Faecal Carriage of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella* spp. in Mozambican University Students

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Submitted in fulfillment for the degree of Master of Medical Sciences in the School of Health Sciences, University of KwaZulu-Natal

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

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 Mr. Lourenço Marcos Chirindze Junior.

As the candidate's supervisor, we have approved this dissertation for submission.

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Signed: ---------- Name: **Prof. Gunnar Skov Simonsen** Date: 14/01/2017

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

DECLARATION

I, Mr. Lourenço Marcos Chirindze Junior, declare as follows:

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.

4. Signed _____ Date_____

DEDICATION

I dedicate this research to my mother, an example of a courageous women, faith and a strong warrior, who always shows me the right way to follow when I am in doubt about the right decision to make. I dedicate this work to my daughters, my inspiration to keep walking in every step of my carrier.

ACKNOWLEDGEMENTS

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- To Raúl Antonio Vaz, a Technician that helped me a lot with the media preparation.

LIST OF ACRONYMS/ABBREVIATIONS

API Analytical Profile Index

ATCC American Type Culture Collection

CLSI Clinical and Laboratory Standards Institute

CNS Central Nervous System

DNA Deoxyribonucleic Acid

ESBL Extended-Spectrum β-lactamase

MIC Minimum Inhibitory Concentration

PCR Polymerase Chain Reaction

UTI Urinary Tract Infection

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ABSTRACT

In recent years, the world has seen a surge in extended-spectrum β-lactamase (ESBL)-producing bacteria. Among antibiotic resistance mechanisms, the production of β-lactamase is the most rapidly developing and clinically significant in Gram-negative bacteria. In the present study, a total of 275 stool samples were collected from students of both sexes in three student residencies of Eduardo Mondlane University-Mozambique from January to February 2016. All samples were cultured on MacConkey agar with ceftriaxone (1mg/L) and without ceftriaxone. The isolates were biochemically identified with API20E test. Confirmed *E. coli* and *Klebsiella* spp. isolates were subjected to antimicrobial susceptibility testing by the disc diffusion method and ESBL strains were confirmed with the disc approximation method. From these samples, 56 ESBL positive *E. coli*(n=35) and *Klebsiella* spp. (n=21) strains were isolated. Among the ESBL-positive isolates, 39.3% (22/56) were cefoxitin resistant and none were confirmed as carbapenemase producers. The frequency of ESBL colonization in both sex were similar for *E. coli* and *Klebsiella* spp**.** Among the ESBL-positive isolates, 50% (28/56) of the isolates only contained class A ESBLs, 5.4% (3/56) only class C ESBLs, and 44.6% (25/56) both class A and C ESBLs. Among the *E. coli* strains, 100% were resistance to ampicillin, and both *E. coli* and *Klebsiella* spp. demonstrated69.6% resistance to tetracycline and cotrimoxazole, 62.5% to ceftazidime, 33.9% to ciprofloxacin, and 34.8% to cefoxitin. None of the isolates showed resistance to meropenem. In total, 78.6 % of ESBL strains were defined as multi-resistant. The ERIC-PCR demonstrated low similarity among the strains. This study demonstrated that the carriage rates and the diversity of ESBL genes among the students are high.

Contents

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

In recent years, the world has seen a surge in extended-spectrum β-lactamase (ESBL) producing bacteria (1). Among resistance mechanisms, the production of β-lactamase is the most rapidly developing and clinically significant in Gram-negatives (2,3).

Members of the *Enterobacteriaceae* family are inhabitants of the intestinal flora and are among the most common human pathogens that cause community and hospital-acquired infections. They have the propensity to spread easily between humans through hand carriage, contaminated food and water, and to acquire genetic material through horizontal gene transfer often mediated by plasmids and transposons (4).

The transmission of ESBL-positive bacteria may also occur via the faecal-oral route and is facilitated by overcrowding. Among the risks factors for colonization with ESBL, the literature describes prolonged hospital stays, recent surgery, prior antibiotics use, particularly quinolones and third-generation cephalosporins, but also cotrimoxazole, aminoglycoside, and metronidazole (32,36).

The commensal glut flora is a very highly populated ecosystem and its constituents may, at later stages, become a source of extra-intestinal infections. Resistance determinants may also spread to other members of the micro-biota, including potential pathogens (5).

Among members of the *Enterobacteriaceae* family, the production of extended spectrum β-lactamases (ESBL) is an important mechanism of resistance to β-lactam antibiotics (6).*Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are common opportunistic pathogenic species of *Enterobacteriaceae* that frequently incorporate ESBL-encoding genes (7).ESBL production is often encoded on plasmids (8) that have a high capacity for spread via horizontal gene transfer mechanisms (9).

B-lactamases are a large group of enzymes capable of hydrolyzing the β-lactam ring of various groups of β-lactam antibiotics thus rendering them inactive (10,11,12,35). Among the β-lactamases, extended-spectrum β-lactamases have a broad substrate range including third generation cephalosporins, and are by the classical definition inhibited by clavulanic acid (13).

Since the early 1980s, third-generation cephalosporins have become an important weapon in the treatment of severe bacterial infections, and the acquisition of ESBL enzymes by a high number of bacterial species is thus a cause for great concern (14).

Data on the spread of ESBL-producing bacteria in Sub-Saharan Africa is needed to assess the extent of this emerging health threat in resource-poor settings (1), but information about the epidemiology of ESBL-producing bacteria in this region of Africa is still limited. There are only sporadic reports about the prevalence of ESBL-producing bacteria in clinical isolates, and very few studies have systematically collected data on the prevalence of colonization with these pathogens (15).

Our knowledge about the prevalence of ESBL is even more limited concerning clinical isolates from Mozambique and particularly Maputo province. The prevalence of ESBL colonized subjects still remains unknown.

No study has been done to determine the rate of faecal colonization of antibiotic resistance in *E. coli* and *K. pneumoniae/Klebsiella* spp. among students at Eduardo Mondlane University, Maputo.

Research Question: What is the prevalence of colonization by ESBL-positive *E. coli* and *K. pneumoniae/Klebsiella* spp. among students at Eduardo Mondlane University, Maputo?

1.2. AIM & OBJECTIVES

Aim

- To explore the prevalence of ESBL-positive *E. coli* and *Klebsiella* spp. carriage in students living in the University residence at Eduardo Mondlane University.

Objectives

- To isolate and confirm the identity of *E. coli* and *Klebsiella* spp. from stool samples of University students.
- To correlate the frequency of *E. coli* and *Klebsiella* spp. ESBL colonization with gender, use of antibiotics and hospitalization.
- To ascertain the antibiotic susceptibility of ESBL-positive isolates against an appropriate panel of antibiotics by standardized agar disc diffusion and/or minimum inhibitory concentration (MIC) determinations using CLSI guidelines.
- To phenotypically confirm the presence of ESBLs using the double-disc synergy test/E-test.
- To identify the ESBL genes by PCR and sequencing.
- To undertake strain typing by ERIC-PCR to determine possible clonal relationship.

1.3. LITERATURE REVIEW

1.3.1. Enterobacteriaceae family

The *Enterobacteriaceae* family is the most heterogeneous group of Gram-negative bacilli of medical importance. The *Enterobacteriaceae* are the cause of a large group of diseases in humans, including 30% to 35% of all bacteremias, more than 70% of urinary tract infections (UTI) and many intestinal infections (16). They are transmitted both from other animals or humans and from the inanimate environment. Many infections arise from the body's normal flora when opportunities are provided by medical, surgical or other therapies (17).

E. coli and *Klebsiella pneumoniae* are members of the *Enterobacteriaceae* family and commensal members of the intestinal glut flora that can cause opportunistic infection (16).

1.3.2. *Escherichia coli*

E. coli is a ubiquitous human pathogen (7,18). It is a common cause of urinary tract infections (UTI) (7,12) and bacteremia in humans of all ages. It is a frequent cause of varied organ infections, ranging from the biliary system to the CNS. The spectrum of pathology can range from a spontaneously resolving cystitis to a life-threatening sepsis syndrome.

There is a variety of reasons for the increased prevalence of antibiotic resistant *E. coli*. One of them is that *E. coli* is an organism known for its flexible genome and propensity to exchange genetic material (7).

E. coli is the main aerobic component of the mixed flora in intra-abdominal infections (12). *E coli* has concomitantly become the most prevalent species among ESBL-producing *Enterobacteriaceae* isolates in community. Such isolates have been recognized as a common cause of hospital and community-onset infections.

1.3.3. *Klebsiella pneumoniae*

Klebsiella pneumoniae is an important human pathogen, causing predominantly nosocomial infections (19,20). Its most common mechanism of resistance to oxyimino-cephalosporins is the production of extended-spectrum β-lactamases (19).

In humans, *K. pneumoniae* is present as a saprophyte in the nasopharynx and the gastrointestinal tract. It is estimated that the rate of carriers varies from5-38% in faeces and from 1-6% in the nasopharynx (16).

Klebsiella pneumoniae is responsible for up to 10% of all nosocomial infections, and this proportion has been increasing due to the emergence and progressive spread of multidrug resistance and specifically the ESBL strains in hospital settings (21). In some countries the prevalence of *Klebsiella pneumoniae* ESBL-producer approaches 50%(22).

1.3.4. Mechanism of resistance in *Enterobacteriaceae*

Antibiotic resistance is now regarded as a major public health problem. In comparison with infections caused by susceptible bacteria, those caused by multidrug-resistant bacteria are associated with higher mortality, as well as increased costs because of prolonged hospital stay and the need for more expensive antibiotics as therapy (23).

In many developed countries, the use of antibiotics is to some degree controlled. This is generally not the case in developing countries, where the treatment of bacterial infections is empirical (24,25). Hopefully, interventions to restrict and improve antibiotic use may slow down the problem of resistance.

Enterobacteriaceae may become resistant to all β-lactam antibiotics and frequently co-resistant to most other antibiotics, leaving very few treatment options. Since the 1950s and 60s, when broad-spectrum antibiotics became available for the treatment of Gram-negative infections, *Enterobacteriaceae* have acquired a growing range of mechanisms to evade these agents. In particular, β-lactam antibiotics such as penicillins and cephalosporins are vulnerable to hydrolysis by enzymes called β-lactamases (26).

1.3.5. β-Lactam Antibiotics

The β-lactam class of antibiotics constitutes the largest family of antibiotics, widely used in clinical practice for the treatment of community-acquired and hospital-acquired infections (27). Theβ-lactams are classified into penicillins, cephalosporins, carbapenems, monobactams and β-lactamase inhibitors (28).All β-lactam antibiotics interfere with bacterial cell wall synthesis by inhibiting the transpeptidase enzyme forming cross links between peptide chains linked to the peptidoglycan framework. Inhibition of this function leads to lysis of the bacterial cell.

1.3.6. Extended Spetrum β-Lactamase (ESBL)

ESBLs were initially identified as variants of the common SHV-1 or TEM-1 β-lactamase, often differing from the parent enzymes by only one or two amino acids (27). Based on substrate

specificities, the β-lactamase family is divided into 4 functional groups: penicillinases, extended-spectrum β-lactamases (ESBLs), carbapenemases, and AmpC-type cephalosporinases (24). Alternatively, β-lactamases can be classified on the basis of structural relationship into Ambler class A (serine β-lactamases including classical ESBLs inhibited by clavulanic acid), class B (metallo-β-lactamases), class C (serine β-lactamases including AmpC enzymes not inhibited by clavulanic acid) and class D (OXA β-lactamase). β-lactamase production is the cardinal mechanism of resistance to β-lactams in Gram-negative organisms. Some species produce chromosomal β-lactamases, but plasmid-mediatedβ-lactamases have become prevalent among many Gram-negative bacteria during the past 50 year (29).

Plasmid mediated production of enzymes inactivate modern expanded-spectrum cephalosporins by hydrolyzing their β-lactam ring. This is the most important mechanism of resistance in *Enterobacteriaceae* (24,30). The successful spread of these plasmids is often attributed to selective pressure resulting from long use of antibiotics in clinical and veterinary medicine (31).

More than 200 types of ESBLs, the results of multiple mutations, have been described in various species of the *Enterobacteriaceae* family and other non-enteric organisms, including *Pseudomonas aeruginosa* and *Acinetobacter* spp. (32).High rates of intestinal ESBL colonization have been reported in Asia, with predominance of CTX-M enzymes (33).

In Europe, there was an increase in invasive infections caused by *Klebsiella pneumoniae* and *Escherichia coli* resistant to third-generation cephalosporins between 1999 and 2008. The SMART study concluded that in Europe, the ESBL prevalence among *E. coli* and *K. pneumoniae* was 17.6% and 38.9%, respectively. In North America, the prevalence was 8.5% and 8.8%, respectively. In Asia, the prevalence of ESBL among *E. coli* was found to be 5% and among *K. pneumoniae* 0%, in New Zealand this prevalence varies between 67 and 61%, respectively (34).

Faecal carriage of ESBL in África

In Africa, the prevalence of ESBL has been researched at local levels but not summarized for the continent as a whole (34). Some reported studies conduted in our continent have shown high prevalence of ESBL. A study conducted in Bangui, Central African Republic, revealed 59% of ESBL carriage, one of the highest reported worldwide (37). Another study of ESBL carriage conducted in North Africa (Casablanca, Marrocos), in community setting has found 4.5% of carriage (38).

1.4. Chapter structure

This research presents the following chapters:

Chapter 2. Manuscript:for publication entitled “Faecal Carriage of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella* spp. in Mozambican University Students". The manuscript addresses the objectives stated above.

Chapter 3. Conclusion: This chapter describes the conclusions, the limitations of the study, the recommendations and the significance of the study.

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CHAPTER 2: MANUSCRIPT

The findings are reported in the following manuscript intended for submission to the African Journal of Infections Diseases:

Chirindze LM, Sekyere JO, Govinden U, Simonsen GS, Zimba TF, Chenia HY and Essack SY. Faecal Carriage of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella* spp. in Mozambican University Students

Contributions:

Mr Lourenco Chirindze, as the principal investigator, developed the protocol, undertook the laboratory work and data analysis, and prepared the manuscript.

Dr. Tomas Zimba, as a co-supervisor, facilitated the preliminary laboratory work and contributed to the critical revision of the manuscript.

Professor Gunnar Skov Simonsen, as co-supervisor, co-conceptualized the study, ensured quality control of preliminary laboratory work and undertook critical revision of the manuscript.

Dr. J Osei Sekyere facilitated the laboratory work and data analysis, and contributed to the critical revision of the manuscript.

Dr. Usha Govinden facilitated laboratory work and data analysis and contributed to the critical revision of the manuscript.

Dr. Hafizah Chenia facilitated and assisted with analysis of the ERIC-PCR and contributed to critical revision of the manuscript.

Professor Sabiha Essack, as principal supervisor, co-conceptualized the study, facilitated data analysis and undertook critical revision of the manuscript.

Sequencing of PCR products was done by Inqaba Biotechnology, Pretoria.

Faecal Carriage of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella* spp. in Mozambican University Students

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Running title: Faecal carriage of ESBLs in University students

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INTRODUCTION

In recent years, the world has seen a surge in extended-spectrum β-lactamase (ESBL)-producing bacteria (1). Among antibiotic resistance mechanisms, the production of β-lactamase is the most rapidly developing and clinically significant in Gram-negative bacteria (2,3). ESBLs have a broad substrate range including third and fourth generation cephalosporins and are, by the classical definition, inhibited by clavulanic acid (4). ESBL genes are often carried on plasmids(5) that have a high capacity for spreading via horizontal gene transfer mechanisms(6,7).

The commensal gut flora is a highly populated ecosystem, the bacterial constituents of which carry resistance genes that can spread to other members of the micro-biota (8). These include *Escherichia coli* and *Klebsiella pneumoniae,* which may, at later stages, become a source of extra-intestinal infections. Both *E. coli* and *K. pneumoniae* are common opportunistic pathogens that frequently harbor ESBL-encoding genes (9).

Among the risks factors associated with the colonization with ESBL are prolonged hospital stay, recent surgery, and prior antibiotics use, particularly quinolones, third-generation cephalosporins, cotrimoxazole, aminoglycoside, and metronidazole (10-11).

Data on the epidemiology of ESBL-producing bacteria in Sub-Saharan Africa is still limited. There are only sporadic reports about the prevalence of ESBL-producing bacteria in clinical isolates, and very few studies have systematically collected data on the prevalence of colonization with these pathogens (12).

METHODOLOGY

Ethical considerations

Ethical approval was received from the Biomedical Research Ethics Committee of University of KwaZulu-Natal (BE214/16) and the Bioethical Council ISCISA-Mozambique (TFCMCSCLJ03/15).

Study sample

A total of 275 stool samples were collected from students of both sexes in three student residencies of Eduardo Mondlane University-Mozambique within a six-week period, from January to February 2016. All samples were cultured on MacConkey agar with ceftriaxone 1mg/L and without ceftriaxone. From these samples, 56 ESBL positive *E. coli* and *Klebsiella* spp. strains were isolated. Two strains were isolated from the same student. These putative ESBL-producers constituted the study sample.

Identification and susceptibility test

All the lactose-positive isolates growing on MacConkey agar impregnated with 1mg/L ceftriaxone were subjected to identification tests using API20E. Confirmed *E. coli* and *Klebsiella* spp. were subjected to antimicrobial susceptibility testing by disc diffusion method with the following antibiotics: ampicillin, cefoxitin, ceftazidime, ceftriaxone, meropenem, amikacin, gentamicin, ciprofloxacin and cotrimoxazole. The results were interpreted according to the CLSI breakpoints to determine their susceptibility profile (13).

Phenotypic detection of β-lactamases

The disc approximation method that consists of ceftazidime and ceftriaxone in addition to amoxicillin/clavulanic acid discs was used for ESBL confirmation (14). ROSCO discs (Rosco Diagnostic, Taastrup, Denmark) were used for ESBL and AmpC production confirmation(15). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive quality control strains, respectively.

Genotypic characterization of β-lactamases

For the DNA extraction, 18-24 hour-colonies grown on Muller Hinton agar were inoculated in Luria-Bertani(LB) broth (16) and incubated at 37°C with shaking. After 20 hours of incubation, extraction was done using Fungal/Bacterial DNA MiniPrep kit (Thermo Fisher Scientific, Lithuania). The PCR for detection of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX}$,*bla*$_{CMY}$, *bla*$_{DHA}$, *bla*$_{FOX}$ and *bla*$_{MOX}$ was performed on a ThermalCycler T100^{TM} (Bio-Rad, USA) with a final volume of 50µL (25µL of Master mix, 15µL of water, 4µL of each primer (Inqaba Biotechnology Industries, South Africa) and 2µL of the template DNA), with an initial denaturation temperature of 98^{o}C for 10 seconds, extension at 72^{o}C for 15 seconds and a final extension at 72^{o}C for 1 minute. The annealing temperature for the genes was:*bla*$_{TEM}$60^{o}C, *bla*$_{SHV}$56^{o}C, and *bla*$_{CTX}$57^{o}C. The annealing temperature for *bla*$_{CMY}$ was 57^{o}C, while that of *bla*$_{DHA}$, *bla*$_{FOX}$ and *bla*$_{MOX}$ was 50^{o}C.

The PCR products were loaded on a 1.5 % (w/v) agarose gel. The products were visualized by UV transillumination (Bio-Rad ChemiDoc^{TM}MP System) after staining in 0.1 mg/mL Gel Red for 15 min. PCR products were sent to Inqaba Biotech, South Africa for DNA sequencing.

TABLE 1. Primers used for amplification

Target enzyme	Primers	Sequence (5' to 3')	Anealling temperature
TEM-1	TEMMF	AAA ATT CTT GAA GAC G	
	TEMMR	TTA CCA ATG CTT AAT CA	60^{o}C (29)
SHV	SHVMF	TTA ACT CCC TGT TAG CCA	
	SHVMR	GAT TTG CTG ATT TCG CCC	56^{o}C (29)
CTX-1	CTXMF	GGT TAA AAA ATC ACT GCG TC	
	CTXMR	TTG GTG ACG ATT TTA GCC GC	57^{o}C (27)
CMY	CMYMF	GAT TCC TTG GAC TCT TCA G	
	CMYMR	TAA AAC CAG GTT CCC AGA TAG C	57^{o}C (28)
FOX	FOXMF	CAC CAC GAG AAT AAC CAT	
	FOXMR	ATG TGG ACG CCT TGA ACT	57^{o}C (28)
DHA	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	57^{o}C (28)
MOX	MOXMF	GCT GCT CAA GGA GCA CAG GAT	
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C	50^{o}C (28)

Genomic DNA isolation

Genomic DNA was isolated from 35 *E. coli* and 21*Klebsiella* spp. isolates and purified using the Gene Jet Genomic DNA purification Kit (Thermo Scientific). Antibiotic sensitive *E. coli* ATCC 25922 and β-lactam-resistant *K. pneumoniae* ATCC 700603 were used as controls for comparison.

ERIC-PCR analysis

The total PCR reaction volume was 10 µL, which contained 2 µL of template DNA and 0.1µLprimers and 5 µL of DreamTaq (Thermo Scientific). The primers ERIC 1 and ERIC 2 (Versalovic et al., 1991)were used. 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, in an Applied Biosystems 2720 thermal cycler. The ERIC-PCR products were loaded onto 1% (w/v) agarose gels and subjected to electrophoresis at 80V using 1× TAE buffer. Amplification products were visualized by UV transillumination (Syngene, UK)

after staining in 0.1 mg/mL ethidium bromide for 15 min. Genotypic variation were analyzed using the GelCompareII version 6.0 software package (Applied Maths) by Jacquard and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to produce a dendogram.

RESULTS

Setting

Among 275 collected samples, 159 (57.8%) were collected from male students and 116 (42.2%) from females, all varying from 19 to 32 years old. The students live in separated blocks and/or floors for male and female students. Each floor has one kitchen where students can prepare their own food. There are students from different courses: Engineering, medicine, political science, biology, sociology, and others. As we had to go room by room explaining the student about the research, there were not too much difficulties in giving sample. The challenge was in convincing the students that the samples were only for the objectives stated in the research. The issue was in health sciences students who demonstrated difficulties to give samples, but helped us in recruiting the other students to participate. the student that. All students who signed the informed consent, gave the sample.

Frequency of *E. coli* and *Klebsiella* spp. ESBL colonization

From a total of 275 samples collected, 140 bacterial colonies grew on the MacConkey+ceftriaxone agar (1mg/L). Among them, 25% (35/140) were confirmed as *E. coli* and 15% (21/140) as *Klebsiella* spp. Among the participants in this study, 50% of the carries (28/56) were male and 50% female, and the frequency of colonization in both sex were similar for *E. coli* and *Klebsiella* spp. Thus 56/140 isolates (40%) isolates were used for subsequent tests.

ESBL confirmation by double disc synergy and by ROSCO discs

All the 56 isolates identified as *E. coli* (n=35) and *Klebsiella* spp. (n=21) were confirmed as ESBL producers. Among the ESBL-positive isolates, 39.3% (22/56) were cefoxitin resistant and confirmed with the ROSCO discs containing cefotaxime, cefotaxime+boronic acid, ceftazidime and ceftazidime+boronic acid as AmpC producers.

Carbapenemase confirmation by Carba NP-test

Only two isolates showed reduced susceptibility to imipenem and meropenem by disc diffusion. These were confirmed as carbapenemase negative by the Carba NP-test, which means that among the strains enrolled in this study there were no carbapenemase producers.

ESBL gene identification by PCR

The PCR results are summarized in Table 2.

Table 2: Distribution of the ESBL enzymes according to the species

ESBL Enzymes	*E. coli*	*Klebsiella* spp.	Total
TEM	10(17.9%)	2(3.6%)	12
CTXM1	32(57%)	9(16.1%)	41
SHV	32(57%)	7(5.4%)	39
CMY	9(16.1%)	3(5.4%)	12
FOX	13(23%)	4(7.1%)	17
MOX	7(12.5%)	4(7.1%)	11
DHA	17(30.4%)	4(7.1%)	21
TOTAL	120	33	153

Among the ESBL-positive isolates, 61% were positive for at least two enzymes, 43% were positive for at least three, and 25% positive for at least 4 enzymes. On the other hand, 50% (28/56) of the isolates contained only class A ESBL, 5.4% (3/56) only class C ESBLs, and 44.6% (25/56) both class A and C ESBLs.

Antibiotic susceptibility

The *E. coli* and *Klebsiella* spp. strains showed high resistance rates to ampicilin (100% respectively), followed by tetracycline and co-trimoxazol (69.6%), ceftazidime (62.5%), ciprofloxacin (33.9%), and cefoxitin (34.8%). None of the isolates showed resistance to meropenem.

Table 3: Antimicrobial resistance of ESBL-producing *E. coli* and *Klebsiella* spp.

	Isolates	
Drugs	*E. coli*	*Klebsiella* spp.
Cefoxitin	37%	47%
Ciprofloxacin	37.1%	28.6%
Ceftazidime	71.4%	42.9%
Ampicillin	100%	100%
Gentamicin	14.3%	42.9%
Tetracycline	65.7%	76.2%
Ceftriaxone	100%	100%
Cotrimoxazole	62.9%	76.2%
Imipenem	0%	0%

The percentage of multi-resistance, defined as resistance to three or more antibiotics, was high among the isolates, with 25% (14/56) showing resistance to six antibiotics, 46% (26/56) to five and 19.6% to three antibiotics. In total, 78.6 % of ESBL strains were defined as multi-resistant.

Antibiotic consumption and hospitalization

No participants declared a story of hospitalization within six months prior to the study and 87.5% of them had not consumed any antibiotics for at least three months.

ERIC-PCR results for *E. coli*

Distinct ERIC-PCR profiles were obtained for the 35 *E. coli* isolates from university students residing in the same residencies (Fig. 1), compared to the antibiotic susceptible *E. coli* ATCC

25922 strain. The absence or presence of a band was noted in determining variation among the strains and banding patterns comprised between 2 and 14 individual bands. Polymorphisms based on fragment length were obtained as a means of differentiating *E. coli* isolates. Fragments of different molecular weights were observed in the ERIC-PCR fingerprints, ranging from 0.5 – 20 kb (Fig.1). Amplification of different intensities was observed and visual analysis of the ERIC profiles included primary, secondary and tertiary amplification (Fig. 1). 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 typeable using this fingerprinting technique and band profiles were reproducibly obtained under similar experimental conditions on repeat amplification.

The ERIC–PCR profiles allowed the differentiation of the 35 *E. coli* isolates into 24 ERIC-PCR types which were grouped into 15 clusters (A – O), with each of the clusters being sub-divided into multiple sub-clusters (Fig. 1). Isolates demonstrated up to 35% similarity to *E. coli* ATCC 25922. CTX-M and SHV genes were amplified from isolates in different clusters and were the most prevalent of the β-lactamase genes identified. Isolates with similar profiles demonstrated varying β-lactamase gene content.

ERIC-PCR results for *Klebsiella* spp.

Twenty-three *Klebsiella* spp. isolates were selected for ERIC-PCR analysis in comparison to SHV-containing *K. pneumoniae* ATCC 700603. Distinct profiles were obtained for all isolates tested

using ERIC-PCR fingerprinting (Fig. 2). The absence or presence of a band was noted in determining variation among the strains and banding patterns comprised between 2 and 16 individual bands. Polymorphisms based on fragment length were also used as a means of differentiating *Klebsiella* spp. isolates. Fragments of different molecular weights were observed in the ERIC-PCR fingerprints, ranging from 0.5 – 20 kb (Fig. 2). Amplification of different intensities was 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 than the tertiary amplification products, while the tertiary amplification products are the minor amplification products of low intensity. All isolates were typeable using this fingerprinting technique, and band profiles were reproducibly obtained under similar experimental conditions on repeat amplification.

The ERIC–PCR profiles allowed the differentiation of the 23 isolates into 17 ERIC-PCR types which were grouped into 12 clusters (A – L), with each of the clusters being sub-divided into multiple sub-clusters (Fig. 2). Isolates demonstrated up to 34% similarity to *K. pneumoniae* ATCC 700603. CTX-M and SHV genes were the most prevalent of the β-lactamase genes amplified and were identified in isolates from different clusters, predominantly clusters B-G. Isolates with similar profiles demonstrated varying β-lactamase gene content.

DISCUSSION

Antibiotic resistant microorganisms are an emerging cause of infection in Mozambique and worldwide, but information regarding the resistance mechanisms of extended-spectrum ß-lactamase (ESBL)-producing *Enterobacteriaceae* in our country remains scarce. To our knowledge, there are no previous studies regarding gastrointestinal ESBL colonization that describes the frequency of carriage among students.

We have systematically collected samples from healthy students to determine the level of colonization by *E. coli* and *Klebsiella* spp. ESBL producer and their antibiotic susceptibility. In a total of 140 ceftriaxone resistant isolates from faecal samples, 25% were identified as *E. coli* and *Klebsiella* spp. ESBL producers. Similar results were found in a study conducted in children attending pre-school childcare facilities in the Lao People's Democratic Republic where the prevalence was 23% (17) and in a study conducted in Korea which found 28.2%, reporting the frequency of *E. coli* to be higher (78%) than the frequency of *Klebsiella* spp. (18%) (18).

A study recently conducted in Madagascar (12) demonstrated lower rates of colonization by ESBL Gram-negative bacilli compared to this study despite the fact that more species were included whereas our study worked only with *E. coli* and *Klebsiella* spp.

Colonization in the intestinal gut by ESBL-producing isolates has been associated with a high risk for developing infection due to ESBL producers (19). To screen for carriage is the key to predict the risk of ESBL infection by extra intestinal pathogenic *E. coli* (19). An example of community acquired ESBL infection is *E. coli* community associated strains that can reach high levels of prevalence such as 30% - 60% (20).

This study showed a high percentage of ESBL carriage compared to a study conducted in France where the frequency of ESBL carriage was 5.3% (21). This big difference may be because in developing countries, antibiotic consumption is poorly controlled and hygiene conditions are suboptimal (22). In Mozambique, antibiotic therapy is mostly empirical because of scarce microbiology facilities (30).

A study conducted in United Kingdom (23) demonstrated 31% resistance to cephalosporins, 20% to cotrimoxazole and 79% to tetracycline , while this study revealed higher resistance rates to cephalosporins and cotrimoxazole (81.3% and 69.6% respectively), but lower resistance rates to tetracycline.

We have found 39.9% of co-existence of ESBL and AmpC β-lactamases. Our rate of ESBL/AmpC co-existence in *Enterobacteriaceae* is higher compared to the one clinically reported in Turkey as 13.9% (36) and as 19.5% in Europe (35).

Most of the isolates on this study carried $bla_{\text{CTX-M-15}}$ (71.4%), followed by $bla_{\text{CTX-M-55}}$ (14.3%), $bla_{\text{CTX-M-186}}$ (9.5%) and $bla_{\text{CTX-M-3}}$ (4.8%). These results are different from the findings in a study conducted in Kenya (28) which demonstrated 29% of the isolates carrying $bla_{\text{CTX-M-15}}$, 4% carrying $bla_{\text{CTX-M-3}}$ and no isolates carrying$bla_{\text{CTX-M-55}}$ or $bla_{\text{CTX-M-186}}$. However, our results are similar to the ones in a study conducted in Niger (21) and in Tanzania (33) that found $bla_{CTX\text{-}M\text{-}15}$gene in 90% and 94.7% of the carriers, respectively.

The $bla_{\text{CTX-M-15}}$ seem to be the major type in humans (34) and exhibits enhanced catalytic efficiencies against ceftazidime (25). This fact can justify the reason why we have found high resistance rates to this antibiotic (59.5%), because in our study the $bla_{\text{CTX-M-15}}$ was the most

predominant among the CTX-Ms. The CTX-M is most prevalent in *E. coli*, *Klebsiella* spp. and *Proteus (*26), but is mainly produced by *E. coli* and has become predominant in the community (27).

In this study, we have found that no participants had a story of hospitalization within six months prior to the study and 87.5% of them had not consumed any antibiotics for at least three months. This suggests that the high antibiotic resistance rates found in this study are not related to antibiotic consumption or hospitalization, suggesting that the *E. coli* and *Klebsiella* spp. ESBL producers isolated in this study are probably community acquired.

In the university residencies, there is a mix of students from different courses including medicine course and health sciences. These ESBL-colonized students are going to work in health institutions, which may constitute a reservoir of multi-resistant microorganisms that can spread among patients thus increasing the problem of antibiotic resistance.

To control the rapid spread of ESBL among students and, consequently, among the general population, it is necessary to educate the students about the importance of personal and general hygiene and develop more studies in order to know the prevalence of colonization in different groups of the population. The prevalence in general may vary depending on socioeconomic status of individuals involved (29), which makes it difficult to estimate the prevalence in the general population as a whole. However, one may suppose that the prevalence in the students is high because of the condition they live under (eg.: many individuals sharing the same bath room and kitchen) .

The multiple different strains illustrated in the REP-PCR indicates that there is at present no outbreak at the strain level, although there could be dissemination of one or more plasmids. One may speculate that students are exposed to ESBL strains from some external source like dissemination in the food supply. The students eat food prepared at the general kitchen at the student residence, but there are other alternative kitchens in each residency block to allow the students to cook their own food.

Very limited is known about dissemination of ESBL strains in the food supply in Mozambique, but it is known from other countries that ESBL *E. coli* and *Klebsiella* spp. may disseminate among food animals and environmental sources (31,32,37,38).

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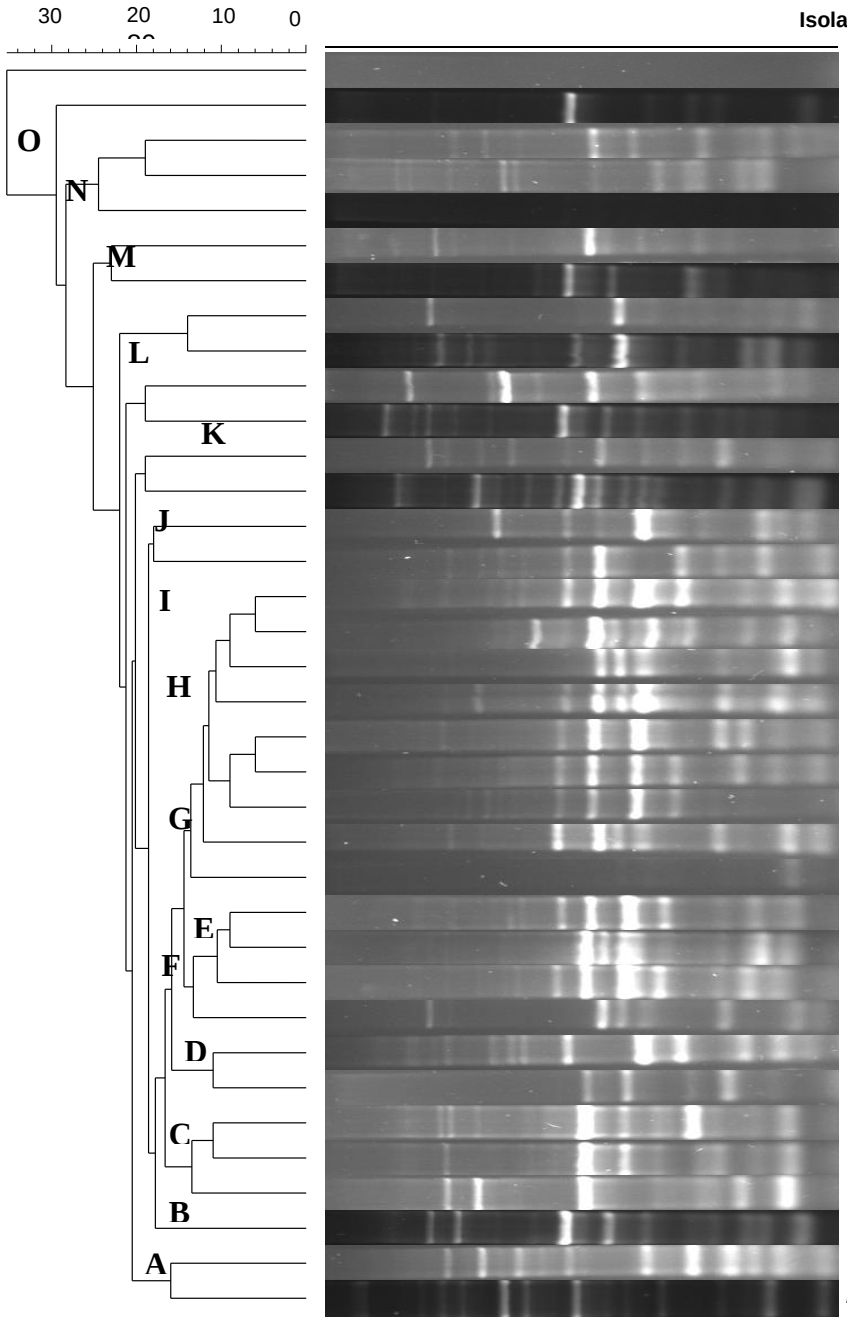
Table 1: Resistance Genes Identified and Sensitivity Results of *E. coli* and *Klebsiella* spp.

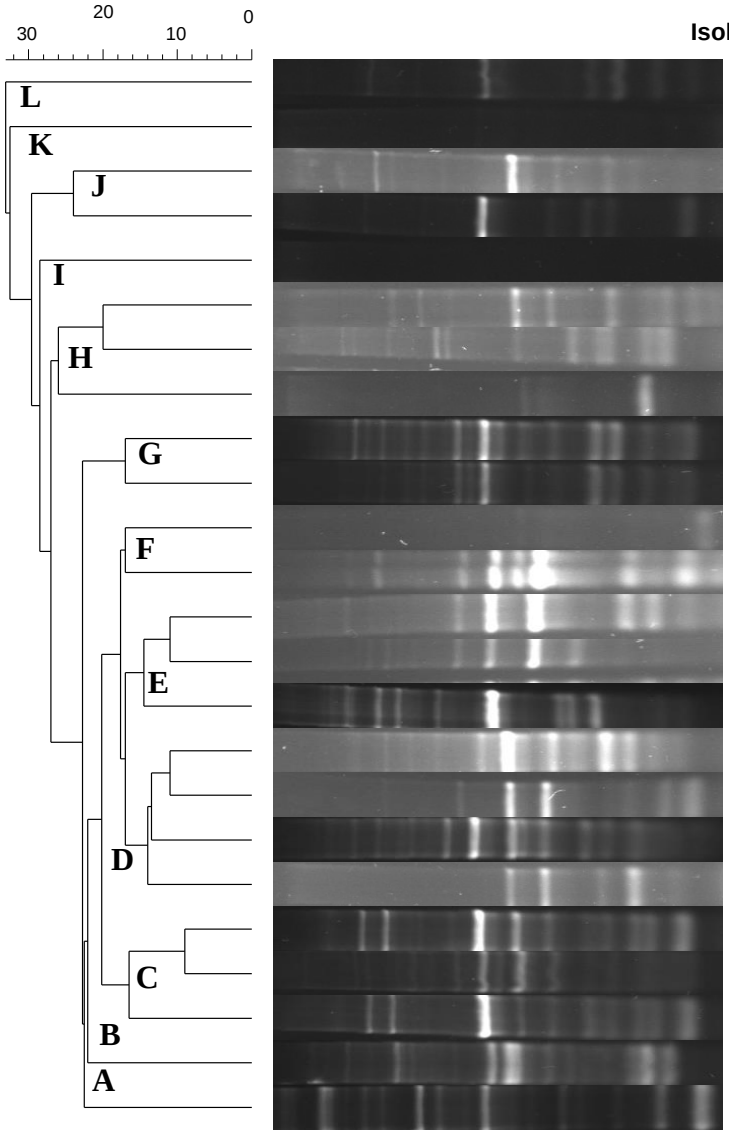
Isolate number	Specie	CTXM1	SHV	TEM	CMY	FOX	MOX	DHA	FOX	CIP	CAZ	AMP	GENT	TET	CRO	COTR	IMIP
2	Klebsiella spp.	+	+	-	-	-	+	+	S	R	I	R	S	R	R	R	S
9	E. coli	+	+	-	-	-	-	-	S	I	R	R	S	R	R	R	S
12	E. coli	+	+	-	-	-	-	-	S	S	R	R	S	R	R	R	S
22	E. coli	+	+	-	+	-	+	+	R	R	I	R	S	R	R	R	S
30	E. coli	+	+	+	-	-	-	-	I	R	R	R	S	S	R	I	S
33	E. coli	-	+	-	-	-	-	-	S	S	S	R	R	S	R	S	S
34	E. coli	+	+	-	-	-	-	-	S	S	I	R	R	R	R	R	S
36	E. coli	-	-	+	-	-	-	-	S	R	R	R	S	R	R	R	S
42	E. coli	+	+	-	-	-	-	+	S	S	R	R	S	S	R	S	S
43	E. coli	-	+	-	-	-	-	-	S	S	R	R	S	R	R	R	S
44	E. coli	+	+	-	-	-	+	+	R	S	R	R	S	R	R	R	S
46	E. coli	+	+	-	+	+	+	+	R	R	R	R	R	R	R	R	S
49	E. coli	-	-	-	+	-	-	-	R	R	R	R	S	S	R	R	S
59	Klebsiella spp.	+	+	-	-	-	-	-	R	S	R	R	S	I	R	S	S
60	E. coli	+	+	-	-	-	-	+	S	S	R	R	S	R	R	S	S
62	E. coli	+	+	+	-	-	-	+	R	S	R	R	S	S	R	S	S
65	Klebsiella spp.	+	+	+	-	-	-	-	S	S	S	R	R	R	R	R	S
66	E. coli	-	-	-	-	-	-	-	S	S	R	R	S	R	R	R	S
67	E. coli	-	+	+	+	+	+	+	R	R	R	R	S	I	R	S	S
68	Klebsiella spp.	-	-	-	-	-	-	+	S	S	S	R	S	R	S	R	S
79	E. coli	+	+	-	-	-	-	+	S	S	R	R	S	R	R	R	S
80	Klebsiella spp.	+	+	-	+	+	+	-	R	R	R	R	S	R	R	R	S
90	Klebsiella spp.	-	-	-	-	-	-	+	S	R	I	R	S	R	R	R	S
92	Klebsiella spp.	+	-	-	-	-	+	+	R	S	S	R	R	R	R	R	S
95	E. coli	+	+	-	-	-	-	-	S	S	S	R	S	R	R	R	S
99	E. coli	+	+	-	-	-	-	-	S	S	R	R	S	R	R	R	S
104	E. coli	+	+	+	-	-	-	-	I	R	R	R	S	S	R	I	S
104/2	Klebsiella spp.	+	+	-	+	-	-	+	R	S	R	R	S	I	R	S	S
110	E. coli	-	+	-	-	+	+	+	R	S	S	R	S	R	S	S	S
112	E. coli	+	-	-	-	-	-	-	S	S	S	R	S	R	S	R	S
120	E. coli	+	-	-	-	-	-	-	S	I	R	R	S	S	R	I	S
138	E. coli	+	+	-	+	+	+	+	R	R	R	R	S	R	R	R	S
142	Klebsiella spp.	+	+	-	-	+	+	+	R	R	R	R	R	R	R	R	S
145	E. coli	+	+	+	-	-	-	-	I	R	R	R	S	S	R	I	S
146	Klebsiella spp.	-	+	-	+	+	+	+	R	S	I	R	S	R	I	S	S
150	Klebsiella spp.	-	+	-	-	-	-	-	S	S	I	R	S	S	R	R	S

152	E. coli	+	-	-	-	-	-	-	S	S	S	I	S	S	S	S	S
161	Klebsiella spp.	+	+	+	+	+	+	+	R	R	R	R	R	R	R	R	S
162	E. coli	+	+	-	-	-	-	-	S	S	I	R	S	S	R	R	S
165	Klebsiella spp.	+	-	-	-	-	-	-	S	I	R	R	R	R	R	R	S
167	Klebsiella spp.	+	-	-	-	-	-	-	R	I	R	R	S	S	R	I	S
169	E. coli	+	+	-	-	-	-	-	S	S	R	R	S	R	R	R	S
171	Klebsiella spp.	+	+	+	-	-	-	-	S	S	S	R	R	R	R	R	S
176	Klebsiella spp.	-	-	-	-	-	-	-	S	S	S	R	S	R	S	R	S
186	Klebsiella spp.	+	-	-	-	-	-	-	R	I	R	R	S	S	R	I	S
195	Klebsiella spp.	+	-	-	-	+	+	+	R	I	R	R	R	R	R	R	S
207	E. coli	+	+	+	+	-	+	+	R	I	R	R	S	R	R	R	S
209	E. coli	+	+	+	-	+	+	+	R	R	R	R	R	R	R	R	S
214	E. coli	+	+	-	+	+	+	+	R	R	R	R	R	R	R	R	S
217	E. coli	+	-	-	-	+	+	+	R	S	R	R	S	R	R	S	S
220	Klebsiella spp.	+	+	-	-	-	-	-	S	I	R	R	R	R	R	R	S
223	E. coli	+	+	+	-	-	-	-	S	R	S	R	S	R	R	R	S
226	Klebsiella spp.	+	+	-	-	-	-	-	S	S	S	R	R	R	R	R	S
228	E. coli	+	+	-	-	-	-	+	R	R	R	R	S	R	R	R	S
277	E. coli	+	+	-	-	-	-	-	S	S	S	I	S	S	S	S	S
317	Klebsiella spp.	+	+	-	-	-	-	-	S	R	I	R	S	R	R	R	S

Figure 1: Dendrogram representing the genetic relatedness and cluster analysis of 35*E. coli*, isolated from stool samples of University residence students, based on ERIC-PCR fingerprinting patterns using Jacquard index and UPGMA algorithm. The scale at the top represents percentage similarity to *E. coli* ATCC 25922



Isolate code	Species	CTX-M	SHV	TEM	CMY	MOX	FOX	DHA
152	*E. coli*	+	-	-	-	-	-	-
223	*E. coli*	+	+	+	-	-	-	-
95	*E. coli*	+	+	-	-	-	-	-
110	*E. coli*	-	+	-	-	+	+	+
214	*E. coli*	+	+	-	+	+	+	+
104	*E. coli*	+	+	+	-	-	-	-
217	*E. coli*	+	-	-	-	+	+	+
36	*E. coli*	-	-	+	-	-	-	-
228	*E. coli*	+	+	-	-	-	-	+
66	*E. coli*	-	-	-	-	-	-	-
207	*E. coli*	+	+	-	+	-	+	+
43	*E. coli*	-	+	-	-	-	-	-
277	*E. coli*	+	+	-	-	-	-	-
33	*E. coli*	-	+	-	-	-	-	-
34	*E. coli*	+	+	-	-	-	-	-
49	*E. coli*	-	-	-	+	-	-	-
60	*E. coli*	-	-	-	-	-	-	-
12	*E. coli*	+	+	-	-	-	-	-
42	*E. coli*	-	-	-	-	-	-	-
62	*E. coli*	+	+	+	-	-	-	+
67	*E. coli*	+	+	-	-	-	-	-
79	*E. coli*	+	+	-	-	-	-	+
30	*E. coli*	+	+	+	-	-	-	-
9	*E. coli*	+	+	-	-	-	-	-
99	*E. coli*	+	+	-	-	-	-	-
169	*E. coli*	+	+	-	-	-	-	-
120	*E. coli*	+	-	-	-	-	-	-
44	*E. coli*	+	+	-	-	-	+	+
22	*E. coli*	+	+	-	+	-	+	+
112	*E. coli*	-	-	-	-	-	-	-
138	*E. coli*	+	+	-	+	+	+	+
162	*E. coli*	+	+	-	-	-	-	-
145	*E. coli*	+	+	+	-	-	-	-
209	*E. coli*	+	+	+	-	+	+	+
46	*E. coli*	+	+	-	+	+	+	+
***E. coli* ATCC 25922**								



Isolate code	Species	CTX-M	SHV	TEM	CMY	FOX	MOX	DHA
227	*Klebsiella* spp.	-	-	-	-	-	-	-
167	*Klebsiella* spp.	+	-	-	-	-	-	-
176	*Klebsiella* spp.	-	-	-	-	-	-	-
171	*Klebsiella* spp.	+	+	+	-	-	-	-
68	*Klebsiella* spp.	-	-	-	-	-	-	-
90	*Klebsiella* spp.	-	-	-	-	-	-	-
146	*Klebsiella* spp.	-	+	-	+	+	+	+
195	*Klebsiella* spp	.+	-	-	-	-	-	-
2	*Klebsiella* spp.	+	+	-	-	-	+	+
35	*Klebsiella* spp.	-	-	-	-	-	-	-
59	*Klebsiella* spp.	+	+	-	-	-	-	-
65	*Klebsiella* spp.	+	+	+	-	-	-	-
317	*Klebsiella* spp.	+	+	-	-	-	-	-
104/2	*Klebsiella* spp.	+	+	-	+	-	-	+
142	*Klebsiella* spp.	+	+	-	-	+	+	+
150	*Klebsiella* spp	.-	+	-	-	-	-	-
92	*Klebsiella* spp.	+	-	-	-	-	+	+
165	*Klebsiella* spp.	+	-	-	-	-	-	-
226	*Klebsiella* spp.	+	+	-	-	-	-	-
220	*Klebsiella* spp.	+	+	-	-	-	-	-
161	*Klebsiella* spp.	+	+	+	+	+	+	+
***K. pneumoniae* ATCC 700603**								

Figure 1: Dendrogram representing the genetic relatedness and cluster analysis of 21*Klebsiella* spp.,isolated from stool samples of University residence students, based on ERIC-PCR fingerprinting patterns using Jacquard index and UPGMA algorithm. The scale at the top represents percentage similarity to *Klebsiella pneumoniae* ATCC 700603.

CHAPTER 3. CONCLUSION

3.1. Introduction

We have collected 275 stool samples from students living at Eduardo Mondlane University residencies, Maputo-Mozambique. The sample collection was made during six weeks between February and March 2016. The demographic data regarding antibiotic consumption, previous hospitalization, age and gender were collected with a small questionnaire. The samples were tested for presence of ESBL producer *E. coli* and *Klebsiella* spp.

3.2. Conclusion

- This study demonstrated that the prevalence of colonization by ESBL *E. coli* and *Klebsiella* spp. strains among male and female students is high.
- The prevalence of multi-resistance among ESBL-positive *E. coli* and *Klebsiella* spp. is demonstrated to be high.
- The strains demonstrated low genetic similarity among them, what means that they are not related.
- Were identified different ESBL genes with different strains containing both class A and C β-lactamase genes.

3.3. Limitations

The main limitations on this study was that the strain typing was not done in all ESBL isolates and it was not possible to determine Minimum Inhibitory Concentrations (MIC) of isolates.

3.4. Recommendations

Further studies should be carried out to monitor the ESBL carriage among the students living at the University residencies and identify the source of the ESBL. Similar studies should be done in other groups of population to explore the colonization in different groups. Complete sequencing of the strains should be done in all strains.

3.5. Significance

Our results bring new data about the carriage rate among University students in Maputo-Mozambique. This will help to understand the situation of colonization in this group of the population and to design strategies for monitoring this situation.