# Occurrence, identification and antimicrobial resistance profiles of extended-spectrum and AmpC β-lactamase-producing *Enterobacteriaceae* from fresh vegetables retailed in Gauteng Province, South Africa.

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## Abstract

Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase-producing *Enterobacteriaceae* are no longer restricted to the health care system, but represent increased risks related to environmental integrity and food safety. Fresh produce has been increasingly reported to constitute a reservoir of multidrug resistant potential human pathogenic *Enterobacteriaceae*. This study aimed to detect, identify and characterize the antimicrobial resistance of ESBL/AmpC-producing *Enterobacteriaceae* isolates from fresh vegetables at point-of-sale. Vegetable samples [spinach, tomatoes, lettuce, cucumber and green beans (n=545)] were purchased from retailers in Gauteng, the most densely populated province in South Africa. These included street vendors, trolley vendors, farmers' market stalls and supermarket chain stores. Selective enrichment, plating onto chromogenic media and MALDI-TOF MS confirmation of isolate identities, showed that 17.4% (95/545) vegetable samples analyzed were contaminated with presumptive ESBL/AmpC-producing *Enterobacteriaceae*. Dominant species identified included *Escherichia coli*, *Enterobacter*

*cloacae*, *Enterobacter asburiae and Klebsiella pneumoniae.* Phenotypic antibiotic resistance analysis showed that 96.1% of 77 selected isolates were multidrug resistant, while resistance to aminoglycoside (94.8%), chloramphenicol (85.7%) and tetracycline (53.2 %) antibiotic classes were most prevalent. Positive phenotypic analysis for ESBL production were shown in 61 (79.2%) of the 77 isolates and AmpC production in 41.6% of the isolates. PCR and sequencing confirmed the presence of β-lactamase genes in 75.3% isolates from all vegetable types analyzed, mainly in *E. coli*, *Enterobacter* spp. and *Serratia* spp. isolates. CTX-M group 9 (32.8%) was the dominant ESBL type, while EBC (24.1%) was the most prevalent plasmidic type AmpC β-lactamase. Our findings document, for the first time, the presence of multidrug resistant ESBL/AmpC producing *Enterobacteriaceae* in raw vegetables sold at selected retailers in Gauteng Province, South Africa.

## Introduction

Extended-spectrum β-lactamase (ESBL)- and AmpC-producing *Enterobacteriaceae* have increased in occurrence globally in health care systems, agroecosystems and fresh produce, due to the widespread use of broad-spectrum antibiotics (Ye *et al.*, 2017). Dissemination of these antimicrobial resistant microorganisms have been identified as one of the six main antibiotic resistance (AR) related health risks globally (WHO, 2015). If infection by ESBL/AmpC-producing *Enterobacteriaceae* occurs, treatment options become limited as a result of expanded AR of the corresponding isolates (Freitag *et al.*, 2018). Since ESBL/AmpC β-lactamases are capable of inactivating broad spectrum penicillins and cephalosporins, their presence in *Enterobacteriaceae* is of clinical and epidemiological importance (Kolar *et al.*, 2010). Clinically important ESBL-producing *Enterobacteriaceae*

have been reported in different South African provinces [Eastern Cape (Vasaikar *et al.*, 2017); Western Cape (Peirano *et al.*, 2011); KwaZulu-Natal (Mahomed and Coovadia, 2014); and Gauteng Province (Ehlers *et al.*, 2009)]. In 53 clinical isolates from Gauteng, ESBL gene prevalence was reported in 87 % (Ehlers *et al.*, 2009).

ESBLs, classified as Ambler Class A enzymes, include TEM-, SHV- and CTX-M-type enzymes (Östholm, 2014; Ghafourian *et al.*, 2015). More than 200 TEM and SHV variants have been documented, while 90 different enzymes within the CTX-M type have been described (Östholm, 2014). Class A enzymes hydrolyse ampicillin and extended-spectrum cephalosporins (Ghafourian *et al.*, 2015). AmpC β-lactamases, classified as Class C enzymes, are resistant to additional β-lactams, i.e. cephamycins, and are not influenced negatively by class A enzyme inhibitors (Jacoby, 2009; Njage and Buys, 2017). Plasmid-mediated AmpC (pAmpC)-producing strains are distinguished from chromosomal AmpC since they are often not inducible (Mezzatesta *et al*., 2012). Six families of pAmpC-β-lactamases including CIT, FOX, MOX, DHA, EBC and ACC have been described, with DHA, CMY (CIT family member) and FOX most commonly detected (Thomson, 2010). Co-occurrence of β-lactamase enzymes, especially AmpC β-lactamases and ESBLs, are common (Thomson, 2010).

*Salmonella* spp., pathogenic *Escherichia coli* and *Shigella* spp. have been implicated in foodborne disease (FBD) outbreaks, while *Klebsiella pneumoniae*, *Serratia marcescens*, *Citrobacter freundii*, and *Enterobacter* spp. are regarded as opportunistic human pathogenic bacteria (Baylis *et al.*, 2011). The presence of ESBL/AmpC-producing *Enterobacteriaceae* on fresh produce has been studied worldwide (Kim *et al.*, 2015; Nüesch-Inderbinen *et al.*, 2015; Zurfluh *et al.*, 2015). Transfer of multidrug resistant (MDR) *Enterobacteriaceae* onto fresh produce occur through the use of contaminated irrigation water or during production via animal manure (van Hoek *et al.*, 2015). Subsequent transfer to humans can happen through consumption of raw vegetables, potentially impacting consumer health negatively (Ye *et al.*,

2017). Concomittantly AR genes can easily be transferred to commensal bacteria which typically colonize the human gut.

Fresh vegetables produced in SA are retailed nationally and to the South African Development Community (SADC) countries, Swaziland, the United Kingdom, Middle East and Asian markets (DAFF, 2012a, 2012b, 2016). Current knowledge regarding the occurrence of ESBL/AmpC- producing *Enterobacteriaceae* on fresh vegetables in SA is limited. The aim of this exploratory study was to detect, to identify and to characterize the AR of ESBL- and AmpC-producing *Enterobacteriaceae* isolates from frequently consumed fresh vegetables from selected retailing sites in Gauteng, the most densely populated province in SA.

## Materials and Methods

### Sample collection

A total number of 545 vegetable samples was collected from 10 formal retailers, 10 street trading greengrocers, 10 mobile trolley vendors, and 13 vendors at two farmers' markets in Gauteng, South Africa, from September 2017 to May 2018 (Supplementary Figure S1). In the informal markets, street traders typically display fresh produce on a table, underneath a shade covering, at the roadside or they use mobile trolleys. The vegetable samples included, depending on availability, spinach (bunches, baby leaves, or minimally processed ready-to-eat (RTE) pillow packs) (n=200), tomatoes (n=200), cucumbers (n=45), lettuce (Iceberg lettuce heads or mixed salad leaf RTE pillow packs) (n=50), and green beans (n=50 samples). All samples were transported in cooler boxes and stored at 4 °C until further processing within 24 h.

**Processing of fresh produce**

At least three leaves from one spinach bunch and the inner leaves of three lettuce heads were used to prepare 50 g composite samples of each of the leafy vegetable samples. Each spinach or lettuce sample were aseptically cut into a sterile polyethylene strainer stomacher bag containing 200 ml buffered peptone water (BPW) (3M, Johannesburg, SA) in a 1:4 weight to volume ratio. A 150 g sample of tomatoes and cucumbers (composite of at least three tomatoes or cucumbers) and a 150 g sample of green beans were each placed into a sterile polyethylene stomacher bag containing 150 ml BPW in a 1:1 weight to volume ratio (Xu *et al.*, 2015). Individual vegetable samples were blended for 5 min at 230 rpm in a Stomacher 400 circulator paddle blender (Seward Ltd., London, UK).

**Isolation and identification of presumptive extended-spectrum and AmpC β-lactamase-producing *Enterobacteriaceae***

Each of the BPW-sample mixtures was incubated for 3-4 h at 37 °C after which 1 ml of each sample was added to 9 ml *Enterobacteriaceae* enrichment (EE) broth (Oxoid, Johannesburg, SA) according to ISO 21528-1:2004 and incubated overnight at 30 °C (Blaak *et al.*, 2014). ESBL-producing microorganisms were detected by streaking 10 µl of each of the enriched samples onto ChromID ESBL agar plates (bioMérieux, Midrand, SA) and incubated overnight at 30 °C (Blaak *et al.*, 2014). All presumptive positive ESBL/AmpC- producing *Enterobacteriaceae* colonies based on colony colour, including weakly coloured colonies, on the chromogenic media were isolated and purified. Isolate identities were determined using Matrix-Assisted Laser Desorption Ionisation Time-of-Flight mass spectrometry (MALDI-TOF) (Bruker, Bremen, Germany) to species level as described by Standing *et al*., (2013). A single colony on nutrient agar were transferred to the MALDI-TOF polished steel target plate and further analysed according to manufacturer's instructions (AOAC-OMA#2017.09),

following calibration with the bacterial test standard. Non-*Enterobacteriaceae* isolates were not included in further analysis.

**Antimicrobial susceptibility testing**

A selection of 77 presumptive ESBL producing *Enterobacteriaceae* isolates, representing all unique species per product type from each supplier, were selected for further analysis. The Kirby-Bauer disk diffusion technique was used to determine the resistance patterns of the isolates [Clinical Laboratory Standard Institute (CLSI, 2018)]. All isolates were screened for ESBL production by the double-disk synergy test (DDST) using cefotaxime-30 µg, ceftazidime-30 µg, and cefpodoxime-10 µg, alone or in combination with clavulanic acid-10 µg (Mast Diagnostics, Randburg, SA) (EUCAST, 2013). Zone diameters were compared to the CLSI and EUCAST criteria to determine if isolates were resistant, intermediate or susceptible. Isolates showing resistance to cefoxitin and cefotaxime or ceftazidime were regarded as a phenotypic indicator of AmpC production (EUCAST, 2013). Production of ESBLs were confirmed using the cefepime ESBL disc set (Cefepime-30 µg, cefepime-clavulanic acid-30 µg-10 µg) and AmpC production using the AmpC detection set (Mast Diagnostics, Randburg, SA) (EUCAST, 2013; CLSI, 2018). Additional antimicrobials tested for resistance or susceptibility of isolates included ampicillin-10 µg, amoxicillin-clavulanic acid-20 µg/10 µg, amoxicillin-10 µg, trimethoprim-sulfamethoxazole-1.25µg/23.75 µg, imipenem-10 µg, neomycin-10 µg, tetracycline-30 µg, gentamycin-10 µg, chloramphenicol-10 µg (Mast Diagnostics, Randburg, SA) (CLSI, 2018). Isolates resistant to three or more antimicrobial classes were regarded as MDR. *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* NCTC 13315, *Enterobacter cloacae* NCTC 1406, and *Escherichia coli* ATCC 25922 were included as positive and negative controls as described by the manufacturer (Mast Diagnostics, Randburg, SA).

## Characterization of β-lactamase genes

The presence of ESBL determinants ($bla_{TEM}$, $bla_{SHV}$, $bla_{CTX-M}$, $bla_{OXA}$) and pAmpC resistance genes ($bla_{ACC}$, $bla_{FOX}$, $bla_{MOX}$, $bla_{DHA}$, $bla_{CIT}$, $bla_{EBC}$) in the selected isolates were analysed by PCR and sequencing. Single colonies of each presumptive ESBL-producing *Enterobacteriaceae* isolate were cultured aerobically under shaking conditions at 200 rpm in tryptone soy broth (MERCK, Johannesburg, SA) for 24 h at 30 °C. The cells were pelleted by centrifugation (12,500 *g* for 10 min), DNA was extracted using the Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, USA) and the DNA concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg, SA). PCR was performed using the DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Johannesburg, SA), specific primers, and thermocycling conditions for each of the genes as described in Supplementary Table S1. PCR products were sequenced using BigDye Terminator v3.1 cycle sequencing on an ABI 3500XL sequencer in forward and reverse direction (InquabaBiotec, Johannesburg, SA). The sequences were edited with Chromas 2.6 and BioEdit sequence alignment editor software and consensus sequences were subjected to BLAST nucleotide search analysis to identify the AR genes.

## Results

### Identification of presumptive extended-spectrum and AmpC β-lactamase-producing *Enterobacteriaceae* isolates

Using MALDI-TOF analysis, 122 (28.2 %) of the 432 presumptive extended-spectrum/AmpC β-lactamase-producing isolates obtained from the fresh vegetable samples were confirmed as *Enterobacteriaceae* belonging to ten genera. The 310 non-*Enterobacteriaceae* isolates were predominantly identified as *Pseudomonas* spp. The *Enterobacteriaceae* isolates were identified as *Enterobacter* spp. (28.7 %), including *E.*

*cloacae*, *E. asburiae*, *E. cowanii*, and *E. ludwigii*; *Serratia* (18.9 %), including predominantly *S. fonticola*; *Escherichia coli* (18 %); *Klebsiella* spp. (14.8 %), including *K. pneumoniae* and *K. oxytoca*; *Rahnella aquatilis* (9 %); *Proteus* spp. (4.9 %), including *P. penneri* and *P. mirabilis*; *Citrobacter* spp. (2.5 %), including *C. farmeri* and *C. freundii*; *Kluyvera ascorbata* (1.64 %); *Achromobacter xylosixidans* (1.6 %) and *Raoultella ornithinolytica* (0.8 %). Presumptive ESBL/AmpC-producing *Enterobacteriaceae* were isolated from the vegetable types tested.

**Phenotypic antibiotic resistance profiling**

All the 77 selected presumptive ESBL-producing *Enterobacteriaceae* showed resistance to more than one antimicrobial agent, with 96.1 % being MDR (resistant to ≥ 3 antimicrobial classes) (Figure 1). Resistance to the aminoglycoside and chloramphenicol classes were dominant, observed in 94.8 % and 85.7 % of the isolates respectively. All isolates with cephalosporin resistance (CTX30C, CAZ30C, CPD10C or CPM30C) were further screened using DDST, after which 61/77 (79.2 %) were tested positive for ESBL production (Figure 1). All isolates that showed cefoxitin resistance (n=46), were additionally screened with the AmpC detection set. From these 46 isolates, 32/77 (41.6 %) were tested positive for AmpC production. This included 27 isolates showing resistance to cefoxitin, ceftazidime and/or cefotaxime and additionally 5 isolates that showed cefoxitin resistance, but ceftazidime and/or cefotaxime susceptibility. All isolates displaying ESBL or AmpC phenotypes were further characterized for identification of ESBL and/or AmpC resistance genes.

**Genotypic antibiotic resistance profiling**

Genes encoding β-lactamases were detected in 58/77 (75.3 %) isolates obtained from all vegetable types, mainly in *E. coli* (n=20), *Enterobacter* spp. (n=12), and *Serratia* spp. (n=11)

isolates. This included 37 (48 %) broad-spectrum, 39 (51 %) ESBL and 20 (25.9 %) AmpC genetic determinants (Figure 1). The most frequently detected β-lactamase genes were $bla_{CTX-M}$ (n=28), followed by $bla_{SHV}$ (n=22), $bla_{TEM}$ (n=21) and $bla_{OXA}$ (n=5). Extended-spectrum β-lactamases encoded by $bla_{CTX-M}$ included CTX-M-14 (n=15), CTX-M-15 (n=6), CTX-M-27 (n=4), and CTX-M-55 (n=3); $bla_{TEM}$ genes encoded TEM-3 (n=3), while $bla_{SHV}$ genes encoded SHV-18 (n=6), SHV-28 (n=1) and SHV-154 (n=1). All the $bla_{OXA}$, 85.7 % (n=18) of the $bla_{TEM}$, and 63.6 % (n=14) of the $bla_{SHV}$ sequences encoded broad-spectrum β-lactamases OXA-1, TEM-1, TEM-215, SHV-1, SHV-11, or SHV-26 respectively. Three isolates harboured more than one ESBL; one *E. coli* isolate carried the $bla_{TEM-3}$, $bla_{SHV-18}$, and $bla_{CTX-M-14}$ genes, and two isolates (*E. coli* and *E. cowanii*) carried the $bla_{TEM-3}$ gene in association with $bla_{CTX-M-14}$ and $bla_{SHV-18}$ genes, respectively. In 12 isolates [*E. coli* (n=3); *Enterobacter* spp. (n=3); *Serratia* spp. (n=3); *R. aquatilis* (n=2); and *P. mirabilis* (n=1)] ESBL genes in association with broad-spectrum β-lactamases were detected (Figure 1).

AmpC resistance genes were detected in 18/58 (31 %) isolates harbouring β-lactamase genetic determinants (Figure 1). In 17 isolates, only one pAmpC genetic determinant was detected; $bla_{MIR-20}$ (n=4), $bla_{MIR-16}$ (n=3), $bla_{ACT-58}$ (n=2), and one isolate each carried $bla_{CMY-2}$, $bla_{MIR-14}$, $bla_{ACT-29}$, $bla_{ACT-10}$, $bla_{ACT-2}$, $bla_{EC}$, $bla_{CMY-161}$, or $bla_{CMY-87}$ respectively. Among these 17 isolates, five isolates [*Enterobacter* spp. (n=2), *E. coli* (n=1), *R. aquatilis* (n=1), and *S. fonticola* (n=1)] also harboured ESBL genetic determinants. One *Proteus penneri* isolate carried three AmpC genes ($bla_{ACT10}$, $bla_{DHA-18}$, and $bla_{CMY-49}$). The EBC family of the AmpC genetic determinants was the most dominant type.


**Discussion**

MDR ESBL/AmpC-producing *Enterobacteriaceae* were detected, for the first time, in raw vegetables retailed at selected sites in Gauteng Province, SA. Antibiotic resistant

opportunistic pathogens on fresh produce are a serious health concern that contributes towards the burden of AR in different environments leading to increased risk of infection if colonization in humans occurs (Al-Kharousi *et al.*, 2016). *Enterobacteriaceae* regarded as emerging bacterial threats include *E. coli*, *K. pneumoniae* and *Enterobacter* spp. showing resistance to β-lactams and aminoglycosides (Fair and Tor, 2014). Presumptive ESBL-producers, predominantly *E. coli, K. pneumoniae, E. cloacae and E. asburiae*, were detected in 17.4 % of our vegetable samples analyzed. This is lower than the 25.4 % reported by Zurfluh *et al*., (2015) for imported vegetables into Switzerland from the Dominican Republic, India, Thailand, and Vietnam, but higher than the 6 % reported by Reuland *et al.,* (2014) on retail vegetables in the Netherlands.  Similar to Blaak *et al.,* (2014), environmental ESBL-producing *Enterobacteriaceae* isolated from vegetables included *S. fonticola* and *R. aquatilis*.

Phenotypic confirmation of ESBL/AmpC production showed that 61 (79.9 %) of the 77 analysed *Enterobacteriaceae* isolates displayed an ESBL-producing phenotype and 41.6 % an AmpC-producing phenotype, which is higher than results reported by van Hoek *et al*., (2015). Isolates with a combined ESBL- and AmpC-producing phenotype were also observed in 35 % of the isolates. MDR phenotypes (resistance to ≥ 3 antimicrobial classes) were observed in 96.1 % of our analyzed isolates. The most prevalent non-β-lactam resistance profiles showed resistance against aminoglycoside (94.8 %), chloramphenicol (85.7 %) and tetracycline (53.2 %). This is higher than reports from similar studies which showed resistance to aminoglycosides (46.7 % - 66.7 %), chloramphenicol (33.3 %) (Zurfluh *et al.*, 2015; Ben Said *et al.*, 2016), and tetracycline (46.7 %) (Ben Said *et al.*, 2016) in ESBL-producing *Enterobacteriaceae*.

Genes expressing broad-spectrum β-lactamases, ESBLs and/or AmpC β-lactamases were detected in 69.9 % of our MDR isolates. Co-expression of  ESBL and AmpC genes in environmental (van Hoek *et al.*, 2015; Ye *et al.*, 2017) and clinical (Tau *et al.*, 2012; Kharat

*et al.*, 2017) *Enterobacteriaceae* isolates have also been reported. Globally the $bla_{CTX-M-type}$ ESBL genes are predominant in *Enterobacteriaceae*, which was similar in our study, the majority detected in *E. coli* isolates. $bla_{CTX-M-14}$ was the main genetic determinant detected from mostly *E. coli* and *C. freundii* isolates, which corresponds to results obtained from vegetable samples in Tunisia (Ben Said *et al.*, 2016). Isolates harboring $bla_{CTX-M-15}$ included *E. coli*, *E. cloacae*, *K. pneumoniae*, *R. aqualtilis*, and *S. fonticola* and were second most prevalent in our study. $bla_{CTX-M-15}$ was the most prevalent gene detected in *E. coli* and *K. pneumoniae* isolates from fresh vegetables imported into Switzerland from India and the Dominican Republic (Zurfluh *et al.,* 2015). This is in agreement with reports that $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ are predominant and have been associated with clinically relevant *Enterobacteriaceae* infections (Ehlers *et al.*, 2009; Zurfluh *et al.*, 2015). In contrast to Njage and Buys, (2014), who predominantly detected $bla_{CTX-M\ Group\ 8/25}$ positive *E. coli* isolates from lettuce in the North West Province (SA), no $bla_{CTX-M\ Group\ 8/25}$ genes were detected in any of our *E. coli* isolates from the vegetable samples analysed. The $bla_{CTX-M-15}$ (CTX-M Group 1) and $bla_{CTX-M-14}$ (CTX-M Group 9) genes detected in our environmental isolates, reported to be closelely related to chromosomally encoded $bla_{FONA}$ and $bla_{RAHN}$ genes of *S. fonticola* and *R. aquatilis*, had no significant similarity in the GenBank database using NCBI BLAST based on total BLAST alignment scores. This contrasts results reported by Raphael *et al.*, (2011) where sequences similar to $bla_{RAHN-2}$ and $bla_{FONA-5}$ was detected using $bla_{CTX-M}$ primers.

In our study, five isolates including *E. coli*, *Enterobacter* spp., *R. aquatilis*, *S. fonticola* simultaneously harboured ESBL and AmpC genes. Environmental isolates are known to carry chromosomally encoded AmpC β-lactamases. However, *Enterobacteriaceae* harbouring both chromosomal and pAmpC β-lactamases are increasingly reported to hydrolyze broad-spectrum cephalosporins more efficiently, resulting in adverse treatment options in clinical settings (Jacoby, 2009; Reuland *et al.*, 2014). The 18 isolates in which

pAmpC resistance genes were detected, predominantly included the EBC type pAmpC β-lactamases (identified as $bla_{ACT}$/$bla_{MIR}$). This contrasts with two previous studies where $bla_{CIT}$, $bla_{DHA}$, or $bla_{ACC}$ pAmpC β-lactamases were mostly detected in *Enterobacteriaceae* isolated from fresh produce and water samples (Njage and Buys, 2014; Ye *et al.*, 2017). $bla_{ACT/MIR}$ genes have been reported to be the dominant AmpC genetic determinants in *Enterobacter* spp. causing intra-abdominal infections (Khari *et al.*, 2016) and were detected in seven of the *Enterobacter* spp. isolates in our study. The fact that fresh produce can serve as a reservoir of MDR ESBL/AmpC-producing *Enterobacteriaceae,* including their genetic determinants, constitute a potential health risk to the consumer as resistance to antimicrobials frequently used to treat human infections were shown.

**Conclusion**

The results obtained from screening at these selected sites indicate that further investigation of different fresh produce types in Gauteng and other provinces in SA is also necessary. Future studies should focus on surveillance of fresh produce production systems from farm to retail to identify potential sources of contamination which contribute to the presence and dissemination of antimicrobial resistant microorganisms and their genetic determinants. Since AR is a worldwide problem, a global solution is required that integrates the contributions from governmental departments as well as from the scientific community.

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