University of Sassari

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PhD Course in Life Sciences and Biotechnologies

Phenotypic and Molecular Characterization of Extraintestinal Pathogenic *Escherichia coli* and other Gram-negative invasive bacteria in Mozambique

by

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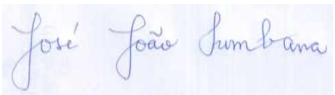
Declaration

I declare that this Ph. D. thesis is my own work and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

October 2019

Jose João Sumbana Signed 

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List of original articles in preparation

- José João Sumbana, Antonella Santona, Maura Fiamma, Elisa Taviani, Massimo Deligios, Tomás Zimba, Gilberto Lucas, Clotilde Nhatave, Alice Manjate, Jahit Sacarlal, Salvatore Rubino, Bianca Paglietti. First occurrence of Carbapenemase resistant Extra-intestinal Pathogenic *Escherichia coli* ST405 isolate co-harboring *bla*NDM-5, *bla*CTXM15 and *bla*TEM1B, in Mozambique.

- José João Sumbana, Antonella Santona, Maura Fiamma, Elisa Taviani, Massimo Deligios, Tomás Zimba, Gilberto Lucas, Clotilde Nhatave, Ventura Relvas Elisa Jaime Azarias Chongo, Jahit Sacarlal, Salvatore Rubino, Bianca Paglietti. Emergence of Multidrug-resistant Extraintestinal Pathogenic *Escherichia coli* ST131 in two Maputo hospitals, Mozambique: The urgent measures to prevent their expansion in whole country.

- José João Sumbana, Antonella Santona, Maura Fiamma, Elisa Taviani, Massimo Deligios, Tomás Zimba, Gilberto Lucas, Clotilde Nhatave, Samuel Elija Simbine, Fabião Edmundo Maússe, Jahit Sacarlal, Salvatore Rubino, Bianca Paglietti. CMY-2 β-lactamase among Extra-intestinal Pathogenic *Escherichia coli* ST410 AmpC and Carbapenemase producers in Hospital Central of Maputo, Mozambique: The Great threat to the Public healthy and Urgent need of Laboratory detection in the Country.

Abbreviations

AAC	Aminoglycoside acetyltransferase
A. baumannii	*Acinetobacter baumannii*
AMEs	Aminoglycosides-modifying enzymes
AmpC	Plasmid mediated β-lactamases
AMR	Antimicrobial resistance
ANT	Aminoglycoside nucleotidyltransferase
AslA	Arylsulfatase-like gene
AVF	Antigen 43-like virulence factor
APH	Aminoglycoside phosphotransferase
APEC	Avian pathogenic *E. coli*
BSI	Bloodstream infection
CAUTI	Catheter-associated urinary tract infections
Cg	Core genome
CGE	Center for Genomic Epidemiology
CF	Cystic fibrosis
CSF	Cerebrospinal fluid
CMY	Cephamycinase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CPE	Carbapenemase Producing Enterobacteriaceae
CPOs	Carbapenemase-producing organisms
CPS	Capsular polysaccharide
CRKP	Carbapenem-resistant *K. pneumoniae*
CTX-M	Cefotaximase - Munich
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. cloacae	*Enterobacter cloacae*
E. complex	*Enterobacter complex*
ESBLs	Extended Spectrum β-lactamases
ExPEC	Extraintestinal Pathogenic *E. coli*
FIM	Florence imipenemase

GBS	Group B *Streptococcus*
GIM	Germany imipenemase
GNBP	Gram-negative bacteria pathogens
GNP	Gram-negative pathogens
HCM	Hospital Central of Maputo
HCQ	Hospital Central of Quelimane
HGM	Hospital General of Mavalane
HJM	Hospital General of José Macamo
HMKP	Hypermucoviscous *Klebsiella pneumoniae*
HlyA	Haemolysin A toxin
HPQ	Hospital Provincial of Quelimane
IBD	Invasive bacterial diseases
ICE	Integrative conjugative elements
ICU	Intensive care unit
IMP	Imipenemase metallo-β-lactamase
Inc	Incompatibility groups
iNTS	non typhoidal *Salmonella*
IPEC	Intestinal Pathogenic *Escherichia coli*
IS	Insertion sequence
IS*Aba1*	Insertion sequence *Acinetobacter baumannii*
IS*Ecp1*	Insertion sequence *Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
K. oxytoca	*Klebsiella oxytoca*
K. variicola	*Klebsiella variicola*
KpVP-1	*Klebsiella pneumoniae* virulence plasmid 1
LPS	Lipopolysaccharide
MBLs	Metallo-β-lactamases
MDR	Multi-drug resistant
MDR-GNB	Multi-drug resistant Gram-negative bacteria
MGE	Mobile genetic elements
MIC	Minimum inhibitor concentration

MISAU	Ministério da Saúde
MLMF_UEM	Microbiology Laboratory of Medicine Faculty of Eduardo Mondlane University
M. morganii	*Morganella morganii*
MLST	Multi-locus sequence typing
MLQ	Microbiology Laboratory of Quelimane
MLVT	Multi-Locus Variable Number of Tandem
NBCI	National Center for Biotechnology Information
NDM	New Delhi metallo-β-lactamase
NMEC	Newborn meningitis *Escherichia coli*
ompA	Outer membrane protein A
OMPs	Outer-membrane proteins
OM	Outer-membrane
OXA-48	Oxacillinase-48
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
P. otitidis	*Pseudomonas otitidis*
P. stutzeri	*Pseudomonas stutzeri*
PLA	Pyogenic liver abscess
PMDR	Plasmid-mediated quinolone resistance
qnr	Plasmid-mediated quinolone resistance
SEPEC	Sepsis associated *Escherichia coli*
SHV	Sulfhydryl reagent variable
SNPs	Single nucleotide polymorphisms
S. Isangi	*Salmonella enterica* serovar Isangi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SLV	Single locus variant
S. maltophilia	*Stenotrophomonas maltophilia*
SPM	São Paulo metallo-β-lactamase

SSA	Sub-Saharan Africa
ST	Sequence type
tnp	Transposase
TRs	Tandem repeats
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
USA	United States of America
usp	Uropathogen-specific protein
UTI	Urinary Tract Infection
VAG	Virulence associated genes
VIM	Verona integron-encoded metallo-β-lactamase
VNTR	Variable number of tandem repeats loci
WGS	Whole genome sequencing
WHO	Word Health Organization
wt	wild type
XDR	Extensively drug-resistant

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Abstract

There is limited information is available regarding the population structure of extraintestinal pathogenic *Escherichia coli* (ExPEC), and other Gram-negative bacteria (GNB) and their susceptibility profile in Africa, although the increasing prevalence of Extended Spectrum β-lactamases (ESBL), Plasmid mediated β-lactamases (AmpC) and Carbapenemase producing bacteria. Characterization of Gram-negative bacteria pathogens (GNBP) is pivotal in selection of empiric antimicrobials, while awaiting bacterial culture results with suspected invasive bacterial and infection preventive measures worldwide. In the majority of low-income countries like Mozambique, despite the increasing of GNB that produces ESBLs, Plasmid mediated β-lactamases (AmpC) and Carbapenemase and their association with high morbidity-mortality, not all antibiotics agents are available to treat these pathogens and most clinical diagnostic laboratories may not attempt to detect these three major groups of enzymes leading to difficulties in the hospital control of resistant microorganisms and antibiotics misuse. The overall purpose of this study was to evaluate the epidemiology and antimicrobial resistance of ExPEC and other GNB associated with invasive infections such as urinary tract infection, intra-abdominal infection, osteomyelitis, soft-tissue infection, pneumonia, sepsis, meningitis and other infections in Mozambique, through phenotypic and molecular approach. From February 2016 to July of 2018, mainly in Hospital Central of Maputo (HCM) and in other four hospitals: Hospital General of Macamo (HGM), Hospital General of José Macamo (HJM) Hospital Provincial of Quelimane (HPQ) and Hospital Central of Quelimane (HCQ) (n=159) samples were collected. Bacterial identification was done using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). Antibiotic Susceptibility Testing (AST) was done phenotypically by VITEK 2 compact system (bioMérieux) and by genotypic approaches through PCR (n=77) and Whole Genome Sequencing (WGS) (n=82) DNA of Gram-negative bacteria extracted using Wizard® Genomic DNA Purification Kit (Promega). Full genome sequences of the isolates were obtained using WGS technology and were analyzed *in silico* for molecular characterization. To our best knowledge, this is the first depth study of ExPEC and other GNB based on WGS in Mozambique. Overall, 159 GNB were isolated, of which 81% were from the main Departments of HCM (Pediatric, Medicine, Surgery and Gynecology and Obstetrics) and 19% from other hospitals (HGM and HJM,

and HPQ and HCQ), in particular from Pediatric Departments. Of the 128 GNB from HCM, 59% were isolated from blood and 38% and 2% from pus and cerebrospinal fluid (CSF), respectively. The most frequent isolates in HCM were *K. pneumoniae* (27%), followed by ExPEC and *Acinetobacter* spp. (20%). Other GNB as *Pseudomonas* spp., *P. mirabilis*, *Enterobacter* spp., *Salmonella* spp., *M. morgani*, *K. oxytoca*, *K. variicola*, and *S. maltophilia* were less observed (ranging from 8% to 1%). The *Acinetobacter* spp. included (*n*=23) *A. baumannii*, and (*n*=1) *A. complex*; *Pseudomonas* spp. included (*n*=17) *P. aeruginosa*, (*n*=1) *P. otitidis* and (*n*=1) *P. stutzeri*; *Enterobacter* spp. included (*n*=4) *E. complex* and (*n*=1) *E. cloacae*; and *Salmonella* spp. included, (*n*=2) *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and (*n*=1) *S.* Isangi. From other hospitals, seven species of GNB from blood samples were isolated, including (*n*=2) *Salmonella* spp., (*n*=8) *S.* Typhimurium, (*n*=1) *Salmonella enterica* serovar Thompson and *Salmonella enterica* serovar Tallahassee, (4) *E. coli* and *K. pneumoniae*, (3) *E. cloacae*, (2) *P. aeruginosa*, (*n*=2) *A. baumannii* and *A. complex*, (*n*=1) *S. complex*, *S. marcescens* and *P. septica* only in Pediatric departments.

Among ExPEC and other GNB, the resistance to the commonly antibiotics, including penicillins, cephalosporins, trimethoprim-sulfamethoxazole, gentamicin and ciprofloxacin used in Mozambique is high and being associated with different mechanisms, including narrow β-lactamases (TEM-1B and OXA-1), ESBL (CTX-M-9, 15, -27, -88), AmpC (CMY-2 and DHA-13) and carbapenemases (NDM-5, OXAs, NDM and VIM). These mechanisms were found associated with others depending on the species, namely: chromosomal mutations fluoroquinolone mutations in *gyrA, parC* and *parE*, *acrR*, *ompK36*, *ompK37* and *ramR*; efflux pumps *oqxA* and *oqxB*, aminoglycosides and fluoroquinolones genes [*aac(6')-Iaa*, aac*(6')Ib-cr*, *aac(6')-Ian*, *aac(6')-IIC, aac(3)-Ia, aac(3)-IIa, aac(3)-IId, aph(3')-Ia*, *aph(3'')-I*, *aph(6)-Id* and *ant(2'')-Ia*, 16S rRNA methylases (*rmtB*)], sulfonamides (*sul1* and *sul2*), phenicols (*catA1, catA2, catA3, catB3*, *catB7*, *cmlA1* and *floR*), macrolides [*mdf(A), mph(A)*, *mph(E)* and *msr(E)* and *ereA*), tetracycline [*tet(A)*/*(B)*, *tet(D)*, *tet(J)* and *tet(39)*], fosfomycin (*fosA*), rifampicin (*ARR-2* and *ARR-3*), plasmid mediated quinolone resistance (*qnrB1, qnrB6* and *qnrS1*), colistin (*mcr-9*) and *dfr* genes, mainly belonging to class 1 and 2 integrons (*dfrA1, drfrA5, dfrA7, dfrA8, dfrA12, dfrA14, dfrA17, dfrA16*, *dfrA19, dfrA27*) encoding resistance to trimethoprim-sulfamethoxazole.

Several GNB including ExPEC*, K. pneumoniae, P. mirabilis, K. oxytoca,* and *E. complex*, harbored the ESBL CTX-M-15 downstream of IS*Ecp1* on IncF and A/C plasmids, that plays an important role in the dissemination of resistance genes among GNB in HCM. The presence of pandemic or/and emerging ExPEC (mainly ST131, ST69, ST410, ST405, ST38), *K. pneumoniae*, *K. oxytoca*, *E. cloacae* ST84, *P. mirabilis*, *A. baumanii* and *P. aeruginosa* carrying CTX-M-15 and IS*Ecp1* linked to IncF, A/C and Col plasmids represent a high risk for the country due to rapidly dissemination and evolution of diverse multiresistant plasmids and also for treatment failure with antibiotics. Additionally, the detection of the hypervirulent and hypermucoviscous *K. pneumoniae* ST23 constitutes a worrisome situation due to its ability to generate invasive community-acquired infections. Therefore, prudent use of antibiotics is advocated, and a systematic national surveillance system of antibiotic resistance is urgently need to overcome the dissemination of ESBL, AmpC, and carbapenemases containing GNB in Mozambique. The presence of *E. coli* ST405 and ST410 carrying carbapenemase (NDM-5) and AmpC (CMY-2), respectively, both located on IncF plasmid highlight the need of strict adherence to infection prevention and control policies by healthcare workers to prevent further dissemination within HCM. Early detection of ESBL, AmpC and carbapenemase genes would be important for the reduction of mortality rate and spread of MDR organisms in studied hospitals. Although the study found evidence of antimicrobial resistance of ExPEC and other GNB, the data collected did not represent the complete picture of the situation in the whole studied hospitals due to small number of samples related to blood and CSF culture contaminations.

CHAPTER ONE

INTRODUCTION

Invasive bacterial diseases (IBD) are conditions during which microorganisms are identified in bodily fluids that are usually sterile due to bacterial penetration through anatomical barriers with the possibility of developing sepsis and focal infections in various tissues and organs (Azzari et al., 2015). The most reason that constitute a worrisome case of these agents is their multi-drug resistance (MDR), where Gram-negative bacteria (GNB) have increasingly been reported with high morbidity and mortality among IBD. Multi-drug resistant Gram-negative bacteria (MDR-GNB) that cause invasive infections, such as urinary tract infection, pneumonia, sepsis, meningitis are increasingly being reported from many regions of the world with high burden in low-income countries including Mozambique. Infection caused by MDR organisms are more likely to prolong the hospital stay, increase the risk of death and require treatment with more expensive antibiotics (Jo, 2009).

MDR-GNB such as *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* spp., *Haemophilus influenza*, *Neisseria meningitidis, Serratia marcescens*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* have been associated with invasive infections and high morbiditymortality rate, especially in children under-five and intensive care unit (ICU) (Onipede et al., 2009; Zingg et al., 2017; Didovic et al., 2018). In Mozambique, a recent study conducted at the Hospital Central of Maputo (HCM) identified 79.3% of GNB *versus* 20.7% of Gram-positive (Mahaluça, 2018a) from ICU patients.

Among invasive infections caused by MDR-GNB in human, *E. coli* and *K. pneumoniae* are the commonest pathogens of urinary tract infection (UTI), and have significantly increased worldwide (Mazzariol et al., 2017). Additionally, *E. coli* are the leading cause of Gram-negative bloodstream infections (de Kraker et al., 2013) and the major causative agent of extraintestinal invasive infections, such as neonatal meningitis, bacteremia, pyelonephritis, cystitis, prostatitis, and sepsis (Kaper et al., 2004), mainly in women, newborns, elderly, and immunocompromised individuals (Mellata, 2013). However, *E. coli* are commensal, promoting normal intestinal homeostasis and preventing colonization by pathogens. *E. coli* can acquire or carry a combination of virulence genes that enable them to cause intestinal and extraintestinal infections in humans and homoeothermic animals (Delmas et al., 2015; Sarowska et al., 2019).

The pathogenic *E. coli* are classified based on clinical and genetic criteria into Intestinal Pathogenic *E. coli* (IPEC) and Extraintestinal Pathogenic *E. coli* (ExPEC) (Kaper et al., 2004; Quinn et al., 2011). Pathogenic *E. coli* are able to transfer their virulence and resistance genes through horizontal mechanisms intra and inter species (e.g., plasmid or lysogenic phage, integrons, transposons) (Kaper et al., 2004).

ExPEC had emerged during the 2000s and are facultative pathogens having a great impact on public health with an economic cost of several billion dollars annually (Ron, 2006; Fakruddin et al., 2013). Based on mechanisms of pathogenicity, clinical and epidemiological manifestations, ExPEC are organized in four groups: Uropathogenic (UPEC), Newborn meningitis and septicemia (NMEC/SEPEC) and Avian pathogenic (APEC) (Sarowska et al., 2019).

ExPEC infections have previously been easily treatable with first line antibiotics (ampicillin and trimethoprim-sulfamethoxazole) (Mellata, 2013). From 1990s, the management of infections caused by ExPEC has been complicated due to the novel emergence of resistance to cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole, antibiotic used to treat community and hospital infections caused by *E. coli* that increase morbidity, mortality and economic cost worldwide (Pitout and Laupland, 2008). The emergence of resistance to these antibiotic is associated with delays in appropriate therapy (Tumbarello et al., 2007) and the increasing recognition of ExPEC isolates producing 'newer β-lactamases' consisting of plasmid-mediated AmpC β-lactamases_AmpC (e.g., CMY), extended-spectrum β-lactamases_ESBL (e.g., CTX-M) and carbapenemases (e.g., NDM, KPC and OXA-48) (Pitout, 2012).

The CTX-M-producing *E. coli* are important causes of community-onset UTIs, bacteremia and intra-abdominal infections, where $bla_{\text{CTX-M-15}}$ is the most widespread and prevalent type (Doumith et al., 2015). ExPEC producing $bla_{\text{CTX-M-15}}$ often belong to the international UPEC sequence type (STs) ST131 by Multi locus sequence type (MLST) technique, and to a lesser extend ST38, ST405, and ST648. These *E. coli* STs and other, such as ST69, ST73, ST95 have been isolated in different patients with extraintestinal infections across the world (Banerjee et al., 2013; Doumith et al., 2015).

Studies suggest that some STs and mobile genetic elements (MGE) plays a role in worldwide dissemination of CTX-M-producing *E. coli*, for instance *E. coli* ST131 (Pitout, 2012). *E. coli* ST131 due to the possession of $bla_{\text{CTX-M-15}}$ and also $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$,

aac(6')Ib-cr, *catB4*, *tetA* are associated with MDR (Smet et al., 2010). The *bla*$_{\text{CTX-M-15}}$ and other resistance genes have been found in incompatibility groups F/F31:A4:B1 and F/F36:A4:B1 conjugative plasmids (Rafaï et al., 2015), but also on other plasmid types (Carattoli, 2013). Additionally, from IncF replicon plasmid virulence genes such as *iutA*, *ompT*, *hlyF*, *iss*, and *iroN* have been found (Mathers et al., 2015). Thereby, the combination of virulence genes and antimicrobial resistance may be responsible for the epidemiological success of this ST (Pitout, 2012).

The *E. coli* ST38 has association with *bla*$_{\text{OXA-48}}$ (Poirel et al., 2011), *bla*$_{\text{NDM-1}}$ (Yamamoto et al., 2011), *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{CTX-M-14}}$, and *bla*$_{\text{CTX-M-15}}$. This clone has been isolated in different countries, such as Japan (Suzuki et al., 2009), Netherlands (van der Bij et al., 2011), Korea (Kim et al., 2011), Tanzania (Mshana et al., 2011).

E. coli ST405 and ST648 are also associated production of CTX-M types and with NDM β-lactamases. The ST405 has a worldwide distribution and ST648 has been found in poultry from Spain, humans in China and the Netherlands (van der Bij et al., 2011).

This study is important to describe the ExPEC and other GNB in Mozambique in order to understand the phenotype and resistance determinants through molecular approach, especially in HCM, which is quaternary and referral hospital for the country. Additionally, the integration of molecular approach to traditional microbiological diagnostic could play a significant role in the detection of MDR pathogens, enhancing laboratory diagnostic efficiency in Mozambique.

BACKGROUND

Escherichia coli

In 1885, the German-Austrian pediatrician Theodor Escherich discovered this organism in the feces of healthy individuals. He called it *Bacterium coli commune* due to it was found in the colon. Then, was named *Bacterium coli* with missing. Following a revision of Bacterium, it was reclassified as a *Bacillus coli* by Migula in 1895 and later newly created genus Escherichia, named after its original discoverer as an honor (Croxen et al., 2013).

Microbiology characteristics of *Escherichia coli*

E. coli is a Gram-negative bacillus, member of family *Enterobacteriaceae* that grows readily on simple culture media with minimal nutrients. This microorganism is typically first identified in the microbiology laboratory as a lactose fermenting GNB rod that can grow both aerobically and anaerobically conditions, preferably at 37ºC, and can be either non motile or motile. It is oxidase negative, produces indole, does not ferment citrate, and demonstrates a positive methyl red test and a negative Voges-Proskauer reaction (Tenaillon et al., 2010; Hufnagel et al., 2015).

Genomic diversity of *Escherichia coli*

The pangenome of *E. coli* represents all genes (4.5Mbp - >5.5Mbp), whether constant or variable, including core genome (common set of genes of the species) which is associated with genetic information for most essential in cellular processes; and a flexible gene pool (accessory and regulatory genome regions) that allow their adaptation in specific environment (Dobrindt, 2005). The variability between *E. coli* strains can occurs due to several of reasons, including point mutations, insertions, deletions, genome rearrangements and transfer of exogenous DNA (and extrachromosomal elements_MGE), such as plasmids, phages, genomic islands, transposons, insertion elements (Dobrindt, 2005).

Point mutations include single nucleotide polymorphisms (SNPs) and single nucleotide insertions or deletions at variable mutation (Bryant et al., 2012). Due to these mutations, *E. coli* can settlement in different habitats as a commensal or pathogenic on human or

animal hosts. For instance, study showed less factors associated with colonization in environmental isolates when compared with human isolates (Oh et al., 2012).

The *E. coli* pangenome represent more than 15000 unique genes where many of which are virulence factors despite of not being completely characterized. This richness of *E. coli* in virulence genes is an evidence of how can be ecologically adapted in stressed environmental perturbations, such as antimicrobial (Rasko et al., 2008; Oh et al., 2012).

According to the genetical features, *E. coli* can be classified as a commensal or pathogenic. The pathogenic group includes, (i) Intestinal Pathogenic *E. coli* (IPEC), namely: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggegrative *E. coli* (EAEC) and Diffusely adherent *E. coli* (DAEC) (Figure 1); and (ii) Extraintestinal Pathogenic *E. coli* (ExPEC), namely: Uropathogenic *E. coli* (UPEC), Neonatal Meningitis *E.* coli (NMEC), Sepsis-associated *E. coli* (SEPEC), and Avian Pathogenic *E. coli* (APEC) (Kaper et al., 2004; Sarowska et al., 2019) (Figure 1 and 2).

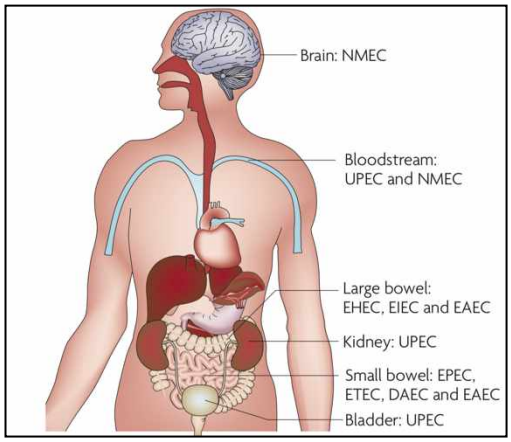


Fig. 1. Sites of pathogenic *E. coli* colonization. EPEC, ETEC and DAEC colonize the small bowel and cause diarrhoea, while EHEC and EIEC colonize the large bowel; EAEC can colonize both the small and large bowels. UPEC use urinary tract as a gateway and travels to the bladder to cause cystitis and, if left untreated, can ascend further into the kidneys to cause pyelonephritis. Septicaemia can occur with both UPEC and neonatal meningitis *E. coli* (NMEC), and NMEC can cross the blood-brain barrier into the central nervous system, causing meningitis. Adapted from (Croxen and Finlay, 2010).

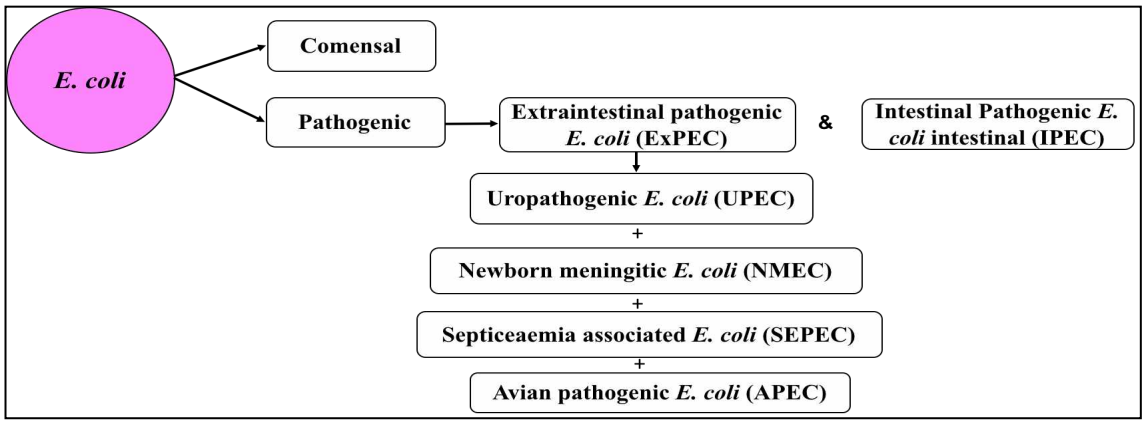


Fig. 2. *E. coli* diversity based on pathogenicity. Adapted from (Sarowska et al., 2019).

E. coli is organized in 8 phylogroups (phylotypes): A, B1, B2, C, D, E, F (*Sensu stricto*) and *Escherichia* cryptic clade I (Clermont et al., 2000). The commensal most often belongs to the group A or B1 and pathogenic are associated with intestinal infection represent phylogroups A, B1 or D. ExPEC frequently belong to the phylogroups B2 and, to a lesser extent to group D. The Group E (has largely been ignored) is related to the group D and group F to group B2 (Sarowska et al., 2019). The *E. coli* phylogroup C are closely related to, but distinct from phylogroup B1 (Clermont et al., 2013).

Extraintestinal Pathogenic *Escherichia coli* (ExPEC)

ExPEC is a facultative pathogen belonging to the normal gut microbiome of human and warm-blooded, namely following the infection site and the presence of virulence factors (Köhler and Dobrindt, 2011; Sarowska et al., 2019). ExPEC has genome plasticity due to the wide range of genes related to bacteria colonization and virulence factors, such as, adhesins, toxins, iron acquisition factors, lipopolysaccharides, polysaccharide capsules, and invasins. The virulence genes are usually found on MGE and can be horizontally transferred between species, genus and kingdom (Ron et al., 2006; Sarowska et al., 2019). According to the molecular features, ExPEC is defined as isolates having at least two of the following virulence factors (VFs) within their genome: *papA* and/or *papC*, *sfa*/*foc*, *afa*/*draBC*, *kpsM II* and *iutA* (Köhler and Dobrindt, 2011) (Table 1). Frequently, ExPEC are more associated with infections originating from abdominal and pelvic sources and less with skin and soft tissue infections, neonatal meningitis and hospital-acquired pneumonia (Sarowska et al., 2019). Related to the ExPEC ability in creating certain

serious diseases, several virulence genes have been linked with different pathovars. Actually, UPEC is mainly linked with *usp* as a major virulence factor (Yamamoto et al., 1995). In addition, type 1 and P fimbriae are common among cystitis and pyelonephritis-associated of UPEC strains (Gao et al., 2017). ST131-O25b is a more common cause of UTI and have been associated with several virulence genes (*fimH, fyuA, iutA, traT, malX*), (*pap, iutA, traT, kpsII*) (Clark et al., 2012; Jeong et al., 2012).

NMEC and SEPEC have shown K1 as etiological factor and associated with other virulences, such as *ibeA, B, C, traT, iss, colV, cvaC, gimB*, *sfa/foc* genes encoded on plasmids to cause meningitis, bacteremia/sepsis (Schmidt and Hensel, 2004; Sarowska et al., 2019).

APEC is like UPEC, harboring diverse armamentarium of virulence factors (*HlyE/hlyF, cvaC, iss, fimbriae type I (fimC)/fimH, iucC, sitA, Tsh, ompT/traT, iroN*) (Li et al., 2005 Ewers et al., 2009; Jeong et al., 2012).

Regarding phylogroup of ExPEC, the group B2 has shown more association with (*usp, cnf1, hlyD, papA* and *ibeA*) than A, B1 and D ExPEC strains (Zhu et al., 2017).

Until the late 1990s, ExPEC was susceptible to ampicillin and trimethoprim-sulfamethoxazole (SXT), however currently, the MDR-resistant ExPEC infections constitute a huge issue due to extension of hospital staying and higher mortality rates. Additionally, ExPEC are important reservoir of resistance to first-line ATBs, including cephalosporins, fluoroquinolones (Gastmeier et al., 2012).

The spreading of CTX-M-15, CMY-1, and NDM-producing *E. coli* is a huge public health and the economy due to they often confer resistance to multiple other antibiotic classes, leaving few or no other treatment options (Pitout, 2012).

Table 1. Extraintestinal Pathogenic *Escherichia coli* virulence factors.

Functionally category	Virulence fator	Reference
Adhesins	Type 1 fimbriae (*fimH*)	Pitout, 2012.
	P fimbriae (*papACEFG*)	Pitout, 2012 and Koga et al., 2014.
	S fimbriae (*sfa/sfaS*)	Koga et al., 2014.
	F1C fimbriae (*foc*)	Riegman et al., 1990.
	N-acetyl D-glucosamine-spefic fimbriae (*gaf*)	Koga et al., 2014.
	Adhesion siderophore (*iha*)	Pitout, 2012.
	Afimbrial adhesion (*afa*/*draBC*)	Pitout, 2012.
	Temperature sensitive hemagglutinatinin (*tsh*)	Pitout, 2012.
	E. coli common pilus (*ecpA*)	Saldaña et al., 2014.
	Heat-resistant haemagglutinin (*hra*)	Pitout, 2012.
	M fimbriae (*bmaE*)	Kudinha et al., 2012.
Invasins	Invasion of brain endothelium (*ibeA*)	Köhler and Dobrindt, 2011.
Iron acquisition systems	Siderophore receptor (*ireA*)	Pitout, 2012.
	Aerobactin receptor (*iutA*)	Pitout, 2012.
	Yersiniabactin (*fyuA*)	Köhler and Dobrindt, 2011.
	Salmochelin receptor (*iroN*)	Pitout, 2012.
	Periplasmic iron binding protein (*sitA*)	Köhler and Dobrindt, 2011.
Toxins	α-hemolysin (*hlyD*)	Pitout, 2012.
	α-hemolysin (*hlyA*)	Croxen and Finlay, 2010.
	Cytolethal distending toxin (*cdtB*)	Williamson et al., 2014.

	Cytotoxic necrotizing factor 1 (*cnf1*)	Wang and Kim, 2013.
Toxins	Secreted autotransporter toxin (*sat*)	Pitout, 2012.
	Serine protease (*pic*)	Pitout, 2012.
	Vacuolating toxin (*vat*)	Pitout, 2012.
Protectins	K1/K2/K5 group 2 capsule variants (*K1/K2, K5*)	Pitout, 2012.
	Conjugal transfer surface exclusion protein (*traT*)	Köhler and Dobrindt, 2011.
	Outer membrane protease T (*ompT*)	Pitout, 2012; Köhler and Dobrindt, 2011.
	Increased serum survival (*iss*)	Pitout, 2012; Köhler and Dobrindt, 2011.
	Colicin V (*cva*)	Köhler and Dobrindt, 2011.
	kpsM II group 2 capsule (*kpsM I*)	Pitout, 2012.
	Group 3 capsule (*kpsMT II*)	Pitout, 2012.
Other	D-Serine deaminase (*dsdA*)	Köhler and Dobrindt, 2011.
	Maltose and glucose-specific PTS transporter subunit IICB (*malX*)	Köhler and Dobrindt, 2011.
	Flagellin variant (*usp*)	Pitout, 2012.
	Glucoronidase (*uidA*)	Köhler and Dobrindt, 2011.
	Colibactin synthesis (*clb* and *clbB*)	Williamson et al., 2014.

Uropathogenic *Escherichia coli* (UPEC)

UPEC is the common uropathogen from intestinal microbiome, can be transmitted through the fecal-oral route or through sexual contact (Terlizzi et al., 2017). This subtype is a primary cause of community-acquired UTIs being responsible for 70-95% of community-onset UTIs and approximately 50% of nosocomial UTIs (Foxman, 2010); and is associated with systemic infections in humans (bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis, infectious arthritis) (George and Manges, 2010).

UPEC has a plasticity of pangenome, possessing different types of virulence genes lipopolysaccharide (LPS), polysaccharide capsule, flagella, outer-membrane vesicles, pili, curli, non-pilus adhesins, outer-membrane proteins (OMPs), as well as secreted toxins, secretion systems, and TonB-dependent iron-uptake receptors, including siderophore receptors (Figure 3). However, the determinant of pathogenicity is the ability to adhere and invade to the host epithelial cells of the urinary tract through P (*pap*) and S (*sfa*) fimbriae (Baldy-Chudzik et al., 2015).

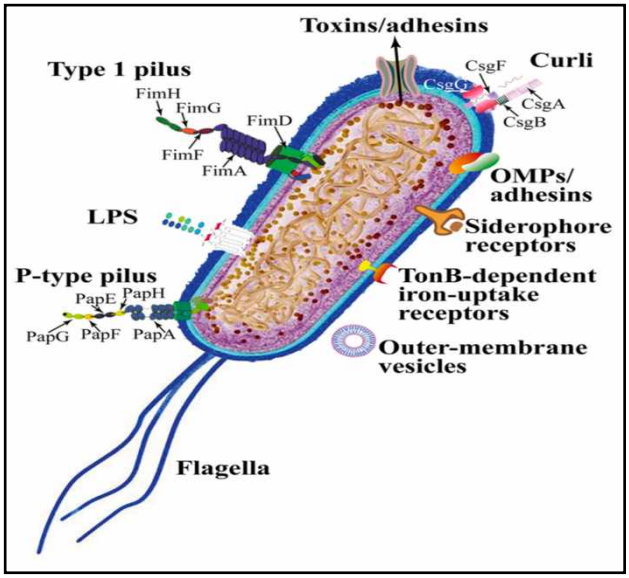


Fig. 3. *E. coli* adhesins and other virulence determinants (Terlizzi et al., 2017).

UPEC first colonize the perineum, overcome the natural host defenses, traverse the urethra, and then infect the bladder, causing cystitis. Additionally, can infect the kidneys creating pyelonephritis, which can result in organ damage, then can gain access to the

bloodstream, causing sepsis and sometimes death (Figure 4) (Al- Hasan et al., 2010; Bien et al., 2012).

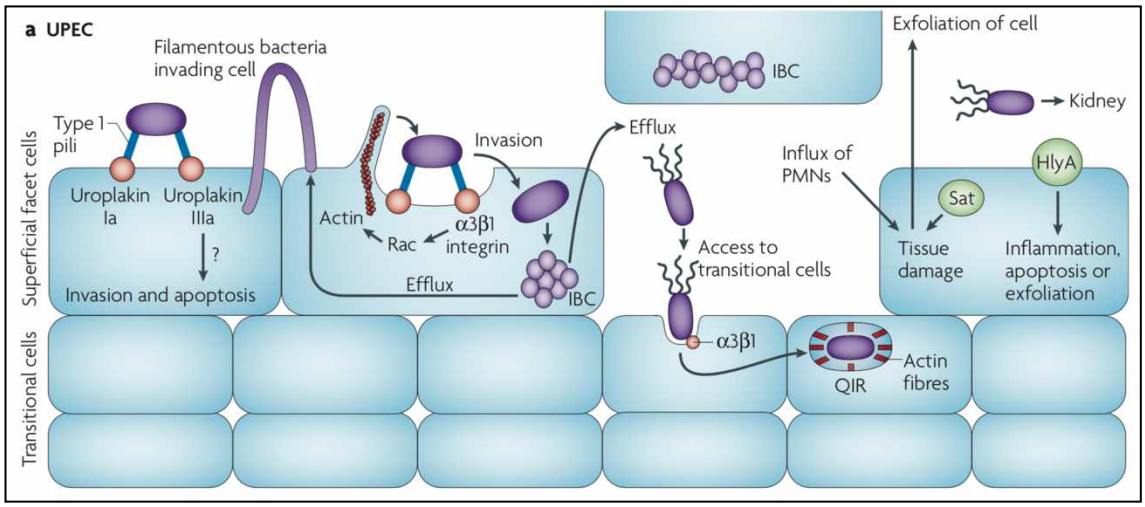


Fig. 4. Pathogenesis mechanisms of UPEC. UPEC attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa; this binding stimulates unknown signaling pathways (indicated by the question mark) that mediate invasion and apoptosis. Binding of type 1 pili to α3β1 integrins also mediates internalization of the bacteria into superficial facet cells to form intracellular bacterial communities (IBCs) or pods. Sublytic concentrations of the pore-forming haemolysin A (HlyA) toxin can inhibit the activation of Akt proteins and leads to host cell apoptosis and exfoliation. Exfoliation of the uroepithelium exposes the underlying transition cells for further UPEC invasion, and the bacteria can reside in these cells as quiescent intracellular reservoirs (QIRs) that may be involved in recurrent infections. Adapted from (Croxen and Finlay, 2010).

UPEC is distributed in several serogroups such as O1, O2, O4, O6, O18, O75 and belong to the phylogroup B2 and D (Mellata, 2013). These serotypes and phylogroups have been associated with resistance of UPEC. UPEC often is resistant to fluoroquinolones and SXT that has been used as a first choice for the treatment of acute uncomplicated cystitis and pyelonephritis. In addition, the resistance to the third-generation cephalosporins (3rdGC) has been observed among ExPEC, mostly from ExPEC producing ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ types), AmpC plasmid mediated resistance (e.g., cephamycinase [CMY] types) and carbapenemase (Verona Integron-encoded Metallo-β-lactamase_VIM, New Delhi metallo-b-lactamase_NDM-types) (Pitout, 2012).

Sepsis associated *Escherichia coli* (SEPEC) and Newborn meningitis *Escherichia coli* (NMEC)

The newborn is particularly susceptible to ExPEC infections (Sepsis associated *E. coli* and Newborn meningitis *E. coli*) due to the premature immune system that are acquired shortly before, during, and after delivery. The pathogenicity of SEPEC/NMEC consists on adhesion, invasion, and fitness. The gateway of SEPEC to the blood is associate with renal cells where grow (bacteremia), following by adhesion and invasion of human brain microvascular endothelial cells, and traversal of the blood–brain barrier to cause inflammation in the central nervous system, resulting in meningitis (Figure 5) (Kim, 2012). They possess the following adhesin-encoding genes (*fimH*, *flu*, *csgA*, *mat*, and *iha*) and other are invasin-encoding genes such as *ibeA*, *tia* and *gimB* (Conceição et al., 2012). SEPEC/NMEC are considered as serum resistant with ability of evading the host complement system through the presence of capsules (mainly K1) and lipopolysaccharide (Siegfried et al., 1994; Conceição et al., 2012). This subtype is distributed in different serogroups, such as O1, O2, O4, O6, O7, O18, O45, O83 and mainly belong to the Phylogenetic group B2 and D (Mellata, 2013).

Due to the initiation of intrapartum antimicrobial prophylaxis for *Streptococcus agalactiae* in the early 2000s the AMR to ampicillin in SEPEC/NMEC was also observed (Schrag et al., 2002). Currently, the SEPEC/NMEC producing CTX-M types is common. For instance, the SEPEC/NMEC caused by a CTX-M-15-producing *E. coli* strain was isolated by Boyer-Mariotte et al., (2008) in a fatal case. Additionally, SEPEC/NMEC possessing a TEM-52 ESBL encoded by an IncI1 replicon and belonged to rare phylogenetic group C was isolated in France (Moissenet et al., 2010). The most common plasmid-mediated β -lactamase (CMY-2) family β-lactamase from the blood and cerebrospinal fluid (CSF) was also isolated from newborn in the United States (Fakioglu et al., 2006).

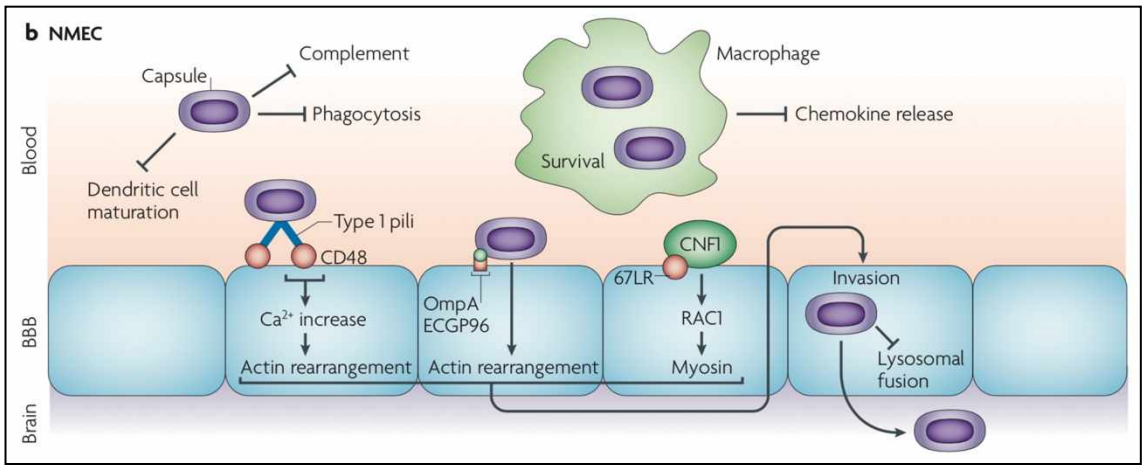


Fig. 5. Pathogenesis mechanisms of SEPEC/NMEC. SEPEC/NMEC using K1 capsule invade the macrophages and can replicated creating a high bacteremia. This growing allows the generation of sufficient bacteria to cross the blood-brain barrier (BBB) into the central nervous system. Attachment of SEPEC/NMEC is mediated by type 1 pili binding to CD48 and OmpA binding to ECGP96. Invasion involves cytotoxic necrotizing factor 1 (CNF1) binding to 67 kDa laminin receptor (67LR; also known as RPSA, as well as type 1 pili and OmpA binding their receptors. PMN, polymorphonuclear leukocyte; Sat, secreted autotransporter toxin. Once in the central nervous system NMEC leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. Adapted from (Croxen and Finlay, 2010).

Avian pathogenic *Escherichia coli* (APEC)

Avian pathogenic *E. coli* (APEC) usually is hosted in intestine, respiratory tract and in the skin feathers of avian causing infection (Ewers et al., 2004). The infection caused by this ExPEC subtype are associated with several virulences genes carried on colicin V plasmids (*iss*, *tsh*, *iucC*, *cvi*, *iutA*, *hylA*, *iroN*, and *ompT*) (Johnson et al., 2008), toxin genes (*astA*, *vat*), iron acquisition system genes (*irp2* and *iucD*), adhesin genes (*papC* and *tsh*), and the ColV genes (*cva-cvi*) (Ewers et al., 2005). Commonly belongs to serogroup O1 (O1:K1), O2 (O2:K1), O18 (O78:K80) and (Rodriguez-Siek et al., 2005; Ron et al., 2006).

Composition of ExPEC populations

Currently, with development of typing methods like Multi-locus Sequence Type (MLST) several STs have been reported worldwide and, unfortunately, certain lineages characterized by virulence genes and MDR are responsible for major outbreaks at different locations simultaneously. The following *E. coli* STs have been reported worldwide: ST10, ST12, ST38, ST69, ST73, ST95, ST127, ST131, ST393, ST405, ST410 and ST648 (Pitout, 2012; Johnson et al., 2013; Roer et al., 2018). Related to AMR, is well known that the initial descriptions of ESBL-producing *E. coli* showed indications of a non-clonal distribution, but nowadays, due to worldwide dissemination of the CTX-M β-lactamases, and, especially CTX-M-15, closely related groups of *E. coli* have been reported.

ST69 *E. coli* has appeared associated with trimethoprim-sulphamethoxazole resistance since 1999; ST393 emerged as a MDR ExPEC in 1986-87 in London, associated with CTX-M-type ESBLs, without losing virulence; ST131 was identified in the mid-2000s and has been associated with ESBL-production and quinolone resistance (Johnson et al., 2013; Riley, 2014); Other very common ExPEC lineages such as ST73 and ST95 were associated with UTI, but rarely carries ESBLs (Rogers et al., 2011; Johnson et al., 2013). ST410 is also an ExPEC associated with resistance to fluoroquinolones, 3rdGC, and carbapenems (Roer et al., 2018). Other sequence types such as ST38, ST405 and ST648 have shown resistance to cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole, as the well successful disseminated ST131 (Pitout, 2012).

Other Gram-negative bacteria involved in invasive infections

Klebsiella pneumoniae

K. pneumoniae is an opportunistic pathogen forming the second most common Gram-negative pathogen after *E. coli* associated with a wide spectrum of infections, such as urinary tract infection, pneumonia, intra-abdominal infection, bloodstream infection (BSI), meningitis. Additionally, has also been identified as a causative agent of other less common, yet serious, infections, such as, liver abscess and invasive syndrome (Hsueh et al., 2002; Siu et al., 2012), septic arthritis or generalized pustulosis (Huang et al., 2013). Meningitis and PLA diseases are more associated with hypervirulent variant of *K. pneumoniae* (hvKP) than classic *K. pneumoniae* strain (cKP) (Bialek-Davenet et al., 2014; Ku et al., 2017). Among these range of disease related to *K. pneumoniae* is noteworthy that has emerged as an important cause of two distinct public health threats: multi-drug resistant (MDR) healthcare-associated infections and drug susceptible community-acquired invasive infections. Invasive community-acquired isolates are associated with capsular serotypes K1 and K2 and appear to differ in clonal background from MDR isolates. Additionally, virulence *K. pneumoniae* has shown less trend to acquire virulence genes than drug-resistance *K. pneumoniae* strains (Wyres et al., 2019). Traditionally, MDR and virulent *K. pneumoniae* are typically observed in distinct populations (Lam et al., 2019) distinguished by the presence of acquired resistance genes and several key virulence loci and this has been largely attributed to the acquisition of large, self-conjugating plasmids. During the last decades, *K. pneumoniae* have steadily accumulated plasmids that carry virulence factors, especially capsule polysaccharides, adhesins, and determinants for iron acquisition, and resistance genes that kept increasing its ability to persist after antimicrobial treatment with cephalosporins, carbapenems, penicillins, aminoglycosides or fluoroquinolones (Boucher et al., 2009; Patel and Bonomo, 2013; Pendleton et al., 2013). However, the clonal groups (CGs) corresponding to these high-risk strains have remained imprecisely defined (Bialek-Davenet et al., 2014).

The *K. pneumoniae* resistance varies considerably among countries, where carbapenemases (KPC) and cefotaximases (CTX-M) enzymes are associated with rapid worldwide spread of *K. pneumoniae* ESBL producers posing a serious threat to global health. For instance, Eastern and South-Western Europe, as well as Mediterranean

countries, are endemic to MDR *K. pneumoniae*, due to ESBL production while from 2015 carbapenem-resistant *K. pneumoniae* (CRKP) has emerged in several countries, such as Romania, Italy and Greece (Navon-Venezia et al., 2017).

Phylogenetic analysis identified some *K. pneumoniae* CG, such as CG23 (ST23, ST26, ST57, and ST163), CG258 (ST258, ST11, ST512, ST394), CG15 (ST15, ST14), CG147 (ST147), CG37 (ST37), CG101 (ST101) and CG17 (ST17) (Bialek-Davenet et al., 2014; Yan et al., 2015). Among these STs, the major epidemic clones are ST11, ST15, ST147 and ST258. In addition, these STs are high-risk extremely drug resistant (XDR) *K. pneumoniae* clones causing worldwide outbreaks in humans, especially due to the possession of bla_{KPC} and $bla_{\text{CTX-M}}$ ESBL, where some STs also carry virulence determinants genes (Bialek-Davenet et al., 2014).

K. pneumoniae ST11, ST15 and ST147, often possess two or three mutations causing substitutions in GyrA and ParC together with carriage of the PMQR gene *aac(6′)Ib-cr* (Tóth et al., 2014) and *oqxAB* and porin loss were described for *K. pneumoniae* ST258 (Mathers et al., 2015). ST258 and ST512 mainly are linked with bla_{KPC}, and ST15 is mostly a CTX-M-15 producer, but also encodes all types of carbapenemases genes: bla_{KPC}, $bla_{\text{OXA-48-like}}$, bla_{NDM}, bla_{VIM} and bla_{IMP} (Navon-Venezia et al., 2017).

CG23 are hypervirulent associated with the highly serum-resistant K1 capsule and carry genotoxin colibactin, microcin E492, and the iron-scavenging siderophores aerobactin, yersiniabactin and salmochelin. These virulence factors are linked with conjugative element ICEKp encoding the siderophore yersiniabactin and genotoxin colibactin (Bialek-Davenet et al., 2014; Lam et al., 2018). Actually, STs from this CG like ST23 has been associated with severe liver abscess infections due to these repertoire of virulence factors. Although traditionally, ST23 is not related to MDR, CTX-M-15-producing has already seen in this ST in South Korea (Leski et al., 2012).

Recently, plasmid harboring resistance and virulence determinants were found. For instance, *K. pneumoniae* virulence plasmid 1_KpVP-1 including aerobactin synthesis locus *iuc* fused with sequences typical of IncFIIK conjugative AMR plasmids encoding the ESBL gene $bla_{\text{CTX-M-15}}$ and seven other AMR genes (bla_{TEM}, *aac3'-IIa*, *dfrA1*, *satA2*, bla_{SHV}, *sul1* and *aadA1*) and other carrying bla_{TEM} and *aac3'-IIa*, and $bla_{\text{CTX-M-15}}$ have been isolated. The presence of KpVP-1 with AMR plasmids enables simultaneous transfer in a single event of hv-MDR *K. pneumoniae* clones (Lam et al., 2019). In

addition, cases of *K. pneumoniae* harboring *bla*$_{\text{CTX-M-15}}$ and PMQR determinants provides like *aac(6')Ib-cr* have been reported from different world regions (Bado et al., 2016).
ESBL and virulence determinants in *K. pneumoniae* are often located on IncFII plasmid as occur in *E. coli* contributing to their evolutionary success (Mathers et al., 2015). Aminoglycoside, fluoroquinolone and *bla*$_{\text{NDM}}$ resistance have been found, especially on IncA/C plasmids that contribute to their selection and spread among *K. pneumoniae*; and *bla*$_{\text{OXA-48}}$ have been identified on IncL plasmids (Lee et al., 2016; Navon-Venezia et al., 2017).

Enterobacter spp.

Enterobacter genus include several species, such as *Enterobacter cloacae* (*E. cloacae*), *Enterobacter asburiae*, *Enterobacter aerogenus*, *Enterobacter dissolvens*, *Enterobacter kobei*, and *Enterobacter nimipressuralis* showing the genomic diversty (O'Hara et al., 1989; Hoffmannand and Roggenkamp, 2003). Among these species, *E. cloacae* and *E. aerogenes* are the main pathogens that are responsible for nosocomial infection (Kang et al., 2004; Davin-Regli and Pages, 2015; Malekzadegan et al., 2017).
E. cloacae are commensal flora of human and animal gastrointestinal tracts (Davin-Regli and Pagès, 2015) and have become increasingly important as a nosocomial pathogen. *E. cloacae* can be responsible of bacteremia, pneumonia, urinary tract and wound infections and infections of the central nervous system, particularly affecting patients in the neonatal ICUs (Sanders and Sanders, 1997), accounting for up to 5% of hospital-acquired septicemias, 5% of nosocomial pneumonias, 4% of nosocomial urinary tract infections, and 10% of postsurgical peritonitis cases (Sanders and Sanders, 1997; Hoffmannand and Roggenkamp, 2003).
E. cloacae can create infections because have many virulence factors such as the ability to secrete cytotoxin, enterotoxin, haemolysin and biofilm formation (Mortazavi et al., 2018).
Due to intrinsic β-lactam resistance conferred by chromosomal AmpC genes acquired ESBLs and carbapenemase enzymes associated with other mechanisms of resistance, the treatment of *E. cloacae* infections can be problematic, where increasing levels of resistance to different antimicrobial agents, especially to quinolones, aminoglycosides and β-lactams have been found. Therefore, WHO include *Enterobacter* spp., in the first

priority tier, named “Priority 1: Critical”, which shows the significance burden of infections and antibiotic-resistant (Dimitrova et al., 2015), especially due to the emergence of resistance to the last-resort carbapenems meropenem, imipenem, and ertapenem (Annavajhala et al., 2019).

As occur in *E. coli* and *Klebsiella* spp., CTX-M and SHV-type β-lactamases have been the predominant ESBLs in *Enterobacter* spp. (Kim and Lim, 2005; Cheong et al., 2012). ESBL *E. cloacae* producers have been associated with successful intrahospital dissemination of CTX-M-15 which constitute a major mechanism of resistance to 3rdCG (Dimitrova et al., 2015).

From *E. cloacae*, *bla*$_{\text{IMP-8}}$ (QnrB2, SHV-12), *bla*$_{\text{VIM-1}}$, ArmA, *bla*$_{\text{CTX-M-9}}$ and *bla*$_{\text{CTX-M-15}}$ have been described and associated with plasmids belonging to the HI2 and Y incompatibility groups (Carattoli, 2009; Robin et al., 2017). In addition, *bla*$_{\text{VEB-1}}$ (QnrA1) in A/C; *bla*$_{\text{IMP-4}}$ (QnrB2, QnrB-4, ArmA) in L/M and A/C, QnrA1 [*bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-9}}$, AAC(6')- IB-CR], QnrS1 [LAP-2, AAC(6')-IB-CR], *bla*$_{\text{SHV-5}}$, *bla*$_{\text{CTX-M-3}}$ (ArmA) in L/M; *bla*$_{\text{VIM-4}}$ (CMY-4) in A/C; QnrB4 [ArmA, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{DHA-1}}$, *bla*$_{\text{SHV-12}}$, AAC(6')-IB-CR] in FIIAs and FIA; and *bla*$_{\text{GES-5}}$ in Q incompatibility groups, have been described worldwide, mainly in human (Carattoli, 2009).

Various STs, ST66, ST78, ST114 and ST108 of *E. cloacae* are the most widespread and epidemic potential associated with resistance spread worldwide, where CTX-M-15 production is linked with ST66, ST78 and ST114; and several STs produce carbapenemase VIM-1 or KPC-2 (Dimitrova et al., 2015).

E. cloacae is the third most common species of enterobacteria carrying the class D β-lactamase OXA-48, after *K. pneumoniae* and *E. coli* (Potron et al., 2013), and OXA-48 has been found on IncF and IncL plasmids of *E. cloacae* (Marti et al., 2017).

Proteus mirabilis

Enteric bacterium *Proteus mirabilis* (*P. mirabilis*) is a Gram-negative rod-shaped bacterium most noted for its swarming motility and urease activity associated with healthcare infections, particularly of wounds, the abdominal cavity and the catheter-associated urinary tract infections (CAUTI) (Armbruster et al., 2018). In addition, CAUTI is the most common source of bacteremia in nursing homes, involving *P. mirabilis*

leading a high mortality rate (Watanakunakorn and Perni, 1994; Kim et al., 2003; Daniels et al., 2014).

P. mirabilis to create these diseases have an impressive arsenal of virulence factors, where the main critical feature of this species is urease. But the bacterium also expresses a startling number of adhesins and other virulence determinants (Figure 6).

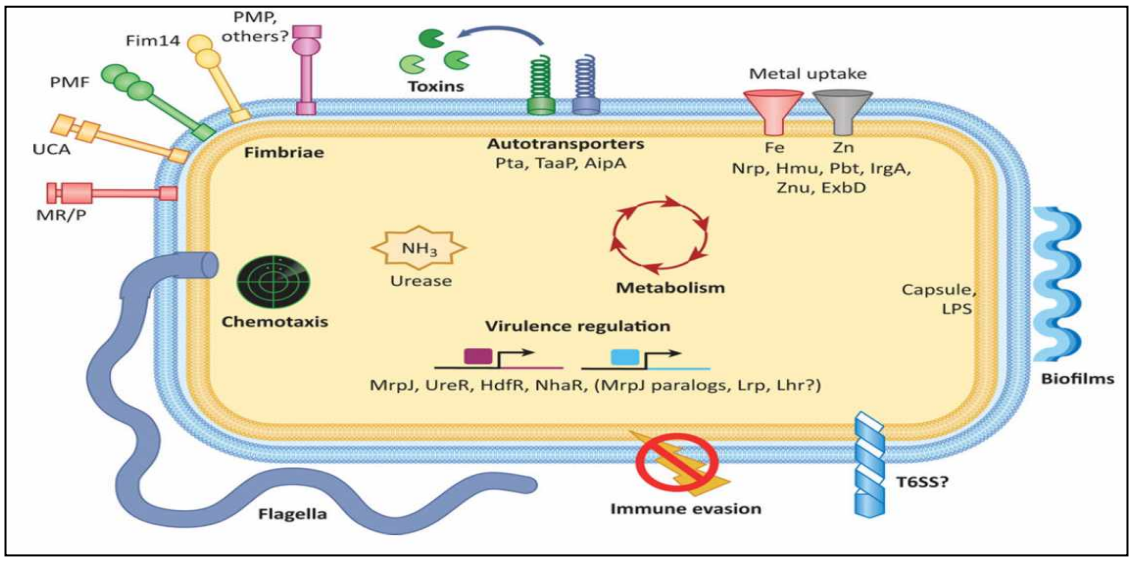


Fig. 6. *Proteus mirabilis* pathogenesis during urinary tract infection (UTI). The mechanism of *P. mirabilis* pathogenicity include consists in (i) adherence (chaperone-usher fimbriae and autotransporter adhesins) by binding catheters, host tissues, and neighboring bacteria may all contribute to disease; (ii) urease: involved in stones, crystalline biofilms, and possibly nutrition or host sensing; (iii) motility: *P. mirabilis* swarms across catheters and may ascend to the kidneys using swimming motility using flagella; (iv) chemotaxis proteins allow the bacteria to follow chemical gradients; (v) metabolism: likely permits establishment of a nutritional niche, competition with other species, and response to host cues; (vi) metal scavenging: iron and zinc uptake are essential for growth, but are sequestered by the host; therefore, specialized proteins are required for bacteria to scavenge these metals; (vii) toxins: proteins such as HpmA and Pta may aid in nutrient accessibility, immune evasion, or provision of surfaces to colonize; (viii) biofilm formation: crystalline biofilms readily form on catheters, and bacterial clusters in the bladder may be a biofilm-mediated process; (ix) immune evasion: this can include antibody and antimicrobial peptide degradation, polymyxin resistance, lipopolysaccharide (LPS) variation, and physical obstruction of phagocytosis; (x) virulence regulation: required to coordinate all steps of infection; (xi) type 6 secretion system (T6SS): involved in self-recognition; unknown role during UTI. Adapted from (Norsworthy and Pearson, 2016).

Like other members of *Enterobacteriaceae* family, *P. mirabilis* can harbor numerous plasmids and integrons as determinants of AMR due to simultaneous production of enzymes, such as ESBLs, AmpC, and rarely carbapenemases (Pagani et al., 2002;

Tibbetts et al., 2008; Tenover et al., 2009), making infections being difficult to treat. However, increasing resistance to carbapenem in *P. mirabilis* has been reported worldwide, including India (Gupta et al., 2006).

The most predominant ESBL are CTX-M enzymes, with other β-lactamases, such as TEM, VEB, and PER types, being identified at lower frequencies (Aragon et al., 2008). The following subtypes of CTX-M enzymes: CTX-M-1 group (CTX-M-3, -12, -15, -32, and -55/57), the CTX-M-2 group (CTX-M-2), the CTX-M-9 group (CTX-M-9, -14, and -90), and the CTX-M-25 group (CTX-M-25 and -41) have been reported in *P. mirabilis* (Navon-Venezia et al., 2008; Aragon et al., 2008; Song et al., 2011; Huang et al., 2015). Recently, *P. mirabillis* with coproduction of plasmid-mediated $bla_{\text{IMP-1}}$, $bla_{\text{KPC-2}}$, and *rmtB*-1 genes have been found that is very worrisome, due to serious challenge to clinicians and infection control team (Ramos et al., 2018).

The IncL/M plasmids have been found possessing the following determinants: *aac(6')Ib-cr*, $bla_{\text{CTX-M-1-3-15-42}}$, $bla_{\text{TEM-3-10}}$, $bla_{\text{SHV-5}}$, $bla_{\text{IMP-4-8}}$, *armA*, *qnrA1*, *qnrB1*, *qnrB2*, *qnrB4*, *qnrS1* in *P. mirabillis* and also in other *Enterobacteriaceae* isolated, such as *E. cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *M. morganii*, *S. enterica*, *S. flexneri* and *S. marcescens* worldwide from human and animal sources (Carattoli, 2009).

Invasive non Typhoidal *Salmonella* (iNTS)

Salmonella enterica include several subspecies that are clinically important, including typhoidal *Salmonella* and non-typhoidal *Salmonella* (Achtman et al., 2012). Invasive non-typhoidal *Salmonella* (iNTS) serovars are a major cause of bloodstream infections in specific geographical regions, posing a particular threat to those individuals affected by HIV or malaria. If left untreated iNTS infection can be often fatal, and it is estimated responsible of 93 million enteric infections and 155,000 associated deaths annually (Crump and Heyderman, 2015; Uche et al., 2017). Among the iNTS *Salmonella* serovars Enteritidis, Dublin and Typhimurium are most commonly isolated. The association of these serovars is mainly due to selective pressure from sustained antibiotic exposure associated with opportunistically invasive in the presence of *Plasmodium falciparum* malaria and HIV (Balasubramanian et al., 2019).

Salmonella Typhimurium (*S.* Typhimurium) sequence type ST313 is prevalent in Africa and has shown a concern handling more invasive disease in humans compared to other

sequence types. Additionally, is more likely to be faecal-orally transmitted and has acquired antimicrobial resistance genes becoming MDR (Almeida et al., 2017). In other countries like Europe and North America *S.* Typhimurium ST19 is also an emergent clone responsible for invasive human disease (Balasubramanian et al., 2019).

The pathogenicity of *S.* Typhimurium ST313, is essentially related to gene *pgtE* (up-regulated its expression), which could potentially promote bacterial survival during human infection (Balasubramanian et al., 2019).

Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) is ubiquitous Gram-negative bacterium belonging to the family *Pseudomonadaceae* and is a major opportunistic human pathogen, responsible for nosocomial infections (outbreaks) leading cause of morbidity and mortality in impaired immune individuals and cystic fibrosis patients that increase medical and veterinary importance (Lister et al., 1999; Silby et al., 2011). Additionally, *P. aeruginosa* is responsible for over 5% of infectious exacerbations in patients with chronic obstructive pulmonary disease (COPD) and has been associated with increased mortality of these patients (Murphy, 2009).

P. aeruginosa is resistant to antibiotics because of the following features: (i) harbor high intrinsic resistance to antibiotics due to low permeability of its cell wall, (ii) has an extraordinary capacity to acquire new resistance mechanisms, (iii) has the genetic capacity to express a wide repertoire of resistance mechanisms and (iv) has able to make mutation in chromosomal genes which regulate resistance genes (Figure 7). All these features increase the prevalence of MDR (Estepa et al., 2014), especially in cystic fibrosis disease (CF). The MDR *P. aeruginosa* represent a global health problem, because of the limitation in clinical treatment options (Zowalaty et al., 2015).

For treatment of MDR *P. aeruginosa*, carbapenems remain effective antimicrobials although the development of high carbapenem resistance rates in *P. aeruginosa* isolates has been reported worldwide with substantial increase of MBLs among carbapenem-resistant from 1990s (Hammami et al., 2011; Yoo et al., 2012).

The acquisition of resistance genes in *P. aeruginosa*, include aminoglycoside and β-lactam resistance. For instance, six types of *P. aeruginosa* MBLs, have been described, including imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), Sao Paulo metallo-β-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo-β-lactamase (NDM) and Florence imipenemase (FIM) (Hong et al., 2015) that are the most prevalent and widespread in *P. aeruginosa* (van der Bij et al., 2012; Khosravi et al., 2012). Associated with β-lactam resistance, two novel aminoglycoside resistance genes, *aac*A29a and *aac*A29b, which are located with VIM-2 gene cassette, in class I integrons of *P. aeruginosa* clinical isolates were described (Poirel et al., 2001). In addition, in a few *P. aeruginosa* isolates, the presence of ESBLs typical to the

Enterobacteriaceae family, such as TEM, SHV (Bae et al., 2014; Potron et al., 2015) and CTX-M-type (Potron et al., 2015), have been identified.

The resistance of the most *P. aeruginosa* to carbapenem agents is due to the loss of the outer membrane protein OprD, especially to imipenem, which requires this porin to cross the outer membrane and over-expression of efflux systems.

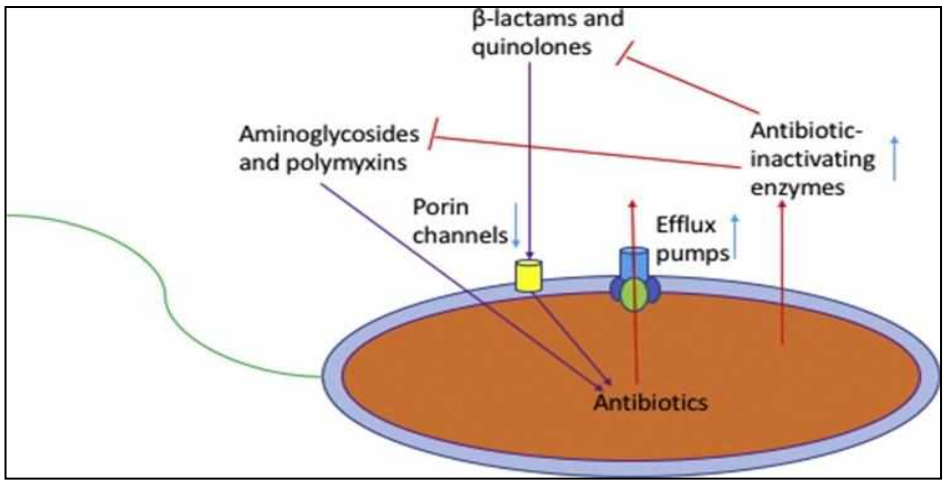


Fig. 7. Intrinsic antibiotic resistance in *P. aeruginosa*. The mechanisms of intrinsic antibiotic resistance possessed by *P. aeruginosa* include restricted outer-membrane permeability, efflux systems that pump antibiotics out of the cells and production of antibiotic-inactivating enzymes. Quinolones (Ciprofloxacin) and β-lactams (e.g. Piperacillin, Ceftazidime, Imipenem, Meropenem and Aztreonam) penetrate the outer membrane through porin aqueous channels. Aminoglycosides (Gentamicin, Tobramycin, Amikacin) and polymyxins (Colomycin and Colistin) promote their own uptake by interacting with *P. aeruginosa* LPS on the outer membrane destroying the permeability barrier of the outer membrane and allows the antibiotics to penetrate through the wall to the cytoplasmic membrane. Adapted from (Pang et al., 2019).

The pathogenesis of *P. aeruginosa* is considered to be multi-factorial consisting in wide array of virulence factors, such as, flagella, pili, alginate, and extracellular proteases (Kipnis et al., 2006; Driscoll et al., 2007). The toxic effects are largely mediated by secreted virulence factors including pyocyanin, elastase (LasB) and alkaline protease (AprA) (Bleves et al., 2010; Rada and Leto, 2013) using type III secretion system to circumvent the host immune system and establish infection; and four exotoxins (ExoS, ExoT, ExoU, ExoY), where ExoU, is the most virulent, potent phospho-lipase that disrupts the plasma membrane and leads to rapid cell death (Sato et al., 2003).

The *P. aeruginosa* epidemic population structure include several STs (ST111, ST175, ST235, ST244 and ST395) distributed worldwide and frequently associated with outbreaks (Ruiz-Roldán et al., 2018). Among these STs, ST235 is the most prevalent

called ‘international’, ‘high-risk’, or ‘widespread’ clone associated with poor clinical outcomes in part due to multi-level and high-level antibiotic resistance (Treepong et al., 2018) and is involved in the dissemination of genes encoding IMP-6 and VIM-2 (Seok et al., 2011).

Acinetobacter baumannii

Acinetobacter baumannii (*A. baumannii*) is associated with serious illness (notorious nosocomial infections), such as bacteremia, pulmonary infections, meningitis, endocarditis, UTI, burn and surgical wound infections primarily in ICU or in immunocompromised patients (Doughari et al., 2011). Among these diseases, pneumonia has been the main manifestation of nosocomial infections caused by *A. baumannii*, resulting in a significant impact on the mortality rate of patients (Villegas and Hartstein, 2003).

A. baumannii, is associated with MDR and XDR due to intrinsic and acquired antimicrobial resistance, often limiting effective therapeutic options and forcing clinicians to use last resort antibiotics such as colistin (Chopra et al., 2013; Chang et al., 2015) although his efficacy and safety is yet to be defined.

A. baumannii is considered as a low virulence pathogen consisting in outer membrane porins (mainly in OmpA), phospholipases, proteases, LPS, capsular polysaccharides (O-pentasaccharide, *pglC* or *pglL*) (Lees-Miller et al., 2013) and extracellular polysaccharide (Phospholipase D, *pld*), protein secretion systems, and iron-chelating systems (Choi al., 2008; Russo et al., 2010; Stahl et al., 2015) (Figure 8). Its capacity to withstand desiccation, starvation and ability to form biofilms by aromatic compounds, *paaE* are other potential virulence factors that contribute to persist in the hospital environment and also the spreading in this environment (Jawad et al., 1998).

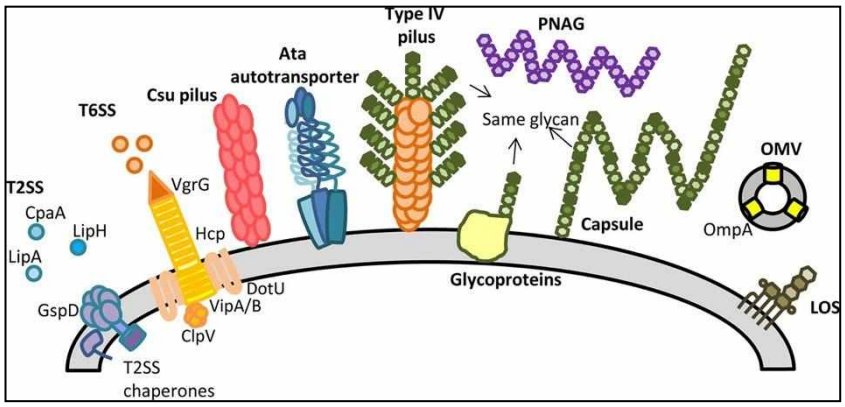


Fig. 8. Pathogenicity of *Acinetobacter* spp. Cell surface components and secretion systems. Adapted from (Weber et al., 2016).

The resistance mechanisms to different classes of antibiotics in *A. baumannii*, include enzymatic degradation of drugs, target modifications, multi-drug efflux pumps, and permeability defects (Figure 9) (Asif et al., 2018). Among these mechanisms, inactivation of β-lactams by β-lactamases is a major antibiotic resistance mechanism in *A. baumannii* and all four classes (A, B, C, and D) of β-lactamases have been identified in *A. baumannii* (Jeon et al., 2015). For instance, TEM-1, CARB-4, and SCO-1 (narrow-spectrum β-lactamases), PER-1, TEM-92, CARB-10, SHV-5, PER-2, CTX-M-2, CTX-M-15, VEB-1, GES-14, and PER-7) are ESBLs; and some carbapenemases, such as GES-14, OXA-like, KPC-2, IMP, VIM, NDM and SIM have been detected in *A. baumannii* (Moubareck et al., 2009; Bogaerts et al., 2010). Carbapenem resistance in *Acinetobacter* spp. is usually mediated by class D β-lactamase, mainly codified by the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-143-like}}$ genes (Zarrillii et al., 2009). In addition, resistance to other classes of antibiotics (aminoglycosides and sulfamethoxazole), tigecycline and colistin have been described (Tavares et al., 2019).

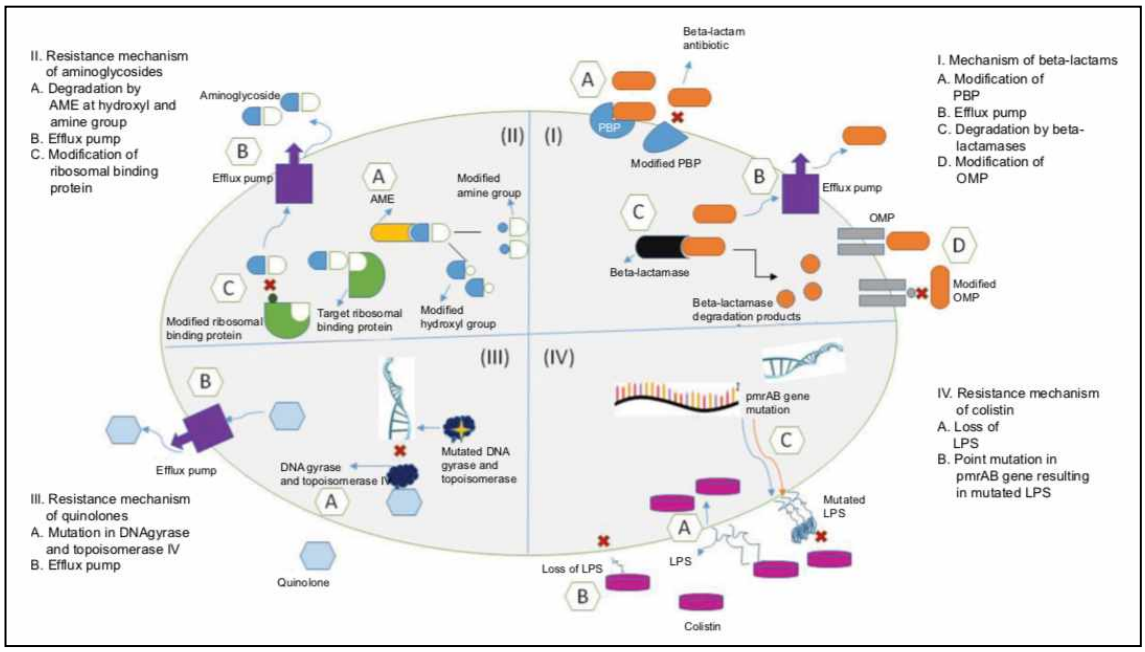


Fig. 9. Mechanisms of resistance in *A. baumannii*: (i) beta-lactams; (ii) aminoglycosides; (iii) quinolones; (iv) colistin. **Abbreviations:** AME, aminoglycoside modifying enzyme; LPS, lipopolysaccharide; OMP, outer membrane porin; PBP, penicillin-binding protein. Adapted from (Asif et al., 2018).

A. baumannii epidemic strains mainly belong to three international clonal complex (Clonal Complex CC1: ST1, ST7, ST8, ST19 and ST20) found worldwide, while international clones 2 (CC2: ST2, ST45 and ST47) and 3 (CC3: ST3 and ST14) are prevalent in Europe and in the United States (Zarrilli et al., 2009; Diancourt et al., 2010; Karah et al., 2012; Rafei et al., 2014). Clone I have been associated with production of the carbapenemase OXA-58 and OXA-23 with international clone II (Clonal complex 92) (Jamal et al., 2018). Additionally, *bla*$_{\text{OXA–23–like}}$, have been associated with IS*Aba1* (Tavares et al., 2019).

DISSEMINATION OF ANTIMICROBIAL RESISTANCE IN GRAM-NEGATIVE BACTERIA

The spreading AMR strains responsible for community or hospital-acquired infections is associated with inter- and intra-specific DNA exchange through plasmids, transposons, insertion, being the horizontal transfer the common mechanism (Figure 10) (Rankin et al., 2011). The genetic exchange occurs through three mechanisms, namely, conjugation/mobilization (mediated by plasmids and integrative conjugative elements,

ICE), transduction (mediated by bacteriophages), and transformation (uptake of extracellular DNA) (Partridge et al., 2018).

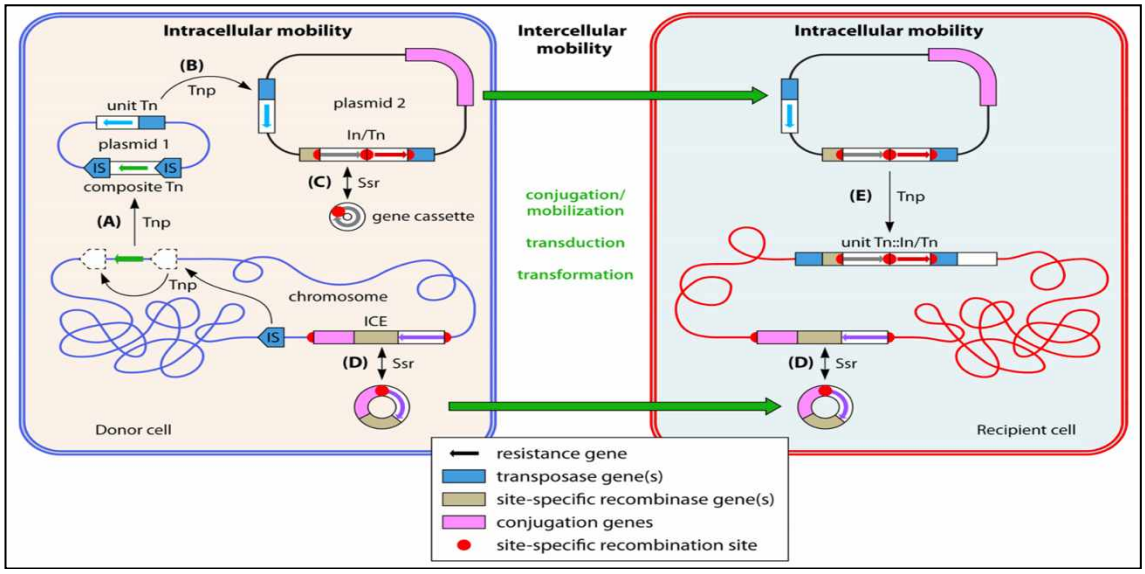


Fig. 10. Mobile genetic elements (MGE) and their intracellular mobility or intercellular transfer of antibiotic resistance genes. The exchange process of MGE is illustrated by two cells of different strains or species, with one acting as donor (envelope and chromosome shown in blue; contains two plasmids) and the other as recipient (shown in red). Various MGE are shown, with the functions of the genes they carry color coded as shown in the key. Different resistance genes associated with different MGE are represented by small arrows of various colors. Thin black arrows indicate intracellular processes, with those mediated by a transposase protein labeled Tnp and those mediated by a site-specific recombinase protein labeled Ssr. Thick green arrows represent intercellular (horizontal) transfer. (A). A unit Tn carrying a resistance gene may transpose between plasmids (B) or from a plasmid to the chromosome or vice versa. A gene cassette may move between In (a class 1 In/Tn structure is represented here) via a circular intermediate (C). An ICE can be integrated into the chromosome or excised as a circular element that can then conjugate into a recipient cell and integrate (reversibly) into the chromosome at a specific recombination site (D). A plasmid may be able to mediate its own intercellular transfer by conjugation or, if it lacks a conjugation region, be mobilized by another plasmid (or, alternatively, move horizontally by phage transduction or transformation). Tn and/or In and associated resistance genes on an incoming plasmid may move into the chromosome or other plasmid(s) in the recipient cell (E), as illustrated here for class 1 In/Tn, which are known to target unit Tn. See relevant sections of the text for further details. Adapted from (Partridge et al., 2018).

Among GNB, plasmids are playing a big role within epidemic pathogens collectively named ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* and *E. coli* (Mahmood et al., 2016; Partridge et al., 2018). These pathogens have been identified

as one of the greatest threats to human health by the WHO due to they cause the majority of hospital infections and they "escape" the antibiotic treatment by becoming resistant or persistent to antibiotic treatment (Rice, 2010; Spellberg et al., 2013; Boucher et al., 2013).

Plasmids

Plasmids are extrachromosomal DNA molecules with ability of autonomous replication and mobilize virulence and resistance genes among different species, genera and kingdoms (Thomas and Nielsen, 2005).

As the plasmids commonly carry multiple resistance determinants (this role was first recognized in Japan in patient with dysentery disease) and virulence genes, a single plasmid conjugation may offer advantage to their host, allowing the plasmid to evolve as an integral part of the bacterial genome (Thomas and Nielsen, 2005; Ramirez et al., 2014).

The antimicrobials resistance found on plasmids, include β-lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides and quinolones resistance genes (Carattoli, 2009). The fact that plasmids carries genes for resistance to β-lactams and other types of antibiotics limit the treatment.

The plasmid is formed by replicon that is a minimal part that replicates with the characteristic copy number of the parent plasmid. From replicon there is the origin of replication (ori) that regulated the plasmid replication (Carattoli, 2009, Rozwandowicz et al., 2018).

The control of plasmid replication occurs by phenomenon known as plasmid incompatibility (Incompatibility groups, Inc) of which two plasmids that share the same replicon, cannot to be propagated stably in the same cell line (Carattoli, 2010). The following plasmids have been associated found in *Enterobacteriaceae*: F plasmids, HI plasmids, I2 plasmids, L/M plasmids, N plasmids, P/P-1 plasmids, T plasmids, U and G/P-6 plasmids, W plasmids, X plasmids, Y plasmids, Q plasmids, ColE1 and related plasmids (Table 2). These plasmids are associated with 23 plasmid incompatibility groups: B, C, D, E, FI, FII, FIII, FIV, H, Ia, I2, Ic, Id, If, J, K, M, N, P, T, V, W and X (Caratolli, 2009). Plasmids with IncF, IncI, IncA/C, IncL (or IncL/M), IncN and IncH are the ones that bear the greatest variety of resistance genes (Rozwandowicz et al., 2018).

Table 2. Main characteristics of known resistance plasmids in *Enterobacteriaceae*. Table adapted from (Partridge et al., 2018; Rozwandowicz et al., 2018).

Inc	Size (kb)	Relaxase type	Replicon(s)	Copy no	Host range	Conjugation or pilus description
A/C (P-3)	18-230	MOBH	A/C	L[c]	Broad	Thick and flexible
F	45-200	MOBF	FII	L	*Enterobacteriaceae*	Thick and flexible
			FIA			
			FIB			
G (P-6)			G	L	Broad	Mobilizable
HI1	75-400		HI1A	L	*Enterobacteriaceae*	Thick and flexible
			HI1B	L	*Enterobacteriaceae*	Thick and flexible
			FIA-like replicon			
I complex	50-250	MOBP	I1/Iγ/B/O/K/Z	L	*Enterobacteriaceae*	Rigid plus thin and flexible
I2			I2	L	*Enterobacteriaceae*	Rigid plus thin and flexible
J (ICE)			J			Thick and flexible
L/M	50-80	MOBP	L/M	L	Broad, α, β, γ	Rigid
N	30-70	MOBF	N	L	Broad	Rigid
P (P-1)	70-275	MOBP	P	L	Broad, α, β, γ	Rigid
Q-1	8-14	MOBQ	Q-1	H[d]	GNB[a] and GPB[b]	Mobilizable
Q-3	8-14	MOBQ		H	GNB[a] and GPB[b]	Mobilizable
R	40-160	Not included	R	L	Broad	Mobilizable
T	~217	MOBH	T	L	Narrow	Thick and flexible
U	29-60	MOBP	U	L	Broad, α, β, γ	Rigid
W	up to 40	MOBF	W	L	Broad, α, β, γ	Rigid
X	30-50	MOBP	X	L	*Enterobacteriaceae*	Thick and flexible
Y			Y	L	*Enterobacteriaceae*	Plasmid-like prophage
Col	6-40	MOBP	Col	H		Mobilizable

[a] GNB- Gram negative bacteria; [b] Gram positive bacteria; [c] Low; [d] High.

Insertion sequences

Insertion sequence (IS) are generally small mobile elements that typically carry little more than one (sometimes two) transposase (*tnp*) gene that are able to move themselves (and associated resistance genes) almost randomly to new locations in the same or different DNA molecules within a single cell (Partridge et al., 2018). The most common type of IS are generally possess inverted repeats (IR) that are designated left (IRL) and right (IRR) with respect to the direction of transcription of the *tnp* gene (Figure 11) (Partridge et al., 2018).

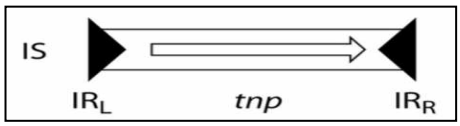


Fig. 11. The insertion sequences structure. Adapted from (Partridge et al., 2018).

GNB possess several IS (Table 3) that mobilize an adjacent region including one or more resistance genes. IS have a strong promoter that drives expression of the captured gene and insertion upstream of an intrinsic chromosomal gene can also influence antibiotic resistance (e.g., IS*Aba1* with $bla_{\text{OXA-51-like}}$ genes in *A. baumannii*) giving carbapenem resistance (Turton et al., 2006). In addition, IS has a capacity to create a hybrid promoter resistance because has a –35 region only, which can combine with an adjacent –10-like (Vandecraen et al., 2017).

Table 3. Examples of IS (Insertion sequence) and composite transposons associated with resistance genes in Gram-negative bacteria. Table adapted from (Partridge et al., 2018).

IS	Tn	Determinant	Resistance
IS*1*	Tn*9*	*catA1*	Chloramphenicol
IS*10*	Tn10	*tet(B)*	Tetracycline
IS*26*	Tn*4352*	*aphA1*	Kanamycin
	Tn*6020*	*aphA1*	Kanamycin
		tet(C)	Tetracycline
		tet(D)	Tetracycline
		catA2	Chloramphenicol
	Tn*2003*	*bla*SHV	β-Lactams
		cfr	Phenicols/lincosamides/oxazolidinones/ pleuromutilins/streptogramin A
IS*256*[a]		*Cfr*	Phenicols/lincosamides/oxazolidinones/ pleuromutilins/streptogramin A
IS*50*	Tn*5*	*aph*(*3'*)-*IIa*-*ble*-*aph*(*6*)-*Ic*	Kanamycin, bleomycin, streptomycin
IS*903*	Tn*903*	*aphA1*	Kanamycin
IS*1999*	Tn*1999*	*bla*OXA-48-like	Carbapenems
IS*Apl1*	Tn*6330*	*mcr-1*	Colistin
IS*Ec69*		*mcr-2*	Colistin
IS*As2*		$bla_{\text{FOX-5}}$	BLBLI[b]
IS*Aba14*	Tn*aphA6*	*aphA6*	Kanamycin
IS*Aba1*	Tn*2006*	$bla_{\text{OXA-23}}$	Carbapenems
		$bla_{\text{OXA-237}}$	Carbapenems
IS*Aba125*	Tn*125*	bla_{NDM}	Carbapenems

[a] Is normally associated with Gram-positive bacteria; [b] BLBLI, β-lactam–β-lactamase inhibitor.

From *E. coli* was identified IS*Ecp1* (IS*1380* family; encodes a DDE-type transposase) that appears to be able to use IRL in combination with a sequence beyond its IRR end to move an adjacent region. For instance, insertion of IS*Ecp1* upstream of a chromosomal $bla_{\text{CTX-M-2}}$ gene in *Kluyvera* and subsequent movement to a plasmid have been demonstrated (Lartigue et al., 2006). IS*Ecp1* appears to be associated with capturing many different resistance genes (Table 4) using at least one promoter or two (Poirel et al., 2003; Kurpiel and Hanson, 2012).

Table 4. Examples of resistance genes associated with IS*Ecp1*. Table adapted from (Partridge et al., 2018).

Insertion sequence	Determinant(S)	Resistance(S)
IS*Ecp1*	*bla*CTX-M-1 group	3rdGC[a]
	*bla*CTX-M-2 group	3rdGC
	*bla*CTX-M-9 group	3rdGC
	*bla*CTX-M-25 group	3rdGC
	*bla*ACC	3rdGC, BLBLI[b]
	*bla*CMY-2-like genes	3rdGC, BLBLI
	*bla*OXA-181-like genes	Carbapenems
	Some *qnrB* genes	Fluoroquinolones (low level)
	qnrE1	Fluoroquinolones (low level)
	rmtC	Fluoroquinolones (low level)

[a] Third generation cephalosporins. [b] BLBLI, β-lactam-β-lactamase inhibitor combinations.

Transposons

There is several families of AMR transposons and the resistance genes are often associated with Tn*3* family transposons. Transposons use site-specific recombination to move resistance genes between defined sites. The Tn*3* family (Tn*1*, Tn*2*, and Tn*3***)** is generally characterized by ~38-bp terminal IR, with IRL and IR with *tnpA* transposase gene, larger than those of IS (~3 kb) and *tnpR* resolvase gene (Figure 12) (Nicolas et al., 2015).

The bla_{TEM} genes, including those encoding ESBL or inhibitor-resistant (IRT) variants, have always been found within Tn*1*, Tn*2*, Tn*3*, or variants, hybrids, or fragments of these transposons. The family Tn*3* transposons can make hybrids such as Tn*1331*-like elements with better matches to Tn*1* or Tn*2* and common in bla_{KPC} genes (Partridge, 2015).

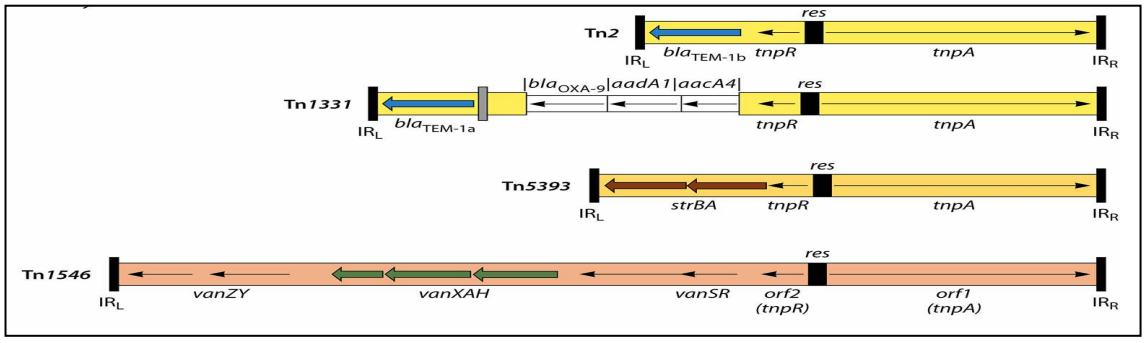


Fig. 12. Tn*3* family transposons. The arrows show the orientations of genes and the thick shows antibiotic resistance genes. Terminal IR in black bars and putative ancestral IR relics by gray bars. *res* sites are indicated by black boxes. The gene cassettes in Tn*1331* are shown as narrower boxes. Adapted from (Partridge et al., 2018).

Gene cassettes and Integrons

A gene cassette is defined as a small mobile element (~0.5 to 1 kb) consisting of a single gene (occasionally two), typically lacking a promoter, and an *attC* recombination site. Can exists in free circular form but are nonreplicative and are usually found inserted into an integron. The cassette insertions into the integron is in the orientation that allows expression of the cassette-borne gene from the Pc promoter. There are several classes of integron of IntI (called IntI1, IntI2, IntI3, etc., with cognate *attI1*, *attI2*, and *attI3* sites), with class 1 being the first reported and most common in antibiotic-resistant clinical isolates (Escudero et al., 2015).

MECHANISM OF ANTIMICROBIAL RESISTANCE IN GRAM NEGATIVE BACTERIA

The emergence of antibiotic resistant pathogenic bacteria poses a serious public health challenge worldwide. In generally, resistance has eventually been seen to nearly all antibiotics that have been developed (Figure 13) attributed to overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry (Rahman et al., 2018).

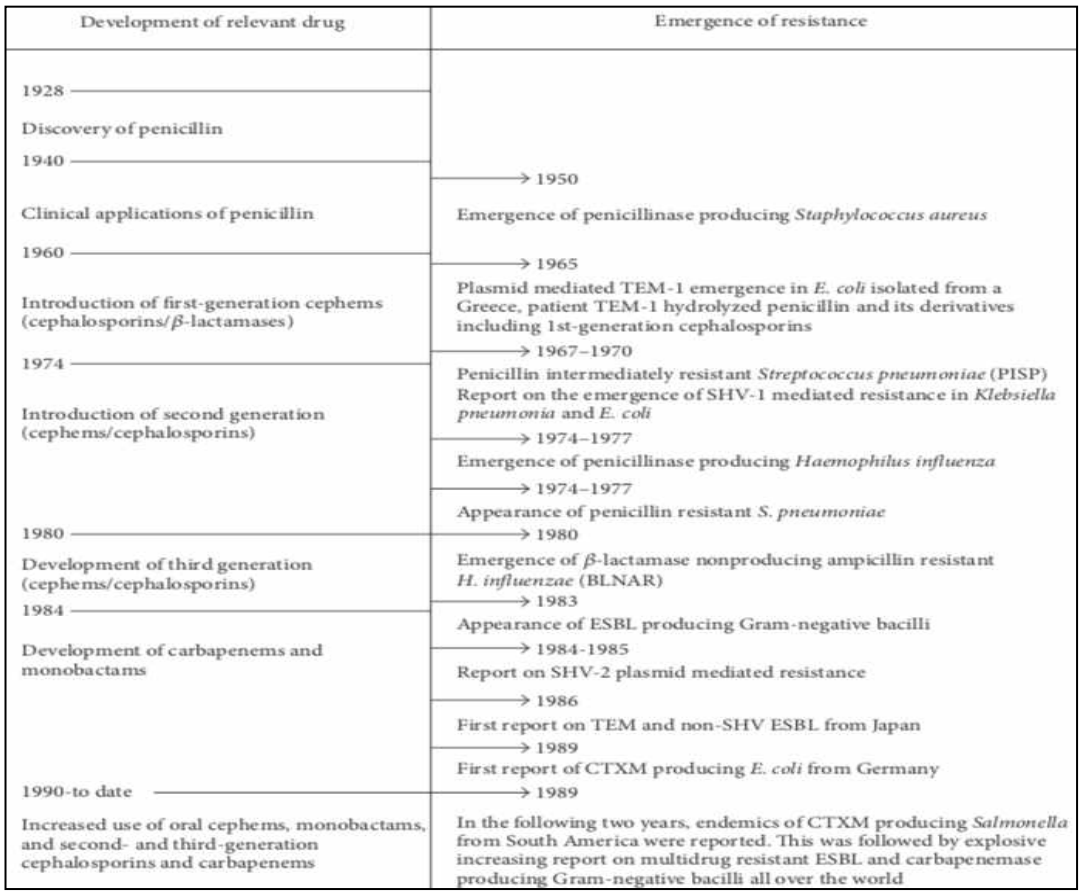


Fig. 13. Emergence of antimicrobial resistance, ESBL. Trend of development of antibiotics and emergence of resistance with emphasis on ESBL, showing that resistance eventually occur after their development and introduction (Rahman et al., 2018).

GNB have a variety of sophisticated mechanisms for self-defense against antibiotics that consists in expression of antibiotic-inactivating enzymes and non-enzymatic mechanisms (Alekshun and Levy, 2007). The antibiotic-inactivating enzymes through β-lactamase production constitute the main mechanism of β-lactam resistance in *Enterobacteriaceae* (Ruppé et al., 2015).

Both mechanisms (antibiotic-inactivating enzymes and non-enzymatic) can occur by (i) mutations in chromosomal genes that result in an increase of expression of intrinsic resistance mechanisms, permeability alterations by loss of outer membrane porins, or target modifications; and (ii) horizontal transfers of MGEs carrying resistance genes, most notably plasmid-encoding β-lactamases, aminoglycosides-modifying enzymes (AMEs), or non-enzymatic mechanisms such as plasmid-mediated quinolone resistance (*qnr*) genes for fluoroquinolone resistance (Ruppé et al., 2015). All mechanisms of bacterial resistance are resumed in three lines (Figure 14).

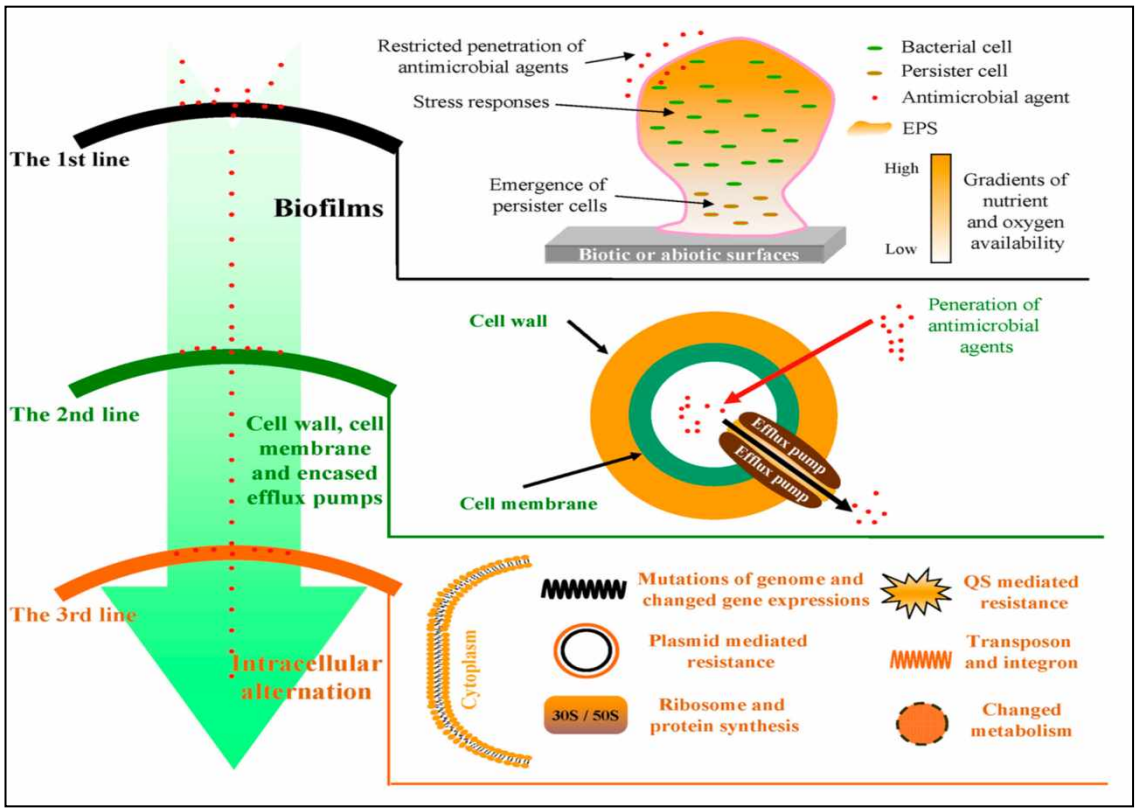


Fig. 14. Mechanisms of bacterial resistance to antibiotics. The three lines of defense for bacterial cells to overcome death by antimicrobial agents consists in: (i) production of bacterial biofilms, which limits the penetration of antimicrobial agents); (ii) the second line, bacteria uses cell wall, cell membrane and the encased efflux pumps construct to reduce the antimicrobial concentration into the cell; (iii) third line of defense, involve the alteration of target sites, regulation of gene expression and production of certain enzymes. Adapted from (Zhou et al., 2015).

For *E. coli* and *Klebsiella* the main mechanism of antibiotic resistance is almost always plasmidic consisting in co-transference of genes encoding an AME, a quinolone resistance determinant, and an ESBL on the same resistance plasmid. Porin/efflux-mediated resistance is not a major determinant mechanism among *E. coli* and *Klebsiella* (Cag et al., 2016); while for *Acinetobacter*, *P. aeruginosa* and *S. maltophilia*, the major mechanism of MDR is the porin/efflux pumps through occurrence of sequential chromosomal mutations (Rumbo et al., 2013). However, *Acinetobacter* and *P. aeruginosa* have ability to acquire MGE encoding resistance determinants, including carbapenemases (Ruppé et al., 2015) becoming difficult to treat infections associate with these bacteria. For instance, *A. baumannii* naturally produces a non-inducible AmpC type cephalosporinase (ACE-1 or ACE-2) and an OXA-51-like oxacillinase which confer, at basal levels of expression, intrinsic resistance to aminopenicillins, first generation

cephalosporins (1GC), second generation cephalosporins (2GC) and aztreonam (Munoz-Price and Weinstein, 2008); and also *P. aeruginosa* harbors an inducible AmpC-type cephalosporinase that confer resistance to amoxicillin (with or without clavulanate), 1GC, 2GC, cefotaxime, ceftriaxone and ertapenem (Lister et al., 1999). *S. maltophilia* also have intrinsic multi-drug resistance phenotype involves several chromosomal determinants (efflux systems_SmeDEF pump, AAC(6′)-Iz, thermo-dependent permeability of its outer membrane). Additionally, *S. maltophilia* can acquire *sul* genes that confer resistance to sulfonamides (Looney et al., 2009).

Traditionally, *S. maltophilia* is highly susceptible to the trimethoprim-sulfamethoxazole (SXT) combination, which is considered as the cornerstone of therapy and fluoroquinolones, (ciprofloxacin, levofloxacin and moxifloxacin) are active despite the low-level expression of a qnr protein encoded by the chromosomal *SmQnr* gene (Sanchez and Martinez, 2010).

Decreased uptake of antibiotics

This mechanism is very important in regulation of the antibiotic concentration into the cell. They reduce the antibiotic absorption or increasing the discharge of them, or by employing both mechanisms simultaneously using the outer-membrane (OM). As the OM is constitute by lipid bilayer and porins, theorically, hydrophobic antibiotics, such as quinolones and macrolides, pass through the lipid bilayer while hydrophilic antibiotics, such as β-lactams, pass through porins (Chapman and Georgopapdakou, 1988). Thereby, the porins mutation can mediate the antibiotic uptake. Porin-efflux mechanisms are activated by spontaneous modifications of inherited structures (Cag et al., 2016).

Target site modification

This mechanism consists by changing the target site that is the antibiotic attack site. Bacteria replace or change target molecules in order to avoid the harmful effects of antibiotics. It is well known that β-lactam antibiotics inactivate PBPs, and initiate dysregulation of peptidoglycan synthesis that end up with bacteria death (Peterson and Kaur, 2018). Apart of modification of PBP, bacteria can alter 50S subunit of rRNA, methylation of ribosomal genes and plasmid-mediated quinolone resistance (PMQR)

where some protein protect gyrase from quinolones. Resistance to macrolide is mostly caused by this mechanism (Leclercq, 2002).

The *Enterobacteriaceae* are naturally susceptible to quinolones and fluoroquinolones. However, resistance can emerge through high-level fluoroquinolone monotherapy, especially when the bacterial *inoculum* is high. The resistance can occur by successive chromosomal mutations in DNA gyrase and topoisomerase IV-encoding genes (*gyrA* and *parC*), that are the target sites for fluoroquinolones such as ciprofloxacin, levofloxacin and moxifloxacin. These mutations can accumulate, resulting in an increasingly higher level of resistance or minimum inhibitor concentration. Additionally, strains with a single mutation can appear susceptible to fluoroquinolones but highly resistant to quinolones (Deguchi et al., 1997).

In parallel with chromosomal mutation, plasmid-encoded resistance has emerged in the 2000s with Qnr (A, B, C, D and S subtypes), that is a small DNA-mimicking protein conferring low-level fluoroquinolone resistance (Allou et al., 2009), *aac(6')Ib-cr* and QepA efflux pump (Périchon, 2007).

Efflux pumps

Efflux pumps seen to be a last resistance mechanism in GNB and usually occurs in conjunction with other mechanisms, such as modification of the antibiotic or the target (Amaral et al., 2014). The pumps are cell wall proteins that can take substances from the bacteria and expel them outside the bacterial cells decreasing their concentration into the cell. In generally, pumps are active simultaneously against several different classes of antibiotics, compared with the other mechanisms that are active against only a single antibiotic or a few from the same class (Poole, 2004). For instance, the MexXY-OprM efflux pump in MDR *P. aeruginosa* decreases its susceptibility to meropenem, aminoglycosides, fluoroquinolones, as well as penicillins and cephalosporins (Poole, 2004).

Antibiotic sequestration

Sequestration of antibiotics involves the function of drug-binding proteins, which prevent the antibiotic from reaching its target. Some AmpC-producers bacteria using antibiotic

sequestration mechanism associated with loss of outer membrane porins are resistant to carbapenem. For instance, clinical *E. coli* isolate carrying bla_{CMY-2} on IncIγ plasmid are resistant to increasing concentrations of meropenem due to CMY-2 can covalently bind carbapenem in the periplasm and prevent them from accessing their targets (van Boxtel et al., 2017).

Enzymatic inactivation of antibiotics

This group of enzymes consists in AMEs and β-lactamases. The AME hamper antibiotic activity by engrafting various radicals aminoglycoside phosphotransferase, APH, aminoglycoside nucleotidyltransferase, ANT and aminoglycoside acetyltransferase, AAC which constitutes the main mechanisms of *Enterobacteriaceae* resistance to aminoglycoside (Chaudhary and Payasi, 2014). *Providencia stuartii* and *Serratia marcescens* possess intrinsic resistance to aminoglycoside AAC(2′) and AAC(6′)-I, respectively (Ruppé et al., 2015).

Extended Spectrum β-lactamases

The name "ESBL" is due to these enzymes have increased spectrum of activity, especially against the oxyimino-cephalosporins (Bradford, 2001). Thereby, ESBLs are enzymes that have the ability to hydrolyse the variety of β-lactam including penicillins, cephalosporins, and monobactams (like aztreonam), but not the cephamycins (like cefoxitin) and carbapenems, and are typically inhibited by "classical" β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam and are encoded by mobile genes (Livermore and Hawkey, 2005; Thomson, 2010).

ESBLs are found in GNB, especially in *Enterobacteriaceae* and *P. aeruginosa* (Nordmann and Guibert, 1998; Bradford, 2001) and constitute the common resistance mechanism to β-lactam in GNB (Bush and Jacoby, 2010).

The ESBLs were firstly reported in the early 1980 after introduction of cephalosporins in clinical practice (Figure 13). The following ESBLs have been isolated in clinical isolates: SHV or TEM types, which are evolved from parent enzymes such as TEM-1, TEM-2, and SHV-1; CTX-M β-lactamases in Europe, Africa, Asia, South and North America (Paterson and Bonono, 2005).

ESBLs are classified in two methods: Ambler molecular classification scheme and Bush-Jacoby-Medeiros (traditional) or functional classification scheme (Bush et al., 1995; Bush and Jacoby, 2010).

The Ambler molecular classification scheme is based on protein homology in the following classes: A, B, C, and D. Classes A, C and D are termed as serine β-lactamases and class B enzymes are metallo-β-lactamases (MBLs). The most ESBL belongs to the class A and D that include OXA types (Table 5 and Figure 15) (Paterson and Bonomo, 2005).

According to the Bush-Jacoby-Medeiros (traditional) the ESBL are classified based on functional similarities (substrate and inhibitor profile) in four main groups (Group 1 cephalosporinases, Group 2 serine β-lactamases, Group 3 metallo-β-lactamases and Group 4 β-lactamases) and multiple subgroups. This classification of ESBL belongs to the group 2be or 2d (OXA-type) (Table 5 and Figure 15) (Bush and Jacoby, 2010).

The Group 1 cephalosporinases belongs to the class C and are encoded on the chromosomes of *Enterobacteriaceae* and few other organisms. Often they are more active on cephalosporin than benzyl penicillin and are usually resistant to inhibition by clavulanic acid and are also active on cephamycins (Thomson, 2010).

The ESBL phenomenon began in western Europe related to the use of antibiotics and after that expanded to the United States and Asia. Therefore, the prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution (Bradford, 2001).

Table 5. Classification schemes for bacterial β-lactamases. Table adapted from (Ghafourian et al., 2015 and Bush and Jacoby, 2010).

Bush-Jacoby-Medeiros (2009)	Molecular class	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
			CA[a]/TZB[b]	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β - lactams	GC1, CMY-37
2[a]	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins,early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3[a]	B (B1) (B3)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1 L1, CAU-1, GOB- 1, FEZ-1
3b	B (B2)	Carbapenems			Preferential hydrolysis of carbapenems	CphA, Sfh-1
4	D[c]					

[a] Clavulanic acid; [b] Tazobactam; [c] Miscellaneous enzymes that do not fit into other groups.

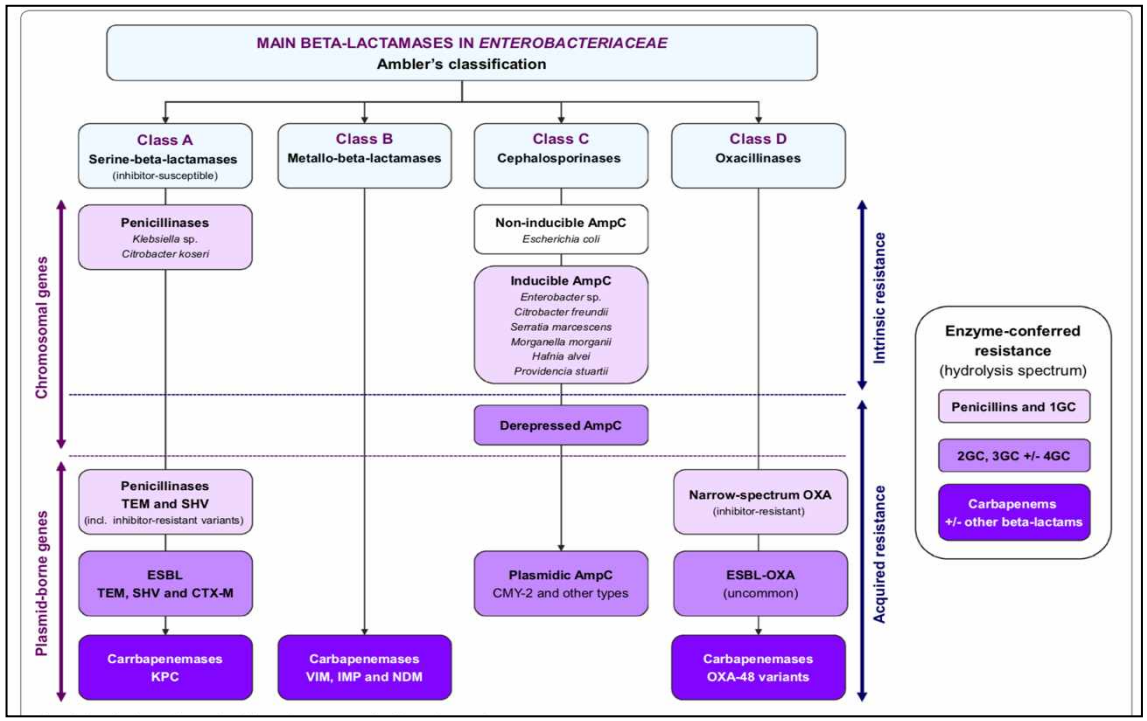


Fig. 15. Classifications of β-lactamases. Intrinsic and acquired β-lactamases in *Enterobacteriaceae* (Ruppé et al., 2015).

TEM β-lactamases

The TEM ESBLs are the derivatives from TEM-1 and TEM-2, and TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient named Temoniera in Athens, Greece, hence the designation "TEM". As are found in plasmid and transposons that facilities the spreading, now is encountered in many different species including *P. aeruginosa, Haemophilus influenzae*, and *Neisseria gonorrhoeae* (Bradford, 2001).

Currently, far 150 TEM types with different spectrum (Table 6) have been isolated where the most often found in *E. coli* and *K. pneumoniae*. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Bradford, 2001).

From early 1990s β-lactamases that were resistant to inhibition by clavulanic acid and sulbactam were discovered from TEM-1 or TEM-2 β-lactamase (Table 6). However, they still showing susceptibility to tazobactam. These inhibitors have been encountered in *K. pneumoniae, Klebsiella oxytoca, P. mirabilis*, and *Citrobacter freundii* (Lemozy, et al., 1995).

Table 6. Characteristics of TEM-type β-lactamases. Table adapted from (Bradford, 2001).

Enzymes	Enzyme type		
	Broad spectrum	**ESBL**	**IRT**
TEM-1	X		
TEM-2	X		
TEM-3[a]		X	
TEM-12, TEM-55, TEM-57, TEM-58		X	
TEM-30, TEM-31, TEM-35, TEM-36, TEM-37, TEM-38, TEM-41, TEM-45, TEM-51, TEM-73, TEM-74			X
TEM-25		X	
TEM-7, TEM-19, TEM-20, TEM-65		X	
TEM-32, TEM-33, TEM-34, TEM-39, TEM-40, TEM-44			X
TEM-29		X	
TEM-5, TEM-17		X	
TEM-9		X	
TEM-10, TEM-11, TEM-13, TEM-26, TEM-63		X	
TEM-50		X	X
TEM-59			X
TEM-68		X	X
TEM-42		X	
TEM-4, TEM-6, TEM-8, TEM-27, TEM-72		X	
TEM-15, TEM-47, TEM-48, TEM-49, TEM-52, TEM-66, TEM-92		X	
TEM-28, TEM-43		X	
TEM-16, TEM-21, TEM-22		X	
TEM-56, TEM-60		X	
TEM-24, TEM-46, TEM-61		X	
TEM-14, TEM-53, TEM-54		X	
TEM-76, TEM-77, TEM-78, TEM-79, TEM-81, TEM-82, TEM-83, TEM-84			X

IRT- Inhibitor-resistant TEM β-lactamase. [a] The first TEM variant with increased activity against ESBL, which was reported in 1987.

SHV β-lactamases

SHV refers to Sulfhydryl variable, the designation was made because it was thought that the inhibition of SHV activity by p-chloromercuribenzoate. The origin of ESBLs SHV is SHV-1 from *Klebsiella* spp. and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. *Citrobacter diversus*, *E. coli*, and *P. aeruginosa* are also possess SHV ESBLs types (El Harrif-Heraud et al., 1997; Rasheed et al., 1997).

In addition, more than 90 SHV varieties are identified, where SHV-5 and SHV-12 are most common variants (Jacoby and Munoz-Price, 2005). Currently, the majority of SHV types derivatives possess the ESBL phenotype and SHV-10 is reported to have an inhibitor-resistant phenotype (Bradford, 2001) (Table 7).

Table 7. Characteristics of SHV-type β-lactamases. Table adapted from (Bradford, 2001).

Enzymes	Enzyme types		
	Broad spectrum	ESBL	IRT
OHIO-1, LEN-1	X		
SHV-3, SHV-14		X	
SHV-24		X	
SHV-1, SHV-11	X		
SHV-2, SHV-2a, SHV-6, SHV-8, SHV-13, SHV-19, SHV-20, SHV-21, SHV-22		X	
SHV-4, SHV-7, SHV-18		X	
SHV-5, SHV-9, SHV-12		X	
SHV-10			X

IRT- Inhibitor-resistant SHV β-lactamase.

CTX-M β-lactamases

From 2000s CTX-M β-lactamases had been identified in different members of the *Enterobacteriaceae*, however especially in *E. coli* have become the most widespread and common type of ESBL (Doi et al., 2017). The name CTX is related to the enzyme activity, that is greater against cefotaxime when compared with other oxyimino-β-lactam substrates such as ceftazidime, ceftriaxone, or cefepime. Regarding the inhibitor, CTX-M are inhibited better by tazobactam than by sulbactam and clavulanate (Bush and Jacoby, 2010).

The $bla_{\text{CTX-M}}$ are normally found on the chromosome of *Kluyvera* spp., a group of rarely pathogenic commensal organisms, and give evidence of plasmid acquisition of β-lactamase genes. More than 80 types of CTX-M β-lactamases are well-known, mainly found in *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae* (Sykes and Bush, 1982).

The CTX-M β-lactamases *E. coli* are associated with primary community-onset UTIs, and bacteremia and intra-abdominal infections (Canton and Coque, 2006). This type of *E. coli* is resistante to cephalosporins included trimethoprim-sulfamethoxazole, tetracycline, gentamicin, tobramycin, and ciprofloxacin that is an alarming worldwide (Pitout and Laupland, 2008).

According to the phylogenetic tree (based on the amino acid sequence) of CTX-M family there are six sublineages or groups: (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and KLUC (Rahman et al., 2018). Each group share >94% identity, whereas 90%

identity is observed between the members belonging to distinct groups (Bonnet, 2004; Paterson and Bonomo, 2005). In addition, there are some hybrid groups (about four CTX-M variants), namely, CTX-M-45 (formerly Toho-2), which is a hybrid of CTX-M-14 with a protein of unknown origin, and CTX-M-64, CTX-M-123, and CTX-M-132 that are hybrids of CTX-M-15 with different segment CTX-M-14 (Zhao and Hu, 2013).

These CTX-M groups are related with different genetic elements such as IS*Ecp1*-like insertion, transposons, or within mobile gene cassettes (Figure 16), that help the spreading of resistance (Lartigue et al., 2006). Additionally, CTX-M-15 and CTX-M-14 are the most common variants detected globally in important microbes, followed by CTX-M-2, CTX-M-3, and CTX-M-1 which their spread has been reported from early 1990s (Levy, 2002).

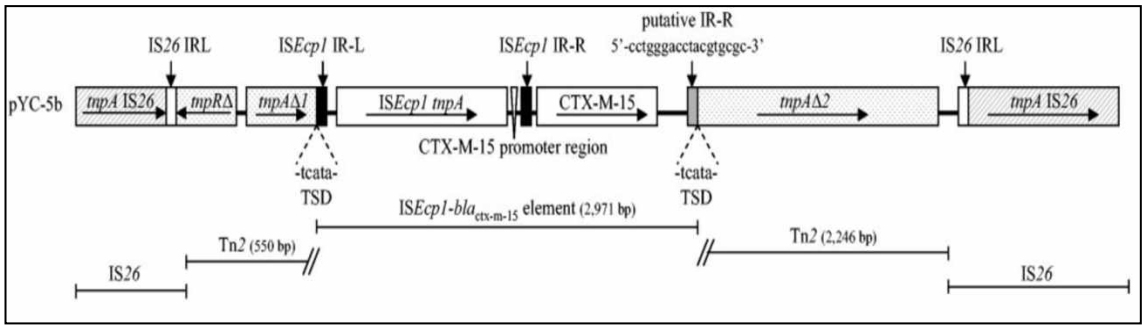


Fig. 16. Schematic representation of the IS*Ecp1-bla*$_{\text{CTX-M-15}}$ containing IS*ECP1*-like insertion and transposons. The presence of a 5-bp duplication at the boundaries of the IS*Ecp1-bla*$_{\text{CTX-M-15}}$ element and the resemblance of its right end to the IRR of IS*Ecp* are indicative of transposition. Transposon-related genes [tnpA, tnpR, tnpM]. Adapted from (Gangoue-Pieboji et al., 2005).

CTX-M enzymes are the most increasingly reported in many countries mostly from South America, Mediterranean and Eastern European countries as well as East Asia (Hopkins et al., 2006; Chong et al., 2018). Especially, in Africa the first report of CTX-M-type-lactamase (CTX-M-12) was from Kenya in 2001 (Govinden et al., 2007). Currently, CTX-M type enzymes are encountered in several African countries.

Plasmid mediated AmpC β-lactamases

AmpC β-lactamases have been known since 1989. These enzymes are able to hydrolyze narrow-, broad-, and expanded-spectrum cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam, and tazobactam. Many GNB produce a chromosomal mediated AmpC at low level while hyperproduction can cause resistance to penicillins, aztreonam, cephamycins, and narrow-, broad-, and expanded-spectrum cephalosporins (Thomson, 2010).

The common transmissible AmpC belong to the ACT, CMY, FOX, MIR, DHA families and have been detected in isolates of *Klebsiella* spp., *Salmonella* spp., *C. freundii*, *E. aerogenes*, *P. mirabilis*, and *E. coli* (Jacoby, 2009). 100% of *E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens*, *Providencia* sp., *Morganella morganii*, *Hafnia alvei*, *Aeromonas* sp., and *P. aeruginosa* isolates can be assumed to be AmpC producers. The expression is low but inducible on exposure to certain β-lactams, such as amoxicillin, ampicillin, imipenem, and clavulanic acid. It is also known that when produced in large amounts, especially in a host with reduced β-lactam, accumulation can provide resistance to carbapenems, especially ertapenem (Weber and Sanders, 1990; Quale et al., 2006).

Carbapenemases

Carbapenemases belong to the molecular classes A, B, and D. The class A enzymes are most commonly produced by members of the family *Enterobacteriaceae,* that include KPC, IMI, SME, NMC-A, and some GES enzymes which are characterized by their high hydrolytic activity against oxacillin and cloxacillin, and poorly inhibited by clavulanic acid and usually hydrolyze penicillins (ampicillin) or cephalosporins (cephalothin) more efficiently than carbapenems. On the contrary of Class A, the Class B enzymes are MBLs which typically hydrolyze carbapenems except aztreonam and resist to currently available β-lactamase inhibitors but are inhibited by chelating agents such as EDTA (Thomson, 2010). This class include Verona integron-encoded metallo-β-lactamase (VIM), Imipenemase metallo-β-lactamase (IMP) families and SPM-1 which have been detected in strains of *P. aeruginosa*, members of the family *Enterobacteriaceae*, and *A. baumannii* (Thomson, 2010). Among the MBLs, NDM, (Figure 17B), VIM and IMP enzymes are the most frequently identified worldwide (Palzkill, 2013).

The class D weekly hydrolyze the carbapenem antibiotics and are inhibited poorly by clavulanate. This class of enzymes belong to the OXA family and are most commonly produced by *Acinetobacter* spp. but have also been reported in some *P. aeruginosa*, *K. pneumoniae*, and *E. coli* strains (Thomson, 2010). OXA-48 and derivatives (e.g., OXA-181 and OXA-232) have been detected in *Enterobacteriaceae* (Poirel et al., 2012), and endemic in Turkey (since 2004) and are frequently encountered in several European countries (e.g., France and Belgium), and across North Africa (Potron et al, 2016).

Carbapenemase-producing organisms (CPOs), are globally distributed in many genera of bacteria. However, certain carbapenemases are typically associated with specific regions or countries (Figure 17A, B and C). KPC-producing *K. pneumoniae* is common in the United States, but is also endemic in some European countries such as Greece and Italy (Munoz-Price et al., 2013). MBL-producing CPE, for instance, NDM producers, are common in Indian Subcontinent as well as with specific countries in Europe, including Romania, Denmark, Spain, and Hungary and OXA-48-like-producing is in essential in Turkey and surrounding countries (Duin and Doi, 2017).

The CPOs, in hospital settings ranged from 2.3% to 67.7% in North Africa and from 9% to 60% in Sub-Saharan Africa (SSA) (Manenzhe et al., 2015).

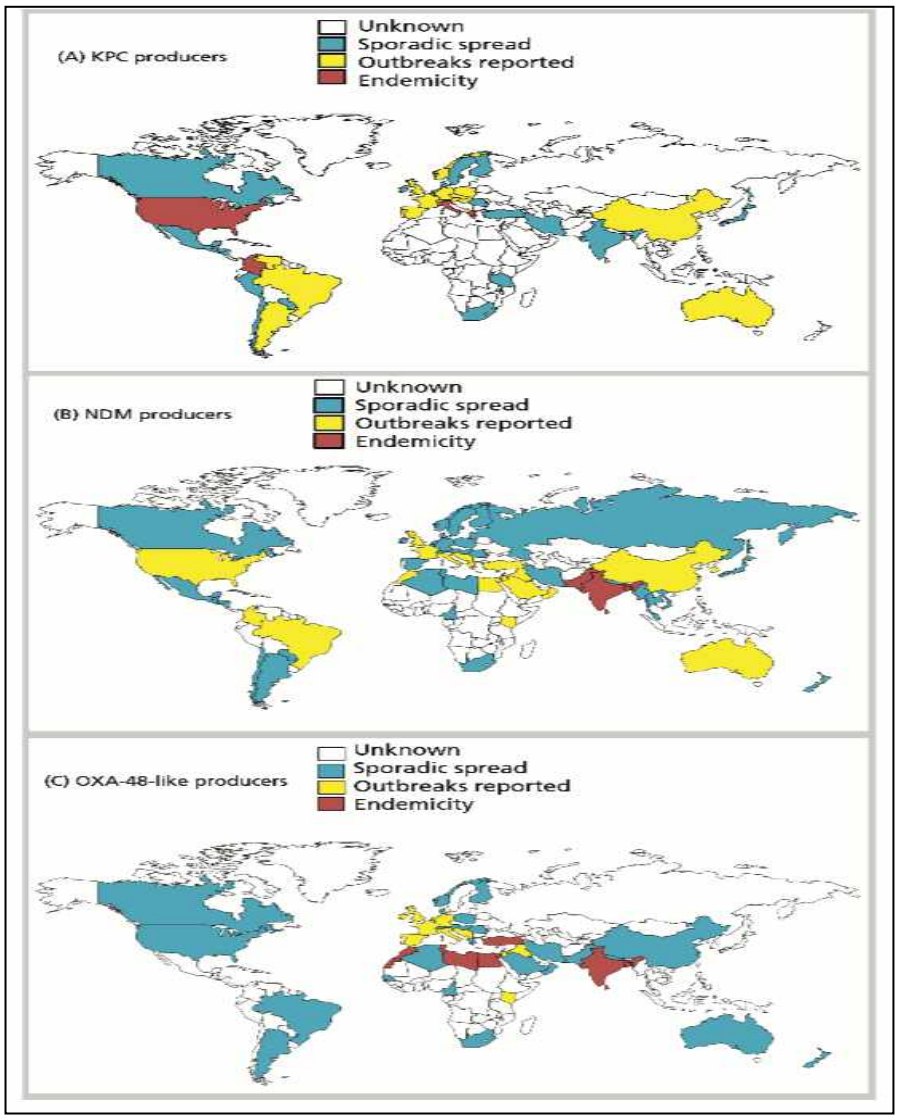


Fig. 17. Worldwide distribution of carbapenemases. A, *K. pneumoniae* carbapenemase producers in Enterobacteriaceae and *P. aeruginosa*. B, New Delhi metallo-β-lactamase producers in Enterobacteriaceae and *P. aeruginosa*. C, OXA-48–like producers in Enterobacteriaceae. Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA-48, oxacillinase-48 (Bonomo et al., 2018).

MOLECULAR TYPING

Is well known that *E. coli* can be commensal or pathogenic. Thereby to determining whether an *E. coli* strain is an ExPEC and whether it is pathogenic is based on its source, O:K:H serotype, phylogenetic background, virulence factor profile, and experimental virulence in an animal model that involves typing methods (Fratamico et al., 2016). Additionally, is clear that certain ExPEC lineages or clonal groups are responsible for a large fraction of human extraintestinal *E. coli* infections, and these lineages are becoming increasingly MDR (Manges and Johnson, 2012). For instance, through typing it has been established that worldwide emergent clone *E. coli* O25:H4-ST131 is as a dominant host of ESBLs posing a huge public health challenge (Coque et al., 2008).

Phylogrouping by PCR

Polymerase chain reaction (PCR) has been useful technique in typing *E. coli* into the phylogroups A, B1, B2 and D. The first PCR protocol was a triplex PCR developed by Clermont and colleagues (2000) with targets *chuA* and *yjaA* genes and a particular DNA fragment known as TSPE4.C2. The other phylogroups (C, E, F and clade I) are miss-assigned by this method and will rest on the composition of the collections evaluated, but an estimated 79% of isolates are correctly assigned (Clermont et al., 2013). In order to correctly discriminate (especially between phylogroup B2 and D) this method was adjusted by Gordon and colleagues and further revised by Clermont and colleagues. Currently, the PCR detection of three marker genes (*chuA, yjaA* and TSPE4.C2) (Figure 18) is followed by potential subsequent detection of a fourth gene *ibeA.* The presence of *ibeA* assign the isolate to group B2 and strains failing to yield any PCR products of the three primary genes remain unassigned as non-type able (Gordon et al., 2008).

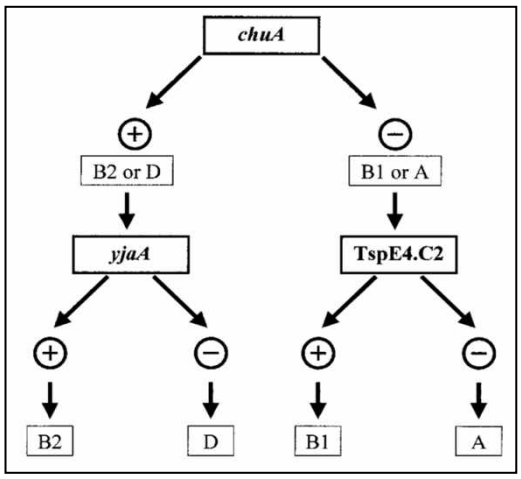


Fig. 18. *E. coli* phylogrouping by PCR. Dichotomous decision tree to determine the phylogenetic group by using the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2 (Clermont et al., 2000).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a powerful method used for the separation of large DNA molecules (entire genomic DNA) using restriction enzymes and gel matrix where the electric field is periodically changed the direction (Sharma-Kuinkel et al., 2016). Provides a good resolution of the entire bacterial chromosome in a single gel with a highly reproducible restriction profile being a useful to discriminate between outbreak and sporadic strains (Bender et al., 1997; Sharma-Kuinkel et al., 2016). Several days to complete the typing, produces results that are suboptimal for interlaboratory comparisons, and can be subjective because it is based on banding patterns, constitute the disadvantages of this method (Noller et al., 2003).

Multi-Locus Sequence Typing

Multi-locus Sequence Typing (MLST) was first proposed in 1998 as a typing approach using the human pathogen *Neisseria meningitidis* (Belén et al., 2009).

MLST is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of six or seven (usually) well-conserved, house-keeping genes or loci within the bacterial genome. The ST is assigned by comparing the set of alleles in the database through allelic variation. This method is becoming powerful tools in molecular epidemiology due to successful for the differentiation of other organisms and has easily standardized and automated. Additionally, has proved portable

and easily comparable between laboratories for evolutionary and epidemiological studies (Noller et al., 2003).

Multi-locus Variable Number of Tandem Repeat Analysis

Multi-locus Variable Number of Tandem (MLVT) is commonly PCR-based approach that requires only a small amount of DNA for analysis simple sequence repeats (SSRs), or microsatellites at a locus. Specifically, this method involves determination of the number of repeats at multiple loci. The repeated regions are found throughout most bacteria as well as mammals, but only identical cells will have the same number of repeats in each of the targeted regions. The variable number of tandem repeats loci (VNTR) can be assigned a number depending on size and one isolate will consequently be assigned a code useful for typing. Thereby, supply a powerful tool for assessing outbreak investigation, forensic investigation, pathogen-source tracking and disease surveillance (Noller et al., 2003; Cooley et al., 2007).

The process of exploring VNTRs can be done *in silico* searching for Tandem repeats (TRs) within the genome(s) of interest, selecting highly potent loci from the thousands of TRs, and then examining the polymorphisms (Cooley et al., 2007).

Serotyping

The serological typing of *E. coli* was proposed by Kauffman in 1944 and is based on surface antigen profiles, namely, O (lipopolysaccharide) and H (flagellar). Both antigens are stable that make the methods reliable being useful for epidemiological studies of pathogenic *E. coli* providing the routes, sources, and prevalence (Banjo et al., 2018). The type K antigen has not been used due to the capabilities of the many laboratories to carry out. To date, 53 *E. coli* H flagellar antigens types, from H1 to H56 (H-types 13, 22, and 50 are not in use), including three missing numbers: H13, H22, and H50 and 186 O-antigens (O1-O188) have been recognized (Orskov and Orskov, 1975; Fratamico et al., 2016). Four groups of O-antigens have been divided into subtypes, namely, O18ab/ac, O28ab/ac, O112ab/ac, and O125ab/ac (Fratamico et al., 2016).

These serotyping require a pool of antisera hat were generated in rabbits against each of the O-groups followed by agglutination with the corresponding single antiserum. This

method is not useful due to time costly, labor-intensive and time consuming, cross reactivity of the antisera with different serogroups occurs, antisera are available only in specialized laboratories, batch-to-batch variations in antibodies can occur, and many *E. coli* strains isolated from various sources are non-typeable (Lacher et al., 2014). These disadvantages open the place for molecular serotyping methods of *E. coli.*

Whole Genome Sequencing

The whole genome sequencing (WGS) of *E. coli* nowadays is replacing established subtyping methods such as PFGE, supplying a major advancement information related food-borne disease outbreaks and for trace-back to sources. This method is also known as core genome (cg) MLST (or extended MLST) due to comparisons of hundreds to more than a thousand genes allowing interlaboratory reproducibility (Schürch et al., 2018).

WGS for typing and epidemiologic analysis consist in management of SNP and/or gene-by-gene (e.g., cgMLST). Core genome as sets of orthologous (i.e. common ancestor) sequences conserved in all aligned genomes is useful for typing. This typing needs mapping of either reads or assembled contigs and various tools available online such as Snippy, NASP, SNVphyl, CFSAN SNP Pipeline, or Lyve-SET (Sahl et al., 2016). The SNP approach can give very high resolution, however, needs a reference genome in order to avoid mismapping. The recent approach related to WGS typing trend to use all the pan-genome to provide a super resolution view into the epidemiology of bacterial populations (McNally et al., 2016).

To analyze the sequences tools and bioinformatic pipelines are being developed in order to obtain specific information such as O- and H-group determination, virulence genes and other genetic markers (Fratamico et al., 2016).

Current situation in Mozambique

Pneumonia, meningitis, bacteremia, enteric infections are a major issue in Mozambique, where the bacterial agents are commonly associated, including *E. coli*, non-typhoidal *Salmonella*, *Shigella* and *Vibrio cholerae* (GARP, 2015). Infections caused by bacteria and especially members of the *Enterobacteriaceae* are among the major causes of hospital admission and associated with morbidity and mortality in children, particularly in Africa (Bryce et al., 2005). Mozambique as each low-income country has been facing an issue related to bacterial infections and antimicrobial resistance (AMR) (Sigaúque et al., 2009). Two studies carried out at the Intensive Care Unit (ICU) of Maputo Central Hospital (HCM), showed high prevalence of GNB, including *Klebsiella* spp., *Acinetobacter* spp., *Enterobacter* spp., *P. aeruginosa*, and *E. coli*, and highlighted high level antimicrobial resistance to penicilins, cephalosporins, aminoglycosides, quinolones and macrolides; and least resistance to carbapenem agents (Mahaluça et al., 2018a; Mahaluça et al., 2018b). Actually, a recent study conducted in Maputo showed that the knowledge about antibiotic use is poor in population (Mate et al., 2019). Worldwide, irrational antibiotic use, self-medication, sub-optimal dosage, overuse, and prescription/use of inappropriate antibiotics are the main keys of emergency and spread of AMR strains (Tenover, 2006).

In Africa, some attention has been given to investigate the prevalence of ESBL mainly in *Enterobacteriacae* at community level and hospital settings. The ESBLs classes of A and D are common in Africa with *bla*$_{\text{CTX-M-15}}$ being most prevalent (Cotton et al., 2008; Muthupandian et al., 2018).

In Mozambique, the prevalence pathogens ESBL producers pathogens is extremely high, although the scarce data (Pons et al., 2015). Some molecular studies have showed the presence of GNB ESBL producers, mainly in *E. coli* and *K. pneumoniae* harboring *bla*$_{\text{CTX-M-15}}$ (Pons et al., 2015; Guiral et al., 2018). This ESBL has been associated with MDR strains worldwide. Recently, a study conducted in a rural hospital at Manhiça District in Southern Mozambique, reported the prevalence of *E. coli* harboring the ESBL gene *bla*$_{\text{CTX-M}}$ group I, especially *bla*$_{\text{CTX-M-15}}$ from blood and urine cultures (Guiral et al., 2018).

RESEARCH OBJECTIVES

The overall purpose of the Ph. D. project was to study the epidemiology and antimicrobial resistance of Extraintestinal Pathogenic *E. coli* and other Gram-negative invasive bacteria associated with invasive infections in Mozambique. Resistance surveillance is also important in light of the rapid emergence and diffusion of multi resistant strains.

The specific studies focused on the following objectives:

- To isolate and identify ExPEC and other GNBs strains from human invasive infections, in 2 Mozambique provinces (3 Maputo and 2 Quelimane hospitals).
- To determine the prevalence of antimicrobial resistance in isolates using phenotypic and genotypic approaches.
- To genotype the ExPEC and other GNBs using pathogenicity and phylogenetic markers.
- To molecular type and perform phylogenetic analysis of ExPEC isolates by WGS comparing isolates from different sources and hospitals.

CHAPTER TWO

MATERIAL AND METHODS

Study sites

The study was conducted from February 2016 to July of 2018, mainly in HCM located in the capital Maputo, in the South of Mozambique. Other four hospitals, two in Maputo Province (Hospital General of Mavalane, HGM and Hospital General of José Macamo, HJM) and two in Zambézia Province (Hospital Provincial of Quelimane, HPQ and Hospital Central of Quelimane, HCQ) located in the Center of Mozambique were included in the study (Figure 19). In HCM the samples were collected from Pediatric, Medicine, Surgery and Gynecology and Obstetrics departments, while samples from HPQ, HCQ, HGM and HJM Hospitals were only from Pediatric departments.

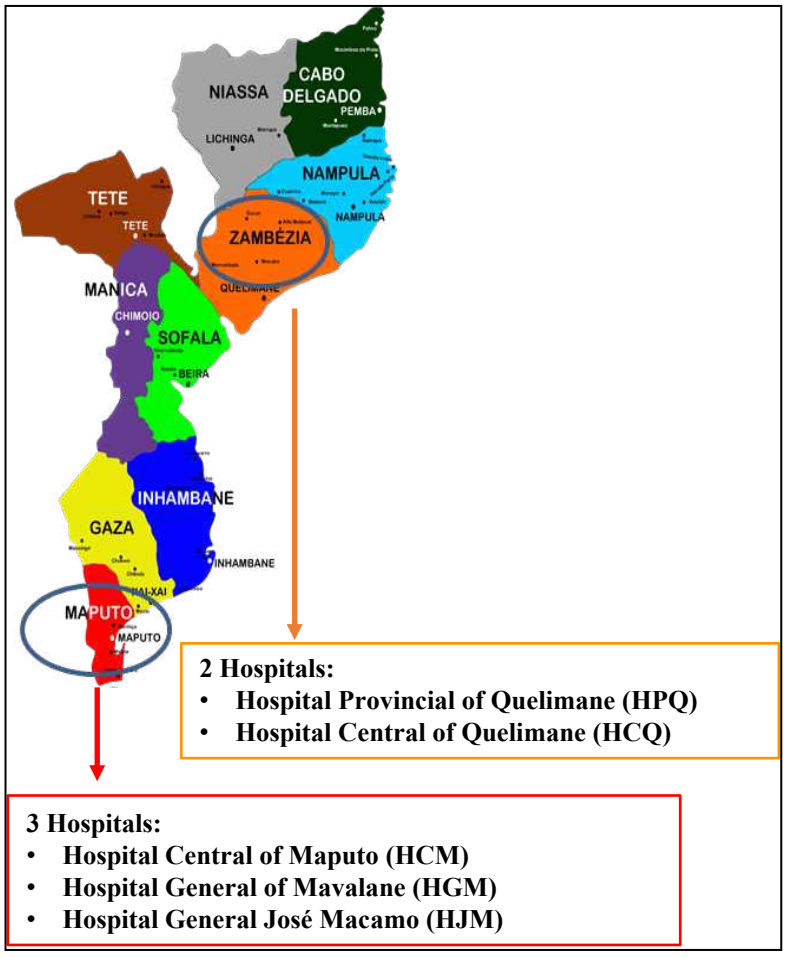


Fig. 19. Map of Mozambique showing the Provinces and hospitals where the samples were collected.

Bacterial isolation and identification

Plates of Blood, Pus and Cerebrospinal fluid (CSF) from patients admitted at HCM were collected in Microbiology Laboratory of HCM. Then, the plates were transported to the Microbiology Laboratory of Medicine Faculty of Eduardo Mondlane University (MLMF_UEM) and subcultured for preliminary microbiological identification. From Pediatric Departments of HGM and HJM and HPQ and HCQ only blood samples were collected by venipuncture aseptically in aerobic flasks (Becton-Dickinson, Franklin Lakes, NJ) from children with suspicion of bacteremia. Blood flasks were transported within 5 hours to the MLMF or the Microbiology Laboratory of Quelimane (MLQ) hospitals for subculture in BACTEC 9050 (Becton-Dickinson) during 5 days. From the positive cultures were made Gram stain and the sample were subcultured on MacConkey, Chocolate and Blood agar at 37°C overnight. Positive cultures we tested for catalase, oxidase and coagulase test.

A collection of GNB was prepared in double in soft agar stabs, one was sent to Italy for further investigations and the other stored at the MLMF_UEM. Isolates were identified by mass spectrometry using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) at the San Francesco hospital laboratory, Nuoro, Italy.

Antimicrobial resistance phenotyping

Antibiotic susceptibility Testing (AST) was performed using VITEK 2 compact system (bioMérieux) including specific card GN377. Pure isolates colonies of GNB were taken from MacConkey fresh culture and the 0.5–0.63 McFarland bacterial suspension was made with 0.45% saline. Cards were automatically filled, sealed, and loaded into the Vitek 2 instrument for incubation and reading. The following antimicrobials were tested Amoxillicin-Clavulanic acid (AMC), Piperacillin-Tazobactam (TZP), Cefotaxime (CTX), Ceftazime (CAZ), Ertapenem (ETP), Meropenem (MEM), Gentamicin (GEN), Amikacin (AMK), Ciproflaxacin (CIP), Tigecycline (TGC), Fosfomycin (FOF), Colistin (CST) and Trimethoprim-Sulfamethoxazole (SXT).

DNA Extraction of Gram-negative bacteria

Chromosomal DNA were extracted from all isolates using Wizard® Genomic DNA Purification Kit (Promega). DNA was quantified using NanoDrop Microvolume Spectrophotometer (ThermoFisher) and QBIT (ThermoFisher).

PCR detection of β-lactamase genes

Seventy-seven GNB were tested by PCR for several resistance genes: ESBL (TEM, SHV, CTX-M, CTX-M-2, CTX-M-9, CTX-M-15, GES, VEB, PER), AmpC (MOXM, CITM DHAM, ACC, EBCM, FOXM) and Carbapenemase (KPC, OXA-48-like, IMP, VIM, NDM) with specific primers reported in Table 8, following previously published protocols (Ellington et al., 2007; Pérez-Pérez and Hanson, 2002; Dallenne et al., 2010; Hijazi et al., 2016).

Table 8. Oligonucleotides used for the detection of ESBL, AmpC and Carbapenemase antibiotic resistance genes in ExPEC and other Gram-negative bacteria.

Resistance genes	Oligonucleotides	Sequence (5'-3')	Size of Product (bp)	References
bla$_{TEM}$	TEM_F	AGT GCT GCC ATA ACC ATG AGT G	431 bp	Hijazi et al., (2016)
	TEM_R	CTG ACT CCC CGT CGT GTA GAT A		
bla$_{SHV}$	SHV_F	GAT GAA CGC TTT CCC ATG ATG	214 bp	
	SHV_R	CGC TGT TAT CGC TCA TGG TAA		
bla$_{CTX-M}$	CTX_F	ATG TGC AGY ACC AGT AAR GT	593 bp	
	CTX_R	TGG GTR AAR TAR GTS ACC AGA		
bla$_{CTX-M-2}$	CTX-M-2_F	AAA CAG AGC GAG AGC GAT AAG	720pb	
	CTX-M-2_R	GGG TAA AGT AGG TCA CCA GAA C		
bla$_{CTX-M-9}$	CTX-M-9_F	GGA TTA ACC GTA TTG GGA GTT T	164 pb	
	CTX-M-9_R	GAT ACC GCA GAT AAT ACG CAG G		
bla$_{CTX-M-15}$	CTX-M-15_F	CAC GTC AAT GGG ACG ATG T	410 pb	
	CTX-M-15_R	GAA AGG CAA TAC CAC CGG T		
bla$_{MOXM}$	MOXM_F	GCT GCT CAA GGA GCA CAG GAT	520 pb	Pérez-Pérez and Hanson (2002)
	MOXM_R	CAC ATT GAC ATA GGT GTG GTG C		
bla$_{CITM}$	CITM_F	TGG CCA GAA CTG ACA GGC AAA	462 pb	
	CITM_R	TTT CTC CTG AAC GTG GCT GGC		
bla$_{DHAM}$	DHAM_F	AAC TTT CAC AGG TGT GCT GGG T	405 pb	
	DHAM_R	CCG TAC GCA TAC TGG CTT TGC		
bla$_{ACC}$	ACC_F	AAC AGC CTC AGC AGC CGG TTA	346 pb	
	ACC_R	TTC GCC GCA ATC ATC CCT AGC		

Resistance genes	Oligonucleotides	Sequence (5'-3')	Size of Product (bp)	References
*bla*EBCM	EBCM_F	TCG GTA AAG CCG ATG TTG CGG	302 pb	
	EBCM_R	CTT CCA CTG CGG CTG CCA GTT		
*bla*FOXM	FOXM-F	AAC ATG GGG TAT CAG GGA GAT G	190 bp	
	FOXM-R	CAA AGC GCG TAA CCG GAT TGG		
*bla*GES	GES_F	AGTCGGCTAGACCGGAAAG	399 pb	Dallenne et al., (2010)
	GES_R	TTTGTCCGTGCTCAGGAT		
*bla*PER	PER_F	GCTCCGATAATGAAAGCGT	520 pb	
	PER_R	TTCGGCTTGACTCGGCTGA		
*bla*VEB	VEB_F	CATTTCCCGATGCAAAGCGT	648 pb	
	VEB_R	CGAAGTTTCTTTGGACTCTG		
*bla*OXA-48	OXA-48-like_F	GCTTGATCGCCCTCGATT	281 pb	
	OXA-48-like_R	GATTTGCTCCGTGGCCGAAA		
*bla*GES	GES_F	AGTCGGCTAGACCGGAAAG	399 pb	
	GES_R	TTTGTCCGTGCTCAGGAT		
*bla*IMP	IMP_F	TTGACACTCCATTTACDG*	139 pb	
	IMP_R	GATYGAGAATTAAGCCACYCT		
*bla*VIM	VIM_F	GATGGTGTTTGGTCGCATA	390 pb	
	VIM_R	CGAATGCGCAGCACCAG		
*bla*KPC	KPC_F	CATTCAAGGGCTTTCTTGCTGC	538 pb	
	KPC_R	ACGACGGCATAGTCATTTGC		
*bla*IMP	IMP_F	GGA ATA GAG TGG CTTAAY TCT C	188 pb	Ellington et al., (2007)
	IMP_R	CCA AAC YAC TAS GTT ATC T		
*bla*VIM	VIM_F	GAT GGT GTT TGG TCG CAT A	390 pb	
	VIM_R	CGA ATG CGC AGC ACC AG		
*bla*NDM	NDM_F	GGTTTGGCGATCTGGTTTTC	699 pb	
	NDM_R	CGGAATGGCTCATCACGATC		
*bla*KPC	KPC_F	CGTCTAGTTCTGCTGTCTTG	798 pb	
	KPC_R	CTTGTCATCCTTGTTAGGCG		

*D = A or G or T.

Whole Genome Sequencing and *in silico* analysis

Eight two GNB isolates, including all isolated *E. coli*, were selected for WGS, Quantified DNAs were diluted for library and sent for Next generation sequencing using Illumina NextSeq platform, at a 30x coverage (NGS Bio, San Francisco).

Genomes were assembled using de novo assembly, SPAdes 3.13.0. web-based tool. The 82 GNB genome assemblies were then subjected to ResFinder 3.2 (Zankari et al., 2012), VirulenceFinder 2.0 (Joensen et al., 2015); MLST 2.0 (Larsen et al., 2012), pMLST 2.0 and PlasmidFinder 2.0 (Carattoli et al., 2014), SerotypeFinder 2.0 (Joensen et al., 2015), FimTyper 1.0 and CHTyper 1.0 (Camacho et al., 2009) analysis through in *Silico* Resource platform CGE online platform: (http://www.genomicepidemiology.org/). In addition, other virulence factors, typically of ExPEC (*F1C fimbriae*, *sfaS*, *fimH*, *papA*, *papC*, *papG*, *iucC*, *iutA*, *fyuA*, *traT*, *maXL*, *cvaC*, *ompT*, *crl*, *sitA*, *foc, afa*, *cdtB, KpsM*, *K1*, *ibe10*), were identified using Geneious R11 tool, which the accession numbers are shown in Appendix 1. Additionally, for *E. coli* Phylogroups typing the http://clermontyping.iame-research.center/ and https://nickp60.pythonanywhere.com/ web sites were used. Basically, for MLST the Achtman scheme for *E. coli* characterization was used, of which the identification of allelic profiles of seven different housekeeping genes coding for fundamental metabolic functions: *adk, fumC, gyrB, icd, mdh, purA* and *recA*. Each allele is given a number and the combination of numbers is used in an MLST scheme to give each isolates a unique ST producing high levels of discrimination between isolates.

For virulence identification of *K. pneumoniae*, Kleborate open source https://github.com/katholt/Kleborate and Klebsiella H typing http://kaptive.holtlab.net/jobs were used. In addition, *rmpA, rmpA2, KpnO porin, iutA* and *magA* associated virulence genes with the following accession numbers: AY059958, AB289643, KT276272, NC_005249.1 and DQ677561.1, respectively, were detected using Geneious R11 tool.

For A. *baumannii* mutations in topoisomerase genes *parC* and *gyrA* were searched by comparison with *gyrA* and *parC* sequences of wild type (wt) *A. baumannii* (Accession number X094850.1 and EU886740.1) using Geneious R11 tool.

Core genome SNP Phylogenetic Tree

In this study a core genome SNP-based Phylogenetic Tree by ParSNP (https://github.com/marbl/parsnp) was build using all our sequenced 29 ExPEC strains and other 13 WGS of ExPEC isolates of different STs from United States of America (USA), India, United Kingdom (UK), Australia, Lebanon and Tanzania available from NBCI database with the following accession numbers: HG941718, JSLB01, JSQO01, JNPU01, JSPS01, AYNJ01, JTGI01, AXLI01, KI929774, CP006784, JSXJ01, JSXN01 and JSXO01. Phylogenetic reconstruction is a key in many studies which use whole genome sequence data from bacterial populations (Lees et al., 2018). There is various method to build the Phylogenetic Tree, including, Mauve, Mugsy, kSNP, Smalt, BWA and parsnp. In this study parsnp was used based on SNP clustering of 29 *E. coli* WGS.

CHAPTER THREE

RESULTS

Gram-negative bacteria (GNB) isolates

During the study period, 159 GNB were isolated, of which 128 (81%) were from the main Departments of HCM and 31 (19%) from other hospitals (HGM, HJM, HPQ and HCQ), in particular from Pediatric Departments. Of these 128 GNB, 29% were from Pediatric Department and 71%, from other departments, including Medicine, Surgery and Gynecology and Obstetrics, 59% (*n*=76) were isolated from blood and 38% (*n*=49) and 2% (*n*=3) from pus and cerebrospinal fluid (CSF), respectively. From the other hospitals, 31 GNB were isolated only from blood, where (26%, *n*=8), (13%, *n*=4), (32%, *n*=10) and (29%, *n*=9) were from HGM, HJM, HPQ and HCQ, respectively (Table 9).

Table 9. Gram-negative bacteria isolated from five hospitals in Mozambique.

HCM[a]	**GN isolates**	**Blood**	**Pus**	**CSF**
Pediatric Department	37	27 (73%)	9 (24%)	1 (3%)
Other Departments[b]	91	49 (54%)	40 (44%)	2 (2%)
Total in HCM	**128**	**76 (59%)**	**49 (38%)**	**3 (2%)**
Other hospitals Pediatric Department				
HGM[c]	8 (26%)	8	-	-
HJM[d]	4 (13%)	4	-	-
HPQ[e]	10 (32%)	10	-	-
HCQ[f]	9 (29%)	9	-	-
Total in other hospitals	**31**	**31**	**-**	**-**
Total of GNB in all hospitals	**159**	**107 (67%)**	-	-

[a] Hospital Central of Maputo; [b] Pediatric, Medicine, Surgery, Gynecology and Obstetrics. [c] Hospital General of Mavalane; [d] Hospital General of José Macamo; [e] Hospital Provincial of Quelimane; [f] Hospital Central of Quelimane.

Frequency of Gram-negative bacterial species isolated in HCM

Of 128 GNB from HCM, the most frequent species isolated were *K. pneumoniae* (35/128), Extra-intestinal *E. coli* ExPEC (25/128), *Acinetobacter* spp. (25/128) and *Pseudomonas* spp. (19/128). Other GNB less observed were *P. mirabilis* (7/128), *Enterobacter* spp. (5/128), *Salmonella* spp. (4/128), *M. morgani* (2/128), *K. oxytoca* (1/128), *K. variicola* (1/128), and *S. maltophilia* (4/128) (Figure 20). The *Acinetobacter* spp. included 23 *A. baumanni*, and 1 *A. complex*; *Pseudomonas* spp. included 17 *P. aeruginosa,* 1 *P. otitidis* and 1 *P. stutzeri*; *Enterobacter* spp. included 4 *Enterobacter complex* and 1 *E. cloacae*; and *Salmonella* spp. included 2 *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and 1 *S.* Isangi.

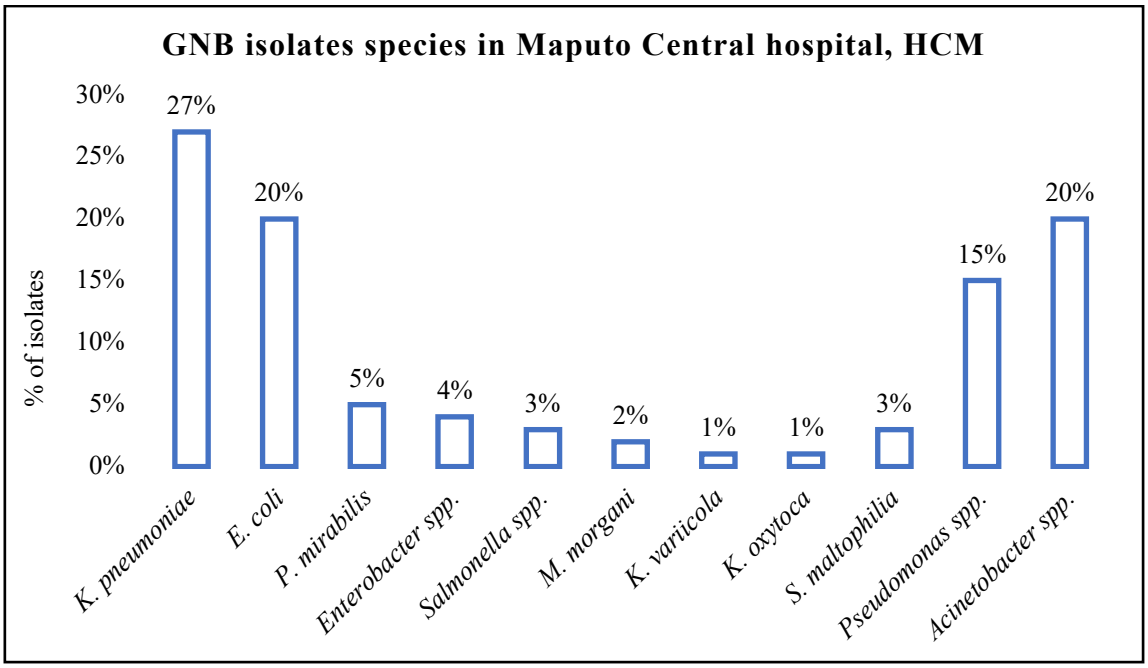


Fig. 20. Gram-negative bacterial species isolated at HCM.

Frequency of Gram-negative bacterial species isolated from Pediatric Department and Other Departments of HCM

ExPEC (30%) and *Acinetobacter* spp. (22%) were mostly isolated from Pediatric department compared to other departments of HCM. On the contrary, in other departments *K. pneumoniae* (32%) was the most abundant. No particular differences among departments were observed in isolation of *Pseudomonas* spp. (16% vs 14%),

Enterobacter spp. (4% vs 3%), *P. mirabilis* (5%), *Salmonella* spp. (3% vs 3%) and *Morganella morgani* (*M. morgani*) (3% vs 1%). *K. oxytoca* was only isolated in Pediatric department while *K. variicola* and *Stenotrophomonas maltophila* (*S. maltophilia*) were isolated in other departments than pediatrics (Figure 21).

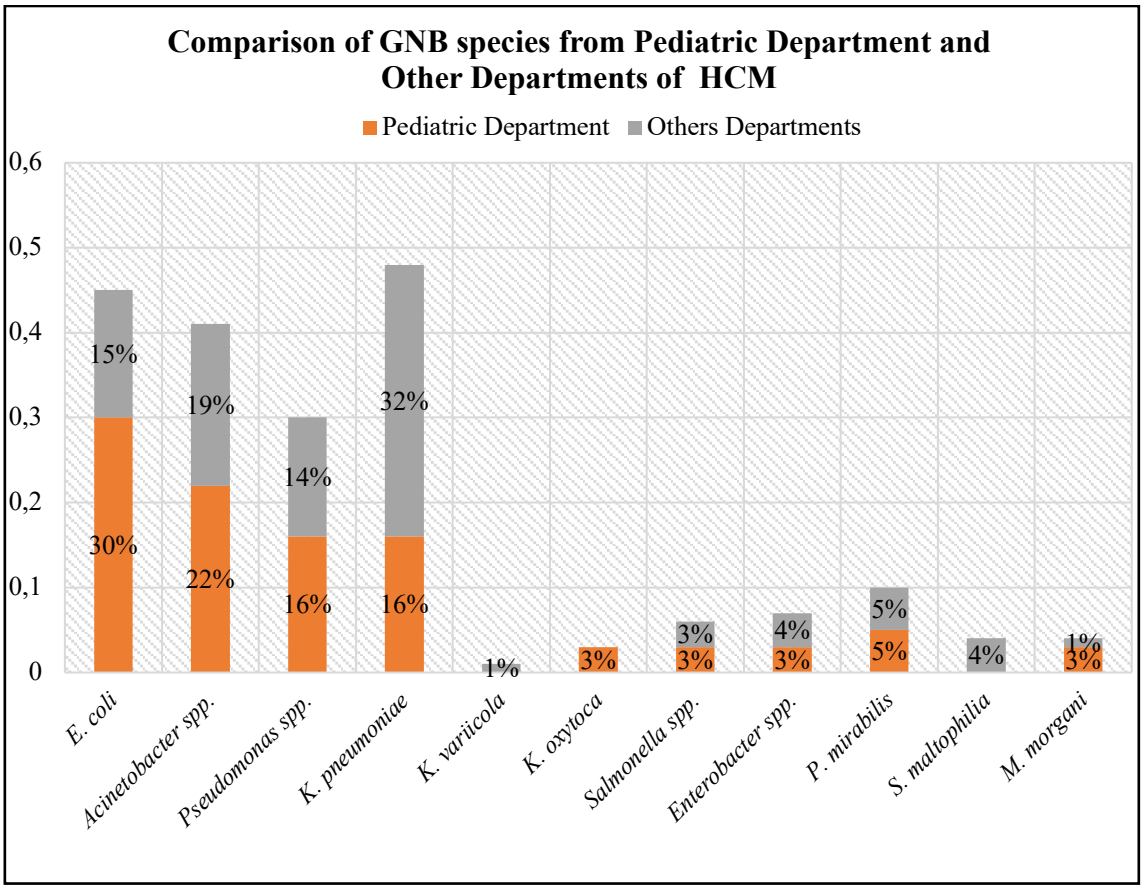


Fig. 21. Gram-negative bacteria species isolated in Pediatric department and other Departments of HCM.

Frequency of Gram-negative bacterial species isolated from Blood, Pus and CSF in HCM

Of the GNB isolated at HCM, *K. pneumoniae* (26/128) and *E. coli* (19/128) were most frequently isolated from blood, while *Acinetobacter* spp. (13/128), *Pseudomonas* spp. (12/128), and *P. mirabilis* (6/128) were more isolated from pus samples; from CSF only *K. pneumoniae* (2/128) and *Pseudomonas* spp. (1/128) were isolated. Moreover, *Enterobacter* spp., (5/128), *Salmonella* spp. (4/128), *M. morgani* (2/128), and *K. oxytoca* (1/128) were found only in blood while *K. variicola* (1/128), and (4/128), *S. maltophilia* only in pus samples (Figure 22).

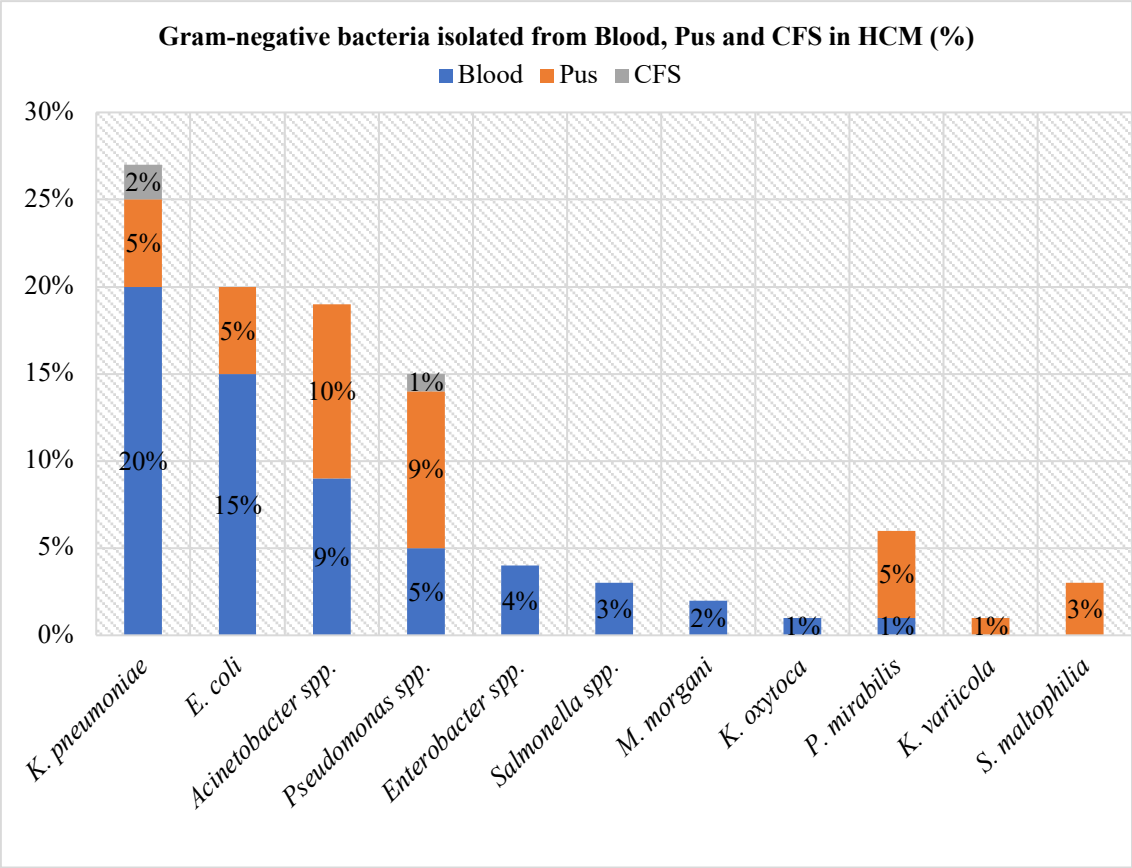


Fig. 22. Gram-negative bacterial species isolated from blood, pus and CSF in HCM.

Characteristics of Extraintestinal Pathogenic *Escherichia coli* ExPEC isolates

ExPEC (*n*=25) were together with *A. baumannii* the second most isolated bacterial species, mostly from Pediatric department and from blood culture (Figure 21 and 22).

Antimicrobial Susceptibilities of ExPEC

The antimicrobial susceptibility to 13 antimicrobials were evaluated using the Vitek 2 automated system. All ExPEC isolates were susceptible to tigecycline and colistin (Figure 23). The highest resistances were seen for trimethoprim-sulfamethoxazole (88%, 22/25), amoxicillin-clavulanic acid (84%, 21/25), cefotaxime (68%, 17/25), ceftazidime (68%, 17/25), gentamicin (60%, 15/25) ciprofloxacin (52%, 13/25), while meropenem and fosfomycin were only 4% (1/25) as showed in Figure 23.

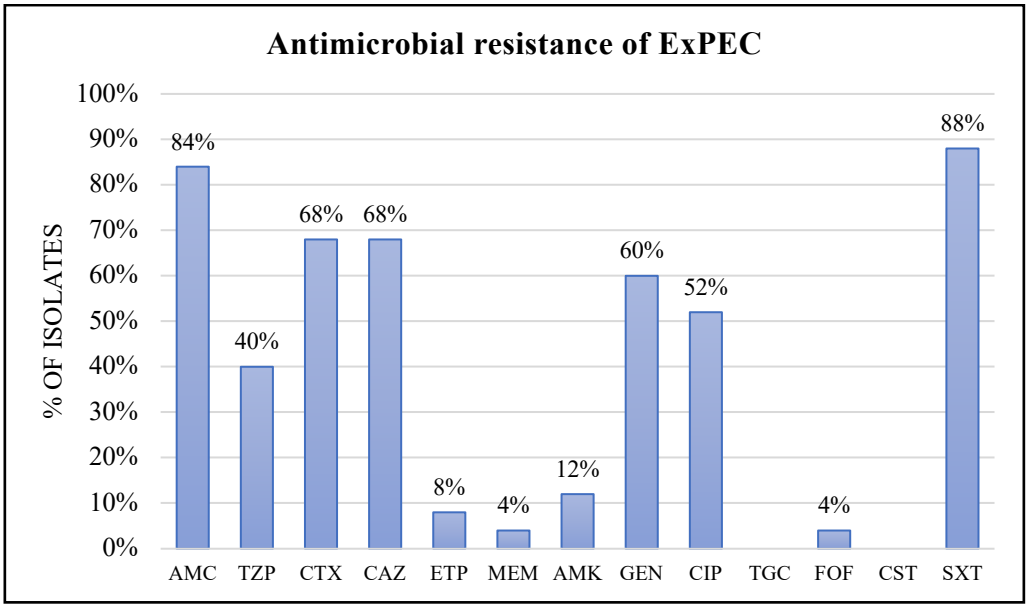


Fig. 23. Phenotypic resistance of ExPEC isolates at Central Hospital of Maputo, HCM.

Of the 25 ExPEC 72% (18/25) were MDR, and 40% (10/25), 20%, (5/25) and 8% (2/25) were ESBL, AmpC, and Carbapenemase potential producers, respectively (Figure 24).

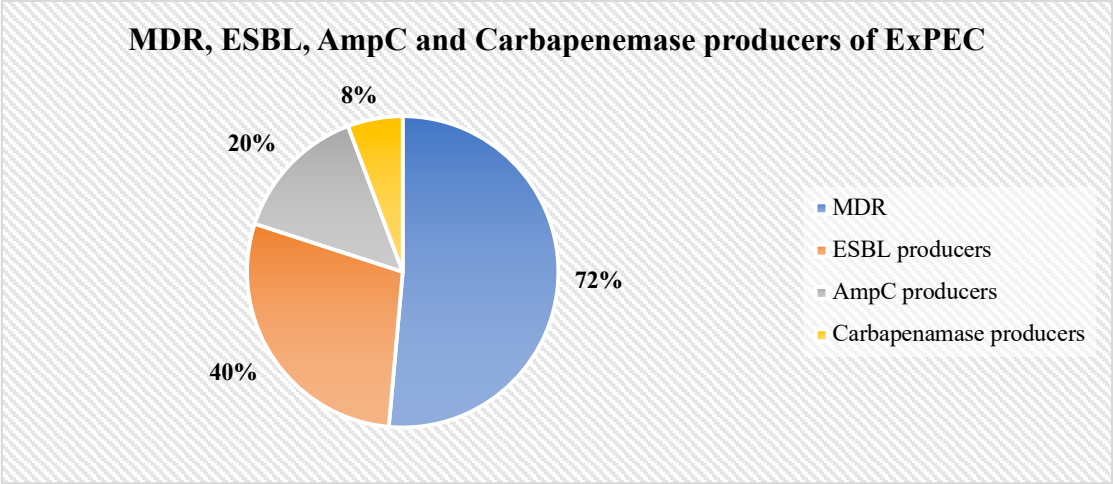


Fig. 24. Percentages of MDR, ESBL, AmpC and Carbapenemase producers in ExPEC from HCM.

Among the 17 β-lactamase producers ExPEC (10 ESBL, 5 AmpC and 2 Carbapenemase producers), (88%, 15/17) were also MDR suggesting the possibility of treatment failures. Most of these ExPEC β-lactamase producers were isolated in Pediatric department (53%, 9/17) from blood (*n*=8) and pus (*n*=1); 24% (4/17) in Medicine ward, all from blood, 18% (3/17) in surgery ward, all of them from pus samples; at last 6% (1/17) were from not specified department, especially from blood cultures.

Genetic determinants associated with antimicrobial resistance of ExPEC

By analyzing WGS a CTX-M type β-lactamase gene was found in nearly all β-lactamase producers ExPEC isolates (16/17), of which 70% harbored CTX-M-15 and 24% CTX-M-27. In addition, 32% (6/17) harbored AmpC CMY-2 and 6% (1/17) carbapenemase NDM-5 genes. ESBL, AmpC and carbapenemase were found associated with other extended- and narrow-spectrum β-lactamases. 59% (10/17) β-lactamase ExPEC producers showed different assortment of ESBL with *bla*$_{\text{CTX-M-15/OXA-1}}$ profile the most represented (40%), followed by *bla*$_{\text{CTX-M-27/TEM-1B}}$ (30%), *bla*$_{\text{CTX-M-15/TEM-1B/OXA-1}}$ (10%) and *bla*$_{\text{CTX-M-15/SCO-1/TEM-63-133}}$ (10%) (Table 10). Regarding AmpC ExPEC producers (29%), all possessed *bla*$_{\text{CTX-M-15/CMY-2/TEM-1B/OXA-1}}$. The two carbapenemase ExPEC producers showed different profiles of β-lactamases, *bla*$_{\text{CTX-M-15/NDM-5/TEM-1B}}$ and *bla*$_{\text{CMY-2/TEM-1B/OXA-1}}$. The 32% of ExPEC isolates were non-β-lactamase producers of which 75% harbored *bla*$_{\text{TEM-1B}}$ gene (Table 10).

Table 10. Sources and phenotypic resistances profile of ExPEC and ESBL, AmpC and Carbapenemase determinants isolated in HCM.

ExPEC isolate	Source	Department	ST	ESBL, AmpC or Carb producers	ESBL determinant genes	Other β-lactamase	Resistance profile	MDR
SS13	Blood	Pediatric	ST617	ESBL	CTX-M-15	OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	Yes
SS39	Blood	Pediatric	ST457	ESBL	CTX-M-27	Nil	CTX-CAZ-SXT	
SS50	Blood	Pediatric	ST131	ESBL	CTX-M-27	TEM-1B	AMC-CTX-CAZ-CIP-SXT	Yes
SS61	Blood	Pediatric	ST69	ESBL	CTX-M-15	OXA-1/TEM-1C	AMC-TZP-CTX-CAZ-GEN-SXT	Yes
SS101	Blood	Medicine	ST38	ESBL	CTX-M-27	TEM-1B	AMC-CTX-CAZ-SXT	Yes
SS101A	Blood	Medicine	ST38	ESBL	CTX-M-27	TEM-1B	AMC-TZP-CTX-CAZ-SXT	Yes
SS45A	Blood	Medicine	ST131	ESBL	CTX-M-15	TEM-1B/OXA-1	AMC-CTX-CAZ-AMK-GEN-CIP-SXT	Yes
SS53B	Blood	Pediatric	ST361	ESBL	CTX-M-15	TEM-63,-133/SCO-1	CTX-CAZ-GEN	
SS37P	Pus	Pediatric	ST131	ESBL	CTX-M-15	TEM-1B	AMC-CTX-CAZ-GEN-CIP-SXT	Yes
SS46P	Pus	Surgery	ST131	ESBL	CTX-M-15	TEM-1B	AMC-CTX-CAZ-GEN-CIP-SXT	Yes
SS6	Blood	Pediatric	ST410	AmpC	CTX-M-15/CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	Yes
SS26	Blood	NA	ST410	AmpC	CTX-M-15/CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	Yes
SS30	Blood	Pediatric	ST410	AmpC	CTX-M-15/CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	Yes
SS49	Blood	Pediatric	ST410	AmpC	CTX-M-15/CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	Yes
SS13P	Pus	Surgery	ST410	AmpC	CTX-M-15/CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-AMK-GEN-CIP-SXT	Yes
SS100	Blood	Medicine	ST405	Carbapenemase	CTX-M-15/NDM-5	TEM-1B	AMC-TZP-CTX-CAZ-ETP-MEM-AMK-GEN-CIP-SXT	Yes
SS34P	Pus	Surgery	ST410	Carbapenemase	CMY-2	TEM-1B/OXA-1	AMC-TZP-CTX-CAZ-ETP-GEN-CIP-SXT	Yes
SS67	Blood	Pediatric	ST131	*	Nil	TEM-1B	AMC-GEN-SXT	Yes
SS48C	Blood	Pediatric	ST131	*	Nil	TEM-1B	AMC-GEN-STX	Yes
SS21P	Pus	Medicine	ST1177	*	Nil	TEM-1B	AMC-CIP-SXT	Yes
SS117	Blood	NA	ST394	*	Nil	TEM-1B	AMC-SXT	
SS38A	Blood	NA	ST59	*	Nil	TEM-1B	AMC-SXT	
SS106	Blood	Medicine	ST62	*	Nil	TEM-1B	AMC	
SS116	Blood	NA	ST394	*	Nil	Nil	SXT	
SS14P	Pus	Medicine	ST648	*	Nil	Nil	Nil	

Abbreviations: AMC, amoxillicin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime, CAZ, ceftazidime; ETP, ertapenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; ESBL, extended-spectrum β-lactamases; AmpC, Plasmid mediated β-lactamases; Carb, carbapenemase; * Non-ESBL/AmpC/Carbapenemase producer; NA, not available; ST, sequence type by MLST.

52% of ExPEC isolates (13/25) were resistant to ciprofloxacin (CIP MIC >= 4) of which 85% (11/13) and 92% (12/13) were also resistant to aminoglycoside and β-lactamase producers, respectively. Resistance to ciprofloxacin were due to chromosomal mutations in topoisomerase genes, two in *gyrA* at codon S83L and D87N, one or two mutations in *parC* (S80I or E84V) and one in *parE* (S458A or I529L). Six isolates carried also *aac(6')Ib-cr* gene cassette (Table 11). Seven of the isolates encoded its fluoroquinolone-resistant *aac(6')Ib-cr.*

Table 11. Quinolone resistance phenotype and genotype of ExPECs isolated in HCM.

Isolate	ESBL, AmpC or Carb producers	CIP MIC (µg/mL)	Resistance gene	Aminoacid mutations					
				gyrA S83L	*gyrA D87N*	*parC S80I*	*parC E84V*	*parE S458A*	*parE I529L*
SS6*	AmpC	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS13*	ESBL	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS26*	AmpC	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS30*	AmpC	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS34P*	Carbapenemase	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS13P*	AmpC	>= 4	*aac(6')Ib-cr*	+	+	+		+	
SS50	ESBL	>= 4		+	+	+	+		+
SS100*	Carbapenemase	>= 4		+	+	+	+		+
SS37P*	ESBL	>= 4		+	+	+	+		+
SS46P*	ESBL	>= 4		+	+	+	+		+
SS49*	AmpC	>= 4		+	+	+		+	
SS21P	**	>= 4		+		+			
SS45A*	ESBL	>= 4	*aac(6')Ib-cr*	+	+	+	+		+

Abbreviations: * ExPEC isolates also resistant to aminoglycosides; ** Non-ESBL/AmpC/Carbapenemase producer; ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb, carbapenemase; MIC, minimum inhibitory concentration; CIP, ciprofloxacin; +, positive.

Regarding aminoglycoside resistance, 60% (15/25) of isolates showed reduced susceptibility to gentamicin and 12% (3/25) to amikacin. Noteworthy of these 15 ExPECs resistant to aminoglycoside 73% (11/15) were also resistant to ciprofloxacin and were β-lactamase producers showing the coexistence of these mechanisms. The 15 ExPECs encoded at least 3 aminoglycoside-modifying enzymes (AMEs) with different assortment and profiles. The most represented combination was *aac(3)-IId/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id* in 27% (4/15); *aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id* or *aac(3)-IIa/aph(3'')-Ib/aph(6)-Id* in 20%; *aac(3)-IId/aph(3'')-Ib/aph(6)-Id* in 13%; *aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id* or *aac(3)-IId/aac(6')Ib-cr/aph(3')-IIb/aph(6)-Id* or *aac(3)-IIa/aph(3')-Ia/aph(3'')-Ib/aph(6)-Id/aadA2/rmtB* in 7% of isolates. The lack of *aac(6')Ib-cr* was associated with decreased MIC of amikacin (<= 1), while the presence of *rmtB*

gene (16S rRNA methyltransferase) with increased MIC to amikacin (=32). In addition, 67% (10/15) of ExPEC isolates possessed *aadA5*, another determinant of resistance to aminoglycoside (streptomycin-spectinomycin) (Table 12).

Table 12. Aminoglycoside susceptibility phenotype and genotype of ExPECs isolated in HCM.

ExPEC Isolate	ESBL, AmpC or Carb producers	AMK MIC (µg/mL)		GEN MIC (µg/mL)		Aminoglycoside resistance enzyme gene (AME)	Other Aminoglycoside resistance determinants
SS13*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS61	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	Nil
SS45A*	ESBL	16	I***	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS53B	ESBL	<= 1	S	>= 16	R	*aac(3)-IIa/aph(3'')-Ib/aph(6)-Id*	Nil
SS37P*	ESBL	<= 1	S	>= 16	R	*aac(3)-IId/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS46P*	ESBL	<= 1	S	>= 16	R	*aac(3)-IId/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS6*	AmpC	4	S	>= 16	R	*aac(3)-IId/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS26*	AmpC	4	S	>= 16	R	*aac(3)-IId/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS30*	AmpC	4	S	>= 16	R	*aac(3)-IId/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS13P*	AmpC	8	I***	>= 16	R	*aac(3)-IId/aac(6')Ib-cr/ aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS49*	AmpC	4	S	>= 16	R	*aac(3)-IId/aac(6')Ib-cr/aph(3')-IIb/aph(6)-Id*	*aadA5*
SS100*	Carbapenemase	32	R	>= 16	R	*aac(3)-IIa/aph(3')-Ia/aph(3'')-Ib/aph(6)-Id/rmtB*	*aadA2*
SS34P*	Carbapenemase	4	S	>= 16	R	*aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id*	*aadA5*
SS67	**	<= 1	S	>= 16	R	*aac(3)-IId/aph(3'')-Ib/aph(6)-Id*	Nil
SS48C	**	2	S	>= 16	R	*aac(3)-IId/aph(3'')-Ib/aph(6)-Id*	Nil

Abbreviations: ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb, carbapenemase; * ExPEC isolates also resistant to ciprofloxacin; ** Non-ESBL/AmpC/Carbapenemase producer; AMK, amikacin; GM, gentamicin; *** Intermediate but considered resistant; MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible.

Of the 25 ExPECs, 22 (88%) were resistant to trimethoprim-sulfamethoxazole with >= 320, where *dfrA17* was found alone (54%, 12/22) or associated with *dfrA8* in 3 cases. 18% possessed *dfrA7*, and 5% *dfrA12*. All ExPECs resistant to trimethoprim-sulfamethoxazole were also β-lactamases producers (ESBL, AmpC and Carbapenemases producers) (Table 13).

Table 13. Trimethoprim-sulfamethoxazole susceptibility phenotype and genotype analysis.

Isolate	ESBL, AmpC or Carb. Producers	Trimethoprim-sulfamethoxazole MIC (µg/mL)		Trimethoprim-sulfamethoxazole resistance mechanism
SS13	ESBL	>= 320	R	*dfrA17*
SS39	ESBL	>= 320	R	*dfrA17*
SS50	ESBL	>= 320	R	*dfrA17*
SS61	ESBL	>= 320	R	*dfrA7*
SS101	ESBL	>= 320	R	*dfrA8/dfrA17*
SS101A	ESBL	>= 320	R	*dfrA8/dfrA17*
SS45A	ESBL	>= 320	R	*dfrA17*
SS37P	ESBL	>= 320	R	*dfrA17*
SS46P	ESBL	>= 320	R	*dfrA17*
SS6	AmpC	>= 320	R	*dfrA17*
SS26	AmpC	>= 320	R	*dfrA17*
SS30	AmpC	>= 320	R	*dfrA8/dfrA17*
SS49	AmpC	>= 320	R	*dfrA17*
SS13P	AmpC	>= 320	R	*dfrA17*
SS100	Carbapenemase	>= 320	R	*dfrA12*
SS34P	Carbapenemase	>= 320	R	*dfrA17*
SS67	*	>= 320	R	*dfrA7*
SS21P	*	>= 320	R	*dfrA17*
SS117	*	>= 320	R	*dfrA8*
SS38A	*	>= 320	R	*dfrA7*
SS116	*	>= 320	R	*dfrA8*
SS48C	*	>= 320	R	*dfrA7*

* Non-ESBL/AmpC/Carbapenemase producer; ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb., carbapenemase; MIC, minimum inhibitor concentration; R, resistant.

Other Resistance determinants mechanisms in ExPEC

All of 25 ExPEC, possess *mdf(A)* determinant gene which encode resistance to chloramphenicol, erythromycin and certain aminoglycosides and fluoroquinolones. In addition, *mdf(A)* were found associated with other determinants genes such as *mph(A)*, *sul1/2*, [*catA1/B3*, *floR*] and *tet(A)/(B)*, encoding resistance to macrolides, sulfonamides, phenicol and tetracycline, respectively (Table 14).

Table 14. Other resistance determinants genes found in ExPEC isolated in HCM.

Isolate	Source	ESBL, AmpC or Carb producers	Other Resistance determinants
SS13	Blood	ESBL	*mdf(A)/mph(A)/catB3/sul1/tet(A)*
SS39	Blood	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS50	Blood	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS61	Blood	ESBL	*mdf(A)/catB3/sul1/sul2/tet(A)*
SS101	Blood	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS101A	Blood	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS45A	Blood	ESBL	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(A)*
SS53B	Blood	ESBL	*mdf(A)/mph(A)/sul2/tet(A)/floR*
SS37P	Pus	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS46P	Pus	ESBL	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS6	Blood	AmpC	*mdf(A)/mph(A)/sul1/sul2/tet(A)/tet(B)*
SS26	Blood	AmpC	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(B)*
SS30	Blood	AmpC	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(B)*
SS49	Blood	AmpC	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(B)*
SS13P	Pus	AmpC	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(B)*
SS100	Blood	Carbapenemase	*mdf(A)/catA1/sul1/sul2/tet(B)*
SS34P	Pus	Carbapenemase	*mdf(A)/mph(A)/catB3/sul1/sul2/tet(B)*
SS67	Blood	*	*mdf(A)/catA1/sul1/sul2*
SS21P	Pus	*	*mdf(A)/mph(A)/sul1/sul2/tet(A)*
SS117	Blood	*	*mdf(A)/sul2*
SS38A	Blood	*	*mdf(A)/sul1/sul2*
SS106	Blood	*	*mdf(A)/tet(B)*
SS116	Blood	*	*mdf(A)/sul2/tet(B)*
SS14P	Pus	*	*mdf(A)*
SS48C	Blood	*	*mdf(A)/catA1/sul1/sul2*

Abbreviations: ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb, carbapenemase; * Non-ESBL, AmpC or Carb. producers.

MOLECULAR CHARACTERIZATION OF ExPEC

WGS of each strain was analized to determine ST (using MLST) and CHtype, phylotype, serotype, Replicon typing of plasmids and virulence profile.

By Multi Locus Sequence Typing analysis, 13 different STs were found among ExPEC isolates. The majority belonged to ST131 (*n*= 6), ST410 (*n*= 6), ST38 (*n*=2), and ST394 (*n*= 2), others STs as ST405, ST361, ST617, ST1177, ST69, ST457, ST59, ST62, ST648 were found in unique isolates (Table 15). The STs associated with the different resistance determinants are shown in Table 10 and 15).

CH typing of 25 ExPEC identified 14 clonotypes, with *fumC4_fimH24* being the most prevalent, which was present only in ST410 (*n*=6) AmpC-producing isolates carrying CMY-2 (Table 15). On the contrary to the ST410, ST131 (*n*=6) were associated with three clonotypes, first *fumC40_fimH30* both belonging to H4:O25 serotype and ESBL producers carrying different CTX-M type (CTX-M-15 and CTX-M-27) (*n*=2), second *fumC40_fimH41* belonging to H5:O16 and ESBL producers harboring CTX-M-15 (*n*=2), and third *fumC40_fimH27* associated with two serotypes, H4 O25 and H4 O18-O18ac, both non-ESBL producers (Table 15). Other STs showed specific clonotype, except ST394 that was untypeable.

E. coli is phylotyped into 7 major phylogroups A, B1, B2, C, D, E, F of which pathogenic ExPEC are represented by A, B2, C, D and F.

Five phylotypes were found among our isolates, 8% (2/25) were of phylogroup A, 24% (6/25) B2, 24% (6/25) C, 28% (7/25) D, and 16% (4/25) F. All phylotypes included more than one serogroup, except for phylogroup C which included only ST410-H9:O8 serogroup isolates (Table 15).

Overall, 109 genes commonly found in ExPEC were detected. The predominant virulence gene was *fimH* (60%, *n*=15/25), encoding for Type 1 fimbriae followed by *traT* (44%, 11/25), *iha* and *sitA* (36%, 9/25), *papA*, *iss*, *kpsM*, *iutA*, and *sat* (32%, 8/25), *fyuA* (24%, 6/25), *iucC* (20%, 5/25), *maxL* (16%, 4/25), *usp* (10%, 3/25), *hlyD* (8%, 2/25), and *papC*, *ompT*, *ireA*, *iroN* and *vat* (4%, 1/25). Other virulence genes, namely: *papG*, *sfaA*, *sfaS*, F1C fimbriae (*foc*), *cvaC*, *K1* and *ibeA* were not found. *usp* was found associated only with ST131 in HCM (Appendix 2).

Further 88 genes, not typically of ExPEC, were detected in *E. coli* isolates, the majority was *crl* and *gad* (64% 16/25), followed by *eilA* and *senB* with (40%, 10/25) and (32%, 8/25), respectively (Appendix 3). Notably, one strain (B2-ST131-O18 O18ac) was hybrid harboring also typically virulence genes of Intestinal pathogenic *E. coli* (IPEC), namely *agg3B, agg3C, agg3D* and *agg5A*.

The isolates that accumulated virulence genes (>= 6) belonged to ST131, ST405, ST62, ST69 and ST1177. Other STs (ST361, ST38, ST394, ST410, ST457, ST59, ST617, ST648) showed <= 3 virulence genes.

Of the 18 ExPEC MDR, 44% (8/18) showed >= 6 ExPEC virulence associated genes (VAG). Sequence types ST131 80% (5/6), ST1177, ST405 and ST69 harbored >= 6 virulence and were also MDR. ST38, ST410 and ST617 were only MDR with <= 3 VAG (Table 15).

Table 15. Typing, and Resistance phenotype of ExPEC isolated in HCM.

Isolate	ST	Phylotype	Serotype	CH	R type	N of Typical VAG
SS53B	ST361	A	H30:O9	*fumC99_fimH398*	ESBL	2
SS13	ST617	A	H10	*fumC11-fimH0*	ESBL/MDR	2
SS50	ST131	B2	H4:O25	*fumC40_fimH30*	ESBL/MDR	13
SS37P	ST131	B2	H5:O16	*fumC40_fimH41*	ESBL/MDR	12
SS45A	ST131	B2	H4:O25	*fumC40_fimH30*	ESBL/MDR	10
SS46P	ST131	B2	H5:O16	*fumC40_fimH41*	ESBL/MDR	6
SS67	ST131	B2	H4:O25	*fumC40_fimH27*	MDR	1
SS48C	ST131	B2	H4:O18-O18ac	*fumC40_fimH27*	MDR	14
SS30	ST410	C	H9:O8	*fumC4_fimH24*	AmpC/MDR	1
SS34P	ST410	C	H9:O8	*fumC4_fimH24*	Carb/MDR	1
SS6	ST410	C	H9:O8	*fumC4_fimH24*	AmpC/MDR	1
SS26	ST410	C	H9:O8	*fumC4_fimH24*	AmpC/MDR	1
SS49	ST410	C	H9:O8	*fumC4_fimH24*	AmpC/MDR	0
SS13P	ST410	C	H9:O8	*fumC4_fimH24*	AmpC/MDR	1
SS101	ST38	D	H18:O66	*fumC26-fimH0*	ESBL/MDR	1
SS101A	ST38	D	H18:O86	*fumC26-fimH0*	ESBL/MDR	3
SS117	ST394	D	H18:O44-O77-O17	-	-	0
SS116	ST394	D	H18:O44-O77-O17	-	-	1
SS21P	ST1177	D	H15:O1	*fumC26_fimH65*	MDR	10
SS100	ST405	D	H6:O102	*fumC37_fimH29*	Carb/MDR	10
SS61	ST69	D	H18:O15	*fimH27-fimH0*	ESBL/MDR	6
SS39	ST457	F	H25:O11	*fumC88_fimH145*	ESBL	1
SS38A	ST59	F	O6	*fimC0_fimH41*	-	2
SS106	ST62	F	O7	*fimC0_fimH44*	-	9
SS14P	ST648	F	H42:O83	*fumC4_fimH58*	-	1

Abbreviations: ST, sequence type; CH, *fumC* and *fimH* type; R-type: Resistance type; ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb, carbapenemase producer; MDR, multi-drug resistant; VAG, virulence associated genes.

Main features of ExPEC carrying CTX-M type

In total 64% (16/25) ExPECs isolates from HCM possessed *bla*$_{\text{CTX-M}}$ types ESBL, *bla*$_{\text{CTX-M-15}}$ (75%, 12/16) was predominant followed by *bla*$_{\text{CTX-M-27}}$ (25%, 4/16). Of the 16 ESBL producers 88% were also MDR.

The *bla*$_{\text{CTX-M}}$ type was found in different phylotypes, STs and serotypes. Among 12 ExPEC that possess *bla*$_{\text{CTX-M-15}}$, 42% (5/12) belong to C-ST410-H9:O8 (phylotype-ST-serotype); 17% (2/15) B2-ST131-H5:O16; 8% (1/12) of B2-ST131-H4:O25; D-ST69-H18:O15; D-ST405-H6:O102; D-ST607-H10; and A-ST361-H30:O9.

ST38-H18:O66_O86, B2-ST131-H4:O25, F-ST457-H25:O11.

Among the four ST131 carrying *bla*$_{\text{CTX-M}}$ types, two clonotypes were found: *fumC40_fimH30* and *fumC40_fimH41*, in isolate SS45A (subclone *H*30Rx, clade C2) and in SS37P and SS46P isolates (subclone *H*41, clade A) respectively. Other clonotypes were found associated with *bla*$_{\text{CTX-M-15}}$ including *fumC99_fimH398* and *fumC0_fimH27* from ST361and ST69, respectively.

bla$_{\text{CTX-M-27}}$ was found associated with ST131 *fumC40_fimH30* (subclone *H*30R-nM27, clade C1-M27), ST38 *fumC26-fimH0* and ST457 *fumC88_fimH145* clonotypes.

bla$_{\text{CTX-M-15}}$ was associated with IncF (92%, 11/12) and IncA/C2 (8%, 1/12) plasmid families, while *bla*$_{\text{CTX-M-27}}$ was found only associated with IncF plasmid family (100%, 4/4). IncFII plasmid replicon (15/15) was predominant. Of the 15 FII replicon, F1 allele were predominant among the 14 FAB formula found, where the majority IncF FAB was (36%, 5/14) [F1:A1:B49]; (29%, 4/14) [F1:A2:B20]; (21%, 3/14) [F2:A-:B10]; and (14%, 2/14) [F31:A4:B1].

The IncF pMLST [F1:A1:B49] were more common in C-ST410-O8 and [F1:A2:B20] in B2-ST131-O16-25. Other plasmid family were found in theses CTX-M types of ExPECs (Table 16 and Appendix 4).

Table 16. Main features of ExPECs carrying CTX-M type isolated in HCM.

Isolate	Source	Department	ESBL, AmpC or Carb. producers	ST	Phylotype	Serotype	CTX-M-type	Plasmid typing associated with CTX-M-type	IncF FAB	Other Plasmid replicon found	MDR
SS61	Blood	Pediatric	ESBL	ST69	D	H18:O15	CTX-M-15	IncFII	-	Col, HI1B, I1	Yes
SS45A	Blood	Medicine	ESBL	ST131	A	H4:O25	CTX-M-15	IncFII	[F1:A2:B20]	Col, I1	Yes
SS37P	Pus	Pediatric	ESBL	ST131	B2	H5:O16	CTX-M-15	IncFII	[F1:A2:B20]	Col	Yes
SS46P	Pus	Surgery	ESBL	ST131	B2	H5:O16	CTX-M-15	IncFII	[F1:A2:B20]	Col	Yes
SS6	Blood	Pediatric	AmpC	ST410	C	H9:O8	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F1:A1:B49]	Col, Q1, p0111	Yes
SS26	Blood	-	AmpC	ST410	C	H9:O8	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F1:A1:B49]	Col, Q1, p0111	Yes
SS30	Blood	Pediatric	AmpC	ST410	C	H9:O8	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F1:A1:B49]	Col, Q1, p0111	Yes
SS49	Blood	Pediatric	AmpC	ST410	C	H9:O8	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F1:A1:B49]	Col, Q1, p0111	Yes
SS13P	Pus	Surgery	AmpC	ST410	C	H9:O8	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F31:A4:B1]	Col, Q1, p0111	Yes
SS100	Blood	Medicine	Carbapenemase	ST405	D	H6:O102	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F1:A1:B49]	Col, I1, I2, Q1, X4	Yes
SS13	Blood	Pediatric	ESBL	ST617	A	H10	CTX-M-15	IncFII(pAMA1167-NDM-5)	[F31:A4:B1]	I1, I2, Q1, X4	Yes
SS53B	Blood	Pediatric	ESBL	ST361	A	H30:O9	CTX-M-15	IncA/C2	-	-	No
SS50	Blood	Pediatric	ESBL	ST131	B2	H4:O25	CTX-M-27	IncFII	[F1:A2:B20]	Col	Yes
SS101	Blood	Medicine	ESBL	ST38	D	H18:O66	CTX-M-27	IncFII	[F2:A-:B10]	Col, Y	Yes
SS101A	Blood	Medicine	ESBL	ST38	D	H18:O86	CTX-M-27	IncFII	[F2:A-:B10]	Y	Yes
SS39	Blood	Pediatric	ESBL	ST457	F	H25:O11	CTX-M-27	IncFII	[F2:A-:B10]	-	No

Abbreviations: ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases; Carb., carbapenemase; MDR, Multi-drug resistant; IncF FAB, FII, FIA and FIB incompability replicon; ST, Sequence type; -, negative.

Phylogenetic analysis of ExPEC isolated in all hospitals

In this study a core genome SNP-based Phylogenetic tree was constructed using 31 genomes including our ExPEC of 16 STs and *E. coli* of different STs from India, USA, UK, Australia, Lebanon and Tanzania with the following accession numbers: HG941718, JSLB01, JSQO01, JNPU01, JSPS01, AYNJ01, JTGI01, AXLI01, KI929774, CP006784, JSXJ01, JSXN01 and JSXO01.

On the cgSNP Tree, ST131 detected in this study formed 4 clusters. The same ST131 clone was found in Pediatric (SW37P) and Surgery (46P) departments of HCM (Figure 25). All the ST410 isolates clustered together, of which only two isolates were clonal (JW6 from pediatric and 26 from unknown ward).

Moreover, ST394 isolates (116 and 117) from unknown wards and ST38 (101 and 101A) from Medicine were clonal (Figure 25). ST4085 (JW522) from healthy people, clustered with ST156 (127A).

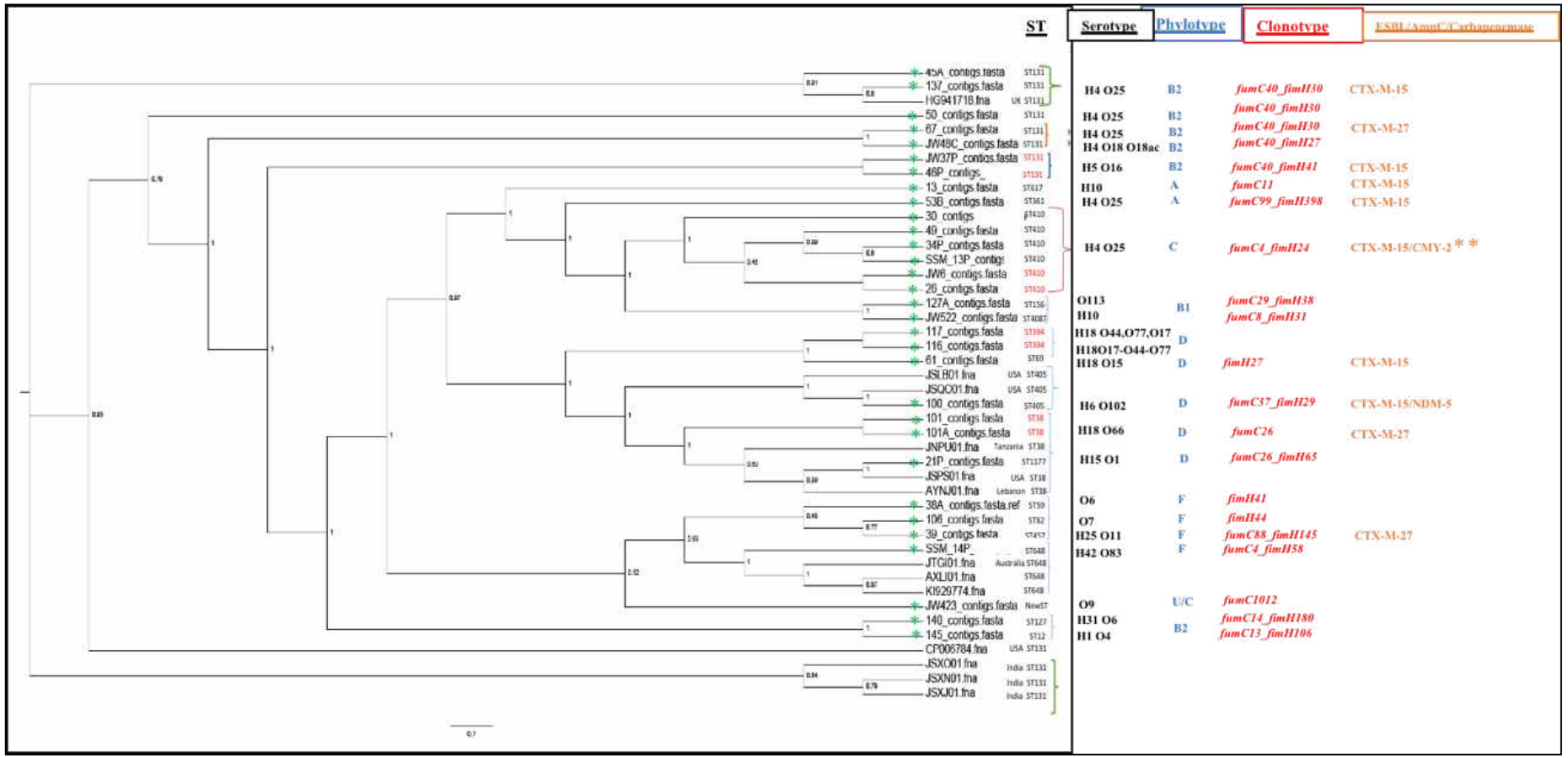


Fig. 25. Core genome SNP-based Phylogenetic Tree of ExPEC isolated in all hospitals and other (*n*=13) isolates from India, USA, UK, Australia, Lebanon and Tanzania (HG941718, JSLB01, JSQO01, JNPU01, JSPS01, AYNJ01, JTGI01, AXLI01, KI929774, CP006784, JSXJ01, JSXN01 and JSXO01). * *E. coli* isolates from our study. ** 34P *E. coli* isolate harbored only CMY-2 AmpC; U/C, U/cryptic phylotype; In brackets are shown *E. coli* isolates belonging to the same clone or clurster; ST, Sequence Type; Serotype, Phylotype, Clonotype, ESBL, AmpC and Carbapenemase are reported for isolates from this study.

Phenotypic and genotypic characterization of Intestinal *Escherichia coli* isolated from Healthy people in Maputo

Three additional strains isolated in feces of healthy people were included in this study due to *E. coli* are facultative pathogens being part of the normal human intestinal microbiome. The following profiles (phylotype-ST-serotype), B2-ST569-H31:O46-O134 and B1-ST4086-H10 both carrying narrow β-lactamase (TEM-1B) and one clone (new ST) harboring ESBL (SHV-185) were found. In addition, these β-lactamases were found in association with other determinants mechanisms conferring resistance to macrolide [*mdf(A)* and *mph(A)*] aminoglycoside [*aph(3'')-Ib*, *aph(6)-Id* and *aadA5*], fluoroquinolone (*oqxA* and *oqxB*), fosfomycin (*fosA*), sulphonamide (*sul1* and *sul2*) and trimethoprim (*dfrA7, dfrA14* and *dfrA17*). ST569 carried TEM-1B associated with IncF[F29:A-:B10] pMLST and possessed more several resistance and virulence associated genes than other clones. Interestingly, efflux pumps (*oqxA* and *oqxB*) were only found in new ST from feces of heathy people (Table 17). Additionally, all ExPEC found in present study did not harbor any efflux pumps mechanism.

Table 17. Main features of 3 *E. coli* isolated in feces in Maputo.

Isolate	Source	ST	VAG	Phylotype	Serotype	β-lactamases type	Other mechanisms	IncF RST	Other Plasmid replicon	Resistance profile*
SS423	Feces	New alleles	*fumC1012*	U/C	O9	SHV-185	*oqxA/oqxB/fosA*	-	-	AMP-AMC-CAZ-NIT-STX
SS453	Feces	ST569	*fumC38_fimH386/mchB/vat/senB/usp*	B2	H31:O46-O134	TEM-1B	*aph(3'')-Ib/aph(6)-Id/aadA5/mdf(A)/mph(A)/sul1/sul2/dfrA7/dfrA17*	[F29:A-:B10]	Col156, FIB, FII, Q1	AMP-AMC-FOX-NIT-NAL-STX
SS522	Feces	ST 4086	*fumC8-fimH31*	B1	H10	TEM-1B	*aph(3'')-Ib/aph(6)-Id/mdf(A)/mph(A)/sul2/dfrA14*	-	-	AMP-AMC-FOX-CAZ-CRO-NIT-NAL-STX

Abbreviation: * Antimicrobial susceptibility test was done by Kirby Bauer; AMP, ampicillin; AMC, amoxillicin-clavulanic acid; CAZ, ceftazidime; SXT, trimethoprim-sulfamethoxazole; FOX, cefoxitin; CRO, ceftriaxone; NIT, nitrofurantoin; NAL, nalidixic acid; ST, sequence type; VAG, virulence associated genes; RST, replicon sequence type.

Characteristics of *Klebsiella pneumoniae* isolates

Antimicrobial Susceptibilities of *Klebsiella pneumoniae* isolates

K. pneumoniae represent the 27% of GNB isolated in this study, mainly from pediatric departmernts of HCM and predominantly from blood. In total 35 *K. pneumoniae* were isolated, of which 83% (29/35) showed high resistance to amoxicillin-clavulanic acid, 77% to cefotaxime and ceftazidime (27/35) and, trimethoprim-sulfamethoxazole, 74% to gentamicin (26/35), and 71% to piperacillin-tazobactam and ciprofloxacin (25/35). The least resistance was seen to tigecycline and fosfomycin, (3%, 1/35) and (29%, 10/35), respectively. All *K. pneumoniae* were susceptible to ertapenem, meropenem, amikacin and colistin (Figure 26).

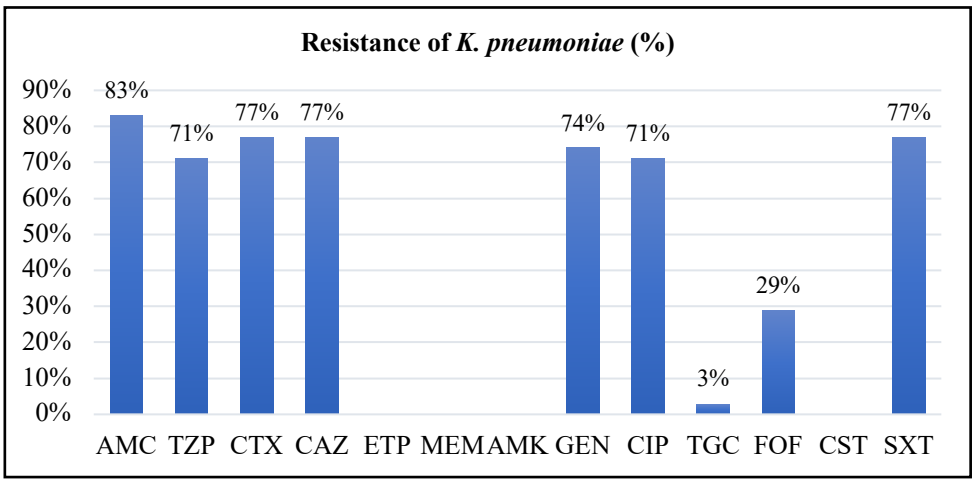


Fig. 26. Resistance profile of *K. pneumoniae* isolated in HCM.

Additionally, 80% (28/35) and 77% of *K. pneumoniae* isolates (27/35) were MDR and ESBL producers, respectively. None of isolates were AmpC or carbapenemase producers.

Genotypic antimicrobial resistance of *K. pneumoniae*

To study the determinants of resistance, PCR and WGS were performed on *n*=20 and *n*=15 *K. pneumoniae* isolates, respectively. 80% and 77% of *K. pneumoniae* isolates were MDR and ESBL producers, respectively. In all ESBL *K. pneumoniae* a β-lactamase of CTX-M group (22 $bla_{\text{CTX-M-15}}$, 10 $bla_{\text{CTX-M-9}}$ and 1 $bla_{\text{CTX-M-88}}$) was found except for one isolate which harbored a bla_{SHV}. $bla_{\text{CTX-M}}$ were found in association with other β-lactamases, mainly bla_{SHV} (*n*=22) and bla_{TEM} (*n*=23) (Table 18).

All 15 WGS of *K. pneumoniae* were analyzed in order to identify determinants of fluoroquinolone resistance and virulence factors. *K. pneumoniae* (*n*=11) possessed *oqxA* and *oqxB* genes encoding for efflux pumps, mainly associated with *aac(6')Ib-cr* and *acrR/ompK36/ompK37/ramR* porins genes that mediate resistance to ciprofloxacin and also resistance to β-lactam agents. In addition, plasmid mediated quinolone resistance PMQR genes *qnrB1* and *qnrB6* were identified in (6/11) and (2/11) isolates, respectively. Additionally, (10/11) *K. pneumoniae* resistant to ciprofloxacin were also resistant to aminoglycosides (Table 19). Conversely to ExPEC, mutations in genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) were not found in *K. pneumoniae*.

Table 18. ESBL analysis and resistance profile of *K. pneumoniae* isolated in HCM.

Isolate	Source	Department	ST	ESBL producer	ESBL Enzyme	Other β-lactamase	Resistance profile
SS1	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	Nil	AMC-TZP-CTX-CAZ-GEN-SXT
SS55	Blood	NA	NA	Yes	CTX-M	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS58	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS69	Blood	Medicine	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT
SS77	Blood	NA	NA	Yes	CTX-M	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT
SS80	Blood	NA	NA	Yes	CTX-M	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS112	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	Nil	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS113	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS115	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS12P	Pus	Medicine	NA	Yes	CTX-M	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT
SS20P	Pus	Medicine	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS54A	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS26_1P	Pus	Pediatric	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-CTX-CAZ-GEN-CIP-SXT
SS54C	Blood	NA	NA	Yes	CTX-M/CTX-M-9/CTX-M-15	TEM/SHV	AMC-CTX-CAZ-GEN-CIP-SXT
SS59A	Blood	Gyn./Obstretics	NA	Yes	-	SHV	AMC-TZP-CTX-CAZ-GEN-SXT
SS51	Blood	NA	NA	No	-	-	FOF
SS65	Blood	NA	NA	No	-	-	-
SS27L	CSF	NA	NA	No	-	-	FOF
SS57A	Blood	Medicine	NA	No	-	-	-
SS4P	Pus	NA	NA	No	-	TEM/SHV	AMC-CIP-SXT
SS35*	Blood	Medicine	ST13	Yes	CTX-M-15	OXA-1/SHV-101	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT
SS37*	Blood	NA	ST607	Yes	CTX-M-15/SHV-5, -98	TEM-1B/LEN12	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS64*	Blood	Pediatric	ST607	Yes	CTX-M-15/SHV-2	TEM-1B	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS73*	Blood	Medicine	ST711	Yes	CTX-M-15	TEM-1B/OXA-1/SHV-187	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT
SS79*	Blood	Pediatric	ST14	Yes	CTX-M-15	TEM-1B/OXA-1/SHV-28	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS85*	Blood	Medicine	ST48	Yes	CTX-M-15	TEM-1B/SHV-1/OXA-1	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS114*	Blood	Pediatric	ST607	Yes	CTX-M-15	TEM-1B/LEN12	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS10P*	Pus	NA	ST985	Yes	CTX-M-15/CTX-M-88	TEM-1C/OXA-1/SHV-187	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS52A*	Blood	NA	ST831	Yes	CTX-M-15/SHV-187	TEM-1B/OXA-1	AMC-CTX-CAZ-CIP-SXT
SS52B*	Blood	NA	ST831	Yes	CTX-M-15/SHV-187	OXA-1	AMC-TZP-CTX-CAZ-GEN-SXT
SS56P*	Pus	Medicine	ST607	Yes	CTX-M-15/SHV-65	TEM-1B	AMC-TZP-CTX-CAZ-GEN-CIP-SXT
SS58P*	Pus	Medicine	ST394	Yes	CTX-M-15/SHV-81, -110	TEM-1B	AMC-TZP-CTX-CAZ-GEN-CIP-TGC
SS63*	Blood	Pediatric ICU	ST17	No	Nil	TEM-1B/SHV-94, -96, -172	AMC-FOF
SS56*	Blood	Medicine	ST23	No	Nil	SHV-190	FOF
SS25L*	CSF	Pediatric ICU	ST23	No	Nil	SHV-190	FOF

Abbreviations: * Isolates subjected to Whole Genome Sequencing; ST, sequence type; NA, not available; AMC, amoxillicin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime, CAZ, ceftazime; GEN, gentamicin; CIP, ciprofloxacin; FOF, fosfomycin; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole; ESBL, extended-spectrum β-lactamases; Gyn, Gynecology; ICU, intesive care unit.

Table 19. Fluoroquinolone resistance phenotype and genotype of *K. pneumoniae* isolated in HCM.

Isolate	ESBL producers	CIP MIC (µg/mL)		Resistance genes										
				aac(6')Ib-cr	*oqxA*	*oqxB*	*qnrB1*	*qnrB6*	*qnrS1*	*acrR*	*ompK36*	*ompK37*	*ramR*	
SS35*	ESBL	>= 4	R	+		+	+	+			+	+	+	+
SS79*	ESBL	>= 4	R	+		+	+	+			+	+	+	+
SS10P*	ESBL	>= 4	R	+		+	+	+			+	+	+	+
SS37*	ESBL	>= 4	R	+		+	+		+		+	+	+	+
SS64*	ESBL	>= 4	R	+		+	+		+		+	+	+	+
SS114*	ESBL	>= 4	R	+		+	+		+		+	+	+	+
SS56P*	ESBL	>= 4	R	+		+	+		+		+	+	+	+
SS52A	ESBL	>= 4	R	+		+	+				+	+	+	+
SS85*	ESBL	>= 4	R	+		+	+				+	+	+	+
SS58P*	ESBL	>= 4	R			+	+				+	+	+	+
SS73*	ESBL	>= 4	R			+	+	+						

Abbreviations: ESBL, extended-spectrum β-lactamases; * *K. pneumoniae* isolates also resistant to aminoglycosides; MIC, minimum inhibitory concentration; CIP, ciprofloxacin; +, positive;

In ten of the 11 *K. pneumoniae* analyzed by WGS resistance to gentamicin was associated to *aac(3)-IIa* and other determinants: *aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id* (73%, 8/11). *aac(3)-IIa/aac(6')Ib-crb* (18%, 2/11) and *aac(3)-IIa* (9%, 1/11), 55% of them harbored resistance genes encoding for streptomycin-spectinomycin *aadA2, aadA14* and *aadA16* (Table 20).

Table 20. Aminoglycoside resistance phenotype and genotype of *K. pneumoniae* isolated in HCM.

Isolate	ESBL producers	AMK MIC (µg/mL)		GEN MIC (µg/mL		Aminoglycoside resistance genes	Streptomycin/spectinoomycin resistance gene
SS58P*	ESBL	<= 1	S	>= 16	R	*aac(3)-IIa*	-
SS10P*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr*	*aadA2*
SS52B	ESBL	<= 1	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr*	*aadA2*
SS35*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	-
SS64*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	-
SS73*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	-
SS85*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	-
SS79*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	*aadA14*
SS37*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	*aadA16*
SS114*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	*aadA16*
SS56P*	ESBL	4	S	>= 16	R	*aac(3)-IIa/aac(6')Ib-cr/aph(3")-Ib/aph(6)-Id*	*aadA16*

Abbreviations: * *K. pneumoniae* isolates also resistant to ciprofloxacin; ESBL, extended-spectrum β-lactamases; MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; -, negative.

11 out of 15 (73%) *K. pneumoniae* were resistant to trimethoprim-sulfamethoxazole encoded by *dfrA14* (27%, 3/11)*, dfrA7/dfrA27* and *drA5/dfrA12* (18%, 2/11), *dfrA7*, *dfrA12* and *dfrA27* (9%, 1/11) (Table 21).

All of 15 *K. pneumoniae* analyzed possessed *fosA* that encode resistance to fosfomycin and it is associated with other resistance determinants such as (*sul1* and *sul2*); (*catA1*, *catA2*, *catA3*, and *catB3*); [*mph(A)* and *mdf(A)*]; [*tet(A)* and *tet(D)*] and *ARR-3* encoding resistance to sulfonamides, phenicol, macrolides, tetracycline and rifampicin resistance, respectively (Table 21).

Table 21. Trimethoprim-sulfamethoxazole resistance and other determinants of *K. pneumoniae* in HCM.

Isolate	MIC (µg/mL)		ESBL producers	STX resistance mechanism	Other Resistance determinants
	STX	Phenotype			
SS35	>= 320	R	ESBL	*dfrA14*	*catB3/sul2/tet(A)/fosA*
SS73	>= 320	R	ESBL	*dfrA14*	*catA3/sul2/tet(A) fosA*
SS79	>= 320	R	ESBL	*dfrA14*	*catA3/sul2/fosA*
SS37	>= 320	R	ESBL	*dfrA7/dfrA27*	*mph(A)/catA2/sul1/sul2/ARR-3/fosA*
SS114	>= 320	R	ESBL	*dfrA7/dfrA27*	*catA2/ sul1/sul2/ARR-3/fosA*
SS52A	>= 320	R	ESBL	*drA5/dfrA12*	*mdf(A)/mph(A)/catA2/catA3/sul1/sul2/tet(D)/fosA*
SS52B	>= 320	R	ESBL	*drA5/dfrA12*	*mph(A)/catA2/catA3/sul1/sul2/ tet(D)/fosA*
SS64	>= 320	R	ESBL	*dfrA7*	*catA2/sul1/sul2/ARR-3/fosA*
SS85	>= 320	R	ESBL	*dfrA12*	*mph(A)/catA1/catA3/sul1/sul2/tet(A)/fosA*
SS10P	>= 320	R	ESBL	*dfrA12/dfrA14*	*mph(A)/catA2/catA3/sul1/sul2/tet(A)/fosA*
SS56P	>= 320	R	ESBL	*dfrA27*	*catA2/sul1/sul2/ ARR-3/fosA*
SS58P	<= 20	S	ESBL	-	*fosA*
SS56	<= 20	S	*	-	*fosA*
SS25L	<= 20	S	*	-	*fosA*
SS63	<= 20	S	*	-	*catA1/tet(D)/fosA*

Abbreviations: * Non-ESBL, extended-spectrum β-lactamases; SXT, trimethoprim-sulfamethoxazole; MIC, minimum inhibitory concentration; R, resistant; S, susceptible; -, negative.

Molecular characterization of *Klebsiella pneumoniae*

By MLST *in silico* analysis, 10 different sequence types were assigned proportionally distributed among ST13, ST48, ST985, ST711, ST394, ST17, ST14, while ST831 and ST23 (2), ST607 (4) were the most represented. 12 were ESBL producers, all of them harboring $bla_{\text{CTX-M-15}}$. One strain $bla_{\text{CTX-M-15}}$ has also CTX-M-88. The ST13, ST607, ST48, ST985, ST831, ST394, ST711 and ST14 harbored $bla_{\text{CTX-M-15}}$ and they were not hypervirulent, while ST23 (n=2) do not have $bla_{\text{CTX-M-15}}$, but show high virulence score due to KL1 capsule and conjugative element (ICEKp10) encoding siderophore yersiniabactin and colibactin. One ST23 isolate possessed both transcriptional regulators of capsular polysaccharide (CPS) biosynthesis (*rmpA* and *rmpA2*), *iutA* encoding aerobactin, *magA* encoding for the K1 mucoviscous serotype and *KpnO* porin, whereas another one harbored only *KpnO* porin. *KpnO* porin was also found in 5 STs non-hypervirulent, including ST13, ST607, ST394, ST17 and ST711. The IncFII(K) plasmid replicon was related to $bla_{\text{CTX-M-15}}$ in the majority of STs (83%, 10/12). Only ST14 and ST711 possess $bla_{\text{CTX-M-15}}$ linked with IncFIB(K) and Col(BS512) plasmid replicon,

respectively. Twelve FAB formula were detected, 5 belonging to [K5:A-:B-], 4 to [K13:A13:B-] and 1 to [K7:A13:B-], [K9:A-:B-] and [K8:A21:B-] (Table 22).

Table 22. Main features of *K. pneumoniae* carrying ESBLs in HCM.

Isolate	Source	Department	ST	ESBL producer	ESBL Enzyme	Plasmid typing associated with CTX-M	Other plasmid replicon	IncF RST	K type	Virulence -score	Yersiniabactin
SS35	Blood	Medicine	ST13	Yes	CTX-M-15	IncFII(K)	Col, FIB	[K5:A-:B-]	KL3	1	ybt 10; ICEKp4
SS37	Blood	NA	ST607	Yes	CTX-M-15	IncFII(K)	FIA, FIB, R	[K13:A13:B-]	KL25	1	ybt 14; ICEKp5
SS114	Blood	Pediatric	ST607	Yes	CTX-M-15	IncFII(K)	FIA, FIB, R	[K7:A13:B-]	KL25	1	ybt 14; ICEKp5
SS56P	Pus	Medicine	ST607	Yes	CTX-M-15	IncFII(K)	Col3M, FIA, FIB, R	[K13:A13:B-]	KL25	1	ybt 14; ICEKp5
SS64	Blood	Pediatric	ST607	Yes	CTX-M-15	IncFII(K)	FIA, FIB, R	[K13:A13:B-]	KL25	1	ybt 14; ICEKp5
SS85	Blood	Medicine	ST48	Yes	CTX-M-15	IncFII(K)	Col, FIB	[K5:A-:B-]	KL62	1	ybt 14; ICEKp5
SS10P	Pus	NA	ST985	Yes	CTX-M-15,M-88	IncFII(K)	FIB, HI1B	[K5:A-:B-]	KL39	1	ybt 16; ICEKp12
SS52A	Blood	NA	ST831	Yes	CTX-M-15	IncFII(K)	FIB, HI1B	[K5:A-:B-]	KL18	1	-
SS52B	Blood	NA	ST831	Yes	CTX-M-15	IncFII(K)	FIB, HI1B	[K5:A-:B-]	KL18	1	-
SS58P	Pus	Medicine	ST394	Yes	CTX-M-15	IncFII(K)	Col, FIB, Q1	[K13:A-:B-]	KL3	0	-
SS73	Blood	Medicine	ST711	Yes	CTX-M-15	Col(BS512)	-	-	K54	1	ybt 9; ICEKp3
SS79	Blood	Pediatric	ST14	Yes	CTX-M-15	IncFIB(K)	FII	[K9:A-:B-]	KL2	1	ybt 9; ICEKp3
SS63	Blood	Pediatric	ST17	No	-	-	FIA, FIB, FII	[K8:A21:B-]	KL25	0	-
SS56	Blood	Medicine	ST23	No	-	-	HI1B	-	KL1	5	ybt 1; ICEKp10
SS25L	CSF	Pediatric	ST23	No	-	-	HI1B	-	KL1	5	ybt 1; ICEKp10

Abbreviations: ST, Sequence type; ESBL, extended-spectrum β-lactamases; IncF RST, IncF replicon sequence type; -, negative.

Phenotypic and genotypic characterization of *Klebsiella variicola* and *Klebsiella oxytoca*

The *K. variicola* isolate was susceptible to all antibiotics tested while *K. oxytoca* isolate was resistant to amoxicillin-clavulanic acid, cefotaxime, ceftazidime, fosfomycin and trimethoprim-sulfamethoxazole; 100% were susceptible to piperacillin-tazobactam, ertapenem, meropenem, amikacin, ciprofloxacin, tigecycline and colistin. *K. oxytoca* was ESBL producers and MDR harboring $bla_{\text{CTX-M-15/TEM-1B/OXA-1/OXY-4-1}}$ and other determinants. $bla_{\text{CTX-M-15}}$ was associated with IncHI2 plasmid (Table 23).

Table 23. Main features of *K. variicola* and *K. oxytoca* isolated in HCM.

Species	Source	Department	ST	Resistance profile	ESBL producers	β-lactamases Enzyme	Other mechanisms	Plasmid typing associated with CTX-M-type
K. oxytoca	Blood	Pediatric	NST	AMC-CTX-CAZ-GEN-FOF-SXT	Yes	CTX-M-15/TEM-1B/OXA-1/OXY-4-1	*aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id/aadA1/oqxB/catA1/catA3/tet(A)/sul2/dfrA14*	IncHI2
K. variicola	Pus	NA	NA	-	-	-	-	-

Abbreviations: NST, new ST; ST, sequence type; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CAZ, ceftazime; GEN, gentamicin; FOF, fosfomycin; SXT, trimethoprim-sulfamethoxazole; ESBL, extended-spectrum β-lactamases; NA, not available; -, negative.

Phenotypic Antimicrobial resistance of other *Enterobacteriaceae*

The other *Enterobacteriaceae* isolated in this study showed multi-drug resistance, except *Salmonella* spp. that was resistant only to amoxicillin-clavulanic acid and trimethoprim-sulfamethoxazole 75% (3/4), and piperacillin-tazobactam 25% (1/4) (Figure 27).

M. morgani displayed the highest resistance pattern among these *Enterobacteriaceae*. The resistance to piperacillin-tazobactam, gentamicin, ciprofloxacin and colistin were 50% (1/2), and resistance to amoxicillin-clavulanic acid, cefotaxime, ceftazidime, fosfomycin, colistin and trimethoprim-sulfamethoxazole 100% (2/2) (Figure 27). In addition, 100% (2/2) were AmpC producers (Figure 28).

Enterobacter spp. was mostly resistant to amoxicillin-clavulanic acid 100% (5/5), followed by piperacillin-tazobactam, cefotaxime, ceftazidime, gentamicin and trimethoprim-sulfamethoxazole 80% (4/5); only ciprofloxacin and fosfomycin showed least resistance that was 40% (2/5) (Figure 27). 80% (4/5) of *Enterobacter* spp. were MDR and ESBL producers, and 40% (2/5) were AmpC producers (Figure 28).

P. mirabilis showed highest resistance to trimethoprim-sulfamethoxazole and colistin 86% (6/7) (data not confirmed by broth microdilution) followed by gentamicin 71% (5/7), amoxicillin-clavulanic acid, piperacillin-tazobactam, cefotaxime, ceftazidime and ciprofloxacin with 57% (4/7), and least resistance to amikacin and tigecycline 14% (1/7) (Figure 27). Additionally, 43% (3/7) of *P. mirabilis* were ESBL producers (Figure 28).

All 4 *Enterobacteriaceae* species were susceptible to carbapenem agents (Figure 27) and none AmpC and carbapenemase producers *Salmonella* spp., and *P. mirabilis* were found (Figure 28).

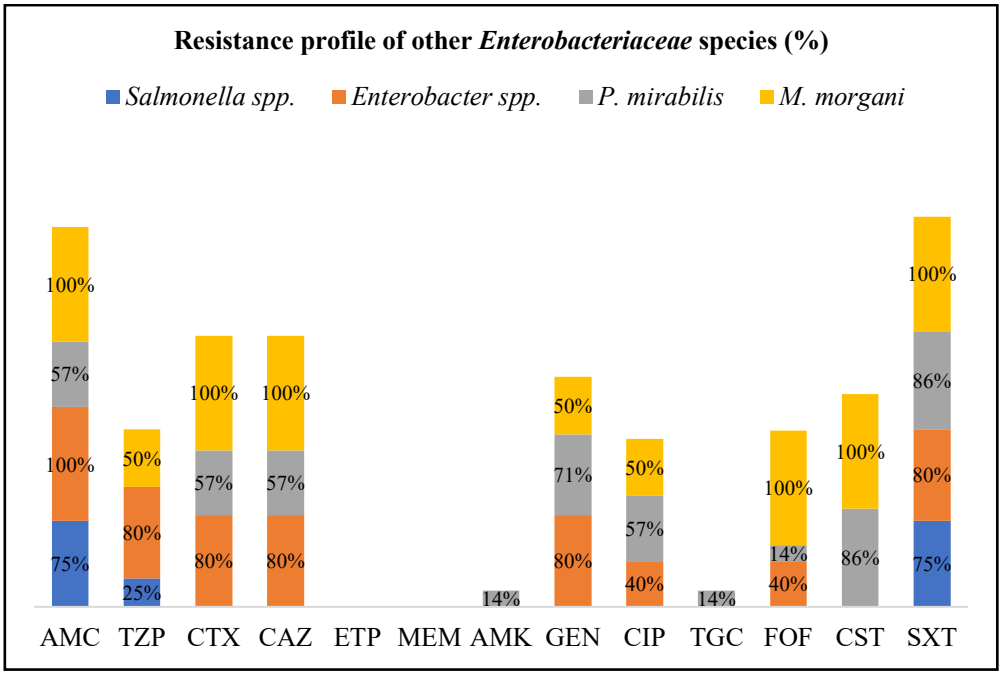


Fig. 27. Resistance profile of other *Enterobacteriaceae* isolated in HCM.

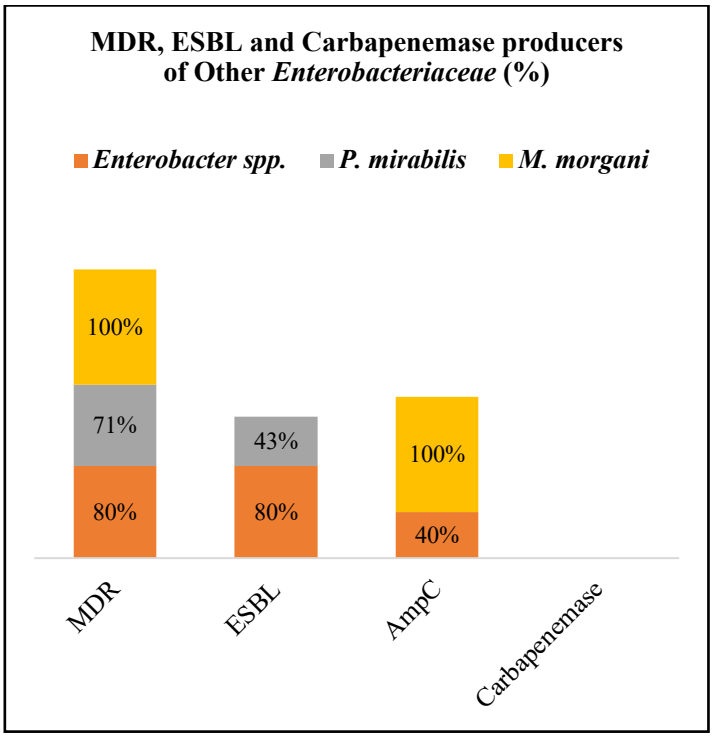


Fig. 28. MDR, ESBL and Carbapenemase producers of other *Enterobacteriaceae* species isolated in HCM.

Genotypic Antimicrobial resistance of *Salmonella* spp.

To study the determinants resistance of 15 *Salmonella* spp. isolated from all hospitals, PCR and WGS of (4/15) and (11/15) strains were performed, respectively. 3 out of 11 WGS of *Salmonella* spp. were isolated in HCM, and identified as *S.* Typhimurium (2) (ST313) and *S.* Isangi (1) (ST335). *S.* Isangi β-lactamase producer was mainly related to $bla_{\text{OXA-10}}$ associated with IncA/C2 plasmid replicon and 3 (1-2-2-2) pMLST while from *S.* Typhimurium mainly $bla_{\text{TEM-1B}}$ was found and associated with FIB(S), FII(S) of IncF pMLST [S1:A-:B17].

Other resistance determinants to macrolides *mph(A)*, aminoglycosides [*aac(6')-Iaa/aph(3'')-Ib/aph(6)-Id, aadA1*], sulfonamides (*sul1* and *sul2)*, tetracycline *tet(A)*, phenicol (*catA1*, *cmlA1* and *floR*), trimethoprim (*dfrA1*, *dfrA14* and *dfrA16*) and rifampicin *ARR-2* were found (Table 24).

Table 24. Main features of *Salmonella* spp. isolated in HCM.

Isolate	Species	Department	Source	ST	Resistance profile	β-lactamases	Plasmid replicon	IncF and A/C2 RST	Other mechanisms
SS32*	*S.* Isangi	NA	Blood	ST335	AMC-SXT	OXA-10	IncA/C2	3 (1-2-2-2)	*mph(A)/aac(6')-Iaa/aph(3")-Ib/aph(6)-Id/sul1/sul2/tet(A)/aadA1/cmlA1/dfrA14/dfrA16/ ARR-2/floR*
SS17*	*S.* Typhimurium	NA	Blood	ST313	AMC-TZP-SXT	TEM-1B	FIB(S), FII(S)	[S1:A-:B17]	*aac(6')-Iaa/aph(3")-Ib/aph(6)-Id/ aadA1/catA1/sul1/sul2/dfrA1*
SS22*	*S.* Typhimurium	Surgery	Blood	ST313	AMC-SXT	TEM-1B	FIB(S), FII(S)	[S1:A-:B17]	*aac(6')-Iaa/aph(3")-Ib/aph(6)-Id/ aadA1/catA1/sul1/sul2/dfrA1*
SS33	*Salmonella* spp.	Pediatric	Blood	-	-	-	-		-

Abbreviations: * Isolates analyzed by WGS (whole genome sequencing); NA, not available; AMC, amoxillicin-clavulanic acid; TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole; RST, replicon sequence type; ST, Sequence type; -, negative.

Genotypic prevalence of Antimicrobial resistance of *Enterobacter* spp.

4 out of 5 *Enterobacter* spp. isolated in HCM analyzed by WGS, were ESBL producers. Of the 4 ESBL *Enterobacter* spp., ST84 and ST125 were found and other isolates showed new STs. ST84, ST125 and other isolates possessed $bla_{\text{CTX-M-15}}$ associated with other β-lactamases (Table 25). Notably, ACT-7 and CMH-3 AmpCs were found from one of new ST and ST84, respectively. In this species, $bla_{\text{CTX-M-15}}$ was found mainly associated with IncFII(Yp) of [Y3:A-:B-] pMLST.

Other resistance determinants to macrolides [*mph(A)* and *ereA*], aminoglycosides [*aac(3)-IId, aac(6')Ib-cr, aac(6')-IIC, aph(3')-Ia, aph(3'')-Ib,* and *aph(6)-Id*] and sulfonamides (*sul1* and *sul2*), tetracycline [*tet(A)* and *tet(D)*], phenicol (*catB3*), fosfomycin (*fosA*), trimethoprim (*dfrA1*, *dfrA14* and *dfrA19*), colistin (*mcr-9*), and quinolone resistance were found (*qnrB1*) (Table 25).

Table 25. Main features of *Enterobacter* spp. isolated in HCM.

Isolate	Department	Source	ST	ESBL Producer	Resistance profile	β-lactamases	Plasmid associated with CTX-M-type	Other plasmid replicon	IncF/HI2 Replicon	Other mechanisms
SS110*	NA	Blood	New ST	ESBL	AMC-TZP-CTX-CAZ-GEN-SXT	ACT-7/TEM-1B/SHV-12	-	HI2, HI2A	HI2 (type 1)	*aac(6')-IIC/aph(3')-Ia/aph(3'')-Ib/aph(6)-Id/aadA2/catA2/sul1/sul2/tet(D)/dfrA19/mcr-9/ereA*
SS74*	Medicine	Blood	ST84	ESBL	AMC-TZP-CTX-CAZ-GEN-CIP-FOF-SXT	CTX-M-15/CMH-3/TEM-1B/OXA-1	IncFII(Yp)	HI2, HI2A, Y	-	*aac(3)-IId/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id/mph(A)/aadA1/catA1/catB3/catB7/tet(A)/dfrA14/qnrB1/fosA/ereA*
SS97*	Pediatric	Blood	ST125	ESBL	AMC-TZP-CTX-CAZ-GEN-CIP-SXT	CTX-M-15/TEM-1B/OXA-1	IncFII(Yp)	FIB, FII, HI2, HI2A	[Y3:A-:B-]	*aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id/catA1/catB3/sul1/sul2/tet(A)/dfrA1/dfrA14/qnrB1/fosA*
SS95*	Medicine	Blood	New ST	Non-ESBL	AMC-FOF	-	-	FIB, FII	-	*fosA*
SS111	NA	Blood	-	ESBL	AMC-TZP-CTX-CAZ-GEN-SXT	CTX-M-15/TEM-SHV	-	-	-	-

Abbreviations: * Isolates analyzed by WGS (whole genome sequencing); NA, not available; ST, sequence type; ESBL, extended-spectrum β-lactamases; AMC, amoxillicin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime, CAZ, ceftazime; GEN, gentamicin; CIP, ciprofloxacin; FOF, fosfomycin; SXT, trimethoprim-sulfamethoxazole; -, negative.

Genotypic prevalence of Antimicrobial resistance of *Proteus mirabilis* and *Morganella morganii*

A total 7 *P. mirabilis* was isolated, where PCR and WGS was performed in 5 and 2 isolates, respectively. 3 out of 7 were ESBL producers, having mainly CTX-M-15 associated with other β-lactamases mainly (TEM-63/OXA-1). CTX-M-15 was linked with A/C2 plasmid in this species (Table 26).

Other resistance mechanisms, aminoglycosides [*aac(3)*-IIa, *aac(6')Ib-cr*, *aph(3'')-Ib*, *aph(6)-Id*, and *aadA1*], sulfonamides (*sul2*), tetracycline [*tet(A)* and *tet(J)*], phenicol (*catB3*), trimethoprim (*dfrA1*), and quinolone resistance (*qnrD1*), florfenicol (*floR*) were found in *P. mirabilis*.

M. morganii was AmpC producers harboring DHAM gene (DHA-13) associated with OXA-1. Other resistance mechanisms such as aminoglycosides [*aac(6')Ib-cr* and *aadA5*], phenicol (*catA1* and *catA3*), sulfonamides (*sul1*), tetracycline *tet(B)* and trimethoprim (*dfrA17*) were also found (Table 26).

Table 26. Main features of *P. mirabilis* and *M. morganii* isolated in HCM.

Isolate	Species	Department	Source	ESBL/AmpC producers	β-lactamases	Other determinants genes	Plasmid typing associated with CTX-M-type	Other plasmid replicon	Resistance Profile
SS55P*	*P. mirabilis*	Pediatric	Pus	ESBL	CTX-M-15/TEM-63/OXA-1	*aac(3)-IIa/aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id/aadA1/cat/catB3/sul2/tet(A)/tet(J)/dfrA1/qnrD1/floR*	A/C2	Col3M	AMC-CTX-CAZ-GEN-CIP-CST-SXT
SS27P*	*P. mirabilis*	Medicine	Pus	ESBL	CTX-M-15/TEM-63/OXA-1	*aac(6')Ib-cr/aph(3'')-Ib/aph(6)-Id/aadA1/cat/tet(J)/dfrA1/qnrD1/floR*	A/C2	Col3M	AMC-CTX-CAZ-GEN-CIP-TGC-CST-SXT
SS26_2P	*P. mirabilis*	Pediatric	Pus	ESBL	CTX-M	-	-	-	AMC-CTX-CAZ-GEN-CIP-CST-SXT
SS50P	*P. mirabilis*	Medicine	Pus	**	TEM	-	-	-	AMC-GEN-CST-SXT
SS53P	*P. mirabilis*	Medicine	Pus	**	TEM	-	-	-	-
SS54P	*P. mirabilis*	Surgery	Pus	**	-	-	-	-	CTX-CAZ-AMK-GEN-CIP-FOF-CST-SXT
SS105	*P. mirabilis*	Medicine	Blood	**	-	-	-	-	CST-SXT
SS75*	*M. morganii*	Pediatric	Blood	AmpC	DHA-13/OXA-1	*aac(6')Ib-cr/aadA5/catA1/catA3/sul1/tet(B)/dfrA17*	-	-	AMC-TZP-CTX-CAZ-CIP-FOF-CST-SXT
SS81	*M. morganii*	Medicine	Blood	AmpC	DHAM	-	-	-	AMC-CTX-CAZ-CIP-FOF-CST-SXT

Abbreviations: * Isolates analyzed by WGS (whole genome sequencing); ** Non-ESBL producer; AMC, amoxillicin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime, CAZ, ceftazidime; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; FOF, fosfomycin; SXT, trimethoprim-sulfamethoxazole; CST, colistin; ESBL, extended-spectrum β-lactamases; AmpC, plasmid mediated β-lactamases.

Phenotypic Antimicrobial resistance of GNB non-fermenter

Antimicrobial results were only analyzed for meropenem, amikacin, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole and colistin to *A. baumannii*; piperacillin-tazobactam, ceftazidime, meropenem, amikacin, gentamicin, ciprofloxacin and colistin for *P. aeruginosa*; and only trimethoprim-sulfamethoxazole was analyzed for *S. maltophilia* due to the intrinsic resistance in these species to other antibiotics not mentioned (Figure 29).

Of the 25 *A. baumannii*, the highest resistances found were to trimethoprim-sulfamethoxazole 96% (24/25), gentamicin 92% (23/25), ciprofloxacin 76% (19/25) and meropenem 44% (11/25) and least to amikacin 12% (3/25). In addition, among *Acinetobacter* spp., 84% (21/25) and 100% were MDR and carbapenemase producers, respectively (Figure 29).

Regarding *Pseudomonas* spp., a total 19 strains were isolated, and piperacillin-tazobactam and ceftazidime were the highest ineffective 63% (12/19), followed by meropenem 47% (9/19), gentamicin and ciprofloxacin 42% (8/19); and least resistance was observed to amikacin 16% (3/19). All *Pseudomonas* spp. were susceptible to colistin. Additionally, 58% (11/19) and 89% (17/19) of *Pseudomonas* spp., were MDR and carbapenemase producers, respectively (Figure 29).

Colistin was completely effective against *A. baumannii* and *P. aeruginosa*, (data not confirmed by broth microdilution).

A total of 4 *S. maltophilia* were isolated of which 25% (1/4) was resistant to trimethoprim-sulfamethoxazole (Figure 29).

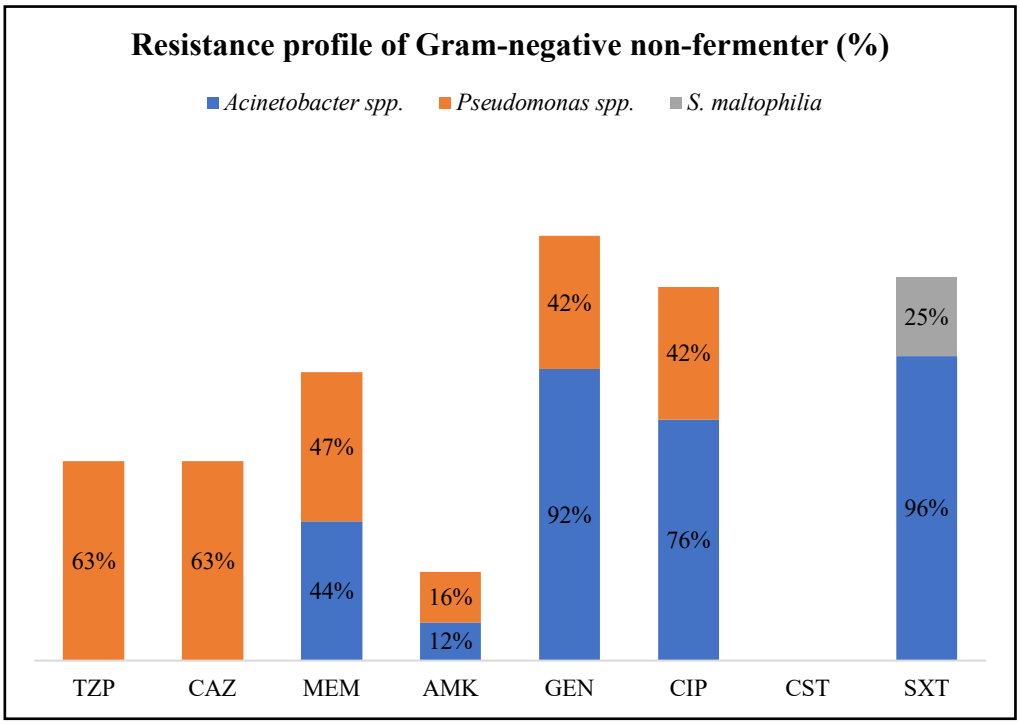


Fig. 29. Resistance profile of Gram-negative non-fermenter isolated in HCM.

Genotypic Antimicrobial resistance of *Acinetobacter baumannii*

6/25 and 19/25 of *A. baumanni* were analyzed by WGS and PCR, respectively. Among 6 WGS of *A. baumannii*, 3 STs were found, namely: ST758 (*n*=2), ST642, and ST405 (*n*=2). One strain showed a new ST.

The resistance phenotype to β-lactam agents, were associated with *bla*$_{\text{OXA}}$ (OXA-23, -25, -58, --65, -68, -69, -366), *bla*$_{\text{VIM}}$, *bla*$_{\text{ADC-25}}$, *bla*$_{\text{CARB-16}}$ and *bla*$_{\text{NDM}}$. In addition, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX}}$ were also found (Table 27).

Other resistance determinants to aminoglycosides [*aac(3)-Ia, aph(3')-Ia, aph(3'')-Ib, aac(3)-IId, aac(6')-Ian, aac(6')-Iaa, aph(6)-Id,* and *ant(2'')-Ia*], macrolides [*mph(A)*, *mph(E)* and *msr(E)*], phenicol (*catA1* and *floR*), sulfonamides (*sul1* and *sul2*), tetracycline [*tet(A), tet(B),* and *tet(39)*] and trimethoprim (*dfrA1*) were also found (Table 27). Additionally, double chromosomal point mutations in *gyrA* and *parC* leading to

substitutions Ser83Leu and Ser80Leu respectively, were found associated to fluoroquinolone resistant *A. baumannii*, mainly ST405 and ST758.

Table 27. Main features of *A. baumanni* isolated in HCM.

Isolate	Department	Source	ST	Carb.	β-lactamases genes	Other determinants genes	Resistance Profile
SS25P*	Surgery	Pus	ST405	Yes	OXA-68, -69/ADC-25	*aac(3)-Ia/aph(3')-Ia/catA1/sul1/tet(A)*	GEN-CIP-SXT**
SS6P*	Surgery	Pus	ST405	Yes	OXA-68, -69/ADC-25	*aac(3)-Ia/aph(3')-Ia/catA1/sul1/tet(A)*	SXT
SS48A*	Pediatric	Blood	NST	Yes	OXA-58/OXA-317	*aac(3)-IId/mph(E)/aph(3'')-Ib/aph(6)-Id/msr(E)/sul2/tet(39)/dfrA1/floR*	GEN-SXT
SS93*	Medicine	Blood	ST758	Yes	OXA-25, -65, -366	*aac(6')-Ian/mph(A)/aph(3'')-Ib/aph(6)-Id/sul2/ant(2'')-Ia*	MEM-GEN-CIP-SXT**
SS94*	Pediatric	Blood	ST758	Yes	OXA-23, -65/ADC-25	*aac(6')-Ian/aac(6')-Iaa/aph(3'')-Ib/aph(6)-Id/ant(2'')-Ia/aph(3')-Ia/sul2*	MEM-GEN-CIP-SXT**
SS53A*	Pediatric	Blood	ST642	Yes	ADC-25/CARB-16	*ant(2'')-Ia/tet(B)*	GEN-CIP-SXT**
SS34	Pediatric	Blood	Nd	Yes	-	-	MEM-GEN-CIP-SXT**
SS68	Pediatric	Blood	Nd	Yes	-	-	MEM-GEN-CIP-SXT**
SS71	NA	Blood	Nd	Yes	-	-	MEM-GEN-SXT**
SS76	Pediatric	Blood	Nd	Yes	-	-	GEN-CIP-SXT**
SS89	Pediatric	Blood	Nd	Yes	-	-	MEM-GEN-CIP-SXT**
SS91	Medicine	Blood	Nd	Yes	VIM	-	GEN-CIP-SXT**
SS103	Medicine	Blood	Nd	Yes	-	-	MEM-GEN-CIP-SXT**
SS11P	NA	Pus	Nd	Yes	-	-	GEN-CIP-SXT**
SS16P	Surgery	Pus	Nd	Yes	-	-	GEN-CIP-SXT**
SS1P	Surgery	Pus	Nd	Yes	-	-	MEM-GEN-CIP-SXT
SS24P	Medicine	Pus	Nd	Yes	TEM	-	MEM-GEN-SXT**
SS32P	Surgery	Pus	Nd	Yes	-	-	GEN-SXT
SS42P	Pediatric	Pus	Nd	Yes	-	-	MEM-GEN-CIP-SXT**
SS49P	Surgery	Pus	Nd	Yes	NDM	-	MEM-GEN-CIP-SXT**
SS59P	NA	Pus	Nd	Yes	-	-	GEN-CIP-SXT**
SS5P	Medicine	Pus	Nd	Yes	CTX-M	-	GEN-CIP-SXT**
SS64P	NA	Pus	Nd	Yes	-	-	GEN-CIP-SXT**
SS8P	Surgery	Pus	Nd	Yes	VIM	-	GEN-CIP-SXT**
SS82	NA	Blood	Nd	Yes	-	-	CAZ

Abbreviations: * Isolates analyzed by WGS (whole genome sequencing); MEM, meropenem; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; Carb. Carbapenemase producers; ** Multi-drug resistant isolate; NA, not available; ST, Sequence type; Nd, not determinated; Carb., carbapenemase; NST, new ST.

Genotypic Antimicrobial resistance of *Pseudomonas aeruginosa*

A total 19 *P. aeruginosa*. were isolated in HCM, of which 9 strains (47%) were analyzed by PCR and 10 through WGS. From 10 WGS analyzed, 5 sequences types of *P. aeruginosa* were found, namely: 3 ST316, 1 ST1047, 1 ST1756, 1 ST274 and 1 ST612.

Related to *P. aeruginosa* antimicrobial results, 89% (17/19) were carbapenemase producers and 2 non-carbapemenase producers but possessed $bla_{\text{TEM/SHV}}$.

Among 17 carbapenemase *P. aeruginosa* producers, β-lactamases were identified in 74%, (13/17) and related to bla_{PAO}, $bla_{\text{OXA-10,-395,- 396,-486,-488}}$, $bla_{\text{VIM-11}}$, bla_{TEM} and bla_{SHV}, with the following profile: (3) $bla_{\text{PAO/OXA-10,-395}}$, (3) bla_{TEM}, (2) $bla_{\text{PAO/OXA-486}}$, (1) $bla_{\text{PAO/OXA-396}}$, (1) $bla_{\text{PAO/OXA-50}}$, (1) $bla_{\text{VIM-11/OXA-488}}$, (1) $bla_{\text{TEM/SHV/VIM}}$ and (1) bla_{VIM}. Among other 4 carbapenemase *P. aeruginosa* producers, β-lactamases related genes were not found (Table 28).

Amost all *P. aeruginosa* harbored *aph(3')-IIb* that encode resistance to aminoglycosides associated with other determinants, namely aminoglycosides [*aph(3'')-Ib, aph(3')-Via, aadA1* and *rmt(B)*]. Macrolides *mdf(A)*, phenicol (*catB3*, *catB7* and *cmlA1*), sulfonamides (*sul1* and *sul2*), tetracycline [*tet(G)* and *tet(39)*], trimethoprim (*dfrA5/7*), fluoroquinolone (*crpP*), fosfomycin (*fosA*) and rifampicin (*ARR-2*) resistance genes were also detected (Table 28).

Table 28. Main features of *P. aeruginosa* isolated in HCM.

Isolate	Species	Department	Source	ST	Carb. producers	β-lactamases	Other determinants genes	Resistance Profile
SS48P*	*P. aeruginosa*	Medicine	Pus	ST316	Yes	PAO/OXA-10,-395	*aph(3')-IIb/aadA1/crpP/catB3/catB7/cmlA1/sul1/tet(G)/dfrA5/fosA/ARR-2*	TZP-CAZ-MEM-GEN-CIP-SXT
SS62P*	*P. aeruginosa*	Medicine	Pus	ST316	Yes	PAO/OXA-10,-395	*aph(3')-IIb/aadA1/crpP/cmlA1/catB3/catB7/sul1/tet(G)/fosA/ARR-2*	TZP-CAZ-MEM-GEN-CIP-SXT**
SS31P*	*P. aeruginosa*	Pediatric	Pus	ST316	Yes	PAO/OXA-10,-395	*aph(3')-IIb/aadA1/crpP/fosA*	TZP-CAZ-MEM-GEN-CIP-SXT**
SS39P*	*P. aeruginosa*	Medicine	Pus	Nd	Yes	TEM-1B	*aac(3)-IId/aph(3'')-Ib/mdf(A)/catA1/sul1/sul2/dfrA7*	TZP-CAZ-SXT**
SS41P*	*P. aeruginosa*	Pediatric	Pus	ST1047	Yes	VIM-11/OXA-488	*aph(3')-IIb/aph(3')-Via/aph(3'')-Ib/rmtB/crpP/catB7/sul1/tet(G)/fosA*	TZP-CAZ-MEM-AMK-GEN-CIP-SXT**
SS33P*	*P. aeruginosa*	Medicine	Pus	NST	Yes	PAO/OXA-396	*aph(3')-IIb/catB7/fosA*	CAZ-SXT
SS30P*	*P. aeruginosa*	Surgery	Pus	NC	No	SHV-50	*aph(3')-IIb/crpP/catB7/fosA*	CAZ-GEN-SXT**
SS52P*	*P. aeruginosa*	Pediatric	Pus	ST1756	Yes	PAO/OXA-486	*aph(3')-IIb/catB7/fosA*	CAZ-SXT
SS98*	*P. aeruginosa*	Pediatric	Blood	ST274	Yes	PAO/OXA-486	*aph(3')-IIb/catB7/fosA*	TZP-CAZ-SXT**
SS107*	*P. aeruginosa*	Medicine	Blood	ST612	Yes	PAO/OXA-50	*aph(3')-IIb/crpP/catB7/fosA*	TZP-CAZ-MEM-SXT**
SS66	*P. aeruginosa*	Medicine	Blood	Nd	Yes	-	-	CAZ-SXT
SS92	*P. aeruginosa*	Pediatric	Blood	Nd	Yes	-	-	TZP- CAZ-MEM-AMK-GEN-CIP-SXT**
SS99	*P. aeruginosa*	Medicine	Blood	Nd	Yes	-	-	CAZ-SXT
SS15P	*P. otitidis*	Surgery	Pus	Nd	Yes	-	-	TZP-CAZ-MEM-SXT**
SS17P	*P. aeruginosa*	NA	Pus	Nd	Yes	TEM	-	CAZ-CIP-SXT**
SS24L	*P. aeruginosa*	NA	CSF	Nd	Yes	TEM	-	TZP-CAZ-SXT**
SS29P	*P. aeruginosa*	Pediatric	Pus	Nd	Yes	TEM/SHV/VIM	-	TZP-CAZ-MEM-AMK-GEN-CIP-SXT**
SS9P	*P. aeruginosa*	Surgery	Pus	Nd	Yes	VIM	-	TZP-CAZ-MEM-AMK-GEN-CIP-SXT**
SS102	*P. stutzeri*	Medicine	Blood	Nd	No	TEM/SHV	-	-

Abbreviations: * Isolates analyzed by WGS (whole genome sequencing); TZP, piperacillin-tazobactam; CAZ, ceftazidime; MEM, meropenem; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; Carb., Carbapenemase producers; ** Multi-drug resistant; ST, sequence type; NC, new combination; NA, not available; Nd, not determined; NST, new ST.

Genotypic characterization of CTX-M-15 among GNB isolates from HCM

Of the 128 GNB isolated from HCM, 67 were analyzed by WGS, these included 25 ExPEC, 15 *K. pneumoniae*, 10 *Pseudomonas* spp., 6 *Acinetobacter* spp., 4 *Enterobacter* spp., 4 *Salmonella* spp., 2 *P. mirabilis*, and 1 *M. morganii* isolates.

29 out of 67 (43%) were ESBL producers and possessed *bla*$_{\text{CTX-M-15}}$, namely: 42% (12/29) *E. coli*, 42% (12/29) *K. pneumoniae*, 7% (2/29) *P. mirabilis*, 3% (1/29) *K. oxytoca*, 3% (1/29) *E. complex* (Figure 30).

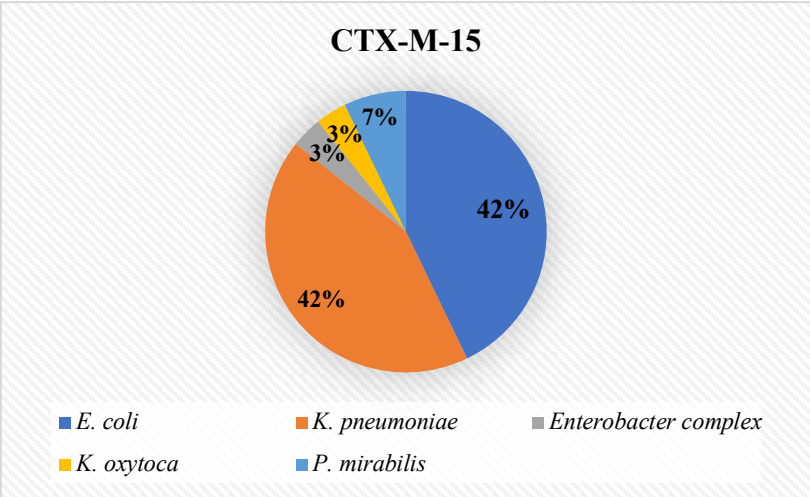


Fig. 30. Percentages of GNB species containing *bla*$_{\text{CTX-M-15}}$ ESBL from HCM.

bla$_{\text{CTX-M-15}}$ in 28 out of 29 GNB showed 100% of identity with *bla*$_{\text{CTX-M-15}}$ sequence (KF727590 accession number) used for comparison (Table 29), only 1 (SS10P) showed 99,9%. In addition, in these 28 isolates the insertion sequence IS*Ecp1*, was found downstream of *bla*$_{\text{CTX-M-15}}$ gene, playing a role in dissemination of this ESBL among GNB. 27 IS*Ecp1* among our isolates showed 100% of identity, except for one isolate (SS100) which shared 99,9% of identity (Table 29 and Figure 31).

Table 29. Characteristics of GNB containing CTX-M-15 isolated in HCM.

Isolate	Department	Source	GNB species	ST	CTX-M-15	IS*Ecp1*
SS61	Pediatric	Blood	*E. coli*	ST69	y	y
SS13	Pediatric	Blood	*E. coli*	ST617	y	y
SS13P	Surgery	Pus	*E. coli*	ST410	y	y
SS26	NA	Blood	*E. coli*	ST410	y	y
SS30	Pediatric	Blood	*E. coli*	ST410	y	y
SS49	Pediatric	Blood	*E. coli*	ST410	y	y
SS6	Pediatric	Blood	*E. coli*	ST410	y	y
SS100	Medicine	Blood	*E. coli*	ST405	y	y *
SS53B	Pediatric	Blood	*E. coli*	ST361	y	-
SS45A	Medicine	Blood	*E. coli*	ST131	y	y
SS37P	Pediatric	Pus	*E. coli*	ST131	y	y
SS46P	Surgery	Pus	*E. coli*	ST131	y	y
SS73	Medicine	Blood	*K. pneumoniae*	ST711	y	y
SS56P	Medicine	Pus	*K. pneumoniae*	ST607	y	y
SS114	Pediatric	Blood	*K. pneumoniae*	ST607	y	y
SS64	Pediatric	Blood	*K. pneumoniae*	ST607	y	y
SS85	Medicine	Blood	*K. pneumoniae*	ST48	y	y
SS58P	Medicine	Pus	*K. pneumoniae*	ST394	y	y
SS35	Medicine	Blood	*K. pneumoniae*	ST13	y	y
SS10P	NA	Pus	*K. pneumoniae*	ST985	y *	y
SS52A	NA	Blood	*K. pneumoniae*	ST831	y	y
SS52B	NA	Blood	*K. pneumoniae*	ST831	y	y
SS37	NA	Blood	*K. pneumoniae*	ST607	y	y
SS79	Pediatric	Blood	*K. pneumoniae*	ST14	y	y
SS90	Pediatric	Blood	*K. oxytoca*	New	y	y
SS97	Pediatric	Blood	*E. complex*	ST125	y	y
SS74	Medicine	Blood	*E. cloacae*	ST84	y	y
SS55P	Pediatric	Pus	*P. mirabilis*	-	y	y
SS27P	Medicine	Pus	*P. mirabilis*	-	y	y

Abbreviations: NA, not available; ST, sequence type; GNB, Gram-negative bacteria. y 100% y*(99,9% of homology).

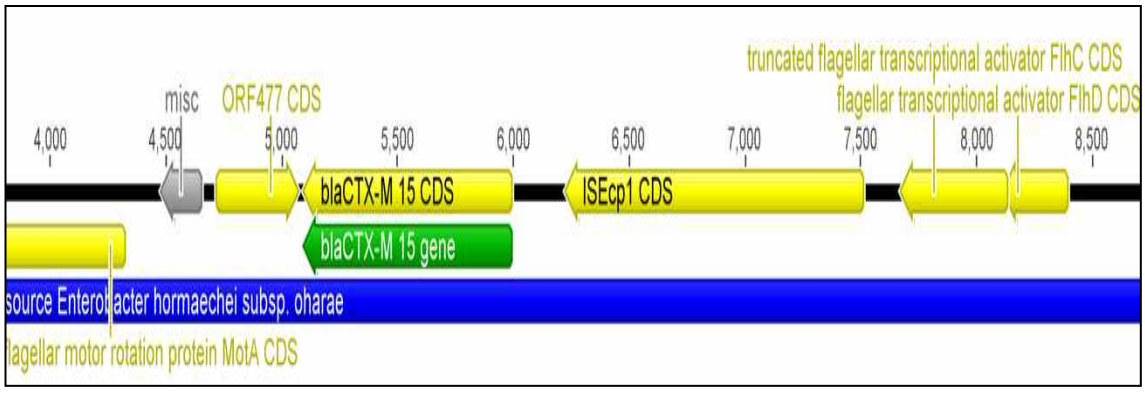


Fig. 31. Schematic representation of *bla*$_{\text{CTX-M-15}}$ identified among GNB isolated in HCM.

Comparison of Sequence types and Resistance determinants of GNB isolated in Pediatric Department of HCM and Other Hospitals

To compare the STs of GNB that circulate in Mozambique and their mechanisms of resistance, few samples were collected in other 4 hospitals, mainly in Pediatric Departments, including HGM, HJM, HPQ and HCQ (Other hospitals). A total 31 GNB were isolated in other hospitals from blood and were compared with other 24 blood isolates (GNB) from HCM. Thus, 55 GNB isolated from blood from Pediatric department were compared (Table 30).

Table 30. Gram-negative isolates from blood in Pediatric Departments.

Hospitals	Hospital localization	GN Isolates
HCM	Maputo Province (City)	24
Other hospitals		
HGM	Maputo Province	8
HJM	Maputo Province	4
HGM	Maputo Province	8
HPQ	Zambezia Province	10
HCQ	Zambezia Province	9

Salmonella spp. were found in all hospitals followed by *E. coli* and *Acinetobacter* spp., *K. pneumoniae*, *Pseudomonas* spp. and *Enterobacter* spp. *P. septica* and *M. morgani* were only isolated in HJM and HCM, respectively (Figure 32).

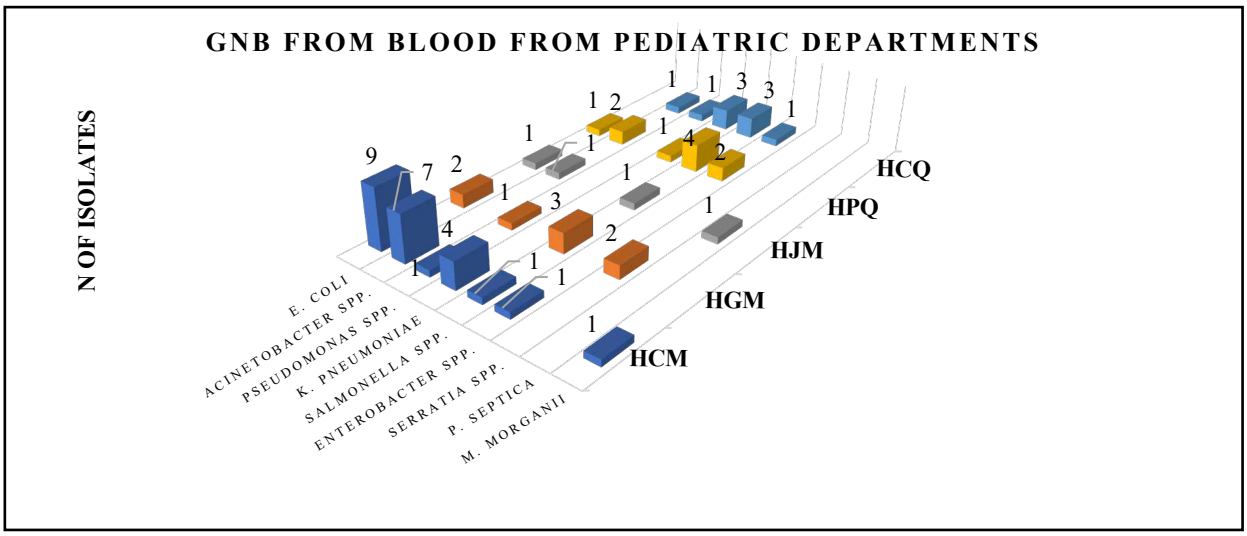
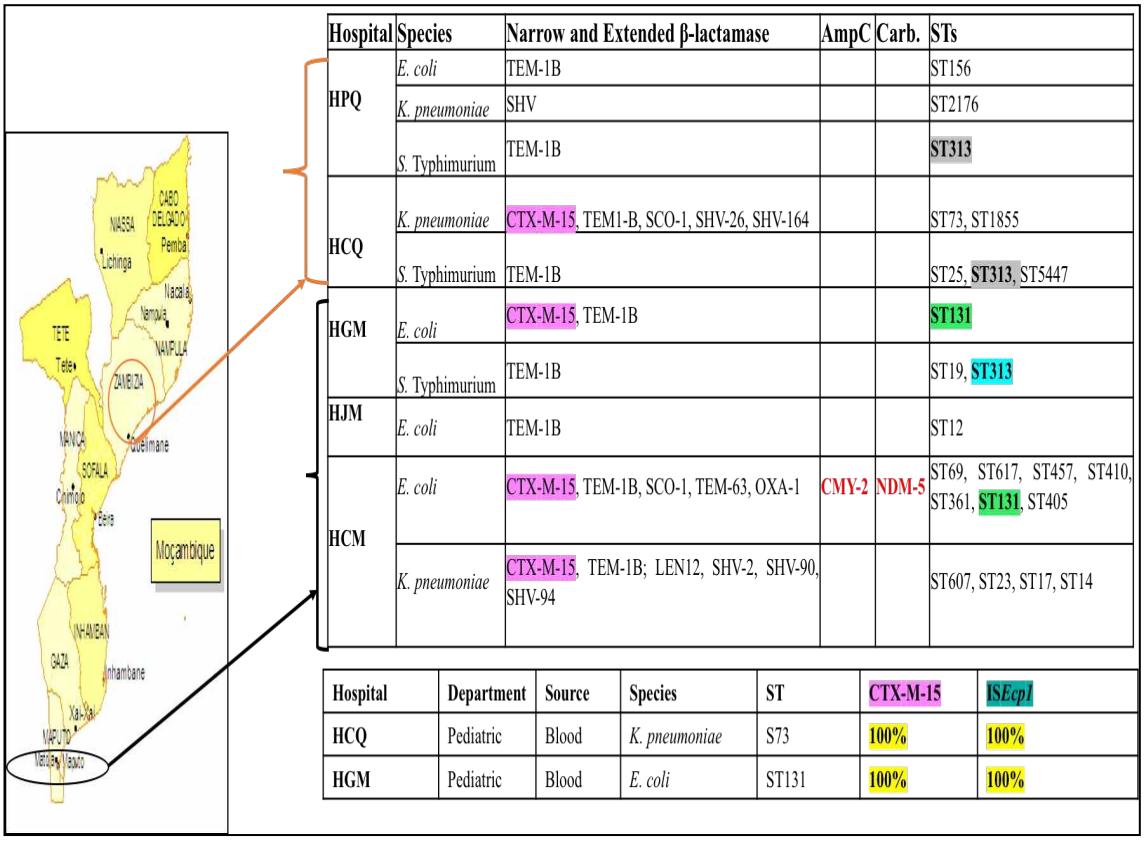


Fig. 32. Gram-negative species isolated from blood from Pediatric Departments of HCM, HGM, HJM, HPQ and HCQ hospitals.

The determinants of resistance of the same species found among Pediatric departments were compared, including *E. coli*, *K. pneumoniae* and *Salmonella* spp.

E. coli ST131 was found only in HGM and HCM. *bla*$_{TEM-1B}$ was found in all *E. coli* from HPQ, HGM, HJM and HCM. However, *bla*$_{CTX-M-15}$ was only found in HCM isolates. Additionally, *bla*$_{CMY2}$ (AmpC) and *bla*$_{NDM-5}$ (Carbapenemase) were found only in HCM isolate. *S.* Typhimurium ST313 possessing *bla*$_{TEM-1B}$ were found in different hospitals namely: HPQ, HCQ and HGM.

Related to *K. pneumoniae*, MLST evidenced different STs among hospitals; *K. pneumoniae* from HCM and HCQ possess *bla*$_{CTX-M-15}$ associated with other β-lactamases while strain from HPQ have only *bla*$_{SHV}$. The *bla*$_{CTX-M-15}$ from *K. pneumoniae* isolated in HCQ and *E. coli* from HGM shared 100% of identity and were associated with IS*Ecp1*. All the features of STs and β-lactamases resistance genes are shown in Figure 33.



Hospital	Species	Narrow and Extended β-lactamase	AmpC	Carb.	STs
HPQ	*E. coli*	TEM-1B			ST156
	K. pneumoniae	SHV			ST2176
	S. Typhimurium	TEM-1B			ST313
HCQ	*K. pneumoniae*	CTX-M-15, TEM1-B, SCO-1, SHV-26, SHV-164			ST73, ST1855
	S. Typhimurium	TEM-1B			ST25, ST313, ST5447
HGM	*E. coli*	CTX-M-15, TEM-1B			ST131
	S. Typhimurium	TEM-1B			ST19, ST313
HJM	*E. coli*	TEM-1B			ST12
HCM	*E. coli*	CTX-M-15, TEM-1B, SCO-1, TEM-63, OXA-1	CMY-2	NDM-5	ST69, ST617, ST457, ST410, ST361, ST131, ST405
	K. pneumoniae	CTX-M-15, TEM-1B; LEN12, SHV-2, SHV-90, SHV-94			ST607, ST23, ST17, ST14

Hospital	Department	Source	Species	ST	CTX-M-15	IS*Ecp1*
HCQ	Pediatric	Blood	*K. pneumoniae*	S73	100%	100%
HGM	Pediatric	Blood	*E. coli*	ST131	100%	100%

Fig. 33. Sequence types and resistance determinants of GNB isolated in blood from Pediatric Departments of all hospitals.

CHAPTER FOUR

DISCUSSION

Several bacteria, including GNB have been found associated with invasive infections and MDR responsible of increased morbidity and mortality, hospital treatment costs and high risk for the community, worldwide (Onipede et al., 2009; Kariuki and Dougan, 2014; Zingg et al., 2017; Mulu et al., 2017; Didovic et al., 2018).

In Mozambique, according to the Global Antibiotic Resistance Partnership (GARP), *E. coli*, non-typhoidal *Salmonella* and other GNB not isolated like *Shigella* and *Vibrio cholerae* are a major contributor to the burden of diseases. Indeed, in a recent study regarding etiology of hospital infections conducted at ICU of HCM, *E. coli*, *K. pneumoniae*, *Klebsiella* spp., *Acinetobacter* spp., *A. baumannii*, *Enterobacter* spp., were found as most etiologic agents isolated from blood followed by pus and urine samples (GARP, 2015; Mahaluça et al., 2018a).

In this study, the majority of GNB (81%) was from HCM and the rest from other four hospitals, including HGM, HJM, HPQ and HCQ, mainly from blood specimen followed by pus and CSF. Among these GNB, 13 species were identified, namely *K. pneumoniae*, *E. coli*, *Acinetobacter* spp., *Pseudomonas* spp., *P. mirabilis*, *Enterobacter* spp., *Salmonella* spp., *M. morganii*, *K. oxytoca*, *K. variicola*, *S. maltophilia*, *S. marcescens* and *Serratia complex*. From HCM, the most isolated species were *K. pneumoniae* (27%), ExPEC (20%), *Acinetobacter* spp. (20%) and *Pseudomonas* spp. (15%). These frequencies are higher when compared with previous study conducted in ICU wards in 2017, from blood, urine and pus in the same hospital, being 13.4% for *K. pneumoniae*, 8.9% for *Acinetobacter* spp., 4.5% for *A. baumanni*, 7.8% for *P. aeruginosa*, 3.9% for *Pseudomonas* spp., and 7.3% for *E. coli* (Mahaluça et al., 2018a). The high prevalence of GNB isolated in our study, may be related to studied departments and to the specimen collected. The present study was carried out including all the main departments (Pediatric, Medicine, Surgery, Gynecology and Obstetrics) and samples from blood, pus and CSF. However, the prevalence of *E. coli* and *Salmonella* spp. found in this study is similar to those reported from previous study conducted in Etiopia from blood, which was 17.4% and 4.3%, respectively (Zenebe et al., 2011).

ExPEC (30%) and *Acinetobacter* spp. (22%) were mostly isolated from Pediatric department while *K. pneumoniae* (32%) was common in other departments. In a previous study Berezin et al., reported high prevalence of *E. coli*, *Pseudomonas* spp. and sporadic *Acinetobacter* spp. in Pediatric department in developing countries (Berezin et al., 2014; Azimi et al., 2019). This prevalence might be given to the ability of these bacteria to colonize almost any habitat and to acquire antimicrobial-resistance mechanisms, as well as virulence. Conversely to our findings, *K. pneumoniae* isolates are becoming an evolving crisis of global dimensions especially for pediatric patients (Medernach and Logan, 2017; Tian et al., 2018). The low prevalence of *K. pneumoniae* in pediatric department in this study can be addressed to the lack of information related to the department provenance of some samples.

K. pneumoniae and *E. coli*, were more isolated from blood cultures in our hospitals, while *Acinetobacter* spp., *Pseudomonas* spp. from pus samples; further *K. pneumoniae* and *Pseudomonas* spp. were found in CSF samples. The high prevalence of *K. pneumoniae* and *E. coli* in blood is consistent with previous study conducted in Zanzibar archipelago in Africa, although they found also *Acinetobacter* spp. as a frequent isolate (Onken et al., 2015). Indeed, *E. coli* and *K. pneumoniae* are the most common gram-negative species causing invasive infections (Vading et al., 2018).

High prevalence of *Acinetobacter* spp. and other GNB including *K. pneumoniae* and *E. coli* were found in previous study carried out in India (Taj et al., 2018). These bacteria species have emerged as the causative agent of important nosocomial infections, especially in the ICUs (Dash et al., 2013).

Phenotypic and Genotypic antimicrobial resistance of ExPEC

Before the 2000s, ExPEC was mostly susceptible to first-line antibiotics (e.g. cephalosporins and fluoroquinolones) that were often used to treat human and animal infections. However, nowadays resistance to the first-line oral antibiotics, such as trimethoprim-sulfamethoxazole, amoxicillin and amoxicillin-clavulanic acid, cephalosporins and ciprofloxacin among *E. coli* are common and very widespread (Nicolas-Chanoine et al., 2014; Pitout and DeVinney, 2017). In line with these findings, in the present study 72% of ExPEC were MDR and more resistant to the commonly used antibiotic agents in Mozambique, including trimethoprim-sulfamethoxazole (88%), amoxillicin-clavulanic acid (84%), 3rdGC cephalosporins (68%), gentamicin (60%) and ciprofloxacin (52%). The tigecycline and colistin were completely effective against ExPEC, while meropenem and fosfomycin were less effective (4%). Trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, gentamicin and ciprofloxacin agents are used as a first line in Mozambique to treat bacteria infections (MISAU, 2017). Additionally, the *E. coli* resistance frequencies found in this study were higher when compared with a mean resistance rate reported from Mozambique and other Africans countries such as Zambia, Democratic Republic of Congo and Tanzania, in 2013, of 2%, 15%, 28%, 37%, 38%, 55%, 57%, 76% and 95% for meropenem, ciprofloxacin, ceftazidime, gentamicin, ceftriaxone, augmentin, tetracycline, cotrimoxazole and ampicillin, respectively. These data display an increasing resistance of *E. coli* that might be exacerbated due to the overuse and misuse of antibiotics that are considered major reasons for the emergence of resistant bacteria in many low-income countries, like Mozambique (Hawkey and Jones, 2009; Lyimo et al., 2016). Indeed, the knowledge about antibiotic use is poor in Maputo City population (Mate et al., 2019). By MLST analysis a high level of diversity among ExPEC was identified at HCM, with 13 different STs, of which the most prevalent were ST131 and ST410 (46%), followed by ST38 and ST394 (15%), while ST361, ST617, ST1177, ST405, ST69, ST457, ST59, ST62, and ST648 were found in unique isolates. This high level of diversity of ExPEC clones has not been previously described in Mozambique and could be due to the different geographical origin of the patients admitted to the HCM in Maputo. ST131 and ST69 found in our study, constitute the major pandemic clonal lineages of ExPEC along with other STs (ST393,

ST95, and ST73) associated with both community-onset and healthcare-associated infections, in particular UTI and BSI (Riley, 2014) that were not found in this study.

E. coli ST131 strains are mostly of serotype H4:O25 (Nicolas-Chanoine et al., 2014). In present study ST131 was associated with different serotype mainly H4:O25, H5:O16 and H4:O18-O18ac. ST131 serotype H5:O16 has recently been identified in Japan, Denmark, Australia, Spain, Pakistan, and France (Nicolas-Chanoine et al., 2014; Riley, 2014).

ST131 belongs to the phylogroup B2 and possess *fimH*, mainly *fimH30* (O25), *fimH41* (O16) and *fimH27* (O18-O18ac and O25) as important factors in translocation and colonization (Pitout and DeVinney, 2017) as we found in present study. ST131 clonal subgroup H30 is responsible for the current pandemic of fluoroquinolone (carrying *gyrA* and *parC* genes) and multi-drug resistant *E. coli* infections mainly due to ESBLs (CTX-M-15 and CTX-M-27) around the globe (Roer et al., 2018) and thus represent a threat. In according to the fact that ST131 *fimH41* subclone is less frequently expanded along with *fimH22* (Mamani et al., 2019) we also did not found these subclones in this study.

Additionally, this ST has been found uniformly carrying *sat* (secreted autotransporter toxin), *fyuA* (yersiniabactin receptor), *kpsM* II (group 2 capsule synthesis), *malX* (pathogenicity island marker), *iha* (adhesin siderophore receptor), *usp* (flagellin variant), *ompT* (outer membrane receptor) *iucD* (aerobactin), *iutA* (aerobactin receptor), and *traT* (serum resistance associated) most of them found in present study. However, the number of identified virulence genes has been increasing (Sarowska et al., 2019). Indeed, in this study were found several virulence genes classically identified in non-ST131 group B2 ExPEC including *papA/C/G* (P fimbrial adhesins), *hlyD* (α-hemolysin), *agg3B, agg3C, agg3D* and *agg5A* [aggregative adherence fimbriae of Enteroaggregative *Escherichia coli* (EAEC)] (Nicolas-Chanoine et al., 2014; Jønsson et al., 2015; Jønsson et al., 2017) mainly in one ST131 isolate (SS48C).

The resistance to β-lactam agents among HCM ExPECs was associated with CTX-M, mainly CTX-M-15 (70%) and CTX-M-27 (24%) found linked with TEM-1B and OXA-1, that mediate resistance to ampicillin or amoxicillin. Interestingly, SHV type were not found associated with our ExPEC. This finding is consistent with previous study

conducted at Manhiça District Hospital, in Southern Mozambique, which found that CTX-M-15 was the most frequently ESBL, accounting for 75% among *E. coli* isolated from blood and urine (Guiral et al., 2018). On the contrary to our findings Guiral et al., (2018), found other β-lactamases including CTX-M-37 and SHV-12, commonly found in *E. coli* ST131.
Two ExPEC ST131 isolates (SS67 and SS48C) were not producing ESBL showing that they have not acquired CTX-M enzymes or other β-lactamases gene.

Among our ExPEC, 46% belongs to the ST131. The successful lineage ST131 was identified in the mid-2000s in UK and Canada with an unknown origin and has been associated with ESBL-production mainly CTX-M-15, which currently has a worldwide distribution as fluoroquinolone resistance mainly encoded by *aac(6')Ib-cr* (Nicolas-Chanoine et al., 2014). In present study ST131 also harbored CTX-M-27 and to our best knowledge this is the first description in Mozambique. However, ST131 harboring CTX-M-27 has been reported from other countries, such as Korea, China, Australia, Nepal, Cambodia, Israel, Czech Republic, Switzerland, Spain, France, Portugal, Netherlands, Canada, and United States and Japan (Nicolas-Chanoine et al., 2014; Matsumura et al., 2016). The presence of CTX-M-27 in Mozambique may reflect travel and trade with these countries.

CTX-M-15 and CTX-M-27 was found associated with incompatibility groups FII conjugative plasmids, pMLST type (IncF[F1:A2:B20]). IncF plasmids with F1:A2:B20 and F2:A1:B replicons have shaped the evolution of ST131 mainly for the C1 and C2 subclades (Pitout and DeVinney, 2017). The plasmids IncF might be also harbored resistance to other classes of antibiotics found in ST131, such as *aac(6')lb-cr* which confers resistance to both (ciprofloxacin) and aminoglycosides (amikacin and tobramycin), [*aac(3)-II, aph(3")-Ib*, and *aph(6)-Id*] aminoglycosides, [*mdf(A)* and *mph(A)*] macrolides, (*catA1* and *catB4*) chloramphenicol, (*tetA*) tetracycline, class I integron *dfrA7/dfrA17* and *aadA5*, conferring resistance to trimethoprim and streptomycin, respectively, and (*sul1* and *sul2*), conferring sulfonamide resistance as we find in our study (Nicolas-Chanoine et al., 2014). These findings suggest that plasmids IncF family has a complex structure and have clearly played a major role in the

dissemination of CTX-M-15 and other resistance mechanism expressed by *E. coli* ST131 strains and also by other GNB.

In *E. coli*, mutational alterations in the DNA topoisomerase II (DNA gyrase) and topoisomerase IV, are recognized to be the major mechanisms through which resistance to fluoroquinolone develops (Karczmarczyk et al., 2011). In this study resistance to ciprofloxacin in ST131 isolates and other STs was also determined by amino acid substitutions on DNA *gyrA* (*S83L* and *D87N*), and topoisomerase IV *parC* (*S80I* and *E84V*) and *parE* (*I529L*) mainly from SS37P-*fimH41*, SS46P-*fimH41*, SS45A-*fimH30* and SS50-*fimH30* isolates. ST131 are usually resistant to fluoroquinolones, owing to chromosomal gene mutations (*gyrA* and *parC*) (Riley, 2014). In our study, ExPEC show the accumulation of fluoroquinolone resistance mechanisms associated with mutations of the *gyrA*, *parC* and *parE* genes in the ST131 subclone *H30* and *H41* that is in accordance with previous studies (Nicolas-Chanoine et al., 2014; Li et al., 2017). No *qnr* determinants were found from all ExPEC including ST131 in our study that is inconsistent with previous studies (Malekzadegan et al., 2019; Sheikh et al., 2019). However, in line with our findings, previous study conducted by Guiral et al., at Manhiça District Hospital, in Southern Mozambique, resistance to ciprofloxacin was associated with mutation in *gyrA* at codon Ser83. In addition, one *E. coli* did not show any of the resistance determinants studied (Guiral et al., 2018). This finding suggests that qnr family is not the main mechanism of quinolone resistance in ExPEC from HCM and other studied hospitals and has been less found in Mozambique.

ST69 has common circulating worldwide, predominantly in the community in human and animals and resistant primarily to trimethoprim-sulphamethoxazole but not regularly as ESBL-producing strains since 1999 (Riley, 2014; Bourne et al., 2019). However, in our study ST69 was found co-harboring CTX-M-15, OXA-1 and TEM-1C β-lactamases, and several virulence genes. Additionally, ST69 isolate was serogroup H18:O15, carrying resistance genes *dfrA7* (trimethoprim resistance), *mdf(A)* (macrolide), *catB3* (chloramphenicol), *sul1* and *sul2* (sulphonamide) and *tet(A)* tetracycline. This finding shows its ability of acquiring resistance and virulence genes becoming pandemic ST as other proficient pathogenic ExPEC like ST131.

E. coli ST410 carbapenemase producers harboring CMY-2, has been reported worldwide, as an extraintestinal pathogen associated with resistance to fluoroquinolones, 3rd Generation Cephalosporins, and carbapenems and poses a high risk in clinical settings (Mavroidi et al., 2012; Schaufler et al., 2016). In our study, ST410 was found carrying several resistance genes, including CTX-M-15 and CMY-2 encoding resistance to cephalosporins, *aac(6')Ib-cr* (ciprofloxacin), *aac(3)-IId*, *aph(3")-Ib*, and *aph(6)-Id* (aminoglycosides), *dfrA8* and *dfrA17* (trimethoprim), *mdf(A)* and *mph(A)* (macrolides), *catB3* (chloramphenicol), sulfonamides (*sul1* and *sul2*), tetracycline [*tet(B)*] associated with pMLST type (IncF[F1:A1:B49]) in accordance with previous study (Carattoli, 2009; Roer et al., 2018). CMY-2 when upregulate, can lead resistance to meropenem and has been found associated with IncF plasmid in *Enterobacteriaceae* including *E. coli* (van Boxtel et al., 2017). Its origin is chromosomal of *Citrobacter freundii*, and had been mobilized onto plasmids of different replicon types (IncK, IncI1, IncA/C and IncFIA-FIB) by the insertion sequence (IS*Ecp1*) that also provides the promotor for high-level expression of CMY-2 (Pietsch et al., 2018).

ST38, ST405 and ST648 despite not considered pandemic represent emergent MDR *E. coli* lineages harboring CTX-M mainly (CTX-M-15), TEM or/and SHV as we displayed in our study (Pitout, 2012; Roer et al., 2018).

Studies have identified ST38 with CTX-M-9, -14, and -15 in clinical isolates from Tanzania and other countries such as Netherlands, Korea and Japan (Mshana et al., 2011). ST38 was also found associated with OXA-48 and NDM-1 (Pitout, 2012). In our study, the presence of ST38 possessing CTX-M-27 ESBL including narrow β-lactamase TEM-1B, *mdf(A)* and *mph(A)* encoding resistance to macrolides, *sul1* and *sul2* to sulfonamides, *tet(A)* to tetracycline*, dfrA8* and *dfrA17* to trimethoprim might represent a spreading and emergence of this ST due to its ability to acquire resistance genes.

In the present study, ST405 apart of CTX-M-15 has already acquired NDM-5 and accumulated typical ExPEC virulence associated genes showing that is a real emergence of MDR *E. coli* lineage. CTX-M-15 and NDM-5 was associated with IncFII incompatibility plasmid but can be found also on IncN and IncX3 plasmids (Zhu et al., 2016; Li et al., 2018; Yoon et al., 2018). NDM-5 has been identified in America,

Australia, China, Denmark and India from clinical specimens but also from animals, such as dogs, cats and cows (Li et al., 2018).
To our knowledge this is the first report of ST405 NDM-5 in Mozambique and the presence, might be related to the travels, mainly medical tourism or trade among these countries due to the less use of carbapenem agents in Mozambican hospitals.

In present study contrary to other studies, ST648 remain susceptible and only resistant to macrolide having *mdf(A)* that encode resistance to macrolide. ST648 ESBL producer has been found globally, including Europe, North and South America, Africa, and Asia in human patients and more incidentally from chicken and pigs in Europe and from wild birds in Germany and Mongolia (Pitout, 2012; Ewers et al., 2014). ST648 was found less virulent than ST131 and other STs. This finding is in accordance with previous study (Sherchan et al., 2015).

ST62 was non-ESBL producers it possessed *mdf(A)* encoding resistance to macrolide, *tet(B)* to tetracycline. As ST62, in our study ST1177 were also non-ESBL producers but possessed *mdf(A)*, *mph(A)*, *sul1* and *sul2* and *tet(A)*. In a previous study, ST1177 carrying ESBL was found in the muddy soil around Mirongo river in Mwanza, Tanzania (Moremi et al., 2017). These findings show the importance of continuous surveillance.

ST361 and ST617 were less virulent and ESBL producers both harboring CTX-M-15 located on IncA/C2 and IncFII(pAMA1167-NDM-5) plasmid, respectively. In particular case CTX-M-15 can be found on other plasmids including IncA/C families and other (IncI1, IncN) (Caratolli, 2009). ST617 ESBL-producing, has also been reported around world, including Nigeria in animals and healthy people (Ewers et al., 2012).

Phylogenetic analysis of ExPEC

In this study ST131 formed 4 clusters, where one share same origin with other ST131 isolated in UK and India. Two clonal ST131 strains carrying CTX-M-15 were found in Pediatric (SW37P) and Surgery (46P) departments of HCM, respectively, suggesting their nosocomial nature and clonal expansion inside of different wards. ST410 in HCM was present with different subclones, 2 clonal isolates (JW6 from pediatric and 26 from

unknown ward) showed different assortment of resistance genes, suggesting their presents in HCM from different time.

Also isolates from ST394 (116 and 117 from unknown wards) and ST38 (101 and 101A from Medicine) were clonal (Figure 25), suggesting their spread among patients.

ExPEC, especially ST131 have adaptive strategies that are driven mainly by gene loss, genetic exchange, and coevolution with an antimicrobial resistance repertoire (Roer et al., 2018). These data suggest that transfer of accessory elements between pathotypes and recombination (homologous and nonhomologous recombination) in the core genome is playing important role in pathogenesis or niche of ExPEC (Corander et al., 2012; McNally et al., 2013).

ST4086 (JW522) clustered with ST156 (127A) these two STs were detected recently in China (Ali et al., 2017; Tang et al., 2019) from bovine and poultry, respectively. The large migration of Chinese to Mozambique could explain the presence of these clones, one of the 2 coming from a healthy person, generating concern since he was also capable of acquiring antibiotic resistance including CTX-M-15 (Tang et al., 2019).

Phenotypic and Genotypic characterization of *E. coli* isolated in Feces of Healthy people in Maputo

Three strains isolated from feces of healthy people mainly resistant to penicillins, cephalosporins, nalidixic acid and trimethoprim-sulfamethoxazole were included in our study. Two strains assigned to B2-ST569 and B1-ST4086 carried narrow β-lactamase (TEM-1B) and one strain non-typed (new alleles) harbored ESBL (SHV-185). Resistance determinants to other mechanism among these strains were also found. Notably, ST569 carried more VAG including *usp*, that is the major virulence factor of UPEC (Yamamoto et al., 1995), showing the dissemination of this gene in the community. High prevalence of fecal carriage narrow and ESBL associated with other classes of antibiotic resistance mechanisms have been reported worldwide, especially in Mozambique (Woerther et al., 2013; Hazirolan et al., 2018; Chirindze et al., 2018). This finding reinforces the necessity of continuous surveillance of resistance mechanism from healthy people because *E. coli* are facultative pathogens that are part of the normal human intestinal microbiome (Sarowska et al., 2019).

77% and 80% of *K. pneumoniae* were MDR and ESBL producers respectively. Similar to ExPEC, *K. pneumoniae* showed high resistance to the commonly used antibiotic in Mozambique, including amoxicillin-clavulanic acid (83%), cefotaxime and ceftazidime (77%), trimethoprim-sulfamethoxazole (77%), gentamicin (74%), piperacillin-tazobactam and ciprofloxacin (71%). Least resistance was seen to tigecycline and fosfomycin (3%) and (29%), respectively. Interestingly, ertapenem, meropenem, amikacin and colistin were totally effective against *K. pneumoniae*.
In accordance with our findings, a study carried out at Tertiary Teaching Hospital in Kenya, found most of *K. pneumoniae* as a MDR, where the highest resistance observed was against ceftriaxone, cefepime, gentamycin, chloramphenicol, and ceftazidime with 80% (Apondi et al., 2016).

On the contrary to our findings, study conducted from outpatients in urban and rural districts of Uganda found least antibiotics prevalence of resistance from *K. pneumoniae*, MDR (33%), of which amoxillicin-clavulanic acid (36%), ceftriaxone (3%), cefoxitin (22%), cefepime (15%), trimethoprim-sulfamethoxazole (70%), gentamicin (11%), piperacillin-tazobactam (27%), ciprofloxacin (11%) (Najjuka et al., 2016). These findings showed that the high resistance of *K. pneumoniae* is associated with clinical samples. *K. pneumoniae* is the second GNB invasive after *E. coli* (Vading et al., 2018).

Diversity of *K. pneumoniae* STs (10), assigned to ST13, ST48, ST985, ST711, ST394, ST17, ST14, ST23, ST831, ST607 was seen. All of them were ESBL producers carrying mainly CTX-M-15 (81%), with the expection of ST17 and ST23. ST985 harbored also CTX-M-88. This finding suggests the circulation of multiple *K. pneumoniae* STs ESBL producer in a single hospital although anyone of them belongs to the major epidemic clones that include ST11, ST15, ST147 and ST258 (Yan et al., 2015). CTX-M-9 was also found by PCR showing the diversity of CTX-M type among *K. pneumoniae* in accordance with previous study conducted in hospitalized patients in Brazil (Chagas et al., 2011).

CTX-M-15 was found located downstream of IS*Ecp1* and associated with other mechanism SHV (96%), TEM (88%), OXA-1 (27%) and LEN12 (8%). Moreover, CTX-

M-15 was mainly located on IncF plasmid, especially IncFII(K). In accordance with our findings plasmids belonging to group IncF harbored CTX-M-15 associated with TEM-1, OXA-1, and *aac(6')Ib-cr* resistance genes (Carattoli, 2009; Nicholas et al., 2019). CTX-M-15 from *K. pneumoniae* was first identified in India in 1999, Portugal, Korea and Cameroon, Tanzania and Nigeria with IS*Ecp1* element as we found in the present study (Soge et al., 2006).

The resistance to ciprofloxacin and aminoglycosides in all STs except ST17 and ST23 were mainly encoded by *aac(6')Ib-cr*, efflux pumps (*oqxA* and *oqxB*), porins mutation (*acrR*, *ompK36*, *ompK37* and *ramR*) associated with *qnrB1* (found only in ST13, ST14, ST985 and ST711) and *qnrB6* (found only in ST607). ST711 and ST394 did not possess *aac(6')Ib-cr* and only porin mutations were not found in ST711. In addition, all ESBL producers STs harbored *aac(3)-IIa*.
In present study ST607 possess β-lactamase (CTX-M-15 and LEN12) associated with *aadA16* encoding resistance to streptomycin and spectinomycin. Recently, ST607 harboring CTX-M-15 was found in South Africa, neighboring country of Mozambique (Founou et al., 2019) showing the possibility of regional transmission between these countries.

ST48 has been reported producing TEM-3, SHV-12 in 2003 in Tunisia and CTX-M-15 in Korea. ST13 and its SLV (single locus variant) of ST327 has been described carrying DHA-1 and CTX-M-15, Spain and Israel, respectively (Marcade et al., 2013). ST985 isolated from water and clinical samples has been reported carrying SHV-83 and OXA-10 and carbapenemase VIM-1 and has been reported in Australia (Lepuschitz et al., 2019). These findings show the spreading of STs associated with endemicity of some resistance genes.

K. pneumoniae ST14 has previously been described as a host lineage for the NDM-1 and is also a frequent host of CTX-M enzymes in India, Sweden, and the UK (Giske et al., 2012) and KPC-3 enzyme in Portugal (Caneiras et al., 2018).

K. pneumoniae ST831 has been identified in South Africa (Lowe et al., 2019) and also isolated in urine samples in Canada harboring OXA-48, SHV-11 and OXA-1 (Robaina et

al., 2013), whereas ST394 is pandemic along other STs (ST131, ST69, ST95, ST10, ST117) (Manges, 2016) and was found carrying many enteroaggregative *E. coli* virulence genes without CTX-M-15 in Pakistan (Zahara et al., 2018). Additionally, was reported also in Nigerian children (Okeke et al., 2010).

K. pneumoniae ST17 is considered international clones and has been found in the post-craniotomy meningitis isolates in Taiwan, China and other various geographic areas in the world which could produce ESBL/AmpC β-lactamases (DHA-1, KPC-2 or NDM-1metallo-β-lactamase) (Ku et al., 2017). In addition, recently was isolated in clinical specimens, patients hospitalized from KwaZulu-Natal, South Africa (Founou et al., 2019).

K. pneumoniae ST23 was identified in mid-1980s and 1990s, from Taiwan. This is hypermucoviscous *Klebsiella pneumoniae* (HMKP) that cause invasive community-acquired infections and is spreading worldwide (Cheong et al., 2016). Indeed, currently it was reported in Asian Pacific Rim (e.g Korea, Vietnam and Japan) North America, South America, the Caribbean, Europe, the Middle East, Australia, Africa and South Africa (Shon et al., 2013). In accordance to previous studies, our ST23 (n=2) isolates showed high virulence score due to KL1 capsule and conjugative element (ICEKp10) encoding siderophore yersiniabactin and colibactin. Notably, one of ST23 was hypervirulent harboring a complex of virulence factors including *rmpA* and *rmpA2* transcriptional regulators of CPS biosynthesis, *iutA* encoding for aerobactin, *magA* encoding for the K1 mucoviscous serotype and *KpnO* porin. The other ST23 harbored only *KpnO* porin found also in other STs less virulent. These hypervirulent variants of *K. pneumoniae* belonging to clonal group 23 being ST23 K1 serotype predominant have been reported worldwide. Hypervirulent phenotype seems to be associated with a complex interplay of several genetic determinants rather than a phenomenon originated by a single gene (Catalán-Nájera et al., 2017; Shankar et al., 2018). However, strains hypervirulent lacking the *rmpA/rmpA2* but with siderophores were also described causing invasive infection as we found in this study (Catalán-Nájera et al., 2017).

Although in present study ST23 strains was susceptible to most tested antibiotics there is the possibility to become MDR due to epidemic ESBL plasmids dissemination. Indeed,

in other countries, including Korea, ST23 carrying CTX-M-15 was reported (Leski et al., 2012; Lam et al., 2018). These findings show the importance of a continuous stewardship of antimicrobial resistance in the country.

K. oxytoca is emerging as an important bacterial isolate causing hospital-acquired infection in adults and having multiple drug resistance to commonly used antibiotics (Singh et al., 2016). In our study, *K. oxytoca* was isolated from blood in pediatric department showed that is resistant to commonly antibiotics and constitutes a particular concern, although the less number of isolates. These results were also found in previous studies (Singh et al., 2016).

K. oxytoca was MDR and harbored several β-lactamases including CTX-M-15, TEM-1B, OXA-1 and OXY-4-1 associated with other resistance mechanisms. CTX-M-15 was found linked to IncHI2 plasmid. The plasmid-encoded CTX-M, TEM, SHV and DNA chromosomal OXY (OXY1-4) type ESBLs have been increasingly transmitted among *K. oxytoca* (Monstein et al., 2009). Despite not being found (KPC, IMP, VIM, OXA-48 and NDM-1) carbapenemase-encoding genes, these have been reported among these isolates (Ghasemian et al., 2018) representing any risk for the country due to resistance genes dissemination among bacteria.

K. variicola its growing clinical importance because of an increasing in the number of reports of MDR isolates (Rodríguez-Medina et al., 2019). Fortunately, the isolated *K. variicola* was susceptible to all antibiotic tested in our study. Although susceptibility this bacterium reports of ESBL- and carbapenemase-producing *K. variicola* isolates have been increasing (Potter et al., 2018). This finding highlights the importance of continual surveillance in the country.

Phenotypic and Genotypic characterization of *Salmonella* spp.

Salmonella isolates were only resistant to amoxicillin-clavulanic acid and trimethoprim-sulfamethoxazole (75%) and piperacillin-tazobactam (25%). Non typhoidal *Salmonella* (NTS) isolates causing BSI have been increasing in developing countries, especially among HIV/AIDS patients. In sub-Saharan Africa, invasive NTS is consistently the most common bacterial bloodstream and it has been showing resistance to ampicillin, cotrimoxazole and chloramphenicol, while being susceptible to ciprofloxacin (Feasey et al., 2012; Mshana et al., 2013). Currently, these isolates can be resistant to ciprofloxacin due to co-selection of resistance genes phenomenon and they are spreading worldwide (Chong et al., 2018; Guiral et al., 2018).

In the present study, although *Salmonella* spp. not being ESBL producers, *S.* Isangi ST335 possessed OXA-10 associated with IncA/C2 plasmid and 3 (1-2-2-2) pMLST type, while *S.* Typhimurium ST313 possessed TEM-1B associated with FIB(S), FII(S) with [S1:A-:B17] IncF pMLST type were found.

Recently *S.* Typhimurium ST313 sublineage II.1 was reported as extensive drug resistance including ESBL production, azithromycin on IncHI2 plasmid in Democratic Republic of Congo and driving chloramphenicol resistance in other countries (Puyvelde et al., 2019; Canals et al., 2019), showing dissemination of resistance among *Enterobacteriaceae* and the role of continuous antimicrobial stewardship.

Phenotypic and Genotypic characterization of *Enterobacter* spp.

80% *Enterobacter* spp. were MDR and ESBL producers, where the highest resistance was found to amoxicillin-clavulanic acid (100%), piperacillin-tazobactam, cefotaxime, ceftazidime, gentamicin and trimethoprim-sulfamethoxazole (80%). Close to our findings, Dimitrova et al., (2015), described *E. cloacae* isolated from blood with the following resistance percentages: amikacin, (97.8%), levofloxacin (76.6%), trimethoprim-sulfamethoxazole (40.4%), ciprofloxacin (19%), gentamicin (8.4%), cefepime (4.2%), and piperacillin-tazobactam, tobramycin (2.1%) (Dimitrova et al., 2015).

In present study, the high prevalence of resistance in *Enterobacter* spp. might be related to the ESBLs genes. Notably, *mcr-9* determinant encoding resistance to colistin were

found only in *Enterobacter complex* among our GNB. Colistin resistance is considered a serious problem, due to a lack of alternative antibiotics (Wang et al., 2019; Aghapour et al., 2019). However, acquired colistin-resistance mechanisms have been recognized in some members of *Enterobacteriaceae* family, including *Enterobacter* spp., *E. coli*, *Salmonella* spp., *Klebsiella* spp. *P. aeruginosa*, *A. baumannii* (Aghapour et al., 2019). Although *mcr-9* was found only in *Enterobacter* spp., this finding constitutes a worrisome situation due to its dissemination through MGE. There is a need of surveillance studies in the country.

E. complex ST84 (not considered widespread) possessed CTX-M-15 and other resistance genes CMH-3, TEM-1B, and OXA-1 associated with IncFII(Yp) and pMLST type [Y3:A-:B-]. *E. cloacae* carrying other resistance genes colistin-resistant coharboring *mcr-4.3* and NDM-1 was found from a patient in China. *E. cloacae*, including *E. coli* and *K. pneumoniae*, possess a number of lineages, spreading on a wide geographical scale and acquiring resistance traits, such as various ESBLs and carbapenemases (Woodford et al., 2011; Izdebski et al., 2015).

Phenotypic and Genotypic characterization of *Proteus mirabilis*

71% and 43% of *P. mirabilis* were MDR and ESBL producers, respectively. The highest resistance was found to trimethoprim-sulfamethoxazole and colistin (86%), amoxicillin-clavulanic acid, ceftazidime, cefotaxime and ciprofloxacin (57%) and gentamicin (71%). Resistance to trimethoprim-sulfamethoxazole, chloramphenicol, cefazoline, moderate resistance to gentamycin and tobramycin, and activity of imipenem, amikacin, azetronam, azithromycin, ciprofloxacin was reported from *P. mirabilis* isolates (Al-Jumaily and Zgaer, 2016). CTX-M-15 was the main mechanism found and linked to A/C2 plasmid. IncA/C plasmid is frequently associated with *Enterobacteriaceae* and harbor multiple resistance genes including resistance for extended-spectrum cephalosporins and carbapenems (Lyimo et al., 2016).

Phenotypic and Genotypic characterization of *Morganella morganii*

M. morganii were (100%) MDR and AmpC producers with high resistance to amoxillicin-clavulanic acid, ceftazidime, cefotaxime, fosfomycin, colistin and trimethoprim-sulfamethoxazole (100%), and piperacillin-tazobactam, gentamicin, ciprofloxacin, fosfomycin, colistin (50%). Carbapenem, amikacin and tigecycline agents were completely effective. This finding might be due to *M. morganii* has intrinsic resistance (DHAM, especially DHA-13) associated with OXA-1 that was found in this study (Liu et al., 2016).

Phenotypic and Genotypic characterization of *Acinetobacter baumannii*

84% and 100% of *A. baumannii* were MDR and carbapenemase producers, respectively. *A. baumannii*, showed highest resistance to trimethoprim-sulfamethoxazole (96%), gentamicin (92%), ciprofloxacin (76%) and meropenem (44%) and least to amikacin (12%). Colistin rendered effective in consistent with previous study from South Africa (Lowings et al., 2015). Uwingabiye et al., (2016), also obtained high frequency of *A. baumannii* resistance to all several antibiotics when compared with our findings, namely 87%, 86%, 79%, 76%; 52%, 43%, 33% 32% and 1.7% to ciprofloxacin, ceftazidime, piperacillin-tazobactam, imipenem, amikacin, tobramycin, netilmicin, rifampicin and colistin, respectively.

ST405, ST758 and ST642 in this study harbored OXAs β-lactamases, except ST642. OXAs type mainly OXA-23, -25, -65, -366 (ST758), OXA-68, -69 (ST405) and OXA-58 (New combination clone) was associated with ADC-25 as a common mechanism of carbapenemase resistance of *A. baumannii* (D'Souza et al., 2019). In addition, ST642 instead of having OXA type harbored especially CARB-16 associated with ADC-25.

Recently, ST758 isolated from urine, sputum and blood cultures in Tshwane region, South Africa neighbouring of Mozambique was found harboring OXA-23. The occurrence of the OXA-23 gene has been reported since 1985 in *A. baumannii* isolates and has now disseminated worldwide (Lowings et al., 2015). ST405 was reported in Brazil possessing OXA-23 associated with IS*Aba1*, sequence which the presence confer resistance to carbapenem agents such as imipenem than meropenem, ertapenem and

doripenem (Carvalho et al., 2011; Royer et al., 2018). ST642 have been reported in Asian countries including, Indonesia, China, Malaysia, and Japan as a carbapenem-nonsusceptible determined by OXA-23-like (Saharman et al., 2018).

Some isolates of *A. baumannii* not whole genome sequenced harbored TEM, CTX-M, VIM and NDM β-lactamases which is consistent with other studies (Ghaima, 2018). TEM and CTX-M are commonly found in *Enterobacteriaceae* (Shaikh et al., 2015) showing the dissemination of this mechanism through MGE, among GNB in HCM.
The resistance to other antibiotic agents such as aminoglycosides, phenicols, trimethoprim, florfenicol, tetracycline, sulfonamides and macrolides were found and distributed according to the STs. Interestingly, *ant(2'')-Ia* determinant encoding resistance to aminoglycosides was found only in this species among all GNB studied and mainly from ST758 and ST642. In addition, *aac(6')-Ian* encoding also resistance to aminoglycosides was found in this species, especially in ST758. These findings show the coexistence of carbapenemase mechanisms associated with other mechanism encoding resistance to several classes of antibiotics, mainly aminoglycoside in accordance with previous study (Nie et al., 2014; Tada et al., 2016).

Phenotypic and Genotypic characterization of *Pseudomonas aeruginosa*

Infections caused by *P. aeruginosa* are notoriously difficult to treat due to its intrinsic ability to resist many classes of antibiotics as well as its ability to acquire resistance. In the present study, 74% and 90% of *P. aeruginosa* were MDR and carbapenemase producers, respectively. *P. aeruginosa* showed highest resistance to trimethoprim-sulfamethoxazole (95%), followed by piperacillin-tazobactam and ceftazidime (63%), meropenem (47%), gentamicin and ciprofloxacin (42%) and least resistance was observed to amikacin (16%) and all *Pseudomonas* spp. were susceptible to colistin. Our results are close to another study that found sensitive to colistin (95.4%), polymyxin B (95%), piperacillin-tazobactum (64.5%), and gentamicin (45%) (Sharma et al., 2016).
Several *P. aeruginosa* STs were found in present study including ST316, ST1047, ST1756, ST274 and ST612 all of them MDR except ST1756. The β-lactamase genes OXA types was found in all ST VIM-11, TEM and SHV in consistent with previous study (Hussain et al., 2017). PAO and OXA types, *aph(3'')*-Ib, *catB7* and *fosA* were found as

the main mechanisms encoding resistance to β-lactams, aminoglycosides, phenicols and fosfomycin, respectively in all *P. aeruginosa* STs. Additionally, OXA-488 and *rmtB*, *aph(3')-Via/aph(3'')-Ib* encoding resistance to β-lactams and aminoglycosides were found only in ST1042, whereas OXA-10, -395 and *ARR-2* encoding resistance to β-lactams agents and rifampicin, respectively, was found in *P. aeruginosa* ST316. *crpP*, is a new mechanism of ciprofloxacin resistance in *P. aeruginosa* (Chávez-Jacobo et al., 2018) found only in *P. aeruginosa* ST316, ST612 and ST1042 and also was not found in GNB species isolated in the present study.

P. aeruginosa ST274 has been reported in European countries and Australia, Spain as a MDR strain (Fernández-Olmos et al., 2013). ST1047, was originally obtained in Norway and found to produce VIM-type MBLs (Tada et al., 2017). ST612 fluoroquinolone resistant have also been reported in Asia continent including Korea Daejeon, Korea (Cho et al., 2013). The presence of these STs of *P. aeruginosa* in HCM might be related to the medical tourism and also trade between Mozambique with other countries.

Phenotypic and Genotypic characterization of *Stenotrophomonas maltophilia*

S. maltophilia has been sensitive to trimethoprim-sulfamethoxazole and to other antibiotics such as minocycline, and levofloxacin (Chung et al., 2012). Unfortunately, in our study, *S. maltophilia* showed (25%) of resistance to trimethoprim-sulfamethoxazole representing an alarming concern to treat infections associated with this isolate and needs of antimicrobial surveillance. No ESBL, AmpC and carbapenemase PCR were done because of its phenotypic susceptibility.

Genotypic characterization of CTX-M-15 among isolates from HCM and Spreading of resistance mechanism

In Zambia, Democratic Republic of Congo, Mozambique and Tanzania, MDR *E. coli*, *K. pneumoniae*, *V. cholerae*, iNTS and other pathogens responsible for nosocomial infections represent a major increase concern due to ESBL producers in confirming the spread of these clones worldwide (Mshana et al., 2013). In our study, CTX-M-15 was found in various GNB species including *E. coli, K. pneumoniae, P. mirabilis, K. oxytoca,* and *E. complex* with 100% of similarity to another CTX-M-15 used to compare from

database with KF727590 (accession number). In addition, CTX-M-15 were found associated with insertion sequence IS*Ecp1* in (97%) of GNB species linked mainly with IncF conjugative plasmid family that has been found playing a role in dissemination of this ESBL. This finding suggests that the main mechanism of CTX-M dissemination is related to MGE not only as a result of particular clones dissemination (Guiral et al., 2018). IncF plasmid and other carrying ESBL genes, mainly CTX-M-15 commonly possess the capability of getting transferred between strains or even species, favoring their dissemination among the bacterial population and from region to region (Coque et al., 2008; Carattoli, 2013).

Sequence types and Resistance determinants of GNB isolated in Pediatric Department of HCM and other Hospitals

Plasmids mainly F, A/C, L/M, I1, HI2 and N family, especially IncF group (FIA, FIB and FII), IncK and IncI1 largely contribute to the dissemination of ESBL genes, primarily of $bla_{\text{CTX-M-15}}$ (Zhao and Hu, 2013). In addition, high risk or pandemic clones are playing a role in dissemination of resistance mechanism in *Enterobacteriaceae* (Caratolli, 2009). *E. coli* ST131, *S.* Typhimurium ST313 and *K. pneumoniae* were found among hospitals possessing the same or different mechanisms of resistance. Several reports have described the isolation of clonal ExPEC and other isolates from non-human sources, including food products, food animals, and companion and wild animals rather than local selection by antimicrobial drugs (Riley, 2014). However, in present study although there are needs of more research to better understand the main mechanism of clones disseminations, this fact might be related to patients transference or it could be healthcare workers rotating between hospitals. The HCM is a referral hospital in the country the reason that receive patient from all Mozambican provinces. These finding suggest the necessity of "One Health approach" in the country in order to better understand the dissemination of resistance mechanism among GNB.

Conclusion

According to the findings presented above, phenotypic and genotypic information of Extraintestinal Pathogenic *Escherichia coli* (ExPEC) and other GNB in Mozambique have been provided.

The most frequent isolates in HCM were *K. pneumoniae* (27%), followed by ExPEC and *Acinetobacter* spp. (20%). Other GNB as *Pseudomonas* spp., *P. mirabilis*, *Enterobacter* spp., *Salmonella* spp., *M. morgani*, *K. oxytoca*, *K. variicola*, and *S. maltophilia* were less observed (ranging from 8% to 1%).

In other hospitals, seven species of GNB from few blood samples were isolated, including *Salmonella* (Typhimurium and Isangi), *E. coli, K. pneumoniae*, *E. cloacae*, *P. aeruginosa, Acinetobacter* spp., *S. complex*, *S. marcescens* and *P. septica* only in Pediatric departments.

Regarding ExPEC isolates 72%, 40%, 20% were MDR, ESBL, and AmpC producers, respectively, displaying high resistance to trimethoprim-sulfamethoxazole, penicillins, cephalosporins, aminoglycosides and ciprofloxacin, ranging from 52% to 88%. Further, in 8% of isolates resistance to carbapenem agents was found associated with NDM-5 and/or CMY-2.

The main mechanisms conferring resistance in ExPEC were CTX-M-15 (70%) and CTX-M-27 (24%) in ESBL producers, TEM-1B and OXA-1 for narrow β-lactamases, *aac(6')Ib-cr*, chromosomal mutations *gyrA* (S83L and D87N), *parC* (S80I or E84V) and *parE* (S458A or I529L) for fluoroquinolone resistance, *aac(3)-II*, *aph(3")-Ib/aph(6)-Id* for aminoglycosides. Further, many other resistance genes were identified in ExPEC as *dfrA7, dfrA8*, *dfrA17*, *mdf(A)*, *mph(A)*, *catB3*, *sul1* and *sul2*, *tet(A)* and *tet(B)*. IncF plasmid family, especially IncFII replicon drives the dissemination of these mechanism in HCM.

The MLST showed high diversity identifying 13 STs, of which ST131 and ST410 were the most represented accounting each for 46% of isolates, followed by ST38 and ST394 (15%), while others STs, ST361, ST617, ST1177, ST405, ST69, ST457, ST59, ST62, and ST648 were identified in unique isolates. Notably, the ST131 clonal subgroup H30,

responsible for the current pandemic of fluoroquinolone and multi-drug resistant *E. coli* infections around the globe was found among Mozambique ExPEC. ST405 represent emergent MDR *E. coli* lineages and harbored NDM-5 encoding resistance to carbapenem agents associated with CTX-M-15. This ST constitute a high risk for the country because carbapenems agents are considered last resort treatment for severe infected diseases caused by MDR gram-negative bacilli. Additionally, due to the localization of NDM-5 on MGE there is the risk of its dissemination among GNB in the country where most of carbapenem agents are often unaffordable.

K. pneumoniae was the most abundant among GNB in this study displaying high level of MDR (77%) and ESBL (80%) producing, with high resistance to trimethoprim-sulfamethoxazole, penicillins, cephalosporins, gentamicin, and ciprofloxacin, ranging from 71% to 83%. CTX-M-15 (81%), CTX-M-9 (37%) and CTX-M-88 (4%) were the most represented ESBLs, found also in association with SHV, TEM, OXA-1 and LEN12. These mechanisms were found linked to IncF plasmid, especially IncFII(K) replicon. *K. pneumoniae* was assigned to 10 STs, including ST13, ST48, ST985, ST711, ST394, ST17, ST14, ST23, ST831, ST607, all of them ESBL producers carrying mainly CTX-M-15, except for ST17 and ST23.

Notably, a ST23 hypervirulent strain due to KL1 capsule and conjugative element (ICEKp10) encoding siderophore yersiniabactin and colibactin, transcriptional regulators capsular polysaccharide biosynthesis *rmpA* and *rmpA2*, *iutA* encoding aerobactin, *magA* encoding for the K1 mucoviscous serotype and *KpnO* porin was found among our isolates.

Other *Enterobacteriaceae*, including *K. oxytoca*, *Salmonella* spp., *Enterobacter* spp., *P. mirabilis* and *M. morganii* isolates were MDR, with the exclusion of *Salmonella* spp. Only carbapenem agents were completely effective against all these *Enterobacteriaceae*. *K. oxytoca*, and *Enterobacter* spp., ESBL producers harbored mainly CTX-M-15 associated with IncFII, whereas in *P. mirabilis* producers CTX-M-15 was linked to A/C2 plasmid family. AmpC *M. morganii* producers was encoded by DHAM, mainly DHAM-13. *mcr-9* plasmid gene linked to colistin resistance was found in 1 *Enterobacter complex*. 84% and 100% of *A. baumannii* were MDR and carbapenemase producers respectively, and colistin was completely effective against *A. baumannii* including *P. aeruginosa*.

MLST assigned ST405, ST758 and ST642 to *A. baumannii* isolates, in which OXAs β-lactamases was the main mechanism encoding resistance to carbapenem agents, except for ST642. ST758 harbored OXA-23, -25, -65, -366; ST405, OXA-68, -69 and a new ST clone, OXA-58. All STs possessed ADC-25 β-lactamase.

74% and 90% of *P. aeruginosa* were MDR and carbapenemase producers, respectively. ST316, ST1047, ST1756, ST274 and ST612 were assigned by MLST. The β-lactamase OXA types was found in all STs associated with carbapenemase (VIM-11) and ESBL (TEM and SHV) found in some strains.

Among different species from HCM and other hospitals, including ExPEC*, K. pneumoniae, P. mirabilis, K. oxytoca,* and *E. complex*, CTX-M-15 was found mainly linked to IncF and A/C plasmid with 100% of similarity and associated with the insertion sequence IS*Ecp1* which has played an important role in dissemination of resistance genes among GNB species in HCM.

The results presented above support the fact that the resistance to the commonly antibiotic, including penicillins, cephalosporins, trimethoprim-sulfamethoxazole, gentamicin and ciprofloxacin used in Mozambique is high, and associated with different mechanisms ESBL (CTX-M-9, -15, -27, -88), AmpC (CMY-2) and carbapenemases (NDM-5, OXAs, VIM) although the less use of carbapenem agents in the country. The presence of pandemic or/and emerging ExPEC (mainly ST131, ST69, ST410, ST405, ST38), *K. pneumoniae* (ST23), *K. oxytoca*, *E. cloacae* (ST84), *P. mirabilis* carrying CTX-M-15 upstream of IS*Ecp1* on IncF and A/C plasmid family represent a high risk for the country due to rapidly dissemination and evolution of diverse multiresistant plasmids, and also for treatment failure with antibiotics. Additionally, *A. baumanii* (ST405 and ST758) and *P. aeruginosa* (ST274) resistant to carbapenem agents mainly due to OXA types, inclunding OXA-23 constitute a high risk associated with location of this mechanism on MGE.

Hypervirulent ST23 *K. pneumoniae* constitute a worrisome situation due to its ability to generate invasive community-acquired infections. Therefore, prudent use of antibiotics is advocated, and a systematic national surveillance system of antibiotic resistance is urgently needed to overcome the dissemination of producing ESBL, AmpC, and carbapenemases producing GNB in Mozambique. The presence of *E. coli* ST405 and

ST410 carrying carbapenemase (NDM-5) and AmpC (CMY-2), respectively, both located on IncF plasmid highlight the needs of stricter adherence to infection prevention and control policies by healthcare workers to prevent further dissemination in HCM. Early detection of ESBL, AmpC and carbapenemase genes would be important for the reduction of mortality rate and spread of MDR organisms in studied hospitals.

Limitations of the study

Unfortunately, detailed information related patient data, health condition of patients and provenience of some department were not all obtained from HCM.

Although the study found evidence of antimicrobial resistance of ExPEC and other GNB, it doesn't give the complete picture of the situation particularly in Maputo (HCM, HGM, HJM) and Quelimane hospitals (HPQ and HCQ), due to the small size of samples related to high contaminations during blood and CSF sampling and isolations of strains.
Additionally, as the isolation of the strains was conducted in a developing country, storing conditions of isolates were not appropriate, which resulted in the loss of many isolates. Furthermore, the lack of whole genome sequences of all GNB, except *E. coli* did not permit us to have all the further information related mainly to virulence associated genes, sequence types and antimicrobial resistance determinants of all isolates found in this study.

CHAPTER FIVE

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APPENDICES

APPENDIX 1

Table 1. Function and Accession numbers of Virulence associated genes of *E. coli* searched on Geneious R11 tool.

Gene	Protein Function	Acession number/NCBI
Crl	Enable biofilm formation and promote pathogenicity	X67207.1
cvaC	Colicin V	JN704082.1
F1C fimbriae (focA)	Adhesin	AF298200.1
fimH	Adhesin	JX847135.1
Usp	Flagellin variant	AB027193.1
fyuA	Yersiniabactin	KP657549.1
hlyD	Hemolysin	Y13891.1
ibeA	Cell invasion into the host tissues	AY248744.1
iucC	Aerobactin synthase	MK941173.1
iutA	Aerobactin receptor	JX466848.1
K1	Capsule (protectins)	AY779018.1
KpsM	The protection factor against phagocytosis and the spreading factor	M57382.1
malX	Maltose and glucose-specific PTS transporter subunit IICB	MH753045.1
ompT	Enable intracellular survival, evasion from the body's defense.	HM210637.1
papA	Stimulate the production of cytokines by T lymphocytes, colonization factor in extraintestinal infections	NC_011750.1
papC	Stimulate the production of cytokines by T lymphocytes, colonization factor in extraintestinal infections	JX485631.1
papG	Stimulate the production of cytokines by T lymphocytes, colonization factor in extraintestinal infections	AF237474.1
sitA	Transportation of Fe, Mn	KP657545.1
traT	Inhibition of the classical pathway of complement activity	KY020407.1

Abbreviation: NCBI, National Center for Biotechnology Information.

APPENDIX 2

Table 2. Study of pathogenicity phylogenetic classification, and virulence genes in 29 ExPEC strains (25 isolated in HCM and 4 isolated in other Hospitals).

Isolate	ST	*fimH*	*papA*	*papC*	*papG*	*sfaA*	*sfaS*	*F1C*	*iss*	*traT*	*cvaC*	*ompT*	*KpsM*	*iha*	*ireA*	*iroN*	*iucC*	*iutA*	*sitA*	*fyuA*	*vat*	*sat*	*hlyD*	*maxL*	usp	Total
SS21P	ST1177	+	+	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	+	+	-	+	-	-	-	10
SS145*	ST12	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	19
SS140*	ST127	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	20
SS46P	ST131	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	6
SS50	ST131	+	+	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+	+	+	-	+	-	+	+	13
SS45A	ST131	+	+	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	10
SS37P	ST131	+	+	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	+	+	-	+	-	+	+	12
SS67	ST131	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS137*	ST131	+	+	-	-	-	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	15
SS48C	ST131	+	+	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	+	+	+	14
SS127A*	ST156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
SS53B	ST361	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	2
SS101	ST38	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS101A	ST38	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	3
SS116	ST394	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS117	ST394	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
SS100	ST405	+	+	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	+	-	+	-	+	-	10
SS34P	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS13P	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS26	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS30	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS49	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SSJ6	ST410	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS39	ST457	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS38A	ST59	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	2
SS13	ST617	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
SS106	ST62	+	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	-	-	+	-	-	-	9
SS 4P	ST648	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
SS61	ST69	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-	+	-	-	6
Total	**29**	**19**	**11**	**3**	**1**	**1**	**1**	**2**	**11**	**12**	**1**	**3**	**11**	**12**	**2**	**4**	**8**	**11**	**12**	**9**	**3**	**10**	**4**	**7**	**6**	**164**

Abbreviations: ST, sequence type; * Isolates from other hospitals (HGM, HJM and HPQ); -, negative; + positive.

APPENDIX 3

Table 3. Study of pathogenicity phylogenetic classification, and virulence genes in 29 ExPEC strains (25 isolates in HCM and 4 isolates in other Hospitals).

Isolate	ST	ORF3	ORF3	Aar	Air	aatA	agg3B	agg3C	agg3D	agg5A	aggR	Aap	astA	eatA	eilA	capU	cnf1	Cma	mchB	mchC	mchF	mcmA	nfaE	senB	gad	ipfA	pic	crl	Total
SS21P	ST1177	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	4
SS145*	ST12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	+	7
SS140*	ST127	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	4
SS46P	ST131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	2
SS50	ST131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	3
SS45A	ST131	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	4
SS37P	ST131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	2
SS67	ST131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	1
SS137*	ST131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+	4
SS48C	ST131	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	14
SS127A*	ST156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	3
SS53B	ST361	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	2
SS101	ST38	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+	4
SS101A	ST38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
SS116	ST394	-	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	6
SS117	ST394	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	7
SS100	ST405	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	3
SS34P	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	1
SS13P	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	2
SS26	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	3
SS30	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2
SS49	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2
SS6	ST410	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	2
SS39	ST457	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	3
SS38A	ST59	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	2
SS13	ST617	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	2
SS106	ST62	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	-	-	5
SS14P	ST648	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	5
SS61	ST69	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-	+	6
Total	**29**	**1**	**1**	**1**	**5**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**3**	**1**	**10**	**2**	**3**	**1**	**1**	**1**	**2**	**2**	**2**	**8**	**19**	**13**	**1**	**20**	**106**

Abbreviations: ST, sequence type; * Isolates from other hospitals (HGM, HJM and HPQ); -, negative; + positive.

APPENDIX 4

Table 4. Plasmid characterization and epidemiological relationship between the ExPEC isolates (25 isolates in HCM and 4 isolates in Other Hospitals).

Isolate	Hospital	Department	Source	ST	ESBL/AmpC/Carb	Other ESBL	Plasmid replicon	pMLST IncF RST or A/C2
SS13	HCM	Pediatric	Blood	ST617	CTX-M-15	OXA-1	FIA, FIB, FII, I1, I2, Q1, X4	[F31:A4:B1]
SS61	HCM	Pediatric	Blood	ST69	CTX-M-15	OXA-1	Col, FIB, FII, HI1B, I1, Q1	-
SS46P	HCM	Surgery	Pus	ST131	CTX-M-15	TEM-1B	Col, FIA, FIB, FII	[F1:A2:B20]
SS37P	HCM	Pediatric	Pus	ST131	CTX-M-15	TEM-1B	Col, FIA, FIB, FII	[F1:A2:B20]
SS30	HCM	Pediatric	Blood	ST410	CTX-M-15	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS137*	HGM	Pediatric	Blood	ST131	CTX-M-15	TEM-1B/OXA-1	FIA, FIB, FII, I1, X1	[F36:A6:B1]
SS45A	HCM	Medicine	Blood	ST131	CTX-M-15	TEM-1B/OXA-1	Col, FIA, FIB, FII, I1	[F1:A2:B20]
SS53B	HCM	Pediatric	Blood	ST361	CTX-M-15/SCO-1	TEM-63,-133	A/C2	3 (1-2-2-2)
SS26	HCM	NA	Blood	ST410	CTX-M-15/CMY2	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS49	HCM	Pediatric	Blood	ST410	CTX-M-15/CMY2	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS6	HCM	Pediatric	Blood	ST410	CTX-M-15/CMY2	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS13P	HCM	Surgery	Pus	ST410	CTX-M-15/CMY2	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS100	HCM	Medicine	Blood	ST405	CTX-M-15/NDM-5	TEM-1B	Col, FIA, FIB, FII, I1, I2, Q1, X4	[F1:A1:B49]
SS34P	HCM	Surgery	Pus	ST410	CMY2	TEM-1B/OXA-1	Col, FIA, FIB, FII, Q1, p0111	[F1:A1:B49]
SS39	HCM	Pediatric	Blood	ST457	CTX-M-27	-	FIB, FII	[F2:A-:B10]
SS50	HCM	Pediatric	Blood	ST131	CTX-M-27	TEM-1B	Col, FIA, FIB, FII	[F1:A2:B20]
SS101	HCM	Medicine	Blood	ST38	CTX-M-27	TEM-1B	Col, FIB, FII, Y	[F2:A-:B10]
SS101A	HCM	Medicine	Blood	ST38	CTX-M-27	TEM-1B	FIB, FII, Y	[F2:A-:B10]
SS127A*	HPQ	Pediatric	Blood	ST156	-	TEM-141,-206,-214,-216	Col, FIB, FII, Q1	[F-:A-:B28]
SS48C	HCM	Pediatric	Blood	ST131	-	TEM-1B	FIB, FII, Q1,	[F1:A-:B63]
SS38A	HCM	NA	Blood	ST59	-	TEM-1B	Col, FIB, FII, Q1	[F95:A-:B10]
SS140*	HGM	Pediatric	Blood	SS127	-	TEM-1B	Q1	-
SS145*	HJM	Pediatric	Blood	ST12	-	TEM-1B	FIA, FIB, FII, Q1	[F1:A1:B20]
SS106	HCM	Medicine	Blood	ST62	-	TEM-1B	Col, FIB, FII	[F29:A-:B10]
SS117	HCM	NA	Blood	ST394	-	TEM-1B	FII	[F11:A-:B-]
SS67	HCM	Pediatric	Blood	ST131	-	TEM-1B	FIB, Q1	[F-:A-:B63]
SS21P	HCM	Medicine	Pus	ST1177	-	TEM-1B	Col, FIB, FII, I1	[F29:A-:B10]
SS14P	HCM	Medicine	Pus	ST648	-	-	Col3M	[F-:A-:B-]
SS116	HCM	NA	Blood	ST394	-	-	FII	[F11:A-:B-]

Abbreviations: ST, sequence type; * Isolates from other hospitals (HGM, HJM and HPQ); NA, not available; ESBL, extended-spectrum β-lactamases; AmpC, Plasmid mediated β-lactamases; Carb, carbapenemase -, negative; IncF RST, replicon sequence type.