RESISTANCE TO β-LACTAM AND FLUOROQUINOLONE ANTIBIOTICS IN ENTEROBACTERIACEAE FROM CHICKEN: AN OBSERVATIONAL STUDY IN MAPUTO, MOZAMBIQUE

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Submitted in fulfillment for the degree of Master of Medical Science 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 Science (Medical Microbiology).

This is the dissertation in which the research is written as a research publication, with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss Sara Lino Faife.

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

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i. Declaration

I, Miss Sara Lino Faife, 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.

Signed Sara Lino Faife Date: 02/03/2017

ii. Dedication

This is research is dedicated to my husband, Paulino Armando Timana and my son Adner Paulino Timana.

iii. Acknowledgements

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Content

iv. List of Acronyms/Abbreviations

AMR	Antimicrobial resistance
API	Analytical Profile Index
bla	β-lactamase
CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycinase
CTX-M	Cefotaximase-München
DNA	Deoxyribonucleic Acid
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus- Polymerase Chain Reaction
ESBL	Extended-Spectrum β-lactamase
FAO	Food and Agriculture Organization of the United Nations
GPAs	Growth-promoting antibiotics
MIC	Minimum Inhibitory Concentration
OIE	World Organization for Animal Health
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
QRDR	Quinolone Resistance-Determining Region
Qnr	Plasmid-mediated quinolone resistance
RND	Resistance-nodulation-division
SHV	Sulfhydryl variable
TEM	Temoneira
WHO	World Health Organization

v. Abstract

Extended-spectrum β-lactamase (ESBL) and/or plasmid-mediated AmpC (pAmpC)-producing *Enterobacteriaceae* with co-resistance to fluoroquinolones are increasingly identified in food-borne pathogens globally. This study investigated ESBL- and pAmpC-mediated β-lactam resistance as well as plasmid-mediated fluoroquinolone resistance in 198 samples taken from 99 frozen chickens (99 samples from the liquid thaw and 99 from carcass swabs), Brazil, South Africa and Mozambique each produced 33 chicken. Species identification was undertaken using in-house biochemical tests and antibiotic susceptibility was ascertained by the disc diffusion test following CLSI guidelines. ESBL production was determined using the double-disk synergy test and isolates were subjected to PCR to detect the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{DHA}, *qnrB*, *qnrD*, *qnrS* and *qepA* genes. A random selection of CTX-M genes was sequenced. The 198 samples yielded 27 putative ESBL-positive isolates: *Citrobacter diversus* (1), *Citrobacter freundii* (3), *Enterobacter aglomerans* (1), *Enterobacter cloacae* (1) and *Escherichia coli* (21). Of the 27 isolates, 19 were from South African chicken, five from Mozambican chicken and three from Brazilian chicken. Resistance to ampicillin was 100,0%, followed by 88.8% to ceftriaxone, 77.8% to trimethoprim-sulphamethoxazole, 74.0% to cefotaxime, 70.4% to ciprofloxacin, 66.6% to ceftazidime, 22.2% to cefoxitin and 7.6% to gentamicin. Multi-drug resistance was evident in 19 (70.4%) isolates, 15 of which were from South Africa. The predominant ESBL gene was bla_{SHV} (85%), followed by $bla_{\text{CTX-M}}$ (62.9%) and bla_{TEM} (44.4%) whilst bla_{MOX} (33, 3%) and bla_{DHA} (33.3%) were the most common pAmpC genes. The predominant plasmid-mediated fluoroquinolone-resistance gene was *qepA* (22.2%). DNA sequencing of the $bla_{\text{CTX-M}}$ genes putatively identified $bla_{\text{CTX-M-55/-79/-101/-164}}$, with $bla_{\text{CTX-M-164}}$ being most common. ERIC–PCR profiles allowed the

differentiation of the *E. coli* and *Citrobacter* spp. isolates. There was no strong evidence of clonality, however, South African isolates shared more similarity. *qnr* and *qepA* genes were predominantly identified in South African *E. coli* isolates while CTX-M and SHV genes were prevalent in isolates from diverse origins. The Mozambican population is thus exposed to a reservoir of plasmid-mediated and hence, mobile β-lactam and fluoroquinolone resistance genes from imported, and to a lesser extent, locally-produced chicken. The complexity and diversity of mobile resistance genes combinations and permutations is of concern in the food safety context.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Antibiotic resistance is considered a global health problem for many decades and has now been escalated by World Health Organization (WHO) to one of the top health challenges of the 21stcentury (CDC, 2010).

One of the most studied and clinically relevant resistant bacteria is extended-spectrum beta-lactamase (ESBL) - producing *Enterobacteriaceae*. Currently, ESBL-producing bacteria are not isolated only in humans but also in food bacteria. According to Platteel *et al.* (2013) two compartments have been suggested as habitats of ESBL-producing *Enterobacteriaceae*: one in hospitals as a result of cross-contamination and unrelated to food consumption, and another in the community as a result of consumption of contaminated food.

Some food animals, including chickens, turkeys, pigs and cattle, may be colonized by ESBL and/or plasmid-mediated AmpC (*pAmpC*)-producing *E. coli* and have been considered potential causes of community-acquired diseases (Carattoli, 2008). The presence of ESBL producing bacteria in food animals has been reported repeatedly and the connection between these bacteria in food animals and humans has been proposed (Doi *et al*. 2010).

Fluoroquinolone resistance in animal food production is also being studied in many countries and the use of fluoroquinolones in poultry has been linked to antibiotic-resistant *Campylobacter* infections in humans. Enrofloxacin is the most commonly used fluoroquinolone for colibacillosis (Chu *et al*. 2004).

Antimicrobial agents are essential tools for protecting animal health and welfare, and they also contribute to satisfying the increasing world demand for safe food of animal

origin, such as milk, meat, fish and eggs. Although, antimicrobial agent use in animal is permitted, it is essential that such use is responsible and prudent (OIE, 2013).

According to the World Organization for Animal Health (OIE) the third and fourth generation cephalosporins, and fluoroquinolones are important for human and animal health and should thus not be used as preventive treatment in feed or water or in absence of clinical signs. They should further not be used as first line treatment unless justified and on bacteriological testing (OIE, 2013).

Most studies about ESBL/p*AmpC*–producing bacteria are related to human samples followed by reports in poultry (Ewers, 2012). Contamination of retail chicken meat with ESBL-producing Gram-negative bacteria has been reported in several countries (Mesa *et al*. 2006).

In Mozambique, chicken is one of the most consumed foods by the population. It is sourced in the formal and informal markets that are supplied by chickens reared nationally by aviaries and internationally by the South African and Brazilian firms. The national producers of broilers use antibiotics as additives to promote growth, and the biggest supplier is Interchemie, from the Netherlands (http://www.interchemie.com/). Given the rise of resistant strains in health care facilities, it is deemed appropriate to carry out a study to detect the presence of ESBL/p*AmpC*-producing and fluoroquinolone-resistant *Enterobacteriaceae* in imported and domestically produced chicken in Mozambique.

1.2. AIM AND OBJECTIVES

Aim

To investigate the presence of the ESBL/p*AmpC*-producing and fluoroquinolone-resistant *Enterobacteriaceae* in imported and locally produced chicken and perform selected phenotypic and genotypic characterization of the resistant isolates.

Objectives

- ✓ To culture bacterial isolates from the liquid thaw and carcass swabs from chicken produced in Mozambique, South Africa and Brazil.
- ✓ To identify bacterial isolates from imported and domestically produced chickens/poultry to species level
- ✓ To ascertain the antibiotic susceptibility of isolates against an appropriate panel of antibiotics by standardized agar disc diffusion
- ✓ To phenotypically confirm the production of:
    - ESBLs using the double-disc synergy test,
    - AmpC β-lactamase using the cefoxitin disc sensitivity test, and,
    - Inducible AmpC β-lactamase production using the disk antagonism test.
- ✓ To identify the EBSL and/or p*AmpC* genes by PCR and sequencing
- ✓ To identify plasmid-mediated quinolone resistance genes (*qnrB*, *qnrD*, *qnrS* and *qepA*) by PCR.
- ✓ To undertake strain typing by ERIC-PCR to ascertain clonal similarities or differences at national and international levels
- ✓ To compare the phenotypic and genotypic differences between imported and domestically produced chickens

1.3. LITERATURE REVIEW

1.3.1. Resistance as a Public Health Problem

After the discovery of penicillin by Alexander Fleming, several other antibacterial agents were developed. Most of them were developed in the 1970s and then new classes in the 1980s. In his Nobel Prize speech, Fleming warned that bacteria could become resistant to penicillin (WHO, 2014).

In 2000, WHO considered the increase of antimicrobial resistance a global crisis and in the following year it released its first global strategy for its containment (WHO, 2001). Antimicrobial resistance is a public health problem that has many implications to the lives of humans, because it is a growing threat to the effective treatment of an ever-increasing range of infections, by bacteria, parasites, virus and fungi which reduce efficacy of antibacterial, anti-parasitic, anti-viral and anti-fungal agents respectively making the treatment of patients difficult, costly, and at times impossible. All these factors can result in prolonged illness and increased mortality (WHO, 2014).

From the 1980s, researchers began to notice that certain bacterial strains had become resistant to antibiotics used in poultry and the continued use of antimicrobials as growth promoters had increased a "pool" of resistance genes in nature (Sader, 2004) with concern on the possibility of transmission of this resistance from poultry to humans.

The World Health Organization (WHO), the World Organization for Animal Health (OiE) and the Food and Agriculture Organization of the United Nations (FAO) entered into a tripartite alliance to address AMR in 2015. This collaboration reflects the "One Health" approach, which emphasizes the interconnectedness of the health of humans, animals and ecosystems. The OIE Strategy on Antimicrobial resistance (AMR) and the Prudent Use of Antimicrobials has four main objectives, viz.,"(1) improve awareness and understanding; (2) strengthen knowledge through surveillance and research; (3) support good governance and capacity building; and, (4) encourage implementation of international standards" (OIE, 2016). The FAO's Action Plan on AMR 2016-2020 also has 4 focus areas, viz., "(1) improve awareness on AMR and related threats; (2) develop capacity for surveillance and monitoring of AMR and AMU (antimicrobial use) in food

and agriculture; (3) strengthen governance related to AMU and AMR in food and agriculture; and, (4) promote good practices in food and agricultural systems and the prudent use of antimicrobials" (FAO, 2016). This study speaks to objective 2 and focus area 2 of the OIE and FAO strategies respectively.

1.3.2. Potential Sources and Reservoirs of Resistant Bacteria

Potential sources for the acquisition/transmission of ESBL and AmpC-producing *Enterobacteriaceae* are: foodborne, direct animal-to-human transmission, human-to-human transmission, environment and infections obtained abroad during travelling (Carmo *et al*. 2014).

The use of antibiotics as growth promoters and to prevent infection in farm animals rather than cure infections is responsible for drug-resistant bacteria emerging on farms which reaches the population through human or animal carriers and food (Todar, 2014). Some of the antibiotic resistance genes identified in food bacteria have also been identified in humans, providing indirect evidence for transfer by food handling and/or consumption (Marshall and Levy, 2011).

The increasing levels of bacterial resistance, in particular strains of *Salmonella typhimurium* observed in 1963 at several British feed lots, led to confirmation that the indiscriminate use of antibiotics in agriculture is a major cause of the increase in antibiotic resistant bacteria (Dewey *et al*. 1997). Anderson *et al*. (2003) reported that using antibiotics similar to those used in human medicine as growth promoters and in the treatment of animals increases the possibility of resistant pathogenic bacteria whose reservoirs in food animals develop cross-resistance to antimicrobial used in the treatment of infectious diseases in humans.

ESBL and AmpC-producing *Enterobacteriaceae* were initially only observed in humans, but in recent studies these bacteria are observed in companion animals and increasingly in livestock (Ewers, 2011). The ESBL and AmpC produced by *E. coli* are being found frequently in food-producing animals (Smet *et al*. 2010). According to Ewers (2012), this finding leads to the hypothesis that animals might become infection sources or even reservoirs contributing to the spread of these resistant bacteria.

Companion animals also represent potential sources of the spread of multidrug-resistant bacteria (Dos Santos *et al*. 2013). This is supported by Meyer and collaborators (2012) in a study which showed that previous contact with pets increases by almost seven-fold the possibility to be colonized with ESBL positive bacteria (Meyer *et al*. 2012). Pets are considered as reservoirs of resistant bacteria (Costa *et al*. 2008). The animals that have close contact with humans, like dogs and cats, may share microbiota with humans suggesting an important reservoir of resistant bacteria (Dos Santos *et al*. 2013).

The resistance observed in chickens, pigs and cattle can be transmitted to humans via the food chain in the human intestine (Salyers *et al*. 2004). The poultry industry has been considered a potential reservoir of ESBL-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated meat (Leverstain-van Hall *et al*. 2011).

In the Netherlands, the prevalence of ESBL-producing *E. coli* in gastrointestinal tract of healthy food-producing animals, especially in poultry, increased from 3% in 2003 to 15% in 2008 and in 2009 ESBL–producing bacteria were detected in all 26 broiler farms studied (Dierikx, 2010).

1.3.3. Beta-lactam Antibiotics

1.3.3.1. Beta-lactam Antibiotics Classes

The beta-lactam antibiotics contain the four-membered, nitrogen-containing, beta-lactam ring at the core of their structure (Forbes *et al*. 2007) and their classes are inter alia penicillin, amino-penicillins, ureido-penicillins, cephalosporins, beta-lactamase inhibitor combinations; monobactams, cephamycins, and carbapenems (CDC, 2013).

The beta-lactam antibiotics are the largest group of antibacterial agents, and dozens of derivatives are available for clinical use. Their popularity results from targeted bacterial action and their low toxicity to humans. Also, their molecular structure can be manipulated to achieve greater activity for wider therapeutic application (Forbes *et al*. 2007).

1.3.3.2. Mechanism of Action

The beta-lactam ring is key to the mode of action of these groups of drugs that target and inhibit cell walls synthesis by binding the enzymes involved in synthesis. The essential enzymes for this action are anchored in the cell membrane and as a group are referred to as penicillin-binding proteins (PBPs). When beta-lactams bind to these PBPs, cell walls synthesis is essentially halted. Death results from osmotic instability caused by faulty cell wall synthesis, or the binding of the beta-lactam to PBP may trigger a series of the events that lead to autolysis and death (Forbes *et al*. 2007).

1.3.3.3. Beta-lactam Antibiotic Resistance

Pathogens create mechanisms that destroy or inactivate the antibiotics to avoid destruction by the antibiotics. According to Zemelman *et al*. (2002) in this type of mechanism, the most classical example is β-lactamase which, acts by destroying the β-lactam ring of penicillins and cephalosporins.

ESBL are plasmid-encoded enzymes most commonly found in the bacterial family *Enterobacteriaceae,* especially in *E. coli* and *Klebsiella pneumonia* (EFSA, 2011). Over time, this type of resistance has been observed in other species of the family *Enterobacteriaceae* (including *Enterobacter spp., Serratia spp.* and *Proteus mirabilis*) and other genera, such as *Pseudomonas aeruginosa* (Winn *et al*. 2010). AmpC beta-lactamases are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria. However, the number of AmpC enzymes that are plasmid borne is increasing (EFSA, 2011). ESBL enzymes and AmpC enzymes differ from each other in their ability to hydrolyze the different beta-lactam antibiotics. ESBLs hydrolyze 3rd and sometimes 4th generation cephalosporins and monobactams and are inhibited by clavulanic acid and cephamycins (Ewers, 2012). AmpC beta-lactamases hydrolyze 3rd generation cephalosporins and cephamycins, but not the 4th generation cephalosporins and they are not inhibited by clavulanic acid (Jacoby, 2009).

Until the 1990s, the ESBL frequently identified in human clinical isolates were SHV or TEM (Pitout, 2012) but CTX-M enzymes have become the most widespread type

(Pitout and Laupland, 2008). Over the last years special genes belonging to the CTX-M family are emerging rapidly worldwide (Ewers, 2012).

Numerous studies have described the occurrence of *E. coli* producing ESBL in food-producing animals, and strains relevant to human health are also increasingly being isolated from companion animals. The most frequent genes associated with this resistance among food producing animals and companion animals encode various CTX-M enzymes, followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ (Ewers *et al*. 2011). Among CTX-M enzymes, the most frequent types found in humans, companion animals, poultry, cattle and pigs are CTX-M-1, CTX-M-14 and CTX-M-15 (Ewers *et al*. 2012).

The most important gene families encoding AmpC beta-lactamases are CMY, ACC, DHA and FOX, with CMY being the most predominant family and *bla*CMY-2 as the most predominant gene (Jacoby, 2009).

1.3.3.4. Resistance to beta-lactam antibiotics in food animals

ESBL and/or pAmpC-producing isolates can be found in nearly all food-producing animals (Carattoli, 2008). One of the earlier descriptions of the poultry carriers of ESBL and pAmpC was made by Briñas and collaborators (2003) who observed CTX-M-14, SHV-12 and CMY-2 producing *E. coli* in the feces of healthy chickens in Spain between 2000 and 2001 (Briñas *et al*. 2003).

In 1999 to 2000, the isolation of bacteria carrying CTX-M-14, CTX-M-2 and CMY-2 from healthy poultry was reported in Japan (Kojima *et al*. 2005). A study performed by Kolar and his collaborators in 2010 which aimed to detect ESBL and AmpC beta-lactamase-producing *Enterobacteriaceae* in poultry in the Czech Republic, showed that the most frequent ESBL types were CTX-M-1 and SHV-12 and the most common AmpC enzymes were the CMY-2 types (Kolar *et al*. 2010).

A study carried out by Leverstain-van Hall and collaborators in 2011, which aimed to determine whether retail chicken shared the same ESBL genes, plasmids and strains as those found in Dutch patients, showed that 94% of a representative sample of chicken meat was contaminated with ESBL-producing *E. coli*, of which 39% belonged to

genotypes also found in human samples (Leverstain-van Hall *et al*. 2011). Similar results were found by Kluytmans *et al*. (2013), in a study performed in Netherlands which aimed to compare the ESBL-producing *E. coli* strains, plasmids, resistance genes and virulence factors from retail chicken meat and humans, where significant genetic similarities among ESBL- producing *E. coli* from chicken meat and humans was observed with respect to mobile resistance elements, virulence genes and the genetic backbone (Kluytmans *et al*. 2013).

E. coli strains are one of ESBL-producing bacteria most often isolated in greater numbers in food-producing animals (Smet *et al*. 2010). Some European countries have reported ESBLs in Salmonella isolates, including CTX-M-2, -9, -14 and TEM-52 (Coque *et al*. 2008a).

Another study on the identification of ESBL in the United Kingdom found that chicken breast fillets imported from Brazil, France, Poland and Netherlands were positive for CTX-M-2 producing *E. coli* (Warren *et al*. 2008). In another study, *E. coli* strains isolated from raw chicken meat imported from Argentina, Chile and Brazil were found to carry $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-8}}$ (Dhanji *et al*. 2010). In Portugal, fecal samples from healthy broilers at slaughterhouses, revealed cefotaxime-resistant *E. coli* producing TEM-52, CTX-M-14 and CTX-M-32 (Costa, 2009). In a study performed on fecal samples of healthy and sick poultry in Spain, *E. coli* strains were found to harbor $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-9}}$, or $bla_{\text{SHV-12}}$ genes (Briñas, 2003). A study performed in Italy in farms *E. coli* isolates with reduced susceptibility to cefotaxime or ceftiofur from healthy broilers confirmed the presence of $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-32}}$, $bla_{\text{SHV-12}}$ (Bortolaia *et al*. 2010). In Switzerland, CTX-M-1, SHV-12 and TEM-52 ESBLs were detected in *E. coli* strains recovered from fecal samples of healthy chickens (Geser *et al*. 2012).

Results from several countries indicate that the bacteria mostly frequently isolated is *E. coli* and the most common genes are CTX, SHV and TEM (Geser *et al*. 2012; Briñas *et al*. 2003). Most of the studies on the presence of ESBL and/or pAmpC-producing bacteria in food-producing animals, especially in poultry, were conducted in Europe and Asia, while such studies in South America and the African continent are scarce (Ewers, 2012).

1.3.4. Fluoroquinolone Antibiotics

1.3.4.1. Fluoroquinolones

Fluoroquinolones, also often simply referred to as quinolones, are potent bactericidal agents. They have a broad spectrum of activity that includes Gram-negative and Gram-positive bacteria (Forbes *et al*. 2007).

Nalidixic acid was the first quinolone introduced to human medicine in 1962. Over the past 20 years numerous fluorinated derivatives of nalidixic acid have been developed. The addition of a fluorine molecule at position 6 of the basic quinolone nucleus enhanced the spectrum and potency of quinolone activity and increased penetration into the bacterial cell (Sweet and Gibbs, 2012).

1.3.4.2. Mechanism of Action of Fluoroquinolones

The fluoroquinolone antibiotics kill susceptible bacteria by inhibiting DNA synthesis secondary to direct effect on two members of the topoisomerase class of enzymes, DNA gyrase and topoisomerase IV, promoting the cleavage in these enzyme-DNA complexes. The spectrum of activity of the fluoroquinolone is wide. *Enterobacteriaceae* family are generally highly susceptible to the fluoroquinolones with MIC values of 0.5µg/mL or less (Sweet and Gibbs, 2012; CLSI, 2014).

1.3.4.3. Fluoroquinolone Resistance

The chromosomally encoded resistance was the first mechanism of quinolone resistance characterized. This mutation is classified in two groups: (1) mutation located within the target genes decreasing the interaction between the quinolone drug and the intermediate DNA-gyrase complex and (2) mutation that affect the intracellular levels of quinolone drugs (Santamaria, 2010).

Quinolone resistance was initially associated with the mutations in the target genes. These chromosomal mutations generally cluster within the quinolone resistance-

determining regions (QRDRs) and the regions that have been characterized in each of the four target genes: *gyrA, gyrB, parC* and *parE* (Eaves *et al*. 2004).

In the *Enterobacteriaceae* family, *E. coli* and *Salmonella typhimurium* are the best characterized for quinolone resistance. In this bacterial group, the most important mutation triggering a quinolone resistance phenotype occurs in the *gyr*A gene (Chen *et al*. 2007).

The second mechanism of resistance is a decrease in intracellular accumulation of the antibiotic. The related modifications trigger further increases in the MICs of quinolones and can modulate the final MIC when one or several QRDR mutations have been initially acquired. This mechanism can be associated with:(1) an up-regulation of efflux pumps, which are energy-dependent efflux systems localized in the cell enveloped that can expel quinolone drugs and (2) a decrease in permeability related to decreased expression of porins, which are outer membrane proteins and are only present in Gram-negative bacteria (Jacoby, 2005).

The horizontal transmission of quinolone plasmid-mediated resistance was reported in the 1990s. There are four types of genes of transferable quinolone resistance characterized, i.e., *qnr*, *aac*(6´)-*Ib-cr*, *qepA* and *oqxAB* related to three different mechanisms, i.e. enzyme protection, drug inactivation and efflux respectively (Strahilevitz, 2009).

The first plasmid-mediated mechanism of resistance to quinolones was described in 1998 (Martinez-Martinez, 1998) which was due to the QnrA protein that belongs to the penta-peptide repeat family (Tran, 2005). The second mechanism of quinolone resistance was described in 2006 as enzymatic inactivation of certain quinolones, representing a new mechanism of quinolone resistance. The *acc* (6´)-*IB* genes encodes an aminoglycoside acetyltransferase that modifies aminoglycosides and fluoroquinolones preventing their interaction with targets (Strahilevitz, 2009).

In 2007, a plasmid-encoded efflux pump, QepA, was found in plasmids from *Enterobacteriaceae*. QepA is associated with an increase of 2-, 32- and 64-fold increase in the MICs of nalidixic acid, norfloxacin and ciprofloxacin, respectively, and differs

from chromosomally encoded quinolone efflux pumps that frequently have a wider range of substrates (Kim, 2009).

OqxAB is another plasmid-encoded quinolone efflux pump that was first described as conferring resistance to the swine growth enhancer olaquindox (Hansen, 2004). This determinant is multidrug efflux pump, belonging to the resistance-nodulation-division (RND) family of bacterial transporters and capable of effluxing quinolones among other drugs (Hansen, 2007).

1.3.4.4. Resistance to Fluoroquinolones in Food Animals

The fluoroquinolones used to treat sick food animals create selection pressure for the consequent development of resistant bacteria in humans, which include resistant strains of *Campylobacter spp., E. coli* and *Salmonella spp*. (Collignon, 2005; Chu *et al*. 2004).

Before fluoroquinolone were used in farming, no humans without prior exposure to the drugs were known to carry fluoroquinolone-resistant *Campylobacter* spp. (Witte, 1998). After the introduction of this antibiotic in food producing animals, fluoroquinolone-resistance was observed with a tendency of increasing over time. For example, in 1991 when fluoroquinolones were first approved for use in poultry in the UK, just 1% of birds carried fluoroquinolone-resistant Campylobacter spp. (Endtz, 1991) and in 2008, 18% of *C. jejuni* and 25 of *C. coli* taken from chickens during an abattoir survey were ciprofloxacin resistant (EFSA, 2010).

In a study performed by Li *et al*. (2014), which aimed to explore prevalence and characteristics of ESBL and plasmid-mediated fluoroquinolone resistance genes in *E. coli* in China involving 202 isolates, 37.1% possessed plasmid-mediated quinolone resistance determinants. In these determinants, positive rates for qnr genes and acc (6´)-IB-cr were 10.4% and 32.2 %, respectively (Li *et al*. 2014).

Antibiotic resistance in recent years has increased dramatically, and is thus considered a public health problem. Initially, the strains were observed only in humans. However, currently, they are observed in food animals, in pets as well as in agricultural settings. Various studies have shown that resistant strains can pass from animals to humans

through the food chain, as well as by contact, like caregivers of animals, however, the mechanism is not known. Several studies in this area have been performed in Europe, America and Asia, but are scarce in Africa and absent in Mozambique. This study will provide a Mozambican perspective on ESBL and pAmpC-producing and fluoroquinolone-resistant bacteria isolated from chickens.

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CHAPTER 2

MANUSCRIPT

The following manuscript emanated from the study:

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- Ms Sara Faife, as the principal investigator, developed the protocol, undertook the laboratory work and data analysis, and, prepared 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. Tomas Zimba, as a co-supervisor, facilitated the preliminary laboratory work 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 Arnfinn Sundsfjord, as co-supervisor, co-conceptualized the study, ensured quality control of preliminary laboratory work and undertook 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.

Running title: ESBL, pAmpC and fluoroquinolone resistance in chicken in Mozambique.

Extended Spectrum β-Lactamase, Plasmid-Mediated AmpC and Plasmid-Mediated Fluoroquinolone-Resistance in *Enterobacteriaceae* from Imported and Locally-Produced Chicken: An Observational Study in Maputo, Mozambique

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Key words: antibiotic resistance; ESBL; pAmpC; CTX-M; Enterobacteriaceae; chicken.

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ABSTRACT

Extended-spectrum β-lactamase (ESBL) and/or plasmid-mediated AmpC (pAmpC)-producing *Enterobacteriaceae* with co-resistance to fluoroquinolones are increasingly identified in food-borne pathogens globally. This study investigated ESBL- and pAmpC-mediated β-lactam resistance as well as plasmid-mediated fluoroquinolone resistance in 198 samples taken from 99 frozen chickens (99 samples from the liquid thaw and 99 from carcass swabs), 33 produced in each of Brazil, South Africa and Mozambique. Species identification was undertaken using in-house biochemical tests and antibiotic susceptibility was ascertained by the disc diffusion test following CLSI guidelines. ESBL production was determined using the double-disk synergy test and isolates were subjected to PCR to detect the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{DHA}, *qnrB*, *qnrD*, *qnrS* and *qepA* genes. A random selection of CTX-M genes was sequenced. The 198 samples yielded 27 putative ESBL-positive isolates: *Citrobacter diversus* (1), *Citrobacter freundii* (3), *Enterobacter aglomerans* (1), *Enterobacter cloacae* (1) and *Escherichia coli* (21). Of the 27 isolates, 19 were from South African chicken, five from Mozambican chicken and three from Brazilian chicken. Resistance to ampicillin was 100%, followed by 88.8% to ceftriaxone, 77.8% to trimethoprim-sulphamethoxazole, 74.0% to cefotaxime, 70.4% to ciprofloxacin, 66.6% to ceftazidime, 22.2% to cefoxitin and 7.6% to gentamicin. Multi-drug resistance was evident in 19 (70.4%) isolates, 15 of which were from South Africa. The predominant ESBL gene was bla_{SHV} (85%), followed by $bla_{\text{CTX-}M}$ (62.9%) and bla_{TEM} (44.4%) whilst bla_{MOX} (33, 3%) and bla_{DHA} (33.3%) were the most common pAmpC genes. The predominant plasmid-mediated fluoroquinolone-resistance gene was *qepA* (22.2%). DNA sequencing of the $bla_{\text{CTX-M}}$ genes putatively identified $bla_{\text{CTX-M-55/-79/-101/-164}}$, with $bla_{\text{CTX-M-164}}$ being most common. ERIC–PCR profiles allowed the differentiation of the *E. coli* and *Citrobacter* spp. isolates. There was no

strong evidence of clonality, however, South African isolates shared more similarity. *qnr* and *qepA* genes were predominantly identified in South African *E. coli* isolates while CTX-M and SHV genes were prevalent in isolates from diverse origins. The Mozambican population is thus exposed to a reservoir of plasmid-mediated and hence, mobile β-lactam and fluoroquinolone resistance genes from imported, and to a lesser extent, locally-produced chicken. The complexity and diversity of mobile resistance genes combinations and permutations is of concern in the food safety context.

INTRODUCTION

Antibiotic resistance is a global health problem that is prevalent in the human, (food) animal and environmental health sectors. Resistance exists to all currently used antibiotics in clinical and veterinary medicine. Of particular concern is the escalating resistance to broad-spectrum β-lactam and fluoroquinolone antibiotics, the former largely attributed to extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC (pAmpC) enzymes and the latter increasingly mediated by plasmid-mediated *qnr* and *qep* genes. These resistance mechanisms to both antibiotic classes have been described in food-borne pathogens (*1, 3, 20*).

The most common ESBL genes detected in bacteria among food-producing animals are $bla_{\text{CTX-M}}$ enzymes, followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ *(8)* and mostly from *E. coli*. Reports on antibiotic resistance in food products from developing countries are minimal. As chicken is one of the most consumed foods by the Mozambican population, we undertook a study to investigate ESBL and pAmpC-mediated β-lactam resistance as well as plasmid-mediated fluoroquinolone resistance in *Enterobacteriaceae* isolated from imported and locally-produced chicken in Maputo, Mozambique.

METHODOLOGY

Ethical Considerations. Ethical clearance was received from Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE473/14) and the Institutional Committee of Bioethics for Health-CIBS (TFCMCSSF01/15).

Study Sample. Ninety-nine frozen chickens, consisting of 33 chicken imported from South Africa, 33 imported from Brazil and 33 locally produced in Mozambique, were purchased from three different supermarkets in Maputo City to form the study sample. Acquisition was done weekly during May 2015 to obtain samples from different batches.

Two samples were taken from each chicken, one from the thaw liquid and the other from a swab of the internal parts of the carcass yielding a total of 198 samples from 99 chickens. The samples were cultured on ESBL-selective media (MacConkey plates supplemented with 2mg/L of ceftriaxone) plates prior to their morphological and biochemical identification to the species level.

Species identification and antimicrobial susceptibility testing. Species identification was undertaken using biochemical tests *(27)*. Antimicrobial susceptibility testing (AST) was undertaken using the agar disc diffusion assay according to CLSI guidelines *(6)* against ampicillin, piperacillin-tazobactam, cefoxitin, cefuroxime, cefotaxime, ceftazidime, gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole. *E. coli* ATCC 25922 and *E. coli* A5-8 (resistant strain) were used as quality controls.

Phenotypic detection of putative ESBLs, and AmpC-mediated resistance. ESBL confirmation was done using the double-disk synergy test (DDST) using ROSCO disks containing cephalosporins (cefotaxime, ceftazidime, cefepime) with and without clavulanic acid *(9)*. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* A-43 were used as controls. The phenotypic AmpC confirmation test was based on inhibition of AmpC by cloxacillin and boronic acid derivatives *(9)* with *E. coli* ATCC 25922 and *E. coli* A5-8 as controls. Results were considered positive when the inhibition zones of the combined disc were ≥ 5mm compared to that of the antibiotic disc alone.

The disc diffusion method was used to determine isolates that were resistant to ciprofloxacin using CLSI breakpoints *(6)*.

DNA extraction, PCR and Electrophoresis. Genomic DNA was extracted from overnight bacterial cultures with the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) as per manufacturer's guidelines.

PCR amplification of ESBL, AmpC, Qnr and QepA genes was done in a final volume of 25 µL, containing 12.5 µL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, USA), 7.5µL of sterilized distilled water, 2 µL of each primer and 1 µL template DNA. PCR was undertaken in a T100^{TM} Thermal cycler (Bio-Rad, USA). The cycle comprised of a preliminary denaturation for 10 s at 98°C, followed by 35 cycles of denaturation at 98°C for 1s, annealing for 5s and elongation at 72°C for 7 min. The annealing temperatures and primers are described in Table 1. The amplicons obtained were visualized by electrophoresis in 1.5% agarose gel for 40 min at 120 V, stained with gel red and detected by ultraviolet transillumination.

The PCR amplicons for a random selection of CTX–M were further purified and sequenced by Inqaba Biotec South Africa, using Sanger Dideoxy sequencing technology. Sequences were analyzed using Basic Local Alignment Search Tool, available on the website of the National Center for Biotechnology Information (http://www.ncbi.nhlm.nih.gov/blast/BLAST.cgi) and BioEdit.

ERIC-PCR was done in a total reaction volume of 10 µL, which contained 2µL of template DNA and 0.1 µL of 100 µM primers ERIC 1 and ERIC 2 *(25)* and 5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific). 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 of 16 min at 65 °C, in an Applied Biosystems 2720 Thermal Cycler. The ERIC-PCR products were loaded into 1.0% (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 variations were analyzed using the Gel CompareII 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

Of the 198 samples, 27 were ESBL positive: 15 from the carcass swabs and 12 from the thaw liquid from a total of 22 chickens. Five chickens (4 from South Africa and 1 from Mozambique) yielded positive results from both the carcass swab and thaw liquid. Of the 27 ESBL-positive isolates, 19, 5 and 3 isolates were from South African, Mozambican and Brazilian chickens, respectively. The isolates were identified as *C. diversus* (n=1), *C. freundii* (n=3), *E. aglomerans* (n=1), and *E. cloacae* ((n=1) and *E. coli* (n=21) (Table 2).

Resistance to ampicillin was 100%, followed by 88.8% to ceftriaxone, 77.8% to trimethoprim-sulphamethoxazole, 74.0% to cefotaxime, 70.4% to ciprofloxacin, 66.6% to ceftazidime, 22.2% to cefoxitin and 7.6% to gentamicin. Multi-drug resistance (resistance to three or more antibiotic classes) was evident in 19 (70.4%) isolates, of which 15 were from South Africa and 4 from

148 Mozambique. Multi-drug resistance was not evident in the three isolates from Brazilian chicken.
149 The predominant ESBL gene was bla_{SHV} (85.0%), followed by bla_{CTX-M} (62.9%) and
150 bla_{TEM} (44.4%). DNA sequencing of a random selection of CTX-M amplicons putatively identified
151 $bla_{CTX-M-55/-79/-101/-164}$, with $bla_{CTX-M-164}$ being most common (Table 2). The most prevalent AmpC
152 genes were bla_{MOX} (33, 3%) and bla_{DHA} (33.3%) whilst *qepA* (22.2%) was the predominant
153 plasmid-mediated fluoroquinolone resistance gene. The majority of genes were isolated from *E.*
154 *coli*, where SHV was the most predominant. CTX-M-55 was only detected in *Citrobacter* spp and
155 $bla_{CTX-M-79/-101/-164}$ were identified in *E. coli*. Resistance gene content ranged from 0-9 in a myriad
156 of permutations and combinations.
157
158 Based on ERIC-PCR profiles, the *E. coli* isolates had similarities in banding patterns varying from
159 4 to 15 fragments, ranging in size from 0.5 to 20 kb in length. Amplification of different intensities
160 was observed and visual analysis of the ERIC profiles included primary, secondary and tertiary
161 amplification. All isolates were typeable using this fingerprinting technique and profiles were
162 reproducible on repeat amplification. The ERIC–PCR profiles allowed the differentiation of the 21
163 *E. coli* isolates into 14 ERIC-types which were grouped into seven clusters (C1-7), with majority
164 of the isolates being found in cluster 3 (Fig. 1; C3). Most of the Brazilian and Mozambican
165 samples were observed in clusters C3-5 while South African samples shared more similarity.
166
167 The ERIC-PCR fingerprint profiles of the four *Citrobacter* spp. isolates are shown in Figure 2.
168 Isolates exhibited banding patterns varying from 5 to 18 fragments of 0.5 to 20 kb in length and the
169 *C. freundii* isolates were not clonal. Although the CTX-M and SHV genes were identified in 75%
170 of these isolates, variation was observed in pAmpC and quinolone resistance gene content.

DISCUSSION

Plasmid-mediated antibiotic resistance to third generation cephalosporins, cephamycins and fluoroquinolones is increasingly being reported in meat products such as chicken *(10, 17, 24)* which is the most popular animal protein in Mozambique. In this study 198 samples, yielded 27 (13.6%) ESBL-positive isolates from 22 chickens. ESBL frequency in this study was higher than the a study from Nigeria where a single isolate expressed the CTX-M-15 gene from a total sample of 96 *E. coli* from 96 chickens (10) and lower than a study in China where 64 (31.7%) of *E. coli* isolates from 202 chickens were ESBL positive (*17)*. The majority of ESBLs were isolated in South African chicken (19) followed by Mozambican (4) and finally Brazilian chicken (3). This may be related to selection pressure of antibiotics used as growth promoters, for prophylaxis and metaphylaxis in South Africa (personal communication – Dr D Petty*)*. *E. coli* was the predominant bacterial species isolated, which is consistent with the observation that *E. coli* are one of ESBL-producing bacteria that are often isolated in greater numbers in food-producing animals *(24)*. Similar results were found in healthy broiler chickens in Germany, where *E. coli* and *Enterobacter cloacae* were identified *(21)*.

Susceptibility testing results showed high levels of resistance to the antibiotics tested. Resistance to ampicillin was 100%, followed by 88.8% to ceftriaxone, 74% to cefotaxime, 70.4% to ciprofloxacin, 66.6% to ceftazidime and 22.2% to cefoxitin similar to that observed in a study carried out in Germany on poultry imported from Italy for ampicillin (100%) but different for cefotaxime which was higher at 94% and for ceftazidime which was lower at 30% *(2)*.The resistance recorded for ceftriaxone was 90.0% in a study carried out in Owerri, Nigeria to determine the presence of ESBL-producing *E. coli* strains from poultry, similar to our results but,

195 in contrast, showed 100% resistance to ceftazidime and cefotaxime *(4)*. In a study involving 163
196 broiler chicken conducted in Italy the resistance rates of cefotaxime and ceftazidime were also
197 higher, reaching 91.7% for both antibiotics (*11*).
198
199 The β-lactam antibiotic susceptibility profiles were putatively corroborated by the ESBLs and
200 pAmpC genes identified in 78.9% (15/19) of the South African isolates, 60.0% (3/5) of the
201 Mozambican isolates 33% (1/3) of the Brazilian isolates (in the absence of definitive gene
202 identification save for some CTX-M genes) (Table 2). Examples of anomalies were *E. coli* A25c
203 which carried the TEM, MOX and DHA genes but was sensitive to ceftazidime and cefoxitin, *E.*
204 *coli* A23c which carried only the TEM gene but was resistant to all the β-lactams tested and *E. coli*
205 M32c, *B18c* and B23d which all carried the CTX-M genes but were sensitive to cefotaxime.
206 These anomalies point to silent or minimally expressed genes in the main while the expression of
207 multiple ESBLs and pAmpCs of the same family cannot be discounted. For example, both
208 inhibitor-resistant TEMs and TEM ESBLs were hypothesized in *E. coli* A17c.
209
210 Fluoroquinolone resistance was evident only in the South African isolates. Resistance to
211 ciprofloxacin was 70.4%, lower than that found in *E. coli* from retail broiler chicken in Italy (88.
212 8%) *(14)* and higher than the *E. coli* from Germany in a study carried out in poultry imported from
213 Italy (39%) (*2,11*). In a study by Fortin *et al*. (2011) conducted in *E. coli* isolates from healthy
214 animals from Nigeria, *qepA*, *qnrB* and *qnrS* were also detected, although at different frequencies
215 with a higher prevalence of *qnrS* (12.5%) and a lower prevalence of *qnrB* (4.1%) *and qepA* (3.1%),
216 *(9)*. In Abidjan, Ivory Coast, *qnrA*, *qnrB* and *qnrS* were also observed and the most prevalent gene
217 was *qnrB* at 14.6% *(12)*. Six isolates exhibited resistance in the absence of *qnr* and *qep* genes

indicating alternative mechanisms of resistance such as the chromosomal mutations in the quinolone resistance determining regions of the *gyrA, gyrB, parC* and *parE* target genes or efflux *(7,13).*

Notwithstanding the need to definitively confirm the genotypic results, ideally by whole genome sequencing, the complexity and diversity of various mobile resistance genes combinations and permutations is of concern in the food safety context. Co-carriage of ESBL, pAmpC and plasmid mediated fluoroquinolone resistance genes have been similarly observed in Italy by Ghodousi *et al*. (2015), albeit not in this complexity *(11).*

In several studies related to the ESBL-producing bacteria in chicken, the gene most frequently isolated is $bla_{\text{CTX-M}}$ *(21, 22)*, which differs from this study where the most common gene was $bla_{\text{SHV.}}$ In a study carried out in Henan Province *(26)*, the most predominant gene was bla_{TEM}. CTX-M-55, CTX-M-79, CTXM-101 and CTX-M-164 genes were detected in this study, differing from other studies where CTX-M-1 was most prevalent *(5, 16, 22)*. CTX-M-79 and CTXM-101were found in Northeast China (Heilongjiang, Liaoning, Jilin) and in the Jiangsu province in a study characterizing ESBLs in *E. coli* from chickens, although with lower frequencies of 3.,6% and 0.5%, respectively *(2)*. CTX-M-55 was only detected in *C. freundii* strains isolated from South African chickens while CTX-M β lactamase was characterized in *E. coli* isolates in a study in China *(17)*. To our knowledge, this is the first report of the putative CTX-M-164 in chicken.

Among the three countries, CTX-M-55, CTX-M-79 and CTX-M-101 were only detected in South African isolates while CTX-M-164 genes were detected in two South African and one Brazilian

chicken. The CTX-M ESBLs found in our study are dissimilar to those isolated in humans as evident from a non-systematic literature review of research published in 2008-2012, which described the prevalence of CTX-M 1, 3, 9, 14a, 14b, 15, 27 and 28 in *Enterobacteriaceae* from hospital and community settings in Africa *(26)*. Other studies have, in contrast, showed similarity between ESBLs isolated from *E. coli* in chicken meat and humans *(14)*.

Overall, the ERIC-PCR dendogram showed extensive diversity of *E. coli* isolates, however, there were some isolates that demonstrated similarity, especially those of South African origin. Mozambican *E. coli* isolates shared some similarity with South African and Brazilian isolates. Interestingly, only South African *E. coli* isolates carried plasmid-mediated quinolone resistance genes while isolates from all three origins harbored ESBL and pAmpC genes in different permutations and combinations. There was no correlation between the ERIC-PCR profiles and resistance genes identified. Isolates with similar profiles demonstrated different resistance gene content, e.g., M27D (SHV) and B18C (CTX-M, TEM, CMY, MOX and DHA) in cluster C3 and A10D (CTX-M, SHV, TEM, MOX, FOX, DHA, *qnrB, qnrS* and *qepA*) and A12C (CTX-M, SHV and TEM) in cluster C1. CTX-M and SHV genes were amplified from isolates belonging to all clusters, however the *qnr* and *qepA* genes were identified in South African isolates clustering in C1-3.

The results of this study showed the presence of ESBL- and pAmpC-producing and fluoroquinolone resistant bacteria in chickens consumed in Mozambique. This represents a food safety concern as the Mozambican population is exposed to a reservoir of plasmid-mediated, and

hence mobile β-lactam and fluoroquinolone resistance genes via imported, and to a lesser extent, locally produced poultry.

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Legends

Table Legends

Table 1. Primers and Annealing Temperatures

Table 2: Susceptibility Results and Resistance Genes

Figure Legends

Figure 1: Dendogram representing the genetic relatedness and cluster analysis of 21 *E. coli* isolated from South African (A), Mozambican (M) and Brazilian (B) chicken samples 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. Included are PCR results for ESBL (CTX-M, SHV, TEM), pAmpC (CMY, MOX, FOX, DHA) and plasmid-mediated fluoroquinolone resistance genes (*qnrB, qnrD, qnrS* and *qepA*), where (+) represents positive results and (-) negative results.

Figure 2: Dendogram representing the genetic relatedness and cluster analysis of three *C. freundii* and a *Citrobacter diversus* isolated from South African (A) and Brazilian (B) chicken samples based on ERIC-PCR fingerprinting patterns using Jacquard index and UPGMA algorithm. Included are PCR results for ESBL (CTX-M, SHV, TEM), pAmpC (CMY, MOX, FOX, DHA) and plasmid-mediated fluoroquinolone resistance genes (*qnrB, qnrD, qnrS* and *qepA*), where (+) represents positive results and (-) negative results.

Table 1. Primers and Annealing Temperatures

Gene	Sequence (5´ to 3´)	Annealing temp (^{O}C)	Reference
CTX-M	F - GGTTAAAAAATCACTGCGTC	53	*(12)*
	R - TTGGTGACGATTTTAGCCGC		
TEM	F - AAAATTCTTGAAGACG	60	*(23)*
	R - TTACCAATGCTTAATCA		
SHV	F - TTAACTCCCTGTTAGCCA	56	*(23)*
	R - GATTTGCTGATTTCGCCC		
CMY	F - GATTCCTTGGACTCTTCAG	50	*(19)*
	R - TAAAACCAGGTTCCCAGATAGC		
MOX	F - GCTGCTCAAGGAGCACAGGAT	50	*(19)*
	R - CACATTGACATAGGTGTGGTGC		
FOX	F - CACCACGAGAATAACCAT	50	*(19)*
	R - ATGTGGACGCCTTGAACT		
DHA	F - AACTTTCACAGGTGTGCTGGGT	50	*(19)*
	R - CCGTACGCATACTGGCTTTGC		
qnrB	F - GGAATCGAAATTCGCCACTG	49	*(17)*
	R - TTTGCCGTTCGCCAGTCGAA		
qnrD	F - AGATCAATTTACGGGAATA	49	*(28)*
	R - AACAAGCTGAAGCGCCTG		
qnrS	F - CACTTTGATGTCGCAGAT	49	*(29)*
	R - CAACATACCCAGTGCTT		
qepA	F - CCGATGACGAAGCACAGGG	49	*(15)*
	R - CTACGGGCTCAAGCAGTTGG		

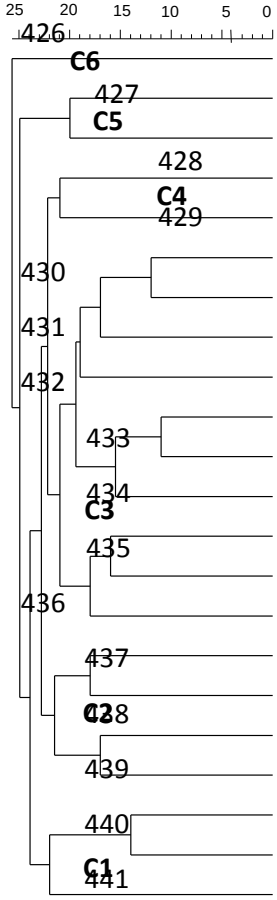
F-Forward; R-Reverse

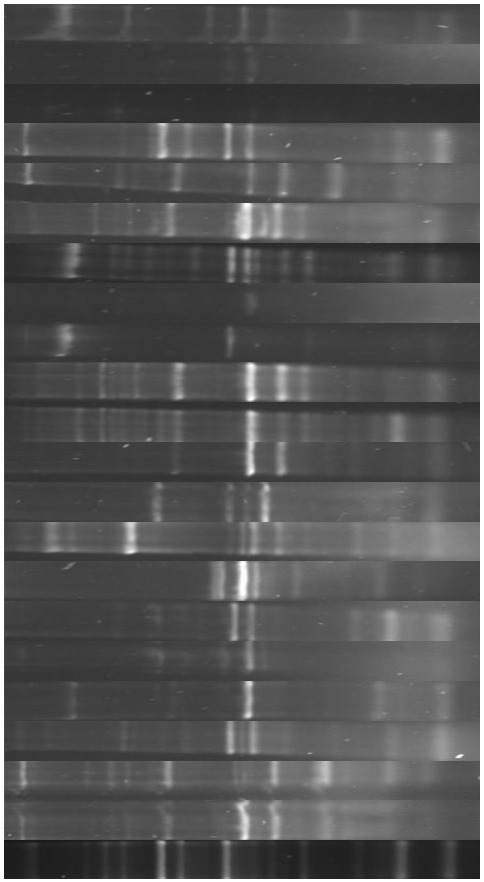
Table 2: Susceptibility Results and Resistance Genes

Country	Code	Species	Source	Antibiotic Susceptibility									B-Lactamase Genes							Plasmid-Mediated Fluoroquinolone Genes			
				AUG	CTX	CAZ	CTR	CFX	GEN	AMP	SXT	CIP	CTX-M	SHV	TEM	CMY	MOX	FOX	DHA	*qnrB*	*qnrD*	*qnrS*	*qepA*
RSA	A3d	*E. aglomerans*	Thaw	S	R	R	R	R	S	R	R	R	-	+	-	-	+	-	+	+	-	+	+
	A10c	*E.coli*	Carcass	R	R	R	R	R	S	R	R	R	+	+	+	+	+	+	+	-	-	-	+
	A10d	*E.coli*	Thaw	S	R	R	R	I	S	R	R	R	+	+	+	-	+	+	+	+	-	+	+
	A12c	*E.coli*	Carcass	S	I	S	R	S	S	R	R	S	CTX-M-101	+	+	-	-	-	-	-	-	-	-
	A13d	*E.coli*	Thaw	S	I	R	R	S	S	R	R	R	CTX-M-79	+	+	-	-	-	-	-	-	-	-
	A17c	*E.coli*	Carcass	I	R	R	R	S	S	R	R	S	-	-	+	-	-	-	-	-	-	-	-
	A19c	*C. diversus*	Carcass	S	R	R	R	S	S	R	R	S	CTX-M-55	+	+	-	-	-	-	+	-	+	+
	A20c	*E.coli*	Carcass	S	R	R	R	S	S	R	R	R	-	+	+	-	-	-	-	-	-	-	-
	A23c	*E.coli*	Carcass	R	R	R	R	R	S	R	R	R	-	-	+	-	-	-	-	+	-	-	-
	A25c	*E.coli*	Carcass	S	I	S	R	S	S	R	R	R	-	-	+	-	+	-	+	-	-	-	-
	A25d	*E.coli*	Thaw	I	R	R	R	S	S	R	R	I	-	+	-	-	-	-	-	-	-	-	-
	A26c	*C. freundii*	Carcass	I	R	R	R	S	S	R	R	R	CTX-M-55	+	-	-	+	-	+	+	+	+	+
	A26d	*C. freundii*	Carcass	S	R	R	R	I	S	R	R	R	+	+	+	-	+	-	+	-	+	-	-
	A27d	*E.coli*	Thaw	S	R	R	R	I	S	R	R	R	-	-	-	-	-	-	-	-	-	-	-
	A29c	*E.coli*	Carcass	S	R	R	R	S	S	R	R	R	+	+	-	-	-	-	-	-	-	-	-
	A30C	*E.coli*	Carcass	S	R	I	R	S	S	R	R	R	+	+	+	-	-	-	-	-	+	+	+
	A30d	*E.coli*	Thaw	R	R	R	R	R	S	R	R	S	+	+	+	+	+	-	+	-	-	-	-
	A33d	*E.coli*	Thaw	S	R	R	R	S	S	R	R	R	CTX-M-164	+	-	-	-	-	-	-	-	-	-
	A32d	*E.coli*	Thaw	S	R	R	R	S	S	R	R	R	CTX-M-164	+	+	-	-	-	-	-	-		-

Brazil	B17c	*E.coli*	Carcass	*R*	*S*	S	S	S	S	R	S	R	-	+	-	-	-	-	-	-	-	-
	B18c	*E.coli*	Carcass	*R*	*S*	R	I	R	R	R	S	S	CTX-M-164	+	+	+	+	-	+	-	-	-	-
	B23d	*C. freundii*	Thaw	S	S	S	S	I	S	R	S	S	+	+	-	-	-	-	-	-	-	-	-
Mozam-bique	M15d	*E. cloacae*	Thaw	S	R	S	R	S	S	R	R	R	-	-	-	-	-	-	-	-	-	-	-
	M27c	*E.coli*	Carcass	R	R	R	R	R	S	R	S	S	-	+	-	-	+	-	+	-	-	-	-
	M27d	*E.coli*	Thaw	S	R	S	R	S	S	R	R	R	-	+	-	-	-	-	-	-	-	-	-
	M30d	*E.coli*	Thaw	S	R	S	R	S	I	R	R	R	+	+	-	-	-	-	-	-	-	-	-
	M32c	*E.coli*	Carcass	S	S	S	R	S	S	R	R	R	+	+	-	-	-	-	-	-	-	-	-

R-resistant; S-sensitive; I-intermediate; AUG - Amoxicillin/ clavulanic acid (≥26-S, 23-25-I, ≤22-R); CTX- Cefotaxime (≥18-S, 14-17-I, ≤13-R); CAZ-Ceftazidime (≥21-S, 18-20-I, ≤17-R); CTR-Ceftriaxone (≥23-S,20-22-I,≤19-R); CFX-Cefoxitin (≥18-S,15-17-I,≤14-R); GEN-Gentamicin (≥15-S,13-14-I,≤12-R), AMP-Ampicillin (≥17-S, 13-16-I, ≤13-R); SXT – Trimethoprim-sulphamethoxazole (≥16-S, 11-15-I,≤10-R); CIP-Ciprofloxacin (≥21-S,16-20-I,≤15-R)

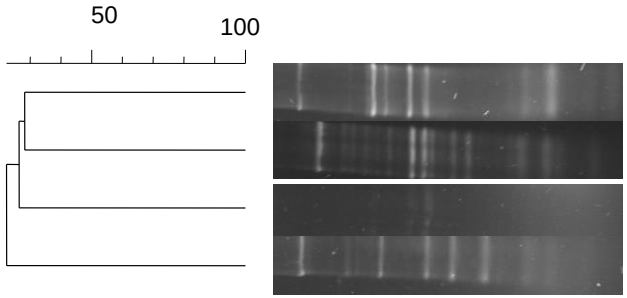




Isolate code	Species	CTX-M	SHV	TEM	CMY	MOX	FOX	DHA	*qnrB*	*qnrD*	*qnrS*	*qepA*
A33D	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
A27D	*E. coli*	-	-	-	-	-	-	-	-	-	-	-
M32C	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
A20C	*E. coli*	-	+	+	-	-	-	-	-	-	-	-
M30D	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
A17C	*E. coli*	-	-	+	-	-	-	-	-	-	-	-
M27C	*E. coli*	-	+	-	-	+	-	+	-	-	-	-
A29C	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
A32D	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
B18C	*E. coli*	+	+	+	+	+	-	+	-	-	-	-
M27D	*E. coli*	-	+	-	-	-	-	-	-	-	-	-
B17C	*E. coli*	-	+	-	-	-	-	-	-	-	-	-
A13D	*E. coli*	+	+	+	-	-	-	-	-	-	-	-
A30C	*E. coli*	+	+	+	-	-	-	-	-	+	+	+
A10C	*E. coli*	+	+	+	+	+	+	+	-	+	-	+
A25C	*E. coli*	-	-	+	-	+	-	+	-	-	-	-
A25D	*E. coli*	+	+	-	-	-	-	-	-	-	-	-
A23C	*E. coli*	-	-	+	-	-	-	-	+	-	-	-
A30D	*E. coli*	+	+	-	+	-	+	+	-	-	-	-
A10D	*E. coli*	+	+	+	-	+	+	+	+	-	+	+
A12C	*E. coli*	+	+	+	-	-	-	-	-	-	-	-
E coli ATCC 25922												

447

448



Isolate code	Species	CTX-M	SHV	TEM	CMY	MOX	FOX	DHA	*qnrB*	*qnrD*	*qnrS*	*qepA*
A19C	*C. diversus*	+	+	+	-	+	-	+	-	+	-	-
B23D	*C. freundii*	+	+	-	-	-	-	-	-	-	-	-
A26D	*C. freundii*	-	-	-	-	-	-	-	-	-	-	-
A26C	*C. freundii*	+	+	-	-	+	-	+	+	+	+	+

CHAPTER 3

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

3.1 Conclusions

Ninety-nine frozen chickens, consisting of 33 chicken imported from South Africa, 33 from Brazil and 33 locally produced in Mozambique, were purchased from three different supermarkets in Maputo City to form the study sample. Acquisition was done weekly during May 2015 to obtain samples from different batches. Two samples were taken from each chicken, one from the thaw liquid and the other from a swab of the internal parts of the carcass yielding a total of 198 samples from 99 chickens. The samples were phenotypically and genotypically tested for the presence of ESBL, pAmpC and plasmid-mediated quinolone resistance genes.

The following were the main findings:

- Five different bacterial species were identified from 27 positive cultures, viz., 21 *E. coli*, 3 *Citrobacter freundii*, 1 *Citrobacter diversus*, 1 *Enterobacter aglomerans* and 1 *Enterobacter cloacae.*

  Of these, 19 were isolated from South African chicken, 5 from Mozambican and 3 from Brazilian chickens.
- Resistance to ampicillin was 100%, followed by 88.8% to ceftriaxone, 77.8% to trimethoprim-sulphamethoxazole, 74.0% to cefotaxime, 70.4% to ciprofloxacin, 66.6% to ceftazidime, 22.2% to cefoxitin and 7.6% to gentamicin.

  Multi-drug resistance (resistance to 3 or more antibiotic classes) was evident in 19 (70.4%) isolates, of which 15 were from South Africa and 4 from Mozambique.
- The predominant ESBL gene was bla_{SHV} (85%), followed by $bla_{\text{CTX-M}}$ (62.9%) and bla_{TEM} (44.4%) whilst bla_{MOX} (33, 3%) and bla_{DHA} (33.3%) were the most common AmpC genes.

- DNA sequencing of the *bla*$_{\text{CTX-M}}$ genes putatively identified *bla*$_{\text{CTX-M-55/-79/-101/-164}}$, with *bla*$_{\text{CTX-M-164}}$ being most common. To our knowledge, this is the first report of the putative CTX-M-164 from chicken in Africa.
- The predominant plasmid-mediated fluoroquinolone-resistance gene was *qepA* (22.2%).
- The complexity and diversity of mobile resistance genes combinations and permutations is of concern in the food safety context.
- Isolates did not appear to be clonal, but shared similarity particularly *E. coli* of South African origin. No one specific ERIC-PCR profile was linked to carriage of plasmid-mediated resistance genes.
- The Mozambican population is thus exposed to a reservoir of plasmid-mediated and hence, mobile β-lactam and fluoroquinolone resistance genes from imported, and to a lesser extent, locally-produced chicken.

3.2 Limitations

- The small sample size precluded conclusions on the true prevalence of ESBL, pAmpC-producing Enterobacteriaceae with co-resistance to fluoroquinolones in chicken locally produced in Mozambique and imported from South Africa and Brazil.
- Limited resources precluded the definitive identification of all but some of the CTX-M genes by DNA sequencing.

3.3 Recommendations

The following recommendations emanated from this study:

- The study should be repeated with a larger sample size to obtain representative results.
- A similar study should be undertaken on chickens sold in informal markets to ascertain the nature and extent of resistance in this sector.
- All resistance genes should be definitively identified by DNA sequencing.
- Alternative mechanisms of resistance should be investigated as appropriate.

- This baseline data should engender further research to generate evidence to inform AMR policy in Mozambique, specifically to improve/inform agricultural practice related to growth promoters in poultry

Appendix: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 May 2016

Mrs Sara Lino Faife
School of Health Sciences
Westville Campus

Dear Mrs Faife,

Protocol reference number: AREC/064/015
Project title: Prevalence of ESBL-producing Enterobacteriaceae in Poultry

Full Approval – Research Application

With regards to your revised application received on 03 May 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 May 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor Sabiha Essack
Cc Academic Leader Research: Professor Mershen Pillay
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc ISCISA

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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