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Original Article

β-lactam and fluoroquinolone resistance in *Enterobacteriaceae* from imported and locally-produced chicken in Mozambique

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Abstract

Introduction: Plasmid-mediated resistance to β-lactam and fluoroquinolone antibiotics was investigated in *Enterobacteriaceae* isolated from retailed frozen chickens from Brazil, South Africa and Mozambique.

Methodology: Carcass swabs and the liquid thaw of 33 chickens from each of the three countries constituted the total sample size of 198. Isolates were identified by biochemical tests, antibiotic susceptibility was ascertained by the disc diffusion assay and β-lactamases were detected using the double-disk synergy test. PCR was used to detect the presence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{CMY}}$, *bla*$_{\text{MOX}}$, *bla*$_{\text{FOX}}$, *bla*$_{\text{DHA}}$, *qnrB*, *qnrD, qnrS* and *qepA* genes. A random selection of CTX-M genes was sequenced.

Results: The 198 samples yielded 27 (13.6%) putative extended-spectrum β-lactamase (ESBL)-positive isolates, 15 from carcass swabs and 12 from the liquid thaw from 22 chickens with 19, 5 and 3 isolates from South African, Mozambican and Brazilian chicken, respectively. Isolates exhibited the following resistance: ampicillin 100%, ceftriaxone 89%, trimethoprim-sulfamethoxazole 78%, cefotaxime 74%, ciprofloxacin 70%, ceftazidime 67%, cefoxitin 22% and gentamicin 8%. The predominant putative ESBL gene was *bla*$_{\text{SHV}}$ (85%), followed by *bla*$_{\text{CTX-M}}$ (62.9%) and *bla*$_{\text{TEM}}$ (44.4%) whilst *bla*$_{\text{MOX}}$ and *bla*$_{\text{DHA}}$ were the most common pAmpC genes at 33.3%. The predominant plasmid-mediated fluoroquinolone-resistance gene was *qepA* (22.2%). DNA sequencing identified *bla*$_{\text{CTX-M-55/-79/-101/-164}}$. ERIC–PCR profiles did not show strong evidence of clonality.

Conclusion: The Mozambican population is exposed to a reservoir of plasmid-mediated, and hence mobile β-lactam and quinolone resistance genes *via* imported, and to a lesser extent, locally produced poultry. This presents a food safety concern.

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

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Introduction

Antibiotic resistance is a global health problem in humans, (food) animals and the environment. Resistance exists to all currently used antibiotics in human and veterinary medicine [1]. The use of antibiotics for growth promotion, prophylaxis and metaphylaxis in addition to the treatment of infections in food animals results in the emergence of antibiotic resistant bacteria which can reach humans through zoonosis and food [2]

Although antimicrobial use in food animals is permitted, responsible and prudent use is recommended by the World Organization for Animal Health (OIE) [3]. For example, as the third- and fourth-generation cephalosporins and fluoroquinolones are important for both human and animal health, it is recommended that they should not be used as preventive treatment in feed or water nor in the absence of clinical signs, nor as first line, unless justified by bacteriological testing [3].

Notwithstanding the above OIE recommendation, 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 [4–6].

In Africa, the most common ESBL genes detected in bacteria among food-producing animals are $bla_{\text{CTX-M}}$, followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ genes and mostly in *Escherichia coli*. Antibiotic resistance in food products in Africa have also been reported [7-10].

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 [11]. 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

Study Sample

Ninety-nine frozen chickens, consisting of 33 chickens imported from each of South Africa and Brazil, and 33 locally produced in Mozambique, were purchased from three different major supermarkets in Maputo City. Eleven chickens from each country were randomly purchased weekly during May 2015 from each of the three supermarkets selected as they carried chickens produced in all three countries. The chickens were from a single producer in each country.

Two samples were taken from each chicken, one from the liquid thaw considered a proxy for the carcass rinsate and the other from a swab of the carcass yielding a total of 198 samples from 99 chickens. One milliliter of liquid thaw was collected in a sterile tube and used for culturing procedures. The carcass sample was taken by passing the swab into different internal parts of the carcass. Samples were cultured on ESBL-selective media, i.e., MacConkey agar (Oxoid Ltd, Basingstoke, Hampshire, England) plates supplemented with 2 mg/L of ceftriaxone using the streak method [12].

Species identification and antimicrobial susceptibility testing

All Gram-negative, oxidase-negative bacilli were identified to species level using biochemical tests (sugars with Kligler iron agar, motility-indole-ornithine agar, citrate agar, urea agar, lysine agar) [12]. After overnight incubation at 37°C, lactose and glucose fermenters, gas producers, indole-positive, lysine and ornithine-positive and motile isolates were identified as *E. coli*. *Citrobacter* spp. were identified as all Gram-negative bacilli which were lactose and glucose fermenters, gas producers, citrate and urease-positive and motile. The production of sulfuric acid served as a differentiator between *Citrobacter diversus* and *Citrobacter freundii*. *Enterobacter* spp. were identified as lactose and glucose fermenters, gas producers, citrate- and ornithine-positive and motile isolates. *Enterobacter* spp. which were urease-positive were identified as *Enterobacter cloacae* and those that were lysine-positive were identified as *Enterobacter agglomerans* [13].

Antimicrobial susceptibility testing (AST) was undertaken using the agar disc diffusion assay according to Clinical and Laboratory Standards Institute (CLSI) guidelines [14] with the antibiotic panel consisting of ampicillin, cefotaxime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, gentamicin, amoxicillin/clavulanic acid and trimethoprim-sulfamethoxazole. *E. coli* ATCC 25922 was used as the control.

Phenotypic detection of putative ESBLs, and pAmpCs

ESBL production was confirmed using the double-disk synergy test (DDST) using ROSCO disks containing cephalosporins (cefepime, cefotaxime, ceftazidime,) with and without clavulanic acid [15]. *E. coli* ATCC 35218 producing TEM-1 and *Klebsiella pneumoniae* ATCC 700603, producing SHV-18 were used as controls. The phenotypic AmpC confirmation test was based on inhibition of AmpC by cloxacillin and boronic acid derivatives [15] with *E. coli* ATCC 25922 and *E. coli* ATCC 35218 as controls. Results were considered positive when the inhibition zones of the combined discs were ≥ 5 mm compared to that of the antibiotic discs alone.

DNA extraction, PCR and electrophoresis

Genomic DNA was extracted from overnight bacterial cultures using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, USA) as per manufacturer's guidelines. PCR amplification of ESBL, pAmpC, *qnr* and *qepA* genes was performed with primers and conditions previously described [16–19], in a final volume of 25 µL, containing 12.5 µL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific,Waltham, USA), 7.5 µL of sterilized distilled water, 2 µL of each primer and 1 µL template DNA. PCR was undertaken in a T100™ Thermal cycler (Bio-Rad, Hercules, USA). The cycle comprised of preliminary denaturation for 10 seconds at 98°C, followed by 35 cycles of denaturation at 98°C for 1 second, annealing for 5 seconds and elongation at 72°C

for 7 minutes. Annealing temperatures and primers are described in the Supplementary Table 1. Amplicons were visualized by electrophoresis in 1.5% agarose gels for 40 minutes at 120 V, stained with gel red and detected by ultraviolet trans-illumination.

Randomly selected CTX–M PCR amplicons were 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 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

ERIC-PCR was undertaken in a total reaction volume of 10 µL, which contained 2 µL of template DNA and 0.1 µL of 100 µM primers ERIC 1 and ERIC 2 [20] and 5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, USA). PCR conditions were as follows: 94°C for 3 minutes, 30 cycles of 30 seconds of denaturation at 94°C, 1 minute of annealing at 50°C, 8 minutes of extension at 65°C and a final elongation of 16 minutes at 65°C, in an Applied Biosystems 2720 Thermal Cycler. The ERIC-PCR products were loaded onto 1.0% (w/v) agarose gels and subjected to electrophoresis at 80V using 1× TAE buffer. Amplification products were visualized by UV trans-illumination (Syngene, Cambridge, UK) after staining in 0.1 mg/mL ethidium bromide for 15 minutes. Genotypic variations were analyzed using the Gel Compare II version 6.0 software package (Applied Maths) by Jacquard and Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a dendrogram. Optimization and band tolerance were set at 1% and 80% similarity cut-off was used to define clusters.

Results

Isolates identified

Of the 198 samples, 27 were putatively ESBL-positive: 15 from the carcass swabs and 12 from the liquid thaw from a total of 22 chickens. Five chickens

Table 1. Antibiotic susceptibility results.

Country	Code	Species	Source	Antibiotic susceptibility								
				AMC	CTX	CAZ	CRO	FOX	GEN	AMP	SXT	CIP
South Africa	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
	A13d	*E.coli*	Thaw	S	I	R	R	S	S	R	R	R
	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
	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
	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
	A32d	*E.coli*	Thaw	S	R	R	R	S	S	R	R	R
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
	B23d	*C. freundii*	Thaw	S	S	S	S	I	S	R	S	S
Mozambique	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; AMC - 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); CRO-ceftriaxone (≥ 23-S,20-22-I, ≤ 19-R); FOX-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).

(4 from South Africa and 1 from Mozambique) yielded positive results from both the carcass swab and the liquid thaw (12 isolates). 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 *Citrobacter diversus* (n = 1), *Citrobacter freundii* (n = 3), *Enterobacter agglomerans* (n = 1), *Enterobacter cloacae* (n = 1) and *E. coli* (n = 21) (Tables 1 and 2).

Susceptibility testing results

Resistance to ampicillin was 100%, followed by resistance to ceftriaxone (89%), gentamicin (78%), trimethoprim-sulfamethoxazole (78%), cefotaxime (74%), ciprofloxacin (70%), ceftazidime (67%), amoxicillin/clavulanic acid (22%) and cefoxitin (2%). Multi-drug resistance (resistance to three or more antibiotic classes) was evident in 19 (70.4%) isolates, 15 and 4 of which were from South Africa and Mozambique respectively.

PCR amplification results

PCR results indicated that the predominant ESBL gene was *bla*$_{SHV}$ (85.0%), followed by *bla*$_{CTX-M}$ (62.9%) and *bla*$_{TEM}$ (44.4%). DNA sequencing of a random selection of CTX-M amplicons identified *bla*$_{CTX-M-55/-79/-101/-164}$, with *bla*$_{CTX-M-164}$ being most common (Table 2). *bla*$_{MOX}$ (33.3%) and *bla*$_{DHA}$ (33.3%) were the most prevalent pAmpC genes and *qnr* and *qepA genes* (22.2%) were the most common fluoroquinolone resistance genes (TEM, SHV and AmpC and *qnr* genes were not sequenced).

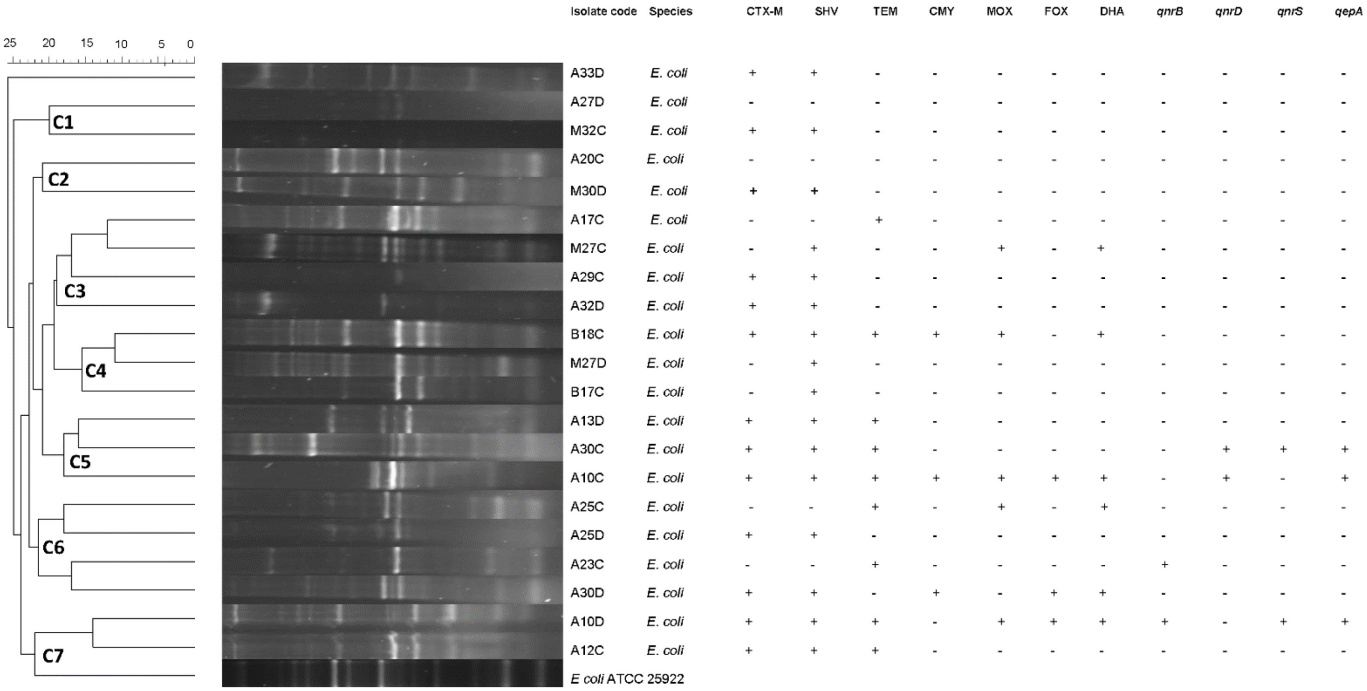
The majority of genes were isolated from *E. coli*, where SHV was the most predominant. *bla*$_{CTX-M-55}$ was only detected in *Citrobacter* spp. While *bla*$_{CTX-M-79/-101/-164}$ were identified in *E. coli*. Resistance gene content ranged from 0-9 in several permutations and combinations (Figure 1)

Based on ERIC-PCR profiles (Figure 1), the *E. coli* isolates had similarities in banding patterns varying from 4 to 15 fragments, ranging in size from 0.5 to 20 kb in length and allowed the differentiation of the 21 *E. coli* isolates into 14 ERIC-types which were grouped into seven clusters (C1-7), with the majority of the

Table 2. Antibiotic Resistance Genes Identified.

Country	Code	Species	Source	β-lactamase genes							Plasmid-mediated fluoroquinolone genes				
				CTX-M	SHV	TEM	CMY	MOX	FOX	DHA	*qnrA*	qnrB	*qnrD*	*qnrS*	*qepA*
South Africa	A10c	*E.coli*	Carcass	+	+	+	+	+	+	+	-	-	-	-	+
	A10d	*E.coli*	Thaw	+	+	+	-	+	+	+	-	+	-	+	+
	A12c	*E.coli*	Carcass	CTX-M-101	+	+	-	-	-	-	-	-	-	-	-
	A13d	*E.coli*	Thaw	CTX-M-79	+	+	-	-	-	-	-	-	-	-	-
	A17c	*E.coli*	Carcass	-	-	+	-	-	-	-	-	-	-	-	-
	A19c	*C. diversus*	Carcass	CTX-M-55	+	+	-	-	-	-	-	+	-	+	+
	A20c	*E.coli*	Carcass	-	+	+	-	-	-	-	-	-	-	-	-
	A23c	*E.coli*	Carcass	-	-	+	-	-	-	-	-	+	-	-	-
	A25c	*E.coli*	Carcass	-	-	+	-	+	-	+	-	-	-	-	-
	A25d	*E.coli*	Thaw	-	+	-	-	-	-	-	-	-	-	-	-
	A26c	*C. freundii*	Carcass	CTX-M-55	+	-	-	+	-	+	-	+	+	+	+
	A26d	*C. freundii*	Carcass	+	+	+	-	+	-	+	-	-	+	-	-
	A27d	*E.coli*	Thaw	-	-	-	-	-	-	-	-	-	-	-	-
	A29c	*E.coli*	Carcass	+	+	-	-	-	-	-	-	-	-	-	-
	A30C	*E.coli*	Carcass	+	+	+	-	-	-	-	-	-	+	+	+
	A30d	*E.coli*	Thaw	+	+	+	+	+	-	+	-	-	-	-	-
	A33d	*E.coli*	Thaw	CTX-M-164	+	-	-	-	-	-	-	-	-	-	-
	A32d	*E.coli*	Thaw	CTX-M-164	+	+	-	-	-	-	-	-	-		-
Brazil	B17c	E.coli	*Carcass*	-	+	-	-	-	-	-	-	-	-	-	-
	B18c	E.coli	*Carcass*	CTX-M-164	+	+	+	+	-	+	-	-	-	-	-
	B23d	C. freundii	*Thaw*	+	+	-	-	-	-	-	-	-	-	-	-
Mozambique	M15d	E. cloacae	*Thaw*	-	-	-	-	-	-	-	-	-	-	-	-
	M27c	E.coli	*Carcass*	-	+	-	-	+	-	+	-	-	-	-	-
	M27d	E.coli	*Thaw*	-	+	-	-	-	-	-	-	-	-	-	-
	M30d	E.coli	*Thaw*	+	+	-	-	-	-	-	-	-	-	-	-
	M32c	E.coli	*Carcass*	+	+	-	-	-	-	-	-	-	-	-	-

Figure 1. Dendrogram 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



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												

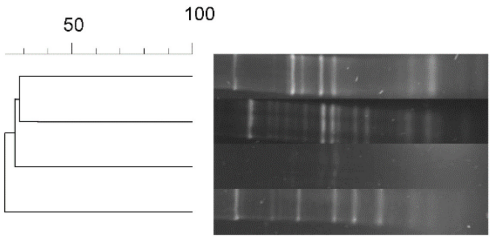
isolates being found in cluster 3 (Figure 1; C3). Most of the Brazilian and Mozambican samples were observed in clusters C3-5 while South African samples shared more similarity.

The ERIC-PCR fingerprint profiles of the four *Citrobacter* spp. isolates are shown in Figure 2. Isolates exhibited banding patterns varying from 5 to 18 fragments of 0.5 to 20 kb in length and the *C. freundii* isolates were not clonal. Although the CTX-M and SHV genes were identified in (3/4) 75% 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 [21–23] which is the most popular animal protein in Mozambique. Susceptibility testing results showed high levels of resistance to the antibiotics tested. Resistance to ampicillin was 100%, followed by 89% to ceftriaxone, 74% to cefotaxime, 70% to ciprofloxacin, 67% to ceftazidime and 22% to cefoxitin. These results were similar to a study on poultry from retail outlets in Hannover, Germany, which had been imported from Italy, where 100% resistance was observed to ampicillin while resistance

Figure 2. Dendrogram 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.



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*	+	+	-	-	+	-	+	+	+	+	+

to cefotaxime was higher at 94% and resistance to ceftazidime was lower at 30% [21]. The resistance recorded for ceftriaxone was 90% in a study carried out in Owerri, Nigeria, which investigated the presence of ESBL-producing *E. coli* from poultry, but, in contrast to our study, 100% resistance to ceftazidime and cefotaxime was observed [22]. In an Italian study, the resistance of bacteria isolated from 163 broiler chickens to cefotaxime and ceftazidime were also higher at 91.7% for both antibiotics [23].

The ESBL frequency of 13.6% in this study was lower than that reported in a study that characterized PMQR determinants, β-lactamases, plasmids and clonality among commensal *E. coli* isolated from 100 healthy chickens at slaughterhouse in Ibadan, Nigeria where ESBL- positive strains were isolated from 15% (15/100) of the chickens [24] as well as a study in Anhui Province, China where 49.5% (100/202) of chickens yielded ESBL-positive *E. coli* isolates [16]. The majority of ESBL-producing *Enterobacteriaceae* were isolated in South African chicken, followed by Mozambican and Brazilian chicken. This may be related to selection pressure of antibiotics used as growth promoters, for prophylaxis and metaphylaxis in South Africa (D. Petty, personal communication, May 14, 2016*). E. coli* was the predominant bacterial species isolated, which is consistent with the observation that *E. coli* is one of ESBL-producing bacteria that is often isolated in greater numbers in food-producing animals [25]. Similar results were found in healthy broiler chickens in Germany, where *E. coli* and *E. cloacae* were identified [21].

The β-lactam antibiotic susceptibility profiles were corroborated by the ESBLs and pAmpC genes identified in 78.9% (15/19) of the South African isolates, 60.0% (3/5) of Mozambican isolates and 33% (1/3) of Brazilian isolates (Table 1). Examples of anomalies were *E. coli* A25c which carried the TEM, MOX and DHA genes but was sensitive to ceftazidime and cefoxitin, *E. coli* A23c which carried only the TEM gene but was resistant to all the β-lactams tested and *E. coli* M32c, B18c and B23d which all carried the CTX-M genes but were sensitive to cefotaxime. These anomalies point to silent or minimally expressed genes in the main while the expression of ESBLs and pAmpCs belonging to other families is also possible.

Fluoroquinolone resistance was evident only in the South African isolates. Resistance to ciprofloxacin was 70.4%, lower than that found in *E. coli* from retail broiler chicken in Italy (88. 8%) [23] and higher than in *E. coli* (39%) from German retail outlets selling poultry of Italian origin [21]. In this study, a higher prevalence of *qepA* (22,2%)*, qnrB* (18,5%) and *qnrS* (18,5%) genes was detected compared to the study in Nigeria where the prevalence was lower at 3,1%, 4,2% and 9,4%, respectively [24] while the study in Anhui Province, China, reported *qnrS* as the most prevalent with none of the isolates being positive for *qnrA*, *qnrB* and *qepA* genes [16]. Six isolates exhibited resistance in the absence of *qnr* and *qep* genes indicating alternative mechanisms of resistance such as chromosomal mutations in the quinolone resistance determining regions of the *gyrA*, *gyrB*, *parC* and/or *parE* target genes or efflux [25,26].

Co-carriage of ESBL, pAmpC and plasmid-mediated quinolone resistance genes observed in this study has been similarly observed in a study on retail broiler chicken in Italy [23], albeit not at the same level. ESBL, AmpC and PMQR determinants were detected at a frequency of 98.5%, 11.2% and 91%, respectively in the Italian study, while in our study ESBL and PMQR determinants were detected at a lower frequency and AmpC frequency was higher at 23%. In several studies related to the ESBL-producing bacteria in chicken, the gene most frequently isolated is $bla_{\text{CTX-M}}$ [21,27], which differs from this study where the most common ESBL gene was bla_{SHV}. $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-79}}$, $bla_{\text{CTXM-101}}$ and $bla_{\text{CTX-M-164}}$ genes were definitively identified in this study, different from other studies where $bla_{\text{CTX-M-1}}$ was most prevalent [10,11,27,28]. CTX-M-79 and CTXM-101 were 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 [29].

Among the three countries, *E. coli* with $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-79}}$ and $bla_{\text{CTX-M-101}}$ genes were only detected in South African isolates while the $bla_{\text{CTX-M-164}}$ gene was detected in two South African and one Brazilian isolates. $bla_{\text{CTX-M-55}}$ was only detected in *C. freundii* isolates from South African chickens. To our knowledge, this is the first report of the $bla_{\text{CTX-M-164}}$ in 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 [30]. Other studies have, in contrast, showed similarity between ESBLs isolated from *E. coli* in chicken meat and humans [11].

Overall, the ERIC-PCR dendrogram 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.

Conclusion

This study describes the presence of β-lactam 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 quinolone resistance genes *via* imported, and to a lesser extent, locally produced poultry.

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Annex – Supplementary Items

Supplementary Table 1. Primers and Annealing Temperatures.

Gene	Sequence (5′ to 3′)	Annealing temp (°C)	Reference
CTX-M	F - GGTTAAAAAATCACTGCGTC R - TTGGTGACGATTTTAGCCGC	53	[17]
TEM	F - AAAATTCTTGAAGACG R - TTACCAATGCTTAATCA	60	[17]
SHV	F - TTAACTCCCTGTTAGCCA R - GATTTGCTGATTTCGCCC	56	[17]
CMY	F - GATTCCTTGGACTCTTCAG R - TAAAACCAGGTTCCCAGATAGC	50	[18]
MOX	F - GCTGCTCAAGGAGCACAGGAT R - CACATTGACATAGGTGTGGTGC	50	[18]
FOX	F - CACCACGAGAATAACCAT R - ATGTGGACGCCTTGAACT	50	[18]
DHA	F - AACTTTCACAGGTGTGCTGGGT R - CCGTACGCATACTGGCTTTGC	50	[18]
qnrB	F - GGAATCGAAATTCGCCACTG R - TTTGCCGTTCGCCAGTCGAA	49	[17]
qnrD	F - AGATCAATTTACGGGAATA R - AACAAGCTGAAGCGCCTG	49	[19]
qnrS	F - CACTTTGATGTCGCAGAT R - CAACATACCCAGTGCTT	49	[20]
qepA	F - CCGATGACGAAGCACAGGG R - CTACGGGCTCAAGCAGTTGG	49	[17]

F: Forward; R: Reverse.