Extended spectrum β-Lactamase and plasmid mediated AmpC resistance in clinical isolates of *Escherichia coli* from the Central Hospital of Maputo, Mozambique

by

Calvina Ernesto Langa Estaleva

214580068

Submitted in fulfilment of the academic requirements for the degree of Master of Health Sciences, University of KwaZulu-Natal, Durban.

Supervisors

Prof. Arnfinn Sundsfjord

Prof. Sabiha Y. Essack

Dr. Tomás Zimba

December 2016

EXTENDED SPECTRUM β-LACTAMASE AND PLASMID MEDIATED AMPC RESISTANCE IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* FROM THE CENTRAL HOSPITAL OF MAPUTO, MOZAMBIQUE

DECEMBER 2016

Calvina Ernesto Langa Estaleva

214580068

A dissertation submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Master of Health Science

This is the dissertation in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss **Calvina Ernesto Langa Estaleva**

As the candidate's supervisor, I have approved this thesis for submission.

Supervisors:

Signed: ..... Name: **Professor Arnfinn Sundsfjord** Date: 11/01/2017

Signed: SYEssack Name: Prof. Sabiha Y. Essack Date ..17.01 2017……

Signed: ..... Name: Dr. Tomás Zimba Data....16:01:2017

DECLARATION

I, **Miss Calvina Ernesto Langa Estaleva,** declare that:

1. That the work described in this dissertation has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

- The research reported in this dissertation, except where otherwise indicated, is my original work
- This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

3. This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

- Their words have been re-written but the general information attributed to them has been referenced.
- Where their exact words have been used, then their writing has been placed in italics, inside quotation marks and duly referenced.

Signed Calvina Ernesto Langa Estaleva Date 16.02.17

DEDICATION

This study is dedicated to Mr Estaleva, my kids Vilma, Edvina, Dennys and Vino; my parents and loved ones who always encourage me to pursue postgraduate studies.

ACKNOWLEDGEMENTS

I am most grateful to the Almighty God for the grace given to pursue this study. I appreciate the support of my supervisors Prof. Arnfinn Sundsfjord and Prof. Sabiha Yusuf Essack and the opportunity granted to me to pursue a research area of interest to me and for the conceptualization of this research topic. Miss Bjorg Haldorsen deserves commendation for his tireless effort in training a novice in phenotypical ESBL identification to carry out such research. I acknowledge Dr. Tomás Zimba for all help for this study.

Raul Vaz, my colleague of the Microbiology Laboratory of Maputo Central Hospital is appreciated for providing some of the materials for this research work.

I also acknowledge ISCISA giving me the opportunity to continue my studies, the Maputo Central Hospital allowing me to carry out the laboratory work in the Department of Microbiology, and the NORHED project making the Masters programme at ISCISA possible.

Finally, I am grateful to the Estaleva family and all friends in Maputo and beyond for their encouragement and support which saw me to the successful completion of my Masters study.

ABBREVIATIONS

CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycinase
CRE	Carbapenem-resistant Enterobacteriaceae
CTX-M	Cefotaximase-München
DNA	Dioxyribonucleic acid
E.coli	*Escherichia coli*
ESBL	Extended Spectrum β-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra intestinal pathogenic *Escherichia coli*
IMP	Imipenemase
KPC	*Klebsiella pneumonia* Carbapenemase
MDR	Multi-Drug Resistant
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
SHV	Sulfhydryl Variable
TEM	Temoneira
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
VIM	Verona Integron-Enconded Metallo-β-Lactamase

ABSTRACT

Antibiotic resistance is one of the main public health problems worldwide, reducing treatment options and increasing morbidity and mortality. The production of extended-spectrum β-lactamases (ESBLs), plasmid mediated (pAmpC) β-lactamases are the most important resistance mechanisms that hamper antimicrobial treatment of infections caused by Enterobacteriaceae. This study describes the detection and characterization of pAmpC- and/or ESBL-producing clinical isolates of *Escherichia coli* (n=230) from urine and blood samples at the Central Hospital of Maputo, Mozambique from mid-August to mid-November 2015. Antimicrobial susceptibility testing was performed by the disc diffusion method. Isolates with reduced susceptibility to cefotaxime and/ or ceftazidime (n=75) were subjected to phenotypic AmpC- and/or ESBL testing as well as PCR-detection of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{CMY}, bla_{MOX}, bla_{FOX} and bla_{DHA}. A total of 75/230 (32.6 %) isolates were ESBL positive, and twenty-five of these were pAmpC positive. The most prevalent ESBL and pAmpC were CTX-M (77%) and FOX (32%), respectively. Most CTX-M negative ESBL-strains were bla_{SHV} positive indicating a SHV-ESBL-type. The presence of co-resistance (R and I) to clinically important antibiotics were also frequent; blood ciprofloxacin (CIP; n= 12/17: 70.6%), gentamicin (GEN; n= 8/17: 47.1 %) and trimethoprim-sulfamethoxazole (SXT; n= 17/17; 100%) and urine CIP (n=40/58; 68.9%), GEN (n= 27/58; 46.5 %) and SXT (n= 55/58; 94.8%). Multidrug resistance was observed in 17/17 (100 %) and 58/58 (100 %) blood and urinary isolates, respectively. ERIC-PCR analysis revealed a large genetic diversity of strains with some minor clusters indicating intra hospital spread.

The study has shown that: (i) a large proportion of clinical isolates of *E. coli* from the urinary tract and blood cultures from the Central Hospital are pAmpC and/or ESBL-producing. (ii) CTX-Ms and FOX were the dominant ESBL- and pAmpC-types, respectively. (iii) All ESBL- and pAmpC-producing strains were MDR-strains only susceptible to antibiotics that are not easily available in the current location. The overall findings strongly support the urgent need for strengthened and rapid diagnostic services to guide correct treatment of serious life-threatening infections and improved infection control measurements.

Key words: *E.coli*; antibiotic resistance; extended-spectrum β-lactamase (ESBL); plasmid mediated AmpC

CONTENT

DECLARATION iii
DEDICATION iv
ACKNOWLEDGEMENTS v
ABBREVIATIONS ii
ABSTRACT iii
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW 1
1.1 INTRODUCTION 1
1.2 LITERATURE REVIEW 2
1.2.1 *Escherichia coli*: basic characteristics and pathogenicity 2
1.2.2 Multi- Drug Resistant *Escherichia coli* 3
1.2.3. β-Lactam antibiotics, their use and mechanism of action 4
1.2.4 Mechanisms of resistance to β- Lactams 5
1.2.4.1 Beta- Lactamase- Mediated Resistance 6
1.3 RESEARCH QUESTION 7
1.3.1 AIM AND STUDY OBJECTIVES 7
1.4. CHAPTER STRUCTURE 8
CHAPTER 2. MANUSCRIPT 13
CHAPTER 3. CONCLUSION 34
3.1 Introduction 34
1.5 Limitations 35
1.6 Recommendations 35
1.7 Significance 35
APPENDIX 1 37
Ethical clearance letter 37

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Antimicrobial resistance (AMR) is a global public health threat making commonly used antibiotics inefficient in the treatment of infectious diseases (WHO 2015). Beta-lactam have since the 1940s been our most successful antibiotics in the treatment of various bacterial infections. The global expansion of different resistance mechanisms towards beta-lactams are observed in both Gram-positive and Gram-negative bacteria.

The production of β-lactamases is the most common mechanism of bacterial resistance to β- lactam antibiotics in Gram-negative bacteria. Beta-lactamases are a large group of enzymes that are capable of hydrolyzing the beta-lactam ring of beta-lactam antibiotics. β- lactamase genes are widespread and mutate in response to continuous antibiotic exposure [Nkrumah *et al.*, 2013]. The production of extended-spectrum β-lactamase (ESBLs), plasmid-mediated AmpC (pAmpC) enzymes and carbapenemases are the most important resistance mechanisms that hamper the antimicrobial treatment of infections caused by *Enterobacteriaceae* [Paterson and Bonomo 2005].

Eschericia coli and *Klebsiella pneumoniae* are amongst the most commonly encountered bacteria producing ESBLs, pAmpC and carbapenemases, but nowadays detection of these enzymes has also been observed in several other species of *Enterobacteriaceae* and *Pseudomonas species* [Paterson 2014].

The first description of *K. pneumoniae* and *E. coli* resistant to third generation cephalosporins was in Germany in 1983 [Trabulsi e*t al*., 2004]. Since then, numerous types of enzymes have been reported in many different countries at all continents [Trabulsi *et al.*, 2004]. Moreover, the beta-lactamases are most often encoded by genes on mobile genetic elements that also confer other resistance mechanisms [Coque *et al.*, 2008]. This notion explains that ESBL strains have been associated with resistance to non-β-lactam antibiotics like the aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole and/or chloramphenicol [Khurana *et al.*,2002].

1.2 LITERATURE REVIEW

Infectious diseases continue to account for a larger percentage of the global disease burden, affecting more people in developing countries. Urinary tract infection (UTI) is a disease of high incidence in both community and nosocomial settings [Peirano e*t al.*,2014]. UTIs cause significant morbidity and mortality, with approximately 150 million cases globally per year. Uropathogenic *E. coli* (UPEC) is the primary cause of UTI and is generally treated empirically according to their predicted antibiotic susceptibility profile [Trabulsi *et al.,* 2004]. This section reviews important characteristics of *E. coli*, infections caused by *E. coli*, beta-lactam antibiotics and their role in the treatment of *E. coli* infections, some aspects of multi drug resistant *E. coli*, and resistance mechanisms against beta- lactam antibiotics.

1.2.1 *Escherichia coli*: basic characteristics and pathogenicity

Escherichia coli

E. coli is one of the most extensively studied Gram-negative bacteria belonging to the family *Enterobacteriaceae* [Bang 2016, Nicolau 2008]. This species is widely dispersed in nature and found in soil, water and has been associated with intestinal and extra intestinal infections in humans and many animals. *E. coli* is facultative anaerobic and thus preferably uses aerobic respiration in oxygen containing environments [Nicolau 2008].

E. coli contains an inner membrane, a peptidoglycan layer and an outer membrane. *E. coli* has a genome with a common backbone of genes (core genome), but among the different types of *E. coli* there is great genomic diversity [Bang 2016].

Certain strains have acquired specific virulence factor which are associated with a spectrum of disease. Currently, six major groups of intestinal pathogenic *E. coli* have been recognized: entero-pathogenic *E. coli*; Shiga toxin-producing *E .coli*, entero-aggregative *E. coli*, entero-toxigenic *E. coli*, entero-invasive *E. coli*, and diffusely adherent *E. coli* [Tang *et al.,* 2016]. It has been estimated that catheter-associated UTIs caused by *E. coli* represent one of the most common causes of nosocomial infection in United States [Tang *et al.,* 2016].

E. coli can also cause diseases such as urinary tract infections, diarrhoea, hemorrhagic colitis and hemolytic uremic syndrome [Paterson 2014]. The number of extra intestinal pathogenic *Escherichia coli* (ExPEC) infections is increasing worldwide, leading to a tremendous burden on public health [Mellata 2013]. In addition to causing human diseases, ExPEC strains are responsible for significant economic losses in animal production, particularly within the poultry industry as one of the fastest growing industries in the United States and worldwide [Paterson and Bonomo 2005].

The widespread use of antimicrobials, coupled with the transmission of genetic elements carrying resistance determinants in the form of plasmids, transposons, and integrons, are major contributors to the increasing worldwide prevalence of antimicrobial resistance [Trang *et al.,* 2013]. *E. coli* is heavily exposed to antimicrobial selection as a major human and animal commensal pathogen. Combined with its large genomic diversity including mobile genetic elements, *E. coli* is a significant hub for acquired multidrug resistance.In Africa, there is a disproportionate burden of global childhood mortality caused by infectious diseases. Invasive bacterial infections are major contributors to this excess mortality among children and *Streptococcus pneumoniae*, *Staphylococcus aureus*, *E. coli*, are the most common bacterial pathogens [Mandomando *et al.,* 2010].

Africa in general and Mozambique in particular is often limited by infrastructure, cost, and human resource constraints [Moon *et al.,* 2010]. The absence of microbiologic capacity for diagnosis and surveillance outside the main research centres and teaching hospitals forces many clinicians to practice without any locally relevant data about distribution of pathogens, antimicrobial susceptibility patterns or their evolution in time [Moon *et al.,* 2010].

1.2.2 Multi- Drug Resistant *Escherichia coli*

Antimicrobial resistance hinders effective treatment of some of the leading causes of morbidity and mortality in the developing world. Antibiotics given empirically without proper antibiotic susceptibility testing are one of the major causes for the continuous selection of multi-drug resistant bacteria [Nkrumah *et al.,* 2013].

Antibiotics most often target cell wall biosynthesis, protein synthesis or DNA replication and repair in bacteria [Moon *et al.,* 2010]. Antibiotic use in the society has contributed to selection pressure which gives rise to antibiotic-resistant microbes. Therefore antibiotic stewardship, implementation of basic hygiene procedures and sanitations are important factors in limiting the spread of resistant microbes [Bang 2016].

The extended-spectrum beta-lactamases (ESBLs) are class A plasmid mediated enzymes that hydrolyse oxyimino cephalosporin and monobactam antibiotics but are inhibited by clavulanic acid in vitro. *E. coli* harbouring ESBLs confer resistance to penicillin, narrow and extended-spectrum cephalosporin, and aztreonam antibiotics. They also frequently show up as multidrug resistant expressing co-resistance to commonly used important antibiotics such as aminoglycosides, trimethoprim/sulfamethoxazole, and quinolones [Rezai *et al.* 2014, Moon *et al.* 2010].

Thus, infections caused by ESBL-producing *Enterobacteriaceae* have serious implications for both public health and infection control practices [Nicolau 2008]. These infections often undermine empirical treatment regimens and cause delays in the administration of effective therapy [Nicolau 2008].

1.2.3. β-Lactam antibiotics, their use and mechanism of action

The β-lactams are a broad class of antibiotics that include the penicillins, cephalosporins, cephamycins, monobactams and carbapenems [Tang 2016]. They have an important place in the therapy of Gram- negative organisms including infections caused by *E. coli*, either as mono therapy or in combination with other antibiotics [Tang 2016]. Beta-lactams kill susceptible bacteria by specifically inhibiting the transpeptidases that catalyzes the final step in cell wall biosynthesis, the cross-linking of peptidoglycan [Yocum *et al.,* 1980].

The introduction of penicillin, in the early 1940s, was perceived as marking the end of infectious diseases but penicillin resistant *Staphylococcus aureus* strains was reported just a few years after its use [Francisco *et al.,* 2014]. Penicillin was the first of the β-lactam antibiotics consisting to thiazolidine ring connected to a β – lactam ring with an

acyl side chain. Penicillin G and penicillin V, were the first natural synthetic penicillin, have a little activity against Gram negative bacteria and are susceptible to hydrolysis by β – lactamases [Drawz and Bonomo 2010, Francisco *et al.,* 2014].

The third- generation cephalosporin have become an important tool in the treatment of severe infection caused by *E. coli* and other *Enterobacteriaceae*. Unfortunately the ESBLs are now the major mechanism behind the increasing resistance against penicillin, and all cephalosporins (except cephamycins) and monobactams [Coque *et al.,* 2008]. pAmpC β lactamases are sensitive to 4^{th} generation cephalosporins (cefepime, cefpirome) and carbapenems, but confer resistance to second and third cephalosporins as well as cephamycins. Carbapenemases have the ability to hydrolyze virtually all betalactam drugs [Datta *et al.,* 2014].

Historically, the carbapenems have been reserved as last-resort agents in the treatment of serious or highly resistant infections as they are not compromised by ESBLs or pAmpCs. ESBL and AmpC enzymes generally lack the ability to hydrolyze the carbapenems, but reduced susceptibility or resistance to carbapenems can arise when such enzymes are accompanied by decreased outer membrane permeability [Datta *et al.,* 2014]. Importantly, b-lactamase enzymes with carbapenemase activity have now spread worldwide and are beginning to compromise the use of even carbapenems [Doi and Paterson, 2015].

Carbapenems are stable against most β-lactamases mainly due to the structural modifications, such as a carbon at position C-1 and a hydroxyethyl R-2 side chain. Modifications in structure of the first carbapenem, thienamycin, led to the development of imipenem, meropenem, ertapenem and more recently, doripenem [Pérez and Hanson 2002].

1.2.4 Mechanisms of resistance to β- Lactams

Antibiotic-resistant bacteria are difficult to treat and can be associated with high morbidity and mortality. Therefore, they pose a great threat to public health. There is no exception for Enterobacteriaceae, and their ability to acquire resistances to broad-spectrum beta-lactam antibiotics [Tang *et al.* 2016].

1.2.4.1 Beta- Lactamase- Mediated Resistance

ESBLs are enzymes capable of hydrolysing oxyimino-β-lactams, such as third generation cephalosporins, which include the commonly used antimicrobials, ceftriaxone, ceftazidime and cefotaxime. The β-lactamases are grouped into four main classes, namely: A, B, C and D by the Ambler classification based on their biochemical characteristics. Classes A, C and D are serine β-lactamases and class B enzymes are metallo- β-lactamase [Sonda *et al*., 2016].The dissemination of ESBLs is a global problem, particularly for *K. pneumonia* and *E. coli* [Nasser and Sundsfjord 2011]. CTX-M-type β-lactamases are the dominant ESBL-type worldwide. CTX-Ms, which preferentially hydrolyze cefotaxime were first reported in the late 1980s and have since undergone a rapid, global spread. The spread of CTX-M-type β- lactamases has been dramatic and greater than the impact of the TEM- and SHV-type ESBLs [Nasser and Sundsfjord 2011].

Plasmid mediated AmpC β lactamases (pAmpC) are enzymes which belong to class C of Ambler. These enzymes confer resistance to a wide variety of β lactam drugs including cephamycins and β lactamase inhibitors like clavulanic acid, sulbactam and tazobactam to which class enzyme (ESBLs) are sensitive [Masud *et al.*, 2014].

For a long time, carbapenems have been considered as an important antibiotic for the treatment of enterobacteriaceae infection. However, now carbapenem-resistant Enterobacteriaceae (CRE) has become a global issue. [Tang *et al.* 2016].

The development of resistance to carbapenems among *E. coli* is of particular concern because these agents are often the last line of effective therapy in invasive infections. New Delhi metallo-β-lactamase (NDM) and carbapenem-hydrolyzing class D β-lactamases such as oxacillinase-48 (OXA-48) are the most common carbapenemases among *E. coli* worldwide. [Peirano *et al.,* 2014, Poumaras *et al.,* 2013].
Carbapenemase identified in Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are placed in three classes of β-lactamase as Ambler class A, class B metallo β-lactamases, and carbapenem hydrolyzing class D oxacillinases [Ronald *et al.,* 2011]. Carbapenemase are classified into two major types based on the active site of the enzyme as serine carbapenemase (KPC- *Klebsiella pneumonia* Carbapenemase and OXA type β-lactamase) and metallo-β-lactamase (IMP-

Imipenemase, VIM- Verona Integron-Encoded Metallo-β-Lactamase, and NDM-New Dehli Metallobetalactamase). [Reich *et al.*, 2013]

1.3 RESEARCH QUESTION

Considering that *E. coli* is the most common bacteria isolated in the microbiology laboratory of Maputo Central Hospital , it is imperative to undertake the phenotypic and genotypic characterization of resistance mechanisms in these *E. coli* using a prospective study with a view of informing empiric therapy and treatment guidelines. For that, we designed the following research question:

What is the occurrence of and the phenotypic and genotypic profile of ESBL- and/or pAmpC-producing clinical isolates of *E. coli* in samples processed in the Microbiology Laboratory of Central Hospital of Maputo in August to November-2015?

1.3.1 AIM AND STUDY OBJECTIVES

To delineate the occurrence of and the pheno-/genotypic characteristics of ESBL- and/or pAmpC-producing *E. coli* from the microbiology laboratory of Maputo Central Hospital - the study had the following objectives:

1- Isolate and confirm the identity of clinical isolates of *E. coli* processed from urinary tract and blood culture samples at Maputo Central Hospital in a defined time period.
2- Ascertain the antibiotic susceptibility of isolates against an appropriate panel of antibiotics by agar disc diffusion according to the CLSI method.
3- Examine for ESBLs and/or increased AmpC-production using specific double-disc synergy tests in isolates with reduced susceptibility to cefotaxime and/or ceftazidime.
4- Examine for carbapenemase production in isolates with reduced susceptibility to meropenem.
5- To definitively identify the ESBL-, pAmpC- and carbapenemase encoding genes by PCR and sequencing and assess the genetic relatedness between pAmpC- and/or ESBL-positive strains by ERIC-PCR.

6- To provide these data in the process of establishing empirical antibiotic treatment guidelines used at Maputo Central Hospital.

1.4. CHAPTER STRUCTURE

This research is presented in the following chapters:

Chapter 2. Manuscript intended for submission to the Journal of Infection in Developing Countries: Extended spectrum β-Lactamase and plasmid AmpC mediated resistance in clinical isolates of *Escherichia coli* from the Central Hospital of Maputo – Mozambique 2015. This manuscript addresses objectives 1-6 as stated above.

Chapter 3. Conclusion. This final chapter indicates the extent to which the objectives have been met, outlines limitations and recommendations, and highlights the significance of the study.

REFERENCES

1. Ahmed SF, Ali MMM, Mohamed ZK, Moussa TA, Klena JD (2014) Fecal carriage of extended-spectrum β-lactamases and AmpC-producing *Escherichia coli* in a Libyan community. Ann of Clin Microbiol and Antimicrob 18: 13-22.

2. Ashley EA, Lubell Y, White NJ (2011) Antimicrobial susceptibility of bacterial isolates from community acquired infection in Sub-Saharan Africa and Asian low and middle income countries. Trop Med and International Health 16: 1167-1179.

3. Bang SC (2016) Carbon monoxide and nitric oxide as antimicrobial agents- focus on ESBL – producing uropathogenic *E. coli.* Doctoral Dissertation, Orebro University: ISBN 978-91-7529-119-2. p. 18-30.

4. Barton MD, Wilkins J (2001) Antibiotic resistance in bacteria isolated from Poultry. Rural Industries Research and Development Corporation Publication 15: 14-19.

5. Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L (2008) Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum β-lactamase CTX-M-15. Emerg Infect Dis 14: 195–200.

6. Datta P, Gupta V, Arora S, Garg S, Chander J (2014) Epidemiology of extended-spectrum b-Lactamase, AmpC and carbapenemase production in Proteus mirabilis. J Infect. Dis. 67: 44-46.

7. Doi Y, Paterson DL (2015) Carbapenemase-Producing *Enterobacteriaceae.* Semin Respir Crit Care Med. 36: 74-84.

8. Drawz SM, Bonomo RA (2010) Three decades of β-lactamase inhibitors. Clin Microbiol Rev 23:160-201.

9. Embil JM, Plourde P (2009) Prevention and Control of Infection in the home. Canadian Medical Association or its licensors 180: 82-86.

10. Francisco CD, Köhler UV, Florian W, Alexandre M, Angelika F (2014) Characterization of *Escherichia coli* isolates from Hospital inpatients or outpatients with Urinary Tract Infection. J Clin Microbiol 52: 407–418.

11. Gupta V, Garg R, Garg S, Chander J, Attri AK (2013) Coexistence of extended spectrum beta-lactamases, AmpC beta-lactamases in *Acinetobacter baumannii* from burns patients: A report from Tertiary care centre of India. Annals of Burns and Fire Disasters 4 Suppl 25: 189-192.

12. Hegstad K, Samuelsen Ø, Hegstad J, Sundsfjord A (2015) Molecular methods for detection of antibacterial resistance genes: rationale and applications. In: Antibiotics in laboratory medicine. Wolters Kluwer ISBN 978-1-4511-7675-9. p. 408-449.

13. Khurana S, Taneja N, Sharma M (2002) Extended Spectrum β-lactamase mediated resistance in urinary tract isolates of family Enterobacteriaceae. J Med Res 116: 145-149.

14. Kumar D, Singh AK, Ali MR, Chander Y (2014) Antimicrobial susceptibility profile of extended spectrum β-lactamase (ESBLs) producing *Escherichia coli* from various clinical samples. Infect Dis Res and Treat 11: 71–78.

15. Mandomando I, Sigaúque B, Espasa LMM, Valles X (2010) Antimicrobial drug resistance trends of bacteremia isolates in rural hospital in southern Mozambique. Amer J Trop Med Hyg 83: 152–157.

16. Mansouri S, Neyestanaki DK, Shokoohi M, Halimi S, Beigverdi R, Rezagholezadeh F, Hashemi A (2014) Characterization of AmpC, CTX-M and MBLs types of β-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* producing extended spectrum β-lactamases in Kerman, Iran. Jundishapur J Microbiol 7: 1-7.

17. Maraskolhe DL, Deotale VS, Mendiratta DK, Narang P (2014) Comparision of three laboratory tests for detection of AmpC β lactamases in *Klebsiella* species and *E. coli*. J Clin Diag Res 8: 5-8.

18. Masud MDR, Afroz HA, Fakruddin MD (2014) Prevalence of extended-spectrum β-lactamase positive bacteria in radiologically positive urinary tract infection. Springer Plus 3: 210- 216.

19. Mellata M (2013) Human and Avian Extraintestinal Pathogenic Escherichia coli: Infections, Zoonotic Risks, and antibiotics Resistance Trends. Foodborne Pathogens and disease 10: 916- 932.

20. Moon TD, Silva P, Buene M, Morais L, Valverde E, VermundSten H, Brentlinger PE (2010) Bacteremia as a Cause of Fever in Ambulatory, HIV-Infected Mozambican Adults: Results and Policy Implications from a Prospective Observational Study. Amer J Trop Med Hyg 25: 110- 160.

21. Naseer U, Sundsfjord A (2011) The CTX-M Conundrum: Dissemination of Plasmids and Escherichia coli Clones. Microbial Drug Resistance 10: 1-15.

22. Ndir A, Diop A, Faye PM, Cisse MF, Ndoye B, Astagneau P (2016) Epidemiological and Burden of Bloodstream Infections Caused by Extencted- Spectrum

Beta-Lactamase Producing Enterobacteriaceae in a Pediatric Hospital in Senegal. PLoS One 2: 1-13.

23. Nicolau DP (2008) Carbapenems: a potent class of antibiotics. Expert Opinion on Pharmacotherapy. Clin Microbiol Rev 9: 23-37.

24. Nkrumah NO, Twum- Danso K, Krogfelt KK (2013) High levels of Extended-Spectrum Beta-lactamases in a major teaching hospital in Ghana: The need of regular monitoring and evaluation of antibiotic resistance. Amer J of Trop Med Hyg. 89: 960-964.

25. Oplustil CP, Zoccoli CM, Toboit NR (2010) Procedimentos basicos em Microbiologia clinica. 3ª Edição S. Paulo: ISBN 978-85-7378-215-8. p315-356.

26. Paterson DL, Bonomo RA (2005) Extended spectrum B-Lactamases: a clinical updata. Clin Microbiol Rev 14: 657–686.

27. Paterson, DL (2014) Resistance in Gram-negative bacteria: Enterobacteriaceae. Association for professionals in infection control and Epidemiology. J Clin and Diag Res 8: 5-8.

28. Peirano G, Bradford AP, Kazmierczark KM, Badal RE, Hackel M, Hoban D (2014) Global incidence of Carbapenemase-producing *Escherichia coli* ST131. Emer Infect Dis 28: 1928-1931.

29. Pérez F, Hanson N (2002) Detection of Plasmid-Mediated AmpC -Lactamase Genes in Clinical Isolates by Using Multiplex PCR. J Clin Microbiol 20: 2153–2162.

30. Poumaras S, Zarkotou O, Poulou A, Kristo I, Vrioni G, Digalaki KT (2013) A Combined Disk Test for Direct Differentiation of Carbapenemase- Producing Enterobacteriaceae in Surveillance Rectal Swabs. J Clin Microbiol 51: 2986–2990.

31. Rajabnia R, Asgharpour F, Shahandashti F, Moulana Z (2015) Nosocomial emerging of (VIM1) carbapenemase-producing isolates of *Klebsiella pneumonia* in North of Iran. J Infect Dis and Trop Med Res Center, Babol University of Medical Sciences, Department of Microbiology 6: 115-122.

32. Reich F, Atanassova V, Klein G (2013) Extended-spectrum ß-Lactamase– and AmpC-producing Enterobacteriaceae in healthy broiler Chickens. Amer J Trop Med hyg. 19: 1253-1259.

33. Rezai M, Salehifar E, Langaee T (2014) Characterization of Multidrug Resistant Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* among Uropathogens of Pediatrics in North of Iran. BioMed Research International8: 115-125.

34. Ronald NJ, Mariana C, Bijie H, Yuxing N, Stephen SFL, Rodrigo EM, Yao W (2011) Update of contemporary antimicrobial resistance rates across China: reference testing results for 12 medical centers. Microbiol Rev 13: 305-309.

35. Sonda T, Kumburu H, Zwetselaar MV, Alifrangis M, Lund O, Kibiki G, Aarestrup FM (2016) Meta-analysis of proportion estimates of Extended-Spectrum-Beta-Lactamase producing *Enterobacteriaceae* in East Africa hospitals. Antimicrobial Resistance and Infection Control 8: 1-9.

36. Tang H, Hsieh CF, Chang PC (2016) Clinical Significance of Community- and Healthcare-Acquired Carbapenem-Resistant Enterobacteriaceae Isolates. J Clin Microbiol 8: 89- 120.

37. Tenover FC, Emery SL, Spiegel CA, Bradford PA, Eells S, Endimia A, Bonomo RA McGowan JE (2009) Identification of plasmid-mediated AmpC _-Lactamases in Escherichia coli, *Klebsiella* spp. and *Proteus* species can potentially improve reporting of cephalosporin susceptibility testing results. J Clin Microbiol 13: 294-299.

38. Thakuria B, Lahon K (2013) .The beta lactam antibiotics as an empirical therapy in a developing Country: An update on their current Status and recommendations to counter the resistance against them. J Clin Diag Res 6: 1207-1214.

39. Trabulsi LR, Campos LC, Franzolin MR (2004) Diarrheagenic Escherichia coli Categories among the Traditional Enteropathogenic E. coli O Serogroups - A Review. Mem Inst Oswaldo Cruz, Rio de Janeiro 99: 545-552.

40. Trang NHT, Nga TVT, Campbell JI, Hiep NT, Farrar J, Baker Stepher, Duy PT (2013) The characterization of ESBL genes in Escherichia coli and Klebsiella pneumoniae causing nosocomial infections in Vietnam. J Infect Dev Countries 7: 922-928.

41. Yadav KK, Adhikari N, Khadka R, Pant A, Shah B (2013) Multidrug resistant Enterobacteriaceae and extended spectrum β-lactamase producing *Escherichia coli*: a cross-sectional study in National Kidney Center, Nepal. BioMed Central 4: 1-7.

42. Yocum RR, Rasmussen JR, Strominger JL (1980) The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase. J Biol Chem. 255: 3977-86.

43. Wassef M, Behiry I, Younan M, Guindy NE, Sally M, Abada E (2014) Genotypic identification of AmpC β-Lactamases production in Gram-negative Bacilli isolates. Jundishapur Journal Microbiol. 7:1-8.

CHAPTER 2. MANUSCRIPT

The findings are reported in the following manuscript intended for submission to the Journal of Infection in Developing Countries:

Estaleva CEL, Zimba TF, Sekyere JO, Govinden U, Chenia HY, Essack SY, and Sundsfjord A. Extended spectrum β-lactamase and plasmid mediated AmpC resistance in clinical isolates of *Escherichia coli* from the Central Hospital of Maputo - Mozambique

Contributions:

- Estaleva CEL, as the principle investigator, developed the protocol, undertook the laboratory work and data analysis, and drafted the manuscript.
- Dr. Zimba TF, as co-supervisor took part in study design, facilitated laboratory work, took part in data analysis and drafting the manuscript.
- Drs. Sekyere JO, Govinden U, and Chenia HY, as co-authors, facilitated laboratory work, took part in data analysis and drafting the manuscript.
- Professor Essack SY. as co-supervisor, designed the study, facilitated laboratory work and data analysis, and contributed to the writing and critical revision of the manuscript.
- Professor Sundsfjord A, as principle supervisor, conceptualized the study, performed data analysis, and undertook critical revision of the manuscript.
- Bacterial culture, identification and initial susceptibility testing was undertaken at the Microbiology laboratory Maputo Central Hospital.
- Detection of genes encoding ESBL and plasmid-mediated AmpC and ERIC-PCR was carried at the Antimicrobial Research Unit, UKZN.
- Sequencing was carried out by Inqaba Biotechnology, Pretoria.

Extended spectrum β-lactamase and plasmid mediated AmpC resistance in clinical isolates of *Escherichia coli* from the Central Hospital of Maputo, Mozambique

Calvina E. L. Estaleva[1, 2], Tomas F. Zimba[1, 2], John, Osei Sekyere[2], Usha Govinden[2], Hafizah Y.Chenia[3], Sabiha Y. Essack[2], and Arnfinn Sundsfjord[4, 5]

[1]Microbiology Laboratory, Maputo Central Hospital, Mozambique.[2]Anitimicrobial Research Unit, School of Health Science, University of KwaZulu-Natal, Durban, South Africa.

[3] Discipline of Microbiology School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa

[4] Department of Medical Biology, Faculty of Health Sciences, University of Tromsø – Norwegian Arctic University, and [5] the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Clinical Microbiology and Infection Control University Hospital of North Norway, Tromsø, Norway

Corresponding author: Professor MD, Arnfinn Sundsfjord
Department of Medical Biology, Faculty of Health Sciences, University of Tromsø – Norwegian Arctic University, NO-9037 Tromsø, Norway
Email: arnfinn.sundsfjord@uit.no

Running title: Extended spectrum β-Lactamase and plasmid mediated AmpC resistance in clinical isolates of *Escherichia coli* from the Central Hospital of Maputo - Mozambique

Key words: *E. coli*; antibiotic resistance; extended-spectrum β-lactamase (ESBL); plasmid mediated AmpC.

Abstract

Antibiotic resistance is one of the main public health problems worldwide, reducing treatment options and increasing morbidity and mortality. The production of extended-spectrum β-lactamases (ESBLs), plasmid mediated AmpC β-lactamases (pAmpC) and linked co-resistances are the most important resistance mechanisms that hamper antimicrobial treatment of infections caused by Enterobacteriaceae. This study describes the detection and characterization of pAmpC- and/or ESBL-producing clinical isolates of *Escherichia coli* (n=230) from urine and blood samples at the Central Hospital of Maputo, Mozambique from mid-August to mid-November 2015. Antimicrobial susceptibility testing was performed by the disc diffusion method. Isolates with reduced susceptibility to cefotaxime and/ or ceftazidime (n=75) were subjected to phenotypic AmpC- and/or ESBL testing as well as PCR-detection of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{CMY}, bla_{MOX}, bla_{FOX} and bla_{DHA}. A total of 75/230 (32.6 %) isolates were ESBL positive, and twenty-five of these were pAmpC positive. The most prevalent ESBL and pAmpC were CTX-M (77%) and FOX (32%), respectively. Most CTX-M negative ESBL-strains were bla_{SHV} positive indicating a SHV-ESBL-type. The presence of co-resistance (R and I) to clinically important antibiotics were also frequent; blood ciprofloxacin (CIP; n= 12/17: 70.6%), gentamicin (GEN; n= 8/17: 47.1 %) and trimethoprim-sulfamethoxazole (SXT; n= 17/17; 100%) and urine CIP (n=40/58; 68.9%), GEN (n= 27/58; 46.5 %) and SXT (n= 55/58; 94.8%). Multidrug resistance was observed in 17/17 (100 %) and 58/58 (100 %) blood and urinary isolates, respectively. ERIC-PCR analysis revealed a large genetic diversity of strains with some minor clusters indicating intra hospital spread.

The study has shown: (i) a large proportion of ESBL- and/or pAmpC-producing clinical isolates of *E. coli* from the urinary tract and blood cultures from the Central Hospital with CTX-Ms. (ii) CTX-Ms and FOX were the dominant ESBL- and pAmpC-types, respectively. (iii) All ESBL- and pAmpC-producing strains were MDR-strains only susceptible to antibiotics that are not easily available in the current location. The overall findings strongly support the urgent need for strengthened and rapid diagnostic services to guide correct treatment of serious life-threatening infections and improved infection control measurements.

Key words: *E. coli*; antibiotic resistance; extended-spectrum β-lactamase (ESBL); plasmid mediated AmpC

Introduction

There is an increasing awareness about the growing worldwide problem with antimicrobial-resistant bacteria in the community and health care facilities. Resistance to antimicrobial agents occurs in all parts of the world with an increasing prevalence and threatens human and animal health [WHO 2015]. Thus, the World Health Organization (WHO) now recognizes antimicrobial resistance (AMR) as one of the principal public health problems of the 21st centuries threatening the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi [Prestinaci *et al*., 2015].

The production of β-lactamases is the most common mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria. Beta-lactamases are a large group of enzymes that are capable of hydrolyzing the β-lactam ring of β-lactam antibiotics. β-lactamase genes are widespread, mutate and adapt in response to continuous antibiotic exposure [Nkrumah *et al.*, 2013].

Extended spectrum β-lactamases (ESBL) are enzymes that hydrolyse an extended spectrum of β-lactam antibiotics including penicillins and cephalosporins with an oxyimino side chain [Paterson and Bonomo 2009]. These cephalosporins include cefotaxime, ceftriaxone and ceftazidime as well as the oxyimino-monobactam aztreonam. The most widely distributed ESBLs enzymes, are CTX-M-type β-lactamases, which preferentially hydrolyze cefotaxime. CTX-Ms were first reported in the late 1980s and have since undergone a rapid, global spread. The dissemination of CTX-M-type β- lactamases has been dramatic and greater than the impact of the TEM- and SHV-type ESBLs [Paterson and Bonomo 2009].

AmpC β-lactamases are also an important cause of beta- lactam resistance in Gram-negative bacteria [Jacoby 2009]. Many Gram-negative bacteria including most *Enterobacteriaceae* have chromosomally encoded AmpC-betalactamases. Some AmpC-genes have been mobilized from their chromosomal origin and may be spread between bacteria by plasmids. They are named plasmid-mediated AmpC (pAmpC), and include MOX, FOX, DHA and CMY, which are the most prevalent [Jacoby 2009]. AmpC-

betalactamases confer resistance to a wide variety of β-lactam drugs including penicillins, 1-3 generation cephalosporins as well as β-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam to which class enzyme (ESBLs) are sensitive [Paterson 2014]. ESBLs and pAmpCs may be present together in the bacteria. The effect of pAmpC β-lactamase expression may phenotypically mask the presence of ESBLs which may result in failure to detect these enzymes [Prestinaci *et al.*, 2015].
Among the beta-lactamases, the emerging carbapenemases, especially transferrable metallo-beta-lactamases (MBLs) are of particular concern because of their ability to hydrolyze virtually all beta-lactams, including the carbapenems [Datta *et al.*, 2014]. *Escherichia coli* and *Klebsiella pneumoniae* are amongst the most commonly encountered clinical isolates producing ESBLs, pAmpCs or carbapenemases, however, they have been observed in other species of Enterobacteriaceae, as well as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [Datta *et al.*, 2014].

In the context of developing countries, there is limited data from large surveillance studies on antimicrobial resistance [Mandomando *et al.*, 2010, Moon *et al.*, 2013]. In addition, few clinical studies document the susceptibility pattern of common causative pathogens in humans; partly due to the lack of microbiological facilities in many health facilities [Datta *et al.*, 2014]. This study delineates plasmid AmpC- and ESBL-mediated resistance in clinical isolates of *E. coli* routinely processed in the Maputo Central Hospital, Mozambique.

Material and Methods

Bacterial strains

The bacterial strains were collected at Maputo Central Hospital, a 1000-bed reference hospital in Maputo, Mozambique. We included urine and blood culture samples received at the Department of Clinical Microbiology from mid-August and through mid-November 2015. The urine samples were obtained from both in- and outpatient whereas blood cultures were only from inpatients.

Ethical considerations

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE 030/16) and the Institutional Bioethics Committee for Health-CIBS- ISCISA (TFCMCSCE 02/15)

Identification and susceptibility testing

Bacterial identification was performed using standard biochemical tests including API-20E kit (Biomerieux, la Balme-les-Grottes, France). Strains identified as *E.coli* were further characterized with antimicrobial susceptibility testing (AST). AST was performed using the Kirby-Bauer disk diffusion method according to CLSI guidelines [CLSI 2015]. Multidrug-resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [Magiorakos *et al.*, 2011].

Internal quality assurance was performed weekly during the whole study period using the wild type *E. coli* ATCC 25922 strain. In addition the *K. pneumoniae* ATCC 700603 (ESBL-type SHV-18 positive) and the *E.coli* A5-8 (bla_{DHA}) were used as ESBL- and AmpC-positive controls, respectively. The A5-8 strain was obtained from the Norwegian Advisory Unit for Detection of Antimicrobial Resistance. Strains were stored in skimmed milk broth with 10% glycerol at -70˚C until they were subjected to molecular analysis.

Phenotypic detection of β- lactamase

Strains that showed reduced susceptibility (I or R) to cefotaxime and/or ceftazidime were examined for ESBL-production. Strains with reduced susceptibility to cefotaxime and/or ceftazidime and cefoxitin were examined for increased AmpC-production. [http//www.eucast.org/fileadmin/src/med 2016]. The mechanisms of resistance were examined using ROSCO combined disc tablets (ROSCO Taastrup, Denmark) for ESBL-A, AmpC- detection and interpreted according to instructions of the manufacturer.

Genotypic characterisation of β-lactamases

The genetic characterization was performed at the Anitimicrobial Research Unit, UKZN. All isolates were screened for the presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY}, bla_{DHA}, bla_{FOX} and bla_{MOX} genes using PCR with the specific primers given in Table 1. Briefly, the bacterial strains were grown on nutrient agar overnight at 35±2˚C for 16-18h. DNA extractions were performed using GeneJet Purification kit (Thermo Scientific) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until used as DNA template for gene amplifications using PCR.

The PCR amplification mixture was prepared in a final volume of 25µL: 7.5µL sterilised distilled water; 1µL template DNA, 2µL of each primer; forward and reverse primers (Inqaba Biotechnology Industries, South Africa) and 12.5µL of master mix (Applied Biosystems, USA). Amplification were undertaken using T100^{TM} Thermal cycler (Bio-Rad, USA) and comprised of a preliminary denaturation at 98˚C for 10s, followed by denaturation at 98˚C and extension of 72˚C for 15s for 30 cycles, with a final extension of 72˚C for 1 min. The same PCR conditions were used for the other genes but with the following annealing temperatures: TEM (56 ˚C), SHV (56 ˚C), and CTX-M (57 ˚C).

The products were run on 1.5% agarose gel at 120 V for 45 min, stained with gel red and visualized using Bio-Rad's ChemiDoc^{TM} MP System. CTX-M-positive PCR products were sent for DNA sequencing at Inqaba Biotec.

Genomic profiling by ERIC-PCR

The clonal relatedness of ESBL-positive isolates was examined by ERIC- PCR [Versalovic *et al.,* 1991]. The total reaction volume was 10 µL, which contained 2µL of template DNA and 0.1µL primers and 5 µL of DreamTaq mastermix (Thermo Scientific). The primers ERIC 1 and ERIC 2 were use [Versalovic *et al.,* 1991] PCR conditions were as follows: 94 °C for 3 min, 30 cycles of 30 s of denaturation at 94 °C, 1 min of annealing at 50 °C, 8 min of extension at 65 °C and a final elongation at 16 min at 65 °C. The ERIC-PCR products were loaded on a 1% (w/v) agarose gel. The products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/mLgel red for 15 min. Genotypic variation were analysed using the GelComparII version 6.0 software package (Applied Maths) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to produce a dendrogram.

Results

Strain selection and antimicrobial susceptibility testing

Clinical samples (n=3326) were received during the study period from in-patients at different departments, Maputo Central Hospital (n= 1841; 55.4 %) as well as from outpatients (n=1485; 44.6%). A total of 1078/3326 (32.4%) samples were from urine (n=823; 24.7 %) and blood (n=255; 7.7 %). *E. coli* strains were identified from urine (n=199/823; 24.1%) and blood (n=31/255; 12.1%) culture samples. A total of 140/230 (60.8%) and 90/230 (39.1%) *E.coli* strains were obtained from in- and outpatients,

respectively, including urinary strains from in- (n=109) and outpatients (n=90). All blood culture isolates were from inpatients.

A total of 17/31 (55 %) blood culture and 58/199 (29 %) urine isolates showed reduced susceptibility (R or I) to cefotaxime and/or ceftazidime (Tables 2 nr 3). The ESBL confirmation test was positive for all 75/230 (32.6 %) isolates. Among the urinary samples (n=199) the ESBL-positive rates were 8/90 (8.8 %) and 50/109 (45.8 %) for out- and inpatients, respectively.

Reduced susceptibility to meropenem was not observed. Reduced susceptibility to cefoxitin was observed in 41/75 (54.6 %) of the isolates and further examined for increased AmpC-production; 7/17 and 34/58 blood and urine strains, respectively (Table 2 and Table 3). A total of 25/41 (60.9 %) isolates were confirmed AmpC positive using the ROSCO combined disc method, representing 25/75 (33 %) of the total ESBL-positive population.

The presence of co-resistance (R and I) to clinically important antibiotics were also examined (Tables 2 and 3); blood ciprofloxacin (CIP; n= 12/17: 70.6%), gentamicin (GEN; n= 8/17: 47.1 %) and trimethoprim-sulfamethoxazole (SXT; n= 17/17; 100%) and urine CIP (n=40/58; 68.9%), GEN (n= 27/58; 46.5 %) and SXT (n= 55/58; 94.8%). Interestingly, most of the isolates, even pAmpC-negative, expressed resistance to betalactamase inhibitors (amoxicillin-clavulanic acid and piperacillin-tazobactam) indicating additional resistance mechanisms beyond ESBL.

Multidrug resistance defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [Magiorakos *at al.,* 2011], was observed in 17/17 (100 %) and 58/58 (100 %) blood and urinary isolates, respectively.

Detection of genes encoding ESBL and plasmid-mediated AmpC.

A total of 58/75 (77 %) and 39/75 (52 %) ESBL-positive strains scored positive for $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} by PCR, respectively (Fig 1). The $bla_{\text{CTX-M}}$ amplicons were sequence-typed with the following results: CTX-M 15 (n=24), CTX-M 28 (n=2), CTX-M 117 (n=1), CTX-M 36 (n=1), CTX-M 164 (n=1) and CTX-M no typing (n=29). Available resources did not allow sequence typing of SHV-amplicons. But, a total of 13/17 $bla_{\text{CTX-M}}$ negative ESBL-specimen were bla_{SHV}-positive, indicating a SHV-ESBL-type. Only one isolate was bla_{TEM} positive, and this strain was positive for $bla_{\text{CTX-M 15}}$. Surprisingly, a total of 25/75 (33 %) strains examined scored positive for one of more

plasmid mediated AmpC genes (Fig 1); *bla*$_{CMY}$ (n=1), *bla*$_{DHA}$ (n=13), *bla* $_{MOX}$ (n=22), and *bla*$_{FOX}$ (n=24). The strains contained two (n=15) or three (n=10) pAmpC genes: *bla*$_{CMY;DHA}$ (n=1); *bla*$_{DHA;FOX}$ (n=2), *bla*$_{MOX;FOX}$ (n=12) and *bla*$_{DHA;MOX;FOX}$ (n=10). Importantly, only phenotypical AmpC-positive strains scored positive for *bla*AmpC.

ERIC – PCR Results

Distinct profiles were obtained for the 75 isolates tested using ERIC-PCR fingerprinting (Fig. 1). Fragments of different molecular weights were observed and polymorphisms based on fragment length were used as a means of differentiating *E. coli* isolates. The absence or presence of a band was also noted in determining variation among the isolates. ERIC-PCR profiles comprised between 4 and 12 individual bands, varying in size from 0.5 – 10 kb (Fig. 1). Amplifications of different intensities were observed and visual analysis of the ERIC profiles included primary, secondary and tertiary amplification. Primary amplification products refer to those products of high intensity, which appear extremely bright on the gels. Secondary amplification products are those products that are not as bright as the primary amplification products but more intense that the tertiary amplification products, while the tertiary amplification products are the minor amplification products of low intensity. All isolates were type able using this fingerprinting technique and band patterns were reproducibly obtained under repeat amplification.

The ERIC–PCR profiles allowed the differentiation of the 75 isolates into 50 ERIC-PCR types, which were grouped into eight main clusters (A – H), with each of the clusters being sub-divided into two or three main sub-clusters (Fig. 1). The urine isolates were diverse and distributed in all eight identified clusters (clades A-H) with characteristics as outlined below. Isolates with similar profiles demonstrated different β-lactamase gene content. The blood isolates were more clonal in nature and mostly observed in clusters F and G, but predominantly in cluster G. Although closely related, they did demonstrate differences in their β-lactamase resistance gene content.

Clade A comprised of 10 strains; urine (n=9) and blood (n=1), and CTX-M positive (n=8). These strains were from samples collected in patients admitted at the departments

of pediatrics (n=5), internal medicine (n=2) as well as outpatients (n=3). The eight CTX-M positive strains were from pediatrics (n=5), internal medicine (n=1) and outpatients (n= 2). One strain was positive for pAmpC-genes. Twelve strains were clustered in clade B; urine (n=11) and blood (n= 1). The strains were from samples collected in internal medicine (n=2), pediatrics (n=9) and one outpatient. There were CTX-M (n=9) positive strains from pediatrics (n= 7), internal medicine (n=1) and one outpatient. Seven strains showed the presence of pAmpC genes in various combinations.

Clade C clustered eight strains from urine (n=7) and blood (n=1) cultured from patients at internal medicine (n= 1), pediatrics (n= 6) and surgery (n= 1). The CTX-M positive strains were from pediatrics (n= 6). Six strains were pAmpC positive with 13 blaAmpC genes in total. Clade D comprised of 12 strains; urine (n=11) and blood (n=1) from patients hospitalized in pediatrics (n= 9), internal medicine (n= 2) as well as one outpatient. The eight CTX-M positive strains were from pediatrics (n=6), internal medicine (n=1) and one outpatients. Six strains showed the presence of a total of 13 pAmpC genes.

The clade E strains (n=11) were from urine (n=9) and blood (n=2) and were all CTX-M positive. These strains were from samples collected in patients admitted at internal medicine (n= 3), pediatrics (n= 6) and outpatients (n= 2). Five pAmpC genes were detected from 2 strains. Clade F clustered 12 strains; urine (n=6) and blood (n=6) from samples collected in patients at pediatrics (n= 7), internal medicine (n= 4) and surgery (n= 1). Nine CTX-M positive strains were from the departments of pediatrics (n=5), internal medicine (n=3) and surgery. Six pAmpC genes were detected in 2 strains.

The clade G strains (n=7) were from urine (n=2) and blood (n=5). Five strains were CTX-M positive. These strains were from samples collected in patients admitted at pediatrics (n= 4) and internal medicine (n= 3). The five CTX-M positive strains were from pediatric (n=3) and internal medicine (n=2). One strain was positive for two pAmpC genes. The clade H strains (n=3) were all urine isolates and the CTX-M positive strains (n=2) were from samples collected in patients at pediatrics and medicine. Plasmid mediated AmpC genes were not observed.

Discussion

To our knowledge this is the first study describing the presence of plasmid-mediated AmpC- and/or ESBL-producing clinical isolates of *E. coli* in Mozambique. Recent literature reviews show that most African studies on the presence of ESBL-producing *Enterobacteriaceae* have been conducted in Northern and Eastern Africa, with a relative lack of data in Sub- Saharan Africa with a few exceptions [Mshana *et al.,* 2013, Sangare *et al.,* 2015, Sonda *et al.,* 2016). There are very limited data from surveillance studies and a few clinical studies documenting the susceptibility pattern of common pathogens from human and animals. All studies underline the threat of antimicrobial resistance in general and ESBL-producing *Enterobacteriaceae* in particular. Moreover, several studies underline the free availability of antibiotics from private pharmacies and drug shops for self-medication before seeking medical service, as part of the complexity. The overall findings reveal a great variety in proportions of ESBL-producing Enterobacteriaceae between countries, underlining the importance of active surveillance studies and local data in order to guide antimicrobial therapy and infection control [Mshana *et al*., 2013, Sonda *et al*., 2016].

The overall presence of plasmid-mediated AmpC- and/or ESBL-producing in clinical isolates of *E. coli* was (n=25/230; 10.8%) and (n=75/230; 32.6%), respectively. They were present in both urinary tract and blood culture isolates and with a higher prevalence in the latter and in urine samples from inpatients. CTX-Ms were the most dominant ESBL-type, with CTX-M 15 as the major subtype. Most of the *bla*CTX-M negative ESBL-positive strains were negative for *bla*TEM, but positive for *bla*SHV, indicating an SHV-ESBL-type. This is in accordance with the international situation [Paterson *et al.,* 2009, Umaer and Sundsfjord 2011]. A 2010-11 survey in the United States showed that CTX-M type accounted for 85% of ESBL-producing *E. coli* strains [Laxminarayn *et al.,* 2013].

In China, CTX-M-type ESBLs accounted for more than 70% ESBL-producing *E.coli* over the past 10 years and CTX-M-14 was the most abundant genotype, although the detection rate of CTX-M-15 showed a continuously increasing trend in recent years [Li *et al.,* 2012, Tian *et al.,* 2012]. Moreover, a study in Sub-Saharan Africa of ESBL-

producing *Enterobacteriaceae* in stool samples in Mali, Niger and Cameroon showed that CTX-M was the dominant ESBL-type [Sangare *et al.*, 2015].

A total of 25/230 (11 %) of the strains expressed an AmpC phenotype and all contained pAmpC. Surprisingly, all strains contained two to three pAmpC genes. Bla_{Fox} was the most prevalent in our strains. These observations contrast the worldwide observations of bla_{CMY} as the most prevalent pAmpC gene in *E. coli* populations [Jacoby 2009]. CMY-2 has the broadest geographic spread among pAmpCs and is an important cause of extended beta-lactam resistance in *E. coli* as well as in nontyphoid *Salmonella* strains in many countries [Egorova *et al.*, 2008]. The finding of multiple pAmpC-bla genes in single strains has recently been reported in a Tunisian study. A total of 11 out of 75 pAmpC positive clinical strains of Enterobacteriaceae were shown to contain up to three different pAmpCs [Cherif *et al.*, 2016]. In contrast to our study CMY-2 was the most common pAmpC-type in this strain collection. Moreover, the combination of MOX-, FOX- and CMY-2 type enzymes was dominant in their strains, in contrast to our strains that mostly contained MOX- and FOX-types in combination with DHA.

ERIC-PCR has been a useful rapid method in various molecular epidemiological studies to describe the genetic relatedness in Enterobacteriaceae strain collections [Ranjbar *et al.* 2014]. Our ERIC-PCR results revealed an overall genetic diversity of ESBL- and/or pAmpC-positive *E. coli* strains at the Maputo Central Hospital. The results indicate that there is not a dominant clone of ESBL-/pAmpC positive *E. coli*. However, there are several clades with clonal relatedness indicating minor outbreaks between patients at specific departments. This notion is supported by the isolation of CTX-M-15 producing strains with similar resistance patterns from the Pediatric department linked in time within clade B (data not shown).

The observation of multidrug resistant pAmpC- and/or ESBL-producing *E. coli* in a high proportion of clinical strains during a period of three months is a major concern. *E. coli* is the most prevalent cause of urinary tract infections and Gram-negative bacteremia. A large proportion of the ESBL-producing strains also expressed resistance to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole. In such cases of MDR-septicemia the last resort antibiotics are carbapenems, piperacillin-tazobactam, colistin or tigecycline. Those drugs are not easily available at Maputo

Central Hospital and in developing countries in general. Thus, a significant amount of clinical strains of *E. coli* at the Maputo Central Hospital is in fact not treatable with the current available antibiotics.

In conclusion, our study has shown: (i) a high proportion of ESBL- and/or pAmpC-producing clinical isolates of *E. coli* from the urinary tract and blood cultures at the Central Hospital with CTX-Ms. (ii) CTX-Ms and FOX/MOX were the dominant ESBL- and pAmpC-types, respectively. (iii) All ESBL- and pAmpC-producing strains were MDR-strains only susceptible to antibiotics that are not easily available in the current location. (iv) Studies of the genetic relatedness between ESBL- and/or pAmpC-producing strains show genetic diversity and some clusters indicating within-hospital spread of strains. The overall findings strongly support the urgent need for accurate and rapid diagnostic services to guide correct treatment of serious life-threatening infections and improved infection control measurements. The findings have a probable transfer value to other hospitals in Mozambique as the Central hospital has reference functions with transfer of patients from and to other hospitals.

Acknowledgments

The authors are grateful to the Norwegian Agency for Development Cooperation for funding this study under the Norwegian Program for Capacity Development in Higher Education and Research for Development (NORHED) Grant QZA 0484 RSA 13/0010 on Antibiotic Stewardship and Conservancy. The authors acknowledge the Microbiology Laboratory of Maputo Central Hospital for providing isolates, Microbiology Laboratory of School of Health Science, University of KwaZulu-Natal, Durban, South Africa for providing space and reagents in the accomplishment of the molecular diagnosis. Special thanks goes to Bjorg Christina Haldorsen Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Clinical Microbiology and Infection Control University Hospital of North Norway, Tromsø, Norway.

Conflict of interest

No conflict of interest

REFERENCES

1. Antimicrobial Susceptibility Test Tables (2016). Available from: http//www.eucast.org/fileadmin/src/med.Accessed 14 April 2016.

2. Cherif T, Saidani M, Decre D, Boubaker IB, Arlet G (2016) Cooccurrence of Multiple AmpC β-Lactamases in *Escherichia coli, Klebsiella pneumonia* and *Proteus mirabilis* in Tunisia. Antimicrobial Agents Chemother. 1: 1- 49.

3. Clinical and Laboratory Standards Institute (2015) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. M100-S25.

4. Datta P, Gupta V, Arora S, Garg S, Chander J (2014) Epidemiology of extended-spectrum B-lactamase, AmpC, and Carbapenemase production in Proteus mirabilis. J Infect Dis 67 Suppl 4: 44-46.

5. Egorova SM, Timinouni M, Demartin SA, Granier JM, Whichard V, Sangal L, Fabre A, Delaune´ M, Pardos Y, Millemann E, Espie´ M. (2008) Ceftriaxone-resistant Salmonella enterica serotype Newport, France. Emerg Infect Dis. 14: 954–957.

6. Feglo P, Adu-Sarkodie Y, Ayisi L, Jain R, Spurbeck RR, Springman AC (2013) Emergence of a novel extended-spectrum-beta-lactamase (ESBL)–producing, fluoroquinolone-resistant clone of extraintestinal pathogenic Escherichia coli in Kumasi, Ghana. J Clin Microbiol 51: 728-730.

7. Jacoby GA (2009) AmpC betalactamases. Clin Microbiol Rev. 22: 161-182.

8. Javier FP, Hanson ND (2002) Detection of plasmid- mediated AmpC β-Lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 40 Suppl. 6: 2153-2162.

9. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF (2013) Antibiotic resistance- the need for global solutions. Lancet Infect Dis. 13: 1057-1098.

10. Li B, Sun JY, Liu QZ, Han LZ, Huang XH, Ni YX (2011) High prevalence of CTX-M beta-lactamases in faecal Escherichia coli strains from healthy humans in Fuzhou, China. Scand J Infect Dis 43: 170-174.

11. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y (2011) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert

proposal for interim standard definitions for acquired resistance. Clin Microbial Infect 18: 268-281.

12. Mandomando I, Sigaúque B, Espasa LM, Valles X (2010) Antimicrobial drug resistance trends of bacteremia isolates in rural hospital in southern Mozambique. Amer J Trop Med Hyg; 83 Suppl 1: 152–157.

13. Moon TD, Silva P, Buene M, Morais L, Valverde E, Vermund SH, Brentlinger PE (2013) Bacteremia as a Cause of Fever in Ambulatory, HIV -Infected Mozambican Adults: Results and Policy Implications from a Prospective Observational Study; PLoS One 8: 1-13.

14. Mshana SE, Matee M, Rweyemamu M (2013) Antimicrobial resistance in human and animal pathogens in Zambia, Democratic Republic of Congo, Mozambique and Tanzania: an urgent need of a sustainable surveillance system. Ann Clinical Microbiol Antimicrob 12: 1-10.

15. Ndir A, Diop A, Faye PM, Cisse MF, Ndoye B, Astagneau P (2016) Epidemiological and Burden of Bloodstream Infections Caused by Extencted- Spectrum Beta-Lactamase Producing Enterobacteriaceae in a Pediatric Hospital in Senegal. PLoS One 2: 1-13.

16. Nkrumah NO, Danso K, Krogfelt KK (2013) High levels of Extended-Spectrum Beta-lactamases in a major teaching hospital in Ghana: The need of regular monitoring and evaluation of antibiotic resistance. Amer J Trop Med Hyg 89 Suppl 5: 960-964.

17.Oplustil CP, Zoccoli CM, Toboit NR (2010) Procedimentos basicos em Microbiologia clinica. 3ª Edição S. Paulo: ISBN 978-85-7378-215-8. p- 212- 217.

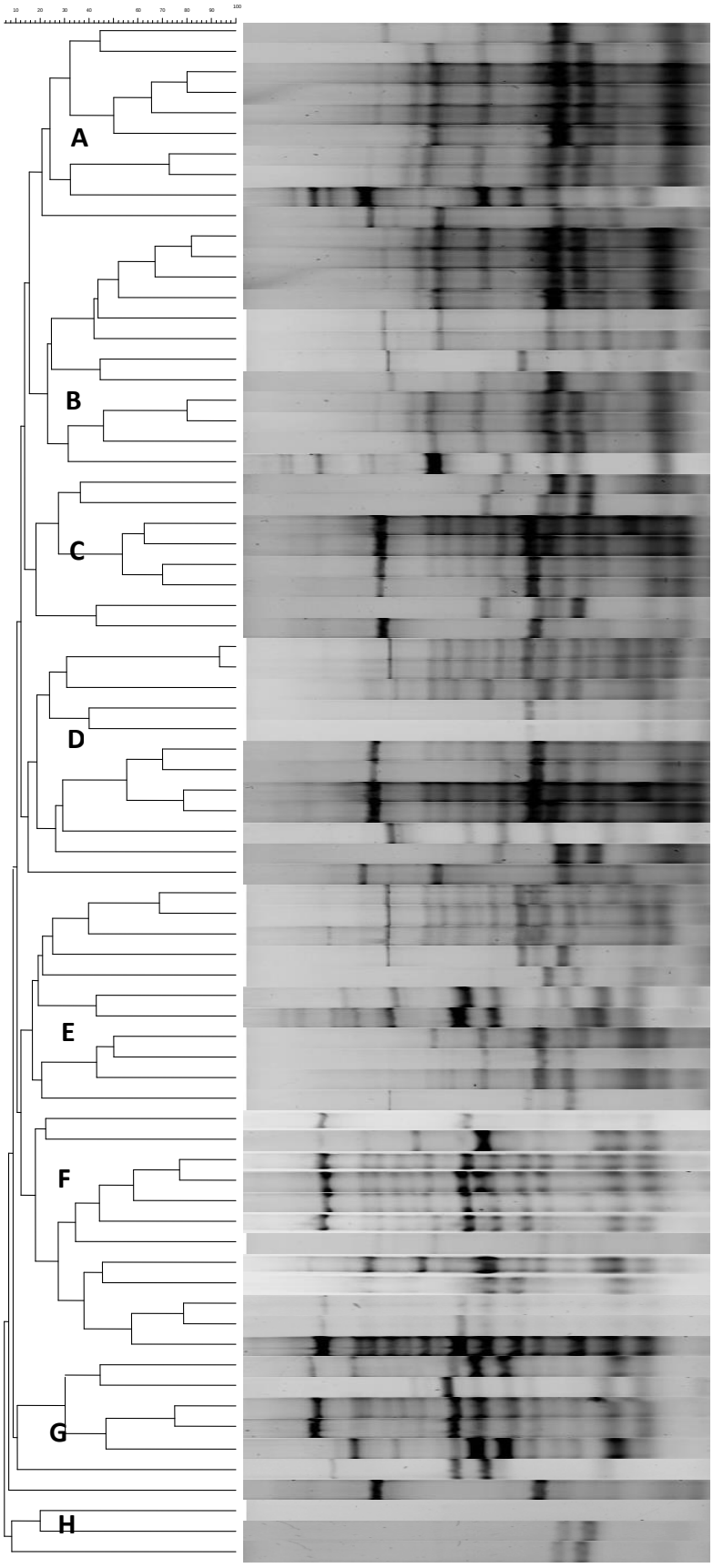
18. Paterson DL (2014) Resistance in Gram- negative bacteria: Enterobacteriacea. Association for professionals in infection control and Epidemiology. J Clin Diag 8 Suppl 6: 5-8.

19. Paterson DL, Bonomo RA (2009) Extended Spectrum B-Lactamases: a clinical update. Clin Microbiol Rev 4: 657-686.

20. Prestinaci F, Pezzotti P, Pantosti A (2015) Antimicrobial resistance: a global multifaced phenomenon. Department of Infections, Parasitic and Immunomediated Diseases, Instituto Superiore di Sanita, Rome, Italy. Pathogens and Global Health. 7: 309-317.

21. Ranjbar R, Karami A, Farshad S, Giammanco G, Mammina C (2014) Typing methods used in the molecular epidemiology of microbial pathogens: a how – to guide. New Microbiologica 37: 1-15.

22. Sangare SA, Maiga AI, Guindo I, Maiga A, Camara N, Savadogo S (2015) Prevalence of extended- spectrum beta-lactamase- producing Enterobacteriaceae isolated from blood cultures in Africa. Medicine et maladies infectieuses 45: 374-382.
23. Sharma J, Sharma M, Ray P (2010) Detection of TEM and SHV genes in *Escherichia coli* and *Klebsiella pneumoniae* isolates in a tertiary care hospital from India. Indian J Med Res 132, 332-336.
24. Tian GB, Wang HN, Zhang AY, Zhang Y, Fan WQ, Xu CW, Zeng B, Guan ZB, Zou LK (2012) Detection of clinically important beta-lactamases in commensal Escherichia coli of human and swine origin in western China. J Med Microbiol 61: 233-2338.
25. Umaer Naseer U, Sundsfjord A (2011).The CTX-M Condrum: Dissemination of Plasmids and Escherichia coli Clones. Microb Drug Res. 20: 1-16.
26. Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes, Nucleic Acids Res. 19; 6823-6831.
27. World Health Organization (WHO). (2015) Global Action Plan on Antimicrobial Resistance. ISBN 9789241509763.



ISOLATE	CTX-M	SHV	TEM	CMY	DHA	MOX	FOX
10156 (U) (P)	+	+	-	-	-	-	-
10642 (U) (O)	-	+	-	-	-	-	-
7421 (U) (P)	+ 15	+	-	-	-	-	-
9481 (U) (P)	+	+	-	-	-	-	-
10088 (U) (M)	+ 15	+	-	-	-	-	-
6187 (U) (P)	+	-	-	-	-	-	-
10646 (U) (P)	+ 15	-	-	-	+	+	+
10822 (U)(O)	+	-	-	-	-	-	-
5848 (B) (M)	-	+	-	-	-	-	-
10846 (U) (P)	+ 15	+	-	-	-	-	-
13040 (U) (P)	+ 15	-	-	-	+	+	+
13304 (U) (P)	+	-	-	-	-	-	-
13302 (U) (P)	+	-	-	-	-	-	-
10869 (U) (P)	+ 15	-	-	-	-	-	-
3490 (U) (P)	-	-	-	-	+	+	+
4980 (U) (M)	-	+	-	-	+	-	+
1000 (U) (O)	+	-	-	-	-	-	-
13305 (U) (P)	+ 28	+	-	-	-	+	+
13309 (U) (P)	+ 15	+	-	-	-	+	+
13310 (U) (P)	+ 15	+	-	-	+	+	+
13308 (B) (M)	+ 36	+	-	-	+	+	+
12900 (B) (P)	+ 15	-	-	+	+	-	-
13301 (U) (P)	+15	-	+	-	-	+	+
6715 (U) (M)	-	+	-	-	-	+	+
11460 (U) (P)	+ 15	-	-	-	-	+	+
12394 (U) (P)	+	-	-	-	-	-	-
10864 (U) (P)	+ 15	+	-	-	-	-	-
11598 (U) (S)	-	-	-	-	-	+	+
12958 (U) (P)	+	+	-	-	+	+	+
13036 (U) (P)	-	+	-	-	-	-	-
4557 (U) (P)	-	+	-	-	-	+	+
5102 (U) (P)	+ 15	-	-	-	-	-	-
5920 (U) (P)	+ 28	+	-	-	+	+	+
5917 (U) (P)	-	+	-	-	-	-	-
6002 (U) (P)	+	+	-	-	-	+	+
6057 (U) (P)	+ 15	-	-	-	+	-	+
6539 (U) (P)	+ 117	+	-	-	-	-	-
6514 (U) (P)	+ 15	+	-	-	-	+	+
6670 (U) (O)	+ 15	-	-	-	-	-	-
12999 (B) (P)	-	-	-	-	-	+	+
9479 (U) (M)	-	+	-	-	-	-	-
6003 (U) (P)	+	+	-	-	-	-	-
1482(U) (M)	+ 15	+	-	-	-	-	-
980 (U) (O)	+	-	-	-	-	-	-
2596 (U) (P)	+	+	-	-	-	-	-
1022 (U) (P)	+	-	-	-	-	-	-
5100 (U) (P)	+	+	-	-	-	-	-
13311 (B) (P)	+ 15	-	-	-	-	-	-
13313 (B) (M)	+	-	-	-	-	-	-
1037 (U) (P)	+	-	-	-	+	+	+
1620 (U) (M)	+	+	-	-	-	+	+
996 (U) (O)	+ 164	+	-	-	-	-	-
3463 (U) (P)	+	-	-	-	-	-	-
13316 (U) (M)	+	-	-	-	-	-	-
2560 (B) (P)	+ 15	-	-	-	-	-	-
13314 (U) (P)	+	+	-	-	-	-	-
1181 ((B) (P)	+	-	-	-	-	-	-
1001 (B) (P)	-	+	-	-	-	-	-
13335 (U) (P)	+	-	-	-	-	-	-
1438 (U) (P)	-	+	-	-	-	-	-
13315 (U) (M)	-	-	-	-	+	+	+
13320 (U) (S)	+ 15	+	-	-	+	+	+
1010 (B) (M)	+	+	-	-	-	-	-
1568 (B) (M)	+	-	-	-	-	-	-
3507 (B) (M)	+	-	-	-	-	-	-
8810 (B) (P)	+ 15	-	-	-	-	-	-
12968 (B) (M)	-	+	-	-	-	-	-
6389 (B) (M)	+	-	-	-	-	-	-
7017 (B) (P)	+ 15	+	-	-	-	+	+
9344 (B) (P)	-	+	-	-	-	-	-
13313_b(U) (M)	+	-	-	-	-	-	-
6818 (U) (O)	+	-	-	-	-	-	-
3450 (U) (P)	+ 15	+	-	-	-	-	-
7922 (U) (P)	-	+	-	-	-	-	-
13088 (U) (M)	+ 15	+	-	-	-	-	-

Figure 1: Dendrogram showing the cluster analysis of *Escherichia coli* isolated from blood and urine samples based on ERIC-PCR fingerprinting patterns using Jacquard index and UPGMA clustering. The scale at the top represents percentage similarity. Isolate columns: identification number is given for each isolate with origin of the isolate in brackets: O = outpatient, P = paediatrics, M = medicine, S = surgery, U = urine, and B= blood. Amplification of CTX-M (and sequence type), SHV, TEM, CMY, DHA, MOX and FOX β-lactamase gene are also indicated for each strain.

Table1. Primer sequences for PCR amplification of ESBL and pAmpC genes

Genes	Primers	Sequence(5' to 3')	Annealing (°C)	Reference
TEM	1	AAAATTCTTGAAGACG	60	[Javier 2002]
	2	TTACCAATGCTTAATCA		
CTX-M	1	GGTTAAAAAATCACTGCGTC	57	[Javier 2002]
	2	TTGGTGACGATTTTAGCCGC		
SHV	1	TTAACTCCCTGTTAGCCA	56	[Javier 2002]
	2	GATTTGCTGATTTCGCCC		
CMY	1	GAT TCC TTG GAC TCT TCA G	50	[Sharma2010]
	2	TAA AAC CAG GTT CCC AGA TAG C		
FOX	1	CAC CAC GAG AAT AAC CAT	50	[Sharma2010]
	2	ATG TGG ACG CCT TGA ACT		
MOX	1	GCTGCTCAAGGAGCA CAG GAT	50	[Sharma2010]
	2	CACATTGACATAGGT GTG GTGC		
DHA	1	AACTTTCACAGGTGTGCT GGGT	50	[Sharma2010]
	2	CCGTACGCATACTGGCTT TGC		

Table2. Antimicrobial susceptibility results for ESBL positive *E. coli* blood isolates (n=17)

ID-NUMBER	WARD	CAZ	CTX	AMC	SXT	CIP	AMP	MER	GEN	FOX	PTZ	CRO
1001	**Pediatric**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**S**	**S**	**S**	**R**
1010	**Medicine**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**S**	**R**
1181	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**S**	**R**
1568	**Medicine**	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
2560	**Pediatric**	**R**	**R**	**I**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
3507	**Medicine**	**R**	**R**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**S**	**R**
5848	**Medicine**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**R**	**R**
6389	**Medicine**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
7017	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**I**	**I**	**R**
8810	**Pediatric**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**
9344	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**I**	**R**	**R**
12900	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**I**	**I**	**R**
12968	**Medicine**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**
12999	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
13308	**Medicine**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
13311	**Pediatric**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
13313	**Medicine**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**

Abbrevations: S- susceptible; I- intermediate; R-resistant, CAZ- Ceftazidime; CTX- Cefotaxime; AMC- Amoxicilin Clavulanic acid;SXT- Trimethoprim-sulfamethoxazole; CIP- Ciprofloxacin; AMP- Ampicilin; MER-Meropenem; GEN- Gentamicin; FOX- Cefoxitin; PZT- Piperacilin-tazobactam; CRO- Ceftriaxone.

Table 3: Antimicrobial susceptibility results for ESBL-positive *E. coli* urine isolates (n=58)

ID-NUMBER	WARD	CAZ	CTX	AMC	SXT	CIP	AMP	MER	NIT	GEN	FOX	PTZ	CRO
980	*Outpatient*	R	R	R	R	I	R	S	S	S	S	R	R
996	*Outpatient*	R	R	R	S	S	R	S	S	R	S	R	R
1000	*Outpatient*	R	R	R	R	R	R	S	R	R	S	S	R
1022	*Pediatric*	R	I	R	R	I	R	S	S	R	S	S	R
1037	*Pediatric*	R	R	R	R	R	R	S	S	R	R	R	R
1438	*Pediatric*	R	R	R	R	S	R	S	S	R	S	S	R
1482	*Medicine*	R	R	R	R	S	R	S	S	S	I	S	R
1620	*Medicine*	R	R	R	R	R	R	S	R	S	R	I	R
2596	*Pediatric*	R	R	R	S	S	R	S	S	R	S	S	R
3450	*Pediatric*	R	R	I	R	S	R	S	S	R	S	S	R
3463	*Pediatric*	R	R	R	R	S	R	S	S	R	S	S	R
3490	*Pediatric*	R	I	R	S	S	R	S	S	S	I	R	R
5917	*Pediatric*	R	R	R	R	R	R	S	R	R	S	I	R
4557	*Pediatric*	R	R	R	R	S	R	S	S	S	I	S	R
4980	*Medicine*	R	R	R	R	S	R	S	S	S	R	S	R
5100	*Pediatric*	R	R	R	R	R	R	S	S	R	S	I	R
5102	*Medicine*	R	R	S	R	S	R	S	S	S	S	S	R
5920	*Pediatric*	R	R	R	R	R	R	S	R	S	R	I	R
6002	*Pediatric*	I	R	R	R	R	R	S	S	S	R	S	R
6003	*Pediatric*	R	R	R	R	R	R	S	R	R	S	R	R
6057	*Pediatric*	R	R	R	R	S	R	S	S	S	R	R	R
6187	*Pediatric*	R	R	R	R	R	R	S	S	S	S	I	R
6514	*Pediatric*	I	R	R	R	R	R	S	R	S	R	S	R
6539	*Pediatric*	R	R	R	R	R	R	S	S	R	S	R	R
6670	*Outpatient*	R	R	R	R	R	R	S	S	R	S	S	R
6715	*Medicine*	R	R	R	R	S	R	S	S	S	I	I	R
6818	*Outpatient*	R	R	R	R	R	R	S	R	R	S	S	S
7421	*Pediatric*	R	R	R	R	R	R	S	S	S	S	R	R
7922	*Pediatric*	R	R	R	R	R	R	S	I	S	S	R	R
9479	*Medicine*	R	R	R	R	R	R	S	S	S	R	R	R
9481	*Pediatric*	R	R	R	R	S	R	S	S	S	R	R	R
10156	*Pediatric*	R	R	R	R	R	R	S	S	R	S	R	R
10642	*Outpatient*	R	I	I	R	S	R	S	S	S	S	S	R
10646	*Pediatric*	R	R	R	R	R	R	S	S	S	R	S	R
10822	*Outpatient*	R	R	R	R	S	R	S	S	S	R	S	R
10846	*Pediatric*	R	R	R	R	S	R	S	S	R	R	R	R
10864	*Pediatric*	R	R	R	R	R	R	S	S	R	R	R	R
10869	*Pediatric*	R	R	R	R	R	R	S	S	S	R	R	R
11460	*Pediatric*	R	R	R	R	R	R	S	R	R	R	S	R
11598	*Surgery*	R	R	R	R	R	R	S	R	S	R	I	R
12394	*Pediatric*	R	R	R	R	S	R	S	R	S	S	S	R
12958	*Pediatric*	R	R	R	R	R	R	S	R	R	R	I	R
13036	*Pediatric*	R	R	R	R	R	R	S	S	R	R	S	R
13088	*Medicine*	R	R	R	R	R	R	S	I	R	R	R	R
13040	*Pediatric*	R	R	R	R	R	R	S	S	S	R	R	R
13301	*Pediatric*	R	R	R	R	R	R	S	R	R	R	S	R
13302	*Pediatric*	R	R	R	R	S	R	S	S	S	R	I	R
13304	*Pediatric*	R	R	R	R	R	R	S	R	S	I	R	R
13305	*Pediatric*	R	R	R	R	R	R	S	S	R	R	I	R
13309	*Pediatric*	R	R	R	R	R	R	S	R	I	R	R	R
13310	*Pediatric*	R	R	R	R	R	R	S	R	R	R	R	R
13314	*Pediatric*	R	R	R	R	R	R	S	R	S	R	R	R
13315	*Medicine*	R	R	R	R	R	R	S	R	S	R	R	R
13316	*Medicine*	R	R	R	R	R	R	S	R	S	S	S	R
13320	*Surgery*	R	R	R	R	R	R	S	R	S	R	R	R
13335	*Pediatric*	R	R	R	R	R	R	S	R	R	R	S	R
13313	*Medicine*	R	R	R	R	R	R	S	S	R	S	R	R
10088	*Medicine*	R	R	R	R	R	R	S	S	S	S	S	R

Abbreviotins: S- susceptible; I- intermediate; R-resistance; CAZ- Ceftazidime; CTX- Cefotaxime; AMC- Amoxacilin Clavulamic acid; SXT- Trimethoprim-sulfamethoxazole; CIP- Ciprofloxacin; AMP- Ampicilin; MER- Meropenem; NIT- Nitrofurantoin; GEN- Gentamicin; FOX- Cefoxitin; PTZ- Piperacilin-tazobactam; CRO- Ceftriaxone

CHAPTER 3. CONCLUSION

3.1 Introduction

Clinical isolates of *E. coli* (n=230) were obtained from urine and blood culture samples collected from in- and outpatient at Central Hospital of Maputo, Mozambique during mid- August to mid- November, 2015. Seventy-five (32.6 %) isolates shown to be resistant to cefotaxime and/ or ceftazidime were selected for further studies. The resistance mechanisms in these isolates towards third generation cephalosporins were investigated using pheno- genotypic methods. The genetic relatedness between strains was delineated.

The following main conclusions emanated from the study with respect to the study objectives.

Objective 1: Clinical isolates of *E. coli* (n=230) were identified using biochemical tests and examined by antimicrobial susceptibility testing (AST).

Objective 2: Seventy-five (32.6 %) of the isolates were resistant to ceftazidime and/or cefotaxime and selected for further studies.

Objective 3: The presence of increased AmpC- and/or ESBL-production was observed using specific double – disc synergy tests in isolates with reduced susceptibility to cefotaxime and/or ceftazidime.

Objective 4: None isolates expressed reduced susceptibility to meropenem. Thus, no strains were further examined for carbapenemase activity.

Objective 5: The ESBLs and pAmpCs at the Central Hospital of Maputo are mediated by CTX-M, SHV and FOX, MOX and CMY betalactamases, respectively. CTX-M-15 is the dominant ESBL and FOX is the dominant pAmpC.

Objective 6: These results will be important in the process of establishing empirical antibiotic treatment guidelines used at the Maputo Central Hospital

1.5 Limitations

1. The strains were collected during a short period of time. There seems to be a preference for sample collection at specific departments (Pediatrics) at the hospital. Thus, the overall prevalence of ESBL- and pAmpC-producing strains may be questioned.

2. Limited resources precluded the final identification of some *bla*$_{\text{CTX-M}}$ and all *bla*$_{\text{SHV}}$ genotypes as well as more extensive comparative genetic analysis of strain relatedness.

1.6 Recommendations

- The study confirmed a relative high occurrence of ESBL- and pAmpC-producing clinical strains of *E. coli* at the hospital. The strains are multidrug resistant. The findings underline the importance of bacterial culture and susceptibility testing to be able to choose the correct antimicrobial treatment. Physicians must request bacteriological samplings for AST to ensure correct use of antibiotics.
- The study found some genetic clusters of pAmpC- and/or ESBL-producing strains supporting strain transfer between departments and patients. This observation strongly supports the need for appropriate infection control measures within the hospital at all level.
- The overall recommendation underline the urgent need for accurate and rapid diagnostic services to guide correct treatment of serious life-threatening infections and improved infection control measurements;

1.7 Significance

- This is the first antimicrobial resistance study conducted at the Central Hospital of Maputo confirming antimicrobial resistance as a serious health problem with the presence of multidrug resistant *E. coli* in all hospital departments.
- Due to a very high occurrence of resistance to trimethoprim-sulfamethoxazole, ampicillin and cephalosporins it is important to review their use in relevant empiric treatment guidelines.
- Considering that the Maputo Central Hospital is a referral hospital within the national health system in Mozambique, appropriate diagnostic services and infection control measures should be established in order to avoid the spread of

resistant strains within the hospital as well as to other hospitals and in the community.

APPENDIX 1

Ethical clearance letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

18 October 2016

Ms CEL Estaleva (214580068)
Discipline of Pharmaceutical Sciences
School of Health Sciences
calylanga@yahoo.com.br

Dear Ms Estaleva

Protocol: Beta lactamase mediated resistance in Escherichia coli from Maputo Central Hospital.
Degree: M H Sc
BREC reference number: BE030/16

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 19 January 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 13 October 2016 to BREC correspondence dated 24 February 2016 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 18 October 2016.

This approval is valid for one year from 18 October 2016. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290108-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be RATIFIED by a full Committee at its next meeting taking place on 08 November 2016.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: essackr@ukzn.ac.za
cc postgrad: nehep19@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8486 Facsimile: +27 (0) 31 260 4609 Email: brec@ukzn.ac.za