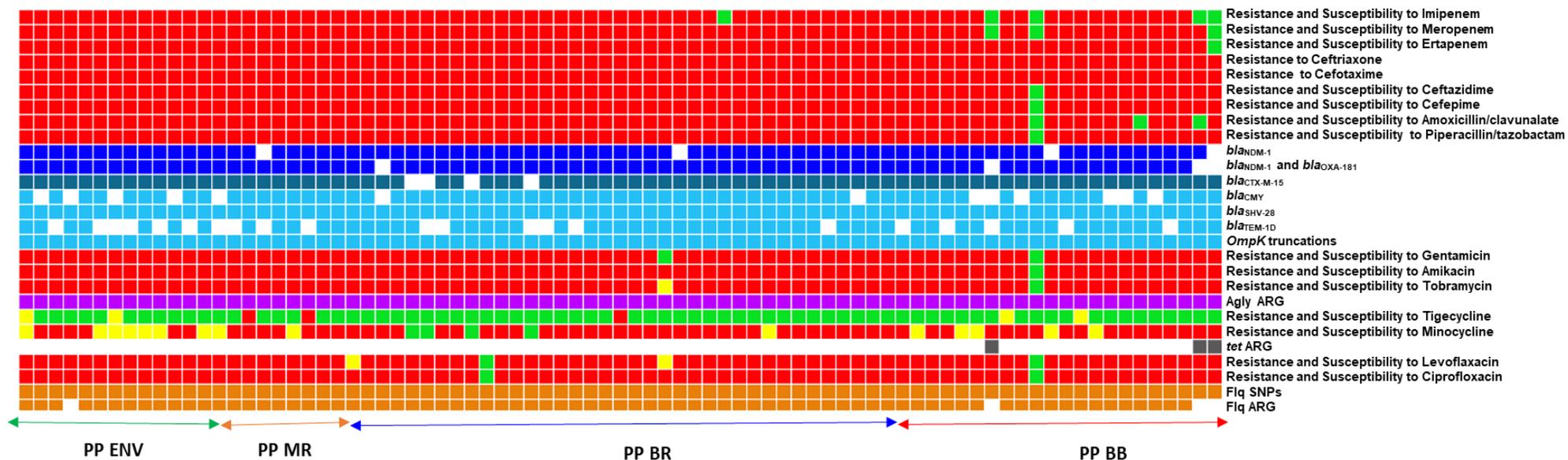


Fig. 46: Antibiotic resistance (red) and susceptibility (green) to carbapenems (imipenem, meropenem and ertapenem), cephalosporins (ceftazidime and cefepime), penicillin treatments (amoxicillin/clavulanate and piperacillin/tazobactam), aminoglycosides (gentamicin, amikacin and tobramycin), tetracyclines (tigecycline and minocycline) and fluoroquinolones (levofloxacin and ciprofloxacin) along with presence of resistance markers responsible for resistance to such antibiotic treatments. Carbapenemases *bla*_{NDM-1} and *bla*_{OXA-181}, broad spectrum β -lactamases such as *bla*_{SHV-28} and *bla*_{TEM-1D}, ESBL *bla*_{CTX-M-15} and *OmpK* truncations. Aminoglycoside (Agly) ARG, *tet* ARG and Flq SNPs and ARG

6.2 Resistome of PP *K. pneumoniae*

The total PP *K. pneumoniae* (n=284;100%) carried several acquired ARG that confer resistance to β -lactam, aminoglycosides, tetracyclines and fluoroquinolones tested in this study. In addition, SNPs that confer resistance to fluoroquinolones and truncations that confer antibiotic resistance and to specifically colistin were found too. Fig. 45 summarises the percentages of the described ARG and other ARG found. In Appendix 3 the ARG carried by each PP *K. pneumoniae* are listed.

ARG that confer resistance to the tested β -lactams included *bla*_{OXA}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{NDM} among the most found. PP *K. pneumoniae* frequently carried ESBL and carbapenemase ARG that are responsible for resistance to cephalosporins and carbapenems, respectively. PP *K. pneumoniae* frequently harboured *bla*_{CTX-M-15} only (n=176; 62%) but nine (3%) PP *K. pneumoniae* carried *bla*_{CTX-M-15} and *bla*_{SHV-27} simultaneously, three (1%) harboured *bla*_{CTX-M-15} and *bla*_{VEB-5}. In addition, *bla*_{SHV-27} and *bla*_{SHV-42} were harboured by one isolate each, respectively, these being ESBLs too. *bla*_{NDM} ARG which are carbapenemases, were frequently harboured by PP *K. pneumoniae* too. The most frequently found were *bla*_{NDM-1} (n=84;30%) and 83 (30%) PP *K. pneumoniae* carried both *bla*_{NDM-1} and *bla*_{OXA-181} which is carbapenemase as well. Other *bla*_{NDM} ARG were found too, including *bla*_{NDM-4} (n=1;0.4%), *bla*_{NDM-7} (n=6;2%), One (0.4%) PP *K. pneumoniae* carried *bla*_{OXA-181} only and two (0.7%) isolates harboured *bla*_{OXA-48}. In relation, other *bla*_{OXA} ARG *bla*_{OXA-1} (n=62; 22%) and *bla*_{OXA-10} (n=67;24%) were the more common ARG.

*bla*_{SHV} ARG included *bla*_{SHV-28} (n=103,36%) as the most frequently found, and *bla*_{SHV-187} (n=50;18%). But other commonly found *bla*_{SHV} ARG included *bla*_{SHV-11} (n=55;19%) and *bla*_{SHV-26} (n=15;5%). Regarding, *bla*_{TEM} ARG the most frequently found included *bla*_{TEM-1D} harboured by 167 (60%) PP *K. pneumoniae*. In addition, PP *K. pneumoniae* also frequently carried *bla*_{CMY-6} which was carried by 89 (31%) PP isolates.

As other antibiotics tested included aminoglycosides (gentamicin, amikacin, and tobramycin) the frequencies of Agly ARG harboured by PP *K. pneumoniae*, such as *rmt*, *aac*, *aad* and *aph* were determined. Among PP *K. pneumoniae* *rmtC* (n=99;35%) was frequently found but other *rmt* ARG were

found including *rmtB* (n=2;0.7%) and *rmtF* (n=3;1%). *aph3-VIb* was frequently found as well, being harboured by 56 (20%) PP *K. pneumoniae*, in contrast, with *aph* ARG such as *aph3-Ia* (n=1;0.4%), *aph3-IIb* (n=1;0.4%) and *aphA6* (n=1;0.4%). Also, the majority of PP *K. pneumoniae* harboured *aac6-Ib* (n=89;31%) and *aac3-IIId* (n=61;21.5%). *aad* ARG were carried by 64 (22.5%) PP *K. pneumoniae* that in majority carried *aadA1* and *aadA16* simultaneously. In addition, five (2%) PP isolates carried *armA* ARG.

Other antibiotics tested included fluoroquinolones (ciprofloxacin and levofloxacin) thus not only Flq ARG frequencies were determined but also the frequency Flq resistance conferring SNPs. In relation to PP *K. pneumoniae* carrying Flq ARG only *qnrB1* (n=86;30%) was the most frequent ARG. However, *qnrS1* (n=9;3%) and *qnrB4* (n=3;1%) were found as well, but in smaller number. Additionally, other PP isolates were found to harbour *qnrS1* and *qnrB1* (n=7;2.5%), simultaneously, and three (1%) PP *K. pneumoniae* carried *qnrB1* and *qnrB4*. Regarding, Flq SNPs these were commonly carried simultaneously with *qnr* ARG and the frequencies determined refer to PP *K. pneumoniae* that carried Flq SNPs and Flq ARG. Also, despite these PP isolates carrying Flq ARG these were not included in the frequencies of PP isolates that carried Flq ARG only. Thus, in total, 82 (29%) PP *K. pneumoniae* displayed *gyrA-83F*, *gyrA-87A*, *parC-80I* and *qnrS1*, this being the most frequently found combination of Flq SNPs and Flq ARG. Contrary to combinations such as *gyrA-83F*, *gyrA-87A*, *parC-80I* and *qnrS1*, *qnrB1* (n=1;0.4%), *gyrA-83I*, *parC-80I* and *qnrS1*; *qnrB1* (n=2;0.7%), *gyrA-83L*, *gyrA-87N*, and *qnrB1* (n=2;0.7%) and *gyr-83I*, *parC-80I* and *qnrB1*. PP *K. pneumoniae* displaying Flq SNPs only were found as well. PP *K. pneumoniae* (n=19; 7%) showed *gyr-83F*, *gyrA-87A* and *parC-80I* only and two (0.7%) PP isolates carried *gyr-83T* and one (0.4%) PP isolate showed *parC-80I* only.

tet ARG frequencies were considered too because tigecycline and minocycline were tested as well. Most PP *K. pneumoniae* harboured *tetA* and *tetB* (n=54;19%) followed by PP isolates carrying *tetA* (n=26; 9%) and by PP *K. pneumoniae* that carried *tetB* (n=15;5%). In addition, 11 (4%) PP isolates harboured *fosA2* ARG that confer resistance to fosfomycin.

Furthermore, PP *K. pneumoniae*, not only harboured ARG but also *OmpK* truncations that confer antibiotic resistance as well. Among these isolates the most frequently found was *OmpK35* truncation that was displayed by 106 (37%)

PP *K. pneumoniae* followed by five(2%) PP isolates displayed *OmpK35* and *OmpK36* truncations and one (0.4%) PP *K. pneumoniae* displaying an *OmpK36* truncation. Furthermore, PP *K. pneumoniae* (n=3;1%) showed *mgrB* and one PP isolated displayed *pmrB* truncations these truncations being responsible for resistance to colistin.

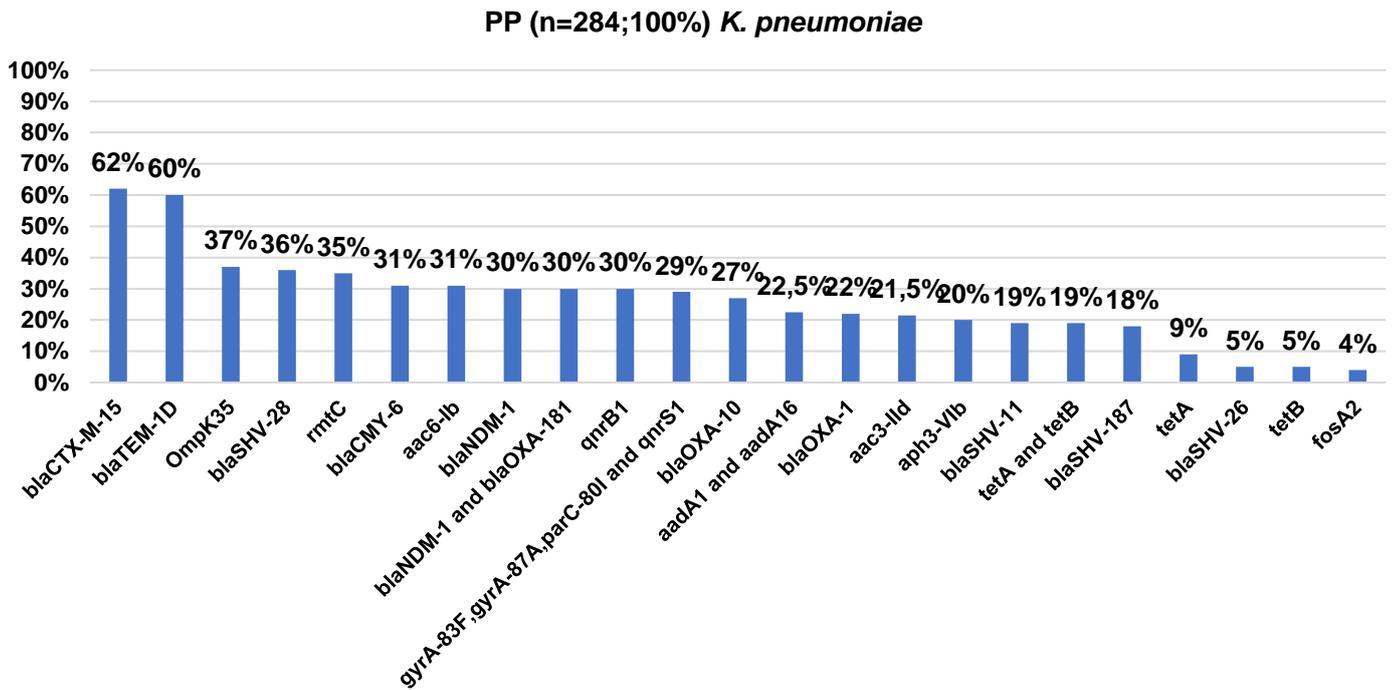


Fig. 45: Percentages of frequent of ARG and antibiotic resistance conferring mutations carried by *K. pneumoniae* from Pakistan (PP). Most PP *K. pneumoniae* carried *bla*CTX-M-15 (62%) and *bla*TEM-1D (60%). *bla*SHV-28 was carried by 36% of PP isolates followed by *rmtC* carried by 35% of isolates. Carbapenemase *bla*NDM-1 was carried by 30% and both *bla*NDM-1 and *bla*OXA-181 were carried by 30% of PP *K. pneumoniae*. In addition, *OmpK35* was harboured by 37% of PP isolates and 29% of PP *K. pneumoniae* carried *gyrA-83F, gyrA-87A, parC-80I and qnrS1*. In contrast, *tetA* was carried by 9% of PP *K. pneumoniae*, *bla*SHV-2 and *tetB* by 5% of PP isolates and *fosA2* by 4%.

6.2.1 Resistome of PP *K. pneumoniae* across PP-BB, BR, MR and ENV samples

Frequencies of ARGs were determined for PP-BB, BR, MR and ENV separately as they were for ES *K. pneumoniae* in order to understand if there are differences in percentage of the ARG described among different sample types of origin. This is particularly important for PP-BB because these caused sepsis in PIMS, thus, knowledge of the common ARGs would help inform appropriate treatment options, and the common ARGs in BR, MR and ENV would help to identify possible sources of these ARG.

PP-BB isolates (n=42;100%) commonly carried *bla*_{CTX-M-15} (n=33;79%) (Fig. 46a). In addition, *bla*_{NDM-1} and *bla*_{OXA-181} were harboured simultaneously by 19 (45%) and 11 (26%) PP-BB *K. pneumoniae* harboured *bla*_{NDM-1} only. Other common *bla* ARG included *bla*_{SHV-28} carried by 27 (64%) of PP-BB *K. pneumoniae* and *bla*_{TEM-1D} (n=33;79%)

Regarding, Agly ARG, the most frequently found was *aac6-Ib* harboured by 21 (50%) PP-BB *K. pneumoniae* followed by *rmtC* (n=20; 48%) and *aac3-IId* (n=11;26%).

WGS revealed that most PP-BB isolates (n=19; 45%) harboured Flq ARG and SNPs such as *gyrA-83F*, *gyrA-87A*, *parC-80I* and *qnrS1* simultaneously and 11 (26%) PP-BB isolates harboured *qnrB1* only. Also, results showed that *tetA* and *tetB* (n=11; 26%) ARG were more commonly found simultaneously among PP-BB *K. pneumoniae*. No fosfomycin ARG were harboured by PP-BB *K. pneumoniae*. In relation to, resistance conferring truncations such as *OmpK* and colistin resistance conferring truncations PP-BB *K. pneumoniae* only displayed *OmpK35* truncations in 23 (55%) PP-BB isolates

Considering ARG harboured by PP-BR *K. pneumoniae* (n=106;100%) (Fig. 46b) *bla*_{CTX-M-15} (n=80;75.5%) was the most frequently *bla*_{CTX-M} ARG found among PP-BR isolates likewise among PP-BB isolates. In addition, *bla*_{NDM} ARG were frequently found among PP-BR as well. *bla*_{NDM-1} was harboured by 39 (37%) PP-BR *K. pneumoniae* and other 41 (39%) harboured *bla*_{NDM-1} and *bla*_{OXA-181}. Other common *bla* ARG carried by PP-BR isolates included for example *bla*_{OXA-10} (n=33;31%), *bla*_{OXA-1} (n=28;26%), *bla*_{SHV-28} (n=44;41.5%) and *bla*_{TEM-1D} (n=81;76%).

In relation to Agly ARG found among PP-BR *K. pneumoniae* *rmtC* was the most frequent being harboured by 51 (48%) isolates followed by *aac6'-Ib* (n=42;40%) and *aph3-VIb* (n=28;26%). Also, *aadA1* and *aadA16* were more commonly carried simultaneously among PP-BR *K. pneumoniae* (n=29;27%) and *aac3-III d* was carried by 23 (22%) of PP-BR isolates. In relation to fluoroquinolone resistance PP-BR were shown to carry *qnrB1* (n=41;39%) and *qnrS1* simultaneously showing *gyrA-83F*, *gyr-87A* and *parC-80I* Flq SNPs (n=40;38%). Also, regarding resistance to tetracyclines 22 (21%) PP-BR *K. pneumoniae* harboured *tetA* and *tetB* simultaneously and fosfomycin ARG *fosA2* was carried by five (5%) of PP-BR isolates. Furthermore, PP-BR *K. pneumoniae* also commonly displayed *OmpK35* (n=51;48%) truncation in contrast to colistin resistance conferring truncations, in this case, an *mgrB* truncation, that was found only among two (2%) PP-BR isolates.

The frequencies of ARG determined for PP-MR *K. pneumoniae* (n=91;100%) (Fig. 46c) showed that ESBL and carbapenemase ARG commonly included *bla_{CTX-M}* and *bla_{NDM}* ARG respectively, but other ARG were found too as among PP-BB and PP-BR. PP-MR isolates in majority carried *bla_{CTX-M-15}* (n=29;32%) and *bla_{NDM-1}* (n=12;13%). Other commonly found ARG *bla* ARG included *bla_{SHV-187}* (n=18;20%), *bla_{SHV-28}* (n=14;15%) and *bla_{SHV-11}* (n=25;27.5%) and *bla_{TEM-1D}* (n=25; 27.5%). Among the Agly ARG results showed that PP-MR frequently carried *aac3-III d* (n=10;11%), *aac6'-Ib* (n=10;11%), *aadA1* and *aadA16* simultaneously (n=8;9%), *rmtC* (n=12;13%) and *aph3-VIb* (n=6;7%). Additionally, PP-MR isolates were shown to in majority harbour Flq ARG *qnrB1* (n=18;20%) and *gyrA-83F*, *gyr-87A*, *parC-80I* and *qnrS1* (n=9;10%). The most common *tet* ARG was *tetA* (n=14;15%). And five (5.5%) PP-MR harboured *fosA2*. Resistance conferring truncations included was *OmpK35* (n=14;15%) but colistin *pmrB* and *mgrB* truncations were displayed by one (1%) PP-MR isolate each.

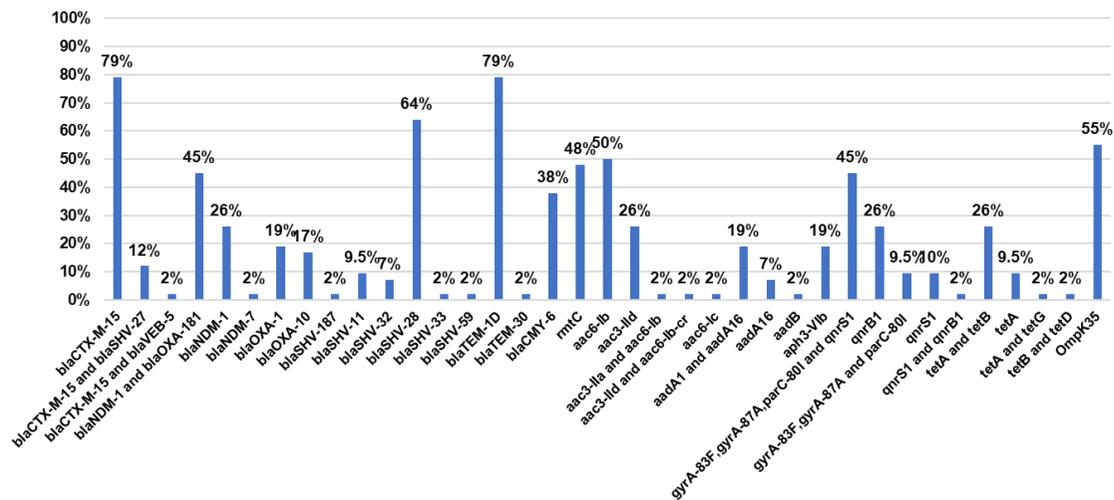
Lastly, PP-ENV *K. pneumoniae* (n=44;100%) (Fig. 46d) isolates harboured *bla_{CTX-M-15}* (n=34;77%), carried *bla_{NDM-1}* (n=22;50%) and *bla_{NDM-1}* and *bla_{OXA-181}* (n=14;32%), simultaneously. In addition, *bla_{TEM-1D}* (n=28;64%) was frequently found too. Concerning Agly ARG most PP-ENV *K. pneumoniae* also harboured *aac6II-Ib* (n=16;36%), *aac3-III d* (n=17;39%), *rmtC* (n=16;36%) and *aph3-VIb* (n=14;32%) and 18 (41%) PP-ENV *K. pneumoniae* carried *aadA1* and *aadA16* simultaneously. In relation to resistance to fluoroquinolones and

tetracyclines Flq ARG and SNPs and *tet* ARG PP-ENV *K. pneumoniae* in majority harboured *qnrB1* (n=16;36%) and *gyrA-83F*, *gyrA-87A*, *parC-80I* and *qnrS1* (n=14;32%). Most PP-ENV isolates (n=16;36%) harboured *tetA* and *tetB* simultaneously. Only one (2%) PP-ENV isolate harboured fosfomycin ARG *fosA2* and the only *OmpK* truncations shown by PP-ENV *K. pneumoniae* were *OmpK35* truncations showed by 18 (41%) PP-ENV isolates

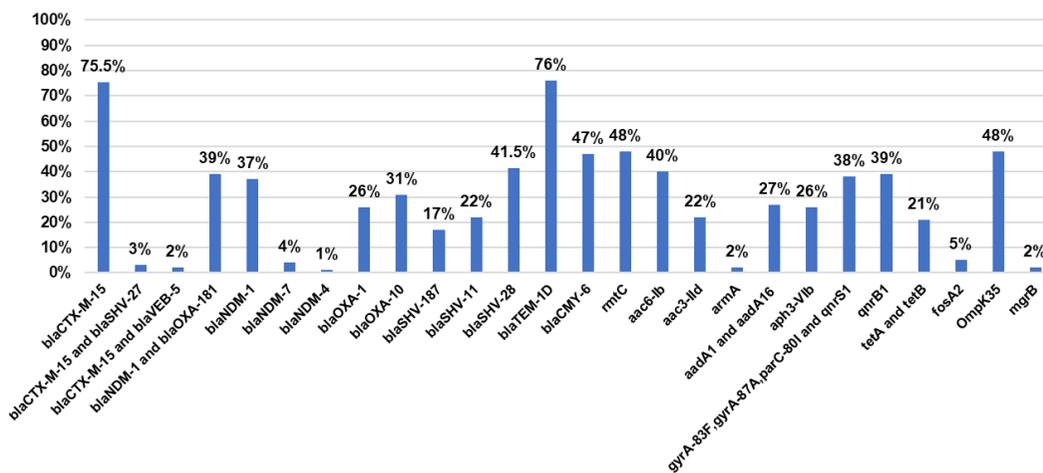
Likewise, ES *K. pneumoniae* the percentages of ARG and diversity of ARG among PP isolates varied across diversity sample types of origin and percentages were higher among PP-BB followed PP-BR, PP-ENV and PP-MR (Fig. 48). Again *bla_{CTX-M-15}* was the most frequent across the different sample type of origin, but, more importantly, PP *K. pneumoniae* also carried *bla_{NDM-1}* and *bla_{OXA-181}* carbapenemases, which as other ARG showed different percentages when considering the different sample types of origin. *bla_{NDM-1}* was only found among PP-BB (26%) followed by PP-ENV (50%), PP-BR (37%) and PP-MR (13%). *bla_{NDM-1}* and *bla_{OXA-181}* carried concurrently was more frequent again in PP-BB (45%) followed by PP-BR (39%), PP-ENV (32%) and PP-MR (10%).

More importantly, high percentages of ARGs were found among PP *K. pneumoniae* that caused sepsis (PP-BB) but also among PP-BR and PP-ENV indicating that PP-ENV could possibly be acting as a reservoir of ARG though PP-BR could later on be responsible for sepsis. (6.6 Plasmid replicon harboured by PP *K. pneumoniae*) (6.1 Results – Phylogeny and population of *K. pneumoniae*)

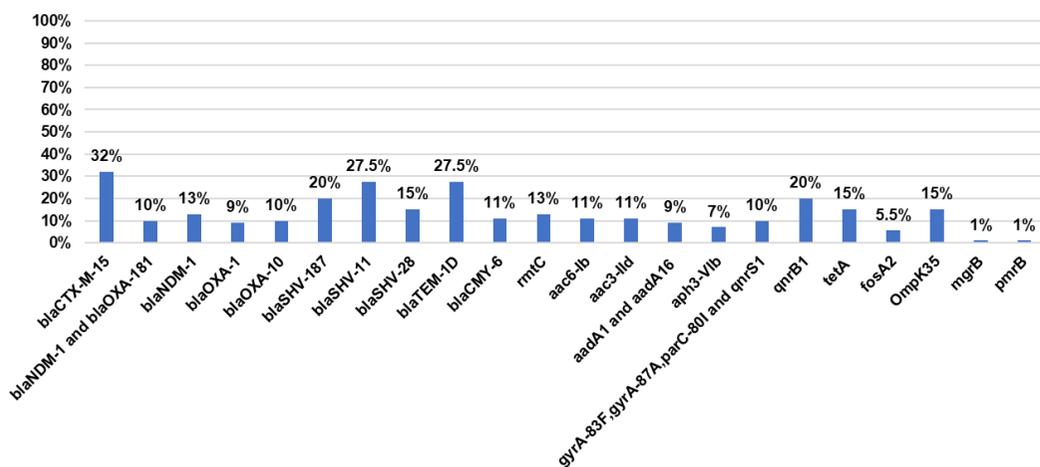
a. PP-BB (n=42;100%) *K. pneumoniae*



b. PP-BR (n=106;100%) *K. pneumoniae*



c. PP-MR (n=91;100%) *K. pneumoniae*



d. PP-ENV (n=44;100%) *K. pneumoniae*

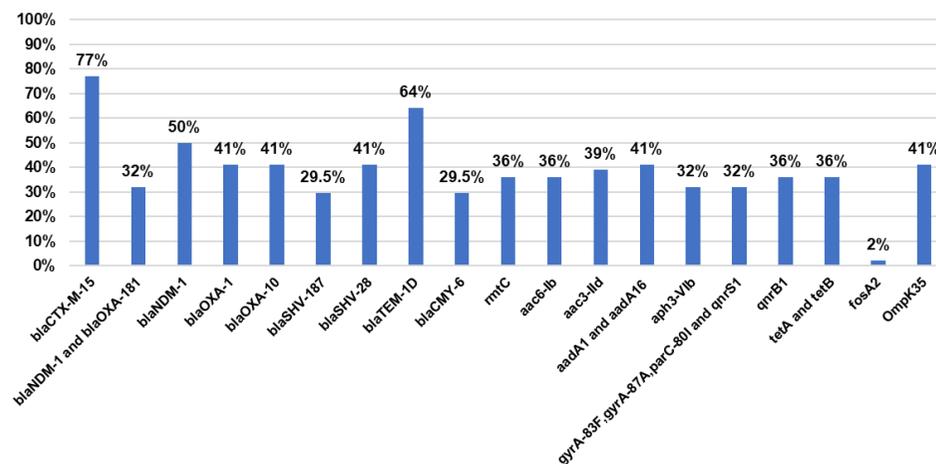


Fig. 46: Percentages of frequent of ARG and antibiotic resistance conferring mutations among *K. pneumoniae* from Pakistan (PP) across the different sample types of origin. a. Most PP-BB *K. pneumoniae* carried *bla*_{CTX-M-15} (79%) and *bla*_{TEM-1D} (79%) followed by *bla*_{SHV-28} (64%) and *aac6I-b* (50%). In addition, *OmpK35* was carried by 55% of PP-BB isolates and 45% carried *gyrA-83F*, *gyrA-87A*, *parC-80I* and *qnrS1*. Contrarily, *qnrS1* was carried by 10% PP-BB isolates and *bla*_{SHV-11} by 9.5% of PP-BB *K. pneumoniae*. **b.** In majority PP-BR *K. pneumoniae* carried *bla*_{TEM-1D} (76%) followed by *bla*_{CTX-M-15} (75.5%) and *rmtC* (48%). Also, *bla*_{NDM-1} was carried 39% of PP-BR *K. pneumoniae* and 37% of PP-BR isolates carried *bla*_{NDM-1} and *bla*_{OXA-181}. In addition, PP-BR carried *OmpK35* (45%) and *mgrB*(2%). In contrast, *bla*_{NDM-7} was carried by 4% PP-BR *K. pneumoniae* and *fosA2* by 5% of PP-BR isolates. **c.** PP-MR *K. pneumoniae* carried in majority *bla*_{CTX-M-15} (32%) followed by *bla*_{SHV-11} (27.5%) and *bla*_{TEM-1D} (27.5%). In addition, Also, *bla*_{NDM-1} was carried 13% of PP-MR *K. pneumoniae* and 37% of PP-MR isolates carried *bla*_{NDM-1} and *bla*_{OXA-181}. Also, *OmpK35* was carried by 15% of PP-MR isolates, 20% carried *qnrB1* and 10% carried *gyrA-83F, gyrA-87A*, *parC-80I* and *qnrS1*. **d.** PP-ENV *K. pneumoniae* in majority carried *bla*_{CTX-M-15} (77%) followed by *bla*_{TEM-1D} (64%) and *bla*_{NDM-1}(50%). In addition, 41% of PP-ENV isolates carried *aadA1*and *aadA16*, 41% of PP-ENV *K. pneumoniae* also carried *OmpK35*.

6.3 Antibiotic susceptibility profiles of PP *K. pneumoniae*

The antibiotic susceptibility profile of 278 (100%) PP *K. pneumoniae* were determined (Fig. 47) and studied across the different sample types in the following section. Among PP *K. pneumoniae* there was a high percentage of resistance to the β -lactams (ranging from n=178;64% to n=276;99%). Specifically, these included cefotaxime (n=219;79%), ceftriaxone (n=219;79%), ceftazidime (n=219;79%) and cefepime (n=208;75%). Likewise, 64% (n=177) PP *K. pneumoniae* were resistant to ertapenem followed by a percentage of resistance of 45% (n=125) and 45% (n=124) to meropenem and imipenem, respectively. Also, PP isolates displayed a high percentage of resistance to minocycline (n=232;84%), ciprofloxacin (n=184;66%), gentamicin (n=171;62%), amikacin (n=178;64%) and tobramycin (n=172,62%).

Lastly, most PP *K. pneumoniae* were susceptible to fosfomycin (n=223;81%) and colistin (n=269;97%). Also, carbapenemase positive PP *K. pneumoniae* (n=167;100%) were tested against ceftazidime/avibactam and aztreonam/avibactam. These *K. pneumoniae* showed high percentages of resistance to ceftazidime/avibactam (n=159;95%) in contrast to high percentages of susceptibility to aztreonam/avibactam (n=167;100%).

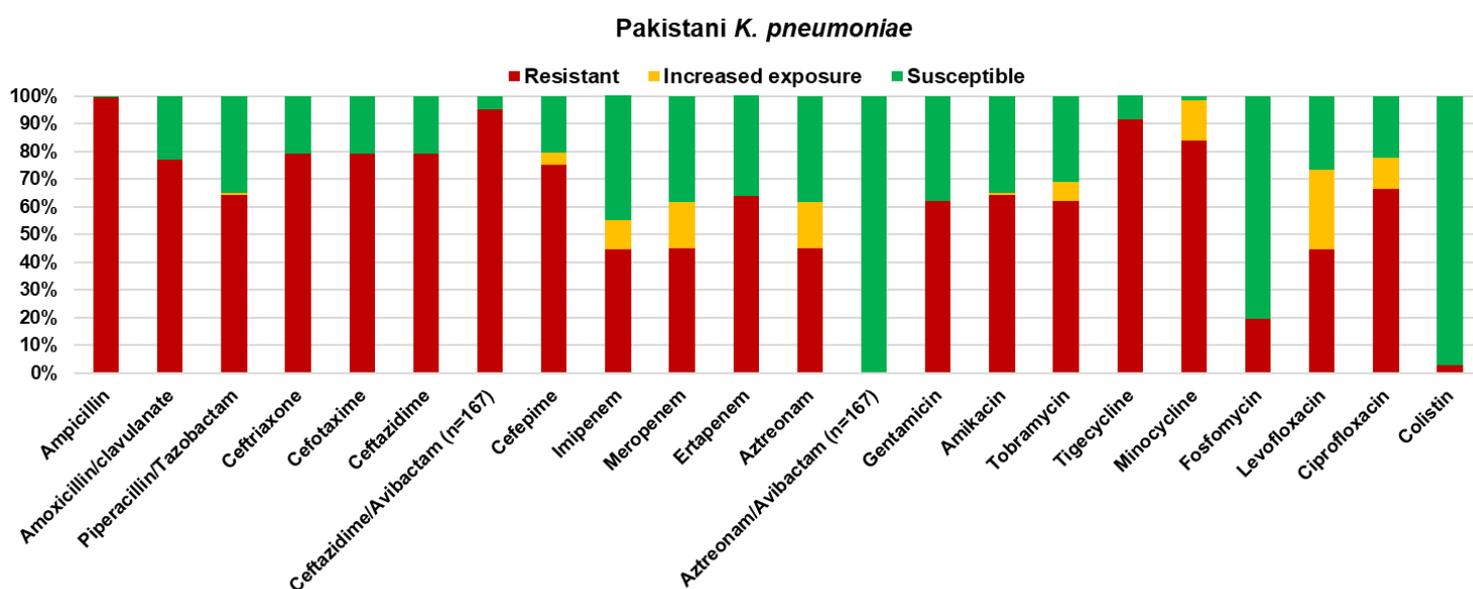


Fig. 47: Antibiotic susceptibility profile of *K. pneumoniae* isolates from Pakistan. *K. pneumoniae* from Pakistan displayed high percentage susceptibility to colistin (97%), fosfomycin (81%) and aztreonam/avibactam (100%) whereas high percentages of resistant to ceftriaxone (79%), cefotaxime (79%), ceftazidime (79%), cefepime (75%). The colour of the bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green). Percentages of susceptibility to ceftazidime/avibactam and aztreonam/avibactam refer to CRE+ *K. pneumoniae* only.

6.3.1 PP *K. pneumoniae* antibiotic susceptibility profiles of BB, BR, MR and ENV isolates

PP-BB *K. pneumoniae* (n=41;100%)(Fig. 48a) displayed high percentage of resistance to β -lactams including cephalosporins (n=35;85% to n=40;98%). PP-BB were resistant to ceftriaxone (n=40;98%), cefotaxime (n=40;98%), cefepime (n=35;85%) and ceftazidime (n=38;93%). Likewise, PP-BR (n=106;100%) (Fig. 48b) and PP-ENV (n=44;100%) (Fig. 50d) also showed high percentages of resistance to the tested cephalosporins, 97% (n=103) and 86% (n=37) to 93% (n=40), respectively. In contrast, PP-MR (n=97;100%) (Fig. 48c) showed 55% (n=53) susceptibility to ceftriaxone and 52% (n=50) susceptibility cefotaxime and cefepime, respectively. In relation to carbapenems 20% (n=8) of PP-BB *K. pneumoniae* were resistant to imipenem, 41% (n=17) resistant to meropenem and 73% (n=30) were resistant to ertapenem. PP-BR (n=51;48%), and PP-ENV (n=25;58%) isolates showed higher percentages of resistance to imipenem when compared to PP-BB *K. pneumoniae*. Likewise, among PP-BR (n=67;63% and n=90;85%, respectively) and PP-ENV (n=24;56% and n=36;84%, respectively) the percentages of resistance to meropenem and ertapenem were higher than among PP-BB isolates. However, PP-MR isolates were in majority susceptible to carbapenems showing 80% (n=78), 73% (n=71) and 74% (n=72) susceptibility to imipenem, meropenem and ertapenem, respectively.

Considering the tested aminoglycosides PP-BB *K. pneumoniae* were frequently resistant to gentamicin (n=31;76%), amikacin (n=32;78%) and tobramycin (n=34;83%) with PP-BR and PP-ENV also showing high percentages of resistance to gentamicin (n=83;78% and n=35,81%,respectively), amikacin (n=92;87% and n=33;77%, respectively) and tobramycin (n=82;77% and n=34;79%,respectively). In contrast, PP-MR showed high percentages of susceptibility to gentamicin (n=67;69%), amikacin (n=71;73%) and tobramycin (n=69;71%) (Fig. 50). Regarding, tetracyclines PP-BB displayed 73% (n=30) of susceptibility to tigecycline and 85%(n=35) of resistance to minocycline. In comparison the percentage of susceptibility to tigecycline among PP BR (n=66;62%), PP-MR (n=60;62%) and PP-ENV (n=24;56%) was lower than among PP-BB isolates. But percentages of resistance to minocycline were higher among PP-BR (n=98;92%) and lower among PP-MR (n=77;79%) and PP-ENV (n=32;74%) (Fig. 48).

PP-BB isolates (n=26;56% and n=34;83%) were resistant to levofloxacin and ciprofloxacin, respectively. Likewise, PP-BR (n=60;57% and n=89;84%, respectively) and PP-ENV (n=27;63% and n=36;84%, respectively) showed high percentages of resistance to levofloxacin and ciprofloxacin these being higher than among PP-BB *K. pneumoniae*. PP-MR isolates were in majority susceptible to levofloxacin (n=53;55%) and ciprofloxacin (n=45;46%). Lastly, most PP-BB (n=39;95%), PP-BR (n=84;79% and n=102;96%), PP-MR (n=83;86% and n=95;98%) and PP-ENV (n=44;100%) were susceptible to fosfomycin and colistin, respectively (Fig. 48).

In summary, PP-BB *K. pneumoniae* showed high percentages of resistances to all β -lactams including cephalosporins and carbapenems which is of concern because these are usually used for sepsis treatment. With PP-BR and PP-ENV also showing high percentages of resistance to the testes antibiotics which is of concern because it showed that the *K. pneumoniae* among resistance to antibiotics used in treatments was established among the neonates microbiota and in the clinical environment. However, the percentages of resistance to the same antibiotics were lower among PP-MR *K. pneumoniae*

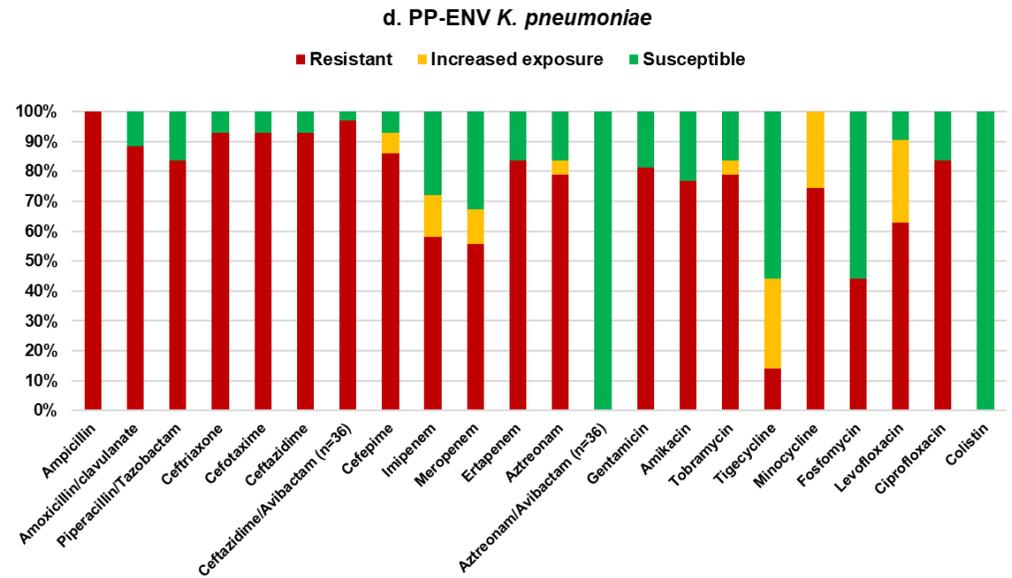
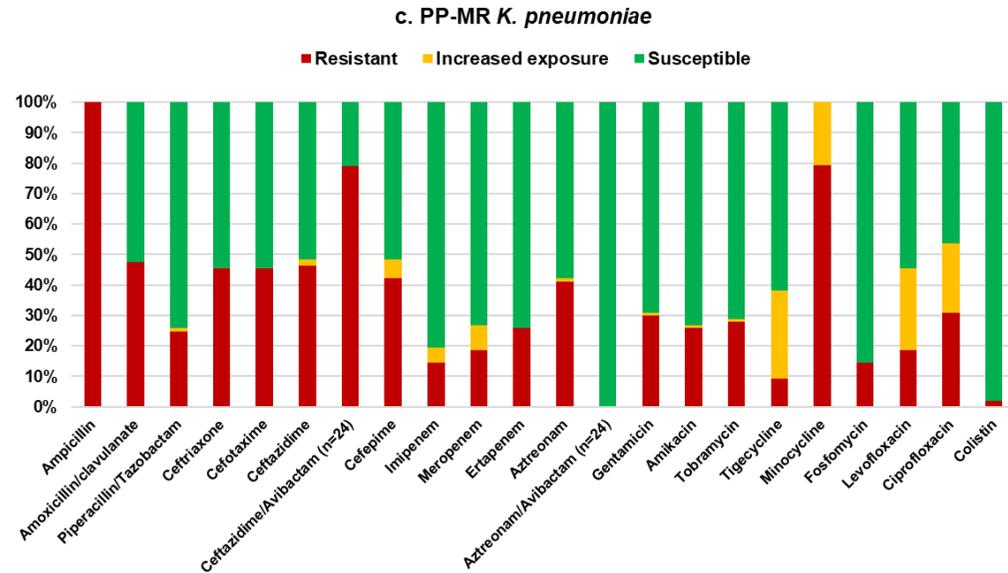
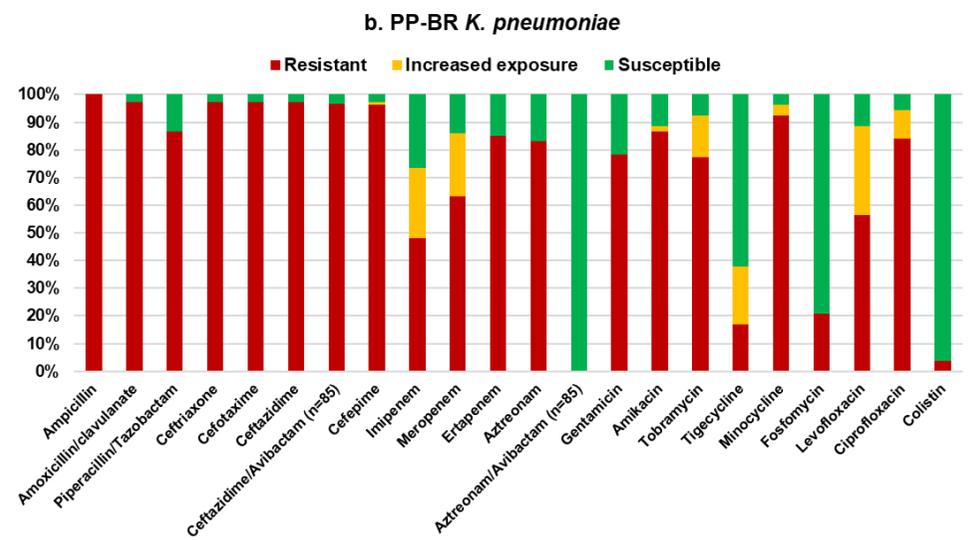
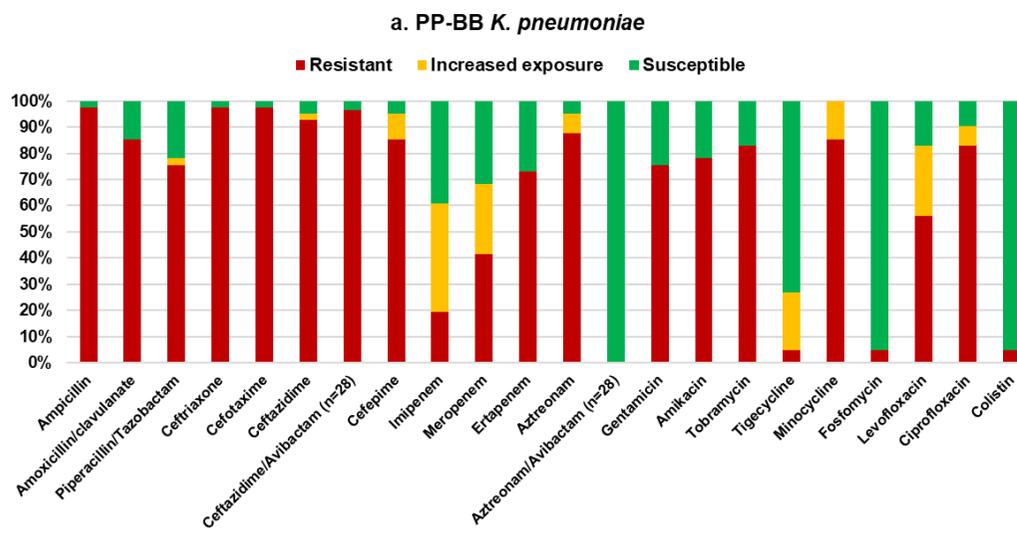


Fig. 48: Antibiotic susceptibility profile of PP-BB, BR, MR and ENV *K. pneumoniae*. **a.** PP-BB isolates displayed high percentages of resistance to ceftriaxone (98%), cefotaxime (98%), ceftazidime (93%), cefepime (75%). PP-BB *K. pneumoniae* were resistance to gentamicin (76%), amikacin (78%) and tobramycin (83%) as well as to levofloxacin (56%) and ciprofloxacin (83%). Most PP-BB were susceptible to colistin (95%), fosfomycin (95%) and aztreonam/avibactam (100%). **b.** Most PP-BR were resistant to β -lactams including cephalosporins ceftazidime (97%), cefotaxime (97%), and cefepime (96%), and resistant to carbapenems, ertapenem (85%) and meropenem (63%). PP-BR (78%) were resistant to gentamicin and tobramycin and 87% and 92% were resistant to amikacin and minocycline, respectively. As well as being resistant to levofloxacin (57%) and ciprofloxacin (84%). In majority, PP-BR *K. pneumoniae* were susceptible to colistin (96%), fosfomycin (79%) and aztreonam/avibactam (100%). **c.** In majority PP-MR were susceptible to the tested antibiotics. PP-MR were in majority susceptible to imipenem (80%) followed by amikacin (73%) and gentamicin (69%) in contrast PP-MR were resistant (79%) or showed increased exposure (21%) to minocycline. Most PP-MR *K. pneumoniae* were susceptible colistin (98%), fosfomycin (86%) and aztreonam/avibactam (100%). **d.** Most PP-ENV were resistant to β -lactams including cephalosporins ceftriaxone (93%), ceftazidime (93%), cefotaxime (93%), and cefepime (85%), and resistant to carbapenem, ertapenem (83%). PP-ENV were in majority resistance to gentamicin (81%), amikacin (77%) and tobramycin (79%) as well as to levofloxacin (63%) and ciprofloxacin (84%) In majority PP-ENV isolates were susceptible to colistin (100%) and aztreonam/avibactam (100%) The colour of the bars represents percentage of susceptibility: resistant (red), increased exposure (yellow) and susceptible (green). Percentages of susceptibility to ceftazidime/avibactam and aztreonam/avibactam refer to CRE+ *K. pneumoniae* only.

6.3.2 Antibiotic susceptibility profile of PP *K. pneumoniae* – discussion

Considering PP *K. pneumoniae* in total most isolates were resistant to most antibiotics tested which was of concern. Resistance rates were highest for PP-BR and PP-ENV samples, but still considerably high for the clinical sepsis isolates (PP-BB). Resistance rates to most antibiotic classes, including cephalosporins, carbapenems and aminoglycosides were much lower for PP-MR isolates. Importantly, the very high rates of resistance for clinical (BB) but also BR and ENV isolates included those usually empirically prescribed (piperacillin/tazobactam and amikacin, cefotaxime and imipenem) at PIMS and those used to treat specifically to treat *K. pneumoniae* sepsis cases at PIMS (piperacillin/tazobactam, amikacin, cefotaxime, imipenem, amoxicillin, imipenem, meropenem, ciprofloxacin). However, PP-BB *K. pneumoniae* were susceptible to colistin which is also part of the antibiotics that were used to treat some *K. pneumoniae* sepsis cases at PIMS. It is also important to notice that PP-BR were resistant to the same antibiotics as PP-BB. More importantly, when considering phylogeny of PP ST15 *K. pneumoniae* it was possible to see that PP-BB and PP-BR isolates that these were able to spread among neonates and cause sepsis (6.1 Phylogeny and population of PP *K. pneumoniae*). This is further supported because WGS of similar PP-BB and PP-BR *K. pneumoniae* isolated from the same neonate confirmed that these carried carbapenemases *bla*_{NDM-1} and *bla*_{OXA-181} as well as ESBL *bla*_{CTX-M-15}. and MIC determination confirmed that these were all resistant to carbapenems and cephalosporins. Concerning Agly ARG these PP *K. pneumoniae* all harboured *aac6-1b* and *rmtC* and through MIC determination it was also possible to confirm that these were resistant to gentamicin, amikacin and tobramycin (165, 166) In addition, other *bla* ARG were harboured by both PP-BB and PP-BR *K. pneumoniae* such as *bla*_{SHV} and *bla*_{TEM} ARG.

Carbapenemase positive PP *K. pneumoniae* were all susceptible to aztreonam/avibactam and colistin in addition to these isolates also showed susceptibility to tigecycline. Hence, colistin and tigecycline could provide patients with a possible treatment because PP *K. pneumoniae* including ST15 isolates were susceptible in majority to both antibiotics. However, in Pakistan, these are paid for by the patients which can impair its availability because buying these antibiotics could place a heavy burden on the average living wage of PIMS patients such as 19% and 73% of the living wage (169).

6.4 Antibiotic susceptibility and ARG among PP *K. pneumoniae* belonging to ST15

In relation, to PP *K. pneumoniae* isolates belonged to ST15 (n=82;100%). These PP isolates were among the group for which MICs were determined and the MIC₅₀ and MIC₉₀ were determined for these isolates as well and shown in Table 9.

PP ST15 include BB (n=22;27%), BR (n=41;50%), MR (n=9;11%) and ENV (n=14;17%). Fig. 51 depicts the antibiotic susceptibility of PP ST15 isolates. Overall, most PP ST15 isolates were resistant to the antibiotics tested. However, high percentages of susceptibility were displayed to colistin (n=81;99%; MIC₅₀ and MIC₉₀ 1 mg/L), fosfomycin (n=66;80%; MIC₅₀ 32 mg/L and MIC₉₀ 64 mg/L) and tigecycline (n=74;90%; MIC₅₀ and MIC₉₀ 1 mg/mL). WGS of these isolates revealed that one (1%) PP-MR and one (1%) PP-BR harboured truncations associated with colistin resistance, namely *pmrB* and *mgrB* truncations, respectively. However, these two (2%) isolates were susceptible to colistin whereas another isolate that tested resistant to colistin did not carry *pmrB* or *mgrB* truncations or other colistin resistance markers such as *mcr* ARG (178).

MIC testing showed that ST15 PP *K. pneumoniae* were in majority susceptible to tigecycline (n=74;90%) but in contrast most were resistant to minocycline (n=62;76%). These results suggested that these isolates would carry *tet* ARG however four (5%) ST15 PP were shown to carry *tet* ARG and among these two (2%) carried *tetA* only other two (2%) isolates carried *tetA* and *tetG* together and *tetD* and *tetG*, each. Also, these four (5%) isolates were susceptible to tigecycline and resistant to minocycline which is in line with the MIC overall results and these ARG confer resistance to minocycline. In contrast no fosfomycin resistance markers were found among PP ST15 isolates even though 20% (n=66) of resistance was displayed. Fig. 49 shows the ARG harboured by these isolates and the antibiotic susceptibility percentages.

Table 9: PP ST15 *K. pneumoniae* MIC₅₀ and MIC₉₀ for the antibiotics tested. In majority among PP ST15 isolates MIC₅₀ and MIC₉₀ were over the clinical break with the exception of the values determined for aztreonam/avibactam and colistin.

Antibiotic	PP ST15	
	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)
Ampicillin	>32	>32
Amoxicillin/clavulanate	>32	>32
Piperacillin/Tazobactam	>32	>32
Ceftriaxone	>4	>4
Cefotaxime	>4	>4
Ceftazidime	>4	>4
Ceftazidime/Avibactam	>4	>4
Cefepime	>4	>4
Imipenem	>8	>8
Meropenem	>8	>8
Ertapenem	>2	>2
Aztreonam	>4	>4
Aztreonam/Avibactam	0.5	0.5
Gentamicin	>8	>8
Amikacin	>32	>32
Tobramycin	>8	>8
Tigecycline	1	1
Minocycline	4	>4
Fosfomycin	32	64
Levofloxacin	>4	>4
Ciprofloxacin	>2	>2
Colistin	1	1

In contrast PP ST15 isolates showed resistance (n=59;72% to n=81;99%) to β -lactams including cephalosporins and carbapenems. These isolates displayed high percentage of resistance to ceftriaxone (n=82;100%), cefotaxime (n=82;100%), ceftazidime (n=81;99%) and cefepime (n=80;98%). Furthermore, ST15 was significantly associated with resistance to all tested cephalosporins (p<0.001). In addition, MIC₅₀ and MIC₉₀ displayed for cephalosporins were all >4 mg/L. Also, high percentage of resistance was displayed to meropenem (n=76;93%) and ertapenem (n=81;99%) with MIC₅₀ and MIC₉₀ of >8 mg/L and >2 mg/L for meropenem and ertapenem, respectively. In addition, ST15 PP isolates showed resistance to imipenem (n=59;72%) and aztreonam (n=80;98%). For these antibiotics both MIC₅₀ and MIC₉₀ of >8 mg/L and >4 mg/L were determined, respectively. This suggested the presence of carbapenemase and ESBL ARGs and this was confirmed by WGS of PP ST15. As most PP ST15 *K. pneumoniae*, including 22 (27%) PP-BB, 40 (49%) PP-BR, nine (n=11) PP-MR and 14 (17%) PP-ENV carried carbapenemases *bla*_{NDM-1} and *bla*_{OXA-181}, simultaneously and, all PP ST15 isolates carried *bla*_{CTX-M-15}, as well. The presence of *bla*_{NDM-1} (p<0.001), *bla*_{OXA-181} (p<0.001) and *bla*_{CTX-M-15} (p<0.001) was significantly

associated with the tested carbapenems (imipenem, meropenem and ertapenem) and cephalosporins, respectively. Furthermore, other β -lactamase ARG were carried by these isolates including *bla*_{TEM}, *bla*_{SHV} and *bla*_{CMY} and in fact most isolates were resistant to amoxicillin/clavulanate (n=79;96%) and to piperacillin/tazobactam (n=81;99%). With the MIC₅₀ and MIC₉₀ values determined for these antibiotic combinations being of >32 mg/L. In addition, *OmpK* truncations, which are associated with resistance to β -lactams, were harboured by all PP ST15 isolates (*OmpK35* n=86; *OmpK35* and *OmpK36* n=1) which is in line with the presence of the β -lactam ARG found. PP ST15 (n=77;100%) *K. pneumoniae* were tested against ceftazidime/avibactam and aztreonam/avibactam as these were carbapenemase carrying isolates. The results showed that among these isolates 96% (n=74) were resistant to ceftazidime/avibactam (MIC₅₀ >4 mg/L; MIC₉₀ >4mg/L) and all were susceptible to aztreonam/avibactam (MIC₅₀ and MIC₉₀ 0.5 mg/L) which is in line with the presence of *bla*_{NDM-1} because this is an MBL (Fig. 49; Table 12).

The percentage of resistance to aminoglycosides were high as well. PP ST15 isolates revealed that 98%(n=80) were resistant to gentamicin and tobramycin (MIC₅₀ and MIC₉₀ > 8 mg/L) and 99% (n=81) were resistant to amikacin (MIC₅₀ and MIC₉₀ >32 mg/L). Thus, the high percentages of resistance to aminoglycosides suggested that Agly ARG were harboured by PP ST15 *K. pneumoniae* and in fact most isolates harboured Agly ARG that included *aac*, *aad* and *armA* and *rmt* ARG which are responsible for resistance to gentamicin, tobramycin and amikacin, respectively (165, 166)(Fig. 49).

Lastly, most isolates including PP-BB, PP-BR, PP-MR and PP-ENV isolates were resistant to fluoroquinolones ciprofloxacin (n=80;98%) and levofloxacin (n=78;95%). ST15 PP isolates displayed MIC₅₀ and MIC₉₀ >2 mg/L for ciprofloxacin and levofloxacin MIC₅₀ and MIC₉₀ were of >4 mg/L. WGS of these isolates showed that most PP ST15 carried *gyrA-83F*, *gyrA-87A*, *parC-80I* fluoroquinolones resistance SNPs and *qnrS1* ARG.

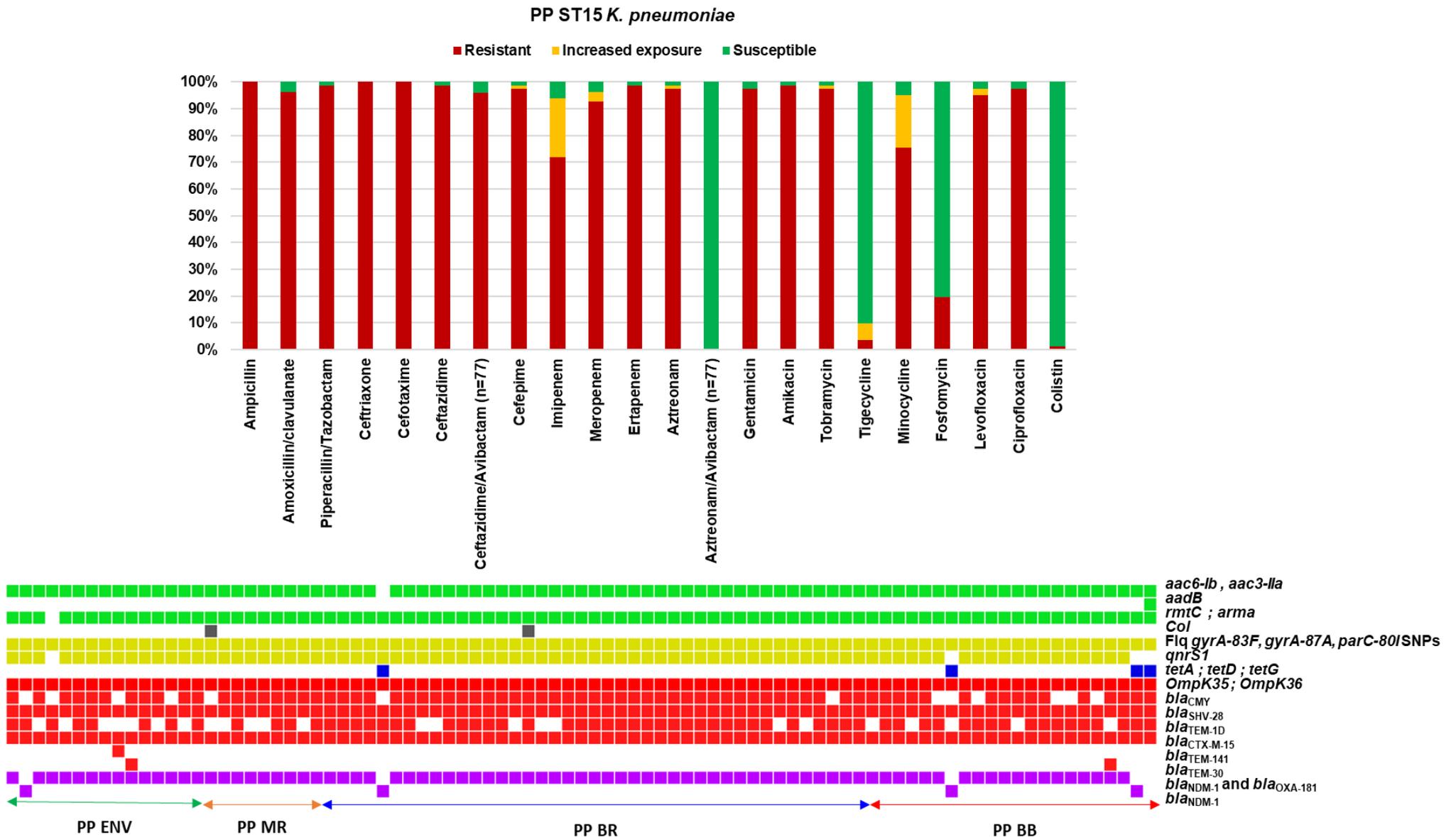


Fig. 49: Antibiotic susceptibility and ARG profile of PP ST15 *K. pneumoniae*. All ST15 *K. pneumoniae* were resistant to ceftriaxone and cefotaxime with most isolates also being resistant to ceftazidime (98%) and ceftazidime/avibactam (95%). Most isolates were resistant to amoxicillin/clavulanate (96%), piperacillin/tazobactam (99%), imipenem (72%), meropenem (93%),ertapenem (98%) and aztreonam (98%). In addition, 98% ST15 *K. pneumoniae* were resistant to gentamicin and tobramycin and 99% were resistant to amikacin. These isolates were resistant to minocycline (76%) but 90% were susceptible to tigecycline, and resistant to levofloxacin (95%) and ciprofloxacin (98%). In majority PP ST15 *K. pneumoniae* were susceptible to fosfomycin (80%), colistin (100%) and to aztreonam/avibactam (100%). Heatmaps show the presence of aminoglycoside ARG (green), tetracycline ARG (blue), fluoroquinolones ARG and SNPs (yellow) and β-lactamase ARG including ESBLs (red) and carbapenemase ARG (purple) carried by ST15 *K. pneumoniae* from ES-BB, ES-BR, ES-MR and ES-ENV.

6.4.1 Antibiotic susceptibility and ARG among ST15 PP *K. pneumoniae* – discussion

In relation to ST15 PP *K. pneumoniae* these isolates showed high percentages of resistance to the antibiotics and these results were in line with ARG content that WGS revealed (Fig. 50). Also, showed high percentages of resistance to most antibiotics including β -lactams and aminoglycosides in addition these values were over the clinical breakpoint for ciprofloxacin and levofloxacin. ST15 *K. pneumoniae* were only shown to be in majority susceptible to colistin, tigecycline and aztreonam/avibactam with MIC₅₀ and MIC₉₀ below the clinical breakpoint for colistin and aztreonam/avibactam but not to tigecycline. Possibly due to the presence of isolates showing increased exposure to tigecycline. In addition, most isolates were susceptible to fosfomycin, MIC₅₀ and MIC₉₀ values were not over the clinical breakpoint. However, these results are particularly important regarding ST15 PP-BB isolates because most carry carbapenemase and ESBL ARG and other β -lactam resistance markers as other β -lactamases and *OmpK* truncations, which could explain why MIC₅₀ and MIC₉₀ values were over the clinical breakpoint for all β -lactam antibiotics which turn reduces the chances of these antibiotics being an option for treatment of neonatal sepsis in PIMS. Likewise, MIC₅₀ and MIC₉₀ values were over the clinical breakpoints for aminoglycosides and fluoroquinolones and ST15 PP-BB *K. pneumoniae* carried not ARG that confer aminoglycoside and fluoroquinolone resistance but also carried fluoroquinolone resistance conferring SNPs (Fig. 49). Nevertheless, another reason for such high percentages of resistance could be linked to antibiotic usage as when empirically prescribing antibiotics to treat neonatal sepsis the most commonly used antibiotics included piperacillin/tazobactam and amikacin, cefotaxime, vancomycin, and imipenem and, treatments used for *K. pneumoniae* infection in PIMS included for example cefotaxime, piperacillin/tazobactam, amoxicillin, imipenem, meropenem, amikacin, ciprofloxacin and colistin (169). All PP ST15 *K. pneumoniae* that showed high percentage of resistance and MIC₅₀ and MIC₉₀ values over the clinical breakpoint with the exception of colistin and tigecycline. This is of concern because these ARG and high antibiotic resistance were not only displayed by PP-BB but also by PP-BR, MR and PP-ENV *K. pneumoniae* suggesting the spread of ARG among the isolates present in the MR, BR, BB and ENV was occurring through HGT seeing that these ARG are often associated with plasmids (179).

ST15 *K. pneumoniae*

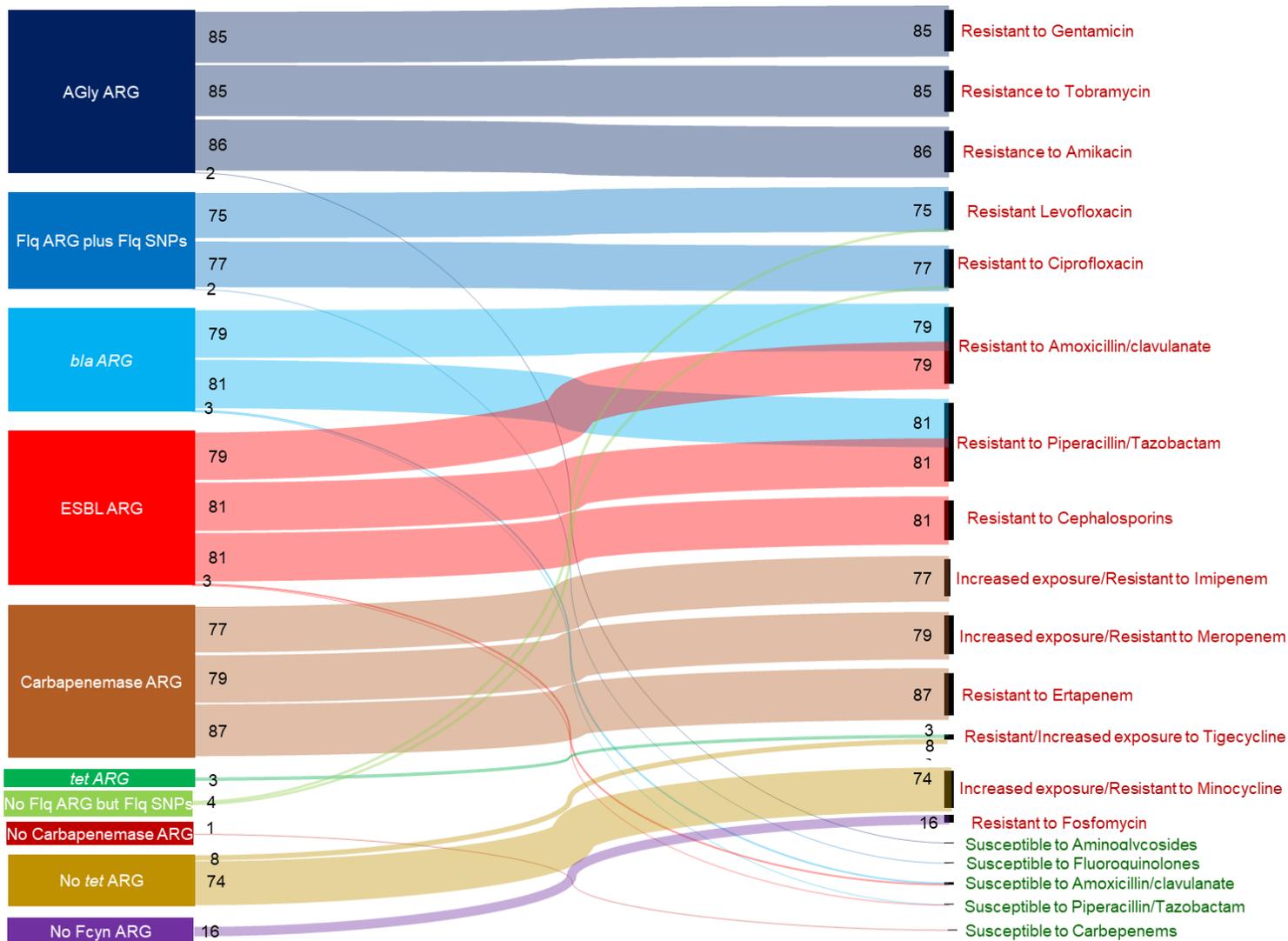


Fig. 50: Sankey diagrams linking ST15 *K. pneumoniae* isolates that carry or not carry ARG with their antibiotic susceptibility profile. The number of isolates that carried ARG (left) are linked to the antibiotic susceptibility profile displayed by these isolates to which each ARG could confer resistance to (right). The number of isolates is indicated in each coloured band, the number of isolates carrying that type of ARG (left) and the number of isolates displaying the respective antibiotic susceptibility (right). The isolates that carried ARG displayed either resistance or increased exposure to the antibiotics that they would confer resistance to and ST15 PP isolates that did not carry ARG showed susceptibility. However, 16 ST15 PP isolates that did not carry Fcyn ARG were resistant to fosfomycin and 74 and 8 *tet* ARG were increased exposure/resistant to minocycline and tigecycline, respectively.

6.5 *In silico* serotyping of PP *K. pneumoniae*

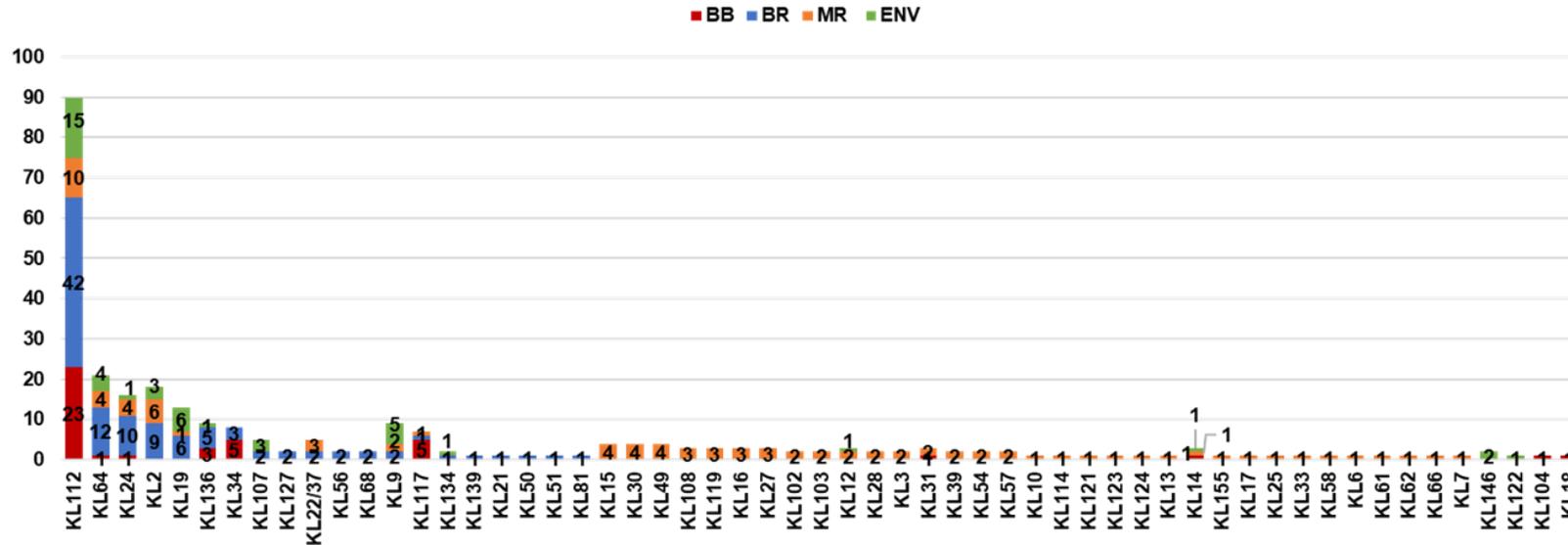
PP *K. pneumoniae* recovered from PP-BB, PP-BR and PP-ENV were studied individually to evaluate differences between the PP *K. pneumoniae* and namely understand the traits of PP-BB isolates

In total PP-BB *K. pneumoniae* (n=42;100%) were studied displaying 10 different KL and seven different OL. PP-BB *K. pneumoniae* showed KL112 in majority (n=23;55%) followed by KL117 (n=5;12%) and KL34 (n=5;12%). PP-BR *K. pneumoniae* showed more diversity than PP-BB isolates, displaying 20 different KL and 11 different OL. However, the dataset of PP-BR *K. pneumoniae* (n=106;100%) was greater in number than PP-BB isolates which could have contributed to the larger diversity in distinct KL and OL. That being said, among PP-BR *K. pneumoniae* there were three main KL identified KL112 (n=42;40%) followed by KL64 (n=12;11%) and KL24 (n=10;9%). Among PP-MR (n=92;100%) KL112 (n=10;11%) was the most showed by isolates followed by KL2 (n=6;6.5%) and KL15 (n=4;4%) In addition, the diversity of KL was higher among PP-MR isolates compared to PP-BB and PP-BR isolates with 38 different KL being identified in total. The distribution of KL is shown in Fig. 51. Within PP-ENV *K. pneumoniae* isolates (n=44;100%) KL112 (n=15;34%) was the most frequently detected, followed by KL19 (n=6;14%) and KL9 (n=5;11%).

Moreover, KL112 (n=90;32%) was the most frequently identified among PP *K. pneumoniae*, from all sample types and other sections explore KL types in relation to STs and population structure. Other KL frequently found across the PP isolates from different sample types of origin were different. PP-BB *K. pneumoniae* showed KL117 and KL34 but were not among the most frequently found among the PP-BR (KL64 and KL24), PP-MR (KL2 and KL15) and PP-ENV (KL19 and KL9).

In relation to the OL found PP-BB *K. pneumoniae* displayed in majority O1v1 (n=23;55%) followed by O2v2 (n=6;14%) and O1v2 (n=5;12%). O1v1 (n=48;45%) was the most frequently showed by PP-BR (n=48;45%), PP-MR (n=22;24%) and PP-ENV (n=16;36%) *K. pneumoniae* as well (Fig. 51). In contrast, the most common OL that followed were different, PP-BR isolates showed O1/O2v1 (n=13;12%) and O2v1 (n=13;12%), PP-MR displayed O2v2 (n=14;12%) and O1/O2v1 (n=10;11%) and PP-ENV *K. pneumoniae* displayed O2v2 (n=9;20.5%), also shown by PP-MR isolates, and O1v2 (n=7;8%).

PP *K. pneumoniae* KL distribution per sample type



PP *K. pneumoniae* OL distribution per sample type

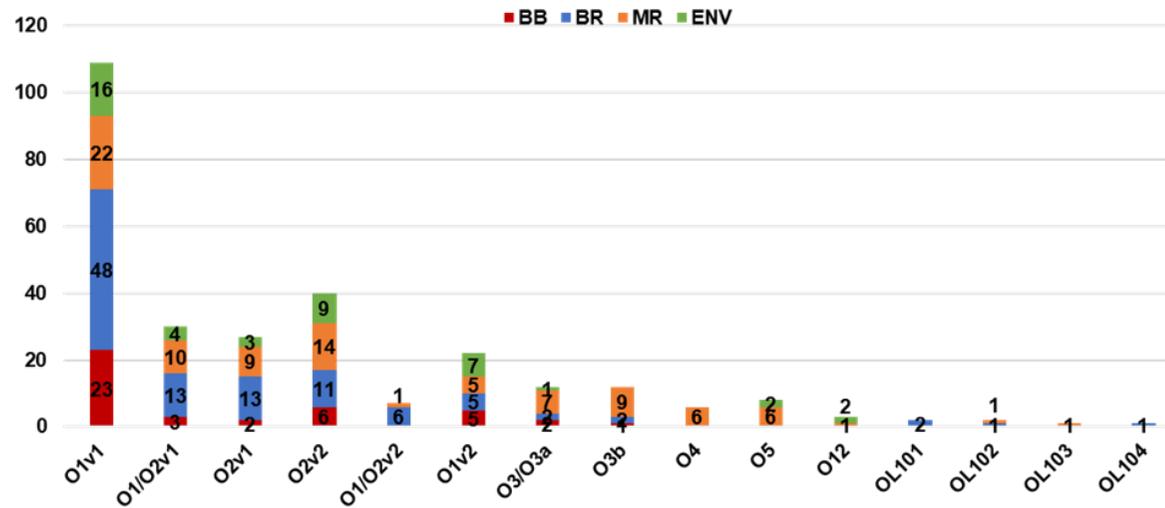


Fig. 51: Distribution of common KL and OL among PP *K. pneumoniae* from different sample types of origin. The most frequently detected KL was KL112 (n=90) followed by KL64 (n=21) and KL24 (n=16). Among PP-BB *K. pneumoniae* the most frequent KL was KL112 (n=23) followed KL34 (n=5) and KL117 (n=5). PP-BR *K. pneumoniae* frequently carried KL112 (n=42) and KL64(n=12) followed by KL24 (n=10). Among PP-MR the most common KL was KL112 (n=10) followed KL2 (n=6) and KL64 (n=4) and KL24 (n=4). PP-ENV *K. pneumoniae* frequently carried KL19 (n=6), KL9 (n=5) and KL64 (n=4) followed by KL15 (n=1). The most frequently detected OL was O1v2 (n=109), followed by O2v2 (n=40) and O1/O2v1 (n=30). In relation to OL among the distinct sample types of origin, PP-BB *K. pneumoniae* most commonly displayed O1v1 (n=23), followed by O2v2 (n=6) and O1/O2v1 (n=3); PP-BR isolates frequently displayed O2v2 (n=48) followed by O1/O2v1 (n=13) and O2v1 (n=13). Among PP-MR *K. pneumoniae* commonly carried O1v1 (n=22) followed by O2v2 (n=14) and O1/O2v1 (n=10) and among PP-ENV isolates the most common OL was O1v1 (n=16) followed O2v2 (n=9) and O1/O2v1 (n=4).

Siderophore loci carried by PP-BB isolates included *ybt* and *iuc* carried by 26 (62%) and one (2%) isolate, respectively. No *iro* loci were harboured by PP-BB isolates. Among *ybt* loci the most found lineage was *ybt* 16 associated with ICEKp12 (n=22;52%). *ybt* 8 associated with ICEKp9 was carried by three (7%) isolates and an unknown *ybt* was found in one (2%) isolate. Similarly, among PP-BR (n=106), PP-MR (n=92) and PP-ENV (n=44) *K. pneumoniae* *ybt* was the most frequent as well, being harboured by 68 (64%) PP-BR isolates, 20 (22%) PP-MR *K. pneumoniae* and 29 (66%) PP-ENV isolates. Also, PP-BR, PP-MR and PP-ENV *K. pneumoniae* isolates harboured *ybt* 16 associated with ICEKp12 (n=21;20%, n=10;11% and n=17;40%, respectively) similarly to PP-BB *K. pneumoniae*. In addition, *ybt* 8 associated with ICEKp9 harboured by PP-BB isolates (n=3;7%) was harboured by PP-BR (n=3;3%) and PP-ENV too (n=1;2%) (Fig. 52). Additionally, PP-BR *K. pneumoniae* commonly carried other *ybt* that included *ybt* 14 associated with ICEKp5 (n=6;6%) and *ybt* 10 associated with ICEKp4 (n=3;3%). PP-MR and PP-ENV *K. pneumoniae* also carried other *ybt* loci. Among PP-MR isolates other *ybt* found included plasmid associated *ybt* 4 (n=3;3%) and *ybt* 10 associated with ICEKp4 (n=2;2%) with other lineages including *ybt* 0 associated with ICEKp12 (n=1;1%), *ybt* 14 associated with ICEKp5 (n=1;1%), *ybt* 9 associated with ICEKp3 (n=1;1%). Additionally, an unknown *ybt* lineage was displayed by two (2%) PP-MR isolates. Other *ybt* loci carried by PP-ENV *K. pneumoniae* three (7%) isolates harboured an unknown *ybt* lineage however six (14%) PP-ENV isolates carried *ybt* 14 associated with ICEKp5. *ybt* 0 associated with ICEKp12 (n=1;2%), *ybt* 9 associated with ICEKp3 (n=1;2%) and an unknown *ybt* lineage was displayed by two isolates (Fig. 52).

In relation, to *iuc* and *iro* loci, only three (7%) PP-BB carried *iuc* 1 loci, likewise, no *iro* loci were harboured by PP-BR and PP-ENV *K. pneumoniae* but three (3%) PP-BR carried *iuc* two (2%) isolates carried *iuc* 1 and one (1%) carried an unknown *iuc*. In addition, PP-MR (n=1;1%) carried an unknown *iuc* and four (4%) PP-MR harboured an unknown *iro*. Lastly, *rmpA* factors were harboured by one (2%) PP-BB *K. pneumoniae* which carried *rmpA2_5*, in contrast, among *K. pneumoniae* from other sample types of origin only two (2%) PP-BR *K. pneumoniae* carried *rmpA2_6*.

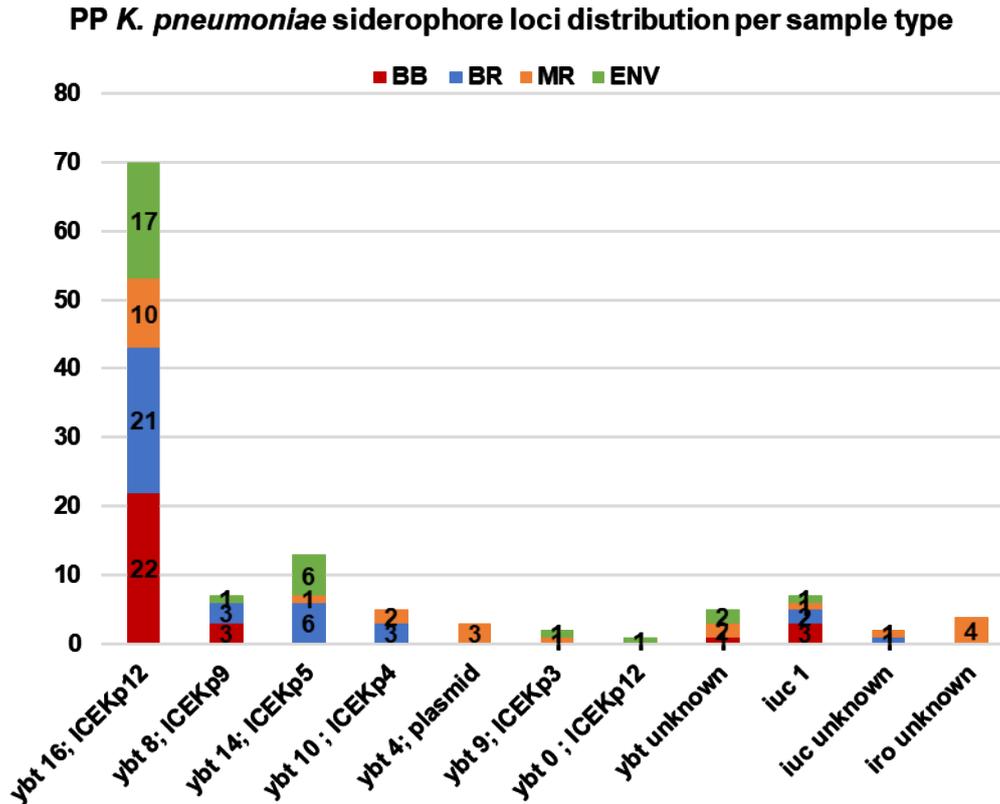


Fig. 52: Distribution of acquired siderophore loci lineages carried by PP-BB, BR, MR and ENV *K. pneumoniae*. *ybt16; ICEKp12* was the most common *ybt* lineage (n=70) among PP isolates being carried by PP-BB (n=22), PP-BR (n=21), PP-MR (n=10) and PP-ENV (n=17). *iuc 1* was carried by PP-BB (n=3), PP-BR (n=2), ES-MR (n=1) and ES-ENV (n=1).

6.6 *In silico* serotyping of PP-BB, BR, MR and ENV *K. pneumoniae* – discussion

PP *K. pneumoniae* isolates carried 61 different KL and 15 different OL were detected. The most frequently found were KL112, KL64, KL2 and O1v1, O2v2 and O1/O2v1. KL112 was found in *K. pneumoniae* from PP-BB, BR, MR and ENV isolates. Furthermore, the distribution of KL and OL was different when considering the different sample types of origin. Despite, KL112 being detected as the most common. Other common KL were detected such as, KL64 and KL24, among PP-BR, KL2 and KL15 among PP-MR and among PP-ENV *K. pneumoniae*, KL9 and KL19. Likewise, the most frequent O type for all samples was O1v1. The distribution of other commonly found OL varied among PP *K. pneumoniae* isolates from different sample type of origin. Since among PP-BB *K. pneumoniae* O2v2 and O1v2 were the most frequently detected following O1v1, with O1/O2v1 and O2v1 following among PP-BR isolates,

and among PP-MR O2v2 and O1/O2v1 were the most commonly identified after O1v1. However, likewise, in PP-BB isolates O2v2 and O1v2 were the most commonly detected after O1v1 among PP-ENV *K. pneumoniae*. Regarding siderophore loci and hypermucoidy factors, *ybt* was the most commonly found siderophore loci but *iuc*, *iro* siderophore loci and *rmpA2* factors were rare. *ybt* was carried by PP isolates from all sample types of origin, *iuc* was carried by PP-BB, PP-BR, and PP-MR isolates and *iro* was found in PP-MR only. *rmpA* factors were only found among PP-BB and PP-MR *K. pneumoniae*.

KL2 associated with hvKP infection (180) (1.7 *K. pneumoniae* virulence determinants, 1.8 Infections caused by virulent *K. pneumoniae*), was found among PP-MR (n=6;6.5%), PP-BR (n=9;8%) and PP-ENV (n=3;7%) suggesting that these *K. pneumoniae* strains presenting with markers of hypervirulence are present in the mothers and neonates' microbiota as well as in the clinical environment. In addition, KL64 described among antimicrobial resistant infection causing *K. pneumoniae* was common particularly among PP-BR. In contrast, other virulence determinants associated with hvKP infection such as *rmpA* and *iuc* and *iro* siderophore loci were rare among PP *K. pneumoniae* and more importantly among PP-BB isolates that caused sepsis. Nevertheless, *ybt* loci were frequently distributed among PP *K. pneumoniae* these being able to improve the ability of these isolates to cause infection too (86, 91, 92).

Also, though KL112, KL117 and KL34 were detected among PP-BB isolates these are less commonly found KL among *K. pneumoniae*. Furthermore, KL112 was detected among all ST15 *K. pneumoniae* and these frequently carried ARG that including ESBLs and carbapenemases (*bla*_{NDM-1} and *bla*_{OXA-181}) (6.2 Resistome of PP *K. pneumoniae*) which are responsible for high percentages of resistance among PP isolates. With this in mind and that less common KL, such as KL64, were described among *K. pneumoniae* causing antimicrobial resistant infection, and that KL variability is wider among resistant *K. pneumoniae*. It would follow that not only ST15 PP-BB *K. pneumoniae* would display KL described as less common, but also, that ST15 PP isolates from PP-MR, PP-BR and PP-ENV would as well since numerous ARG were displayed by ST15 PP *K. pneumoniae* (55).

PP *K. pneumoniae* rarely showed determinants associated with hvKP (58, 86) (1.7 *K. pneumoniae* virulence determinants), indicating that hypervirulent infection could be less frequent among PP *K. pneumoniae*. However, *iuc* and *rmpA* were found

among PP-BB *K. pneumoniae* isolates belonging to ST15 indicating that sepsis causing *K. pneumoniae* in PIMS have the potential to cause hypervirulent infection.

Nevertheless, considering that ARG and antibiotics resistance percentages were high among all PP-BB *K. pneumoniae* including PP ST15 isolates. It would follow that PP-BB *K. pneumoniae* would probably cause antibiotic resistant infection more often rather than hypervirulent infection. Still, virulence determinants as *ybt* and *iuc* loci and *rmpA* were present among ST15 *K. pneumoniae* which concurrently with the numerous ARG carried could exacerbate infection. Thus, ST15 PP-BB *K. pneumoniae* showed the potential of causing hypervirulent infection for which the outcome can be serious. From the available data regarding the outcome of ST15 PP-BB *K. pneumoniae* sepsis cases, 13 neonates survived, five were deceased and for four the outcome was untraceable. Of the neonates with the outcome deceased one case was caused by a PP-BB ST15 isolate harbouring *iuc* 1.

6.6 Plasmid replicon harboured by PP *K. pneumoniae*

In this study 270 (100%) PP *K. pneumoniae* including 40 (15%) PP-BB, 106 (39%) PP-BR, 81 (30%) PP-MR and 43 (16%) PP-ENV harboured plasmid replicons. Different plasmid replicon types were harboured by PP *K. pneumoniae* these included Col, IncF, IncH, IncI, IncL/M, IncN, IncQ, IncR, IncX and TrfA. (Fig. 53).

PP-BB (n=40)	PP-BR (n=106)	PP-MR (n=81)	PP-ENV (n=43)
Col	Col	Col	Col
IncF	IncF	IncF	IncF
IncH	IncH	IncH	IncH
IncX	IncX	IncX	IncX
IncN	IncN	IncN	IncN

Fig. 53 Types of common plasmid replicons harboured by *K. pneumoniae* from Pakistan among the different sample types PP-BB, PP-BR, PP-MR and PP-ENV

Col type plasmids harboured by PP *K. pneumoniae*

Regarding Col type plasmids type ColpVC (n=497;46%) was the most frequent replicon followed by ColRNAI (n=350;32%) and ColKP3 (n=163;15%). ColpVC replicons were harboured by 101 (37%) PP isolates from different sample types of

origin (Fig. 56). PP *K. pneumoniae* that carried ColRNAI and ColKP3 were distributed across PP *K. pneumoniae* from different sample types of origin too (Fig. 54). Additionally, Col (MG828) (n=47;4%), Col (MGD2) (n=13;1%), Col (Ye4449) (n=8;0.7%), Col (BS512) (n=6;0.6%) were also detected. Lastly, PP *K. pneumoniae* (n=46;17%) including eight (3%) PP-BB, 23 (8.5%) PP-BR, six (2%) PP-MR and nine (3%) ENV harboured Col (MGD2) replicons. Col (Ye4449) replicons were harboured by two (0.7%) PP-BB, three (1%) PP-BR and three (1%) PP-MR. Lastly, Col (BS512) replicons were carried by three (1%) PP-BR isolates.

Inc type plasmids harboured by PP *K. pneumoniae*

Among the different Inc replicon types several IncF type plasmids replicons were found including IncFIB(K) (n=229), IncFII (n=206) and IncFIA(HI1) (n=157). IncFIB(K) were carried by 220 (81%) PP *K. pneumoniae* (Fig. 56). Also, 96 (35.5%) PP *K. pneumoniae* harboured IncFIB(pKPHS1) and IncFIB(Mar) was harboured by 87 (32%) PP *K. pneumoniae*. In addition, IncFIB(pQil) was carried by PP-BB (n=2;0.7%), PP-BR (n=12;4%), PP-MR (n=4;1.5%) and PP-ENV (n=2;0.7%) *K. pneumoniae*. Also, IncFIB (AP001918) was carried by three (1%) PP-BR and one (0.4%) PP-MR. Furthermore, PP-BR (n=3;1%) and PP-MR (n=1;0.4%) carried IncFII(K) and IncFII(Yp) was harboured by one (0.4%) PP-BB and two (0.7%) PP-MR isolates. Lastly, regarding IncF replicon plasmids, IncFII was carried by one (0.4%) PP-BB, one (0.4%) PP-BR and one (0.4%) PP-ENV. Additionally, IncFIA was harboured by two (0.7%) PP-BR and IncFIC(FII) was carried by one (0.4%) PP-BR and one (0.4%) PP-BR. IncFII(pRSB107) was carried by one (0.4%) PP-BR isolate.

Moreover, PP *K. pneumoniae* carried plasmids with other replicon types including IncH, IncX and IncN. IncH replicons found included replicons such as IncHI1B(pNDM-MAR) which was harboured by 92 (34%) PP *K. pneumoniae* (Fig. 54). In addition, IncHI2 and IncHI2A replicons were both carried by one (0.4%) PP-BR and one (0.4%) PP-MR *K. pneumoniae*. IncX replicon plasmids were carried by PP *K. pneumoniae* included IncX3 and IncX4, 98 (36%) PP *K. pneumoniae* carried IncX3 and IncX4 replicons. IncN plasmid type replicons were carried by PP-BB (n=3;1%), PP-BR (n=10;4%), PP-MR (n=11;4%) and PP-ENV (n=2;0.7%). Lastly, IncL/M(pOXA-48) replicons was harboured by PP-BR (n=1;0.4%) and one (0.4%) PP-ENV and IncQ

plasmid type replicons IncQ1 and IncQ2 were both carried by one PPMR each. In addition, replicons IncI1 and TrfA were both carried by one PP-BR each.

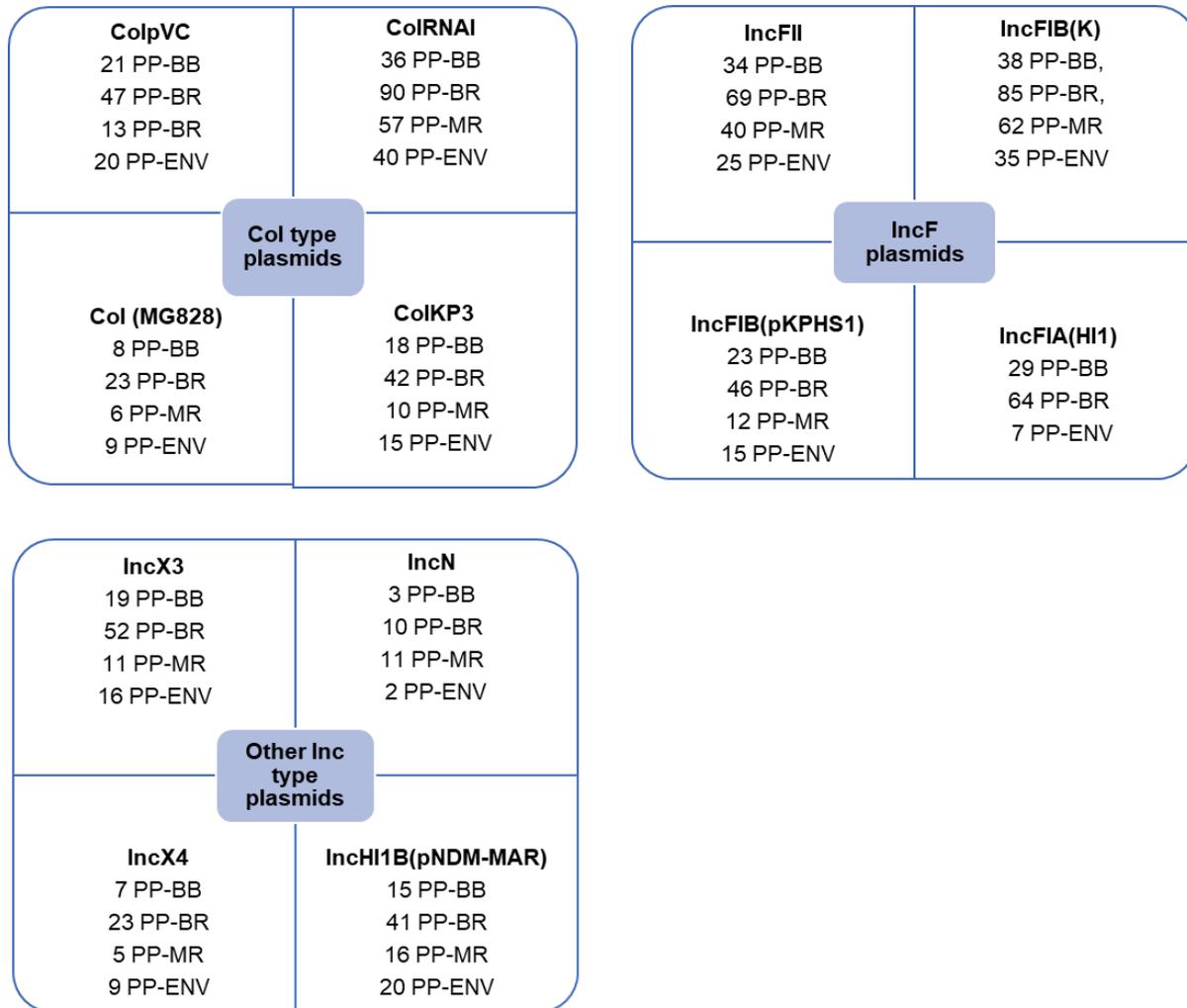


Fig. 54: Col and Inc type plasmid replicons distribution among the PP *K. pneumoniae* across the different sample types of origin. Col type replicons were found among PP-BB, PP-BR and PP-ENV *K. pneumoniae* but were mostly found among PP-BR isolates, with ColRNAI being the most frequently found among PP-BB (n=36), PP-BR (n=90), PP-MR (n=57) and PP-ENV (n=40). **IncF plasmids** were commonly found among PP *K. pneumoniae* with IncFIB(K) being found among 38 PP-BB, 85 PP-BR, 62 PP-MR and 35 PP-ENV. **Other Inc type** plasmids included IncX3, IncX4, IncN and IncHI1B (pNDM-MAR) plasmids, with the most frequently found being IncX3 harboured by 19 PP-BB, 52 PP-BR, 11 PP-MR and 16 PP-ENV.

6.6.1 Inc and ColKP type plasmids carrying *bla*_{NDM-1} and *bla*_{OXA-181} dissemination among PP *K. pneumoniae*

Carbapenemase ARG were frequently found among PP *K. pneumoniae*, thus, long read sequences of plasmids harbouring *bla*_{NDM-1} and *bla*_{OXA-181} were used to construct databases to screen PP *K. pneumoniae* in total and PP ST15 *K. pneumoniae* separately because these PP isolates formed the major cluster among *K. pneumoniae* from PIMS. PP-BB *K. pneumoniae* commonly carried carbapenemase ARG thus understanding its dissemination would help understand the role of plasmids on the burden of resistance among PP *K. pneumoniae* causing sepsis.

In order to screen PP *K. pneumoniae* for plasmids carrying *bla*_{NDM-1} and *bla*_{OXA-181} the long-read sequence available as part of the BARNARDS project of ST15 PP-BB8 *K. pneumoniae* isolate was used for analysis. Analysis using Bandage revealed that this isolate harboured seven plasmids, however, only one harboured *bla*_{NDM-1}, this will be referred to as pKpnST15_PPBB_1, and another plasmid that will be referred as pKpnST15_PPBB_2, carried *bla*_{OXA-181}. (Fig. 55) shows ARG that were among the frequently found in this PhD. The annotation of these plasmids is available in Appendix 12. The sequences of these plasmids were used as the databases to search the PP *K. pneumoniae* in total and PP ST15 *K. pneumoniae*.

In addition to, pKpnST15_PPBB_1 (141kb) carrying *bla*_{NDM-1} and pKpnST15_PPBB_2 (51kb) carrying *bla*_{OXA-181} other ARG were harboured these plasmids as well (Fig. 55; Appendix 12). For example, pKpnST15_PPBB_1 carried Agly ARG, *aac6'-Ib* and *rmtC* in addition to *bla*_{NDM-1} and pKpnST15_PPBB_2 carried *qnrS1* in addition to *bla*_{OXA-181}, indicating that these plasmids could be responsible for different resistance profile among PP *K. pneumoniae*. pKpnST15_PPBB_1 showed an IncA/C replicon system and pKpnST15_PPBB_2 IncX3 and ColKP3 replicon systems suggesting that the impact of these plasmids on the spread of *bla*_{NDM-1} and *bla*_{OXA-181} could be different. Regarding pKpnST15_PPBB_1 carrying *bla*_{NDM-1}, contigs from PP *K. pneumoniae* isolates in total displaying hits with over 10% coverage were found among 21 (7%) PP-BB *K. pneumoniae*, but also among 52 (18%) PP-BR, 10 (3.5%) PP-MR and 14 (5%) PP-ENV isolates.

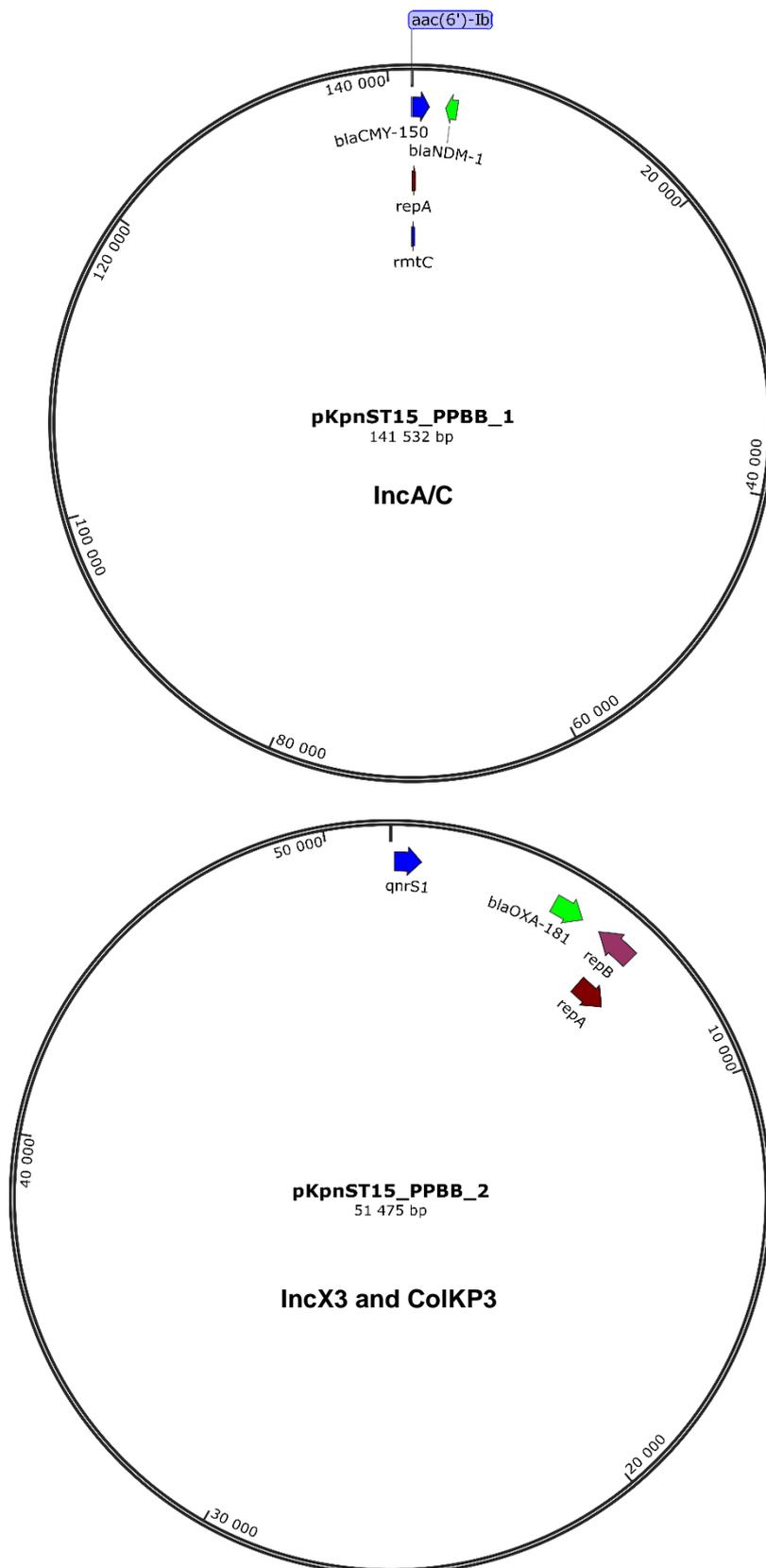


Fig. 55: ST15 PP-BB8 pKpnST15_PPBB_1 and pKpnST15_PPBB_2. pKpnST15_PPBB_1 had 187 893 bp and showed IncA/C replicon. The ARG harboured included *bla*_{NDM-1}, *bla*_{CMY-150}, *aac(6)-Ib* and *rmtC*. pKpnST15_PPBB_2 had 128 136 bp with IncX3 and ColKP3 replicons. The ARG harboured included *bla*_{OXA-181} and *qnrS1*. ST15 PP-BB carried other plasmids too.

Furthermore, considering the PP *K. pneumoniae* in total the results showed that these isolates mostly belonged to ST15 (n=81;28.5%) and PP isolates (n=16;6%) belonged to other STs, including ST11 (n=12;4%), ST20 (n=2;0.7%), ST48 (n=1;0.4%)

and ST1726 (n=1;0.4%). PP. *K. pneumoniae* belonging to these STs were also found among PP-BR (n=12;4%), PP-MR (n=2;0.7%) and PP-ENV (n=2;0.7%).

In relation to pKpnST15_PPBB_2 carrying *bla*_{OXA-181} contigs from PP *K. pneumoniae* sequences with over 10% coverage were found among PP-BB (n=20;7%), PP-BR (n=50;18%), PP-MR (n=10;3.5%) and PP-ENV (n=14;5%) with the average coverage being 31%. In addition, high percentage of coverage (64%) among contigs from PP-BB, PP-BR, PP-MR and PP-ENV *K. pneumoniae* sequences was detected.

Also, among PP *K. pneumoniae* in total the results showed 14 (5%) PP isolates belonged to ST11, including PP-BB (n=1;0.4%), PP-BR (n=10;3.5%), PP-MR (n=2;0.7%) and PP-ENV (n=1;0.4%). However, despite ST11 PP *K. pneumoniae* being found to contain contigs that matched pKpnST15_PPBB_1 and pKpnST15_PPBB_2 these did not carry *bla*_{OXA-181} but only *bla*_{NDM} ARG suggesting that these PP isolate might carry other plasmids similar to pKpnST15_PPBB_2 harbouring *bla*_{NDM}. ARG content. In contrast, most ST15 *K. pneumoniae* were found to carry *bla*_{NDM-1} and *bla*_{OXA-181} suggesting that pKpnST15_PPBB_1 and pKpnST15_PPBB_2 could be among these PP isolates.

The average percentage of coverage among PP *K. pneumoniae* when using pKpnST15_PPBB_1 as the database was 23% with highest percentage of coverage belonging to contigs from PP-BR (for example 65%) and PP-ENV (for example 47%) *K. pneumoniae*, however, among PP-BB isolates coverage of 40% was observed as well.

Considering ST15 *K. pneumoniae* (n=87) the average coverage was of 23% too, with contigs with over 10% coverage being found among PP-BB (n=21;24%), PP-BR (n=40;46%), PP-MR (n=6;7%) and PP-ENV (n=12;14%) *K. pneumoniae*. g pKpnST15_PPBB_2 among ST15 PP *K. pneumoniae* sequences matches to isolates sequences contigs were found in ST15 *K. pneumoniae* from different sample types of origin and the average percentage of coverage was 32%. pKpnST15_PPBB_2 contigs matches were found among 19 (22%) ST15 PP-BB *K. pneumoniae* and among 40 (46%) PP-BR, 8 (9%) PP-MR and 13 (15%) PP-ENV isolates. However, considering that numerous matches among PP *K. pneumoniae* could possibly not correspond to plasmid sequences when screening through a database among ES *K. pneumoniae* the same could happen among PP ST15 *K. pneumoniae*. Therefore, putative plasmid scaffolds were generated from ST15 *K. pneumoniae* short read genome sequences

and were aligned to pKpnST15_PPBB_1 and pKpnST15_PPBB_2 long read sequences.

Mapping pKpnST15_PPBB_1 and pKpnST15_PPBB_2 to ST15 *K. pneumoniae*

The results showed that it was possible to align the reference plasmids to putative plasmid scaffolds harboured among ST15 *K. pneumoniae*. And these did not show frequent variation in relation to pKpnST15_PPBB_1 and pKpnST15_PPBB_2 long read sequences (Fig. 56 and Fig. 57, respectively). More importantly this was seen among not only among PP-BB ST15 but also ST15 *K. pneumoniae* from BR, MR and ENV, suggesting that pKpnST15_PPBB_1 and pKpnST15_PPBB_2 were responsible for spread of *bla*_{NDM-1} and *bla*_{OXA-181} between mothers and neonates but were also present in clinical environment in PIMS which could also have contributed for the spread of *bla*_{NDM-1} and *bla*_{OXA-181}.

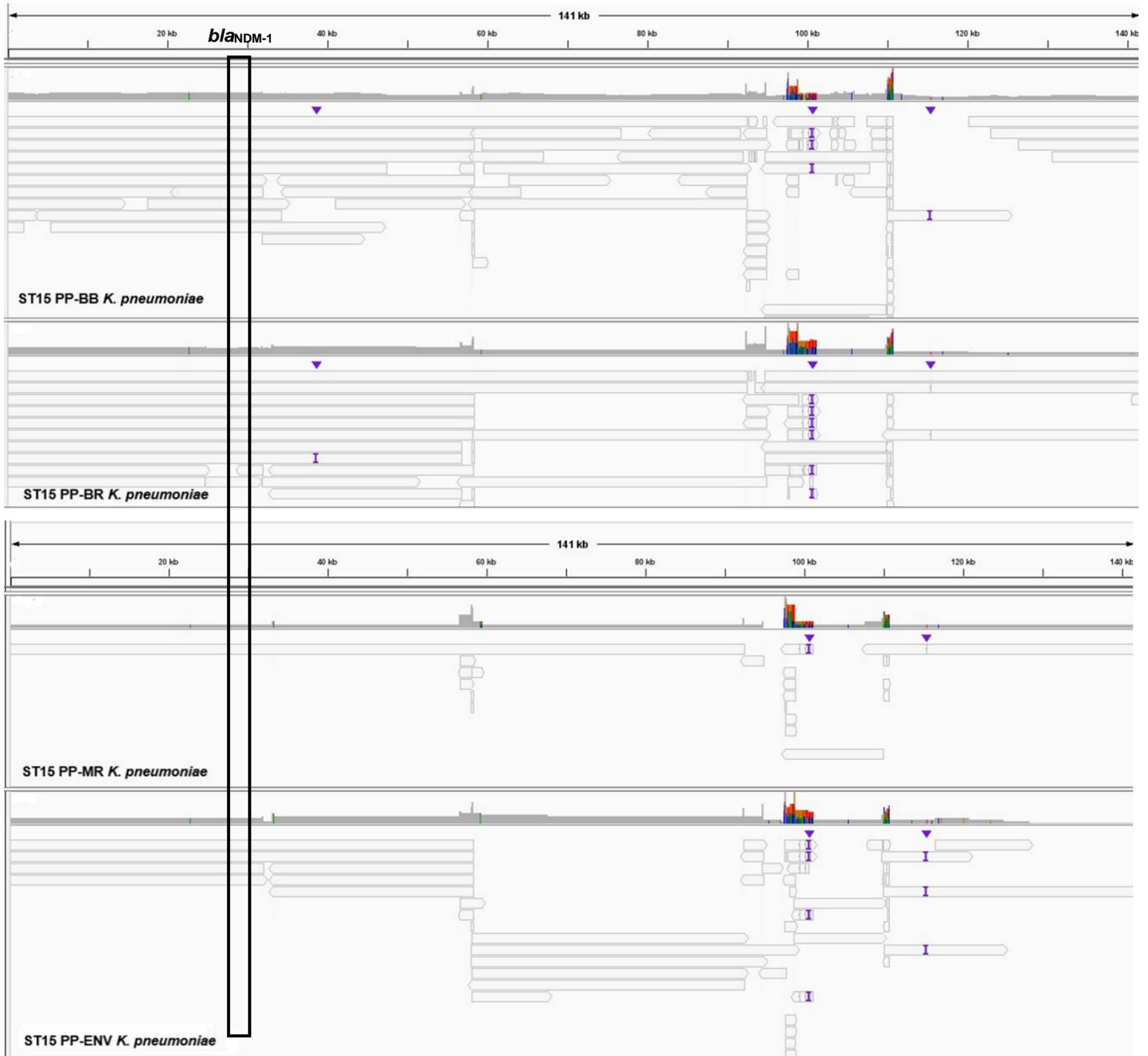


Fig. 56: ST15 *K. pneumoniae* plasmid putative plasmid scaffolds alignment using pKpnST15_PPBB_1 (141kb) as reference. Among PP-BB ST15 *K. pneumoniae* long reads were aligned across the reference plasmid and these displayed few variations in relation to pKpnST15_PPBB_1. The same was found among ST15 PP-BR, PP-MR and PP-ENV however pKpnST15_PPBB_1 was more frequently aligned among PP-BR, PP-BB and PP-ENV. The alignment indicates that pKpnST15_PPBB_1 was harboured by ST15 *K. pneumoniae* from the different sample types of origin. Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). The black rectangle indicates the region of pKpnST15_PPBB_1 (29815bp to 30627bp) in which *bla*_{NDM-1} was found.



Fig. 57: ST15 *K. pneumoniae* plasmid putative plasmid scaffolds alignment using pKpnST15_PPBB_2 (51kb) as reference. Among PP-BB ST15 *K. pneumoniae* long reads were aligned across the reference plasmid and these displayed few variations in relation to pKpnST15_PPBB_2. The same was found among ST15 PP-BR, PP-MR and PP-ENV however pKpnST15_PPBB_2 was more frequently aligned among PP-BR, PP-BB and PP-ENV. The alignment indicates that pKpnST15_PPBB_2 was harboured by ST15 *K. pneumoniae* from the different sample types of origin. Reads that align to the reference are represented by the rectangles and the different colours represent areas of variation (read bases that did not match the reference). The black rectangle indicates the region of pKpnST15_PPBB_2 (12964bp to 13761bp) in which *bla*_{OXA-181} was found.

6.7 Assessing relationships between ARG, virulence determinants and clinical data among PP *K. pneumoniae*

Also, among the ST15 PP-BB (n=22;100%) the effect of antibiotic resistance and acquired virulence determinants in the outcome and onset of infection was studied because these responsible for sepsis in PIMS. Fig. 58 below showing that among these *K. pneumoniae* most sepsis cases outcome was alive (n=13,59%), and the onset of infection was EOS in most cases (n=13;59%).

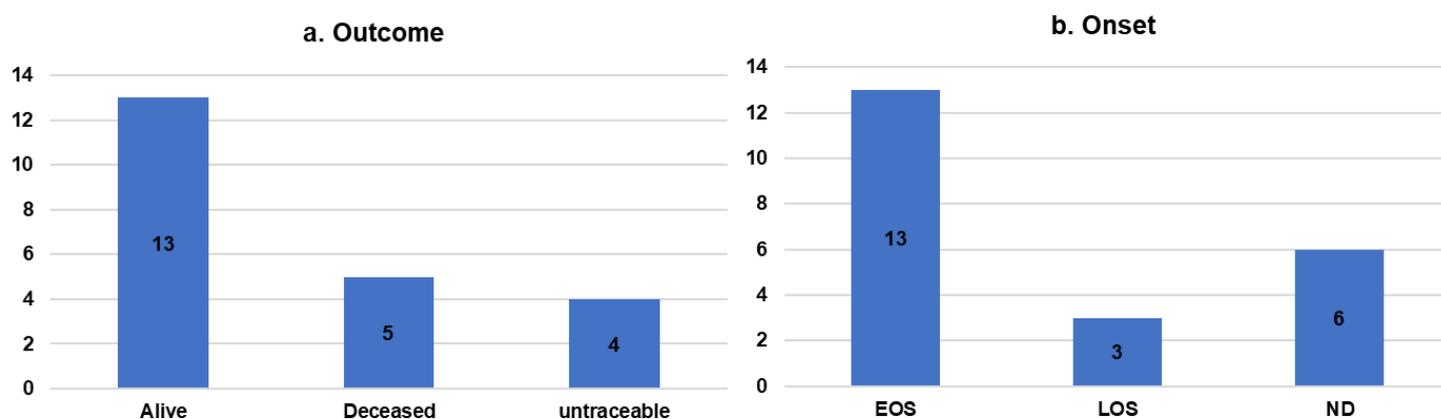


Fig. 58 : Outcome and onset of sepsis caused by PP-BB *K. pneumoniae* belonging to ST15. a. Outcome of sepsis was in majority alive (n=13) with five and four sepsis cases having the outcome of deceased and untraceable, respectively. b. Onset was in majority EOS (n=13) with the onset being LOS for three cases and not determined (ND) for six cases.

ST15 PP-BB *K. pneumoniae* were studied using Kaplan-Meier statistics to assess if carrying *bla*_{NDM-1}, *bla*_{NDM-1} and *bla*_{OXA-181} and siderophore loci affected the outcome of sepsis in PIMS (Fig. 59). Considering the survival plots in Fig. 59 it was possible to understand in overall the cumulative percentage of survival was high (91%,83%,66%,33%) among neonates the suffered from sepsis caused by ST15 PP-BB *K. pneumoniae* which is in accordance with the fact the outcome was alive for most ST15 isolates sepsis cases (n=13;59%) (Fig. 60a). Also, when *bla*_{NDM-1} was not carried the percentage survival was higher (90%, 81% and 41%) than when this ARG was carried by the sepsis causing isolate. In contrast, when ST15 PP-BB isolates carried both *bla*_{NDM-1} and *bla*_{OXA-181} the percentage of cumulative survival was higher (90%) than when these ARG were not present (50%) (Fig. 61). This could be because most ST15 PP-BB isolates (n=19;86%) carried both *bla*_{NDM-1} and *bla*_{OXA-181} and most neonates survived sepsis

(n=13;59%) caused by ST15 isolates including isolates carrying both these ARG. Whereas only two ST15 PP-BB isolates carried *bla*_{NDM-1} only and one neonate suffering from sepsis caused by one of these isolates did not survive. Also, when considering if the presence of siderophore loci affected the outcome of sepsis the survival plot showed that most neonates survived and the percentage of cumulative survival was high (91%,83%,66% and 33%). As expected, seeing that all ST15 PP-BB *K. pneumoniae* carried siderophore loci (6.1 Phylogeny and population of *K. pneumoniae* of PP) and again most neonates survived sepsis caused by ST15 *K. pneumoniae* (Fig. 59).

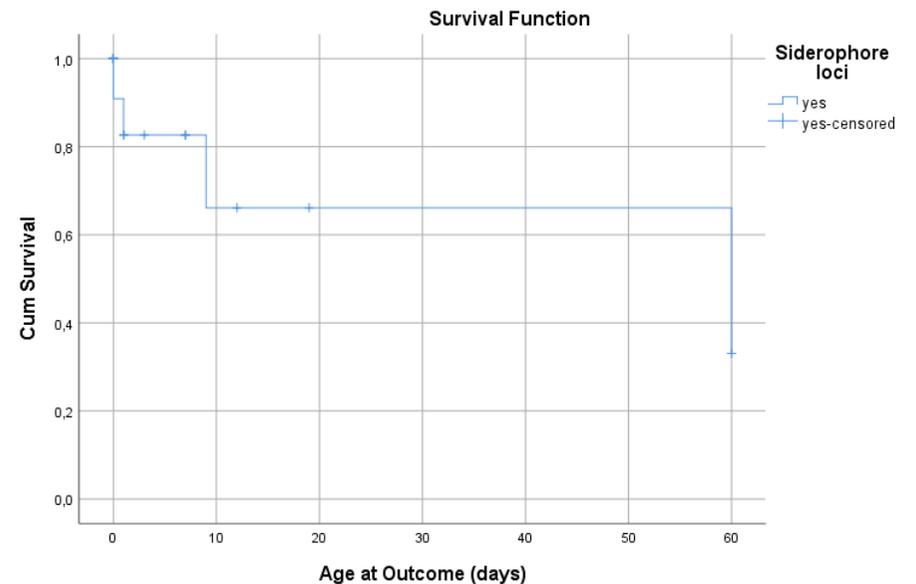
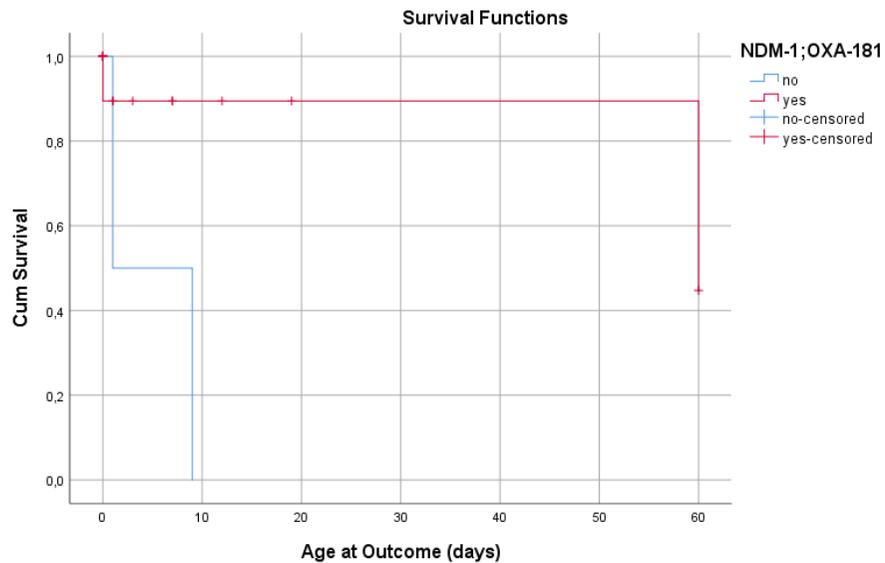
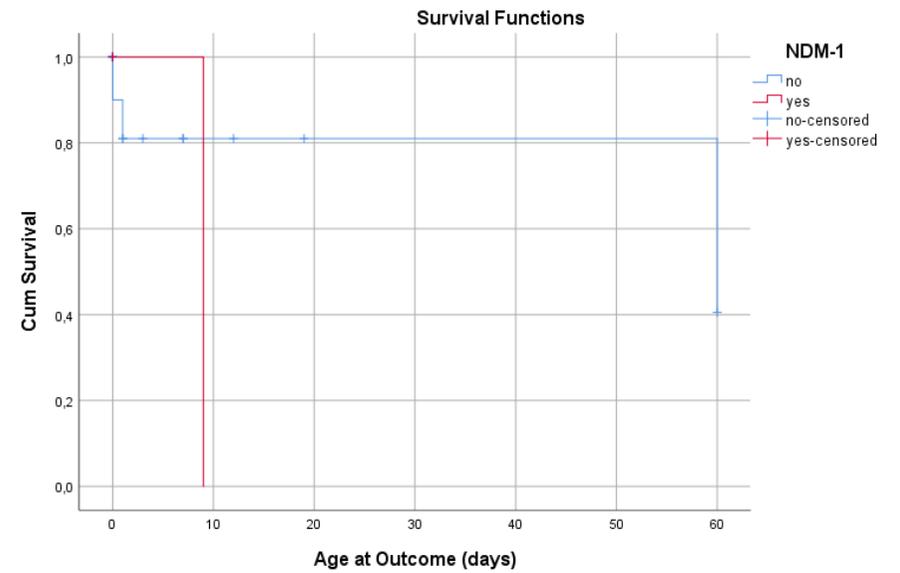
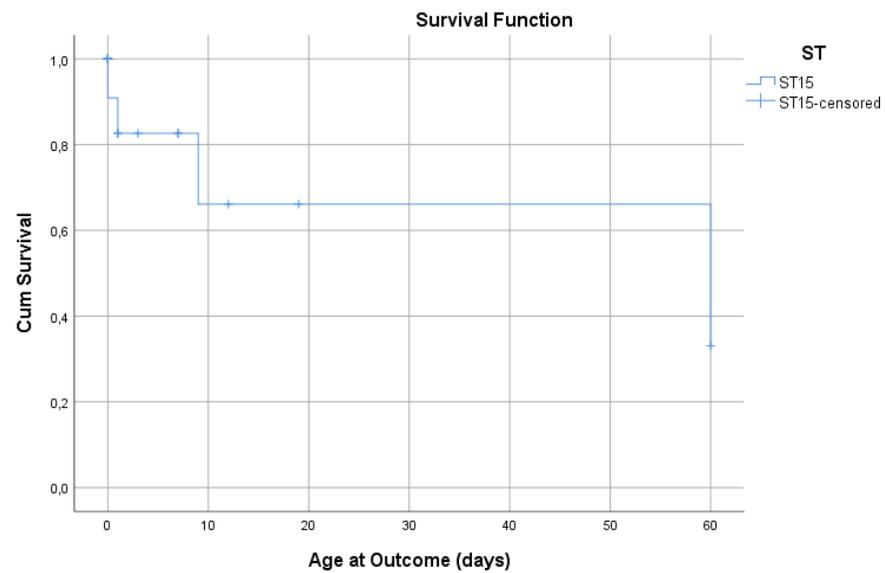


Fig. 59: Cumulative survival plots for sepsis caused by ST15 *K. pneumoniae* and for the presence of bla_{NDM-1} and for the presence of bla_{NDM-1} and $bla_{OXA-181}$ and for the presence of siderophore loci at the age of outcome days. For the ST survival plot the cumulative survival percentage for ST15 was 91%, 83%, 66% and 33% indicating that cumulative percentage of survival was high among neonates the suffered from sepsis caused by ST15 PP-BB *K. pneumoniae* which is in line with the favourable outcome of most ST15 isolates sepsis cases (n=13/22) For the presence of bla_{NDM-1} (yes; red line) 0% and when bla_{NDM-1} was not present (no; blue line) was 90%, 81% and 41%. For the presence of bla_{NDM-1} and $bla_{OXA-181}$ (yes; red line) the cumulative percentages of survival were 90% and 45% and when bla_{NDM-1} and $bla_{OXA-181}$ was not present (no; blue line) the percentage was 50%. When bla_{NDM-1} was not carried the percentage survival was higher than when this ARG was carried contrarily, both bla_{NDM-1} and $bla_{OXA-181}$ the percentage of cumulative survival was higher than when these ARG were not present. For the presence of siderophore loci (yes; blue line) the percentages of cumulative survival were 91%, 83%, 66% and 33% indicating that most neonates survived sepsis caused by these isolates. Cross in blue (no-censored) and red line (yes-censored) indicate where data relating to the sepsis age at outcome was not available, this is indicated in the lines corresponding to the presence or absence of the studied ARG and siderophore loci. For the ST plot cross in blue (ST15-censored), indicate where age at outcome data relating to sepsis caused by isolates belonging to these STs was not available

7. Results - Comparing the phylogeny, resistome, virulome and antibiotic susceptibility profiles of ES and PP *K. pneumoniae*

To understand the extent of relatedness between isolates, core genome phylogenetic analysis of the *K. pneumoniae* population from Ethiopia and Pakistan including BB, BR, MR and ENV isolates was studied (Fig. 60). The pangenome of the overall *K. pneumoniae* population was constituted by 1928 core genes (found in 99% to 100% of isolates) and 102 844 accessory genes in a total of 104 772 genes. In total 116 different STs were identified among the total *K. pneumoniae* isolates (n=465;100%). ST15 (n=87;19%) was the most frequently identified and these isolates were all from Pakistan, followed by ST37 (n=35;7.5%), ST35 (n=35;7.5%) and ST218 (n=22;5%) that were all from Ethiopia. *K. pneumoniae* isolates belonging to the same ST were clustered together despite the country or sample type they were recovered from. MR and BR isolates clustered together within clusters of the country of origin. BB *K. pneumoniae* were also clustered with *K. pneumoniae* recovered from other samples types (Fig. 60). *K. pneumoniae* belonging to ST15, ST35 and ST37 showed different distribution of ARG and virulence markers (Fig. 60). The ARG , virulence determinants and relatedness of Ethiopian and Pakistani *K. pneumoniae* was explored in sections 5.2 and 5.5, 6.2 and 6.5, and 5.1 and 6.1, respectively.

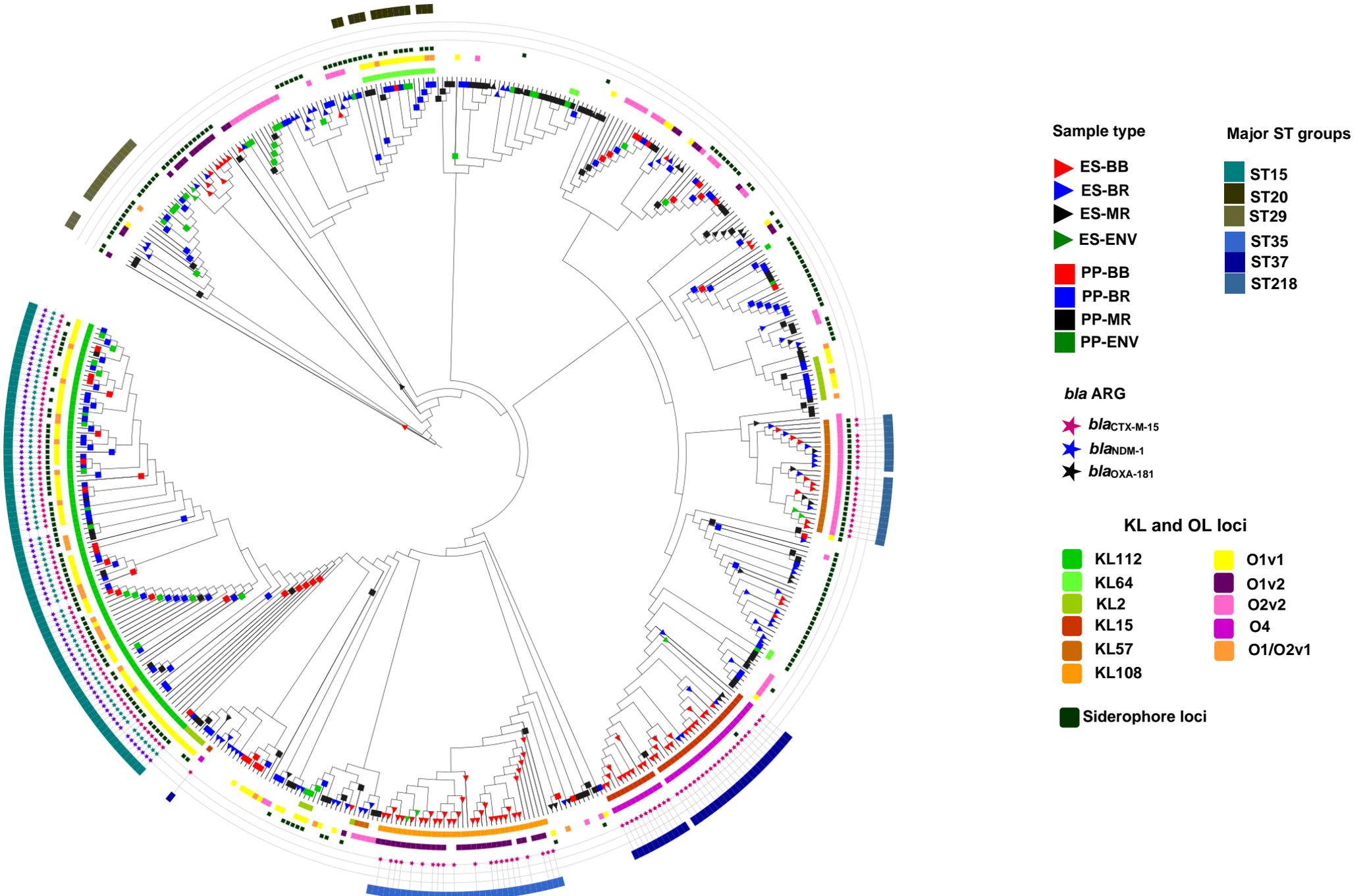


Fig. 60: Core genome maximum likelihood phylogeny of the overall population of *K. pneumoniae* from Ethiopia and Pakistan with distribution the most common ARG, KL, OL, presence of siderophore loci. . Distribution of common *bla* ARG and virulence determinants was different among the most common STs KL112 was shown by ST15 isolates and KL108 and KL15 by ST35 and ST37. ST15 *K. pneumoniae* carried acquired siderophore loci whereas most ST35 and ST37 did not carry siderophore loci. All ST15 *K. pneumoniae* carried *bla*_{NDM-1} whereas ST35 and ST37 did not but *bla*_{CTX-M-15} was commonly carried by *K. pneumoniae* irrespective of country. Sample type of ES (triangle) and PP (rectangle) isolates is shown in different colours BB (red), BR (blue), MR (black) and ENV (green). *bla*_{CTX-M-15} indicates the presence of *bla*_{CTX-M-15} ARG (pink star), *bla*_{NDM-1} the presence of *bla*_{NDM-1} ARG (blue star) and *bla*_{OXA-181} the presence of *bla*_{OXA-181} ARG (dark grey star)

7.1 Resistome of ES and PP *K. pneumoniae*

All isolates (n=465;100%) were studied regarding their acquired ARGs. Most, *K. pneumoniae* carried acquired ARGs to multiple classes of antibiotics concurrently. The most frequently found were β -lactamase ARG (*bla*) and these were carried by 399 (86%) isolates. Similarly, aminoglycosides (Agly) ARG were carried by 388 (83%) isolates. In addition, sulphonamides (Sul) and fluoroquinolones (Flq) ARG and Flq resistance SNPs were found in 371 (80%) and 303 (65%) isolates, respectively. Other ARG carried by the isolates in study included trimethoprim (Tmt) (n=279;60%), tetracyclines (*tet*) (n=229;49%), phenicols (Phe) (n=214;46%), rifampin (Rif)(n=175) and macrolides (MLS) (n=68). These are listed in Appendix 3 and an extensive description of the resistome of the total isolates in study is included in Appendix 10. Fig. 61 shows that most ARG carried were carried simultaneously by the studied isolates shows the distribution of ARG according to antibiotic class for Ethiopia and Pakistan.

Of the chromosomal mutations contributing to increased resistance, the following were screened for (1) fluoroquinolone resistance SNPs, namely *gyrA*-83 & 87 and *parC*80 & 84, (2) the truncation of *mgrB* or *pmrB* which contribute to colistin resistance and (3) *OmpK35* or *OmpK36* genes truncations, which reduce susceptibility to β -lactams (41, 53). *OmpK35/36* truncations (n=119;26%) were the more frequently carried mutations followed by the presence of fluoroquinolone resistance SNPs carried by 113 (24%) isolates. Additionally, six (1%) isolates carried *mgrB* (n=3;0.6%) or *pmrB* (n=3;0.6%) truncations. Regarding the *OmpK35/36* truncations, most isolates showed truncation only of *OmpK35* (n=110;24%). *OmpK36* truncation was present in nine isolates. In addition, among these isolates six (5%) isolates carried both truncations. Among the *K. pneumoniae* isolates with Flq resistance SNPs 92 (81%) carried Flq ARG simultaneously and 23 (20%) isolates carried Flq resistance SNPs only. *gyrA*83, *gyrA*87 and *parC*80 SNPs were found among the studied *Klebsiella* spp. population.

Having characterised both *K. pneumoniae* populations and studying them separately (in sections 5.2 and 6.2) it was possible to identify differences and similarities when comparing them as discussed in the next section.

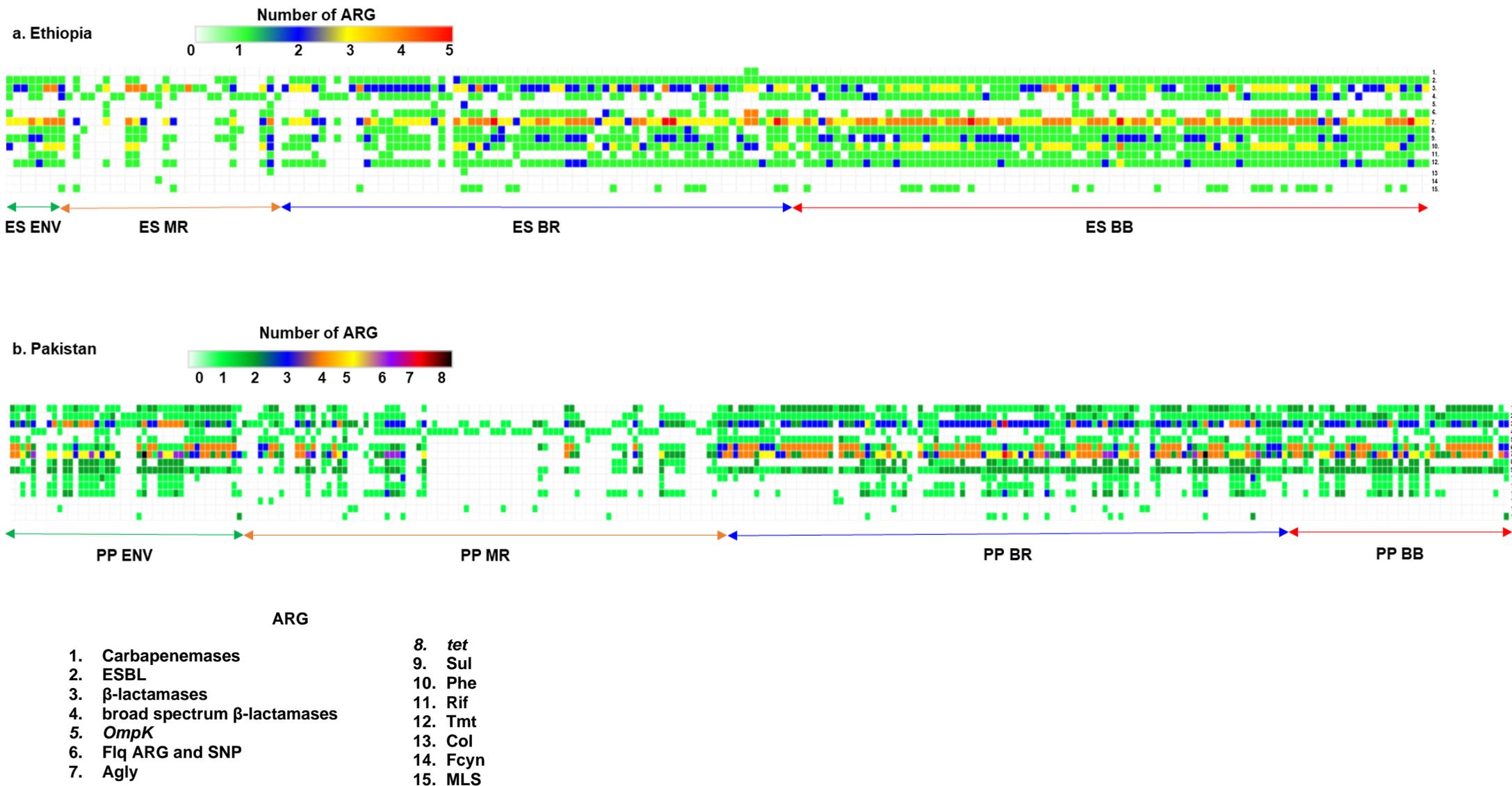


Fig. 61: Distribution of ARG belonging to different classes among *K. pneumoniae* isolates from Ethiopia (a.) and Pakistan (b.) recovered from different sample types (BB, BR, MR and ENV). ARGs belonging to different classes were carried concurrently by the *K. pneumoniae* in study. The more frequently found were β -lactamase ARG and these were carried by 399 isolates. Similarly, aminoglycosides (Agly) ARG were carried by 388 isolates. In addition, sulphonamides (Sul) and fluoroquinolones (Flq) ARG and Flq resistance SNPs were found in 371 and 303 isolates, respectively, were frequently carried as well. Other ARG carried by the isolates in study included trimethoprim (Tmt) (n=279), tetracyclines (*tet*) (n=229), phenicols (Phe) (n=214), rifampin (Rif)(n=175) and macrolides (MLS) (n=68). Carbapenemase ARG were mostly carried by Pakistani *K. pneumoniae* (n=174) and ESBL were by most *K. pneumoniae* from Pakistan (n=190) and Ethiopia (n=166). **1.** Carbapenemases the presence of carbapenemase ARG, **2.** ESBL the presence of ESBL ARG, **3.** β -lactamases the number for β -lactamases, **4.** broad spectrum β -lactamases the number of broad spectrum β -lactamases, **5.** *OmpK* the number *OmpK* truncations, **6.** Flq ARG and SNPs the number of fluoroquinolones plus the number of fluoroquinolone resistance, **7.** Agly indicates the number of aminoglycosides ARG, **8.** *tet* the number of tetracyclines ARG, **9.** Sul the number sulfonamides ARG, **10.** Phe the number of phenicol ARG, **11.** Rif the number of rifampin ARG, **12.** Tmt the number trimethoprim ARG, **13.** Col the number of colistin resistance conferring mutations, **14.** MLS the number of macrolides ARG, **15.** MLS the number of macrolides ARG

7.1.1 Comparing the resistome of ES and PP *K. pneumoniae*

When considering both ES and PP *K. pneumoniae* that the most frequent ARG was the ESBL *bla*_{CTX-M-15} among both populations 85% and 62%, respectively which was expected because *bla*_{CTX-M-15} is widely spread worldwide (11). Also, in relation to ARG such as carbapenemase *bla*_{NDM-1} this was in majority found among PP *K. pneumoniae* (30%) and only carried by two ES-BR *K. pneumoniae* but carried by PP-BR (37%), PP-MR (13%) and PP-ENV (50%). In addition, to *bla*_{OXA-181} concurrently carried with *bla*_{NDM-1} was only carried by PP *K. pneumoniae* (30%).

More importantly, seeing that ESBL and carbapenemase are responsible for resistance to cephalosporins and carbapenems and other β -lactams (9), it was concerning that among PP-BB and ES-BB the percentages of these *bla*_{CTX-M-15} was of 79% and 100%, respectively and the percentage of *bla*_{NDM-1} and *bla*_{OXA-181} concurrently carried with *bla*_{NDM-1} among PP-BB isolates was of 26% and 45%, respectively. These would indicate that infection caused by ES and PP *K. pneumoniae* would be difficult to treat because of the presence of these ARG (18, 84). Since, cephalosporins were used for sepsis treatment in St Paul's hospital, Ethiopia, and both cephalosporins and carbapenems were used in PIMS, Pakistan and most ES and PP *K. pneumoniae* were resistant to the tested cephalosporins and carbapenems (in the case of PP *K. pneumoniae*), it would have followed that the outcome of sepsis would be in majority unfavourable. However, most neonates survived sepsis caused by *K. pneumoniae* in Ethiopia and Pakistan. Nevertheless, it is important to consider that 24% and 17% outcome of *K. pneumoniae* sepsis in Pakistan and Ethiopia was untraceable, therefore, sepsis cases from Ethiopia where more cases were successfully traced a more appropriate indication of the outcome of sepsis was provided

Moreover, despite carrying ESBL and/or carbapenemase ARG and being in majority resistant to cephalosporins and/or carbapenems the outcome of infection was alive, in contrast, with what these results would suggest. This could have possibly been due to the use of other antibiotics such as aminoglycosides or fluoroquinolones. However, as described above ES and PP *K. pneumoniae* carried numerous ARG that would confer resistance to antibiotics belonging to those classes. These ARG were commonly found among ES-BB and PP-BB. In relation to aminoglycoside resistance, ES-BB and PP-BB frequently carried *aac* ARG, *aac3-IIa* (58%) and *aac6-Ib* (50%) were carried by ES-BB and PP-BB, respectively. *aac6-Ib* confers resistance to

amikacin and tobramycin and *aac3-IIa* to gentamicin and tobramycin (165, 166). Hence the difference in antibiotic resistance to amikacin when comparing ES-BB and PP-BB could be partly due to the presence or absence of the ARG. ES-BB and PP-BB *K. pneumoniae* also carried other *aac* variants Agly ARG in addition to *aad* and *aph* ARGs. Also, PP *K. pneumoniae* carried *rmt* and *armA* Agly ARG. The most common was *rmtC* both in total and among PP-BB whereas this ARG was not found among ES *K. pneumoniae*. This is in line with the fact ES-BB were resistant to gentamicin and tobramycin but not to amikacin whereas PP-BB *K. pneumoniae* was resistant to all seeing that *rmtC* is also responsible for amikacin resistance (165, 166).

Fluoroquinolone ARG and SNPs were common among PP and ES *K. pneumoniae* which 30% and 36% carried *qnrS1*, respectively, for example. Nevertheless, Flq SNPs were frequently found among PP *K. pneumoniae* (29%) that carried *qnrS1* concurrently whereas these were not found among ES *K. pneumoniae*. Among PP isolates Flq SNPs carried concurrently were found among PP-BB (45%), PP-BR (38%), PP-MR (10%) and PP-ENV (32%). This could be a possible reason for the higher percentage of resistance to ciprofloxacin and levofloxacin among PP *K. pneumoniae* than ES isolates including among PP-BB and ES-BB *K. pneumoniae*. Also, *qnrB1* was found among ES and PP *K. pneumoniae* across all sample types but more frequently among ES-BR and PP-BR isolates although being found ES-BB and PP-BB *K. pneumoniae* too. *tet* ARG were found among ES and PP *K. pneumoniae* but among ES isolates *tetA* was the most frequent across all sample types of origin. Whereas *tetA* and *tetB* carried concurrently were more frequent among PP *K. pneumoniae* across PP-BB, PP-BR and PP-ENV but not among PP-MR isolates which carried *tetA* only. Also, *tetA* and *tetB* carried concurrently were more frequent among PP-BB and among ES *K. pneumoniae* *tetA* was also more frequent among ES-BB.

Moreover, *OmpK* truncations that confer antibiotic resistance and increase MICs could also have contributed to the differences in resistance antibiotics between ES and PP *K. pneumoniae* (177) because among BB *K. pneumoniae* most *OmpK* truncations were harboured by PP-BB *K. pneumoniae* (n=23;55%) in comparison with ES-BB isolates (n=1; 1%).

Furthermore, WGS of the ARG harboured by ES and PP *K. pneumoniae* from BB, BR, MR and ENV showed that these ARG were frequently found among BR, MR and ENV and not only BB *K. pneumoniae* suggesting the spread of ARG between these isolates. For example, PP-ENV showed high percentages of ARG such as

*bla*_{NDM-1} and PP-BR were indicated to possibly be present in the neonates' microbiota before causing infection (5.1 Phylogeny and population of ES *K. pneumoniae*).

Also, dissemination could have happened between *K. pneumoniae* from other sample types of origin despite the percentages being lower among MR *K. pneumoniae*. Therefore, I explored and discussed the plasmid replicons found among PP and ES *K. pneumoniae* because HGT through plasmids plays an important role in the spread of ARG as for example ESBL *bla*_{CTX-M-15} and carbapenemase *bla*_{NDM-1} (11, 181) as seen in 5.6 and 6.6.

7.2 Comparing the antibiotic susceptibility profiles of ES and PP *K. pneumoniae*

The antibiotic susceptibility profiles of both ES and PP *K. pneumoniae* were described in 5.3 and 6.3. There were marked differences when comparing the antibiotics susceptibility to carbapenems and to aminoglycoside amikacin to which most of ES *K. pneumoniae* were susceptible whereas most PP *K. pneumoniae* were resistant to imipenem, meropenem and ertapenem. Moreover, when considering the ARG WGS results seen in 5.2 and 6.2 these showed that carbapenemase ARG were harboured by PP *K. pneumoniae* and not by ES *K. pneumoniae* a finding which explains the difference in susceptibility. The same was displayed for amikacin to which ES *K. pneumoniae* were susceptible and PP *K. pneumoniae* resistant and the ARG WGS results showed that, for example, *rmt* ARG were carried by PP *K. pneumoniae* and not by ES *K. pneumoniae* (165, 166).

Nevertheless, similarities were seen as well. Firstly, considering BB *K. pneumoniae* ES and PP-BB showed high percentage of resistance to cephalosporins (93% to 98%) and most BB ES and PP *K. pneumoniae* were susceptible to fosfomycin and colistin. With ES and PP *K. pneumoniae* from other sample types following the same trend showing percentages of resistance to cephalosporins and susceptibility to colistin and fosfomycin. However, in relation to β -lactams ES-MR and PP-MR showed susceptibility to cephalosporins (60% to 100%) and carbapenems (52% to 74%), respectively. Also, ES-MR and PP-MR showed high percentage of susceptibility to aminoglycosides (73% to 100% and 69% to 73%, respectively).

7.2.1 Differences in antibiotic susceptibility and ARG among *K. pneumoniae* belonging to major ST groups

Among ES and PP *K. pneumoniae* differences between the antibiotic susceptibility profiles and the ARG carried were noted, particularly, in relation to carbapenems and aminoglycosides. Table 10 describes the resistance that the ARG discussed below confer to antibiotics tested in this PhD. Considering ST35, ST37 and ST218 *K. pneumoniae* from Ethiopia and ST15 *K. pneumoniae* from Pakistan the former were susceptible to carbapenems and the later were mostly resistant. As expected, the MIC₅₀ and MIC₉₀ values for ES ST35, ST37 and ST218 for carbapenems were all below the clinical breakpoint except for the MIC₉₀ for ertapenem of ES ST37 isolates. On the other hand, PP ST15 isolates MIC₅₀ and MIC₉₀ values for

carbapenems were all over the clinical breakpoints. Also, as suggested by contrasting antibiotic susceptibility displayed by these isolates WGS results showed that no ST35, ST37 and ST218 ES *K. pneumoniae* carried carbapenemase ARG and that ST15 PP *K. pneumoniae* often harboured not only one carbapenemase ARG but two carbapenemase ARG. Thus, showing that carbapenems are still an available option for treatment of sepsis caused by *K. pneumoniae* belonging to ST35, ST37 and ST218 in St Paul's Hospital in Ethiopia, however, in PIMS, Pakistan carbapenems would not be able to provide proper treatment to sepsis caused by ST15 *K. pneumoniae*. Regarding, aminoglycosides the results revealed again that PP ST15 were resistant to all aminoglycosides tested but ES *K. pneumoniae* were susceptible to amikacin. Such difference could be explained through the presence of *rmt* ARG, which confer resistance to amikacin, among ST15 *K. pneumoniae* and its absence in ES *K. pneumoniae*. Other differences include the resistance to ciprofloxacin PP ST15 and ES ST37 *K. pneumoniae* and the high rate of susceptible increased exposure results shown by ES ST218 (reflecting that ciprofloxacin remained a viable treatment option but that there were likely to be resistance mechanisms present) in contrast with the susceptibility shown by ES ST35. Likewise, considering levofloxacin PP ST15 isolates were mostly resistant and ES isolates displayed varied susceptibility profiles with most ST35 and ST218 being susceptible, and ST37 showing susceptibility with increased exposure. These differences were reflected in the ARG carried by these isolates with PP ST15 *K. pneumoniae* carrying *qnrS1* ARG in addition to Flq resistance conferring SNPs and only the majority ES ST37 isolates carrying *qnrS1* ARG and few ES ST218 isolates carried *qnrB1*.

Table 10: ARG and Antibiotic resistance by ARG studied in this PhD.

	ARG	Antibiotic resistance conferred by these ARG
Agly	<i>aac6-Ib</i>	High resistance to amikacin and gentamicin Reduced susceptibility to quinolones
	<i>aac3-IIa</i> <i>acc3-IIId</i>	Resistance to gentamicin and tobramycin
	<i>aadA1-pm</i> <i>aadA16</i>	Resistance to spectinomycin and streptomycin
	<i>aph3-Ia</i>	Resistance to kanamycin, neomycin, paromomycin, ribostamycin and lividomycin
	<i>rmtC</i>	High resistance to amikacin, gentamicin and tobramycin
Tet	<i>tetA</i> <i>tetD</i> <i>tetG</i>	Resistance to 2 nd generation tetracyclines such as minocycline Low resistance to 3 rd generation tetracyclines such as tigecycline
Flq	<i>qnrS1</i> <i>qnrB1</i> <i>qnrB38</i>	Low resistance to fluoroquinolones such as ciprofloxacin and levofloxacin
<i>bla</i>	<i>bla</i> _{CTX-M-15}	Resistance to penicillins, cephalosporins (i.e. ceftazidime, cefotaxime and ceftriaxone) and monobactams (i.e. aztreonam)
	<i>bla</i> _{CMY}	Resistance to penicillins, cephalosporins i.e. ceftazidime, cefotaxime and ceftriaxone), monobactams and cephamycins. Not inhibited by classical inhibitors such as clavulanate, sulfabactam and tazobactam.
	<i>bla</i> _{TEM-1D} <i>bla</i> _{TEM-30} <i>bla</i> _{TEM-141}	Resistance to penicillins and first generation cephalosporins (e.g. cefazolin and cephalothin). <i>bla</i> _{TEM-30} confers inhibitor resistance including resistance to amoxicillin-clavulanic acid and intermediate resistance piperacillin
	<i>bla</i> _{OXA-1}	Resistance to penicillins including oxacillin
	<i>bla</i> _{OXA-10}	Resistance to penicillins and low resistance to cefotaxime, ceftriaxone, and aztreonam
	<i>bla</i> _{SHV-28} <i>bla</i> _{SHV-33} <i>bla</i> _{SHV-187}	Resistance to penicillins and first generation cephalosporins
	<i>bla</i> _{OXA-181}	Resistance to penicillins and broad-spectrum carbapenems and are poorly inhibited by β -lactamases inhibitors except for avibactam.
	<i>bla</i> _{NDM-1}	Resistance to most β -lactams including carbapenems (i.e. imipenem, ertapenem and meropenem) except for monobactams

Such differences could have been driven by the antibiotics usually empirically prescribed at PIMS and St Paul's Hospital because at PIMS these included piperacillin/tazobactam and amikacin as a combination, cefotaxime, imipenem and vancomycin these being in accordance with the resistance and ARG displayed by ST15 PP *K. pneumoniae*. And the empirical prescriptions at St Paul's hospital included ampicillin and gentamicin as a combination, cefotaxime, ceftazidime, and vancomycin these also being in line with the ARG and resistance showed by ST35, ST37 and ST218 ES isolates. Furthermore, these antibiotics were included

prescriptions used to treat sepsis caused by *K. pneumoniae*. Also, explaining why BB *K. pneumoniae* from both countries were resistant to cephalosporins and other β -lactam antibiotics only being susceptible to aztreonam/avibactam treatment in the case of ST15 *K. pneumoniae*. Therefore, suggesting that these ARG might be circulating among *K. pneumoniae* carried in the mother and infant microbiota and from the clinical environment. In conclusion, the possible transfer of ARG (sections 5.5 and 6.5) and the possible transmission of *K. pneumoniae* isolates between mother and infant (sections 5.1 and 6.1) may be providing the opportunity for *K. pneumoniae* to cause infection. Moreover, treatment options were becoming further reduced among these isolates because in overall these were only susceptible to colistin, fosfomycin and tigecycline. Except for treatments such as aztreonam/avibactam in the case of PP ST15 *K. pneumoniae* and amikacin regarding ES ST35, ST37 and ST218 *K. pneumoniae*. However, these treatments pose challenges as the former are difficult to obtain in LMICs and amikacin is not available in Ethiopia (169).

7.3 Plasmid replicon content among ES and PP *K. pneumoniae* isolates.

All 465 *K. pneumoniae* with short read WGS data available were screened for plasmid replicons available within the PlasmidFinder database curated by Caratolli et al. (150). In total, 45 different plasmid replicons were carried among the studied *K. pneumoniae* isolates (Table 11). The plasmid replicons found among ES and PP isolates were summarised separately in sections 5.6 and 6.6. Analysing the plasmid content of both clinical sites separately in 5.6.1 and 6.6.1 was performed to characterise any differences in the plasmid population especially in the context of the different ARG such as *bla*_{CTX-M-15} and *bla*_{NDM-1} found among ES and PP *K. pneumoniae*.

Table 11: Types of plasmid replicons harboured by *K. pneumoniae* from Pakistan and Ethiopia.

Plasmid Replicon					
Col	IncF	IncH	IncQ	IncX	Other Replicons
ColpVC_1	IncFII	IncHI1B	IncQ1	IncX1	IncR_1
ColRNAI_1	IncFIB(K)	IncHI2	IncQ2	IncX3	IncA/C2_1
ColKP3_1	IncFIA(HI1)	IncHI2A		IncX4	IncB/O/K/Z_2
Col(MGD2)_1	IncFIB(pQil)				IncI1
Col(Ye4449)_1	IncFIB(pKPHS1)				IncL/M(pOXA-48)
Col(MG828)_1	IncFIB(Mar)				IncN_1
Col(BS512)_1	IncFII(pHN7A8)				rep7_1_repC(Cassette)
Col156_1	IncFII(K)				TrfA_1
Col8282_1	IncFII(Yp)				
	IncFII(pRSB107)				
	IncFIB(AP001918)				
	IncFII(pKPX1)				
	IncFIC(FII)				
	IncFIA				
	IncFII(S)				
	IncFII(pCRY)				
	IncFII(pCoo)				
	IncFIB(pB171)				

7.3.1 Plasmid replicons among ES and PP *K. pneumoniae*

Previous studies showed that ARGs have been associated with certain types of plasmid replicons. For example, ESBL and carbapenems are frequently harboured by IncF plasmids, which are limited to *Enterobacteriaceae* and are conjugative plasmids but, IncA/C have also been associated with carbapenems and ESBL ARG too (179).

The screening of plasmid replicon harboured by ES and PP *K. pneumoniae* revealed that among these *K. pneumoniae* Col, IncF, IncH and IncN were commonly carried by *K. pneumoniae* from Ethiopia and Pakistan. Additionally, IncR and IncQ replicons were only found among ES isolates, and IncX plasmid replicon were only found among PP *K. pneumoniae*.

IncF replicon plasmids are conjugative low copy number plasmids and are the most frequently described plasmid type in *Enterobacteriaceae* in both human and

animal sources (179, 182). Thus, it not necessarily surprising that IncF plasmids would be found among ES and PP *K. pneumoniae*. IncF type plasmids have been shown to frequently carry many different ARG including ESBL and carbapenemase ARG, Agly ARG and quinolone ARG and can encode several replicons including IncFII together with IncFIA and IncFIB. In addition, IncF plasmids have been described to cointegrate IncN replicons (179, 182). So, when considering ES *K. pneumoniae* it is possible that IncN replicons were not harboured by ES-ENV *K. pneumoniae* because the IncF type plasmids did not cointegrate IncN replicons (179, 183, 184). Furthermore, IncF and IncH replicons have been previously associated with ESBL and carbapenemase ARG following that these would be expected to be found among ES and PP *K. pneumoniae* (179). IncR plasmids are more rarely described plasmids, can also carry multiple ARG, but do not have conjugative transfer genes, nevertheless, their broad host range indicates that these are mobilizable (179). Thus, it would follow that IncR type plasmids would be carried among ES *K. pneumoniae* from all sample types of origin.

IncX type plasmids found among PP *K. pneumoniae* are narrow host range plasmids that have been previously associated with carrying carbapenemase ARG, which could offer a possible explanation as to why these were only found among PP *K. pneumoniae*, seeing that these were found to frequently carry carbapenemase ARG contrarily to ES *K. pneumoniae* (179, 185), where no carbapenemase ARG were found.

Moreover, because plasmids replicons such as the ones found in this PhD are important for the spread of antibiotic resistance (179). Sequences of reference plasmids carrying *bla*_{CTX-M-15} were used to screen the ES *K. pneumoniae* population in order to obtain a preliminary assessment of these plasmids' presence and role in the spread of *bla*_{CTX-M-15} and other ARG harboured among the isolates recovered in St Paul's Hospital. The same assessment was performed among PP *K. pneumoniae* but because carbapenemase *bla*_{NDM-1} and *bla*_{OXA-181} were frequent among PP isolates plasmids carrying these ARG were used for the analysis. This analysis would indicate another means of antibiotics resistance spread in addition to transmission of resistant isolates among sepsis causing *K. pneumoniae* and isolates from other sample types of origin belonging to the major STs found in this PhD. Additionally, linking plasmids replicon types with PP and ES *K. pneumoniae* belonging to major STs showed that IncF plasmids were found among all PP ST15 *K. pneumoniae* as well as ES ST35, ES ST37 and ES ST218 *K. pneumoniae* which was not surprising seeing that these were

commonly found among PP and ES *K. pneumoniae* and are frequently described. Also, seeing that IncX were only found in PP *K. pneumoniae* it was not surprising to find them among most PP ST15 *K. pneumoniae* (n=83;95%). Despite ES ST35, ES ST37 and ES ST218 *K. pneumoniae* all harbouring IncF plasmid replicon, ES ST35 *K. pneumoniae* did not harbour IncQ and IncH, which in line with these ES *K. pneumoniae* not carrying pKpnST37_ESBB_1 (Fig. 62).

Moreover, among ES *K. pneumoniae* hits for both pKpnST37_ESBB_1 and pKpnST37_ESBB_2 were found among ES *K. pneumoniae* from ES-BB, ES-BR, ES-MR and ES-ENV which is line with most ES isolates carrying *bla*_{CTX-M-15}. However, it is important to consider that hits could have been found among ES isolates that did not carry *bla*_{CTX-M-15}. For example, due to the presence of other plasmids with similar backbones.

Therefore, having this in mind the alignment of putative plasmids scaffolds harboured by ST35, ST37 and ST218 ES and ST15 PP *K. pneumoniae* using pKpnST37_ESBB_1, pKpnST37_ESBB_2, pKpnST15_PPBB_1 and pKpnST15_PPBB_2 as references were performed. pKpnST37_ESBB_1 and pKpnST37_ESBB_2 harboured different ARG content when compared and showed different replicons. pKpnST37_ESBB_1 showed IncFIB, IncQ and IncH replication systems in contrast pKpnST37_ESBB_2 showed IncR and IncFII replication systems indicating that these are conjugative low copy number plasmids (179). Furthermore, these plasmids were harboured by ES *K. pneumoniae* recovered from BB, BR, MR and ENV indicating that pKpnST37_ESBB_1 was possibly circulating between mother and neonates and the clinical environment, though it was more frequent among ES-BB *K. pneumoniae*. pKpnST37_ESBB_2 screening of ES *K. pneumoniae* revealed that this plasmid was probably carried by ES *K. pneumoniae* from different sample types of origin and among ST37, ST35 and ST218. pKpnST37_ESBB_2 was found among ES-BB more frequently than among other ES *K. pneumoniae* from other sample types of origin. However, likewise pKpnST37_ESBB_1, it was circulating among ES isolates that caused sepsis and were among the mothers and neonates' microbiota. pKpnST37_ESBB_2 was found among ST35 and ST218 *K. pneumoniae* whereas pKpnST37_ESBB_1 was not indicating that different plasmids backbones could be associated to different STs.

pKpnST37_ESBB_1 and pKpnST37_ESBB_2 plasmids both carried *bla*_{CTX-M-15} were more commonly found among ES-BB *K. pneumoniae* and ES-BR *K.*

pneumoniae. This is important because it suggests several plasmids carrying *bla*_{CTX-M-15} and other ARG contributed to the spread of antibiotic resistance mostly among the ES *K. pneumoniae* recovered from the microbiota of neonates (ES-BR) and among *K. pneumoniae* causing sepsis (ES-BB). This being in line with high percentages of *bla*_{CTX-M-15} among ES-BB and ES-BR isolates (5.2 Resistome of ES *K. pneumoniae*). It is worrying that neonates are harbouring bacteria with multiple acquired antibiotic resistance genes in their microbiota from a very early age. In addition, having in mind that pKpnST37_ESBB_1 carried *qnrS1* ARG and it was not carried by ES ST35 *K. pneumoniae* could explain why the higher percentages of susceptibility to levofloxacin and ciprofloxacin showed by these isolates when compared to ES ST37 isolates (5.4 Antibiotic susceptibility and ARG among ES *K. pneumoniae* belonging ST35, ST37 and ST218).

Similarly, in Pakistan, pKpnST15_PPBB_1 and pKpnST15_PPBB_2 showed two different plasmid replicon types and carried different ARG. Both pKpnST15_PPBB_1 and pKpnST15_PPBB_2, were probably carried by PP *K. pneumoniae* from all the different sample types of origin, being often found among PP-BB and PP-BR. Therefore, contributing to carbapenem resistance among PP *K. pneumoniae* causing sepsis in PIMS. Among ST15 *K. pneumoniae* both plasmids were distributed among PP isolates from PP-BB, PP-BR, PP-MR and PP-ENV. However, these plasmids were more commonly found among PP-BB, PP-BR and PP-ENV, suggesting that PP-ENV *K. pneumoniae* harbouring these plasmids probably played a role in spreading ARG such as *bla*_{NDM-1} and *bla*_{OXA-181}. Also, considering that pKpnST15_PPBB_1 and pKpnST15_PPBB_2 were mostly found among ST15 *K. pneumoniae* and the alignments showed slight variation suggested that these plasmids could be associated with ST15 in my PhD. Additionally, seeing that most ST15 *K. pneumoniae* carried both *bla*_{NDM-1} and *bla*_{OXA-181} it was expected that these would carry both plasmids.

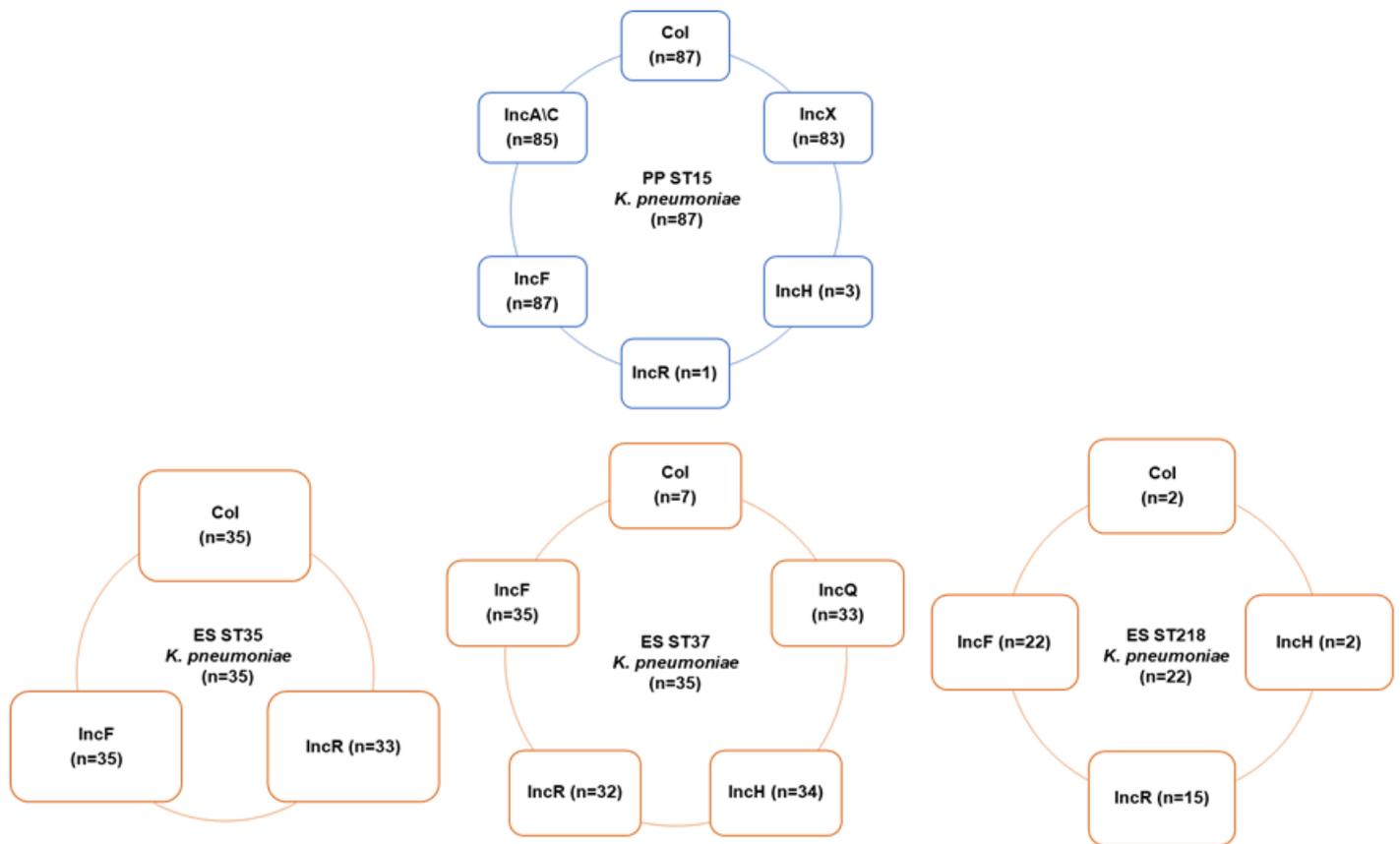


Fig. 62: Number of different plasmids replicon types among PP and ES *K. pneumoniae* belonging to major ST groups. ST15 PP *K. pneumoniae*, ST35, ST37 and ST218 ES *K. pneumoniae* all harboured IncF plasmids. Col type plasmids were found in all ST15 PP *K. pneumoniae* and in all ES ST35 *K. pneumoniae* but only in seven ST37 ES *K. pneumoniae* and two ST218 ES isolates. IncR type plasmids were more commonly found among ES *K. pneumoniae* belonging to ST35 (n=33), ST37 (n=32) and ST218 (n=15) than among ST15 PP *K. pneumoniae* (n=1). IncH type plasmids were more commonly harboured by ES ST37 *K. pneumoniae* (n=34), and only found among two ST218 ES isolates and three ST15 PP *K. pneumoniae*. IncQ type plasmids were only found among ES ST37 *K. pneumoniae* (n=33) and IncX type plasmids were only carried by ST15 PP *K. pneumoniae* (n=83).

7.4 *In silico* serotyping of virulence determinants of ES and PP *K. pneumoniae*

Having characterised both *K. pneumoniae* populations virulome and studying them separately (5.5 and 6.5) it was possible to identify differences and similarities when comparing them as described below and discussed in the next section.

All *K. pneumoniae* isolates were studied in relation to acquired virulence determinants important for clinical infection caused by *K. pneumoniae*. These included capsule polysaccharides as antigen loci (KL) and LPS O antigen as loci (OL), siderophores yersianiabactin (*ybt*), samolchelin (*iro*), aerobactin (*iuc*), and hypermucoidy genes *rmpA* and *rmpA2*.

In total 68 different KLs were found among all the isolates (n=465;100%) in study with KL112 displayed by 93 (20%) isolates followed by KL15 (n=38;8%) and KL108 (n=38;8%). Among the ES *K. pneumoniae* isolates 30 different KLs were found. In majority ES isolates (n=191) showed KL15 (n=35;18%) and KL108 (n=35;18%) followed by KL57 (n=23;5%). PP isolates (n=284;100%) displayed 61 different KLs among these the most found were KL112 (n=90;32%) followed by KL64 (n=19;7%) and KL2 (n=17;6%). Distribution of the ten most frequent KL in overall and for Pakistani and Ethiopian isolates is displayed in Fig. 63. The overall distribution of KL and OL is shown in Supplementary figures – Supplementary Figure 3 and Supplementary Figure 4 in the Appendix 11.

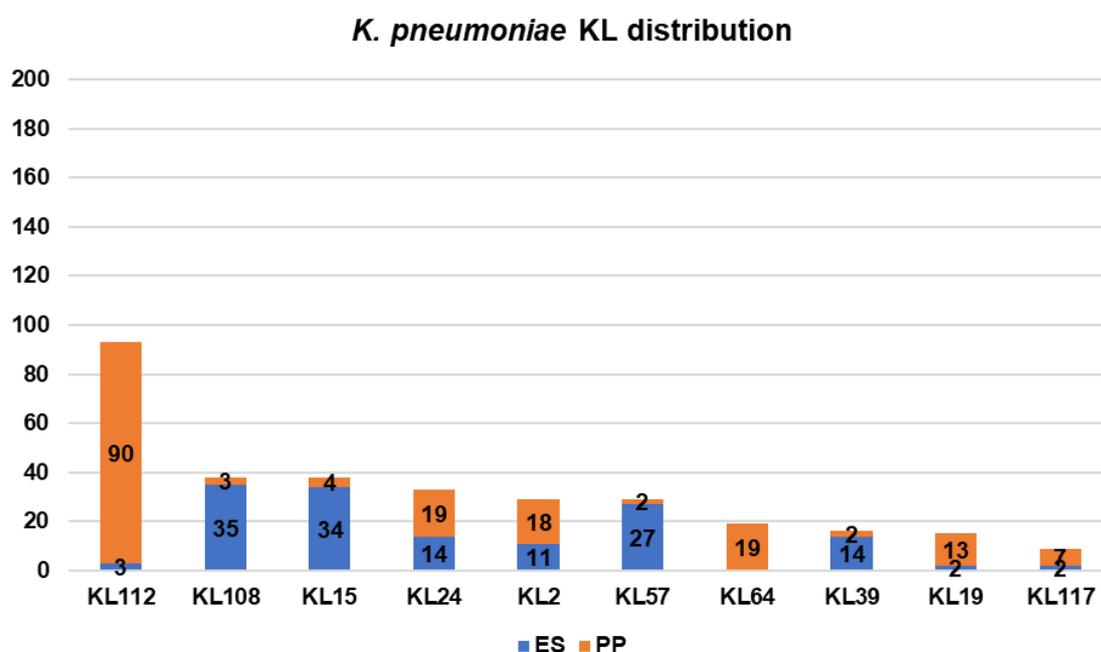


Fig. 63: Distribution of the ten most frequent *in silico* serotypes (KL) carried by *K. pneumoniae* from Pakistan (orange) and Ethiopia (blue), respectively. KL112 was the most common KL found (n=93) being most found in Pakistan (PP; n=90), followed by KL108 (n=38) mostly detected in Ethiopia (n=35) and KL15 (n=38) an mostly detected among *K. pneumoniae* from Ethiopia (ES; n=34).

In relation to OL types overall 15 different loci were found and, the most commonly found OL was O1v1 (n=124;27%) followed by O1v2 (n=76;16%) and O2v1 (n=73;16%). Among the PP isolates 15 different OLs were found. The most frequently found was O1v1 (n=109;37%) followed by O2v2 (n=40;15%) and O1/O2v1 (n=30;10.5%). Whereas for ES *K. pneumoniae* isolates there were 11 different OLs and the most frequently found was O1v2 (n=54;28%) followed by O4 (n=35;18%) and O2v2 (n=33;17%). The distribution of OL in overall and for Pakistani and Ethiopian isolates is displayed in Fig. 64.

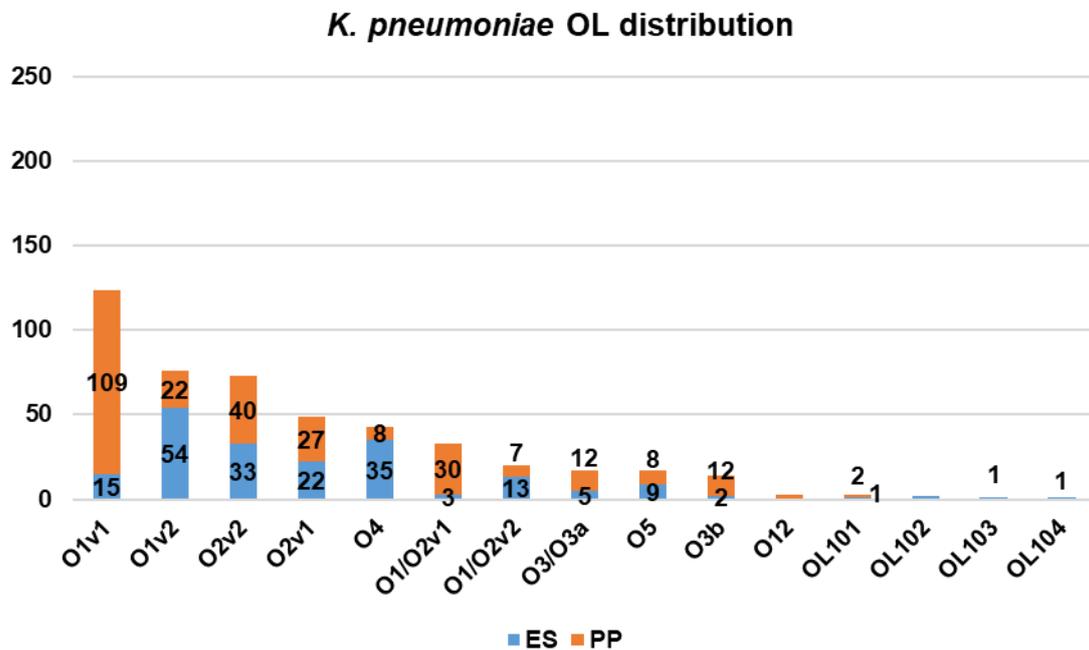


Fig. 64: Distribution of *in silico* serotypes OL carried by *K. pneumoniae* carried by *K. pneumoniae* from Pakistan (orange) and Ethiopia (blue), respectively. The most frequently detected OL was O1v1 (n=124) followed by O1v2 (n=76) and O2v2 (n=73). O1v1 was more frequently detected among PP *K. pneumoniae* (n=109), O1v2 was more frequent among ES *K. pneumoniae* (n=54) and O2v2 was detected in 33 ES *K. pneumoniae* and 40 PP *K. pneumoniae*.

Regarding the studied siderophores *ybt* was the most frequently displayed by isolates (n=225;48%) followed by *iuc* (n=32;7%) and *iro* (n=27;6%). Among the *K. pneumoniae* isolates harbouring *ybt* 30 (6.5%) carried *iuc* simultaneously and 24 (5%) carried *iro* simultaneously. Siderophore distribution for Pakistani and Ethiopian isolates and in overall is shown in Fig. 65.

PP *K. pneumoniae* (n=141;50%) carried *ybt* loci. *iuc* and *iro* were carried by seven (2%) and four isolates (1%), respectively. From the PP isolates carrying *ybt* 6 (4%) harboured *iuc* simultaneously with one (0.7%) isolate carrying both *ybt* and *iro*.

ES isolates (n=84;44%) carried *ybt*, *iuc* and *iro* were carried by 23 (12%) and 25 (13%) ES isolates, respectively. From the ES *K. pneumoniae* isolates carrying *ybt* 24 (28.5%) harboured both *iuc* and *iro* concurrently.

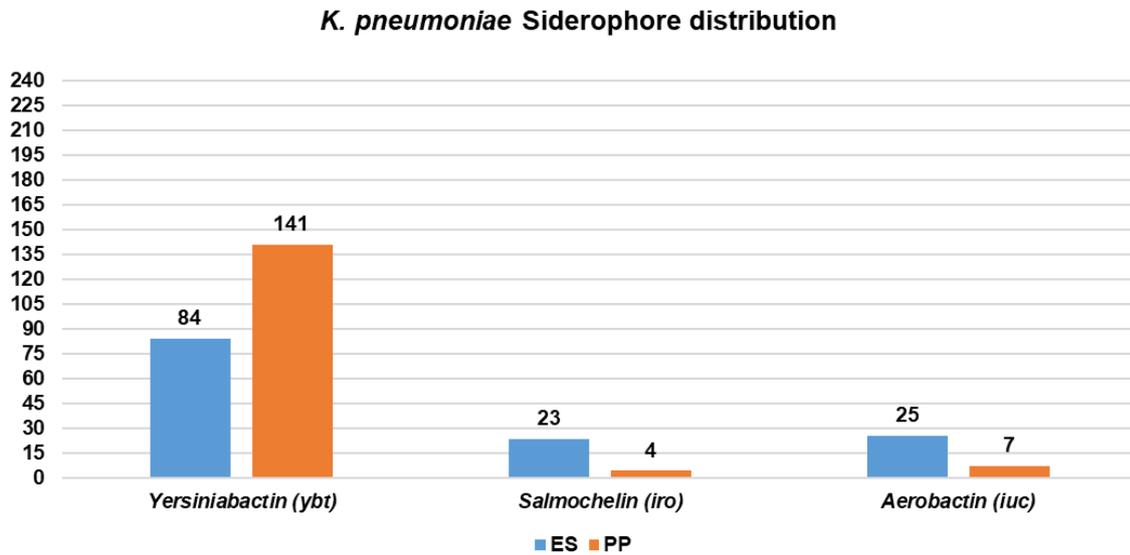


Fig. 65: Distribution of acquired siderophore loci harboured by *K. pneumoniae* from Pakistan (orange) and *K. pneumoniae* from Ethiopia (b). The most common siderophore was yersiniabactin (*ybt*; n=225), in majority carried by PP *K. pneumoniae* (n=141) and carried by 84 ES *K. pneumoniae*. Other siderophore included salmochelin (*iro*) and aerobactin (*iuc*) found in 27 and 32 *K. pneumoniae* among the overall *K. pneumoniae* isolates, respectively. *iro* was more frequently carried by ES *K. pneumoniae* (n=23) and was harboured by four PP isolates. *iuc* was also more frequently found in ES *K. pneumoniae* (n=25) and carried by seven PP *K. pneumoniae*.

Overall, ten different *ybt* lineages were found. An unknown *ybt* lineage was identified in 23 (8%) isolates. The most identified *ybt* lineage was *ybt* 16 associated with ICEKp12 (n=114;24.5%) followed by *ybt* 9 associated with ICEKp3 (n=27;5%) and an unknown *ybt* (n=23;8%). *ybt*16 lineage associated with ICEKp12 was the most frequently identified among PP isolates (n=100;35%), followed by *ybt* 14 associated with ICEKp5 (n=13;4.5%) and an unknown *ybt* (n=9;3%). ES isolates mostly carried *ybt*9 associated with ICEKp3 (n=24;12.5%) followed by *ybt* 16 lineage associated with ICEKp12 (n=14;7%) and an unknown *ybt* (n=14;7%). *ybt* lineage distribution for Pakistani and Ethiopian isolates and in overall is shown in Fig. 66.

iro lineages found included *iro* 1 and unknown *iro* lineage were identified in PP and ES isolates. *iro* 1 was identified in 23 (12%) ES *K. pneumoniae* isolates and *iro* unknown identified in four (1%) PP isolates. In relation to *iuc* lineages *iuc* 1 and an unknown *iuc* lineage were identified in PP and ES isolates. *iuc* 1 was identified in 25 (13%) ES isolates and five (1.8%) PP isolates. The *iuc* unknown was identified in two (1%) ES isolates and in two (0.7%) PP isolates. The unknown *iuc* lineages might be different for PP and ES isolates, respectively. Table 12 shows the distribution of *iro* and *iuc* lineages identified.

In total, 15 (3%) *K. pneumoniae* isolates carried *rmpA* associated with KpVP-1, among these *rmpA*_1 (n=3;0.6%) and *rmpA*_2 (n=12;3%) (Table 13). These were found among ES isolates only. *rmpA*2 was harboured by 20 (10%) isolates including the ES isolates carrying *rmpA* associated with KpVP-1. In addition, three (2%) ES *K. pneumoniae* and three (1%) PP *K. pneumoniae* isolates carrying only *rmpA*2. The *rmpA*2 genes found included *rmpA*2_8 (n=17;4%), *rmpA*2_6 (n=20;4%) and *rmpA*2_5 (n=1;0.2%) variants. *rmpA*2 found in these isolates was often truncated after 60% of the intact amino acid length.

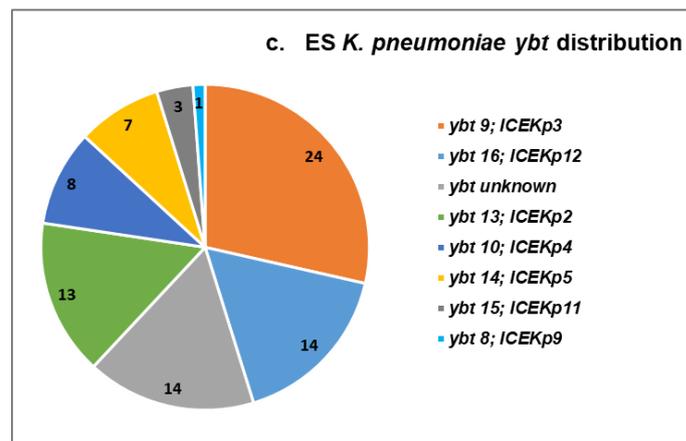
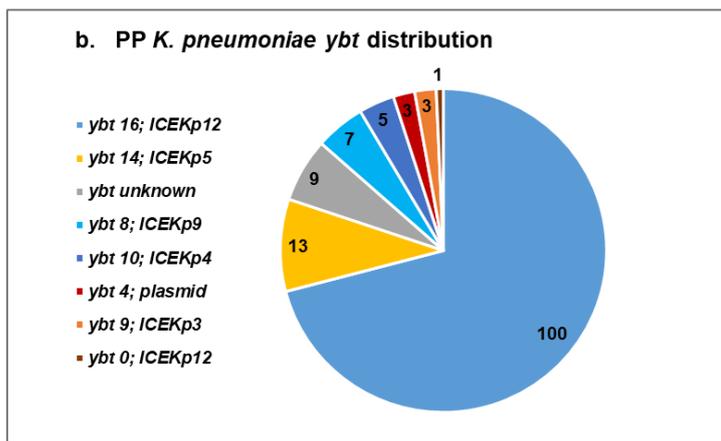
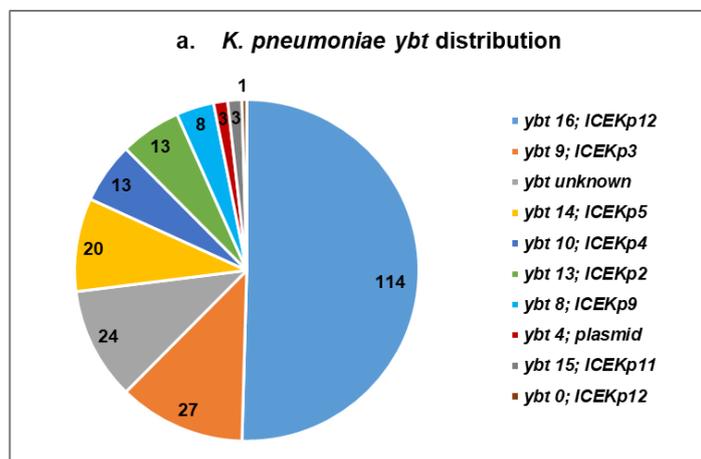


Fig. 66: Distribution of ybt siderophore lineages carried by *K. pneumoniae* in overall (a.), carried by *K. pneumoniae* from Pakistan (b.) and carried by *K. pneumoniae* from Ethiopia (c.). In overall ybt16; ICEKp12 (n=114) was the most common lineage followed by ybt9; ICEKp3 (n=27) and an unknown ybt lineage (n=24). In addition, ybt14; ICEKp5 was carried by 20 *K. pneumoniae* and one isolate carried ybt0, ICEKp12. **b.** Among PP *K. pneumoniae* the most frequently found ybt lineage was ybt16; ICEKp12 (n=100) followed by ybt14; ICEKp5 (n=13) and ybt unknown (n=9). In contrast, ybt0; ICEKp12 was harboured by one PP isolate. **c.** ybt9; ICEKp3 (n=24) was the most common lineage among ES *K. pneumoniae* followed by ybt16; ICEKp12 (n=14) and an ybt unknown (n=14), contrary to ybt8; ICEKp9 that was found in one ES *K. pneumoniae*

Table 12: Distribution of iro and iuc lineages found in the overall *K. pneumoniae* population and in *K. pneumoniae* from Pakistan and Ethiopia, respectively. In overall, iro 1 (n=23) and iuc 1 (n=28) were more frequently found than iro unknown (n=4) and iuc unknown (n=5). Both iro 1 (n=23) and iuc 1 (n=23) were more common among ES *K. pneumoniae* than among PP *K. pneumoniae* (iro 1; n=4 and iuc 1; n=5)

	Salmochelins (<i>iro</i>)		Aerobactins (<i>iuc</i>)	
	<i>iro</i> 1	<i>iro</i> unknown	<i>iuc</i> 1	<i>iuc</i> unknown
Overall	23	4	28	5
PP	-	4	5	2
ES	23	-	23	3

Table 13: Distribution of *rmpA* and *rmpA2* genes carried by the overall *K. pneumoniae* population and by *K. pneumoniae* from Pakistan and Ethiopia, respectively. *rmpA*_1 (n=3) and *rmpA*_2 (n=12) was only found among ES *K. pneumoniae*. *rmpA2*_8 (n=16 and n=1) was found among ES *K. pneumoniae* and *rmpA*_6 (n=2) and *rmpA2*_5 (n=1) was found among PP *K. pneumoniae*.

	<i>rmpA</i>		<i>rmpA2</i>			
	<i>rmpA</i> _1	<i>rmpA</i> _2	<i>rmpA2</i> _8-60%	<i>rmpA2</i> _6-60%	<i>rmpA2</i> _5-54%	<i>rmpA2</i> _8-0%
Overall	3	12	16	2	1	1
ES	3	12	16	-	-	1
PP	-	-	-	2	1	-

7.4.1 Comparing the virulome of *K. pneumoniae* from Ethiopia and Pakistan

By comparing ES and PP *K. pneumoniae* it was possible to identify different traits across the populations. The carriage of siderophore loci such as *iro* and *iuc*, were mostly harboured by ES isolates and not by PP *K. pneumoniae* with the same being found regarding *rmpA* factors. Nevertheless, both ES and PP *K. pneumoniae* frequently carried *ybt* loci which is line with it described as the most frequent among *K. pneumoniae* (91). Also, PP and ES isolates also showed distinct KL, with ES *K. pneumoniae* showing KL15, KL108 and KL57 and PP *K. pneumoniae* KL112, KL64 and KL2, which may align to the most frequently detected STs. Regarding OL variability and differences when comparing PP and ES isolates were noticeable too, however, variability of KL is generally higher than OL variability (89) (1.7 *K. pneumoniae* virulence determinants). Nevertheless, among ES and PP isolates the most frequently identified OL was O1, but different variants were identified among ES and PP *K. pneumoniae* O1v1 and O1v2. Furthermore, KL2 associated with severe infection was found among *K. pneumoniae* populations also showing O1 which was previously described to be associated with K2 serotype. K2 was one of the most common KL among PP *K. pneumoniae* and it was found among ES *K. pneumoniae* as well. In fact, among ES and PP *K. pneumoniae* showing KL2 most showed O1v1. However, among PP isolates no PP-BB were found to show KL2 and O1v1 whereas one ES-BB was found to show both. Additionally, KL2 was detected among PP *K. pneumoniae* from PP-BR, PP-MR and PP-ENV suggesting that isolates that could cause serious infection were present among neonates, mothers in the clinical environment in PIMS. However, KL2 was not harboured by ST15 *K. pneumoniae* but all PP ST15 *K. pneumoniae* carried *ybt* loci.

Acquired virulence determinants such as *ybt* as well as *iuc* and *iro* were circulating among both ES and PP *K. pneumoniae* which is of concern because association with ICE elements and plasmids. Presence of virulence factors positioned on MGE could indicate the ability to spread and disseminate like that of ARG ultimately enhancing pathogenicity (91, 92). *ybt*, *iuc* and *iro* loci were commonly found among ES-BB *K. pneumoniae* but PP-BB *K. pneumoniae* mostly carried *ybt* loci only. Among ES-BB *K. pneumoniae* *iuc* and *iro* loci were harboured as well as *rmpA* factors. These were carried by ST218 ES isolates including ES-BR, ES-MR, and ES-ENV. Nevertheless, these virulence determinants were not found among ST35 and ST37 ES-BB *K. pneumoniae*, despite these being responsible for most sepsis cases in St Paul's Hospital in Ethiopia. Thus, indicating that among ES *K. pneumoniae* plasmid associated *iuc* and *iro* (92) were not spread beyond ST218 *K. pneumoniae*. However, *ybt* loci could have been spreading seeing the same lineages, as for example *ybt* 9; ICEKp3, were found among ES-BB, ES-BR, ES-MR and ES-ENV. The same happening among PP *K. pneumoniae* as for example *ybt* 16; ICEKp12 was found among PP-BB, PP-BR, PP-MR and PP-ENV indicating that potential for serious infection among PP *K. pneumoniae*. Because this PhD focused mainly on antibiotic resistance the impact of virulence associated MGEs was not studied. Nonetheless, ES *K. pneumoniae* carrying *iuc* often carried *iro* and at times *rmpA* suggested that these could be harboured in the same plasmids because these determinants are often associated. Also, the distribution of ARG could have been shifted towards ST15 *K. pneumoniae* isolates seeing that the distribution of acquired ARG in the absence of virulence plasmids was described to be shifted towards genomes carrying ICEKp associated with *ybt* loci (55, 91).

Possible convergence of virulent traits and antibiotic resistance was more concerning among ES *K. pneumoniae* due to the presence of *iuc*, *iro* and *rmpA*. Nevertheless, it was also indicated among PP ST15 *K. pneumoniae* due to the presence of *ybt* loci and cephalosporins and carbapenemase ARG. *iuc*, *iro* and *rmpA* are associated with hypervirulence (86, 93). Therefore, there is a chance of ES *K. pneumoniae* causing severe infection, while also harbouring ARG such as *bla*_{CTX-M-15} that severely reduces treatment options. Even though these virulence determinants were found to be contained among ES ST218 *K. pneumoniae* and not found among ST35 and ST37 isolates that caused most of the sepsis case in St Paul's Hospital it is important to note that these are associated with plasmids. Therefore, the possibility of

spread between other *K. pneumoniae* belonging to other STs and sample types of origin exists. Convergence of virulence determinants and ARG, whether through ICE elements or plasmid spread, as suggested among ST15 PP *K. pneumoniae* and ES ST218 *K. pneumoniae*, respectively, is concerning because the presence of these virulence determinants improves the ability of *K. pneumoniae* to cause severe infection and to resist antibiotic treatment. *K. pneumoniae* can be responsible for diverse and life-threatening infections. Nonetheless, even though, hvKp strains capable to cause invasive infection in healthy people have emerged and are usually associated with pyogenic liver abscess (PLA) (86, 180, 186), cases of neonatal sepsis caused by hypervirulent *K. pneumoniae* were reported in India (187) and in Kazan, Russia (116). Further highlighting the need to acquire knowledge about *K. pneumoniae* virulence determinants that could be used as targets for vaccines and/or antibodies which in turn could held decreasing sepsis cases caused by *K. pneumoniae*.

7.5 Relationships between ARG, virulence determinants and clinical data among ES and PP *K. pneumoniae*

K. pneumoniae recovered from Pakistan and Ethiopia belonging to the dominant STs were studied to assess the extent of relationships between ARG and available clinical data, specifically the outcome of sepsis (5.7 and 6.7). The presence of ARG such as *bla*_{CTX-M-15}, *bla*_{NDM-1} and *bla*_{OXA-181}, and virulence determinants such as acquired siderophores loci factors and their possible association with outcome of sepsis was studied. These were evaluated using the Kaplan-Meier statistical test. (Appendix 5 includes ES and PP *K. pneumoniae* clinical data)

During this PhD it was possible to understand that the population of *K. pneumoniae* from PIMS, Pakistan and from St Paul's Hospital, Ethiopia presented different traits not only regarding, their structure, resistome and virulome but also the regarding antibiotic susceptibility profiles. These differences could be due to the distinct clinical sites these *K. pneumoniae* were recovered from that would represent a set of different pressures these isolates were under, or also, for example, due the different availability or choice antibiotic treatments (169). With that in mind I studied if genomic markers such as ARG and virulence determinants (siderophore loci) contributed for more difficult to treat and more serious infection in PIMS and St Paul's Hospital. The presence of ARG such as *bla*_{CTX-M-15} and *bla*_{NDM-1} and *bla*_{OXA-181} was studied to understand if it affected outcome of sepsis among sepsis cases caused by dominant ST ES-BB isolates and ST15 PP-BB isolates. These ARG were studied because ESBL such as *bla*_{CTX-M-15} and carbapenemase ARG such as *bla*_{NDM-1} and *bla*_{OXA-181} are associated with resistance to β -lactams antibiotics used for treatment in PIMS (for example cefotaxime, imipenem, meropenem) and St Paul's Hospital (for example cefotaxime and gentamicin) (10, 11, 18, 169). In addition, ES-BB and PP-BB isolates belonging to dominants STs in this PhD displayed high percentages of resistance to cephalosporins and carbapenems. Also, the presence of acquired siderophore loci (*ybt*, *iuc* and *iro*) was studied as well because these are associated with virulent *K. pneumoniae* infection and enable *K. pneumoniae* to establish infection (86). ST35, ST37 and ST218 ES-BB *K. pneumoniae* all carried *bla*_{CTX-M-15}. In addition, the age at outcome of most neonates (n=26) that survived sepsis caused by ST35, ST37 and ST218 ES-BB ES *K. pneumoniae* was >30 days and the age at outcome of six neonates who did not survive was lower than seven days. This could indicate that sepsis cases caused by ST35, ST37 and ST218 *K. pneumoniae* carrying *bla*_{CTX-M-15}

could be more difficult to treat. However, in sections 5.7 and 6.7, it was possible to understand that most neonates survived sepsis caused by ES-BB ST35, ST37 and ST218 *K. pneumoniae* and seeing that all these isolates carried *bla*_{CTX-M-15}, it was not possible to ascertain if this ARG influence the age of the neonate at outcome. Likewise, neonates suffering from sepsis caused ST15 PP-BB *K. pneumoniae* in majority survived with age outcome >10 days although these isolates carried *bla*_{NDM-1} and *bla*_{OXA-181} ARG. Nonetheless, this important because it could be linked with spread of ARG and antibiotic resistance between *K. pneumoniae* circulating in neonates' microbiotas that later on could cause sepsis as indicated in 6.2. Also, the mother's microbiota could be important as well seeing that both carried *bla*_{NDM-1}, *bla*_{OXA-181} and *bla*_{CTX-M-15} and were resistant to the tested carbapenems and cephalosporins as seen in 5.4 and 6.4. So, it would be reasonable that treatment would take longer when antibiotics such as piperacillin/tazobactam, amoxicillin/clavulanate, cefotaxime and imipenem were prescribed at PIMS and cefotaxime and gentamicin were usually prescribed at St Paul's Hospital to treat *K. pneumoniae* sepsis.

Also, when considering the presence of siderophore loci carried by these isolates causing sepsis it was not possible to ascertain if this affected outcome of infection seeing that all ST15 PP-BB isolates and among ES isolates only ST218 ES-BB *K. pneumoniae* carried siderophore loci. Moreover, it is important to consider that several factors and not only those studied in this PhD could influence sepsis caused by ES-BB and PP-BB *K. pneumoniae* belonging to these STs as for example other resistance markers as porin truncations (177) or other virulence determinants such as fimbriae (37, 86, 87).

7.6 Intraspecies diversity of *K. pneumoniae* from Pakistan and Ethiopia among isolates from other regions of the world

The Pakistani and Ethiopian isolates characterised during this study were placed in a global context incorporating *K. pneumoniae* sequences from previously published collections and isolates from other BARNARDS sites/countries. In summary, studying the *K. pneumoniae* from Pakistan and Ethiopia aimed to contribute with more information about the population of *K. pneumoniae* in LMICs. which could be helpful when treating sepsis caused by *K. pneumoniae*. The *K. pneumoniae* isolates (n=147;100%) in the global strain collection are described in Appendix 13. *K. pneumoniae* isolates genomes from Europe, USA, South America, and Australia were included in the Global strain collection in order to represent different regions of the globe. These were recovered from blood (n=72;49%) and rectal samples (n=55;37%). The isolates from other BARNARDS sites (n=36;100% were from Bangladesh and Nigeria from MR (n=8;22%), BR (n=26;72%) and ENV (n=2;5.5%) samples. Through, this chapter this group of isolates will be referred to as the Global strain collection (Fig. 67). Global strain collection isolates were chosen from these sample types of origin because these represent infection causing isolates and carriage isolates. These isolates were characterised in relation to specific ARG and virulence determinants, STs and species identified within the *Klebsiella* sp. Fig. 68 shows the distribution of carbapenemase and ESBL ARG (*bla*_{CTX-M-15}, *bla*_{NDM-1}, *bla*_{KPC-3}, *bla*_{KPC-2}, *bla*_{OXA-181} and *bla*_{OXA-48}), the presence of *ybt* loci across the different isolates from the different regions.

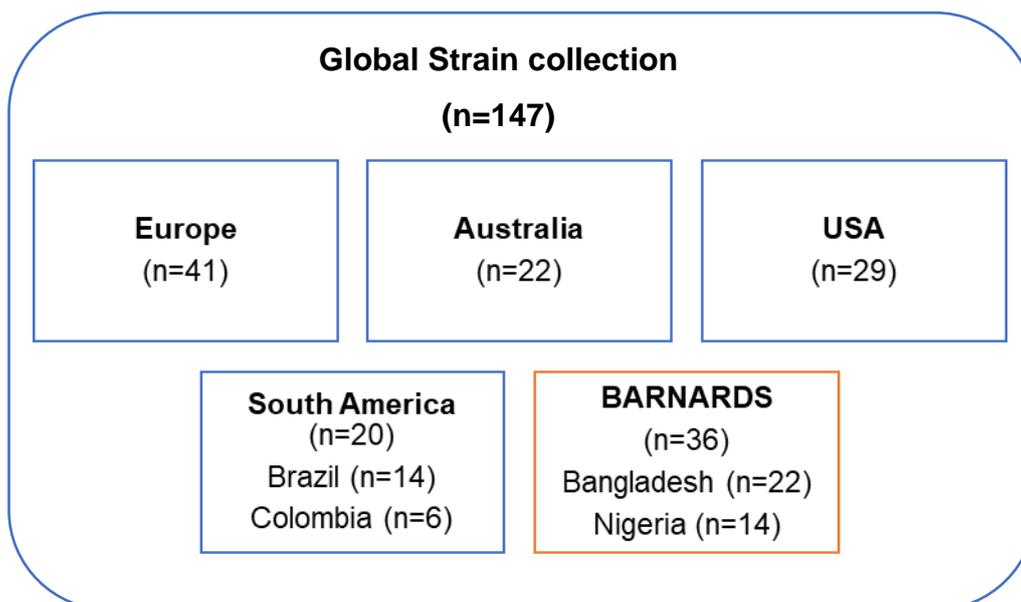


Fig. 67 : Composition of the global strain collection. *K. pneumoniae* isolate sequences from previously published collections from Europe, Australia, USA, and South America were included and, sequences from other BARNARDS clinical sites were included in this collection.

7.6.1 Global strain collection population structure characterisation

The *Klebsiella* sp. included in the Global strain collection belonged to 61 different STs. The most frequently identified were ST258 (n=19; 12.8%) followed by ST15 (n=11; 7.5 %) and ST11 (n=9; 6.1%). Also, ST258 *K. pneumoniae* were found among isolates the USA (n=15; 10%) and from South America, from Brazil (n=2;1.3%) and Colombia (n=2; 1.3%). In contrast, ST258 was not found among *K. pneumoniae* from Pakistan and Ethiopia. However, ST15 *K. pneumoniae* were found, not only, among Pakistani *K. pneumoniae*, but also, among isolates from Europe (n=7; 5%), Australia (n=2, 1.3%), USA (n=1;0.7%) and Nigeria (n=1; 0.7%). In addition, *K. pneumoniae* belonging to ST11 were from Europe (n=5; 3.4%), Brazil (n=3; 2%) and USA (n=1; 0.7%) and ST11 *K. pneumoniae* were also found among isolates from Pakistan (n=14;9.5%).

Klebsiella sp. identified among the Global strain collection (n=147;100%) most were identified as *K. pneumoniae* (n=133, 90.5%) followed by *K. quasipneumoniae* subsp. *similipneumoniae* (n=6; 4%) and *K. quasipneumoniae* subsp. *quasipneumoniae* (n=5; 3%). Additionally, four Global strain collection isolates were identified as *K. variicola* subsp. *variicola*, these were from Australia.

7.6.2 Carbapenemase and ESBL ARG harboured by the Global strain collection isolates

Most *K. pneumoniae* belonging to the Global strain collection harboured carbapenemase ARG. In majority these isolates (n=79; 54%) carried *bla*_{NDM-1} (n=21; 14%) and *bla*_{KPC-3} (n=20; 14%). *K. pneumoniae* carrying *bla*_{NDM-1} were from Bangladesh (n=9; 43%), Nigeria (n=10; 48%), Brazil (n=1; 5%) and Colombia (n=1; 5%) and isolates carrying *bla*_{KPC-3} were from the USA (n=14; 70%), Colombia (n=5; 25%) and Europe (n=1; 5%). *bla*_{KPC-2} (n=15; 10%) was frequently harboured as well being carried by *K. pneumoniae* from Brazil (n=12) and from the USA (n=3). In contrast, no *bla*_{KPC} ARG were harboured by PP and ES *K. pneumoniae*. Other carbapenemase ARG were harboured by the Global strain collection isolates including *bla*_{NDM-5} (n=6; 4%), *bla*_{NDM-7} (n=5; 3%), *bla*_{OXA-48} (n=9; 6%) and *bla*_{VIM-4} (n=1; 0.7%). In majority isolates belonging to the Global strain collection did not harbour ESBL ARG (n=77; 52%). However, 50 (34%) *K. pneumoniae* belonging to this group carried *bla*_{CTX-M-15} ARG. These *K. pneumoniae* were from Europe (n=21; 42%), Bangladesh (n=18; 36%), Nigeria (n=6; 12%), the USA (n=3; 6%), Brazil (n=1; 2%) and Colombia (n=1; 2%). Other ESBL ARG harboured by Global strain collection isolates included *bla*_{CTX-M-12} (n=1; 0.7%), *bla*_{CTX-M-2} (n=4; 3%), *bla*_{CTX-M-9} (n=2; 1.3%), *bla*_{SHV-12} (n=9; 6%), *bla*_{SHV-27} (n=2; 1.3%), *bla*_{SHV-38} (n=1; 1.3%), *bla*_{SHV-40} (n=1; 0.7%), *bla*_{SHV-7} (n=1; 0.7%), *bla*_{TEM-15} (n=1; 0.7%) and *bla*_{SHV-5} (n=1; 0.7%).

7.6.3 Virulence determinants displayed by Global strain collection isolates

Global strain collection isolates were characterised in relation to KL, OL and acquired siderophore loci (*ybt*, *iuc*, *iro* and *clb* loci). These *K. pneumoniae* displayed 56 different KL and 12 different OL. Most isolates displayed KL107 (n=22; 15%) followed by KL64 (n=9; 6%) and KL102 (n=7; 5%). KL107 was displayed by *K. pneumoniae* from the USA (n=14; 9.5%), Colombia (n=5; 3%), Brazil (n=1; 0.7%), Bangladesh (n=1; 0.7%) and Nigeria (n=1; 0.7%). Isolates from Nigeria (n=4; 3%), Bangladesh (n=2; 1.3%), Brazil (n=1; 0.7%) and Europe (n=2; 1.3%) showed KL64 and KL102 was displayed by isolates from Bangladesh (n=4; 3%), Europe (n=3; 2%) and Australia (n=1; 0.7%). The most frequently found OL was O1v1 (n=34; 23%), followed by O2v2 (n=28; 19%) and O2v1 (n=16; 11%). O1v1 was displayed by *K. pneumoniae* from Europe (n=17; 12%), Australia (n=7; 5%), the USA (n=5; 3%),

Nigeria (n=4; 3%) and Bangladesh (n=1; 0.7%). Isolates from Bangladesh (n=6; 4%), Europe (n=5; 3%), Australia (n=3; 2%), Brazil (n=4; 3%), Colombia (n=5; 3%) and the USA (n=5; 3%). Lastly, Global strain collection *K. pneumoniae* frequently harboured *ybt* (n=58, 39%), but also, harboured *clb* (n=8;5%), *iuc* (n=4; 3%) and *iro* (n=3; 2%). Also, the isolates carrying *ybt* were from Europe (n=22; 15%), Australia (n=8; 5%), Brazil (n=3; 2%), Colombia (n=2; 1%), the USA (n=8; 5%), Bangladesh (n=10; 7%) and Nigeria (n=5; 3%). Frequent KL and OL are shown in Fig. 68.

7.7 ES and PP *K. pneumoniae* in the context of the Global strain collection

K. pneumoniae isolates not only formed clusters according to their STs but also according to the countries/sites of origin (Fig. 68). Nevertheless, isolates from different countries were found within the different larger clades. PP and ES *K. pneumoniae* establishing clades according to their most common STs (PP ST15 and ES ST35 and ST37). Therefore, showing that the isolates from the Global strain collection and ES and PP *K. pneumoniae* populations were different. ST15 *K. pneumoniae* formed a clade including isolates PP *K. pneumoniae* majority, but isolates from Nigeria, Europe, and Australia as well. These *K. pneumoniae* created a large clade that comprised three smaller clusters. In relation to ST15 isolates from Europe and Australia these *K. pneumoniae* created a smaller different ST15 cluster within the larger clade indicating that although these belong to the same ST these were not similar to PP *K. pneumoniae*. In addition, *K. pneumoniae* from the Global strain collection were not found among the ST35 and ST37 ES *K. pneumoniae* clusters that included the ES *K. pneumoniae* from this PhD.

K. pneumoniae belonging to the Global strain collection did not establish large clades of isolates belonging to the same ST as these showed high diversity in STs therefore establishing several smaller clades that included *K. pneumoniae* from the same region. Nevertheless, Fig. 68 shows that *K. pneumoniae* belonging to ST258 from the Global strain collection formed a cluster in which most isolates were from the USA but isolates from Brazil and Colombia were also found.

Placing PP and ES *K. pneumoniae* in a global context showed that *K. pneumoniae* population structure is different according to the region of the world with different ARG and virulence determinants being harboured too. Also, because the Global strain collection isolates were isolated from blood samples in majority could

have influenced the ST and ARG and virulence determinants found. As for example *bla_{KPC}* ARG among Global strain collection *K. pneumoniae* is usually found in *K. pneumoniae* causing nosocomial infection (24). In contrast, *bla_{KPC}* was not found among PP and ES *K. pneumoniae* whose sample of origin was collected at hospital admission.

However, differences in geographic distribution of *K. pneumoniae* ARG and STs were previously described in the literature with most ST15 found in South Asia, PP *K. pneumoniae* in the case of *K. pneumoniae* from this PhD, and ST258 being found among *K. pneumoniae* belonging to the Global strain collection, as expected. Since, ST258 *K. pneumoniae* has been described as a main lineage in driving carbapenem resistance in Europe (39) (1.6 Burden of antibiotic resistant *K. pneumoniae* in infection).

In relation to virulence determinants when considering KL typing of Global strain collection *K. pneumoniae* showed different KL when compared with PP and ES isolates, however seeing that KL are extremely variable this was expected. In addition, though PP and ES *K. pneumoniae* did not carry *clb* loci found among Global strain collection isolates, *ybt*, *iuc* and *iro* loci were found among PP and ES isolates and in Global strain collection *K. pneumoniae*. Also, KL64 and O1v1 were found among PP *K. pneumoniae* and O2v2 was displayed by PP and ES *K. pneumoniae*.

In summary, studying the *K. pneumoniae* from Pakistan and Ethiopia in the global phylogeny showed as described previously that *K. pneumoniae* is very diverse but demonstrated a structured population defined by numerous different STs but different in distinct regions of the world (39). This indicated that genomic surveillance studies in addition to the determination of antibiotic susceptibility profiles of the *K. pneumoniae* population, as performed during this PhD, would be a helpful tool to better understand the *K. pneumoniae* present in different clinical settings and communities. So, that more effective and appropriate therapies whether based on antibiotics or other therapeutics such as vaccines or antibodies can be used.

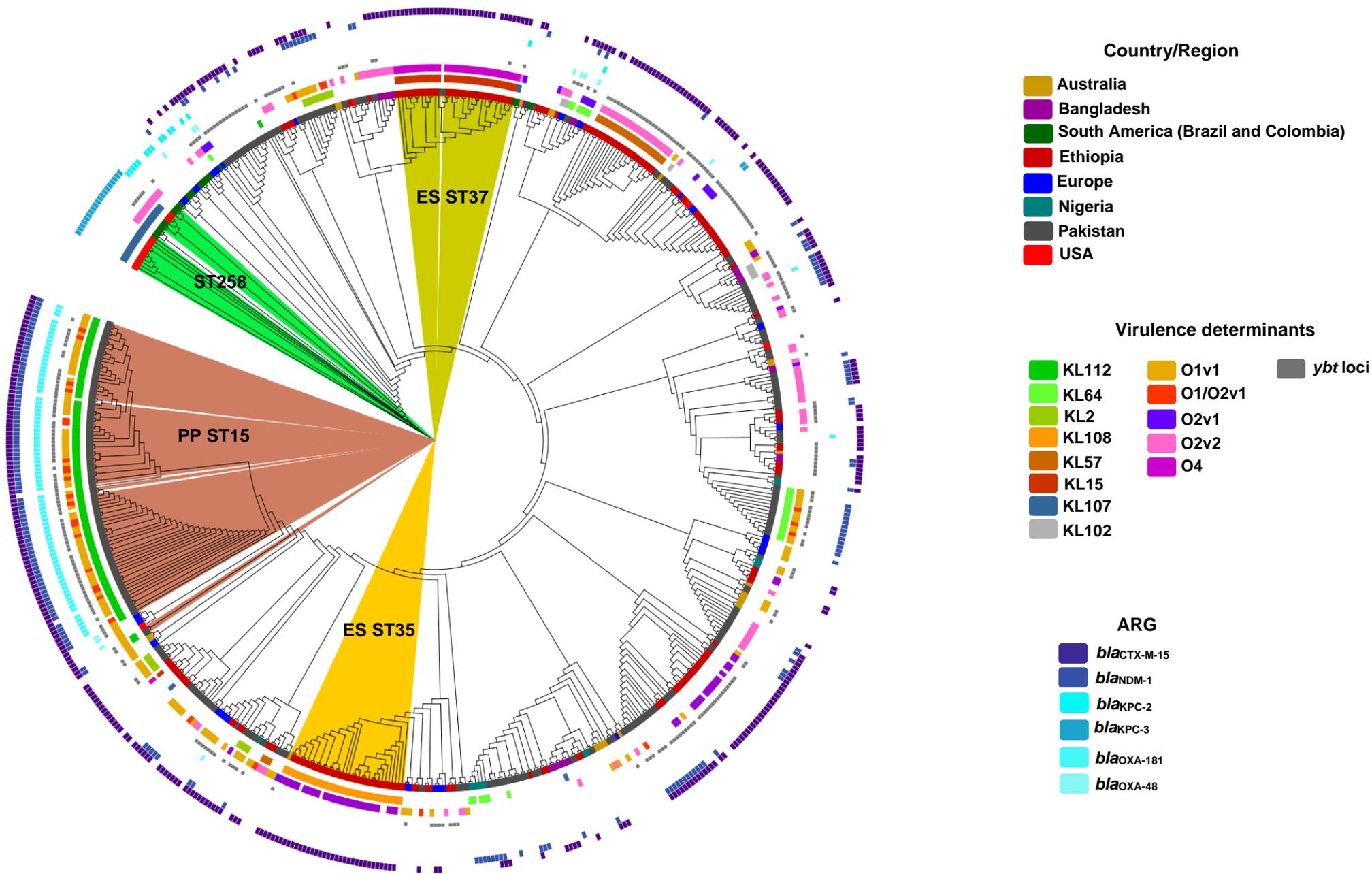


Fig. 68: Core genome phylogeny of *K. pneumoniae* in a global context. STs and distribution the most common ARG, KL, OL, presence of siderophore loci. Distribution of the common ARG and virulence determinants was different among *K. pneumoniae* from different regions/countries. ST258 *K. pneumoniae* created a cluster within which isolates from the USA and South America were found and ST15, ST35 and ST37 from Pakistan and Ethiopia formed other clusters. *bla*_{KPC} ARG were found among isolates from USA, South America and Europe in contrast no *bla*_{KPC} ARG were found among isolates from Pakistan and Ethiopia. *bla*_{NDM-1} was mostly carried by *K. pneumoniae* from Pakistan, Bangladesh, and Nigeria. *bla*_{CTX-M-15} the presence of *bla*_{CTX-M-15} ARG, *bla*_{NDM-1} the presence of *bla*_{NDM-1}, *bla*_{KPC-3} the presence of *bla*_{KPC-3}, *bla*_{KPC-2} the presence of *bla*_{KPC-2}, *bla*_{OXA-181} the presence of *bla*_{OXA-181}, *bla*_{OXA-48} the presence of *bla*_{OXA-48} ARG. The different regions of the globe are indicated in different colours at the end of the phylogeny branches.

8. General Discussion

Neonatal sepsis contributes substantially to mortality and morbidity among children with the majority of the burden being felt in LMICs where lack of healthcare resources and antibiotic resistance is becoming a serious threat (84, 85, 188, 189). The incidence of sepsis during the BARNARDS study further confirmed this by providing data on the burden of antibiotic resistance on LMICs and by reporting on multiple risk factors associated with sepsis and mortality following sepsis (170, 190, 191). A key objective of BARNARDS was to characterise all GNB collected from blood cultures in the seven LMIC within the study to understand the diversity of pathogens causing neonatal sepsis. *K. pneumoniae* was the most frequently recovered GNB responsible for sepsis. This has already been reported, for example, in by Mukherjee et al. in North-East India (73) and in Gaith et al. in Egypt (80), however, these studies are focused on individual sites. In contrast, BARNARDS was a multiple site study with standardised protocols across all enrolled clinical sites that allowed to obtain comparative data from countries in Africa and Asia by blending epidemiological data and molecular/genomic data. During BARNARDS it became apparent quite early on that *K. pneumoniae* was the dominant pathogen in blood cultures in Ethiopia (St Paul's Hospital). Likewise, during the screening of the of mothers' and neonates' microbiota which was performed to detect β -lactamases genes as markers for the resistome high recovery of carbapenemase positive *Klebsiella* in Pakistan (PIMS) was noticed.

For these two reasons my PhD specifically focused on characterising the *K. pneumoniae* populations from these two clinical sites. By performing detailed genomic analysis this PhD aimed to understand whether there was evidence of transmission between the neonate, the mother, and the clinical environment. Building large phylogenies of PP and ES *K. pneumoniae* allowed my analysis to generate a better picture of the role *K. pneumoniae* has in neonatal sepsis in Pakistan (PIMS) and Ethiopia (St Paul's hospital), adding more knowledge on *K. pneumoniae* in LMICs seeing that there are major gaps in knowledge from these countries (163, 192).

8.1 What is the burden of sepsis and antibiotic resistance within PIMS and St Paul's hospital?

It is important to consider that in hospital settings in LMICs such as Pakistan and Ethiopia, where there is a lack of healthcare resources, antibiotic resistance is

becoming a serious threat, with infections becoming exacerbated, neonatal sepsis contributing substantially to mortality and morbidity among children (84, 85, 188, 189).

Risk factors identified during BARNARDS indicated that neonates whose mothers had an infection and used antibiotics three months prior to enrolment had higher odds of biological sepsis with a high proportion of biological sepsis occurring between the first three and seven days of clinical diagnosis. This is worrying considering the increasing prevalence of carbapenemase producing *Enterobacteriaceae*, including *K. pneumoniae*, may contribute to higher morbidity and mortality of infections caused by these bacteria (170, 188, 190, 191). Moreover, the WHO Global Report on the Epidemiology and Burden of Sepsis from 2020 (193) estimated that 84% of neonatal deaths could be prevented through measures as early and timely diagnosis and appropriate clinical management. Such measures would be welcome to prevent neonatal sepsis in LMICS where the burden is highest being estimated at 24%. For example, extensively resistant *K. pneumoniae*, all carrying *bla_{VIM}* and *bla_{NDM}* and, in majority, also carrying *bla_{KPC}* and *bla_{CTX-M}* ARG caused sepsis in a NICU in Egypt (194). Similarly, a study in India reported on resistant *K. pneumoniae* harbouring *bla_{NDM-1}* causing septicaemia in neonates (73). Additionally, as mentioned in the Introduction, *Enterobacteriaceae* as *K. pneumoniae* contribute to antibiotics resistance globally commonly carrying β -lactamases. But they also carry other ARG as *qnrS*, *qnrB* or *qnrA* or Agly ARG as *aac6'-Ib*, *aac6'-Ib-cr*, (73, 80). These reports are in line with the ARG found among ES and PP *K. pneumoniae* studied in this PhD whether these were isolates causing sepsis (BB), carriage, (BR and MR), or isolates recovered from the clinical environmental samples (ENV). In overall, PP and ES isolates harboured high numbers of ARG that confer resistance to a wide variety of antibiotics. β -lactamase and aminoglycoside ARG were harboured commonly by ES and PP *K. pneumoniae*. Among the β -lactamase ARG ESBL and carbapenemase were commonly detected. However, in contrast to PP *K. pneumoniae* that harboured in majority carbapenemase ARG, only two ES-BR *K. pneumoniae* harboured carbapenemase *bla_{NDM}* ARG, but ESBL *bla_{CTX-M-15}* was commonly found among ES and PP *K. pneumoniae* (Fig. 69). Thus, ES and PP *K. pneumoniae* reflect the global widespread of ESBL and carbapenemase ARG but also the geographic differences that exist regarding these ARG (11, 16). Considering that *bla_{NDM}* ARG are frequently found among *Enterobacteriaceae* including *K. pneumoniae* in Asia whereas *bla_{KPC}* is more commonly found among *K. pneumoniae* in the Europe for example (19, 58, 59,

61). Thus, *bla*_{N_{DM}-1 being the most common *bla*_{N_{DM}} ARG among PP *K. pneumoniae* confirms what is described in those reports. *bla*_{N_{DM}-1} was also often carried simultaneously with *bla*_{OXA-181}, among PP *K. pneumoniae*, which could exacerbate the increasingly difficult to treat infections. In this PhD, the presence of these ARG was associated with the outcome of sepsis but most neonates survived and implied longer time until outcome indicating that sepsis caused by these isolates were more difficult to treat (sections 5.7,6.7 and 7.5). Despite, for example, PP ST15 isolates carrying *bla*_{N_{DM}-1} only and isolates carrying both *bla*_{N_{DM}-1} and *bla*_{OXA-181} showing similar phenotypes regarding antibiotics resistance.}

With a high frequency of carbapenemases being mainly found in PIMS, in contrast, to ES *K. pneumoniae* that in majority carried ESBL *bla*_{CTX-M-15}, that were isolated later on for this study while aiming to recover carbapenemase negative isolates. It is important to consider that this PhD studied carbapenemase positive and negative part of the collection of BR, MR and ENV *K. pneumoniae* isolates started as part of BARNARDS which focused on carbapenemase positive isolates then later on for this PhD carbapenemase negative *K. pneumoniae* were collected from both PIMS, Pakistan and St Paul's Hospital, Ethiopia. This could have skewed towards a higher number of carbapenemase positive PP *K. pneumoniae* not only because of the initial collection done during BARNARDS but also because less carbapenemase negative isolates could genuinely be lower in number in PIMS or because over the time the swab samples quality decreased affecting isolation rates. The above is particularly important when considering the different sample type of origin, resistome and the antibiotic susceptibility of ES and PP *K. pneumoniae*. As most PP *K. pneumoniae* were resistant to carbapenems and cephalosporins contrary to ES *K. pneumoniae* that were in majority susceptible to carbapenems. Nevertheless, MR *K. pneumoniae* showed reasonable percentages of susceptibility to cephalosporins and carbapenems in contrast to *K. pneumoniae* recovered from other sample types of origin. (sections 5.3 and 6.3). This could be because MR samples were taken from mothers whom the neonates did not show signs of sepsis and from mothers whom the neonate showed sign of sepsis, so use of antibiotics would mostly affect the former. The difference in antibiotic susceptibility is also important to consider in light of the microbiota contribution to the spread of ARG (43, 45), because if vertical transmission can occur it would follow that the ARG content and antibiotic susceptibility of BB and BR would be similar to that of MR *K. pneumoniae*. However, if the neonate were diagnosed with

sepsis, it would have been exposed to antibiotic therapy and to the clinical environment after admission which can influence the neonates' microbiota concurrently with the mother's microbiota. *K. pneumoniae* often carries acquired ARG following that HGT could have contributed for the difference of antibiotic susceptibility when comparing MR *K. pneumoniae* with BB, BR and ENV *K. pneumoniae* considering that, for example, plasmids carrying carbapenemase ARG were less common among MR isolates. Hence my PhD was able to indicate that differences in plasmid content among different sample types could explain the differences in antibiotic susceptibility. Additionally, ARG that confer resistance to other antibiotics such as aminoglycosides were frequent among both populations of *K. pneumoniae* as well and have probably contributed for resistance to the aminoglycosides tested. The β -lactams, including the carbapenems and cephalosporins, and aminoglycosides tested included antibiotics prescribed at PIMS (cefotaxime, piperacillin/tazobactam, amoxicillin, imipenem, meropenem, amikacin, ciprofloxacin and colistin) and St Paul's Hospital (ceftazidime, cefotaxime, ampicillin, gentamicin) allowing to understand that most PP and ES *K. pneumoniae* displayed high percentages of resistance to antibiotics prescribed. This is particularly worrying among BB *K. pneumoniae* responsible for sepsis seeing that, for example, ST35 and ST37 ES-BB *K. pneumoniae* were responsible for outbreaks carried multiple ARGs including *bla*_{CTX-M-15}, and the presence of this ARG was shown to diminish cumulative survival. Thus, indicating that these infections were more difficult to treat. Furthermore, among the major ST groups found ST15 in Pakistan and ST35 and ST37 in Ethiopia antibiotic susceptibility profiles and ARG content showed differences. There were also differences between the antibiotic susceptibility and the ARG content displayed by ES ST35 and ES ST37, despite both being responsible for outbreaks in Ethiopia. For example, ES ST37 *K. pneumoniae* showed resistance to ciprofloxacin and to piperacillin/ tazobactam and ST35 showed susceptibility. Nevertheless, ES *K. pneumoniae* belonging to these STs are still all resistant to the tested cephalosporins and to other β -lactam treatment such as amoxicillin/clavulanate and in majority susceptible to meropenem, ertapenem, imipenem and amikacin.

When the information of the prescription was available, the outcome of sepsis was alive. Even though the *K. pneumoniae* tested as resistant in this PhD the treatment using the prescriptions above mentioned was in majority successful. Nevertheless, this interpretation must be made carefully seeing that prescription

information was not available for most sepsis cases caused by BB isolates belonging to major STs in PIMS and St Paul's hospital. The MIC determination performed using a range of five concentrations the determination of a more precise MIC was not possible though it still provided valuable information that can indicate more accurate treatment.

Most ES and PP *K. pneumoniae* were susceptible to antibiotics such as colistin and fosfomicin. Likewise, these *K. pneumoniae* were susceptible to combinations as ceftazidime/avibactam, contrarily these were resistant to piperacillin/tazobactam amoxicillin/clavulanate. In this PhD possible treatment options such as ceftazidime/avibactam and aztreonam/avibactam were tested because these could become viable to treat MDR *K. pneumoniae* carrying carbapenemase ARGs. Previous work proposed that aztreonam/avibactam could be a more effective treatment where MBLs carrying *Enterobacteriaceae* are predominant (127). Thus, ceftazidime/avibactam were only tested on carbapenemase positive *K. pneumoniae* in this study. As reported before, the *K. pneumoniae* isolates in this PhD carrying MBLs (in this study *bla_{NDM}*) were resistant to ceftazidime/avibactam in contrast being susceptible to aztreonam/avibactam. Furthermore, high frequency of resistance was noted in Thomson et al to fosfomicin, colistin and gentamicin (169) suggesting that resistance to colistin and fosfomicin among ES and PP *K. pneumoniae* could rapidly increase.

Additionally, PP and ES *K. pneumoniae* harboured ARG that confer resistance to other antibiotics like, for example, fluoroquinolone ARG and other resistance markers as resistance conferring mutations that also contribute to antibiotic resistance, such as SNPs that confer resistance to fluoroquinolones (41), truncation of *OmpK* that confer resistance to β -lactams (177) and truncation that *mgrB* or *pmrB* genes that confer resistance to colistin were found too (41, 53). This is worrying because it might translate into less efficient treatment if these were used for treatment of sepsis caused by *K. pneumoniae* isolates. In this PhD, ST15 PP *K. pneumoniae* in contrast to ST35 and ST37 ES *K. pneumoniae* harboured *OmpK* and Flq SNPs which were often simultaneously harboured with Flq ARG. Also, among ST15 PP *K. pneumoniae* two *K. pneumoniae* (one BR and one MR) harboured colistin resistance conferring mutations, however, all ST15 *K. pneumoniae* were susceptible to colistin. Resistance to colistin was only displayed among PP isolates. These isolates were distributed among two BB, four BR, and one MR *K. pneumoniae* in a total of seven PP *K.*

pneumoniae. The truncation of *mgrB* or *pmrB* was harboured by PP *K. pneumoniae* nevertheless only one of the colistin resistant *K. pneumoniae* harboured a *pmrB* truncation in contrast other PP isolates harboured the truncation of *mgrB* or *pmrB* but did not show resistance to colistin. Also, after a preliminary search two colistin resistant harboured the *mcr-9.1* ARG variant that confers resistance to colistin. This important as colistin resistant KPC, NDM, OXA-48 producing *K. pneumoniae* have been reported already (195, 196) suggesting that the presence of such markers might lead to the convergence of resistance to colistin, cephalosporins and carbapenems among PP *K. pneumoniae*.

Even though this PhD study was able to produce retrospective genomic surveillance and link in antibiotic susceptibility data of the *K. pneumoniae* population within both neonatal sepsis and rectal carriage in PIMS and St Paul's Hospital in order inform diagnosis and antibiotic treatment. It is worth noting that these are settings in LMICs facing challenges as for example overcrowding, lack of health care personnel, long travel distances for patients and many areas lacking paediatricians (189, 197). This PhD defined different profiles of ARG and antibiotic resistance for PIMS and St Paul's Hospital that would help inform prescriptions in the case of *K. pneumoniae* infection, but this would only be effective if a biological sepsis diagnosis can be confirmed in a timely manner but blood sampling among paediatric patients can be difficult. The optimal blood volume is a subject of much discussion due to the needed bacterial load and due to factors as for example low intravascular volume and the risk of causing anaemia. Also, in paediatric patients' low level bacteraemia (≤ 10 CFU/mL) is presumed to be underreported because the amount of blood determines the sensitivity, specificity and time to positivity of the blood culture and approaches deemed adequate call for a sample of 1-1.5mL for children under 11Kg to be drawn (198). Moreover, the small volume of the blood sample taken (1-1.5 mL) could have impaired the diagnosis of sepsis during BARNARDS because if not enough CFUs might have not been collected a negative diagnosis of sepsis might have been incorrect (198). Thus, sepsis caused not only by *K. pneumoniae* but by other agents as well could have been under reported in PIMS and St Paul's Hospital. Also, the possible challenges in acquiring the necessary materials, technology, and trained staff might have affected the implementation of blood culture for diagnosis (199). Taking the above in mind meant that the number of BB *K. pneumoniae* recovered could have been impaired during the timeframe of BARNARDS this being a limitation on this PhD,

because it could impair the ability to assess the diversity and burden of *K. pneumoniae* in the clinical sites. Nonetheless, because BARNARDS suppliers were standardised across all sites it was possible to help all clinical sites implement blood culture for diagnosis with instructions and infrastructure to perform it. Thus, lessening the effects of lack of materials but not of the difficulty of blood sampling.

8.1.1 Could different antibiotic regimens diminish the burden of antibiotics resistance?

With PP ST15 *K. pneumoniae* and the ST35, ST37 and ST218 ES *K. pneumoniae* in mind the MIC₅₀ and MIC₉₀ determined values suggested that high concentrations of antibiotics would be needed for most antibiotics to be effective. The exceptions would be the carbapenems and amikacin among ST35 and ST37 ES *K. pneumoniae* and tigecycline and aztreonam/avibactam among ST15 PP *K. pneumoniae*. When considering antibiotic treatment several factors have to be taken specially when the patient is a neonate whose treatment needs to be done more carefully, seeing that dosing of antibiotics adjusted based on weight when extrapolated from adult studies can on one hand lead to underdosing, which can result in treatment lack of efficacy and on the other hand, underdosing risks toxicity. Also, factors as the lack of data available on dosing antibiotics in neonates have contributed for this challenge, because neonates have the unique physiology that affects drug disposition leading such as the need for higher systemic concentrations and the need for higher initial doses (200). Nevertheless, considering *K. pneumoniae* populations colistin showed low MIC values indicating that it could be a viable treatment. Fosfomycin could also be considered as a possible treatment because it showed low MIC₅₀ and MIC₉₀ values when considering ST35 ES *K. pneumoniae* and ST15 PP *K. pneumoniae*.

Nonetheless, although such treatments may be proposed but availability of antibiotics and their affordability may prove an obstacle in LMICs as Pakistan and Ethiopia as seen in Thomson et al (169). Other antibiotics regimens, such as ceftazidime/amikacin that showed potential target attainment (PTA $\geq 80\%$) of 77.1%, may be proposed seeing that usage could be feasible at PIMS, where the use of amikacin is available. In contrast this would not be possible in Ethiopia where amikacin is not available, therefore, other regimens that showed promising PTAs $\geq 80\%$ in Thomson et al. would be difficult to implement at St Paul's seeing that this included amikacin used in combination with other antibiotics. Only ampicillin and gentamicin

are available through the public system in Ethiopia, and other available antibiotics (ceftazidime and meropenem) have to be paid for, impairing the ability of the average general population to obtain them. Also, while in Pakistan more antibiotics are available to use these are still an economic heavy burden for the average population (169). Even though, fosfomycin and or colistin could be seen as other treatment options considering the MIC₅₀ and MIC₉₀ values in 5.4.1 and 6.4.1 and the high frequency of resistance determined for fosfomycin (68.4%) and for colistin (57.3%) studies in Thomson et al. (169) treatment with these antibiotics should be carefully considered because PIMS and St Paul's Hospital have different profiles of antibiotic resistance. Furthermore, use of ampicillin and gentamicin was common among BARNARDS sites in Africa including Ethiopia, and piperacillin/tazobactam and amikacin where also used in Pakistan which might help explain the differences in antibiotic resistance patterns between PP and ES *K. pneumoniae* (169). In Ethiopia prescriptions using ceftazidime, ceftriaxone, and cefotaxime usually in combination with other antibiotics were used, and in Pakistan besides cephalosporins carbapenems such as imipenem and meropenem were prescribed probably contributing for the carbapenem resistance among PP *K. pneumoniae* and its absence in ES *K. pneumoniae*.

Furthermore, the WHO Global Report on the Epidemiology and Burden of Sepsis, 2020 (193) indicated that EOS causes 8% of all neonatal deaths this being indicative of problems with quality of care including constraints in healthcare settings for care of pregnant women and neonates, delayed diagnosis and poor management of infection. With that mind and that EOS was often the onset of *K. pneumoniae* sepsis in this PhD could possibly reflect lack of appropriate healthcare infrastructure and access to timely diagnosis that would provide better guide for treatment of sepsis in PIMS and St Paul's Hospital. Additionally, most neonates survived sepsis caused by *K. pneumoniae* even though resistance to the antibiotics used for treatment was seen and these *K. pneumoniae* often carried ARG associated with outcome and onset. For example, treatment would often include gentamicin for ES and PP *K. pneumoniae* that were in majority resistant to gentamicin. The same being seen when considering cefotaxime that was used in both clinical sites. Despite the high frequency of resistance to these antibiotics the outcome of infection was in most cases alive when the opposite could have been expected and within the dominant ES and PP STs the cumulative survival was high in the presence of *bla*_{CTX-M-15} and both *bla*_{NDM-1} and

*bla*_{OXA-181}. Still, antibiotic resistance and outcome was not associated in the treatment combinations tested by Thomson et al. (169). The majority of *K. pneumoniae* sepsis had a positive outcome despite the high levels of resistance.

As seen in this PhD ES and PP *K. pneumoniae* carried numerous ARG and were resistant to most tested antibiotics, also, antibiotics that could be used as better options treatments were either not available or would not be affordable. For example, in the case of fosfomycin its frequency of resistance would have to be considered when using it. Nonetheless, in Thomson et al., meropenem, fosfomycin and colistin shown high PTA $\geq 80\%$ when adjusted to patient specific MICs. Meropenem could be considered for treatment of *K. pneumoniae* sepsis in St Paul's Hospital where *K. pneumoniae* belonging to all sample types in study were susceptible to carbapenems, however, the cost to obtain would mean a heavy financial burden(169).

Moreover, having the genomic and antibiotic susceptibility data of this PhD regarding *K. pneumoniae* showed that different clinical sites would need tailored antibiotic regimens, considering the differences in ARG and antibiotic resistance. Despite this being increasingly difficult to select considering the factors that prescribing antibiotics to a neonate include and the efficiency that an available and affordable regimen could have. Thus, when proposing new antibiotic regimens even when supported with genomic surveillance and antibiotic susceptibility data other factors as affordability and availability have to be considered especially in LMICs.

8.1.2 What was the contribution of plasmids to the spread of ARG among PP and ES *K. pneumoniae*?

The important role plasmids played in the dissemination of plasmid encoded carbapenemase ARG among *K. pneumoniae* was previously described in David et al. (181). Therefore, a preliminary analysis of plasmid replicons among PP and ES *K. pneumoniae* was done. In majority, among ES *K. pneumoniae* Inc types F, H, R, Q were found and distributed among ES-BB, BR, MR and ENV sample types and IncN plasmid replicons were harboured by ES-BB and ES-BR as well. Similarly, PP-BB, BR, MR and ENV also harboured IncF, IncH and IncN. PP isolates harboured IncX plasmids as well. In contrast, IncX plasmids were not found among ES *K. pneumoniae* and PP isolates did not harbour IncR and IncQ plasmid type replicons. Therefore, ESBL and carbapenemase ARG carried by *K. pneumoniae* from Pakistan and Ethiopia

might be spread through different plasmids. Nevertheless, both ES and PP *K. pneumoniae* harboured IncF and IncN associated with *bla*_{CTX-M-15}. In 5.6.1, the *bla*_{CTX-M-15} carrying plasmids spread among ES *K. pneumoniae* included IncF plasmid replicons among others such as IncQ, IncR and IncH. However, the plasmids studied among PP *K. pneumoniae* did not carry *bla*_{CTX-M-15}, therefore it was not possible to study what was the effect of HGT on its dissemination in PIMS. Because *bla*_{NDM-1} and *bla*_{OXA-181} were frequently found among ST15 PP *K. pneumoniae*. Furthermore, IncX plasmid replicons being mostly harboured by PP *K. pneumoniae* reflects previously described reports of *bla*_{OXA-181} and *bla*_{NDM} ARG being associated with IncX plasmid replicons. Thus, it followed that it would not be among ES *K. pneumoniae* in which carbapenemase ARG were uncommon. Also, in 6.6.1, IncX3 and ColKP3 replicon type plasmid was found to harbour *bla*_{OXA-181} and an IncA/C replicon type plasmid harboured *bla*_{NDM-1} together with other ARG being in line with previous reports (201-205).

By further studying the plasmids carried by *K. pneumoniae* indicated their role in driving antibiotic resistance between the clinical environment and between mothers and their neonates. Particularly among STs seen as the major causative agents of sepsis and found among different sample types. PP ST15 especially because genomes belonging to CG15 were associated with high frequency of acquired ARG and MDR *K. pneumoniae* with carrying higher number of plasmids than hvKp (55). Among ST15 PP *K. pneumoniae* pKpnST15_PPBB_1 and pKpnST15_PPBB_2, harbouring *bla*_{NDM-1} and *bla*_{OXA-181}, respectively, were found in *K. pneumoniae* recovered from all sample types of origin studied. This is important because it indicated that these plasmid backbones harbouring common ARG were present among ST15 isolates from different sources. Also, IncX3 replicon carrying *bla*_{NDM-1} were previously found among several *Enterobacteriaceae* species enabling them to spread in widespread areas showing that IncX3 plasmids have an important role in disseminating *bla*_{NDM} ARG. These areas included Hong Kong, China, Korea, Myanmar, India, Oman, Kuwait, Italy, and Canada (185). Additionally, in Uruguay plasmids co-harboring *bla*_{CTX-M-15}, *aac6'-Ib-cr* and *qnrB* in *K. pneumoniae*, including ST37 isolates, were found to be the source of resistance to ESBLs in a paediatric hospital. This ARG content is similar to the ARG content of pKpnST37_ESBB_1 and pKpnST37_ESBB_2 that disseminated *bla*_{CTX-M-15}, Agly and Flq ARG in St Paul's Hospital. Thus, these plasmids were a source of resistance to both aminoglycosides

and β -lactams (206). Further highlighting the important role of HGT in the spread of ARG among *K. pneumoniae*.

In this PhD it was possible to study the HGT of specific ARG among *K. pneumoniae* belonging to the most frequent STs in order further understand how the antibiotic resistance was spreading. Also, considering that, pKpnST15_PPBB_1 and pKpnST15_PPBB_2 as well as pKpnST37_ESBB_1 and pKpnST37_ESBB_2 were found among ENV *K. pneumoniae*, it further suggests that IPC measures are important and can help decrease the spread and transmission of resistant *K. pneumoniae* to neonates and their mothers from the clinical environment.

It is also important to consider the role of HGT that can occur in the microbiota seeing that pKpnST15_PPBB_1 and pKpnST15_PPBB_2 as well as pKpnST37_ESBB_1 and pKpnST37_ESBB_2 were found among BR and MR samples. So, HGT that could have occurred in the microbiota included *K. pneumoniae* harbouring plasmids carrying ARG. This important seeing that the neonate's microbiota is not completely developed at birth and resembles that of the mother. Thus, if the neonate is exposed to a clinical environment and mother's microbiota where resistant *K. pneumoniae* are present there is the possibility of HGT of ARG, such as ESBL and carbapenemases, through the clinical environment and through the microbiota.

Furthermore, in *K. pneumoniae* the pattern of clonal replacement and acquisition of MGEs was shown to be linked with the introduction of recognized MDR clones carrying resistance markers on mobile elements (207). Therefore, insight into the ARG, MGEs and phylogeny of ES and PP *K. pneumoniae* populations could provide further knowledge into the population dynamic as well as into the dynamic of the ARG that they harbour. In this PhD both the phylogeny and plasmid content were studied particularly among ST15 *K. pneumoniae* in Pakistan and ST35, ST37 and ST218 *K. pneumoniae* in Ethiopia, according to the timeline these were recovered. This allowed to study possible vertical transmission events but could probably help understand how the ES and PP *K. pneumoniae* population could change over time seeing that plasmid-mediated resistance can lead to a shift from a diverse population to the marked prevalence of few lineages (207). So, by having the timeline of the ES ST35, ST37 and ST218 ES *K. pneumoniae* and considering that ST35 and ST218 did not harbour pKpnST37_ESBB_1 whereas pKpnST37_ESBB_2 was carried by ST35, ST37 and ST218 ES *K. pneumoniae*. It could have been possible to assess if that

difference in plasmid content could lead to the dominance of one of these lineages. However, having a limited timeline with gaps did not allow to understand if the distinct plasmid content influenced the dominance of any ST, but it could offer a possible reason as to why ES ST35 *K. pneumoniae* are susceptible to ciprofloxacin and levofloxacin and ES ST35 isolate were not (207). pKpnST37_ESBB_1 was only found among ST37 ES *K. pneumoniae* indicating that this plasmid backbone could be associated with this ST in this population. In addition, considering that plasmids with similar backbones to the pKpnST37_ESBB_1 backbone were not found among ES ENV suggests an important role of the microbiota in the HGT of these plasmids.

Thus, in light of the role that HGT together with the interaction with the clinical environment and the microbiota had disseminating common ARG among the most frequent found STs in this PhD reinforces that Infection prevention and control (IPC) is valuable as well as care when prescribing and using antibiotics because it can help lessen the spread of ARG and resistant *K. pneumoniae*.

8.2 Could IPC diminish the burden of antibiotic resistance?

Several factors can possibly influence the risk of infection for neonates once the mothers reach the hospital. For example, in Pakistan most mothers of neonates to whom *K. pneumoniae* caused sepsis received private healthcare three months prior to delivery. In Ethiopia, most mothers did not have a prior infection, received private healthcare, or been hospitalised 12 months prior, in addition most had not received antibiotic treatment three month prior to the neonates' birth (170). However, risk factors for sepsis in BARNARDS included, for example, the neonate being in a specific ward or the mother being hospitalised, having an infection, or using antibiotics prior to enrolment (170). These factors could have influenced the mother's microbiota seeing that treatment at an hospital setting and/or antibiotic treatment can affect the microbiota and its acquired ARG (208). In addition, BARNARDS approach of also including sampling of the clinical environment allowed me include ENV *K. pneumoniae* in my PhD. In turn allowing to demonstrate that the clinical environment can participate in the spread of *K. pneumoniae* capable to cause sepsis because PP and ES-ENV isolates were found to be part of major ST clusters.

In this PhD phylogenetic similar ENV *K. pneumoniae* to *K. pneumoniae* from other sample types were found among PP ST15 and ES ST35, ST37 and ST218 in

addition possible transmission of sepsis causing isolates from mother to neonate as well as the possibility of BR *K. pneumoniae* later causing sepsis. Thus, the clinical environment and antibiotic usage are important factors in the spread of resistant *K. pneumoniae* that cause sepsis and that are found in the mothers and neonates' microbiota. Also, the usage of perinatal antibiotics was seen to seriously affect the vertical transmission of the microbiota from mother to child changing the source of colonisation towards the environment (208).

With the above in mind, not only appropriate antibiotic usage, but also IPC measures could help diminish the antibiotic resistance burden and prevent outbreaks considering that the clinical environment can act as a reservoir for resistant *K. pneumoniae*. Thus, strategies to improve and provide higher quality of care were implemented in LMICs because a significant proportion of deaths due to infections occur in hospital settings. Nevertheless, in LMICs lack of health care staff and infrastructure indicates the need for better strategies to prevent, diagnose and treat infections in LMICs (209, 210). In addition, regarding both BARNARDS clinical sites included in this PhD St Paul's Hospital, Ethiopia and PIMS, Pakistan, it is important to recognise that tailored IPC plans would be needed not only because of the distinct *K. pneumoniae* populations but also because of differences in healthcare infrastructure shown in Fig. 70 and Fig. 71. Even so, in Johnson and Milestone, it was referred that efforts to reduce neonatal mortality due to infection have focused primarily in community based interventions and that the implementation of an IPC bundle in a Zambian Hospital reduced the hospital onset and mortality of infections in neonates, despite the challenges that implementing an IPC plan may have in LMICs as for example, the differences in microbiology of infections and the quality of data (211, 212).

Hence with the information from this PhD regarding the different populations of *K. pneumoniae* from PIMS and St Paul's Hospital, would help inform a possible improvement of the IPC plan on both clinical sites that could diminish the incidence of sepsis caused by *K. pneumoniae*.



Fig. 70: St Paul's Hospital, Addis Ababa, Ethiopia.



Fig. 71: PIMS, Islamabad, Pakistan



8.3 Did transmission of *K. pneumoniae* occur between mother, neonate, and the clinical environment in Pakistan (PIMS) and Ethiopia (St Paul's Hospital)?

In addition to being responsible for sepsis PP and ES *K. pneumoniae* were also found among the microbiota and in the clinical environment. Thus, studying the wider population of *K. pneumoniae* would help to understand the dynamic of *K. pneumoniae* causing infection and possible sources of *K. pneumoniae* isolates.

During this study the microbiota of the mothers and the neonates was referred to multiple times because the microbiota provides critical protection against exogenous microorganisms and disturbance of its balance increases susceptibility to sepsis, and the composition of the microbiota is profoundly distorted in patients with sepsis. In addition, drugs as antibiotics can also disturb the microbiota seeing that these alter the availability of nutrients that in turn allows pathogenic bacteria to expand (43).

In this PhD it was possible to see that PP and ES *K. pneumoniae* populations were different. In summary, among ES *K. pneumoniae* ST35, ST35 and ST218 formed clusters that were in majority responsible for sepsis and in Pakistan (PIMS) ST15 formed the more prominent cluster which was more diverse. These *K. pneumoniae* were found among microbiota of mothers and neonates as well as in the clinical environment. Evidence of direct transmission of closely related isolates from mothers to neonates was demonstrated in two cases among the ES *K. pneumoniae* studied during this PhD in which one ES *K. pneumoniae* was responsible for sepsis and the other was found among the neonate's microbiota. Also, in a study by Ferretti et al. that focused on mother-to-infant microbial transmission the mothers' gut microbiome was described to be the main donor of the majority of strains acquired by infants and vertically acquired strains would adapt more easily to the infant microbiome (212). Another study analysing mother-to-child transmission at the strain level also reported strain transmission from the mother to the neonate as well as that ARG can be inherited within families (213). Other studies have also indicated that the maternal bacterial colonisation is a risk factor for infant colonisation and infection (214-216). Two of these studies demonstrated the mother to neonate transmission of ESBL carrying *Enterobacteriaceae* was a risk factor for colonisation of very low birth weight infants (214) and for infants (215), while another study associated EOS with maternal bacterial colonisation (216). Considering these studies, the evidence of two ES *K.*

pneumoniae cases of mother to neonate transmission found in this PhD is further supported. Highlighting that not only the genomic surveillance of *K. pneumoniae* causing sepsis is important but the surveillance of other possible sources of isolates capable to cause sepsis is of importance as well.

BB *K. pneumoniae* were responsible for causing sepsis in neonates these were treated with antibiotics not only after sepsis had been confirmed through blood culture, but also, empirically when the neonate presented with signs of sepsis. This is important because antibiotic therapy could have altered the microbiota of neonates by, for example, enriching certain species of bacteria such as *Klebsiella* sp. and in turn also influence the content of ARG within the microbiota. For example, meropenem, cefotaxime and ticarcillin/clavulanate were associated with decreased species richness of preterm infants' microbiota and *K. pneumoniae* was included among the more abundant species harbouring the higher quantity of ARG (45). In addition, it was also noted that infant's microbiota was either enriched or depleted with certain species according to a specific treatment. For instance, the microbiota of infants after treatment with meropenem was enriched with *S. epidermidis* and after treatment with ticarcillin/clavulanate enriched with *K. pneumoniae* in contrast *E. coli* was depleted after treatment with cefotaxime (45). So, it would follow that the microbiota of neonates admitted at PIMS and St Paul's Hospital treated with the chosen antibiotic regimen at each site would be moulded towards certain bacterial species including *K. pneumoniae* that have caused sepsis or have been recovered from BR samples in this PhD and could have the potential to later on cause sepsis. Furthermore, a study in the UK showed that β -lactamase carrying *Klebsiella* spp. frequently enriched the gut microbiota of either healthy or sick preterm infants indicated that both the microbiomes contribute for the resistome which can be problematic when defining treatment regimens for sepsis (44). This information on effects of antibiotic therapy on the microbiota can also be interpreted in light of the sepsis cases caused *K. pneumoniae* in PIMS and St Paul's Hospital indicating again that vertical transmission can contribute for the dissemination of resistant *K. pneumoniae* and ARG.

Thus, having the above in mind several factors can influence mother to neonate transmission of isolates with transmission occurring at least in two cases in Ethiopia. However, it is also important to consider that the antibiotic treated neonates were described to obtain their microbiota in majority from their clinical environment highlighting the importance of the clinical environment in the spread antibiotic

resistance (208). Though ENV *K. pneumoniae* were not linked to specific BB or BR *K. pneumoniae* in this PhD, two PP ST15 *K. pneumoniae* BB and BR pairs of isolates from the same neonate were similar and ENV ST15 *K. pneumoniae* were found among ST15 *K. pneumoniae* as well possibly indicating the influence of the clinical environment in these cases.

8.3.1 Case study: ST37 and ST218 *K. pneumoniae* causing sepsis phylogenetically similar to isolates recovered from the mother's and neonates' microbiota.

The possibility of mother to child transmission of *K. pneumoniae* has been suggested before in a brief report where a KPC-producing *K. pneumoniae* was responsible for cases of infection in the neonatal intensive care unit in Italy (217). In this PhD possible cases of transmission could have occurred in the case of a pair of ST37 ES-BB and ES-MR *K. pneumoniae*. And a pair comprised of an ES-MR and ES-BR ST218 *K. pneumoniae* suggested that transmission could have occurred from the mothers' microbiota to the neonates' microbiota. In line with these cases, the transmission of mother to child of *K. pneumoniae* was also seen in two cases in a study of a mother-child cohort in Madagascar, and the sustained carriage of *K. pneumoniae* was seen in several neonates as well (218). So, these reports support that events of transmission could have occurred among ST37 and ST218 *K. pneumoniae* pairs. Particularly, in the case of ES-BB17/ES-MR30 in which the pairwise SNPs difference was two and the ES-MR sample was taken prior to blood culture of ES-BB17, further indicating vertical transmission of sepsis causing *K. pneumoniae*. Also, not only has nosocomial transmission of ST218 *K. pneumoniae* been described in another medical centre in Ethiopia (164), but also ST35 and ST37 *K. pneumoniae* have been reported in other hospitals in other regions of the globe. Hence, showing that these *K. pneumoniae* STs can be found in other regions of the world while causing neonatal infection and spread ESBL and carbapenemase ARG. For example, *K. pneumoniae* belonging to these STs have been described among an outbreak of ESBL producing *K. pneumoniae* in a NICU (219). Also, ST37 *K. pneumoniae* carrying *bla*_{NDM-1} were described in a hospital in Shanghai during an outbreak among neonates (220). In addition, specifically exploring the relatedness of ES-BB17/ES-MR30 could substantiate the evidence of vertical transmission. In the case of the ST218 ES-MR/ES-BR *K. pneumoniae* it would be possible study the

intraspecies diversity of *K. pneumoniae* from the same sample and HGT of resistance markers within the neonates' microbiota.

8.3.2 Case study: ST15 *K. pneumoniae* causing sepsis and recovered from the neonates' microbiota

Having in mind that antibiotics usage can influence the ARG found in the neonates' microbiota. And that BB ST15 *K. pneumoniae* were found to be phylogenetically similar to the BR *K. pneumoniae* from the same neonate. It could be possible that the isolate causing sepsis to have been present in neonate's microbiota prior to causing infection. Considering the ST15 PP-BB/PP-BR pairs found in this PhD the day of one of the PP-BR isolate sample was not determined so it was not possible to know this for the PP-BB42/PP-BR91 pair. But in the case of the PP-BB31/PP-BR61 and PP-BB6/PP-BR15 pairs the BR sample was taken prior to blood culture. This would suggest that the *K. pneumoniae* isolate causing sepsis could have been harboured by the neonates' microbiota before causing infection. The PP-BB6/PP-BR15 pair was the less similar (84 pairwise SNPs difference) among the other ST15 PP pairs thus in this case the PP-BR was probably not responsible for sepsis. However, the SNP analysis of PP-BB31/PP-BR61 showed that this was the most similar pair when compared to the others differing in one pairwise SNP. Further indicating that *K. pneumoniae* capable of causing sepsis could be present in the microbiota of the neonate before causing infection. Also, the ST15 SNP phylogeny showed that ENV *K. pneumoniae* were phylogenetically similar to BB and BR isolates which suggests that these *K. pneumoniae* could have spread from the clinical environment. Other reports described transmission of *K. pneumoniae* from the clinical environment. A report from a hospital in France described the link between *K. pneumoniae* carrying OXA-48 that caused outbreaks and single cases with stays at the same hospital room. By examining former occupants of that room, a clone was found, and by investigating the room, a clonal strain was isolated from the sink of that room (221). Another study also described the transmission of carbapenem *K. pneumoniae* between patients and the clinical environmental in intensive care units in Beijing. Possible transmission was found between staff and the clinical environment with another possible route of transmission including and ICU cleaner gown and gauze pads that were around an endotracheal tube comprising a clinical environment

reservoir (222). Taking together these reports with what was found during this PhD further reinforces the need for further studies into environmental reservoirs seeing that transmission of *K. pneumoniae* can occur. Additionally, highlighting the importance of surveillance, outbreak management and IPC measures because these could decrease the transmission of such isolates. Also, reports of ST15 *K. pneumoniae* have been reported in other parts of the world as well, for example ST15 was found to be one of the main STs among a polyclonal outbreak of ESBL and carbapenem carrying *K. pneumoniae* across several wards of a Portuguese hospital (223). In a Vietnamese paediatric hospital ST15 *K. pneumoniae* were also found and these showed carbapenem and colistin resistance (224). So, having the above in mind further work to understand the role of the clinical environment is important. For example, further analysing if the PP reference plasmids have disseminated to other strains and time-measured phylogenies to understand the if these ST15 PP-ENV were established in PIMS and were responsible for the spread to the mothers' and neonates' microbiota and later on cause neonatal sepsis.

8.4 How did *K. pneumoniae* from Ethiopia and Pakistan compare to other *K. pneumoniae* in a global context?

The genomic phylogenetic analysis of ES and PP *K. pneumoniae* allowed for a better understanding of both *K. pneumoniae* populations showing that these populations were very variable displaying numerous STs distributed among the different sample types of origin. Nevertheless, ES and PP *K. pneumoniae* populations had different structures because frequently found STs were distinct when studying the populations separately. In addition, the phylogeny for each population better displayed the combination of clinically important ARG and virulence determinants among *K. pneumoniae* from Pakistan and Ethiopia further supporting that these are distinct populations.

Since, *K. pneumoniae* belonging to the major STs found in this PhD were reported in several regions of the globe it would follow to study them in light of a global context (39). In 7.6, ES and PP *K. pneumoniae* were studied in a global context and shown to cluster together according to their most frequent ST and country and not with isolates from other regions of the world because these would belong to STs such as

ST258 that was found among isolates from the United States and Europe. This is in line David et al where a strong association between genetic and geographic distance was described among carbapenemase positive isolates that cluster at the country and hospital level (24). This would also suggest that PP ST15, and ES ST35, ST37 and ST218 commonly found in this PhD study would only be often found in Asia and Africa. However, ST15 and ST37 that were found among PP and ES *K. pneumoniae*, respectively, are high risk clones and have been reported to be widely spread (53). For example, by searching PathogenWatch (<https://pathogen.watch/> accessed on 02/02/2021;12:13) for ST15 *K. pneumoniae* yielded 802 genomes distributed across the globe with 343 of these *K. pneumoniae* displaying KL112, which is important because it shows that *K. pneumoniae* with similar traits to PP isolates are found in clinical settings globally. By searching ST37, 247 genomes were found and were found distributed through Asia, Europe, North America, Africa, and Australia, and 101 ST35 *K. pneumoniae* genomes were found in Europe, North America and Africa. Only eight genomes of *K. pneumoniae* belonging to ST218 displaying KL57 were found two from the United States, three from Europe and three from Asia. These carried the same KL ST218 ES *K. pneumoniae* from this PhD did even though ST218 *K. pneumoniae* sequences from Africa were not available in PathogenWatch, possibly because none had been submitted yet. The use of PathogenWatch allowed to understand that ST15 *K. pneumoniae* and ST37, ST35 and ST218 *K. pneumoniae* were not restricted to Asia or Africa however ST15 *K. pneumoniae* is still more common in Asia. With carbapenem resistant CG15 *K. pneumoniae* diverse distribution illustrated in Fig. 74 from Wyres et al. showing that these isolates were still more frequent in Asia than for example in the Americas or Europe where CG258 is more frequent. However, it shows that CG15 third-generation cephalosporin-resistant, carbapenem-susceptible *K. pneumoniae* were spread in diverse regions of the world (39). Additionally, searching PathogenWatch allowed to note that there was lack of submitted genomes regarding these continents when compared to the number of genomes that were submitted from Europe or the United States, for example. Thus, this PhD was able to generate not only data that help inform antibiotic treatment but also could contribute with knowledge and data regarding the *K. pneumoniae* population in PIMS in Pakistan and St Paul's Hospital in Ethiopia.

Studying the *K. pneumoniae* from Pakistan and Ethiopia in a global context showed that even though *K. pneumoniae* are usually part of a structured population

geographic differences still exist not only in relation to the structure of the population but also in the resistome and virulome harboured by these isolates. Further highlighting, that genomic and antibiotic surveillance can be useful tools to provide knowledge regarding a specific site in order to give accurate information for therapies such as vaccines and antibodies therapies.

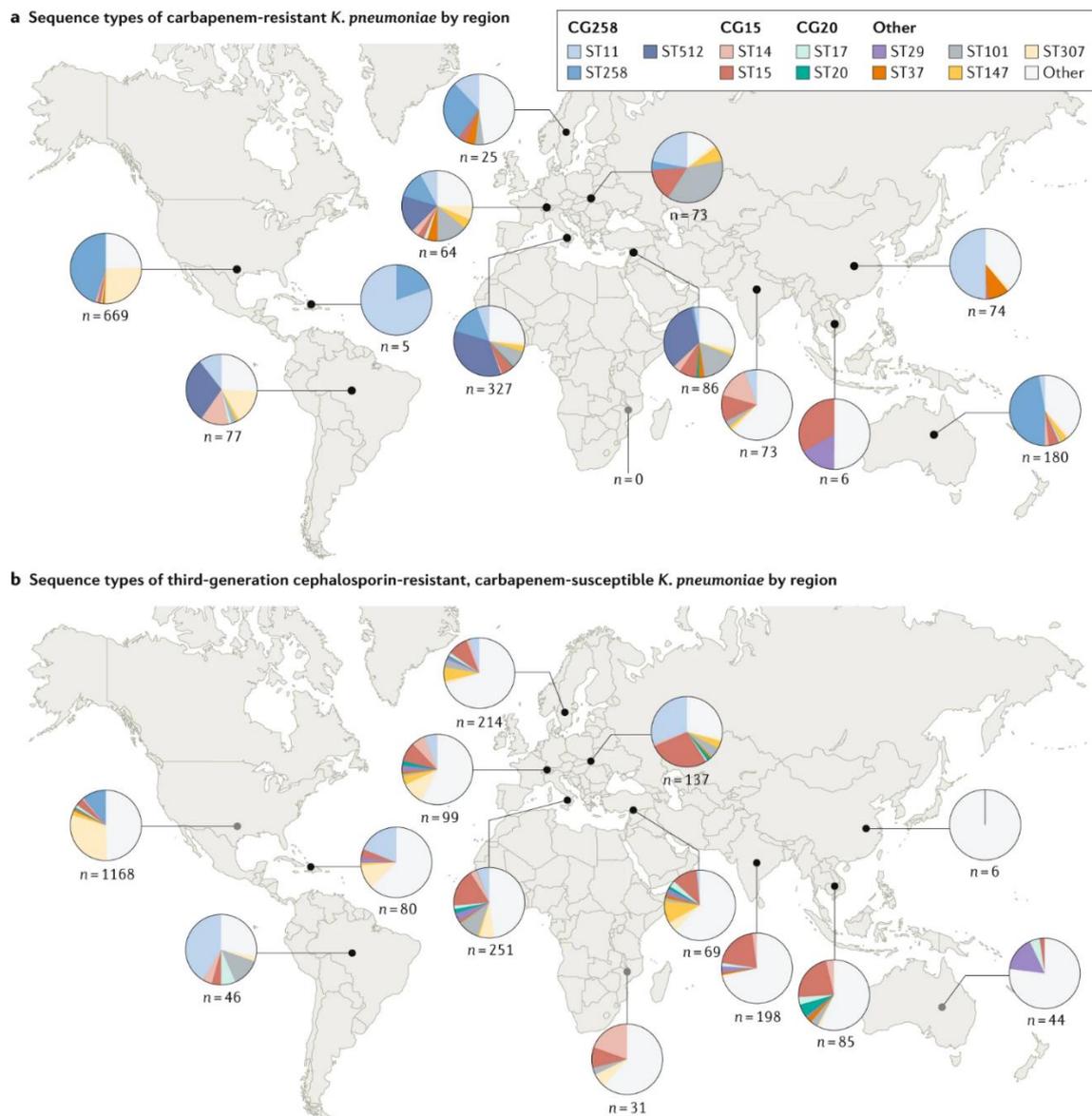


Fig. 72: Geographical distribution of carbapenem resistant *K. pneumoniae* clones and third generation cephalosporin resistant *K. pneumoniae*. The figure shows regional frequencies of carbapenem resistant (a) and third generation cephalosporin resistant, carbapenem-susceptible *K. pneumoniae* (b). The map displays data obtained from several studies summarised in this figure by Wryes et al. (38). The isolates in these studies were not chosen by considering ST, local transmission events or outbreaks. a. CG258 *K. pneumoniae* belonging to ST258 and ST512 are more frequent in the Americas and Southern Europe in comparison being infrequent in other regions of the globe. ST11 is more widely distributed than ST258 or ST512. b. CG15 *K. pneumoniae* were commonly found in distinct regions of the world.

8.5 A *Klebsiella* vaccine and/or antibody for preventing *Klebsiella* neonatal sepsis?

It has been well described that MDR *K. pneumoniae* cause difficult to treat infections but hypervirulent *K. pneumoniae* are also frequently responsible for serious infections such as bloodstream and pyogenic liver infections. Hypervirulence is often associated with specific STs. For example, ST23 is associated with hypervirulent *K. pneumoniae* infection and it also associated with K1 and K2 capsule antigens (180, 186).

During this PhD *in silico* serotype was determined for all *K. pneumoniae*. The population of *K. pneumoniae* displayed high variability in relation to the capsule antigen, typed in as KL, which helps this bacterium establish infection, by, for example, preventing phagocytosis and shielding the LPS from immune cell recognition. Additionally, because the capsule and LPS loci are recombination hotspots in MDR *K. pneumoniae* and because over 138 combinations of genes have been found to compose the *cps* locus it would be expected that the *K. pneumoniae* population in this PhD to display a wide array of different KL (39, 55, 89). However, despite PP *K. pneumoniae* showing a higher number of distinct KL than ES *K. pneumoniae*, the number of PP *K. pneumoniae* in study is higher than the number of ES *K. pneumoniae*. Likewise, the most frequently found OLs showed by PP and ES *K. pneumoniae* were different too. In overall, 68 and 30 different KL were displayed by PP and ES *K. pneumoniae* respectively, KL112, KL15 and KL108 were the most frequently found in overall. KL112 was more frequently found among PP *K. pneumoniae* than among ES isolates. Furthermore, comparing ES-BB and PP-BB, in majority, ES-BB displayed KL108, KL15 and KL57 whereas PP-BB displayed KL112, KL117 and KL34. Thus, showing that the capsular serotypes among *K. pneumoniae* causing neonatal sepsis are different when studying isolates from PIMS in Pakistan and from St Paul's Hospital in Ethiopia. KL112 was mostly found among PP *K. pneumoniae* it was displayed by PP-BR, MR and ENV isolates and mostly found among ST15 *K. pneumoniae*. Of notice is that KL2 associated with hypervirulent *K. pneumoniae* infection (108, 109) was displayed by PP-MR only. The O-antigen responsible for protecting *K. pneumoniae* from the complement and humoral defences was typed as OL. Among PP *K. pneumoniae* the most frequently found OL included O1v1, displayed by most PP *K. pneumoniae* and displayed in majority by PP-BB and PP-BR, MR and ENV. In contrast, O1v2 was the most found among ES *K. pneumoniae* and was the most

frequent OL among ES-BB and ES-ENV whereas ES-MR and ES-BR showed O2v2 and O2v1, respectively.

Moreover, reports of convergence of virulence and ARG have emerged of MDR *K. pneumoniae*, namely carrying *bla*_{NDM} ARG, in Africa and Asia (57). Specifically, in this PhD, a possible occurrence of convergence MDR and hypervirulence is displayed by ES ST218 *K. pneumoniae* that harbour several acquired siderophore loci and hypermucoidy factors associated with hypervirulence (86) in addition to ARG like *bla*_{CTX-M-15}. However, ES ST35 and ST37 *K. pneumoniae*, did not carry siderophore loci, despite being found as one of the main agents of sepsis. Also, if we consider ST15 PP *K. pneumoniae* these carried *ybt* loci as well as *bla*_{NDM-1} and *bla*_{OXA-181} ARG showing that in this PhD convergence of ARG and virulence determinants seems to be occurring. These determinants possibly move between the mothers and neonate's microbiota and between the clinical environment as well because these were found in all sample types of origin particularly among PP ST15 and ES ST218 *K. pneumoniae*. Still, further work would have to be performed to better understand the contribution of hvKp plasmids and *ICEKp* to the spread of *iuc*, *iro* and *rmpA* and *ybt* loci (91, 92), respectively, among neonatal sepsis causing isolates, carriage and environmental *K. pneumoniae*. Also, it is worth noting that by identifying capsule serotypes and O-antigens *in silico* relies on the quality of the sequence, so, if the gene sequences needed for KL and OL typing are not of quality these can be misidentified.

More importantly, convergence of virulence determinants and ARG is of concern seeing that such determinants could aggravate already difficult treat infections caused by *K. pneumoniae*. Furthermore, the most common KL and OL displayed by ES and PP *K. pneumoniae* were in overall significantly associated with the most frequent STs, with siderophore loci being particularly among dominant ST ES isolates contributing for lower percentages of cumulative survival. Thus, having that in mind other therapies as vaccines and antibodies could possibly be helpful to prevent infection caused by MDR *K. pneumoniae* targeting their virulence determinants. Since these have been described as potential targets for glycoconjugate vaccines in a review by Micoli et al. (167) in other bacteria as well as in *K. pneumoniae*. Furthermore, another review by Ahmad et al. (225) has described the development of trials that were underway regarding immunization against *K. pneumoniae* including the active immunisation through a vaccine. This included, for example, killed/attenuated vaccines but these were not safe and partially endotoxic but other safer approaches

using bacterial lysates were shown to be immunogenic. In addition, conjugate vaccines were also seen to be protective against UTI, and septicaemia caused by *K. pneumoniae*. More importantly, the possibility of using polysaccharides as capsule polysaccharides and lipopolysaccharides for vaccine design was included too showing that considerable effort has been put towards developing a vaccine targeting KL and OL (225). For example, a bioconjugate vaccine against K1 and K2 predominant hypervirulent *K. pneumoniae* serotypes was shown to protect mice against lethal infection being immunogenic and efficacious (133). Also, in another study a vaccine targeting ST258 *K. pneumoniae* CPS produced a protective immune response and was indicated that it could prevent, or curb infections caused by ST258 *K. pneumoniae* (226).

Nonetheless, these vaccines focused in K1 and K2 which were not frequent among, ST15 PP *K. pneumoniae* that carried KL112 and ST35, ST37 and ST218 ES *K. pneumoniae* that carried KL108, KL15 and KL57, respectively. Thus, such vaccines would not offer protection against *K. pneumoniae* that were in majority responsible for neonatal sepsis in PIMS or St Paul's Hospital. Even so, knowing the virulence and resistance traits of *K. pneumoniae* through genomic surveillance as done in this PhD could offer more accurate information in order to tailor suitable therapies including, not only, antibiotics regimens, but also, vaccines and/or antibodies. In the case of PIMS a possible vaccine would have to cover KL112, KL64 and KL2, as these were the most commonly found, in the case of St Paul's Hospital, KL108, KL57 and KL15 would have to be covered.

Therapies targeting OL several antibodies targeting LPS specifically have been described to be cross-reactive with common O-antigens and could probably provide protection against clinically important O-antigens serotype *K. pneumoniae* (168). These included O1, O2 and O4 (168) that were also found among ES and PP *K. pneumoniae*, thus, antibodies as those could possibly offer aid for treatment of *K. pneumoniae* infection at PIMS and St Paul's Hospital. Studies have also showed that the antibodies can be seen as therapeutic options as well because they offered protection from infection by carbapenem resistant ST258 *K. pneumoniae* and hypervirulent *K. pneumoniae* (136, 137). In addition, Penini et al. (227) demonstrated that O1 and O2 human monoclonal antibodies could be a therapeutic option because these were shown to offer protection specifically against O1 and O2 serotypes. This protection was synergistic with meropenem which could complement antibiotic therapy

of carbapenem resistant *K. pneumoniae*. Therefore, such antibodies could probably be helpful in the treatment of sepsis caused by *K. pneumoniae* from Pakistan that are carbapenem resistant and in the treatment of ES *K. pneumoniae* sepsis because these were shown to carry O1 and O2 OL too (234).

More importantly, considering the above it would follow that other therapies could possibly help in the treatment of *K. pneumoniae* infection and in turn reduce the burden of antibiotic resistance in *K. pneumoniae*.

8.6. Conclusion

There are still significant knowledge gaps regarding the burden of neonatal sepsis in LMICs resulting from the lack of aetiology and antimicrobial resistance data and epidemiologic data from most LMICs. By characterising the *K. pneumoniae* population in PIMS, Pakistan and St Paul's Hospital in Ethiopia this PhD was able to provide knowledge regarding the burden of antibiotic resistance and the possible routes and spread and transmission of resistant *K. pneumoniae* and spread of ARG (163, 192). Thus, my PhD contributed to further the knowledge regarding antibiotic resistance in LMICs particularly in Pakistan and Ethiopia.

My PhD also highlighted the need for studies that not only focus on clinical isolates responsible for infection but also in assessing the role of other possible reservoirs of resistant isolates. The clinical environment, and both the neonates and mother microbiota influenced the spread of ARG and of resistant *K. pneumoniae*. Nevertheless, it is still difficult to pinpoint the responsible source of a particular sepsis causing *K. pneumoniae*. Although, possible cases of transmission from the mother to neonate were identified in Ethiopia, which would indicate that vertical transmission would be a source of sepsis causing isolates. It was also possible to identify ENV isolates among the major ST clusters. For example, ENV *K. pneumoniae* were found to be similar to *K. pneumoniae* isolates from other sample types of origin including ES-BB ST35 isolates responsible for an outbreak. Additionally, PP-BR *K. pneumoniae* present in the neonate's microbiota could possibly cause sepsis later on suggesting that the clinical environment was also a possible source of sepsis causing isolates. Thus, my PhD showed that either transmission from mother to neonate or spread from the clinical environment could have been the responsible sources of sepsis causing *K. pneumoniae* isolates.

This not only showed that there can be different colonised sources of resistant *K. pneumoniae* isolates but also HGT of resistance markers among the isolate population occurred as well. Considering that similar plasmids carrying *bla*_{CTX-M-15} and *bla*_{NDM-1} and *bla*_{OXA-181} were found among not only *K. pneumoniae* causing sepsis but also in the ENV, MR and BR *K. pneumoniae*. More importantly, clinical data and the data from sections 5.7 and 6.7. It was possible to understand that many factors influence the treatment and outcome of sepsis. Because despite most BB *K. pneumoniae* carrying many ARG and showing resistance to antibiotics used in

treatment the outcome of infection was still favourable and the survival rates were high, despite longer time until outcome. Thus, other factors such as virulence factors or the biology of the neonate will influence the outcome of infection as well as what treatment should or can be administered. In addition, characterising virulence determinants data as done in my PhD could provide possible targets for *K. pneumoniae* vaccines/antibodies that could help diminish the burden of *K. pneumoniae* sepsis in Pakistan and Ethiopia.

To summarise, my PhD highlighted that *K. pneumoniae* caused a heavy burden in sepsis and antibiotic resistance in PIMS (Pakistan) and in St Paul's Hospital (Ethiopia) reinforcing the need for careful use of antibiotics and the adherence to the IPC measures in place. Emphasizing the need for strategies and studies that focus on the contributions of diverse sources considering *K. pneumoniae* was found in the clinical environment, mothers' and neonates' microbiota displaying the ability to easily spread and acquire ARG.

As future work I will aim to build on the work done during PhD particularly aiming to further study the ST15 *K. pneumoniae* cluster found in PIMS as well as the role of the virulence factors among the ST35, ST37 and ST218 clusters found in Ethiopia. To do so to I will aim to determine a possible common ancestor that will indicate from which source and when ST15 isolates were introduced/transmitted among the mothers, neonates from PIMS and/or if these originated from the clinical environment. These analyses will be done together with mapping of the plasmids carrying *bla*_{NDM-1} and *bla*_{OXA-181} ARG aiming to find possible routes of transmission. This analysis will include the plasmids described and mapped in my PhD. However, instead of only using these plasmids backbones long read sequencing of representative genomes from each clade and each sample type of origin (BR; MR and ENV) will be performed. This will also provide a better understating of the variability within the ST15 isolate cluster and improve the study of dissemination of plasmids carrying ARG, such as ESBLs and carbapenemases. A similar approach will be taken to further study the major ST clusters found in Ethiopia to better understand the influence of virulence determinants found in my PhD (because these were more frequent among ES isolates) and to understand if the presence of different plasmids whether carrying ARG or virulence determinants could have a role in the dominance of *K. pneumoniae* belonging to different STs because specific ST were more frequent at different times during the sampling period.

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Appendices

All appendices are included in the Appendices folder accompanying my PhD Thesis.