Epidemiology of extended-spectrum beta-lactamase- and carbapenemase-producing bacteria in stool from apparently healthy children, South Africa

Dissertation submitted for the degree of Master of Science in Medicine (Medical Microbiology)

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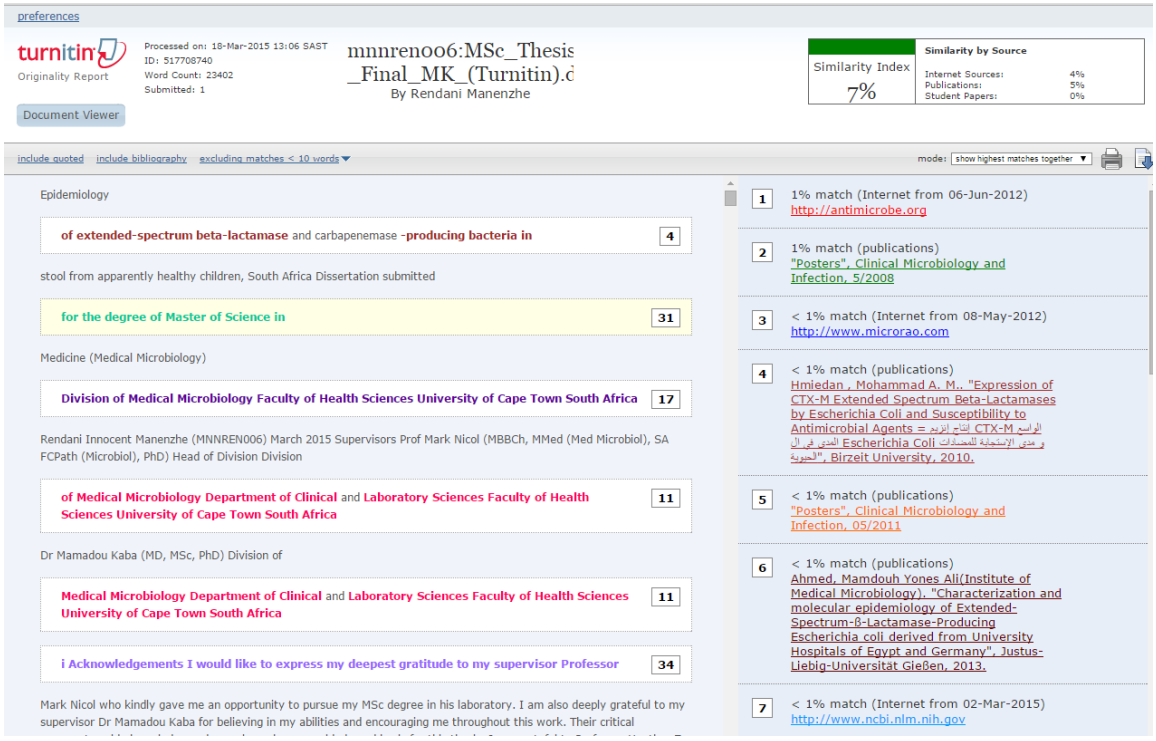

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Foreword

This thesis is submitted for the degree of Master of Science in Medical Microbiology, and was conducted within the Division of Medical Microbiology, Department of Clinical Sciences, University of Cape Town, South Africa.

Multidrug-resistance in Gram-negative bacteria that produce either extended-spectrum beta-lactamases (ESBLs) or carbapenemases is a public health concern due to the limited availability of treatment options. Their actual prevalence in healthy human in the community settings is largely unknown and possibly underestimated. This study aimed to determine their prevalence and genetic characteristics in stools from healthy infants of less than one year of age and their mothers, and to determine the risk factors associated with their carriage.

This pilot study was nested within an ongoing longitudinal prospective birth-cohort study, the Drakenstein Child Health Study (DCHS), located in the Drakenstein sub-district, Western Cape Province, South Africa. The study was approved by the Faculty of Health Sciences (FHS) Human Research Ethics Committee (HREC) of the University of Cape Town, South Africa (HREC reference number: 738/2013).

This work was funded by the Bill and Melinda Gates Foundation Global Health Grant (OPP107641), the Wellcome Trust (United Kingdom), the Carnegie Corporation of New York (United States of America), and the National Research Foundation (South Africa). Any opinions, findings and conclusions, or recommendations expressed in this dissertation are those of the author, and therefore do not represent the official position of the funders.

This thesis was written in a monograph format, and supervised by Dr Mamadou Kaba and Prof Mark Nicol. It consists of scientific contributions or publications co-authored by the MSc candidate. This thesis includes an abstract, general introduction (including the rationale, aim, and objectives), literature review, the methods, the results, discussion, and conclusion and future direction.

Scientific contributions

Articles published in a peer-reviewed journal

Manenzhe RI, Zar HJ, Nicol MP, Kaba M. The spread of carbapenemase-producing bacteria in Africa: a systematic review. *Journal of Antimicrobial Chemotherapy* 2015; **70**: 23–40.

Conference contributions

1. R. I. Manenzhe, H. Zar, M. P. Nicol, M. Kaba. A systematic overview of the epidemiology of carbapenemase-producing bacteria in Africa: 16th International Congress on Infectious Diseases (ICID), Cape Town, South Africa, April 2-5, 2014. Abstract accepted for International Scientific Exchange (ISE).

2. R Manenzhe; C Moodley; M Nicol; H Zar; M Kaba. Faecal carriage of extended-spectrum beta-lactamase- and carbapenemase-producing Enterobacteriaceae in healthy infants and their mothers, South Africa: Keystone Symposia Conference on Gram-negative Resistance, California, United State of America, March 29 - April 2, 2015. Abstract accepted for poster presentation.

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Abstract

Background: The prevalence of extended-spectrum beta-lactamase (ESBL) - and carbapenemase-producing Enterobacteriaceae in healthy humans in the community is largely unknown. We aimed to determine the prevalence and genetic characteristics of ESBL- and carbapenemase-producing Enterobacteriaceae in stools from healthy infants and their mothers, and to determine the risk factors associated with their carriage.

Methods: This study was nested within the Drakenstein Child Health Study, a birth cohort in a semi-rural region of Western Cape Province, South Africa. Maternal and infants faecal samples (including the meconium) were collected at birth and at two additional time-points (5-12 and 20-28 weeks) from the infants only. Samples were screened for ESBLs and carbapenemase-producing organisms using ChromID ESBL and ChromID CARBA media, respectively. Identification of suspect ESBL/carbapenemase-producing isolates and antibiotic susceptibility were determined using the Vitek 2 system. ESBL production was confirmed using the combination disc test, and that of carbapenemase using the modified hodge test. Selected ESBL and carbapenemase genes were evaluated by the singleplex conventional polymerase chain reaction and Sanger sequencing. Risk factors were assessed by univariate analysis using the EPI Info version 7 software.

Finding: We screened 193 stool and 116 meconium samples. Maternal faecal carriage of ESBL-producing organisms was 4.4%; 4/90 (95% confidence interval (CI): 1.5% - 10.2%), and that of infants at birth was 3.5%; 4/116 (95% CI: 1.2% - 8.0%). The infant faecal carriage of ESBL-producing organisms at 5-12 and 20-28 weeks was 4.4%; 3/68 (95% CI: 1.3% - 11.3%) and 5.7%; 2/35 (95% CI: 1.2% - 17.1%), respectively. ESBL genes were detected in six *K. pneumoniae* ($bla_{\text{CTX-M-15}}$), five *E. cloacae* ($bla_{\text{SHV-12}}$ (n= 3) and $bla_{\text{SHV-5}}$ (n= 2)) and three *E. coli* ($bla_{\text{CTX-M-14}}$) isolates. All stool samples tested negative for carbapenemase-producers. Pulsed-field gel electrophoresis showed heterogeneous clones of CTX-M-15-producing *K. pneumoniae* isolates. In contrast, we observed clonal relations among ESBL-producing *E. cloacae* isolates. One mother-infant pair was ESBL-positive at birth with SHV-5- producing *E. cloacae*. In addition, one infant was persistently colonized by SHV-12-producing *E. cloacae* isolate. Infants born to HIV-positive mother, via elective caesarean section, and medication use before discharge were positively associated with ESBL faecal carriage. In contrast, breastfeeding prior to discharge was negatively associated with ESBL carriage.

Interpretation: This is the first study to detect ESBL-producing bacteria in human meconium samples in Africa, and raises questions on the source of such isolates and implications for community transmission. Faecal carriage of ESBL-producing bacteria was common in our study area. Carbapenemase-producing organisms were not detected in stool samples tested. Further research or continuous surveillance is important in order to anticipate future trends in the dissemination of ESBL- and carbapenemase-producing organisms in community settings in South Africa.

1. Introduction

1.1 Problem identification

Multidrug-resistance is emerging worldwide at an alarming rate among Gram-negative bacteria causing both community-acquired and nosocomial infections.[1,2] One of the most important emerging resistance profiles in bacteria belonging to the Enterobacteriaceae family corresponds to resistance to extended-spectrum beta-lactams and carbapenems.[2–4] The major reservoir for such bacteria is the gastrointestinal tract.[5] Prior gastrointestinal colonization by multidrug resistant bacteria has been associated with subsequent infection.[6] A study in the United States of America (USA) has emphasized the importance of identifying individuals carrying antimicrobial-resistant bacteria in both patient and healthy populations.[7] Another study reported that an increase in the proportion of carriage of antimicrobial-resistant bacteria in the community increases the risk that other individuals will also become carriers via human-to-human transmission.[8–10] In addition, the admission into hospital of patients harboring resistant bacteria increases the risk of other hospitalized patients contracting an infection.[8,11,12]

The actual prevalence of extended-spectrum beta-lactamase (ESBL) - and carbapenemase-producing organisms in healthy humans in the community settings is largely unknown and possibly underestimated.[13,14] The acquisition of antibiotic resistance genes at birth has been recently reported, with the mode of delivery affecting rates of acquisition,[15,16] however most studies on the community carriage of antimicrobial-resistant bacteria have been cross-sectional, and targeted adult populations.[14,17–21] In addition, few longitudinal birth-cohort studies describing the acquisition of ESBL- and carbapenemase-producing organisms have been conducted worldwide.[22,23] Therefore, the aim of this project was to study the epidemiology and genetic characteristics of ESBL- and carbapenemase-producing bacteria in stool samples from apparently healthy children of less than one year of age and their mothers, and to determine the risk factors associated with their acquisition.

1.2 Rationale and motivation

Multidrug-resistance in Gram-negative bacteria is a public health concern due to the limited availability of treatment options.[2,24,25] The prevalence of multidrug-resistant

Gram-negative bacteria that produce either extended-spectrum beta-lactamases (ESBLs) or carbapenemases has been increasingly reported over the past years, both in hospitals and communities worldwide.[3,4] The microorganisms producing ESBLs may be responsible for infections that are life-threatening, thus resulting in increased healthcare-associated costs, morbidity and mortality.[26] The first line drugs for the treatment of severe infections caused by ESBL-producing bacteria are the carbapenems,[27] however their efficacy is highly compromised by the emergence and spread of carbapenemase-producing bacteria worldwide.[28,29] The strains that produce either carbapenemases or ESBLs often carry resistance determinants for other classes of antimicrobial agents, and infections caused by these strains are associated with high mortality.[30–32]

A major concern is the coexistence of multiple ESBL and carbapenemase genes that has led to the emergence of organisms that are resistance to nearly all antibiotics.[33] The situation is also complicated by the emergence of a novel carbapenemase, the New Delhi Metallo-beta-lactamase 1 (NDM-1).[34,35] NDM-1-producing bacteria are highly resistant to almost all beta-lactam antibiotics.[34] The $bla_{\text{NDM-1}}$ gene is carried on the large plasmids and is increasingly discovered in diverse bacteria.[36–41] Most organisms producing NDM-1 co-produce the extended-spectrum beta-lactamase CTX-M-15.[13] The identification of NDM-1 in *Escherichia coli* sequence type 131 (ST 131) as a source of community-acquired infection is more concerning.[42] *E. coli* ST-131 is known to effectively mobilize the ESBL CTX-M-15 worldwide.[43]

The epidemiology of ESBL- and carbapenemase-producing organisms has been described in considerable detail in certain parts of the world.[30,32,44] The emergence of ESBLs and that of carbapenemases has had different epicenters, but they are now widespread worldwide.[13,41,45] ESBLs became apparent almost simultaneously in Europe and South America, whereas carbapenemases emerged mainly in Asia, Europe and North America.[13,45] ESBLs have spread widely geographically, causing outbreaks in many parts of the world,[32] and this is likely to be the case with carbapenemases.[45] The prevalence of ESBLs and carbapenemases in the community varies depending on geographical location.[46,47] For example, in community settings, the prevalence of ESBLs in children has been reported to range from 0.1% in Bolivia and Peru,[48] to 10% in

Senegal.[49] The prevalence of bacteria harboring carbapenemases in the community is largely unknown.[13]

In Africa, data on the prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae remain scarce and only few studies have been conducted in the community or hospital settings during non-outbreak situations.[41,49–52] For instance, a study in a Moroccan hospital showed a prevalence of 13% faecal carriage of OXA-48-carbapenemase producing Enterobacteriaceae among hospitalized patients.[50] A study in Niger found ESBL-producing Enterobacteriaceae carriage of 31% within 24h of hospitalization among 55 children,[53] while a study in Gabon found a prevalence of 33.6% of ESBL-producing Enterobacteriaceae within 48h of hospitalization in 110 children, reflecting the likely faecal carriage of ESBL-producing Enterobacteriaceae in community settings.[54] In addition, 2 out of 20 children with no known antibiotic exposure living in a very remote Senegalese village were found to be faecal carriers of a multiresistant *Escherichia coli* clone that produced CTX-M-15, highlighting the spread of ESBLs even in isolated communities.[49]

Colonization of the gastrointestinal tract by resistant organisms has been associated with a high risk for developing infection due to these organisms.[6,44] Several risk factors have been found to be associated with ESBL and carbapenemase carriage, which include prolonged hospitalization, prolonged ICU stay, multiple hospitalization, invasive devices, immunosuppression and recent antibiotic treatment.[55–57] Other risk factors for ESBL rectal carriage are old age (older than 65 years), female sex and MRSA carriage.[57] In the community, risk factors are still not clear but factors that enhance the spread of these organisms include lack of hygiene, overuse and over-the-counter use of antibacterial drugs, and increased worldwide travel.[13]

To date, only few studies have been conducted on ESBL- and carbapenemase-producing bacteria in apparently healthy populations in Africa.[49,54] Carbapenemase- and ESBL-producing bacteria have been shown to be circulating in hospitals in South Africa,[50,55,58,59] Therefore, this study aimed to determine the prevalence and risk factors associated with the acquisition of both extended-spectrum beta-lactamase- and carbapenemase-producing bacteria in stool samples from apparently healthy children of less than one year in the community and their mothers. In addition, the study

assessed mother-to-infant transmission of ESBL- and carbapenemase-producers. Finally, the characterization of resistance genes provided some data on the resistance genes circulating in the study area.

1.3 Aim and objectives

1.3.1 The aim

This study aimed to study the epidemiology and genetic characteristics of ESBL- and carbapenemase-producing bacteria in stool samples from of apparently healthy South African children and their mothers.

1.3.2 Specific objectives

- To determine the prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae in stool samples from apparently healthy children of less than one year of age and their mothers and risk factors associated with their acquisition.
- To characterize the ESBL and carbapenemase genes from ESBL- and carbapenemase-producing isolates.
- To investigate the genetic relatedness of the ESBL- and carbapenemase-producing isolates within infant-mother pairs.
- To determine antibiotic susceptibility profiles of ESBL- and carbapenemase-producing isolates.

2. A review on the epidemiology of extended-spectrum beta-lactamase- and carbapenemase-producing bacteria

The epidemiology of ESBL- and carbapenemase-producing organisms has been substantially described in Europe, North America and Asia.[13,45,60–62] Little is known about their epidemiology in Africa, however, their prevalence is increasingly reported in both hospital and community settings.[41,63,64] Therefore, this review gives an overview of the epidemiology of ESBL- and carbapenemase-producers in Africa. In addition, it aimed to identify research gaps regarding the study of ESBL- and carbapenemase-producing bacteria.

2.1 An overview of antibiotic resistance

2.1.1 Discovery and occurrence of antibiotic resistance

Long before the discovery of penicillin by the British scientist Alexander Fleming in the late 1920s, infectious diseases were the leading cause of mortality worldwide.[65,66] The introduction of penicillin in the 1940s and together with vaccination resulted in improved life expectancy and reduced mortality rate due to infectious diseases during the 20th century.[65,67] However, soon after its introduction penicillin-resistant *Staphylococcus aureus* was detected.[68] The resistance to penicillin was due to bacterial production of penicillinase, a beta-lactamase enzyme capable of hydrolysing penicillin.[68,69] Other antibiotics such as tetracycline, streptomycin and chloramphenicol were introduced in the late 1940s, likewise resistance to these antibiotics was also noted soon after their introduction.[70]

Antibiotic resistance, defined as the ability of bacteria to resist the effects of antibacterial drugs, is one of the world's most pressing public health threats.[70,71] Infections caused by multidrug-resistant (MDR) organisms are increasingly reported worldwide,[1,2,72,73] with very limited treatment options.[74] Lack of effective treatment of patients infected with MDR organisms has led to high mortality and morbidity rates.[74] The rate of novel antibiotic discovery is currently very low compared to the increasing resistance problem.[75] However, it is worth mentioning that a novel antibiotic under development (phase I clinical trial) shows potential for treatment of multidrug-resistant respiratory and skin pathogens.[76] Among other factors, multiple mechanisms of

resistance to antibiotics makes it difficult to develop or discover effective antibiotics.[74,75]

2.1.2 Mechanisms of antibiotic resistance

Resistance can be either an inherent trait of the bacterium (intrinsic) or acquired.[77] A variety of bacterial species possess intrinsic resistance through inherited mechanisms.[78] Some bacteria can mutate to highly resistant forms, however, this accounts for a small proportion of antibiotic resistance.[70] Most bacteria become resistant to antibiotics through the acquisition of resistance-conferring deoxyribonucleic acid (DNA) from other sources.[78–81] Antibiotic resistance genes can be carried on a plasmid, chromosome, or transposon; however, most of these genes are encoded or carried on plasmids, which are self-replicating extrachromosomal pieces of DNA (and which may themselves carry transposons).[78,82–84] Transposons are non-self-replicating pieces of genetic material that can be easily exchanged between plasmid and plasmid, chromosome and chromosome, or between plasmid and chromosome.[82] Transposons also play a major role in disseminating resistance genes among bacterial species.[78,83,84] Resistance genes can be spread from one bacterial species to another by conjugation, transformation, or transduction.[85] Plasmids can transfer genetic material through conjugation.[82] Transduction is a process in which a bacteriophage is needed for exchange of genetic material; whereas, transformation is the uptake of free DNA from the environment.[78–80,83,85]

Bacterial resistance to antibiotics can be caused by either the enzymatic modification of an antibiotic which renders it ineffective, the modification of its target site so that it is unable to exert its effect, active physical removal of the antibiotic from the cell, or by reduction of uptake into the bacterial cell due to changes in membrane permeability.[78,86,87] The main resistance mechanism is the enzymatic inactivation of an antibiotic and the most clinically important example of this is beta-lactamase enzymes, the enzymes that hydrolyze beta-lactam antibiotics.[88,89] This review will focus beta-lactamase enzymes, which are the subject of this thesis.

2.2 Beta-lactams

2.2.1 Classification of beta-lactams

Beta-lactams are a broad class of antibiotics that are widely used for treating bacterial infections.[90] All antibiotics in this class contain, in their molecular structure, a beta-lactam ring which is a four membered lactam (cyclic amide) (Figure 1).[91,92] Beta-lactam antibiotics include: the penicillins, cephamycins, cephalosporins, monobactams, carbapenems and beta-lactamase inhibitors.[90,92]

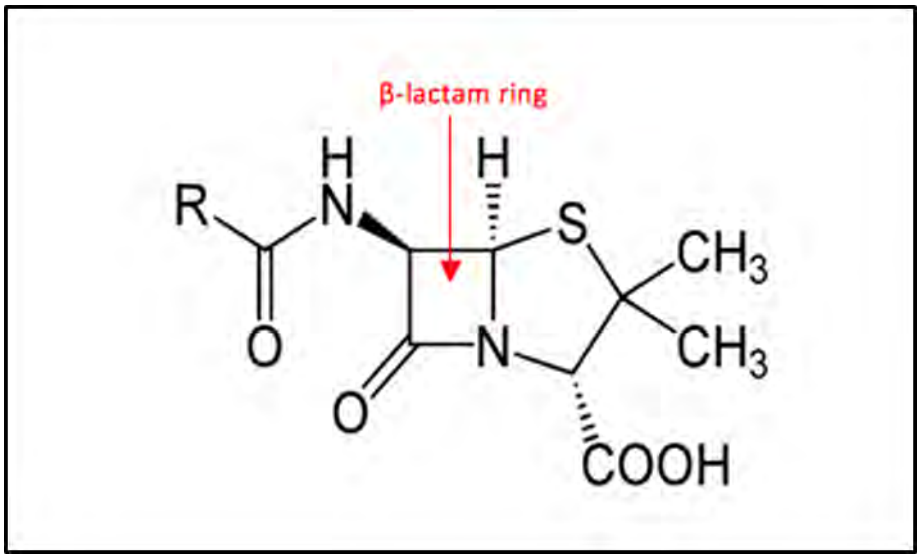

Figure 1. Diagram showing the core structure of penicillin and beta-lactam ring.
The beta-lactam ring is the active site of the beta-lactam antibiotics that irreversibly bind to the penicillin-binding proteins (PBPs) thus blocking their action of synthesizing a peptidoglycan layer. This leads to the weakened bacterial cell wall; and the bacterium eventually bursts.[93]

2.2.1.1Penicillins

Penicillins are effective against most Gram-positive and some Gram-negative strains.[94] Several types of penicillins exist; the natural penicillins (penicillin G), penicillinase-resistant penicillins (e.g. methicillin and oxacillin) and aminopenicillins (e.g. ampicillin and amoxicillin).[94–97] The first two types of penicillins have lethal effects on many Gram-positive organisms, whilst aminopenicillins are effective against a wider range of bacteria including some Gram-negative bacteria.[94,95]

2.2.1.2 Cephalosporins and cephamycins

Cephalosporins are subdivided into five generations including the closely related cephamycin compounds and together they constitute a group of beta-lactams called cephems.[98] The first generation cephalosporins (e.g. cefazolin and cephalothin) were active predominantly against Gram-positive cocci.[99,100] The second generation cephalosporins are more active against Gram-negative bacilli and are somewhat less effective against Gram-positive cocci.[99] Unlike the first and second generation cephalosporins, the third generation cephalosporins have increased activity against both Gram-negative organisms.[100–102] Among the fourth generation cephalosporins, cefepime is the most commonly used antibiotic. The fourth generation cephalosporins have the similar activity against Gram-positive bacteria as the first generation cephalosporins; however, they have a greater activity against Enterobacteriaceae and *Pseudomonas aeruginosa* than the third generation cephalosporins.[103–105] The fifth generation cephalosporins (ceftaroline and ceftobiprole) notably have an improved activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-intermediate *Staphylococcus aureus* strains.[106,107]

2.2.1.3 Monobactams

A currently marketed monobactam is aztreonam, an antibiotic with a very good activity against aerobic and fastidious Gram-negative bacilli such as Enterobacteriaceae and *Pseudomonas aeruginosa* strains.[108,109] Aztreonam has poor activity against anaerobes and Gram-positive organisms.[109]

2.2.1.4 Carbapenems

Carbapenems are normally referred to as the antibiotics of last resort and are bactericidal for both Gram-negative and Gram-positive organisms.[110] They are known to be the most effective antibiotics against many multidrug-resistant Gram-negative bacilli.[111,112] Carbapenems include imipenem, ertapenem, meropenem and doripenem.[110]

2.2.1.5 Beta-lactamase inhibitors

Clavulanic acid, tazobactam and sulbactam are beta-lactamase inhibitors that inhibit a number of plasmid-mediated beta-lactamases. They generally do not inhibit chromosomally-mediated beta-lactamases.[113,114] The combination of beta-lactamase

inhibitors with beta-lactams significantly increases spectrum of activity against many organisms containing plasmid-mediated beta-lactamases.[113,114]

2.2.2 Mechanism of action and resistance

Beta-lactam antibiotics are bactericidal and inhibit the growth of sensitive bacteria by inhibiting the DD-transpetidases, the penicillin-binding proteins (PBPs) that facilitate the final step (transpeptidation) of peptidoglycan synthesis, an important component of the bacterial cell wall.[115–117] Peptidoglycan, a murein, consists of amino acids and sugars that make up an important part of the bacterial cell wall.[115] Beta-lactam antibiotics are analogues of D-alanyl-D-alanine, the terminal amino acid residues on the precursor N-acetylmuramic acid/N-acetylglucosamine (NAM/NAG) peptide subunits of the peptidoglycan layer.[115,117–119] This structural similarity allows the binding of beta-lactams to the active site of PBPs.[118,119] The beta-lactam ring irreversibly binds to the Ser_{403} residue of the PBP active site by acylation thereby preventing the final transpeptidation or crosslinking of nascent peptidoglycan layer by the PBPs, disrupting synthesis of the bacterial cell wall followed by cell death.[119]

The main mechanisms of resistance to beta-lactam antibiotics are decreased penetration of beta-lactams due to the production of modified porins, loss of porins or a shift in the types of porins found in the outer membrane of Gram-negative bacteria, increased efflux from the cell through production of efflux pumps, or inactivation of beta-lactams by chromosome- and /or plasmid-encoded beta-lactamase enzymes (Figure 2). Among the above-mentioned, the most common mechanism of resistance is the production of beta-lactamases.[120,121]

2.2.3 Beta-lactamases

Increased use of beta-lactam antibiotics has been associated with the emergence of beta-lactamase-mediated bacterial resistance.[123] Beta-lactamases are enzymes that inactivate beta-lactam antibiotics by cleaving the beta-lactam ring through an irreversible hydroxylation of an amide bond (Figure 3).[89]

Two classification schemes for beta-lactamases are currently in use, the functional (Bush-Jacoby group) and molecular classification (Ambler classification).[124,125] Functional classification aligns beta-lactamases based on their ability to hydrolyze specific beta-lactams classes and on the inactivation properties of beta-lactamase

inhibitors.[125] The widely used molecular classification (Ambler classification) is based on the amino acid sequences and divides the beta-lactamases into four classes.[124] Class A, C, and D enzymes require serine at their active sites for substrate hydrolysis, whereas class B metallo-beta-lactamases utilize divalent zinc ion for substrate hydrolysis.[125–127]

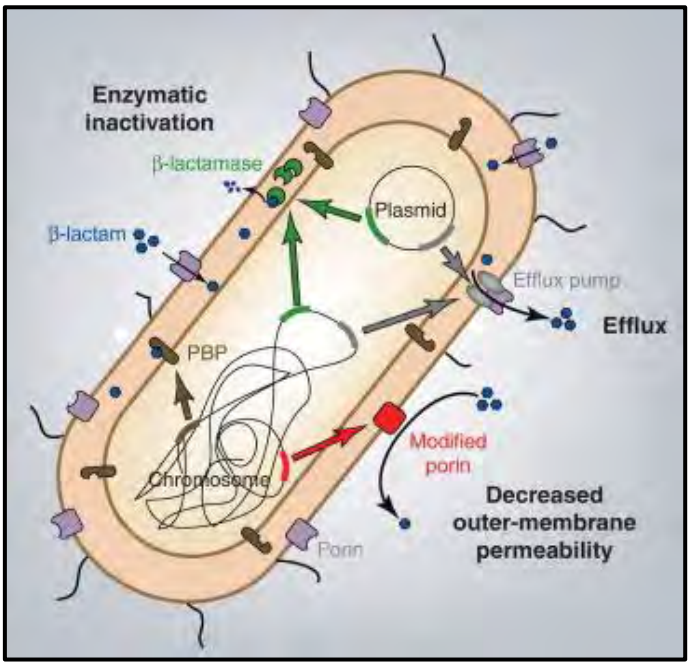

Figure 2. Diagram showing the mechanisms of beta-lactam resistance in Gram-negative bacilli. Hydrophilic channels formed by the porins in the outer membrane of a cell allow the uptake of beta-lactams into the bacterium. In the periplasmic space, beta-lactam molecules bind irreversibly to the penicillin binding proteins (PBPs), leading to the inhibition of the peptidoglycan synthesis. (Taken from Nordmann et al. 2012).[122]

The production of beta-lactamases may be inducible or constitutive.[128] In Gram-positive bacteria, beta-lactamases are generally inducible, resulting in larger amounts of enzymes produced in the presence of an antibiotic.[129] In Gram-negative bacteria, the production of beta-lactamases is frequently constitutive, i.e. the enzyme is produced even when the antibiotic is not present.[129,130] However, ampicillin class C beta-lactamases (AmpCs) are inducible in certain Gram-negative bacteria, such as *Serratia*, *Enterobacter*, *Escherichia* and *Citrobacter* species.[131,132]

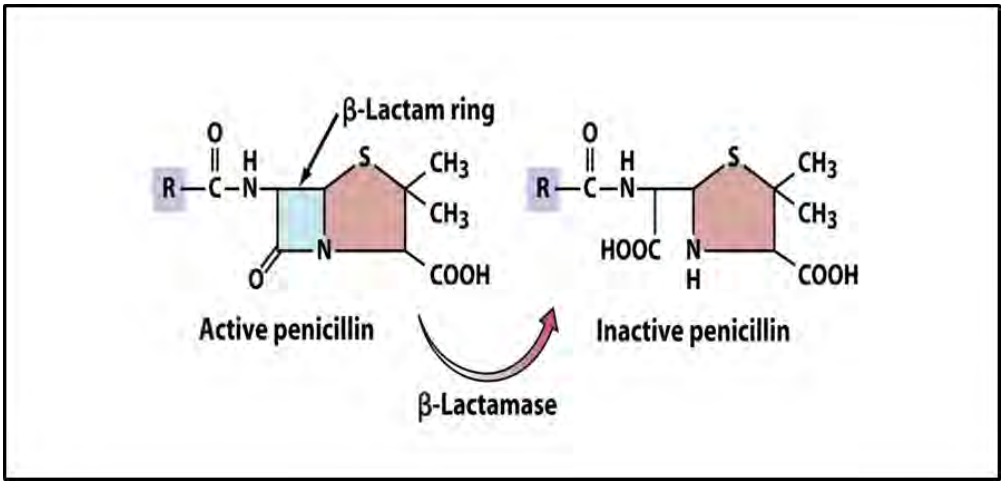

Figure 3. Diagram showing the mechanism of action of beta-lactamases.
Beta-lactamase enzyme causes an opening in the beta-lactam ring rendering an active penicillin ineffective (inactive penicillin), courtesy of www.wiley.com.

Resistance to beta-lactams emerged before the development of penicillin, the first discovered beta-lactam antibiotic. The first beta-lactamase was identified in *E. coli* in the early 1940s before the release of penicillin.[69] After the introduction of penicillin, resistance to this antibiotic emerged in *Staphylococcus aureus* due to a plasmid-mediated penicillinase that specifically hydrolyzed penicillin.[68,69] Penicillinase spread quickly to other species of staphylococci.[133] Resistance to penicillin led to the development of many new beta-lactams; however, with each new class of beta-lactams developed, new beta-lactamases active against that class of antibiotics emerged.[86,134,135] Some of these new classes of antibiotics were the third and fourth generation cephalosporins and carbapenems which became the most common agents for treating infections caused by Gram-negative bacilli.[13,32,33,61,133] However, the rapid emergence of resistance to beta-lactams due to AmpCs, extended-spectrum beta-lactamases (ESBLs) and carbapenemases is of a great public health concern.[13,41,45,88,129] AmpCs are beta-lactamases that hydrolyze broad and extended-spectrum cephalosporins, but are not inhibited by beta-lactamase inhibitors.[132] The following sections focused on the characteristics, epidemiology, and detection methods of the ESBL- and carbapenemase-producing bacteria.

2.2.3.1 Extended spectrum beta-lactamase-producing bacteria

Extended spectrum beta-lactamases are beta-lactamase enzymes that have the ability to hydrolyze penicillins, extended-spectrum cephalosporins (oxyimino-beta-lactams), and

aztreonam, but not carbapenem or cephamycin antibiotics.[136] ESBLs are reported worldwide among different bacterial species, including Enterobacteriaceae and non-fermentative Gram-negative bacteria such as *Pseudomonas* and *Acinetobacter* species.[137]

2.2.3.1.1 Class A extended-spectrum beta-lactamases

CTX-M (cefotaximase, Munich), SHV (sulfhydryl variable) and TEM (Temoneira) types are the most clinically common Ambler class A ESBL enzymes, and are susceptible to beta-lactamase inhibitors (Table 1).[61,129,136,137] The first plasmid-encoded ESBL enzymes were identified among Enterobacteriaceae in the 1980s;[138] they were derived mainly from the narrow spectrum enzymes (TEM-1, TEM-2, or SHV-1) by point mutations resulting in amino acid changes conferring an extended-spectrum of activity against beta-lactams.[139,140] Unlike SHV and TEM enzymes, CTX-M enzymes had an independent evolution.[127] Although ESBLs were first reported as plasmid-encoded, they are not exclusively plasmid-encoded; recent studies have shown that ESBL genes are also carried on chromosomes.[129,141]

- ***CTX-M-type ESBLs***

CTX-M enzymes were derived from the chromosomal cephalosporinase of *Kluyvera* species, and the first CTX-M enzyme was discovered in an *E. coli* clinical isolate from Munich, Germany in 1989.[142] It was suggested that CTX-M genes were captured by mobile elements from the chromosomes of *Kluyvera* species.[143–145]

CTX-M enzymes are classified into five groups; CTX-M-1, -2, -8, -9 and -25. Enzymes within the same CTX-M group share about 94% similarity, whereas ≤90% similarity is observed between members of different groups.[146] CTX-M enzymes have greater hydrolytic activity against cefotaxime than other oxyimino-beta-lactams, hence the name CTX-M (cefotaximase, Munich).[147,148] However, some CTX-M enzymes have greater activity against ceftazidime.[149,150] CTX-M-15, -16, -19, -25, -27, and -32 have higher hydrolytic activity against ceftazidime due to point mutations around their active sites.[149–156] In addition, CTX-M enzymes exhibit higher significant hydrolytic activities against cefepime than observed with other ESBL types.[146] All CTX-M enzymes have ESBL phenotype, and are commonly identified among Enterobacteriaceae, mainly *E. coli* and *K. pneumoniae*.[157]

- ***TEM-type ESBLs***

The first TEM enzyme (TEM-1) was discovered in 1965 in Athens (Greece) from a patient infected with *E. coli*, named Temoneira, hence the name TEM.[158] TEM-1 is the most common plasmid-mediated beta-lactamase causing ampicillin resistance among Enterobacteriaceae; responsible for up to 90% of ampicillin resistance in *E. coli*.[129] Both TEM-1 and TEM-2 have hydrolytic activity against ampicillin and early-generation cephalosporins.[139,140] All TEM-type ESBLs were derived from TEM-1 and TEM-2.[159] TEM-type ESBLs hydrolyze ceftazidime with higher efficiency than other oxyimino-beta-lactams.[160] Some TEM-type ESBL variants possess silent substitutions without amino acid changes (TEM-1a and TEM-1b) and others have single amino acid changes enough to assign a new number (TEM-3, -4, -5, etc.). To date, about 84 TEM variants have an ESBL phenotype. These are commonly identified in Enterobacteriaceae.[161]

- ***SHV-type ESBLs***

SHV-type ESBLs are derived from narrow-spectrum SHV-1 or SHV-11 beta-lactamases as a result of point mutations.[159] Initially, SHV-1 was described as plasmid-encoded and later as chromosomally encoded among *K. pneumoniae* isolates.[162,163] SHV-1 or SHV-11 is responsible for ampicillin resistance and accounts for up to 80% to 90% of resistance in *K. pneumoniae*.[162–164] SHV-type ESBLs have greater hydrolytic activity against ceftazidime than other oxyimino-beta-lactams (cefotaxime, cefepime).[165] SHV-type ESBLs are mainly found in Enterobacteriaceae and *P. aeruginosa* and the most common are SHV-2a, -5 and -12.[166]

2.2.3.1.2 Class D extended-spectrum beta-lactamases

OXA-type ESBLs are the only Ambler class D ESBL enzymes. OXA-type ESBLs are less common and were named OXA for their greater activity against oxacillin or cloxacillin and extended-spectrum beta-lactams.[136] OXA-type ESBLs primarily hydrolyze ceftazidime and are poorly inhibited by clavulanic acid.[127] About 16 OXA-type ESBL enzymes have been discovered; especially in *Pseudomonas aeruginosa* and *E. coli* strains.[129,167]

2.2.3.2 Carbapenemase-producing bacteria

Infections with ESBL-producing organisms has led to increased usage of carbapenems which are the last resort drugs for treatment.[27] However, the efficacy of carbapenems is

compromised by the emergence of carbapenemase-producing organisms.[28,29] Carbapenemases are beta-lactamases that hydrolyze almost all beta-lactam antibiotics.[44,168] Depending on the type or class of carbapenemase, these enzymes hydrolyze penicillins, cephalosporins, monobactams and carbapenems.[44] Carbapenemases are most frequently produced by the Enterobacteriaceae and non-fermenting bacteria such as *Acinetobacter* and *Pseudomonas* species.[41,169] Carbapenemases are classified into to Ambler classes A, B and D (Table 1).[170]

2.2.3.2.1 Class A carbapenemases

Class A enzymes can hydrolyze a wide variety of beta-lactams and are often poorly inhibited by the beta-lactamase inhibitors such as clavulanic acid or tazobactam.[44] Class A carbapenemases include the NMC (not metalloenzyme carbapenemase), IMI (imipenem-hydrolysing beta-lactamase), SFC (*Serratia fonticola* carbapenemase), BIC, and SME (*Serratia marcescens* enzyme) enzymes which are chromosomally-encoded as well as KPC (*Klebsiella pneumoniae* carbapenemase), and GES (Guiana extended-spectrum) carbapenemases which are mostly plasmid-encoded enzymes.[44,171] Among these, KPC enzymes which were first identified in the USA,[172] are the most clinically important and are now spread worldwide.[45,173–177]

2.2.3.2.2 Class B carbapenemases

Class B metallo-beta-lactamases (MBLs) are different from class A enzymes because they can hydrolyze all beta-lactams with the exception of aztreonam, and are resistant to beta-lactamase inhibitors.[44,136] MBLs can be inhibited by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA).[44,136] These enzymes are mostly plasmid-encoded or in some cases chromosomally-encoded and include the NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), IMP (imipenemase), GIM (German imipenemase), SIM (Seoul imipenemase), DIM (Dutch imipenemase), SPM (Sao Paulo metallo-beta-lactamase), and AIM enzymes.[44,169] The most clinically common MBLs are the VIM, IMP and NDM enzymes.[44,45]

2.2.3.2.3 Class D carbapenemases

Like class A carbapenemases, class D enzymes hydrolyze a wide range of beta-lactam antibiotics, and they are poorly inhibited by both beta-lactamase inhibitors and metal ion chelators.[44,136] No specific inhibitor is currently available.[178] Class D

carbapenemases, OXA (oxacillinase), are divided into five subfamilies: OXA-23, OXA-24/40, OXA-48, OXA-51 and OXA-58. The OXA-48-like carbapenemases are predominantly found in Enterobacteriaceae, and the OXA-23-like, OXA-24/40-like, OXA-51-like and OXA-58-like in *Acinetobacter* species.[44] OXA-48-like enzymes represent the main group of enzymes described worldwide.[170] The enzymes belonging to the OXA-23, OXA-24/40, OXA-48 and OXA-58 subfamilies are mainly plasmid-encoded, whilst OXA-51-like enzymes are chromosomally-encoded and intrinsic in *Acinetobacter* species.[44,179]

2.3 Global epidemiology of ESBL- and carbapenemase-producing bacteria

2.3.1 Epidemiology of ESBL-producers

Enterobacteriaceae are the main bacterial species associated with the production of ESBLs.[60] Enterobacteriaceae are commonly found in human gastrointestinal tract (GIT).[180] However, these organisms can cause a variety of extra-intestinal infections in the lower and upper urinary tracts, bloodstreams, central nervous system, pelvis or abdomen and wounds.[60] The most important Enterobacteriaceae species associated with ESBL production are *E. coli* and *K. pneumoniae*.[61] ESBL-producing Enterobacteriaceae (ESBL-PE) often display resistance to multiple drugs and co-resistance to other antibiotics used for treatment (aminoglycosides, fluoroquinolones and trimethoprim) is common.[181] Treatment options for severely ill patients are limited, resulting in increasing morbidity and mortality rates.[182] ESBL-PE species are therefore a growing public health concern worldwide. The prevalence and distribution of ESBLs varies widely in different geographical regions, differs from country to country, as well as within the country between hospital and community settings.[61,183,184]

Table 1. Classification schemes for bacterial beta-lactamases, modified from Bush and Jacoby 2010.[125]

Bush-Jacoby group	Ambler class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
			CA or TZB	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 oxymino-beta-lactams	GC1, CMY-37
2a	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-beta-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-beta-lactams combined with resistance to clavulanic acid, sulbactam and tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis to carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefprirone	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10

Table 1. Classification schemes for bacterial beta-lactamases, modified from Bush and Jacoby 2010 (Continues).[125]

Bush-Jacoby group	Ambler class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
			CA or TZB	EDTA		
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-beta-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-beta-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, NDM-1
3a	B (B3)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	ChpA, Sfh-1

The Bush and Jacoby group or functional classification takes into account the substrate and inhibitor profiles whereas the Ambler or molecular classification is by protein sequence, whereby the beta-lactamases are classified into four molecular classes (A,B, C and D) based on conserved and distinguishing amino acids motifs. CA, clavulanic acid; TZB, tazobactam; EDTA, ethylenediaminetetraacetic acid

2.3.1.1 Epidemiology of ESBL-producing bacteria in Asia, America, and Europe

In Europe, the prevalence of ESBL-PE varies between countries.[62] The prevalence is low in Scandinavian countries but is rapidly increasing.[185,186] A surveillance study conducted in South Western Sweden during 2004 to 2008 reported a greater increase in the prevalence of CTX-M-producing *E. coli* isolates in hospitals (0.2% - 2.5%) than in the community (0.2% – 1.6%).[186] In contrast, the prevalence of ESBL-PE is high in the Southern and Eastern parts of Europe.[62] Seven (6.7%) of 105 healthy humans from Spain were carriers of ESBL-producing *E. coli*, and CTX-M-14 was the most dominant enzyme.[187] A study in Portugal reported the ESBL faecal carriage rate of 2.7% (3/112) in healthy children.[188] In another study conducted in Portugal, the faecal carriage of ESBL-producing *E. coli* in patients with UTI caused by these microorganisms was 68% (36/53), but the household members and the non-household relatives of these patients also had ESBL-producing *E. coli* in faecal samples, 27.4% (20/73) and 15.6% (5/32), respectively, whilst in healthy unrelated controls the faecal carriage rate was 7.4% (4/54).[189] In Germany, ESBL-producers were recovered from animals, including dogs, cats and horse, and the authors also described an active transmission of such organisms between humans and animals.[190]

In America, data on ESBL-PE organisms both in hospital and community settings are scarce. Besides, a recent survey from the USA including 26 hospitals from 20 states reported the prevalence of 6.4% (195/3049) of ESBL-producers among Enterobacteriaceae isolates.[191] Furthermore, surveillance studies have reported high prevalence rates ranging from 45% to 51% in South America.[184,192]

In Asia, a survey conducted in various hospitals in India reported the prevalence of ESBL-producing Gram-negative bacilli ranging from 19% to 60%.[193] In addition, a cross-sectional study conducted in rural community of Thailand also reported a very high ESBL-PE faecal carriage of 58.2% in 160 healthy adult individuals (mean age; 56.0 ± 9.8 years).[194] In the later study, 94.3% of the isolates were positive for CTX-M gene.[194] In China, among 270 healthy adults, the faecal carriage rate of ESBL-producing *E. coli* was 7%.[195] Unlike in most parts of the world where CTX-M-15 is predominant, in South-East Asia the most common ESBL enzyme is CTX-M-14.[195–197]

2.3.1.2 Epidemiology of ESBLs in Africa

Two reviews on ESBL-producing Enterobacteriaceae in Africa have been recently published.[63,64] The prevalence rates of ESBL-PE vary from hospital to hospital and from country to country.[62] In North Africa, a survey conducted in three Egyptian university hospitals reported a very high prevalence of 80% among 160 *K. pneumoniae* strains isolated from blood cultures of hospitalized patients with nosocomial bloodstream infections.[198] In addition, a study conducted in one neonatal ICU in an Egyptian hospital also found a very high proportion of 79% of ESBL-producing *K. pneumoniae* isolates.[199] In another study conducted in nine adult ICUs in Egypt, the authors reported that 76.7% of *K. pneumoniae* and *E. coli* isolates causing bloodstream infections (BSIs) were ESBL-producers.[200] The ESBLs in the three mentioned Egyptian studies were not well characterized.[198–200] The prevalence of ESBLs was also high in Tunisian children hospital, reaching up to 68% among *E. coli* isolates; with CTX-M-15 detected in 97% of such isolates.[201] The proportion rates of 16%,[202] and 31%,[203] of ESBLs among Enterobacteriaceae have been recorded in Libyan and Algerian hospitals, respectively. Studies in North Africa showed the dominance of CTX-M enzymes, particularly CTX-M-15.[63,201–206] Furthermore, there are limited reports of SHV-type ESBLs,[63] and TEM-type ESBLs are very scarce.[63,201]

There are no enough data on the prevalence of ESBL-PE in Eastern Africa,[63] as well as data on the characterization of ESBL genes.[207,208] The hospital prevalence rates of 37%, 38%, and 63% have been recorded in Kenya,[209] Rwanda,[208] and Ethiopia,[207] respectively. As in North Africa, CTX-M enzymes are the most common ESBLs circulating in Kenyan and Tanzanian hospitals.[209,210] Although there are limited data on ESBL-PE in hospital settings from several West African countries, the dissemination of CTX-M enzymes is common in Niger, Nigeria and Cameroon.[53,211–215] The prevalence ranges from 10% in Nigeria,[215] to 83% in Cameroon.[212]

In Malawi, only 0.7% of 1191 Enterobacteriaceae from blood cultures were ESBL-producers.[216] During the study period, ceftriaxone was the only available cephalosporin in Malawi and its use was highly restricted. The predominant ESBLs isolated were SHV-12 (30%) and TEM-63 (20%).[216] Besides Malawi, data on ESBL-producing organisms in Southern Africa are mainly from South Africa.[63] A survey conducted in 59 South African private hospitals in Johannesburg (n= 19), Pretoria (n= 21) and Cape Town (n= 19)

between 2004 and 2009 reported a prevalence of 9% of ESBL-producers among 1218 Enterobacteriaceae strains isolated from complicated intra-abdominal infections.[217] Another study conducted in several private and state hospitals (during 2008 -2009) in Cape Town metropolitan area reported a very low prevalence of ESBL-producing *E. coli* isolates of less than 7%.[218] In the latter study, the most common ESBLs detected were CTX-M-15 (59.1%) and CTX-M-14 (31.8%).[218] Different types of ESBL enzymes have been identified in many other Enterobacteriaceae species in South Africa.[219–222] Based on previously published data, ESBL-producing *Salmonella* species had been a significant public health problem in South Africa, particularly in children whom extended-spectrum cephalosporins were the preferred treatment. SHV and TEM enzymes, including ESBLs were the main enzymes described in such isolates.[223–225]

In the community setting, the prevalence of ESBL-producing Enterobacteriaceae carriage in healthy humans in Africa is poorly studied.[226] However, where studies have been done, the community carriage rates of ESBL-PE in Africa were high, ranging from 7% in Cameroon,[227] to 31% in Niger.[53]

In children, a cross-sectional study conducted in Senegal reported a high ESBL carriage rate of 10% in 20 healthy children (mean age, 6.9 years) living in a remote village with no history of hospitalization or antibiotic usage. CTX-M-15 was the only ESBL enzyme identified among ESBL-positive *E. coli* isolates.[49] The following studies have reflected the likely faecal carriage of ESBL-PE in the community settings among children.[20,53,227,228] A prospective study in Niger reported an ESBL carriage rate of 31% within 24 hours of hospital admission in 55 children of less than five years of age.[53] In the latter study, cross-transmission of ESBL-positive bacteria due to suboptimal hygiene was observed. In addition, CTX-M-15 enzyme was identified in 91% (20/22) of the isolates tested (13 *E. coli*, four *E. cloacae*, two *K. pneumoniae* and one *E. asburiae*), and SHV-2a and SHV-12 enzymes were found in one *K. pneumoniae* each.[53] Furthermore, a cohort study conducted in Madagascar identified ESBL carriage of 21.2% within 48 hours of hospital admission in 244 children (mean age, 38.3 months). The most common ESBL-PE isolates were *K. pneumoniae* (37%) and *E. coli* (36.9%); however, the ESBL enzymes were not characterized.[228] In the latter study from Madagascar, risk factors associated with ESBL-PE carriage were prior hospitalization and use of invasive devices in the last 30 days, poor populations and infection upon hospital admission.[228]

In adults, an ESBL-PE carriage rate of 6.7% was reported in a cross-sectional study conducted in Cameroon among 150 healthy students (mean age, 25 years) attending a clinic for their annual medical examinations.[227] CXT-M-15 enzyme was found in all ESBL-PE isolates (nine *E. coli* and one *K. pneumoniae*).[227] In another cross-sectional study conducted in five Tunisian cities (Tunis, Sfax, El Kef, Gafsa and Kairouan), the authors reported CTX-M-1-producing *E. coli* carriage in 7.3% (11/150) of healthy individuals.[229] CTX-M-1-producing *E. coli* isolates have also been identified in 12.6% of 79 food samples of animal origin in different supermarkets and butcheries in 12 Tunisian cities.[230] In Madagascar, ESBL-PE carriage was reported in 10.1% of 484 patients of all ages (median age, 28 years) attending a health care setting for the first time.[20] As in other studies, CTX-M-15 was commonly identified, 86.8% of ESBL-PE isolates, mainly in *E. coli* and *K. pneumoniae* isolates.[20] Other ESBL-PE isolates identified were SHV-12-producing *E. cloacae*, CTX-M-3-producing *E. coli*, CTX-M-1-producing *E. coli* and SHV-2a-producing *K. pneumoniae*.[20] In the latter study, the occupational status of the head of household and poverty were risk factors for ESBL-PE carriage.[20]

There is still lack of data on the epidemiology of ESBL-producing bacteria in many of African countries. However, based on the data described above, CTX-M-15 is the most predominant enzyme found in ESBL-PE, especially *E. coli* and *K. pneumoniae* isolates, both in hospital and community settings. There is no enough evidence of community carriage of ESBL enzymes belonging to TEM-type in ESBL-PE isolates[20,49,53,227] Furthermore, most of the above-described studies did not report the data regarding risk factors for the acquisition of ESBL-producing bacteria in hospital and community settings.[63,200,204,231] The occurrence, in Tunisia, of CTX-M-1-producing *E. coli* isolates in food samples and healthy pets is of great concern since such organisms can spread to humans through food chain or contact with pets.[230,232,233]It is likely that there could be the transmission of ESBL-producing organisms through food chain or contact with pets in many African countries where studies are lacking.

2.3.2 Global Epidemiology of carbapenemase-producing bacteria

Carbapenemase-producing organisms are a significant and a growing problem worldwide. Although the actual prevalence of carbapenemase-producers is unknown in most parts of the world, their distribution varies by region.[45]

2.3.2.1 Epidemiology of carbapenemase-producing bacteria in America, Asia, and Europe

By far, the most frequent class A carbapenemases are KPC enzymes.[13,45,175,234,235] The KPC enzyme was first identified from a *K. pneumoniae* isolate in North Carolina, USA in 1996 and soon afterwards caused outbreak in New York.[172,177] Since then, outbreaks caused by KPC-producing *K. pneumoniae* have been reported in different countries, including Israel in 2004 and Greece in 2007.[236] In Latin America, KPC enzymes (particularly KPC-2) are endemic in Colombia, mostly among Enterobacteriaceae.[237–239] However, the identification of KPC-2 enzyme in non-fermenters (*P. aeruginosa*) was first reported in Colombia.[240] Of great concern, in China, hospital sewage has been found to harbour KPC-positive *E. cloacae* and *C. freundii*, and community infections with KPC-2 producing isolates have also been documented.[241,242] The mortality associated with infections caused by KPC-producing organisms ranges from 25% to 69%.[175,243–247]

Metallo-beta-lactamases (MBLs) include three globally dominant carbapenemase types, the IMP, VIM and NDM enzymes.[13] Imipenemase-1 (IMP-1) was the first known MBL enzyme identified from *P. aeruginosa* clinical isolate in 1988 in Japan.[248] IMP-producing organisms have been reported to be endemic in Japan, Taiwan, Australia and Eastern China.[249–256] VIM-1 was first identified in a *P. aeruginosa* clinical isolate in 1997 in Italy.[257] VIM-1-producing Enterobacteriaceae are now endemic in Greece.[258,259] However, the VIM-2 enzyme has a worldwide distribution, with endemic spread in Greece, Spain and Italy, where it is predominantly identified in *P. aeruginosa* isolates.[45]

NDM-1 enzyme was first detected in *K. pneumoniae* in 2008 in Sweden from a patient who had been transferred from a New Delhi hospital, in India.[260] Initially many NDM-1-producing Enterobacteriaceae reported in other countries were also isolated in patients with healthcare contact or travel-related to India, Pakistan and Sri Lanka.[261,262] Nonetheless, reports of NDM-1-producers in individuals who had not travelled to South Asia are increasing.[13,263–265] The carriage of NDM-producing organisms in South Asia (India and Pakistan) has been estimated to be 5% to 15% in hospital settings.[34,266] NDM enzymes have now spread into non-Enterobacteriaceae such as *Pseudomonas aeruginosa* and *Vibrio cholera*, and have been identified in the sewage and tap water in India and Vietnam.[262,267,268] NDM-1 has also been found in *A. baumannii* clinical isolates from Germany, France, India, the UK and China.[34,35,269–271] NDM-producing organisms

are also widespread in the USA.[262] To date, there is no evidence that NDM-producers are more virulent than other strains, however, they present limited treatment options.[272] In Greece, the mortality rates associated with MBLs range from 18% to 67%.[13,273]

The most clinically predominant oxacillinases are the OXA-48-like carbapenemases commonly found in Enterobacteriaceae isolates.[13,263–265] The first report of OXA-48 enzyme was from Turkey in 2004 in a *K. pneumoniae* clinical isolate.[274] Since then, OXA-48-producing Enterobacteriaceae have been increasingly reported as a source of outbreaks in Turkey.[275–278] However, there are increasing reports of OXA-48-producing organisms in countries such as France, the Netherland, Germany, Switzerland, Spain and the UK, especially through transfer of patients from areas where the OXA-48 enzyme is endemic.[4,279–283] The mortality rates associated with OXA-producing organisms are still unknown.[170]

2.3.2.2 Epidemiology of carbapenemase-producing bacteria in Africa

A detailed systematic review on the epidemiology of carbapenemase-producing bacteria in Africa has been recently published by our group, in 2014.[41] In Africa, the reports of carbapenemase-producing organisms in hospital setting are increasing; however, reports of such organisms in the community setting are very scarce.[284–286] Figures 4 and 5 show the spread of carbapenemase-producing bacteria in Africa.

Class A carbapenemases

There are still few reported cases of class A carbapenemases in Africa.[55,287,288] KPC-2-positive *K. pneumoniae* and *E. cloacae* have been recovered from adult patients in South Africa. Prolonged hospital stay, multiple antibiotic treatments, use of invasive devices, and immunosuppression were associated with the acquisition of KPC-2-producers.[55] The first identification of KPC enzyme in non-Enterobacteriaceae isolate (*P. aeruginosa*) was recently reported in Tanzania.[287]

Class B carbapenemases

In Africa, VIM enzymes are by far the most reported MBLs.[41] In North Africa, VIM-1,-19, -4 and -29 enzymes seem to be the common VIM enzymes identified in Enterobacteriaceae.[284,289–294] So far, only one study from Tunisia has reported VIM-2 positive Enterobacteriaceae (*E. coli* isolates).[289] VIM-2-producing *P. aeruginosa* isolates have been reported as a cause of outbreaks in South Africa,[59] Tunisia,[295,296] and Côte

d'Ivoire.[297] Since its first identification in Africa,[298] NDM-1 is increasingly reported in both Enterobacteriaceae and *A. baumannii*.[299–304] Of more concern, there are several reports of NDM-producing bacteria identified in patients with no obvious link to South Asia.[290,300,301,298]

Class D carbapenemases

OXA-48 in Enterobacteriaceae and OXA-23 in *Acinetobacter* species are the most prevalent carbapenemases reported in Africa, particularly in North African countries[41]. OXA-48-producing Enterobacteriaceae are thought to be endemic in North African countries.[305] Of note, the identification of OXA-48-producing Serratia marcescens in environmental water samples in Morocco increases the risk that such organisms can spread through contaminated water and poor sanitation.[286]

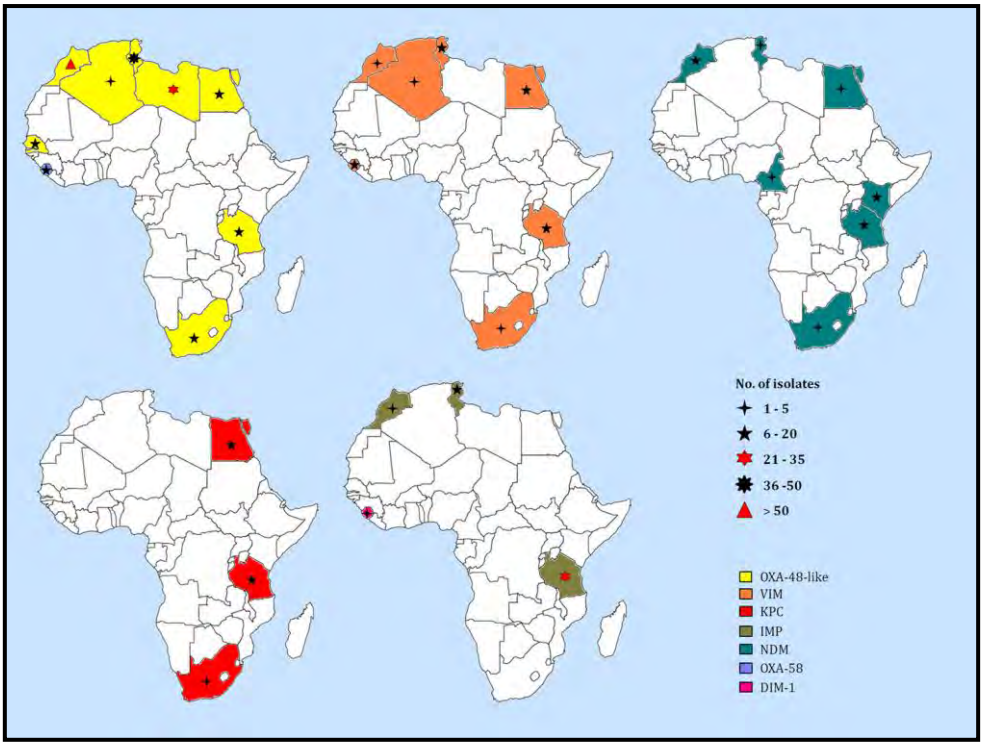

Figure 4. Geographical distribution of carbapenemase-producing Enterobacteriaceae isolates in Africa.

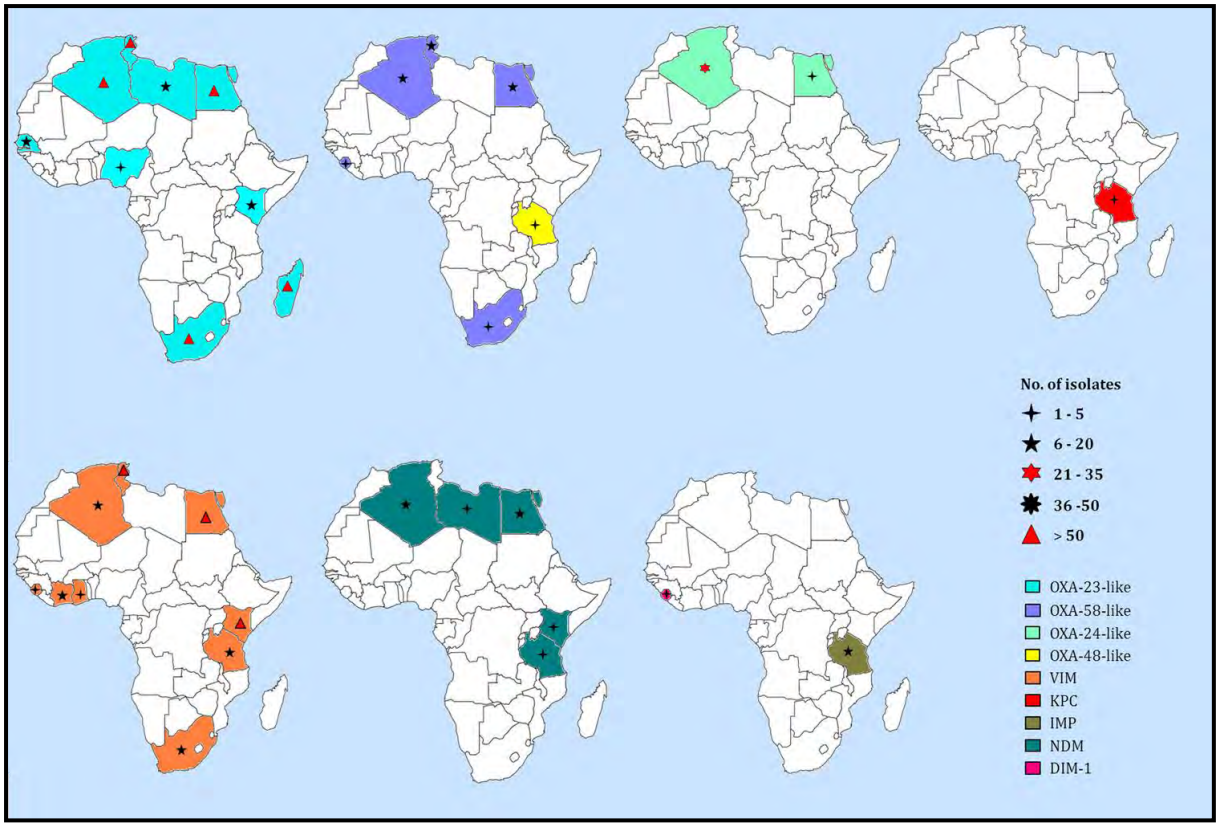

Figure 5. Geographical distribution of carbapenemase-producing non-Enterobacteriaceae in Africa.

2.4 Detection methods for ESBL- and carbapenemase-producers

2.4.1 Detection of extended-spectrum beta-lactamases

Extended-spectrum beta-lactamases can be detected with phenotypic or genotypic approaches.

2.4.3.1 Phenotypic tests

The phenotypic detection of ESBLs includes screening and confirmatory steps.[137] Screening involves testing for reduced susceptibility to extended spectrum beta-lactams, including cefotaxime, ceftazidime, cefpodoxime, ceftriaxone or aztreonam.[306] Cefpodoxime is the most sensitive indicator for detecting ESBLs, because it can be hydrolyzed by all common CTX-M, SHV, and TEM ESBLs;[307] however, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime.[306]

Confirmatory phenotypic tests are based on the demonstration of synergy between the extended spectrum beta-lactams and beta-lactamase inhibitors particularly clavulanic acid.[307] Several confirmatory tests including combination disc test, double-disc synergy test, ESBL E-test or ESBL NDP test are used in different settings.[123,306–309] In addition,

automated systems (the Vitek ESBL and BD Phoenix ESBL tests) that use the above-mentioned detection principle are in use in many clinical microbiology laboratories.[308–312]

2.4.3.1.1 Combination disc test (CDT)

The CDT detects the production of ESBL enzymes based on the principle that they hydrolyze cephalosporin antibiotics and are inhibited by clavulanic acid (Figure 6).[306,307] Cefepime alone and in combination with clavulanic acid is preferred in isolates with inducible AmpC enzymes as this antibiotic is stable to AmpC beta-lactamases.[313] AmpC enzymes interfere with clavulanic acid synergy and detecting ESBL production in such isolates using cefotaxime or ceftazidime is challenging.[314,315] The test is cheap and easy to perform, and interpretation is straightforward. The sensitivity and specificity of this test using cefotaxime and ceftazidime alone and in combination with clavulanic acid have been reported to be 96% and 100%, respectively;[316] however, the sensitivity of this test can be further increased by using cefepime alone and in combination with clavulanic acid.[313]

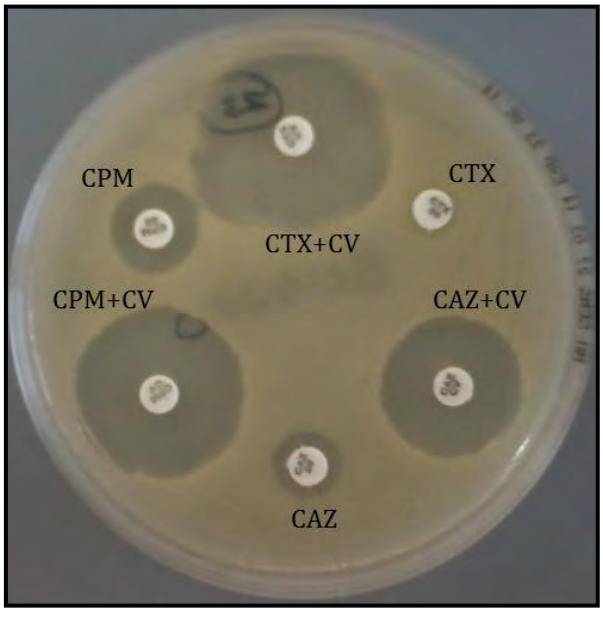

Figure 6. Combination disc test (CDT) showing an increase by at least 5mm for cefotaxime-clavulanic acid, ceftazidime-clavulanic acid and cefepime-clavulanic acid as compared to cefotaxime, ceftazidime and cefepime, respectively. CPM, Cefepime; CPM+CV, Cefepime-clavulanic acid; CAZ, Ceftazidime; CAZ+CV, Ceftazidime-clavulanic acid; CTX, cefotaxime; CTX+CV, Cefotaxime-clavulanic acid.

2.4.3.1.2 Double-disc synergy test (DDST)

The DDST was the first test designed to detect ESBL production in Enterobacteriaceae.[317] The cephalosporin discs are applied to a plate next to a disc containing clavulanate (amoxicillin-clavulanate).[318] A positive result is indicated when the zone of inhibition around any of the cephalosporin discs is augmented in the direction of the disc containing clavulanate.[306] This method has remained a reliable test for the detection of ESBLs in clinical microbiology laboratories.[306] In addition, the DDST is also cheap and easy to perform, however, reading the results is sometime difficult.[307]

2.4.3.1.3 ESBL E-tests

The ESBL E-tests contain the gradients of cephalosporin alone at one end of the strip and combined with 4 μg/ml of clavulanic acid on the other end. The ESBL E-test is considered positive when the minimum inhibitory concentration (MIC) of the cephalosporin combined with clavulanic acid is reduced by ≥8-fold as compared with the MIC of the cephalosporin alone or if a deformed ellipse or phantom zone is present. The ESBL E-test is easy to perform but the interpretation of results is sometimes challenging.[306,312] In 2002, a study conducted in The Netherlands reported failure of laboratory technicians to recognize the phantom zones or ellipse deformation in 30% of cases.[312]

2.4.3.1.4 ESBL Nordmann Dortet Poirel (ESBL NDP) test

This is a newly developed phenotypic test for the detection of ESBLs in Enterobacteriaceae.[319] The ESBL NDP test detects the production of ESBL based on the hydrolysis of cefotaxime. A red phenol solution is used as a pH indicator, and when a protein extract from the bacterium is mixed with red phenol solution containing cefotaxime, ESBLs contained within the protein extract hydrolyze the antibiotic, thus forming an acidic solution which causes the pH indicator to turn from red to yellow or orange. In the presence of tazobactam the activity of ESBLs will be inhibited, and the pH indicator will remain red (unchanged colour), thus confirming the production of the ESBL (Figure 7).[319] Its sensitivity and specificity in detecting CTX-M-type and SHV-12 enzymes have been reported to be 98.0% and 99.8%, respectively.[320] Only few studies have evaluated this test.[319,320]

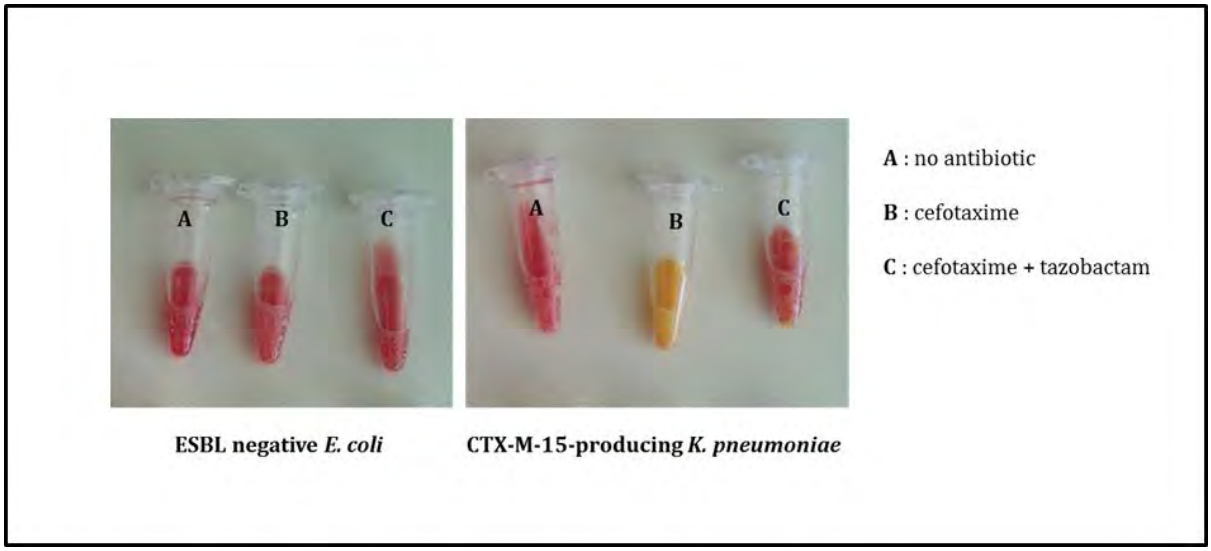

Figure 7. The ESBL NDP test showing the hydrolysis of cefotaxime in CTX-M-15-producing *K. pneumoniae* isolate (tube B). The action of ESBL enzyme (CTX-M-15) is inhibited in the presence of tazobactam (tube C). There is no hydrolysis of cefotaxime in case of ESBL negative *E. coli* (tube B).

2.4.3.2 Genotypic tests

Polymerase chain reaction (PCR) and subsequent sequencing of ESBL genes or DNA microarray-based methods are used for genotypic confirmation of the presence of specific ESBL genes. [61,306] Singleplex or multiplex PCR techniques may be used.[321] Molecular tests remain the gold standard and are technically challenging, however, they have the advantage of identifying the specific type of ESBL gene present in a bacterium.[2,322] These techniques can detect low-levels of resistance, and can be performed without prior culture of the microbiological specimens.[2] The PCR tests are rapid and the results are usually obtained within six hours with excellent sensitivity and specificity.[323] The main disadvantages of molecular techniques are their cost and inability to detect unidentified novel ESBL genes.[323]

2.4.2 Detection of carbapenemases

Rapid detection of carbapenemases is crucial to guide appropriate antibiotic therapy and containment strategies.[323] However, it remains difficult to detect carbapenemase-producing organisms due to the lack of a universal screening medium that is able to detect all types of carbapenemases.[324] A novel screening medium (SUPERCARBA medium) shows promise in this regard, but requires further evaluation.[50,325–327] Most microbiology laboratories rely on routine antibiotic susceptibility test results obtained by either disk diffusion, E-test or automated systems to screen for carbapenemase-

producing organisms.[295,296,328–331] These methods are sometimes unable to detect carbapenemase producers which may show intermediate resistance or susceptibility to carbapenems on routine screening.[13] MALDI-TOF and ultraviolet spectrophotometer are the emerging techniques which allow the detection of carbapenemase within few hours.[306,332–335] Notably, automated systems have been shown to be unreliable in detecting all types of carbapenemase enzymes.[336] Phenotypic tests that are currently used for confirming the production of carbapenemases, include the modified hodge test, carbapenemase inhibitor methods and the Carba NP test.[323,330,337,338]

2.4.2.1 Phenotypic tests

A number of phenotypic tests, most of which are the disc diffusion, have been described and evaluated for detection of carbapenemase-producing organisms.[338]

2.4.2.1.1 The modified Hodge test (MHT)

The clover leaf method or MHT is a recommended phenotypic test (by the clinical and laboratory standards institute) for detection of carbapenemase activity in vitro.[339] The MHT is based on the inactivation of a carbapenem antibiotic by a carbapenemase-producing strain that enables the growth of carbapenem-susceptible indicator strain to extend towards a carbapenem-containing disc, along the streak of inoculum of the tested strain, forming a cloverleaf-like shape or indentation (Figure 8). The MHT detects all types of carbapenemases without classifying the type of enzymes produced by the organisms.[339] However, this test lacks both sensitivity and specificity .[340] In addition, there are reports of false-positive results, involving CTX-M-producing organisms with reduced outer membrane permeability which make the interpretation of the results more challenging.[341]

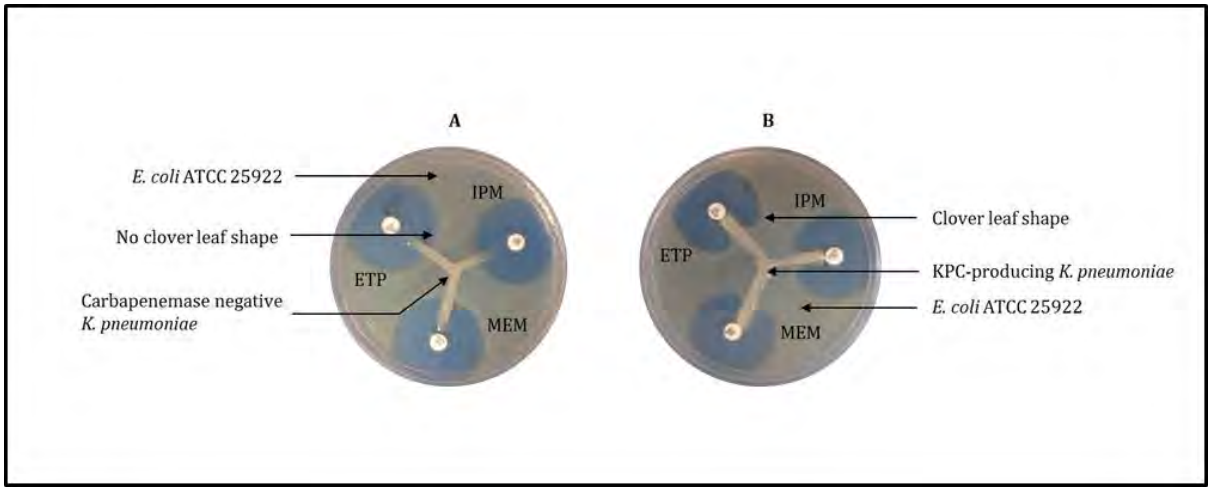

Figure 8. The modified hodge test showing the production of carbapenemase in KPC-producing *K. pneumoniae* by the formation of cloverleaf-like shape or indentation between the test strain and an indicator strain, *E. coli* ATCC 25922 (Figure 8B). In carbapenemase negative strain, there is no formation of cloverleaf-like shape or indentation (Figure 8A). IPM: imipenem, ETP: ertapenem, MEM: meropenem.

2.4.2.1.2 Carbapenemase inhibitor methods (synergy tests)

These methods are based on the synergy between carbapenems and beta-lactamase inhibitors.[342] For detecting MBLs producers, carbapenem disc alone and in combination with EDTA are used.[343] This method uses the chelating agent, EDTA, to inhibit the hydrolysis of carbapenems.[343] An increase, in zone diameter, by at least 5 mm for the carbapenem tested in combination with EDTA as compared to corresponding carbapenem tested alone is an indication of MBL production.[306]

The detection of class A enzymes, particularly KPC, is based on using carbapenem disc alone and in combination with boronic acid.[344] Boronic acid compounds inhibit the hydrolysis of carbapenems by KPC enzymes.[344] They are serine-type beta-lactamase inhibitors that were originally used in detection of AmpCs in Enterobacteriaceae.[344] The test is considered to be positive when there is an increase in the zone diameter of at least 4 mm for carbapenem plus boronic acid as compared to carbapenem alone.[306]

2.4.2.1.3 The Carba NP test

The Carba NP test is recommended by the European committee on antimicrobial susceptibility testing (EUCAST) as a confirmatory test for carbapenemase production.[306] The principle of the Carba NP test is the same as for the ESBL NDP test. The Carba NP test is based on the detection of imipenem hydrolysis (Figure 9).[337] This test was developed to detect carbapenemases in Enterobacteriaceae and *Pseudomonas*

species.[337,345] The Carba NP test cannot determine the types of carbapenemase being detected.[346] Different studies have reported the Carba NP test sensitivities ranging from 80% -100%, with specificity of 100%.[337,347–350] The Carba NP test appear to be easy-to-use, rapid (results obtained within two hours) and cheap, but requires further evaluation.[323]

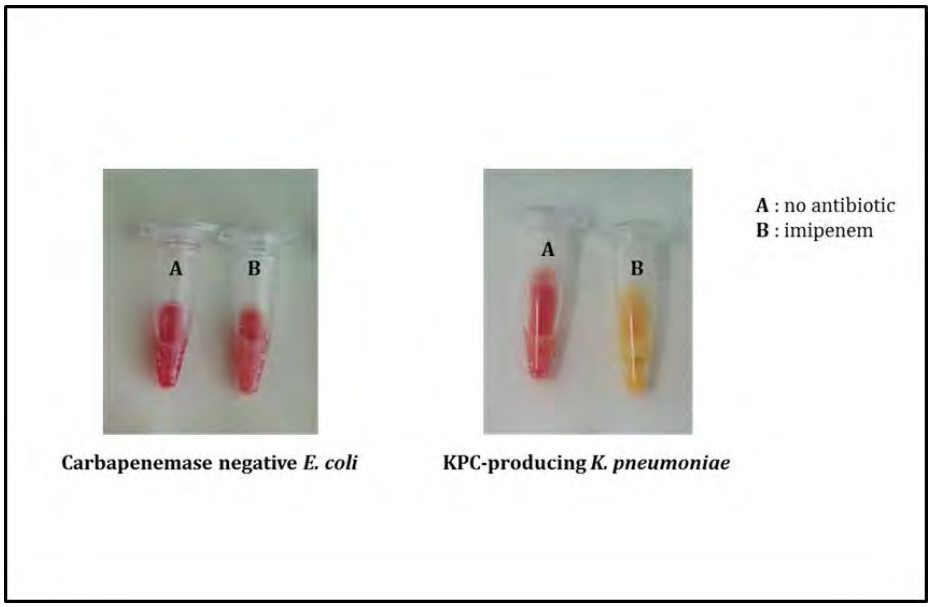

Figure 9. The Carba NP test showing the hydrolysis of imipenem by KPC-producing *K. pneumoniae*. In the absence of carbapenemase enzyme, imipenem hydrolysis is not detected.

2.4.2.2 Genotypic tests

As with the detection of ESBLs, molecular tests remain the reference standard for detecting carbapenemase genes. PCR and subsequent sequencing or DNA microarray-based tests are used.[323] Commercially available DNA microarrays that are able to detect carbapenemase and ESBL genes have been evaluated in different settings worldwide.[351–356] The principle of a DNA microarray is based on the fact that a complementary sequence of DNA will hybridize to immobilized DNA molecules.[357] The immobilized single-stranded of known nucleotides sequence is used to detect and identify beta-lactamase genes. DNA microarrays have excellent sensitivity (100%) and specificity (100%) in detecting different types of known carbapenemases but they are labour-intensive and expensive.[338,351,352]

Conclusion:

The data on the epidemiology of ESBL- and carbapenemase-producing bacteria in the African community setting remain scarce. There are also few studies on the prevalence of ESBL- and carbapenemase-producers in healthy individuals in the community.[49,229] Furthermore, only few studies describing the acquisition of ESBL- and carbapenemase-producing bacteria at birth have been conducted worldwide.[358,359] Therefore, we sought to study the epidemiology of ESBL- and carbapenemase-producing bacteria in a birth-cohort among apparently healthy South African children of less than one year of age and their mothers.

3. Material and Methods

3.1 Study design

This was a pilot study nested within an ongoing longitudinal prospective birth-cohort, the Drakenstein Child Health Study (DCHS). The DCHS was launched in 2011 and is enrolling 1000 mother-infant pairs.

3.2 Study population and sampling

The study was conducted in the Drakenstein sub-district, a peri-rural setting 60 kilometres outside Cape Town, South Africa. This sub-district has a stable but low socioeconomic population status of about 200,000 persons.[360] Pregnant women were enrolled at Paarl Hospital during their 20-24 week ultrasound scan visit and followed until childbirth. Infants were enrolled at birth and the mother-child pairs are being followed until the child is five years of age.[361]

This pilot study was conducted on a subset of mother-infant pairs from whom stool samples were available at the time of this study. Stool samples were collected at delivery from both the mothers and infants and at two further specified intervals (5-12 and 20-28 weeks) from the infants. In addition, data on potential relevant exposures were recorded. Variable included the maternal age, ethnicity, hospitalization in the previous two years, maternal HIV status, medication or supplement administration at birth, mode of delivery, infant gender, breastfeeding prior to discharge, admission to nursery and socioeconomic data.

3.3 Collection, transportation, and storage of stool samples

3.3.1 Maternal and infant faecal sample collection at the clinic

Fifty microliter sterile, dry screw-top containers (Lasec, Cape Town, South Africa) were used for stool sample collection. The maternal stool samples were collected by the study or healthcare staff at birth. Stool passed in a bedpan was collected using the spoon attached to the lid of the stool collection container. For the infants, the meconium samples were collected by a registered nurse into the specimen container using a spatula. When no spontaneous passage of stool had occurred by the time a participant was being weighed, the meconium was aspirated from the rectum using a small soft plastic tube. The sample was aspirated into the tube or syringe and decanted into the specimen collection container. Stool samples were not collected if mixed with urine. The specimens were labelled and placed into the freezer.

3.3.2 Infant stool collection at home

The mothers were supplied with the stool collection containers, a specimen bag labelled with PID, spatula and gloves. The stool collection procedure was explained to the mothers and they were advised to collect a fresh stool sample and deliver to the study staff within four hours of collection. The mothers were also advised not to collect urine, soil, water (from the toilets), or other materials with the stool, as these may contaminate the actual samples. In addition, for infants, collecting samples from the diapers was discouraged; however, the mothers were allowed to place a plastic wrap between the baby's bottom and a diaper (or to put a diaper on inside out). The stool samples were transported in ice box from the study site to the Division of Medical Microbiology laboratory at the University of Cape Town (South Africa) and stored at -80°C without thawing until processing.

3.4 Screening of ESBL- and carbapenemase-producing Gram-negative bacilli

3.4.1 Inoculation of stool samples

Figure 10 summarizes the laboratory procedures performed in this study. The stool samples were screened for the presence of ESBL- and carbapenemase-producing Gram-negative bacilli. Each stool sample was suspended into 100 µl of 1% phosphate-buffered saline (PBS) (containing 137 mM NaCl, 2.7 mM KCL, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4, and distilled water (pH 7.4)) and vortexed until it was clearly suspended. Stool samples were suspended in PBS because it is isotonic and non-toxic to bacterial cells with osmolarity and ion concentrations of solution matching those in human body.[362] Forty microliters of stool suspension was inoculated onto ChromID ESBL agar and ChromID CARBA agar (bioMérieux, Marcy l'Etoile, France); for the detection of presumptive ESBL- and carbapenemase-producing bacteria, respectively. ChromID ESBL agar is a chromogenic medium capable of detecting presumptive ESBL-producing as well as the majority of carbapenemase-producing Gram-negative bacilli.[363] It contains a mixture of antibiotics, including cefpodoxime which is a selective antibiotic used for screening for ESBL production.[363,364] ChromID CARBA agar contains specific antibiotics that select for the growth of presumptive carbapenemase-producing Gram-negatives. Both the ChromID ESBL and CARBA media inhibit the growth of Gram-positive bacteria and yeasts.[363,364]

During each experiment, positive and negative controls were used. Thus, for ChromID ESBL medium, *Escherichia coli* American type culture collection (ATCC) 25922 (negative control) and *Klebsiella pneumoniae* ATCC 700603 (positive control) strains were used. *E. coli* ATCC 25922 does not produce any of the ESBL or carbapenemase enzymes, whilst *K. pneumoniae* ATCC 700603 produces an ESBL enzyme (SHV-18). The ATCC strains were commercially supplied by Davies Diagnostic (Randburg, Johannesburg, South Africa). For ChromID CARBA medium, *E. coli* ATCC 25922 (carbapenemase-negative) and confirmed KPC-, NDM-, and OXA-48-producing *K. pneumoniae* clinical isolates (carbapenemase-producers) were included as quality controls in each run.

The inoculated plates were incubated aerobically at 37°C. The bacterial growth was checked after 24 and 48 hours of incubation as per manufacturer's instructions. According to the manufacturer's specifications, pink to burgundy colonies should be identified as *E. coli* isolates, whereas green or blue colonies should be identified as *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* species. *Proteus*, *Morganella*, and *Providencia* species would appear as dark brown or light brown colonies (bioMérieux, Marcy I'Etoile, France).

3.4.2 Isolation of Gram-negative bacilli

Presumptive ESBL and carbapenemase producers that grew on ChromID ESBL agar and ChromID CARBA agar were subcultured onto MacConkey agar plates (National Health Laboratory Services, Greenpoint, Cape Town, South Africa), which select for Gram-negative bacteria, and differentiate them as lactose or non-lactose fermenters. The plates were aerobically incubated at 37°C for 24 hours, and after overnight incubation, the organisms were Gram-stained to confirm the Gram-negative bacilli.[365]

Pure Gram-negative isolates were preserved in skim-milk tryptone glucose glycerol (STGG) (Wits Health Consortium, South Africa) and stored in -80°C before further analysis.

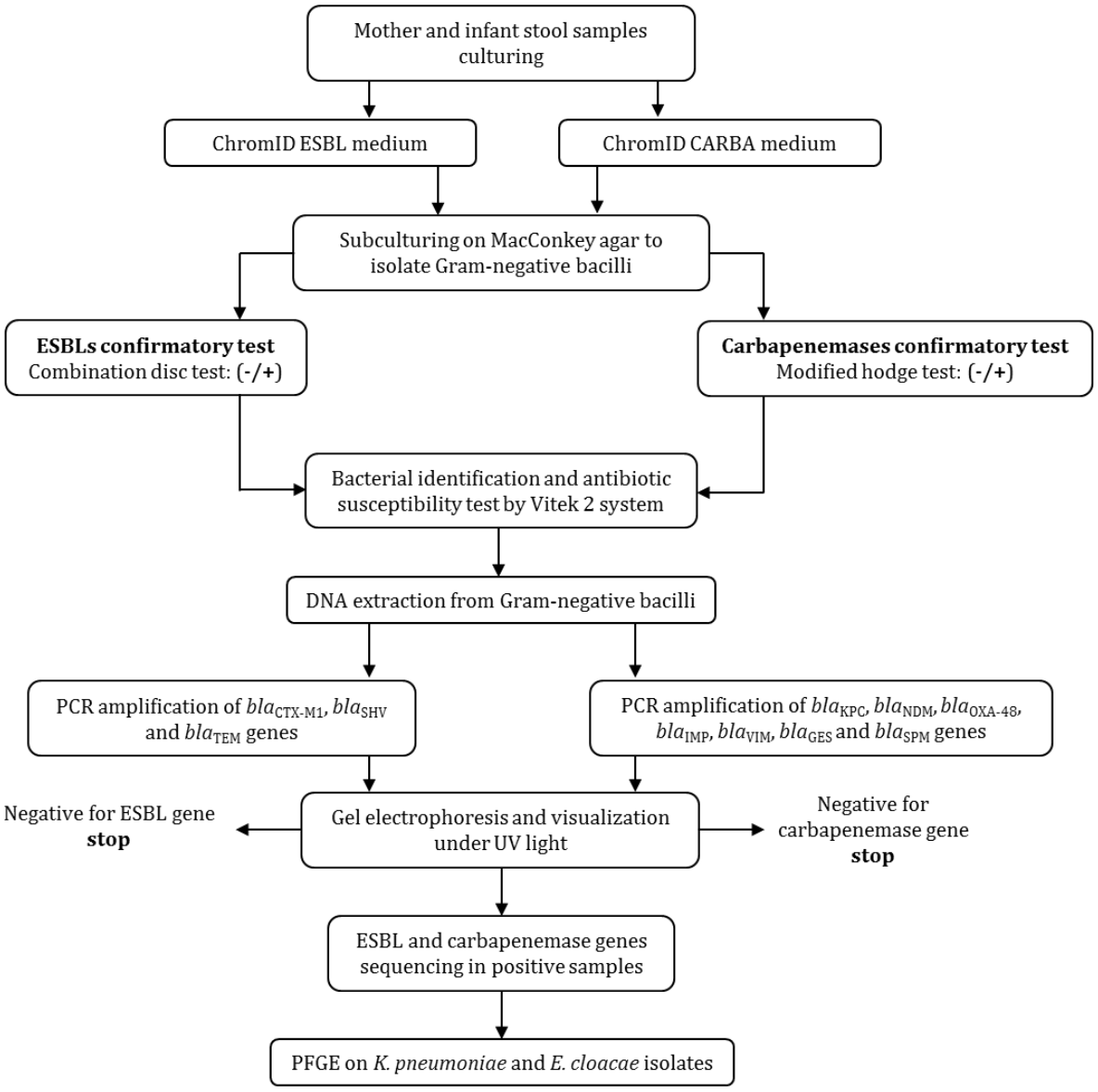

Figure 10. Summary of laboratory procedures performed in this study.
-, Negative; +, Positive; UV, Ultraviolet; PFGE, Pulsed-field gel electrophoresis.

3.5 Identification and antibiotic susceptibility testing

The isolated Gram-negative bacilli were tested with the Vitek 2 system (bioMérieux, Marcy I'Etoile, France) for identification and antibiotic susceptibility testing (AST). Vitek 2 system is an automated system that identifies the bacterial strains and their antibiotic susceptibility profiles (bioMérieux, Marcy I'Etoile, France). The Gram-negative card was used, which is able to identify 135 taxa of the most clinically and significant fermenting and non-fermenting Gram-negative bacilli. Vitek 2 system uses a 64-well card

containing up to 20 antibiotics, each present over a range of concentrations. The system allows kinetic analysis by reading the turbidity of the growth of the tested isolate in the presence of antibiotics to perform linear regression analysis and ultimately determine algorithm-derived minimum inhibitory concentrations (MICs) (bioMérieux, Marcy l'Etoile, France).

Depending on the bacterial strain tested, the MICs for antibiotics shown in Table 2 were determined. Therefore, based on Vitek 2 system results, the isolates were defined as being resistant, intermediate or susceptible to the tested antibiotics according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI).[339]

Table 2. Names and classes of antibiotics tested

Class of antibiotic(s)	Antibiotic(s) tested
1. Penicillins	• Ampicillin
2. Penicillins + beta-lactamase inhibitors	• Amoxicillin + clavulanic acid
3. Antipseudomonal penicillins + beta-lactamase inhibitors	• Piperacillin-tazobactam
4. Non-extended spectrum cephalosporins	• Cefuroxime • Cefuroxime Axetil
5. Cephamycin	• Cefoxitin
6. Extended spectrum cephalosporins	• Cefotaxime • Ceftazidime • Cefepime
7. Carbapenems	• Ertapenem • Imipenem • Meropenem
8. Aminoglycosides	• Gentamicin • Amikacin
9. Fluoroquinolones	• Ciprofloxacin
10. Glycylcyclines	• Tigecycline
11. Polymyxins	• Colistin
12. Nitrofurans	• Nitrofurantoin
13. Folate pathway inhibitors	• Trimethoprim-sulfamethoxazole

3.6 Phenotypic tests for confirmation of suspected ESBL- and carbapenemase-producing bacteria

3.6.1 Confirmatory test for ESBLs

The combination disc test (CDT) was used to confirm the production of extended-spectrum beta-lactamase. Stored bacterial isolates were removed from -80°C and allowed to thaw at room temperature before subculturing. The isolates were subcultured on MacConkey agar (National Health Laboratory Services, Greenpoint-Cape Town, South Africa) and incubated aerobically at 37°C for 24 hours. The bacterial cells were suspended in 0.9% saline at a turbidity equivalent to 0.5 McFarland standard. The discs containing cefotaxime (30 µg), ceftazidime (30 µg), or cefepime (30 µg) alone; and in combination with clavulanic acid (30/10 µg) (Davies Diagnostic, Randburg, Johannesburg, South Africa) were placed on Mueller-Hinton agar plate inoculated with a bacterial suspension and incubated overnight at 37°C. *K. pneumoniae* ATCC 700603 strain (ESBL-producer), and *E. coli* ATCC 25922 strain (ESBL-negative), were used as positive and negative controls, respectively. The test isolates were considered positive for the production of ESBL if the zone of inhibition increased by at least 5 mm for any antibiotic tested in combination with clavulanic acid as compared to corresponding antibiotic tested alone. The test was performed as previously described and the results were interpreted as recommended by the EUCAST guideline.[306]

3.6.2 Confirmatory tests for carbapenemase

The modified Hodge test (MHT) was used to confirm the production of carbapenemase production in Gram-negative isolates which were thawed at room temperature prior to subculture. MHT is a phenotypic test that detects the production of carbapenemase enzymes. When an enzyme is produced by the test strain, it inactivates a carbapenem antibiotic in the medium. This inactivation allows the growth of a carbapenem-susceptible strain towards a carbapenem-containing disc along the streaked line of the test strain forming a cloverleaf-like shape or indentation.[339]

All isolates were subcultured on MacConkey agar (National Health Laboratory Services, Greenpoint, Cape Town, South Africa) overnight and incubated aerobically at 37°C for 24 hours. The test was performed as previously described,[339] with slight modifications. Briefly, the indicator organism, *E. coli* ATCC 25922, was suspended in 0.9% physiological saline at a turbidity equivalent to 0.5 McFarland standard. The bacterial

suspension was then diluted to 1:10 ratio, by adding 1 ml of the 0.5 McFarland to 9 ml of 0.9% physiological saline. A lawn of the 1:10 dilution of an indicator strain (*E. coli* ATCC 25922) was inoculated on Mueller-Hinton agar plate using a sterile swab and allowed to dry. Meropenem (10 µg), ertapenem (10 µg), and meropenem discs (10 µg) (Davies Diagnostic, Randburg, Johannesburg, South Africa) were placed on the swabbed MHA plate. The test organism was streaked in three straight lines from the centre of the MHA plate to the edges of the discs; this was done to give much sharper results than the normal layout. The normal layout inoculates the test organisms in straight lines out from the edge of one disc at the centre of the plate.[339] The following controls were used in each run; carbapenemase-producing *K. pneumoniae* ATCC BAA 1705 (KPC producer) and carbapenem-susceptible *K. pneumoniae* ATCC BAA 1706 strains (Davies Diagnostic, Randburg, Johannesburg, South Africa). The MHT positive test was indicated by the formation of a cloverleaf-like indentation of the indicator strain along the streak of the tested strain within the disc diffusion zone of inhibition (Figure 8).

3.7 Detection of ESBL and carbapenemase genes from bacterial isolates

3.7.1 Total DNA extraction from pure cultures

DNA extraction was performed on 24-hour cultures of Gram-negative isolates grown on MacConkey agar. The total bacterial DNA of presumptive ESBL and carbapenemase producers was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corp., Irvine, California, USA). Briefly, a loopful of bacterial cells was picked from a MacConkey agar plate and suspended in 750 µl of lysis solution in ZR bashing bead ™ lysis tube. Bacterial cells were homogenized and mechanically lysed for 5 minutes using the TissueLyser LT™ (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). DNA was extracted following the manufacturer's instructions and eluted in a total volume of 25 µl. Extracted DNA was stored at -80°C until further processing.

3.7.2 Assessment of bacterial DNA quality

The DNA concentration (ng/µl) and purity (absorbance ratio at 260/280) of extracted DNA were determined by the Biodrop DUO (Whitehead Scientific, Cape Town, South Africa), where pure DNA was defined as having a 260/280 absorbance ratio ranging between 1.7 and 2.0.[366] The quality or integrity of the DNA was confirmed by visualizing approximately 100 ng of DNA on a 2% agarose gel (w/v) containing 0.25 µg/µl of ethidium bromide (EtBr), run in 1X TAE buffer at 100 V for 1 hour. Each DNA sample

was then diluted to a working DNA concentration of 100ng/µl, and stored at 4°C until further analysis.

3.7.3 Amplification of ESBL and carbapenemase genes by singleplex polymerase chain reaction

PCR primers used in this study were synthesized using ABI DNA synthesizer at the Department of Molecular and Cell Biology, University of Cape Town, South Africa. The primers and PCR thermal cycling conditions used for the amplification of selected ESBL genes are shown in Table 3, and those for the carbapenemase genes in Table 4.

The PCR reaction mixture was prepared in a pre-PCR room. A reaction mixture in a final volume of 25µl consisted of nuclease-free water, 1X green GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 0.5 U of GoTaq® G2 Flexi DNA polymerase (Anatech, Johannesburg, South Africa), 2.5mM dNTP mix (Thermo Scientific, Pierce, USA), 0.5 µM of each primer, and 100ng DNA template. The above-described reaction components were used to amplify CTX-M and all carbapenemase genes (Table 4), whilst 2.0 mM $MgCl_2$ was used when detecting SHV and TEM genes. Singleplex conventional PCR amplification of each gene was performed using a PCR Thermal Cycler (Thermo Scientific, Pierce, USA).

Table 3. Primers and conditions for amplification of common ESBL genes

Target	Product size (bp)	Primers	Sequence (5'→3')	Reference	PCR cycles (X 30)	Reference
***bla*$_{CTX-M1}$**	560	CTXM1F	CGCTTTGCGATGTGCAG	[367]	94°C (5 min) 94°C (30 s) 56°C (30 s) 72°C (45 s) 72°C (5 min)	[368]
		CTXM1R	ACCGCGATATCGTTGGT	[367]		
***bla*$_{SHV}$**	1000	SHVC	AGAAGGGTTATTCTTATTTGTCGC	[367]	94°C (5 min) 94°C (30 s) 56°C (30 s) 72°C (60 s) 72°C (5 min)	[368]
		SHVD	TCTTTCCGATGCCGCCGCCAGTCA	[369]		
***bla*$_{TEM}$**	1200	DEB	ATGAGTAAACTTGGTCTGAC	[370]	94°C (5 min) 94°C (30 s) 58°C (30 s) 72°C (60 s) 72°C (5 min)	[368]
		3061TEM	AGGAAGCAAAGCTGAAAGGAATCAAATTTGG	[371]		

Table 4. Primers and conditions for amplification of common carbapenemase genes

Target	Product size (bp)	Primers	Sequence (5'→3')	Reference	PCR cycles (X 30)	Reference
***bla*$_{GES}$**	852	GesF	ATGCGCTTCATTCACGC	[367]	94°C (5 min) 94°C (30 s) 55°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		GesR	GCTCAGGCTGAGTTGTG	[367]		
***bla*$_{IMP}$**	444	ImpXF2	ATTGACACTCCATTTAC	[59]	94°C (5 min) 94°C (30 s) 55°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		ImpXR2	AACAACCAGTTTTGC	[59]		
***bla*$_{NDM}$**	621	NdmF2	GGTTTGGCGATCTGGTTTTC	[368]	94°C (5 min) 94°C (30 s) 55°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		NdmR2	CGGAATGGCTCATCACGATC	[368]		
***bla*$_{OXA-48-like}$**	807	Oxa48F	CGTGTATTAGCCTTATCG	[368]	94°C (5 min) 94°C (30 s) 55°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		Oxa48R	CGCTAACCACTTCTAGG	[368]		
***bla*$_{VIM}$**	656	VimXF	GTGAGTATCCGACAGTC	[59]	94°C (5 min) 94°C (30 s) 55°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		VimXR	GAGCAAGTCTAGACCG	[59]		
***bla*$_{KPC}$**	1011	KpcF	TGTCACTGTATCGCCGTC	[172]	94°C (5 min) 94°C (30 s) 62°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		KpcR	CTCAGTGCTCTACAGAAAACC	[172]		
***bla*$_{SPM}$**	784	SpmF	CTGCTTGGATTCATGGGCGC	[372]	94°C (5 min) 94°C (30 s) 62°C (30 s) 72°C (50 s) 72°C (5 min)	[368]
		SmpR	CCTTTTCCGCGACCTTGATC	[372]		

3.8 Gel electrophoresis and visualization of the amplicons

Ten microliter of PCR products (amplicons) were analyzed on a 2.0% (w/v) agarose gel containing 0.25 µg/ml of ethidium bromide, run in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 100 volts for 1 hour. A 100 bp molecular weight ladder (Thermo Scientific, Pierce, USA) was used as a maker to estimate the size of the amplified fragments in each run. Amplicons were visualized by trans-illuminator, photographed by a digital camera, and transferred to computer data for labelling and storage. The specificity of amplified fragments was then confirmed by DNA sequencing.

3.9 DNA sequencing and analysis of the amplicons

The PCR products were purified using the Zymo research sequencing clean-up kit (Zymo Research Corp., Irvine, California, USA), according to the manufacturer's instruction. The PCR products were sequenced on both strands by using the primers shown in Table 3 for ESBL genes and Table 4 for carbapenemase genes. The purified DNA amplicons were sequenced by Inqaba Biotech (Inqaba Biotech, Pretoria, South Africa) using the ABI Prism BigDye® Terminator (Version 3.1) sequencing kit on ABI Prism 3500XL DNA Analyzer. The nucleotide sequences obtained were assembled using DNA Baser sequence assembler version 4.16.0 (2014, Heracle BioSoft, www.DnaBaser.com). Sequence alignment and analysis were performed online using the basic local alignment search tool (BLAST) available at the national center for biotechnology information (NCBI) (www.ncbi.nil.gov). Similar nucleotide alignment sequences of beta-lactamase genes were searched from the NCBI database. The criteria for the positive hits were the query coverage of ≥ 90% of the reference alignment used and the sequence identity of ≥ 99%.

3.10 Investigation of the relatedness of *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates by pulsed-field gel electrophoresis

3.10.1 Preparation of bacterial cell suspension

Pulsed-field gel electrophoresis (PFGE) was performed on *Klebsiella pneumoniae* or *Enterobacter cloacae* isolates using the procedures described in the PulseNet USA protocols with the following slight modifications.[373] *Klebsiella pneumoniae* ATCC BAA 1705 was included as an internal control in each run to assess the reproducibility and quality of gels. Each of the tested isolates was subcultured on MacConkey agar plate. A 24-hour bacterial culture was picked using a sterile swab and suspended in 1 ml of cell

suspension buffer (100 mM Tris-HCL, 10 mM EDTA). The bacterial suspension concentration was adjusted to 1.0 absorbance (optical density) by measuring at 590 nm wavelength using a Biodrop DUO (Whitehead Scientific, Cape Town, South Africa).

3.10.2 Preparation of plugs

Four hundred microliters of adjusted cell suspension, 20 µl of proteinase K (20 mg/ml) and 5 µl of lysozyme (20 mg/ml) (Inqaba Biotech, Pretoria, South Africa) were combined with 400 µl of melted 1% PFGE plug agarose (w/v): 1% sodium dodecyl sulphate (SDS) (v/v) (Bio-Rad Laboratories, Hercules, USA). The mixture was mixed gently pipetting up and down a few times to prevent DNA shearing by over-pipetting. This mixture was then pipetted into the slots of the PFGE plug mold (Bio-Rad Laboratories, Hercules, USA) by using a pipet tip. The plugs were allowed to solidify at room temperature for about 15 minutes. Three plugs were prepared for each isolate.

3.10.3 Lysis of cells in agarose plugs

After solidification, only two plugs were gently removed from the PFGE mold; and each plug was placed separately in a sterile 2 ml epperndorf tube containing 1.8 ml of cell lysis EC buffer, 25 µl of proteinase K (20 mg/ml) and 50 µl of lysozyme (20 mg/ml). The cell lysis EC buffer contained 6.0 mM Tris-HCl, 1.0 M NaCl, 0.1 M EDTA, 0.5% Brij 35 and 0.5% Sarkosyl (pH 7.5). The epperndorf tubes were incubated in the heating block at 55°C overnight without agitation.

3.10.4 Washing of agarose plugs after cell lysis

Following overnight incubation, all the plugs for the same strain were removed from the cell lysis EC buffer and placed in 50 ml blue topped tube. Enough sterile water and TE buffer (10mM Tris: 1mM EDTA pH 8.0) were pre-heated in a 50°C water bath. Five millilitres of pre-heated sterile water was added into each tube containing a set of plugs for the same strain and shook the tubes at 2500 rpm on the rolling shaker at room temperature for 15 minutes. After 15 minutes, the water was carefully decanted (making sure not to lose the plugs) before adding another 5 ml of sterile water. Each plug set was washed five times with 5 ml of pre-heated sterile water and TE buffer, respectively.

3.10.5 XbaI and SpeI restriction enzyme digestion of DNA in agarose plugs

A prepared plug was cut in 2.0 X 2.0 mm section using a sterile scalpel blade and placed in a sterile 2 ml epperndorf tube. Each plug was digested using a restriction enzyme, XbaI, in a total volume of 200 µl containing 174 µl of nuclease-free water, 4 µl (40 U) XbaI, and 20 µl of 10X Tango buffer and 2 µl of bovine serum albumin (BSA) (Thermo Scientific, Pierce, USA). The plugs were then incubated in the heating block at 37°C for 3 hours without agitation. Similar conditions and reaction components were used when digesting DNA in another 2.0 X 2.0 mm section of the plug using the 4 µl (40 U) SpeI instead of XbaI µl (40 U) enzyme.

3.10.6 Electrophoresis and visualization under UV light

The digested DNA was electrophoresed in 1% of PFGE agarose (w/v) (Bio-Rad Laboratories, Hercules, USA) with a Gene Navigator™ System (Amersham Biosciences, Uppsala, Sweden) for 18 hours with a pulse time of 5-60s at 200 V. PFGE lambda DNA ladder (Bio-Rad Laboratories, Hercules, USA) was included in each gel run. Once the run was complete, the gel was removed from the tank and stained in 400ml 0.5X TBE plus 40ul EtBr (10mg/ml) for 30 minutes, de-stained in water for one hour, changing the water every 30 minutes. The gel was visualized by trans-illuminator, photographed by digital camera and transferred to computer data for labelling and storage. Images were converted to TIFF format for computer analysis. PFGE data were analyzed using BioNumerics software 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were constructed with dice coefficient, represented by the unweighted pair-group method, 1.0% optimization and 1.0% tolerance for the band migration position. Isolates were considered to belong to the same cluster when sharing high levels of pattern similarity (80-100%) and indistinguishable when sharing ≥90% similarity.[141]

3.11 Statistical analysis

The risk factors were statistically analyzed using the environmental performance index (EPI) Info version 7 software (Center for Disease Control and Prevention, Atlanta, Georgia, USA) by univariate analysis. A p-value of ≤ 0.05 was considered statistically significant. The MIC values were analyzed using the WHONET 5.6 software (World Health Organization, Geneva, Switzerland).

3.12 Ethical considerations

This study was approved by the Faculty of Health Sciences (FHS) Human Research Ethics Committee (HREC) of the University of Cape Town, South Africa (HREC reference number: 738/2013). Informed consent was obtained from all participants before enrolment in the broader Drakenstein Child Health Study, for which approval by HREC has previously been given (HREC reference number: 401/2009).

In accordance with the English,[374] Canadian,[375] and United Kingdom (UK) National Health Service (NHS) guidelines;[376] patients who screened positive for colonization were not treated in the absence of infection as further antibiotic resistance could result from treatment. Patients were not informed of the outcome of the testing to avoid unnecessary anxiety.

4. Results

4.1 Characteristics of the study population

Table 5 summarizes the characteristics of the study population. Ninety mothers and 116 infants (90 mother-infant pairs) were included in this study. The longitudinal stool sampling at different time-points is shown in Figure 11.

The mean age of mothers was 26.4 ± 5.5 years. Forty-one (45.6%) mothers were from the Mbekweni area, and the remainder from the TC Newman area (Table 5). Fifty (55.4%) were mixed race and 40 (44.4%) black African. Six (6.7%) of the mothers had been hospitalized in the previous two years, and 19 (21.1%) were HIV positive. At delivery, only 38 (42.2%) mothers were given medications or supplements during hospital stay or at discharge.

The majority of infants were born by normal vaginal delivery (92/116; 79.3%). Seventeen (14.7%) and five (4.3%) infants were delivered by emergency and elective caesarean sections, respectively. The remaining two infants were born through the vaginal vacuum (0.9%) and assisted vaginal breech delivery (0.9%), respectively. Sixty-one of 116 (53%) infants were males. Only seven (6%) infants were immediately admitted to the nursery after birth, and 109 (94%) were roomed with their mothers. The mean birth weight was 3.0 ± 0.5 kilograms (kg). Thirty-four (29.3%) were given medications and only nine (7.8%) had supplements before discharge. Ninety-nine of the infants were breastfed before discharge (Table 5).

Table 5. Characteristics of mothers and infants included in the study

Clinical characteristics	Participants (%)
Mothers (n= 90)	
Location	
Mbekweni	41 (45.6)
TC Newman	49 (54.4)
Maternal age (years), Median [IQR]	25 [22.5 - 31]*
Ethnicity	
Black African	40 (44.4)
Mixed race	50 (55.4)
Hospitalization (in previous two years)	6 (6.7)
HIV positive mothers	19 (21.1)
Medication or supplement at delivery	38 (42.2)
Education	
Primary school	8 (8.9)
High school level	76 (84.4)
Tertiary level	6 (6.7)
Household members, Median [IQR]	5 [3 - 6.5]*
Household adults over 18 years old, Median [IQR]	2 [2 - 4.5]*
Household children under 18 years old, Median [IQR]	1 [0.5 - 1.5]*
Members sharing room with the mother, Median [IQR]	2 [2.5 - 3.5]*
Infants (n= 116)	
Male infant	61 (52.6)
Mode of delivery	
Vaginal delivery	94 (81.0)
Caesarean section	22 (19.0)
Preterm (< 37 weeks)	6 (5.2)
Low birth weight (< 2500g)	15 (12.9)
Birth weight (kg), Median [IQR]	3 [2.7 - 3.3]*
Place of admission immediately after birth	
Nursery	7 (6.0)
Roomed with the mother	109 (94)
Babies given medication before discharge	34 (29.3)
Babies given supplements before discharge	9 (7.8)
Breastfeeding initiated prior to discharge	99 (85.3)

* Median [Interquartile range (IQR)]

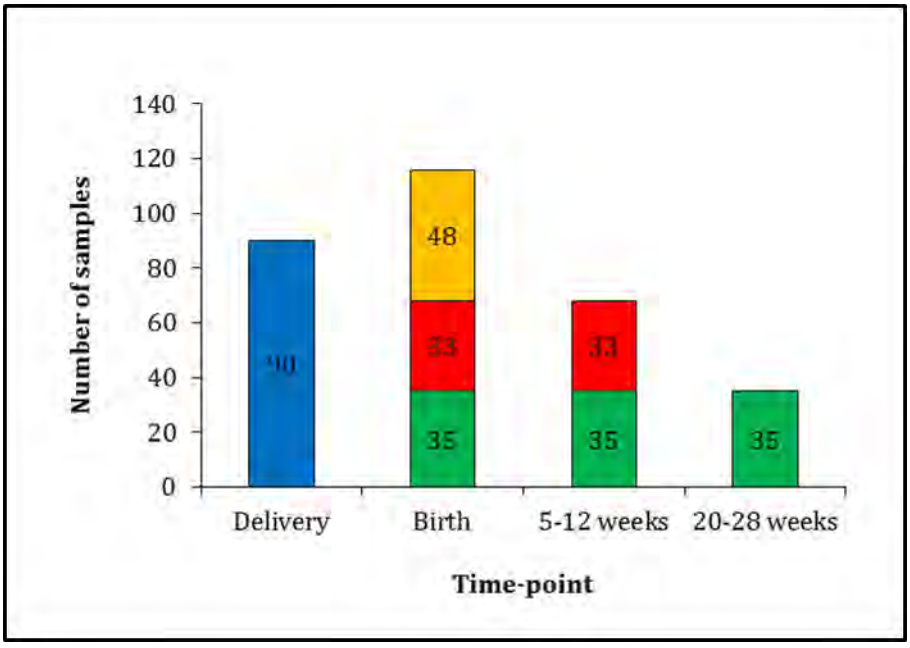

Figure 11. Longitudinal stool sampling in 90 mothers and 116 infants at different time-points.
The red and green colours represent the longitudinal sampling of stool samples among the infants.

4.2 Bacterial isolation and identification

Of the 309 stool samples screened using selective media, resistant Gram-negative bacilli were isolated in 27 stool samples (8.7%) tested. Of these, six were infant stool samples collected at birth, eight at 5-12 weeks and seven at 20-28 weeks, and the remaining six were maternal stool samples collected at delivery. Among the 39 Gram-negative bacilli isolated in this study, seven were from six mothers, and 32 from 20 infants. Table 6 shows the distribution of Gram-negative bacilli in stool samples from the mothers and infants at different time-points.

Table 6. Distribution of Gram-negative bacilli in stool samples from the mothers and infants at different time-points

Time-point(s)	Number of stool samples with:			
	1 Gram-negative bacillus	2 Gram-negative bacilli	3 Gram-negative bacilli	4 Gram-negative bacilli
Delivery	5	1	0	0
Birth	5	1	0	0
5-12 weeks	5	2	1	0
20-28 weeks	5	0	0	2
Total isolates (39)	**20**	**8**	**3**	**8**

Table 7 shows the distribution and frequency of isolated Gram-negative bacilli among the mothers and infants. Among 39 bacterial isolates, the most frequently isolated bacterial species was *Acinetobacter baumannii* (n= 10), followed by *Klebsiella pneumoniae* (n= 7), *Escherichia coli* (n= 5), *Enterobacter cloacae* (n= 5), and *Stenotrophomonas maltophilia* (n= 4). Thirty-two of 39 isolates (82.1%) were recovered from the ChromID ESBL medium; 30 isolates after 24 hours and two after 48 hours of incubation (Table 8). The remaining seven isolates were recovered from the ChromID CARBA medium, after 24 hour (n= 4) and 48 hours (n= 3) of incubation (Table 8).

Table 7. Distribution and frequency of Gram-negative bacilli among the mothers and infants

Bacterial species	Number of isolates		Frequency (%)
	Mothers	Infants	
Enterobacteriaceae			
Enterobacter cloacae	1	4	5 (12.8)
Klebsiella pneumoniae	2	5	7 (18.0)
Escherichia coli	2	3	5 (12.8)
Non-fermenters			
Acinetobacter baumannii	1	9	10 (25.6)
Acinetobacter ursingii	0	2	2 (5.1)
Pseudomonas putida	0	2	2 (5.1)
Pseudomonas luteola	0	1	1 (2.6)
Stenotrophomonas maltophilia	0	4	4 (10.3)
Ochrobactrum anthropi	0	2	2 (5.1)
Cupriavidus pauculus	1	0	1 (2.6)
Total	**7**	**32**	**39 (100)**

Table 8. The number of isolates obtained from different chromogenic screening media

Incubation time	Screening medium	Number of bacterial isolates				
		K. pneumoniae	***E. cloacae***	***E. coli***	**Non-fermenting bacilli**	**Total (39)**
24 hours	ChromID ESBL	7	5	4	14	**30**
	ChromID CARBA	0	0	1	3	**4**
48 hours	ChromID ESBL	0	0	0	2	**2**
	ChromID CARBA	0	0	0	3	**3**

4.3 Antibiograms of Gram-negative bacilli

The minimum inhibitory concentrations (MICs) for 19 antibiotics were determined by the Vitek 2 system (bioMérieux, Marcy I'Etoile, France). The results were categorized using the breakpoints and interpretive criteria provided by the CLSI when available.[339] For tigecycline, the EUCAST clinical breakpoints were used.[377]

4.3.1 Antibiograms of Enterobacteriaceae

Table 9 shows the MIC values for Enterobacteriaceae. All the isolates (n= 17) were susceptible to carbapenems (imipenem, ertapenem and meropenem), colistin and amikacin (Figure 12). High susceptibility rates were also observed for ciprofloxacin (94.1%), Piperacillin-tazobactam (88.2%), Cefoxitin (70.6%) and cefepime (70.6%), (Table 10). In contrast, the susceptibility rate for nitrofurantoin (29.4%) was very low, and 100% of the isolates were resistant to trimethoprim-sulfamethoxazole, with a very high 50% minimum inhibitory concentration (MIC_{50}) of 384 µg/mL. The Enterobacteriaceae isolates were highly resistant to ampicillin (94.1%) and cefotaxime (82.4%), (Table 10). Most of the Enterobacteriaceae isolates (88.2%, 15/17) were resistant to at least one antibiotic from three or more classes of antibiotics and were classified as multidrug-resistant (Table 11). The resistance profiles of 17 Enterobacteriaceae isolates are shown in Table 11.

Table 9. Minimum inhibitory concentrations for different antibiotics in Enterobacteriaceae isolates

Isolate no.	Participant	Isolate	Minimum inhibitory concentrations in µg/mL (breakpoint value)																
			AMP (≤ 8)	AMC (≤ 8)	PZT (≤ 16)	CXM (≤ 8)	CXA (≤ 4)	FOX (≤ 8)	CTX (≤ 1)	CAZ (≤ 4)	FEP (≤ 2)	ETP (≤ 0.5)	IPM (≤ 1)	MEM (≤ 1)	AMK (≤ 16)	GEN (≤ 4)	CIP (≤ 1)	TGC (≤ 1)	NIT (≤ 32)	COL (≤ 2)	SXT (≤ 2)
507393-2	Mother	*E. cloacae*	≥ 32	≥ 32	8	≥ 64	≥ 64	8	8	≥ 64	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≥ 16	≤ 0.25	1	64	≤ 0.5	≤ 20
507394-1	Infant	*E. cloacae*	≥ 32	≥ 32	8	32	32	≤ 4	8	≥ 64	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≥ 16	≤ 0.25	1	128	≤ 0.5	≤ 20
506261-1	Infant	*E. cloacae*	≥ 32	≥ 32	≤ 4	16	16	≥ 64	4	16	≤ 1	≤ 0.5	0,5	≤ 0.25	16	≤ 1	≤ 0.25	1	32	≤ 0.5	≤ 20
506430-1	Infant	*E. cloacae*	≥ 32	≥ 32	≤ 4	32	32	≥ 64	4	16	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	16	≤ 1	≤ 0.25	1	32	≤ 0.5	≤ 20
104650-3	Infant	*E. cloacae*	≥ 32	≥ 32	≤ 4	32	32	≥ 64	4	16	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	16	≤ 1	≤ 0.25	1	64	≤ 0.5	≤ 20
101870-1	Infant	*E. coli*	4	≤ 2	≤ 4	4	4	≤ 4	≤ 1	≤ 1	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	32	≤ 0.5	≤ 20
507214-1	Mother	*E. coli*	≥ 32	8	≤ 4	4	4	≤ 4	≤ 1	≤ 1	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≤ 16	≤ 0.5	≥ 320
502192-1	Mother	*E. coli*	≥ 32	4	≤ 4	≥ 64	≥ 64	≤ 4	4	≤ 1	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≤ 16	≤ 0.5	≤ 20
102461-4	Infant	*E. coli*	≥ 32	16	≤ 4	≥ 64	≥ 64	32	≥ 64	4	8	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	128	≤ 0.5	≥ 320
307338-1	Infant	*E. coli*	≥ 32	16	≤ 4	≥ 64	≥ 64	32	≥ 64	4	16	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	128	≤ 0.5	≥ 320
106906-3	Infant	*K. pneumoniae*	≥ 32	≤ 2	≤ 4	2	2	≤ 4	≤ 1	≤ 1	≤ 1	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	64	≤ 0.5	≤ 20
509599-1	Infant	*K. pneumoniae*	≥ 32	4	8	≥ 64	≥ 64	≤ 4	≥ 64	16	2	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≥ 16	0,5	1	64	≤ 0.5	≥ 320
502317-1	Mother	*K. pneumoniae*	≥ 32	≥ 32	16	≥ 64	≥ 64	≤ 4	≥ 64	16	4	≤ 0.5	≤ 0.25	≤ 0.25	4	≥ 16	1	2	128	2	≥ 320
501940-1	Infant	*K. pneumoniae*	≥ 32	16	8	≥ 64	≥ 64	≤ 4	≥ 64	4	2	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≥ 16	2	≤ 0.5	128	≤ 0.5	≥ 320
506433-1	Mother	*K. pneumoniae*	≥ 32	8	≤ 4	≥ 64	≥ 64	≤ 4	≥ 64	8	2	≤ 0.5	≤ 0.25	≤ 0.25	≤ 2	≥ 16	1	1	64	≤ 0.5	≥ 320
102461-2	Infant	*K. pneumoniae*	≥ 32	16	32	≥ 64	≥ 64	≤ 4	≥ 64	≥ 64	16	≤ 0.5	≤ 0.25	≤ 0.25	8	≥ 16	≤ 0.25	2	64	≤ 0.5	≥ 320
303200-1	Infant	*K. pneumoniae*	≥ 32	16	32	≥ 64	≥ 64	≤ 4	≥ 64	≥ 64	16	≤ 0.5	≤ 0.25	≤ 0.25	8	≥ 16	≤ 0.25	2	64	≤ 0.5	≥ 320

No., Number; AMP, Ampicillin; AMC, Amoxicillin + clavulanic acid; TZP, Piperacillin-tazobactam; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; ETP, Ertapenem; IPM, Imipenem; MEM, Meropenem; GEN, Gentamicin; AMK, Amikacin; CIP, Ciprofloxacin; TGC, Tigecycline; COL, Colistin; NIT, Nitrofurantoin; SXT, Trimethoprim-sulfamethoxazole

Table 10. Antimicrobial susceptibility of the 17 Enterobacteriaceae isolates

Antimicrobial agent	Breakpoint value (µg/mL)	Number (%) of resistant isolates	Number (%) of intermediate isolates	Number (%) of susceptible isolates	MICs (µg/mL)		
					MIC_{50}	MIC_{90}	MICs range
Ampicillin	≤ 8	16 (94.1)	0.0	1 (5.9)	32	32	4 – 32
Amoxicillin/Clavulanic acid	≤ 8	6 (35.3)	5 (29.4)	6 (35.3)	16	32	2 – 32
Piperacillin/Tazobactam	≤ 16	0.0	2 (11.8)	15 (88.2)	4	32	4 – 32
Cefuroxime	≤ 8	13 (76.5)	1 (5.9)	3 (17.6)	64	64	4 – 64
Cefuroxime axetil	≤ 4	13 (76.5)	1 (5.9)	3 (17.6)	64	64	4 – 64
Cefoxitin	≤ 8	5 (29.4)	0.0	12 (70.6)	4	64	4 – 64
Cefotaxime	≤ 1	14 (82.4)	0.0	3 (17.6)	8	64	1 – 64
Ceftazidime	≤ 4	9 (52.9)	1 (5.9)	7 (41.2)	16	64	1 – 64
Cefepime	≤ 2	3 (17.6)	2 (11.8)	12 (70.6)	1	16	1 – 16
Ertapenem	≤ 0.5	0.0	0.0	17 (100)	0.5	0.5	0.5 – 0.5
Imipenem	≤ 1	0.0	0.0	17 (100)	0.25	0.25	0.25 – 0.5
Meropenem	≤ 1	0.0	0.0	17 (100)	0.25	0.25	0.25 – 0.25
Amikacin	≤ 16	0.0	0.0	17 (100)	2	16	2 – 16
Gentamicin	≤ 4	8 (47.1)	0.0	9 (52.9)	1	16	1 – 16
Ciprofloxacin	≤ 1	0.0	1 (5.9)	16 (94.1)	0.25	1	0.25 – 2
Tigecycline	≤ 1	3 (17.6)	0.0	14 (82.4)	1	2	0.25 – 2
Nitrofurantoin	≤ 32	5 (29.4)	7 (41.2)	5 (29.4)	64	128	16 – 128
Colistin	≤ 2	0.0	0.0	17 (100)	0.5	0.5	0.5 – 2
Trimethoprim/Sulfamethoxazole	≤ 2	17 (100)	0.0	0.0	384	384	20 – 320

MIC- Minimum inhibitory concentration, MIC_{50}- Minimum inhibitory concentration required to inhibit the growth of 50% of organisms. MIC_{90}- Minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

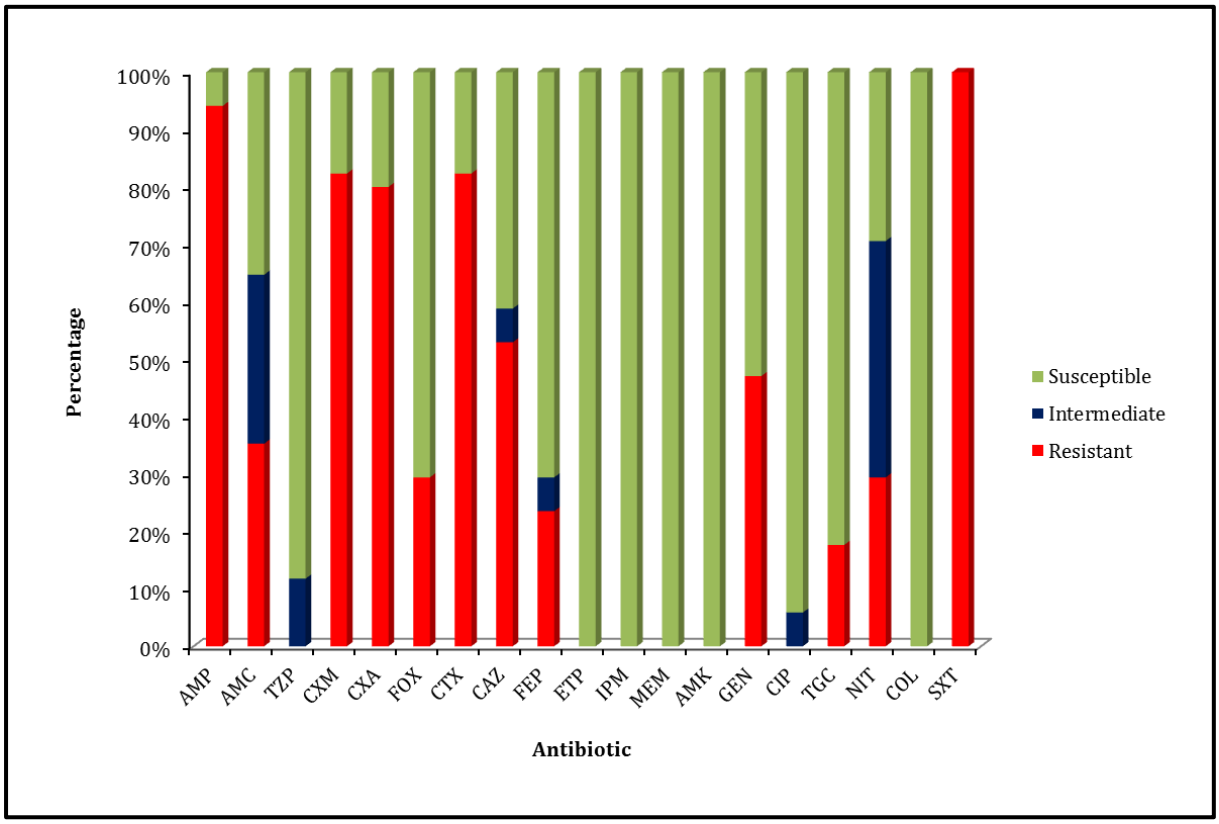

Figure 12. Susceptibility categorization of 17 Enterobacteriaceae isolates to 19 antibiotics by MICs determination.
AMP, Ampicillin; AMC, Amoxicillin + clavulanic acid; TZP, Piperacillin-tazobactam; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; ETP, Ertapenem; IPM, Imipenem; MEM, Meropenem; AMK, Amikacin; GEN, Gentamicin; CIP, Ciprofloxacin; TGC, Tigecycline; NIT, Nitrofurantoin; COL, Colistin; SXT, Trimethoprim-sulfamethoxazole. Findings were categorized using CLSI breakpoints and for tigecycline only, the EUCAST clinical breakpoints were used.

Table 11. Resistance profiles of 17 Enterobacteriaceae isolates to different antibiotics.

Isolate No.	Species	Resistance profile	No. of classes non-susceptible/ No. of classes tested
507393-2	*E. cloacae*	AMP, AMC, CXM, CXA, CTX, CAZ, GEN, NIT, SXT	8/14
507394-1	*E. cloacae*	AMP, AMC, CXM, CXA, CTX, CAZ, GEN, NIT, SXT	8/14
506261-1	*E. cloacae*	AMP, AMC, CXM, CXA, FOX, CTX, CAZ, SXT	7/14
506430-1	*E. cloacae*	AMP, AMC, CXM, CXA, FOX, CTX, CAZ, SXT	7/14
104650-3	*E. cloacae*	AMP, AMC, CXM, CXA, FOX, CTX, CAZ, NIT, SXT	8/14
101870-1	*E. coli*	SXT	1/14
507214-1	*E. coli*	AMP, SXT	2/14
502192-1	*E. coli*	AMP, CXM, CXA, CTX, SXT	5/14
102461-4	*E. coli*	AMP, AMC, CXM, CXA, FOX, CTX, FEP, NIT, SXT	9/14
307338-1	*E. coli*	AMP, AMC, CXM, CXA, FOX, CTX, FEP, NIT, SXT	9/14
106906-3	*K. pneumoniae*	AMP, NIT, SXT	3/14
509599-1	*K. pneumoniae*	AMP, CXM, CXA, CTX, CAZ, GEN, NIT, SXT	7/14
506433-1	*K. pneumoniae*	AMP, CXM, CXA, CTX, CAZ, GEN, NIT, SXT	7/14
501940-1	*K. pneumoniae*	AMP, AMC, CXM, CXA, CTX, GEN, CIP, NIT, SXT	9/14
502317-1	*K. pneumoniae*	AMP, AMC, CXM, CXA, CTX, CAZ, FEP, GEN , TGC, NIT, SXT	10/14
102461-2	*K. pneumoniae*	AMP, AMC, TZP, CXM, CXA, CTX, CAZ, FEP, GEN, TGC, NIT, SXT	10/14
303200-1	*K. pneumoniae*	AMP, AMC, TZP, CXM, CXA, CTX, CAZ, FEP, GEN, TGC, NIT, SXT	10/14

No., Number; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; TZP, Piperacillin-tazobactam; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; GEN, Gentamicin; CIP, Ciprofloxacin; TGC, Tigecycline; NIT, Nitrofurantoin; SXT, Trimethoprim-sulfamethoxazole.

4.3.1 Antibiograms of non-fermenting Gram-negative bacilli

The Vitek 2 system was able to determine the MICs for different antibiotics in 59.1% (13/22) of non-fermenters, mainly *Acinetobacter baumannii* (n= 10) and *Pseudomonas* species (n= 3) (Table 12). MICs for the remaining uncommon Gram-negative bacilli were not determined because the Vitek 2 system used was intended for use in clinical microbiology laboratory to identify and determine the susceptibility of clinically significant aerobic Gram-negative bacilli. All tested non-fermenters (n= 13) were

resistant to nitrofurantoin, trimethoprim-sulfamethoxazole, ampicillin, cefoxitin and cefuroxime axetil (Table 13). However, all isolates remained susceptible to colistin, ciprofloxacin, cefepime and amikacin. Two *A. baumannii* isolates were resistant to both imipenem and meropenem, and two *P. putida* isolates were resistant to both meropenem and tigecycline. Resistance profiles for different antibiotics in 13 non-fermenters are shown in Table 13.

Table 12. Minimum inhibitory concentrations for different antibiotics in non-fermenting Gram-negative bacilli

Isolate no.	Participant	Isolate	Minimum inhibitory concentrations in µg/mL (breakpoint value)																
			AMP (≤ 8)	AMC (≤ 8)	PZT (≤ 16)	CXM (≤ 8)	CXA (≤ 4)	FOX (≤ 8)	CTX (≤ 8)	CAZ (≤ 8)	FEP (≤ 8)	IPM* (≤ 2)	MEM* (≤ 2)	AMK (≤ 16)	GEN (≤ 4)	CIP (≤ 1)	TGC (≤ 1)	NIT (≤ 32)	COL (≤ 2)	SXT (≤ 2)
101277-1	Infant	*A. baumannii*	≥ 32	4	-	32	32	≥ 64	8	8	2	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	1	≥ 512	≤ 0.5	≤ 20
109201-1	Infant	*A. baumannii*	≥ 32	4	-	≥ 64	≥ 64	≥ 64	32	16	8	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
102461-1	Infant	*A. baumannii*	≥ 32	4	-	≥ 64	≥ 64	≥ 64	16	16	8	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
502307-2	Infant	*A. baumannii*	≥ 32	≥ 32	-	-	16	≥ 64	-	4	8	≥ 16	≥ 16	16	≤ 1	≤ 0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
104650-2	Infant	*A. baumannii*	≥ 32	4	-	-	≥ 64	≥ 64	-	8	4	≤ 0.25	0,5	≤ 2	≤ 1	0,5	≤ 0.5	≥ 512	≤ 0.5	160
106983-1	Infant	*A. baumannii*	≥ 32	4	-	-	32	≥ 64	-	4	2	≤ 0.25	≤ 0.25	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
107029-2	Infant	*A. baumannii*	≥ 32	4	-	-	≥ 64	≥ 64	-	8	4	≤ 0.25	0,5	≤ 2	≤ 1	≤ 0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
104650-1	Infant	*A. baumannii*	≥ 32	8	-	-	≥ 64	≥ 64	-	8	4	≤ 0.25	0,5	≤ 2	≤ 1	0,5	≤ 0.5	≥ 512	≤ 0.5	≥ 320
502307-1	Infant	*A. baumannii*	≥ 32	≥ 32	-	-	16	≥ 64	-	4	8	≥ 16	≥ 16	16	≤ 1	<0.25	≤ 0.5	≥ 512	≤ 0.5	≤ 20
507393-1	Mother	*A. baumannii*	16	4	-	-	≥ 64	≥ 64	-	4	4	≤ 0.25	0,5	≤ 2	≤ 1	0,5	≤ 0.5	≥ 512	≤ 0.5	≥ 320
106906-4	Infant	*P. putida*	≥ 32	≥ 32	8	≥ 64	≥ 64	≥ 64	16	4	2	1	4	≤ 2	≤ 1	≤ 0.25	≥ 8	256	≤ 0.5	≥ 320
106906-2	Infant	*P. putida*	≥ 32	≥ 32	≤ 4	≥ 64	≥ 64	≥ 64	16	4	2	1	4	≤ 2	≤ 1	≤ 0.25	≥ 8	≥ 512	1	≥ 320
107029-1	Infant	*P. luteola*	≥ 32	16	≤ 4	-	≥ 64	≥ 64	-	≤ 1	≤ 1	-	-	≤ 2	≤ 1	≤ 0.25	≤ 0.5	256	≤ 0.5	≤ 20

No., Number; * ≤ 1 µg/mL breakpoint value for *Pseudomonas* species; AMP, Ampicillin; AMC, Amoxicillin + clavulanic acid; TZP, Piperacillin-tazobactam; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; ETP, Ertapenem; IPM, Imipenem; MEM, Meropenem; GEN, Gentamicin; AMK, Amikacin; CIP, Ciprofloxacin; TGC, Tigecycline; COL, Colistin; NIT, Nitrofurantoin; SXT, Trimethoprim-sulfamethoxazole; Blank (-) means not tested

Table 13. Resistance profiles of 13 non-fermenting Gram-negative bacilli to different antibiotics.

Isolate No.	Species	Resistance profile	No. classes non-susceptible/ No. classes tested
507393-1	*A. baumannii*	AMP, CXA, FOX, NIT, SXT	5/13
107029-2	*A. baumannii*	AMP, CXA, FOX, NIT, SXT	5/13
106983-1	*A. baumannii*	AMP, CXA, FOX, NIT, SXT	5/13
104650-2	*A. baumannii*	AMP, CXA, FOX, NIT, SXT	5/13
104650-1	*A. baumannii*	AMP, CXA, FOX, NIT, SXT	5/12
101277-1	*A. baumannii*	AMP, CXM, CXA, FOX, NIT, SXT	6/14
502307-2	*A. baumannii*	AMP, AMC, CXA, FOX, IMP, MEM, NIT, SXT	7/13
502307-1	*A. baumannii*	AMP, AMC, CXA, FOX, IMP, MEM, NIT, SXT	7/13
109201-1	*A. baumannii*	AMP, CXM, CXA, FOX, CTX, CAZ, NIT, SXT	7/14
102461-1	*A. baumannii*	AMP, CXM, CXA, FOX, CTX, CAZ, NIT, SXT	7/14
106906-2	*P. putida*	AMP, AMC, CXM, CXA, FOX, CTX, MEM, TGC, NIT, SXT	10/14
106906-4	*P. putida*	AMP, AMC, CXM, CXA, FOX, CTX, MEM, TGC, NIT, SXT	10/14
107029-1	*P. luteola*	AMP, CXA, FOX, NIT, SXT	5/13

No., Number; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; TGC, Tigecycline; NIT, Nitrofurantoin; SXT, Trimethoprim-sulfamethoxazole

4.4 Detection of ESBL- and carbapenemase-producing bacteria

The results obtained by the combination disc test and modified hodge test were consistent with PCR results.

4.4.1 Phenotypic identification

The production of ESBLs among Enterobacteriaceae (n= 17) was confirmed in 14 (82.4%) isolates by the combination disc test (CDT) (Table 14). Using the CDT, cefotaxime with cefotaxime-clavulanate detected ESBLs in 100% (n= 14) of the isolates resistant to cefotaxime and/or ceftazidime. Cefepime with cefepime-clavulanate detected ESBLs in 93% (13/14) of such isolates. The combination of ceftazidime and ceftazidime-clavulanate discs detected ESBLs production in 78.6% (11/14) of Enterobacteriaceae isolates. All Enterobacteriaceae (n= 17) and non-fermenting Gram-negative bacilli (n= 22) were negative for the production of carbapenemase enzymes by the modified hodge test.

Table 14. Detection of ESBLs by phenotypic tests

Isolate no.	Species	MIC values (µg/mL)			Combination disc tests		
		CTX	CAZ	FEP	CTX-CV	CAZ-CV	FEP-CV
507393-2	*E. cloacae*	8	≥ 64	≤ 1	+	+	+
507394-1	*E. cloacae*	8	≥ 64	≤ 1	+	+	+
506261-1	*E. cloacae*	4	16	≤ 1	+	+	+
506430-1	*E. cloacae*	4	16	≤ 1	+	+	+
104650-3	*E. cloacae*	4	16	≤ 1	+	+	+
101870-1	*E. coli*	≤ 1	≤ 1	≤ 1	-	-	-
507214-1	*E. coli*	≤ 1	≤ 1	≤ 1	-	-	-
502192-1	*E. coli*	4	≤ 1	≤ 1	+	-	+
102461-4	*E. coli*	≥ 64	4	8	+	-	-
307338-1	*E. coli*	≥ 64	4	16	+	-	+
106906-3	*K. pneumoniae*	≤ 1	≤ 1	≤ 1	-	-	-
509599-1	*K. pneumoniae*	≥ 64	16	2	+	+	+
506433-1	*K. pneumoniae*	≥ 64	8	2	+	+	+
501940-1	*K. pneumoniae*	≥ 64	4	2	+	+	+
502317-1	*K. pneumoniae*	≥ 64	16	2	+	+	+
102461-2	*K. pneumoniae*	≥ 64	≥ 64	16	+	+	+
303200-1	*K. pneumoniae*	≥ 64	≥ 64	16	+	+	+

No., Number; MIC, Minimum inhibitory concentration; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; CTX-CV, Cefotaxime and cefotaxime-clavulanate; CAZ-CV, Ceftazidime and ceftazidime-clavulanate; FEP-CV, Cefepime and cefepime-clavulanate; +, Positive test result; -, Negative test result.

4.4.2 Genotypic detection

Using the PCR- and sequencing-based method, 14 of 39 (36%) Gram-negative bacilli isolates were positive for ESBL genes. All the *E. cloacae* isolates (n= 5) were positive for ESBL genes. In addition, six *K. pneumoniae* and three *E. coli* isolates carried ESBL genes (Table 15). The PCR test detected ESBL genes in all cefotaxime and/or ceftazidime resistant Enterobacteriaceae, but not in resistant non-fermenters (Table 15). As with phenotypic test, carbapenemase genes were not detected among 39 isolates tested.

Table 15. Detection of ESBLs among Gram-negative bacilli by phenotypic and PCR methods

Name of species (total)	No. of isolates resistant to cefotaxime and/or ceftazidime (%)	No. of isolates positive for ESBL by Combination Disc Test (%)	No. of isolates positive for ESBL by PCR (%)
E. cloacae (5)	5 (100)	5 (100)	5 (100)
E. coli (5)	3 (60.0)	3 (60.0)	3 (60.0)
K. pneumoniae (7)	6 (85.7)	6 (85.7)	6 (85.7)
A. baumannii (10)	2 (20.0)	0	0
P. putida (2)	2 (100)	0	0
P. luteola (1)	0	0	0
A. ursingii (2)	0	0	0
S. maltophilia (4)	0	0	0
C. pauculus (1)	0	0	0
Total (39)	**18 (46.2)**	**14 (35.9)**	**14 (35.9)**

4.5 Diversity of targeted beta-lactamase genes among Enterobacteriaceae

Amplification and sequencing of the full length PCR products identified six beta-lactamase genes; $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-12}}$, $bla_{\text{SHV-5}}$, $bla_{\text{SHV-11}}$ and $bla_{\text{TEM-1}}$. Among 17 Enterobacteriaceae, 14 (82.4%) carried ESBL genes, comprising of six $bla_{\text{CTX-M-15}}$, three $bla_{\text{CTX-M-14}}$, three $bla_{\text{SHV-12}}$ and two $bla_{\text{SHV-5}}$ genes (Table 16). The following narrow-spectrum beta-lactamase (NSBL) genes were also detected: ten $bla_{\text{TEM-1}}$ and six $bla_{\text{SHV-11}}$ genes. All ESBL-producing isolates possessed only one of the targeted ESBL genes. The $bla_{\text{CTX-M-15}}$ gene was detected in all six resistant *K. pneumoniae* isolates only. This gene was detected in combination with the following NSBLs: $bla_{\text{CTX-M-15}}$ + $bla_{\text{SHV-11}}$ + $bla_{\text{TEM-1}}$ (n= 3), $bla_{\text{CTX-M-15}}$ + $bla_{\text{SHV-11}}$ (n= 2) and $bla_{\text{CTX-M-15}}$ + $bla_{\text{TEM-1}}$ (n= 1). One ESBL-negative *K. pneumoniae* isolate carried $bla_{\text{SHV-11}}$ gene. All three ESBL-positive *E. coli* isolates carried both $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-1}}$ genes. The bla_{SHV}-type ESBL genes were not detected in *K. pneumoniae* and *E. coli* isolates. Of five ESBL-positive *E. cloacae* isolates, three harboured both $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-1}}$ genes whereas two carried $bla_{\text{SHV-5}}$ gene (Table 16).

Table 16. Distribution of ESBL and NSBL enzymes among Enterobacteriaceae

Isolate no.	Species	Beta-lactamase contents		Participant	Location	Time-point
		ESBL	NSBL(s)			
507393-2	*E. cloacae*	SHV-5	None	Mother	Mbekweni	Delivery
507394-1	*E. cloacae*	SHV-5	None	Infant	Mbekweni	Birth
506261-1	*E. cloacae*	SHV-12	TEM-1	Infant	Mbekweni	Birth
506430-1	*E. cloacae*	SHV-12	TEM-1	Infant	Mbekweni	Birth
104650-3	*E. cloacae*	SHV-12	TEM-1	Infant	Mbekweni	5 – 12 weeks
101870-1	*E. coli*	None	None	Infant	Mbekweni	5 – 12 weeks
507214-1	*E. coli*	None	None	Mother	Mbekweni	Delivery
502192-1	*E. coli*	CTX-M-14	TEM-1	Mother	TC Newman	Delivery
102461-4	*E. coli*	CTX-M-14	TEM-1	Infant	Mbekweni	20 – 28 weeks
307338-1	*E. coli*	CTX-M-14	TEM-1	Infant	Mbekweni	20 – 28 weeks
106906-3	*K. pneumoniae*	None	SHV-11	Infant	Mbekweni	20 – 28 weeks
509599-1	*K. pneumoniae*	CTX-M-15	SHV-11, TEM-1	Infant	Mbekweni	Birth
506433-1	*K. pneumoniae*	CTX-M-15	TEM-1	Mother	TC Newman	Delivery
501940-1	*K. pneumoniae*	CTX-M-15	SHV-11, TEM-1	Infant	TC Newman	5 – 12 weeks
502317-1	*K. pneumoniae*	CTX-M-15	SHV-11, TEM-1	Mother	Mbekweni	Delivery
102461-2	*K. pneumoniae*	CTX-M-15	SHV-11	Infant	Mbekweni	20 – 28 weeks
303200-1	*K. pneumoniae*	CTX-M-15	SHV-11	Infant	TC Newman	5 – 12 weeks

4.6 Faecal carriage of ESBL-producing bacteria

The prevalence of maternal faecal carriage of ESBL-producing bacteria at delivery was 4.4% (4/90); (95% confidence interval (CI): 1.5% - 10.2%), and that of infants at birth

was 3.5% (4/116); (95% CI: 1.2% - 8.0%). The infant faecal carriage at 5-12 and 20-28 weeks of age was 4.4% (3/68); (95% CI: 1.3% - 11.3%) and 5.7% (2/35); (95% CI: 1.2% - 17.1%), respectively. The mothers were colonized with CTX-M-15-producing *K. pneumoniae* (n= 2), SHV-5-producing *E. cloacae* (n= 1) and CTX-M-14-producing *E. coli* (n= 1). Three out of four infants at birth were colonized with *E. cloacae* isolates which possessed either $bla_{\text{SHV-12}}$ (n= 2) or $bla_{\text{SHV-5}}$ (n= 1) gene. One of the four infants was colonized with a $bla_{\text{CTX-M-15}}$ carrying *K. pneumoniae* isolate (Table 16). At birth, only one mother-infant pair was ESBL-positive with SHV-5-producing *E. cloacae* isolates. Two of the three infants at 5-12 weeks were colonized with CTX-M-15-positive *K. pneumoniae* isolates. Longitudinal carriage of ESBL-producers was observed only in one infant who had SHV-12-producing *E. cloacae*, at birth and 5-12 weeks. Furthermore, only two children carried ESBL-producers at 20-28 weeks. One of the two children was colonized with CTX-M-14-producing *E. coli* isolate. The other child had both *E. coli* ($bla_{\text{CTX-M-14}}$) and *K. pneumoniae* ($bla_{\text{CTX-M-15}}$) and was the only participant being colonized by more than one ESBL-producer.

4.7 Antibiotic susceptibility pattern of ESBL-producing Enterobacteriaceae

We observed very high rates of resistance to different antibacterial agents. All ESBL-producing Enterobacteriaceae (n= 14) were multi-resistant to five or more classes of antibiotics.

In this study, of the 14 ESBL-producing Enterobacteriaceae isolates examined, 64.3%, 64.3%, 78.6%, 85.7% and 92.9% of the isolates were susceptible to cefoxitin, cefepime, tigecycline, piperacillin-tazobactam and ciprofloxacin, respectively (Table 17). Low susceptibility rates were also observed for amoxicillin-clavulanic acid (21.4%), nitrofurantoin (21.4%), ceftazidime (28.6%) and gentamicin (42.9%). All 14 ESBL-PE isolates were susceptible to ertapenem, meropenem, imipenem, amikacin and colistin. Furthermore, all the ESBL-producers were resistant to ampicillin, cefotaxime and trimethoprim-sulfamethoxazole (Figure 13).

The 50% minimum inhibitory concentration (MIC_{50}) for cefotaxime (64 µg/mL) among all ESBL-PE isolates was higher than that for ceftazidime (16 µg/mL) (Table 17). Four of the six CTX-M-15-producing *K. pneumoniae* isolates showed higher MICs for cefotaxime than for ceftazidime. The remaining two isolates had the MIC of ≥64 µg/mL for both

cefotaxime and ceftazidime (Figure 14). One CTX-M-15-producing *K. pneumoniae* isolate was susceptible to ceftazidime (MIC 4 µg/mL) (Figure 14). Of note, three cefepime-resistant isolates were co-resistant to tigecycline (Table 11). Of the three CTX-M-14-positive *E. coli* isolates, one had a low MIC (4 µg/mL) for cefotaxime and the other two had the MICs of ≥ 64 µg/mL. However, all three were susceptible to ceftazidime (Figure 15). Furthermore, all five ESBL-positive *E. cloacae* isolates were resistant to both cefotaxime and ceftazidime and showed higher MICs for ceftazidime than for cefotaxime (Figure 16). Two SHV-5-producing *E. cloacae* isolates had higher MICs for both ceftazidime (≥ 64 µg/mL) and cefotaxime (8 µg/mL), whereas three SHV-12-producing *E. cloacae* isolates had low MICs values of 16 µg/mL and 4 µg/mL, respectively (Table 9).

Table 17. Antimicrobial susceptibility of six CTX-M-15-producing *Klebsiella pneumoniae* isolates

Antimicrobial agent	Breakpoint value (µg/mL)	Number (%) of resistant isolates	Number (%) of intermediate isolates	Number (%) of susceptible isolates	MICs (µg/mL)		
					MIC_{50}	MIC_{90}	MICs range
Ampicillin	≤ 8	14 (100)	0.0	0.0	32	32	32 – 32
Amoxicillin/Clavulanic acid	≤ 8	6 (42.9)	5 (35.7)	3 (21.4)	16	32	4 – 32
Piperacillin/Tazobactam	≤ 16	0.0	2 (14.3)	12 (85.7)	4	32	4 – 32
Cefuroxime	≤ 8	13 (92.9)	1 (7.1)	0.0	64	64	16 – 64
Cefuroxime axetil	≤ 4	13 (92.9)	1 (7.1)	0.0	64	64	16 – 64
Cefoxitin	≤ 8	5 (35.7)	0.0	9 (64.3)	4	64	4 – 64
Cefotaxime	≤ 1	14 (100)	0.0	0.0	64	64	4 – 64
Ceftazidime	≤ 4	9 (64.3)	1 (7.1)	4 (28.6)	16	64	1 – 64
Cefepime	≤ 2	3 (21.4)	2 (14.3)	9 (64.3)	2	16	1 – 16
Ertapenem	≤ 0.5	0.0	0.0	14 (100)	0.5	0.5	0.5 – 0.5
Imipenem	≤ 1	0.0	0.0	14 (100)	0.25	0.25	0.25 – 0.5
Meropenem	≤ 1	0.0	0.0	14 (100)	0.25	0.25	0.25 – 0.25
Amikacin	≤ 16	0.0	0.0	14 (100)	2	16	2 – 16
Gentamicin	≤ 4	8 (57.1)	0.0	6 (42.9)	16	16	1 – 16
Ciprofloxacin	≤ 1	0.0	1 (7.1)	13 (92.9)	0.25	1	0.25 – 2
Tigecycline	≤ 1	3 (21.4)	0.0	11 (78.6)	1	2	0.25 - 2
Nitrofurantoin	≤ 32	5 (35.7)	6 (42.9)	3 (21.4)	64	128	16 – 128
Colistin	≤ 2	0.0	0.0	14 (100)	0.5	0.5	0.5 – 2
Trimethoprim/Sulfamethoxazole	≤ 2	14 (100)	0.0	0.0	384	384	20 – 320

MIC, Minimum inhibitory concentration; MIC_{50}, Minimum inhibitory concentration required to inhibit the growth of 50% of organisms. MIC_{90}, Minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

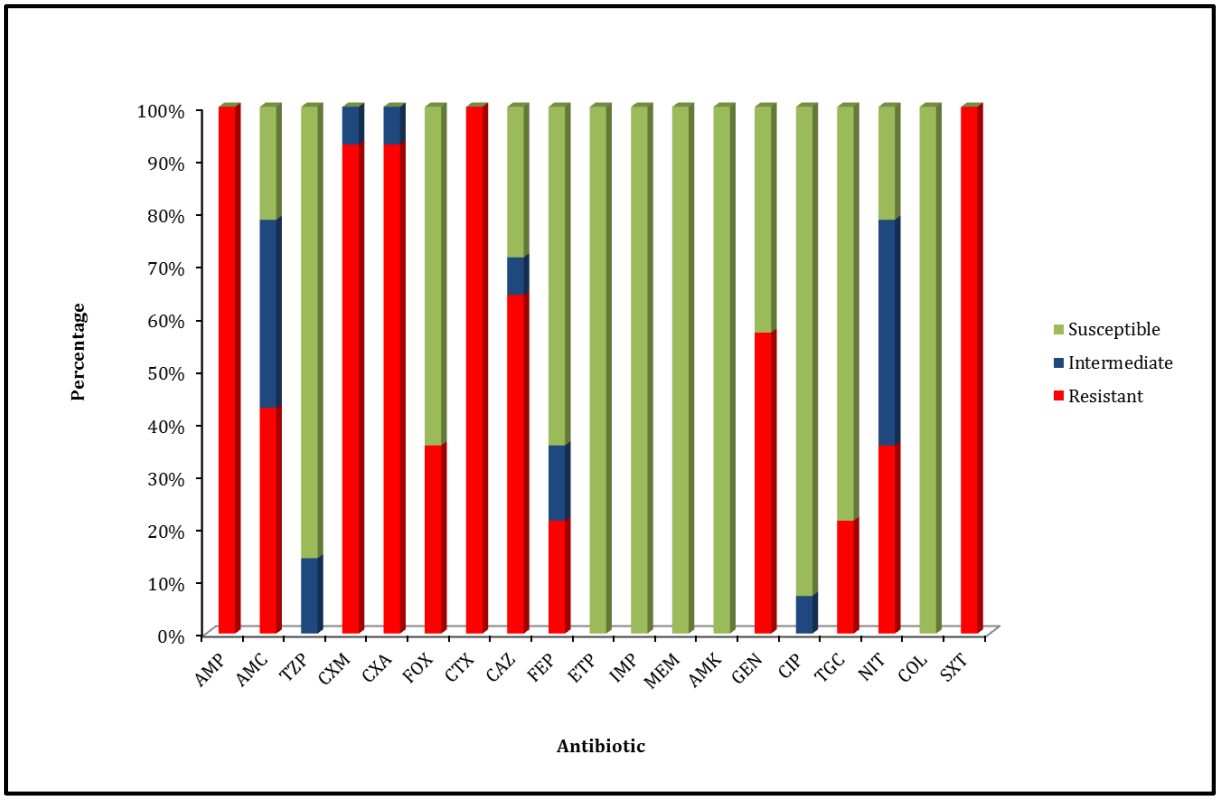

Figure 13. Susceptibility categorization of 14 ESBL-producing Enterobacteriaceae isolates to 19 antibiotics by MICs determination.
AMP, Ampicillin; AMC, Amoxicillin + clavulanic acid; TZP, Piperacillin-tazobactam; CXM, Cefuroxime; CXA, Cefuroxime Axetil; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; ETP, Ertapenem; IPM, Imipenem; MEM, Meropenem; AMK, Amikacin; GEN, Gentamicin; CIP, Ciprofloxacin; TGC, Tigecycline; NIT, Nitrofurantoin; COL, Colistin; SXT, Trimethoprim-sulfamethoxazole. Findings were categorized using the CLSI breakpoints and for tigecycline only, the EUCAST clinical breakpoints were used.

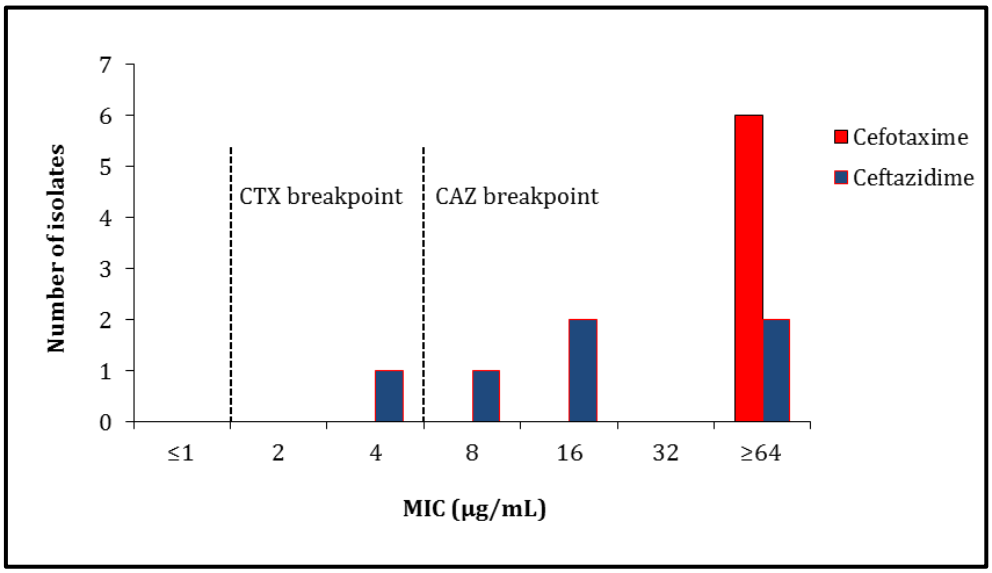

Figure 14. Minimum inhibitory concentration (MIC) for cefotaxime (CTX) and ceftazidime (CAZ) in six CTX-M-15-producing *K. pneumoniae* isolates.

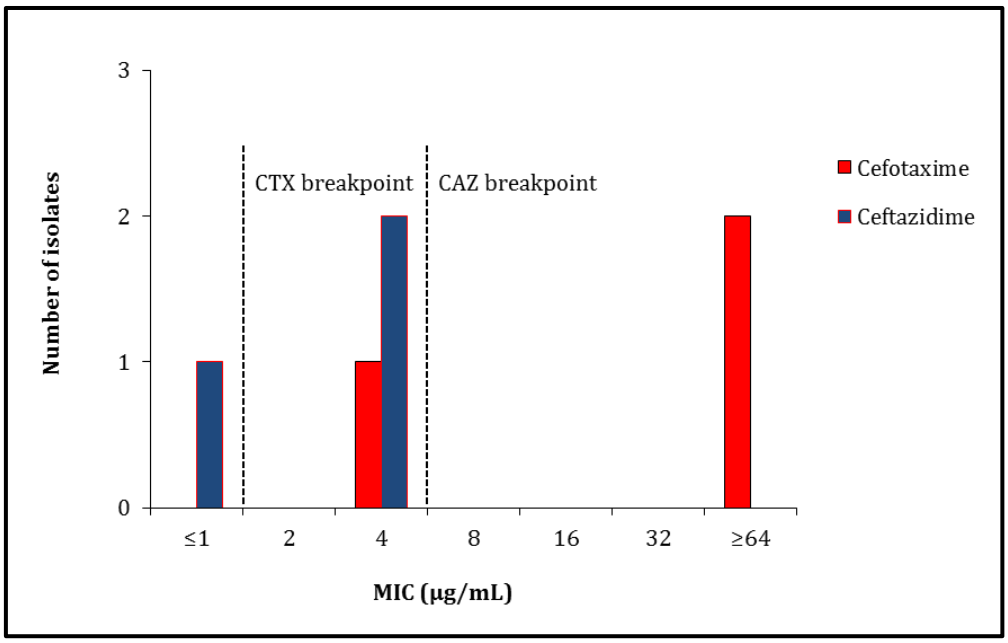

Figure 15. Minimum inhibitory concentration (MIC) for cefotaxime (CTX) and ceftazidime (CAZ) in three CTX-M-14-*Escherichia coli* isolates.

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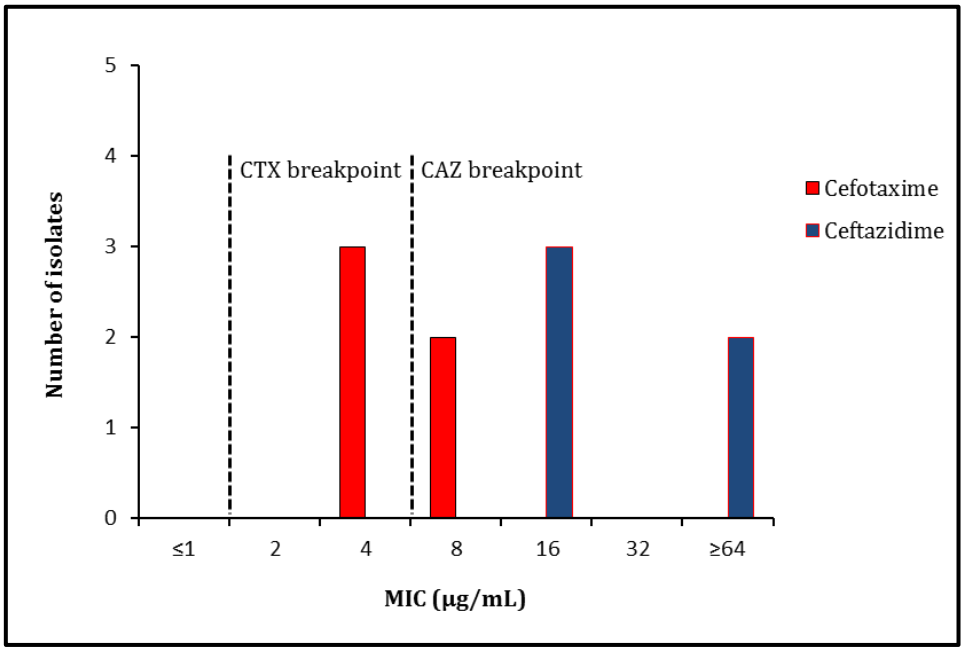


Figure 16. Minimum inhibitory concentration (MIC) for cefotaxime (CTX) and ceftazidime (CAZ) in SHV-producing *E. cloacae* isolates.

4.8 Comparative phenotypic testing for ESBLs detection

Based on the combination disc test, cefotaxime with cefotaxime-clavulanate detected the production of ESBLs in six *K. pneumoniae*, three *E. cloacae*, three *E. coli* and two *E. cloacae* isolates harbouring $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-14,}}$ and $bla_{\text{SHV-5}}$ genes, respectively (Table 18). Ceftazidime with ceftazidime-clavulanate could not detect ESBLs in all three $bla_{\text{CTX-M-14}}$-carrying *E. coli* isolates. In addition, the combination of cefepime and cefepime-clavulanate did not detect ESBLs production in one of the three $bla_{\text{CTX-M-14}}$-positive *E. coli* isolates (Table 18).

Table 18: Comparison of ESBLs and the performance of phenotypic tests

Isolate no.	Species	Beta-lactamase content	Combination disc tests			MIC values (µg/mL)		
			CTX-CV	CAZ-CV	FEP-CV	Cefotaxime	Ceftazidime	Cefepime
507393-2	*E. cloacae*	SHV-5	+	+	+	8	≥ 64	≤ 1
507394-1	*E. cloacae*	SHV-5	+	+	+	8	≥ 64	≤ 1
506261-1	*E. cloacae*	SHV-12, TEM-1	+	+	+	4	16	≤ 1
506430-1	*E. cloacae*	SHV-12, TEM-1	+	+	+	4	16	≤ 1
104650-3	*E. cloacae*	SHV-12, TEM-1	+	+	+	4	16	≤ 1
101870-1	*E. coli*	None	-	-	-	≤ 1	≤ 1	≤ 1
507214-1	*E. coli*	None	-	-	-	≤ 1	≤ 1	≤ 1
502192-1	*E. coli*	CTX-M-14, TEM-1	+	-	+	4	≤ 1	≤ 1
102461-4	*E. coli*	CTX-M-14, TEM-1	+	-	-	≥ 64	4	8
307338-1	*E. coli*	CTX-M-14, TEM-1	+	-	+	≥ 64	4	16
106906-3	*K. pneumoniae*	SHV-11	-	-	-	≤ 1	≤ 1	≤ 1
509599-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	+	+	+	≥ 64	16	2
506433-1	*K. pneumoniae*	CTX-M-15, TEM-1	+	+	+	≥ 64	8	2
501940-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	+	+	+	≥ 64	4	2
502317-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	+	+	+	≥ 64	16	2
102461-2	*K. pneumoniae*	CTX-M-15, SHV-11	+	+	+	≥ 64	≥ 64	16
303200-1	*K. pneumoniae*	CTX-M-15, SHV-11	+	+	+	≥ 64	≥ 64	16

No., Number; MIC, Minimum inhibitory concentration; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; CTX-CV, Cefotaxime and cefotaxime-clavulanate; CAZ-CV, Ceftazidime and ceftazidime-clavulanate; FEP-CV, Cefepime and cefepime-clavulanate; +, Positive test result; -, Negative test result.

4.9 Risk factors associated with the faecal carriage of ESBL-producing bacteria in infants at birth

A univariate analysis comparing ESBL-positive and ESBL-negative infants showed that being born to HIV-positive mother, being born via elective caesarean section and administration of medication before discharge were positively associated with ESBL faecal carriage at birth (Table 19). In contrast, breastfeeding prior to discharge was negatively associated with ESBL faecal carriage. In addition, maternal hospitalization in the previous two years, admission to nursery, female gender and birth via emergency caesarean section were associated with ESBL carriage among infants, but these associations were not statistically significant. The associations were not calculated for the location, maternal ethnicity, vaginal vacuum delivery and supplement administration prior to discharge due to zero positivity (Table 19). Robust multivariate analysis could not be performed due to small numbers of ESBL-PE detected.

Table 19. Risk factors for ESBL carriage in 90 infants at birth: univariate analysis

Variable	No.	ESBL-positive (%)	Risk ratio [95% CI]	*P* value
Gender				
Male	49	2 (4.1)	1.0	
Female	41	2 (4.9)	1.2 [0.18 – 8.12]	0.432
Location				
Mbekweni	41	4 (9.8)	-	
TC Newman	49	0 (0)	-	
Maternal HIV status				
Negative	71	1 (1.4)	1.0	
Positive	19	3 (15.8)	11.3 [1.23 - 101.8]	**0.015**
Maternal hospitalization in the previous two years				
No	84	3 (3.6)	1.0	
Yes	6	1 (16.7)	4.7 [0.57 - 38.4]	0.133
Maternal ethnicity				
Black African	40	4 (10)	-	
Mixed race	50	0 (0)	-	
Mode of delivery				
Normal vaginal	74	2 (2.7)	1.0	
Elective caesarean section	2	1 (50)	18.5 [2.6 - 129.6]	**0.039**
Emergency caesarean section	13	1 (7.7)	2.8 [0.3 - 29.2]	0.223
Vaginal vacuum	1	0 (0)	-	
Place of admission immediately after birth				
Roomed with the mother	83	3 (3.6)	1.0	
Nursery	7	1 (14.3)	3.95 [0.47 - 33.2]	0.155
Medication before discharge				
No	66	1 (1.5)	1.0	
Yes	24	3 (12.5)	8.25 [0.90 - 75.5]	**0.030**
Supplement before discharge				
No	83	4 (4.8)	-	
Yes	7	0 (0)	-	
Breastfeeding prior discharge				
No	9	3 (33.3)	1.0	
Yes	81	1 (1.2)	0.08 [0.01 – 0.31]	**0.001**

No., Number; CI, Confidence interval; HIV, Human immunodeficiency virus; -, Risk ratio not calculated because of zero positivity.

4.10 Molecular typing by DNA macro-restriction analysis of selected beta-lactamase-producing bacteria using PFGE

The genomic DNA from seven *K. pneumoniae* and five *E. cloacae* isolates was digested with XbaI (Figure 17) and SpeI (Figure 18) and analyzed by PFGE.

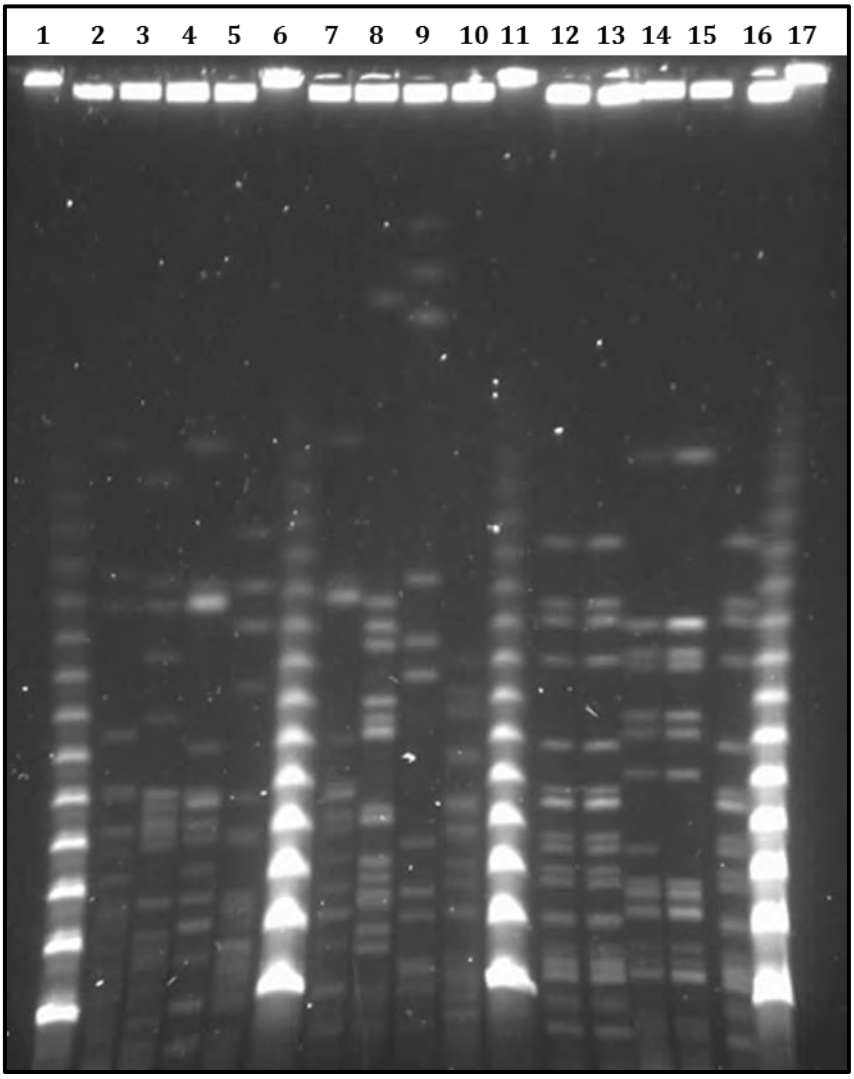


Figure 17. Pulsed-field gel electrophoresis of XbaI-digested genomic DNA from seven *K. pneumoniae* and five *E. cloacae* isolates.
Lanes 1, **6**, **11** and **17**: PFGE lambda marker, **lane 10**: *K. pneumoniae* ATCC BAA 1705 control. Seven *K. pneumoniae* isolates in the following lanes; **lane 2**: 106906-3, **lane 3**: 502317-1, **lane 4**: 102461-2, **lane 5**: 509599-1, **lane 7**: 303200-1, **lane 8**: 506433-1, **lane 9**: 501940-1. Five *E. cloacae* isolates in the following lanes; **lane 12**: 104650-3, **lane 13**: 506261-1, **lane 14**: 507393-2, **lane 15**: 507394-1, **lane 16**: 506430-1.

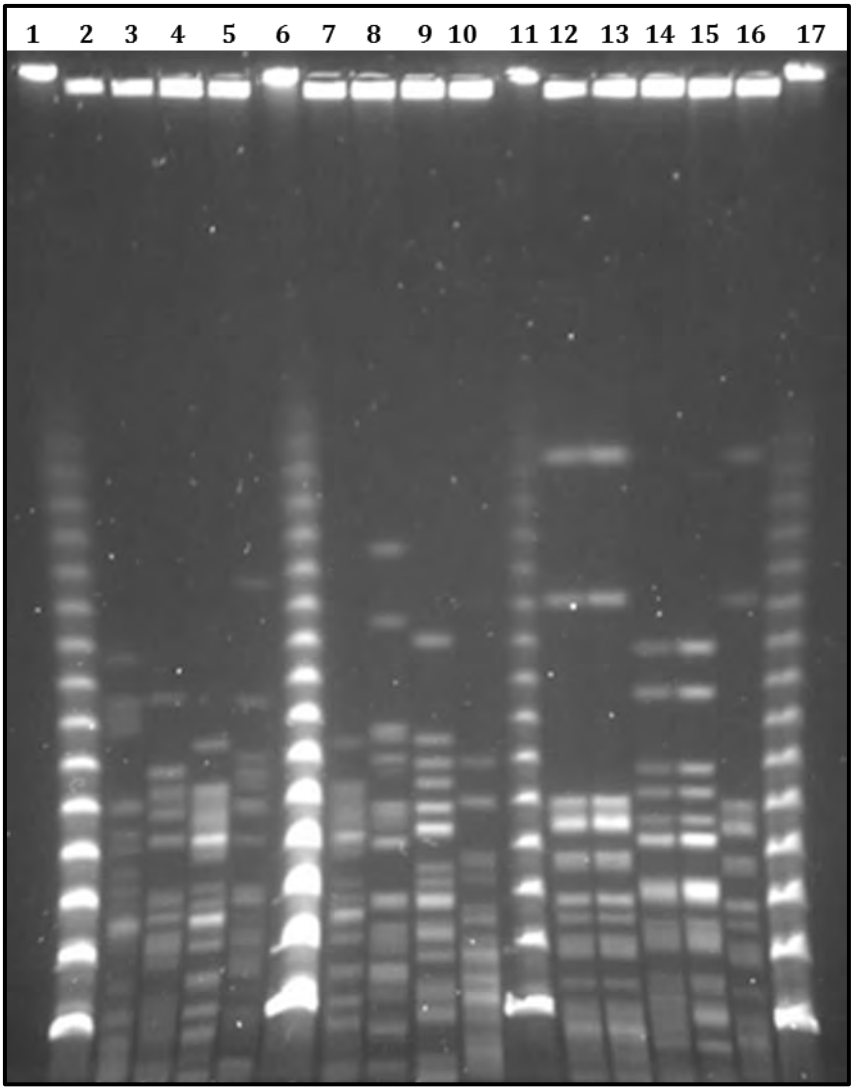


Figure 18. Pulsed-field gel electrophoresis of SpeI-digested genomic DNA from seven *K. pneumoniae* and five *E. cloacae* isolates.
Lanes 1, **6**, **11** and **17**: PFGE lambda marker, **lane 10**: *K. pneumoniae* ATCC BAA 1705 control. Seven *K. pneumoniae* isolates in the following lanes; **lane 2**: 106906-3, **lane 3**: 502317-1, **lane 4**: 102461-2, **lane 5**: 509599-1, **lane 7**: 303200-1, **lane 8**: 506433-1, **lane 9**: 501940-1. Five *E. cloacae* isolates in the following lanes; **lane 12**: 104650-3, **lane 13**: 506261-1, **lane 14**: 507393-2, **lane 15**: 507394-1, **lane 16**: 506430-1.

4.10.1 Genotypic relatedness of ESBL-producing *K. pneumoniae* isolates

The PFGE fingerprint profiling of seven *K. pneumoniae* isolates revealed six different PFGE patterns, A1, A2, B1, C1, D1 and E1 (Figures 19 and 20).

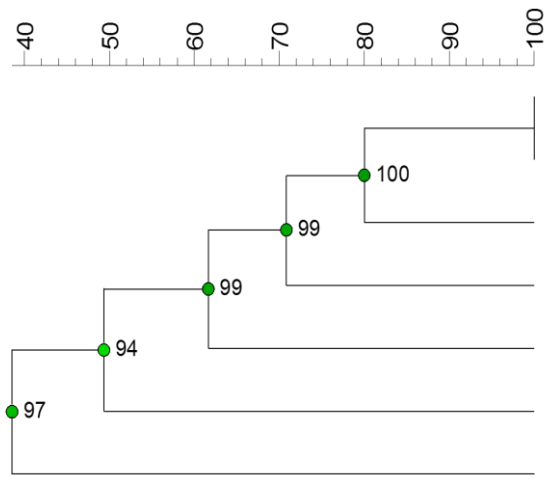
PFGE-XbaI analysis identified only one PFGE cluster composed of three *K. pneumoniae* isolates (102461-2, 303200-1 and 106906-3) sharing ≥ 80% PFGE banding pattern similarity (Figure 19). Isolates 102461-2 and 303200-1 had 100% PFGE banding

pattern similarity. Isolate 102461-2 was identified in stool of 24 week old child from Mbekweni area and isolate 303200-1 in stool of 26 week old child from TC Newman area. These two isolates (102461-2 and 303200-1) produced both CTX-M-15 and SHV-11, and had the same MICs for all tested (n= 19) antibiotics (Table 9).

4.10.2 Genotypic relatedness of ESBL-producing *E. cloacae* isolates

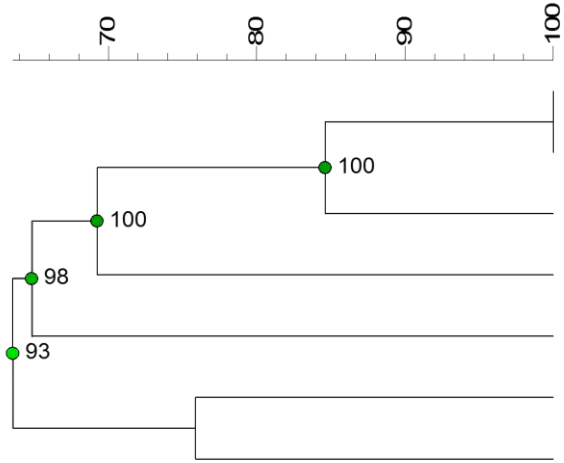
Both PFGE-XbaI and PFGE-SpeI analysis revealed two clusters (Figures 21 and 22). Cluster one contained two isolates (507393-2 and 507394-1), and cluster two contained three isolates (104650-3, 506261-1 and 506430-1). PFGE-XbaI analysis revealed three PFGE patterns (A1, A2 and B1), whereas PFGE-SpeI revealed only two PFGE patterns (A1 and B1).

Two SHV-5-producing *E. cloacae* isolates (507393-2 and 509394-1) showed 100% PFGE pattern similarity by PFGE-SpeI analysis. These isolates (507393-2 and 509394-1) were recovered at birth from a mother-infant pair from Mbekweni area. In addition, these isolates (507393-2 and 509394-1) had the same resistance profile. All three *E. cloacae* isolates positive for both SHV-12 and TEM-1 enzymes (506261-1, 104650-3 and 506430-1) were 100% similar by both PFGE-XbaI and PFGE-SpeI analysis. Isolates 506261-1 (at birth) and 104650-3 (eight week old) were isolated from the same infant from Mbekweni area. Isolate 506430-1 was isolated from a different infant at birth, also from Mbekweni area.



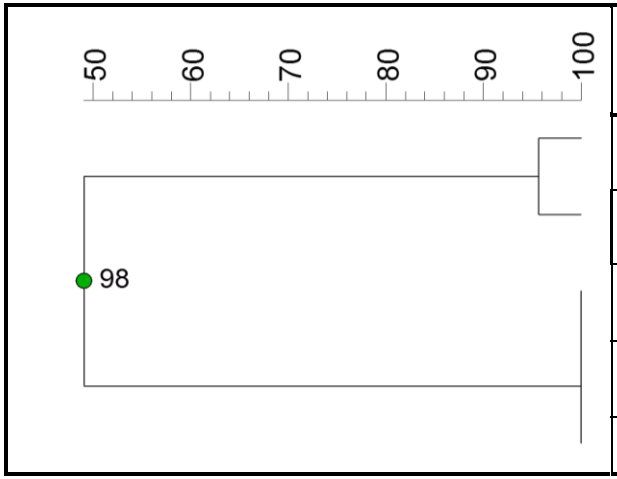
Isolate no.	Species	Beta-lactamases	PFGE	Location	Participant	Time-point
102461-2	*K. pneumoniae*	CTX-M-15, SHV-11	A1	Mbekweni	Infant	20-28 weeks
303200-1	*K. pneumoniae*	CTX-M-15, SHV-11	A1	TC Newman	Infant	5-12 weeks
106906-3	*K. pneumoniae*	SHV-11	A2	Mbekweni	Infant	20-28 weeks
502317-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	B1	Mbekweni	Mother	Delivery
506433-1	*K. pneumoniae*	CTX-M-15, TEM-1	C1	TC Newman	Mother	Delivery
509599-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	D1	Mbekweni	Infant	Birth
501940-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	E1	TC Newman	Infant	5-12 weeks

Figure 19: Pulsed-field gel electrophoresis (PFGE)-XbaI analysis of seven *K. pneumoniae* isolates: one SHV-11-producing *K. pneumoniae* and six CTX-M-15-producing *K. pneumoniae* isolates.
The dendrogram was constructed with dice coefficient, represented by UPGMA, 1.0% optimization and 1.0% position tolerance.



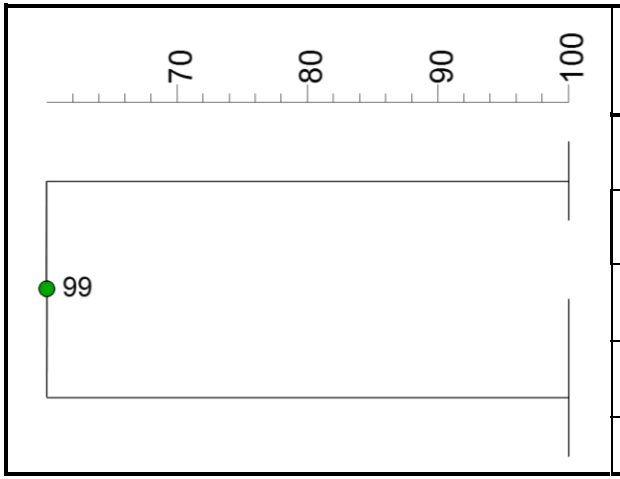
Isolate no.	Species	Beta-lactamases	PFGE	Location	Participant	Time-point
102461-2	*K. pneumoniae*	CTX-M-15, SHV-11	A1	Mbekweni	Infant	20-28 weeks
303200-1	*K. pneumoniae*	CTX-M-15, SHV-11	A1	TC Newman	Infant	5-12 weeks
501940-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	A2	TC Newman	Infant	5-12 weeks
106906-3	*K. pneumoniae*	SHV-11	B1	Mbekweni	Infant	20-28 weeks
506433-1	*K. pneumoniae*	CTX-M-15, TEM-1	C1	TC Newman	Mother	Delivery
502317-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	D1	Mbekweni	Mother	Delivery
509599-1	*K. pneumoniae*	CTX-M-15, SHV-11, TEM-1	E1	Mbekweni	Infant	Birth

Figure 20: Pulsed-field gel electrophoresis (PFGE)-SpeI analysis of seven *K. pneumoniae* isolates: one SHV-11-producing *K. pneumoniae* and six CTX-M-15-producing *K. pneumoniae* isolates.
The dendrogram was constructed with dice coefficient, represented by UPGMA, 1.0% optimization and 1.0% position tolerance.



Isolate no.	Species	Beta-lactamases	PFGE	Location	Participant	Time-point
507393-2	*E. cloacae*	SHV-5	A1	Mbekweni	Mother	Delivery
509394-1	*E. cloacae*	SHV-5	A2	Mbekweni	Infant	Birth
104650-3	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	5-12 weeks
506261-1	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	Birth
506430-1	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	Birth

Figure 21: Pulsed-field gel electrophoresis (PFGE)-XbaI analysis of five *E. cloacae* isolates: two SHV-5-producing *E. cloacae* and three SHV-12-producing *E. cloacae* isolates.
The dendrogram was constructed with dice coefficient, represented by UPGMA, 1.0% optimization and 1.0% position tolerance.



Isolate no.	Species	Beta-lactamases	PFGE	Location	Participant	Time-point
507393-2	*E. cloacae*	SHV-5	A1	Mbekweni	Mother	Delivery
509394-1	*E. cloacae*	SHV-5	A1	Mbekweni	Infant	Birth
104650-3	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	5-12 weeks
506261-1	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	Birth
506430-1	*E. cloacae*	SHV-12, TEM-1	B1	Mbekweni	Infant	Birth

Figure 22: Pulsed-field gel electrophoresis (PFGE)-SpeI analysis of five *E. cloacae* isolates: two SHV-5-producing *E. cloacae* and three SHV-12-producing *E. cloacae* isolates. The dendrogram was constructed with dice coefficient, represented by UPGMA, 1.0% optimization and 1.0% position tolerance.

5. Discussion

The prevalence of extended-spectrum beta-lactamase- and carbapenemase-producing Enterobacteriaceae in healthy individuals in the community is largely unknown.[13,227] However, such organisms are now distributed worldwide and their prevalence is increasing in community settings.[13,41,45,60,126,319,378] This study, nested within a birth-cohort study conducted in Drakenstein sub-district (Western Cape Province, South Africa) aimed to determine the prevalence and genetic characteristics and relatedness of ESBL- and carbapenemase-producing Enterobacteriaceae in stools from healthy infants of less than one years of age and their mothers, and to determine the risk factors associated with their carriage. The present study found that 4.4% of the mothers carried ESBL-PE in their stools at delivery. This rate is similar to the 3.7%,[36] and 4.3%,[379] reported among Spanish and Moroccan healthy adults, respectively. In contrast, the maternal ESBL-PE faecal carriage rate observed in our study was higher than the 1.5% reported in Brazil,[380] and 2.4% in Lebanon,[381] but it was lower than 7% reported in China,[195] 8% in Israel,[57] and 10% in Madagascar,[20] among healthy adults. The ESBL faecal carriage in pregnant women in this study is of specific concern, as they may transfer the ESBL-producing bacteria to the infants. In this study, the infant ESBL-PE faecal carriage at birth was 3.5%. This carriage rate is probably a result of early vertical transmission from the mother-to-infant at delivery.[358,382] All colonized infants at birth were born to the mothers from Mbekweni area and none from TC Newman area. This observation could suggest that the hospital was unlikely to be the source of ESBL-producing bacteria in this study. However, their acquisition from the hospital setting cannot be disregarded.[383] The ESBL faecal carriage in our study is lower than the 9% reported in India among healthy low birth weight (LBW) neonates at day one.[359]

The present study is the first to detect ESBL-producing Enterobacteriaceae in human meconium samples in Africa. The ESBL-producing Enterobacteriaceae was not detected in meconium in a study conducted in France.[358] The infant ESBL-PE faecal carriage of 4.4% at 5-12 weeks in our study was lower than 31% reported in India among LBW infants at day 60 (8.6 weeks).[359] The infant ESBL faecal carriage at 20-28 weeks was 5.7%. This rate is comparable to 4.6%,[47] and 6.4%,[384] reported among healthy French children of ages between 13 and 174 weeks. In contrast, it is higher than 1.7% reported in Peru and Bolivia,[385] and 2.9%[386] in Sweden among healthy children (ages ranging

from 26 to 287 weeks). The increase in ESBL faecal carriage from 3.5% at birth to 5.7% at 20-28 weeks might be due to an increase time of exposure to maternal environment, oral and skin flora.[358,359,382] However, the increase in ESBL faecal carriage might simply reflect sampling error due to small numbers.

No carbapenemase-producing organisms were detected in mothers and infants in this study. Our findings are in agreement to what has been recently reported in Switzerland, where no carbapenemase-producing organisms were found among healthy adults in the community.[17] Nevertheless, a study conducted in an Indian community found that one in 75 infants was colonized by KPC-2-producing *Enterobacter aerogenes* isolate.[359]

The ChromID ESBL and CARBA media were not perfectly selective for ESBL- and carbapenemase-producing isolates, respectively. Non-ESBL- and non-carbapenemase-producers that grew on these media were mainly non-fermenting Gram-negative bacilli. These observations are probably due to isolates harbouring mechanisms of resistance other than ESBL and carbapenemase production.[387] Therefore, complementary tests are required to confirm the production of ESBLs and carbapenemases in isolates growing on these partially selective media.[388] Furthermore, prolonged incubation to 48 hours did not have an effect on the recovery of ESBL-producing bacteria. This was also observed by Glupczynski and colleagues in a study conducted in Belgium.[389] In contrast, a study conducted in France reported an increased recovery of ESBL-producers by 6% from 765 clinical samples after 48 hours of incubation.[363]

The MIC values observed for cefotaxime and ceftazidime among our isolates are typical of ESBL-producers.[330] We observed that a combination of cefotaxime and cefotaxime-clavulanate was more effective in detecting ESBL-producing bacteria. The ability of the combination disc test to detect the production of ESBLs is excellent, and the sensitivity when using both cefotaxime and cefepime alone and in combination with clavulanic acid can reach up to 100%.[316] Although ceftazidime with clavulanic acid detects most ESBL-producers, it could not detect all ESBL-producing bacteria, especially CTX-M-producing isolates,[159] as observed in this study. This might be due to the fact that most of the CTX-M-type enzymes are more active against cefotaxime than ceftazidime.[146]

Furthermore, most of ESBL-producing Enterobacteriaceae in this study were co-resistant to trimethoprim-sulfamethoxazole and gentamicin. Co-resistance to

trimethoprim-sulfamethoxazole and aminoglycosides has been shown to be common in ESBL-producers;[2,390] and this led to increased usage of nitrofurantoin for treatment of urinary tract infections.[391,392] High proportion of isolates resistant to nitrofurantoin (78.6%) in our study is of concern. This rate is higher than reported in other studies, including in Iran (6%),[393] and Cameroon (34.5%).[227] Nitrofurantoin is an alternative, rather than a first-line, therapeutic agent for treatment of urinary tract infections caused by ESBL-producers.[393,395,396] Moreover, tigecycline-resistance (3/14; 21.4%) observed among CTX-M-15-positive *K. pneumoniae* isolates in our study is also worrisome. Resistance to tigecycline among *K. pneumoniae* ranged from 0% to 11.5% in different studies.[397–400] Tigecycline remains as one of the few therapeutic options for infections due to ESBL-producing isolates;[397,400] therefore, the usage and close monitoring of its resistance are important.[401] The ESBL-producing isolates in our study were completely susceptible to ertapenem, meropenem, imipenem, amikacin and colistin. Carbapenems are the first line drugs for the treatment of severe infections caused by ESBL-producing bacteria.[27]

In this study, the bacterial species responsible for ESBLs production were the *K. pneumoniae*, *E. cloacae* and *E. coli* isolates. CTX-M enzymes were the most prevalent ESBLs in our isolates and CTX-M-15 in *K. pneumoniae* isolates was the most common enzyme. This is in accordance with what has been reported in other studies and support the evidence of CTX-M as the most prevalent type of ESBL in community settings worldwide.[30,48,150,152,186,385] Unlike most CTX-M-type enzymes, CTX-M-15 is associated with high level of resistance to both cefotaxime and ceftazidime in *K. pneumoniae* and other Enterobacteriaceae species.[402] This was also observed in CTX-M-15-producing *K. pneumoniae* isolates in our study. Of note, in this study, ESBL-producing *E. coli* isolates carried both CTX-M-14 and TEM-1 enzymes and not CTX-M-15, known to be effectively disseminated by *E. coli* isolates worldwide.[43] CTX-M-14-producing *E. coli* isolates have been isolated from several community hospitals in the Cape Town metropolitan area, and 42.9% of them co-produced TEM-1 and belonged to ST 131, an ST-type clone which is circulating in the community settings worldwide.[43,403] CTX-M-14 enzyme is the most common CTX-M-type in Asia,[195–197] and only few studies have reported its occurrence in Africa.[231,403–406] Studies in Canada and USA showed that CTX-M-14-producing *E. coli* had emerged as an important cause of community-associated bacteraemia and urinary tract

infections.[407,408] TEM-type ESBLs were not detected in all Enterobacteriaceae isolates in this study. In South Africa, the TEM-type ESBLs are commonly identified among isolates in hospital settings.[223–225] The detection of genes encoding *bla*$_{SHV}$-type enzymes in most of the *K. pneumoniae* isolates confirms that they are ubiquitous in such bacterial species.[409] Other important findings in this study were the occurrence of SHV-12 and SHV-5 enzyme in *E. cloacae* isolates. These findings probably suggest the simultaneous community spread of SHV-12 and SHV-5 genotypes in our setting. Our findings are in accordance with the reports from Spain,[410] Bosnia and Herzegovina,[411] Egypt,[141] and Morocco,[36] suggesting that SHV-type ESBLs could be as widespread in community settings as CTX-M enzymes.[36]

In this study, the different PFGE profiles detected among six CTX-M-15-producing *K. pneumoniae* isolates indicates that the spread of *bla*$_{CTX\text{-}M\text{-}15}$ gene was not due to a single clone, but rather likely due to horizontal transfer of *bla*$_{CTX\text{-}M\text{-}15}$-carrying plasmids between isolates with different genetic background.[141] Furthermore, several distinct PFGE profiles suggest possible carriage of the *bla*$_{CTX\text{-}M\text{-}15}$ gene on different antibiotic resistance plasmids.[161,412] Two CTX-M-15-producing *K. pneumoniae* isolates (102461-2 and 303200-1) with similar DNA fingerprint profiles from infants in different areas is of interest and may point to an unidentified epidemiological link. Two clones were observed in five SHV-producing *E. cloacae* isolates in individuals from Mbekweni area. These observations infer that *bla*$_{SHV\text{-}12}$- and *bla*$_{SHV\text{-}5}$-harbouring *E. cloacae* clones are already circulating in Mbekweni area. The CTX-M-15-producing *K. pneumoniae*, SHV-12- and SHV-5-producing *E. cloacae* clones from different locations or families may suggest inadequate hygiene conditions, contamination at the same source or the dissemination of such organisms through the environment as it has been previously described.[413,414]

Several risk factors associated with colonization or infection with ESBL-producing bacteria in the community have been identified in different studies.[20,415–418] In this study, due to the limited number of samples at the later time-points studied (5-12 and 20-28 weeks), we only assessed the risk factors associated with the acquisition of ESBL-producers in 90 mother-infant pairs sampled at birth. Infants born to HIV-positive mother were 11 times more likely to be carriers of ESBL-producing bacteria compared with those born to HIV-negative mothers. This finding likely reflects prior exposure of HIV-infected mothers to the health care services, where the mothers acquired ESBL

carriage and subsequently passed it on to the children. Our data also suggest that ESBL carriage in infants is significantly associated with mode of delivery. Infants born by elective caesarean section were 19 times more likely to be colonized by ESBL-producers compared to those delivered vaginally. Infants born by caesarean section are more exposed to the mother's skin microbiota and bacteria from the hospital environment than those who are born vaginally.[419,420] Another possible explanation could be the intrapartum use of antibiotics by mothers who had caesarean section.[420] Caesarean section influences the composition of intestinal microbiota during the first three days of life.[421] Previous use of antibiotics, especially the third generation cephalosporins, aminoglycosides, fluoroquinolones or trimethoprim-sulfamethoxazole has been associated with the faecal carriage of ESBL-producers in the community settings.[227,383,422–427] The ESBL faecal carriage in our study was significantly associated with administration of medication prior to discharge [risk ratio (RR) 3.95, 95% CI 0.47 - 33.2, *P* = 0.030]. Type of medication given to the infants before discharge was unknown and therefore this association could not be fully understood. Breastfeeding prior to discharge was a protective factor [RR 0.08, 95% CI 0.01 - 0.31, *P* = 0.001] (compared to those who were not breastfed) against colonization with ESBL-producing Enterobacteriaceae. The absence of breastfeeding has been associated with the faecal carriage of ESBL-producing organisms.[428–430] Infants who are breastfed are unlikely to be colonized by ESBL-producers; therefore, possible role of breastfeeding as a protective factor requires further research. HIV exposure and absence of breastfeeding were associated with ESBL faecal carriage. Infants probably had not been breastfed prior to passing meconium and HIV-infected mothers are unlikely to breastfeed. Therefore, we could not determine, by multivariate analysis, which variable was independently associated with this carriage due to small numbers of ESBL-PE detected.

In this study, higher rates of ESBL carriage were observed in participants (both mothers and infants) from Mbekweni area. At delivery, 4.9% (2/41) of the mothers from Mbekweni area, compare to 4.1% (2/49) from TC Newman area, were ESBL carriers. In infants, 9.1% (6/66) and 4% (2/50) of ESBL carriers at three different time-points were from Mbekweni and TC Newman, respectively. Such high rates of ESBL carriage in the mothers and infants from Mbekweni area might be partially explained by the fact that this area has high rates of unemployment, poverty and malnutrition.[361] Poverty was the

main risk factor associated with the carriage of ESBL-producers amongst healthy individuals in Madagascar.[20] Another possible explanation can be the relatively poor sanitation in Mbekweni area, where sewage (possibly contaminated with ESBL-producers) contaminates river water used for watering local agricultural products that are subsequently used for food. In Mbekweni area, a recent study has reported the detection of several bacterial species (*K. pneumoniae*, *K. oxytoca*, *E. coli* and *E. cloacae*) in water used for irrigational and recreational purposes from the Berg River.[431] The latter study did not assess the production of ESBLs.[431] In Norway, recreational swimming in freshwater has been associated with the acquisition of ESBL-producing bacteria.[383] Moreover, the detection of ESBL-producers in environmental water samples has been previously reported elsewhere.[422,432,433]

We found, in this study, that one infant was colonized with SHV-12-producing *E. cloacae* at birth and at eight weeks. The duration and persistence of ESBL faecal carriage can vary greatly.[420,434] Therefore, long-term ESBL faecal carriage in infants may represent a significant reservoir for the spread of ESBL-producing Enterobacteriaceae to household members.[420] Persistence colonization with ESBL-PE isolates has been described in Germany,[435] and Sweden.[436,437] This study also found that one ESBL-positive infant had an ESBL-positive colonized mother. PFGE showed that the SHV-5-producing *E. cloacae* isolates from the mother-infant pair were identical, suggesting that vertical transmission from the mother-to-child at delivery or antenatally was likely. This finding is in agreement with what has been reported in the literature.[438] The maternal vagina, skin and breast milk which have been shown to be the potential source of ESBL-PE during or after birth,[358,382] were not assessed in our study. Therefore, the acquisition of such organisms by the infants from the mothers who screened negative for faecal ESBL carriage cannot be excluded.[358,382]

This study has some limitations. The study only assessed the risk factors associated with the acquisition of ESBL faecal carriage at birth due to limited data for other time-points. The small sample size may limit also the power of the study to recognize other risk factors for acquisition of ESBL-producers; consequently, more studies with large sample size are needed to address this issue. Also, the ESBL faecal carriage might have been underestimated because not all the mothers or infants were sampled at all time-points. In addition, the mothers were only sampled at birth; therefore, the mother-to-

child vertical transmission after birth could not be assessed. Due to insufficient amount of samples, we did not screen for ESBL and carbapenemase genes from the DNA extracted from stool samples. Culture-negative stool samples might contain DNA, with ESBL or carbapenemase genes, from non-viable or less abundant bacteria in the samples.[50]

Plasmids are epidemiologically relevant DNA elements in ESBL transfer; therefore, their role and association with MLST sequence types need to be examined.[439] In this study, MLST was not performed on ESBL-producers. Resistance determinants other than ESBLs and carbapenemases were not determined. This study only screened for the three common ESBL genes, thus compromising the detection of uncommon ESBL genes.[159] Lastly, a large sample size is required to obtain more precise estimates of the prevalence and risk factors. Nevertheless, the results of this study contribute to an improved understanding of the epidemiology of ESBL- and carbapenemase-producing organisms in the community in the Western Cape Province, South Africa.

6. Conclusions and future direction

In conclusion, this is the first study to detect ESBL-producing bacteria in human meconium samples in Africa, and raises questions on the source of such isolates and implications for the transmission of ESBL-producing bacteria in the community. This study also showed that faecal carriage of ESBL-PE among apparently healthy individuals is relatively common in our study setting, the Drakenstein sub-district. CTX-M- and SHV were the main enzymes circulating in our study setting. Person-to-person transmission might enhance their dissemination in the community settings,[427] which would explain the mother-to-infant transmission observed in this study. ESBL faecal carriage is considered to be a risk factor for infections with such bacteria, leading to an increased usage of carbapenems,[6,44] which may contribute to carbapenem resistance due to carbapenemases or porin loss with ESBL production.[27] In addition, multidrug-resistant bacteria may be imported into the hospital settings; therefore, the spread of these organisms in community settings in South Africa merits further surveillance.[440] Another important finding was the occurrence of SHV-12 and SHV-5 enzymes in the Drakenstein sub-district, South Africa. This may suggest that SHV-12 and SHV-5 could be widespread in our community, as it has been observed in Morocco,[36] and Egypt.[141] Further studies are needed to fully understand the epidemiology of SHV-type enzymes in the community settings. Furthermore, research on the persistence and duration of ESBL-PE carriage in the community is needed. Lastly, we did not detect carbapenemase-producing organisms in the stool samples tested. Further research or continuous surveillance is important in order to anticipate future trends in the dissemination of ESBL- and carbapenemase-producing organisms in community settings in South Africa.

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