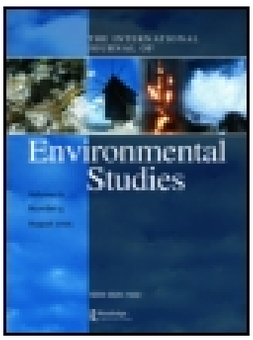

International Journal of Environmental Studies

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/genv20

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To cite this article: Kingsley Ehi Ebomah & Anthony Ifeanyi Okoh (2020): *Enterobacter cloacae* harbouring bla_{NDM-1}, bla_{KPC}, and $bla_{OXA-48-like}$ carbapenem-resistant genes isolated from different environmental sources in South Africa, International Journal of Environmental Studies, DOI: 10.1080/00207233.2020.1778274

To link to this article: https://doi.org/10.1080/00207233.2020.1778274

Published online: 16 Jun 2020.

INTERNATIONAL JOURNAL OF ENVIRONMENTAL STUDIES
https://doi.org/10.1080/00207233.2020.1778274

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ARTICLE

Enterobacter cloacae harbouring bla_{NDM-1}, bla_{KPC}, and $bla_{OXA-48-like}$ carbapenem-resistant genes isolated from different environmental sources in South Africa

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ABSTRACT

Pathogenic bacterial strains that cause serious infections particularly among individuals with immune-deficiency can exhibit antibiotic-resistance against the available therapeutic options thereby constituting a public health concern. In this study, we evaluated the occurrence of carbapenem-resistant *Enterobacter* species isolated from different environmental samples collected from selected study areas in the Eastern Cape Province, South Africa. Our study further aimed to evaluate the antimicrobial-resistant (AMR) profiles of the confirmed isolates recovered from hospital wastewater effluents, wastewater treatment plants and other environmental sources including river water and farm samples. Results revealed out of 142 PCR-confirmed *Enterobacter* spp., 115 isolates (81%) were classified as *Enterobacter cloacae*. The confirmed species exhibited phenotypic resistance in decreasing order for doripenem (80%), meropenem (75%), imipenem (72%) and ertapenem (66%) and carbapenem-resistance genes (CRGs) were detected in 29% of *Enterobacter* spp. isolates. Our findings confirm the presence of CRGs among bacterial isolates populating environmental niches.

KEYWORDS

Bla_{ndm-1}; bla_{KPC}; $bla_{OXA-48-like}$; Enterobacter cloaca

Introduction

Antimicrobial agents have saved many lives but their overuse has produced antimicrobial-resistant (AMR) bacteria. Carbapenems have been the last barrier to antibiotic resistant (AR) microbial infections. They have been the most effective therapeutic options available for treating severe hospital-acquired infections (HAI) triggered by multidrug resistant (MDR) extended-spectrum β-lactamase (ESBL) or possibly AmpC-producing Enterobacteriaceae [1,2]. Carbapenem-resistant Enterobacteriaceae (CRE) have become a global public health concern. There are increasingly annual reports of high prevalence of CRE (including *Enterobacter* spp.) isolated from various environmental samples [3–6].

Several works have demonstrated that CRE cause severe infections (like urinary tract infections and diarrhoea) especially among patients with primary immunodeficiency

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Published online 16 Jun 2020

diseases. These infections lead to increased mortality rates as a result of inadequate treatment options. Recently, there have been reports of decreased susceptibility to carbapenems in hospital acquired *E. cloacae* [7]. Antimicrobial-resistance (AMR) against carbapenem can similarly arise when accumulation of the antibiotics is abridged in bacterial strains that produce ESBLs or AmpC β-lactamases. Carbapenem resistance in bacterial isolates has shown losses of key non-specific porins as well as increased activity of efflux pumps and alterations in some members of Enterobacteriacea group (such as *Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae* and *E. aerogenes*) [8]. There is increasing evidence of antibiotic-resistant bacteria (ARB) from health-care and non-clinical settings. There is concern among medical microbiologists that pharmaceutical developments may be outstripped by such trends [9].

Worldwide, carbapenems are failing to control Enterobacteriaceae and many other species involved in the creation of acquired Metallo-β-lactamases (MBLs) such as New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and Inferred from Mutant Phenotype (IMP), or non-metallo-carbapenemases (NMC) of the imipenemase (IMI), *Serratia marcescens* enzyme (SME), oxacillinase (OXA) or *Klebsiella pneumoniae* carbapenemase (KPC) families [10]. This is alarming.

A recent study in India reported the production of carbapenemases OXA-181 on plasmids among CRE including *Enterobacter absuriae* (an uncommon species) recovered from various environmental samples [11]. This evidence of the detection of ARGs harboured by rare *Enterobacter* species signifies a serious public health problem. It is therefore necessary to apply the use of combination therapy and other reserved antibiotics. But, bacterial resistance against other antimicrobials (such as colistin) has emerged. The increasing incidence of ESBL producers is adequate to drive a greater reliance on carbapenems as well as non-β-lactam antimicrobials [12–14].

The emergence of AMR in Enterobacteriaceae has become a serious public health concern since there is lack of novel antimicrobials. Already *Klebsiella pneumoniae* isolates harbouring bla_{KPC} genes are a major problem in the United States, Greece, and Israel; and there are reports of bla_{VIM} genes distributed amongst *K. pneumoniae* isolates in Greece. CRE is reported to be prevalent in South Africa [15–18], in Egypt [19] and in Uganda [20]. Among the limited studies, de Jager et al. [21] reported clinical isolates of carbapenem-resistant *Enterobacter cloacae* harbouring antimicrobial resistance genes (ARGs) i.e. $bla_{NDM\text{-}1.l.}$ There is insufficient information on the carbapenemase-producing *E. cloacae* isolated from different environmental niches. This research aimed to assess the antimicrobial resistance patterns of carbapenem-resistance isolates of *E. cloacae* recovered from selected environmental samples in three District Municipalities (DMs) located in the Eastern Cape Province (ECP), South Africa.

Materials and methods

Description of study sites and sample collection

The study sites comprise hospitals, wastewater treatment plants (WWTPs), surface waters (rivers, dams and canals) and farms within villages and towns in Amathole DM, Sarah Baartman DM and Chris Hani DM in the ECP, South Africa. Four WWTPs, three hospitals and two farms were used as sampling sites for this study.

Different samples (243 in total) comprising surface water (47), WWTPs final effluents (29), hospital wastewater effluents (27), irrigation water (45), soil (60) and vegetables (35) were collected between the months of January and September 2018. Water samples were collected in duplicate using sterile 1-L glass bottles and transported in an ice box to the laboratory for microbiological analysis within six hours of collection. Vegetable samples including spinach, broccoli and cabbage were randomly collected from selected farms in the study areas. Figure 1 is a map of the study area in the ECP, South Africa.

Sample preparation and processing

Processing of water samples

To isolate the enteric bacteria from the water samples, the membrane filter technique [22] was used. A hundred millilitres of the water samples were filtered through a sterile membrane filter (Merck) of 0.45-µm pore size. Then the filter papers were aseptically picked, placed onto the surface of Eosin methylene blue agar (E.M.B) plates, and incubated at 37°C for 18–24 h. After incubation, presumptive Enterobacter isolates were streaked onto nutrient agar (NA) plates and incubated at 37°C for 24 h. Thereafter, 25% glycerol stock was prepared from the cultured broths and preserved at −80°C for further analyses.

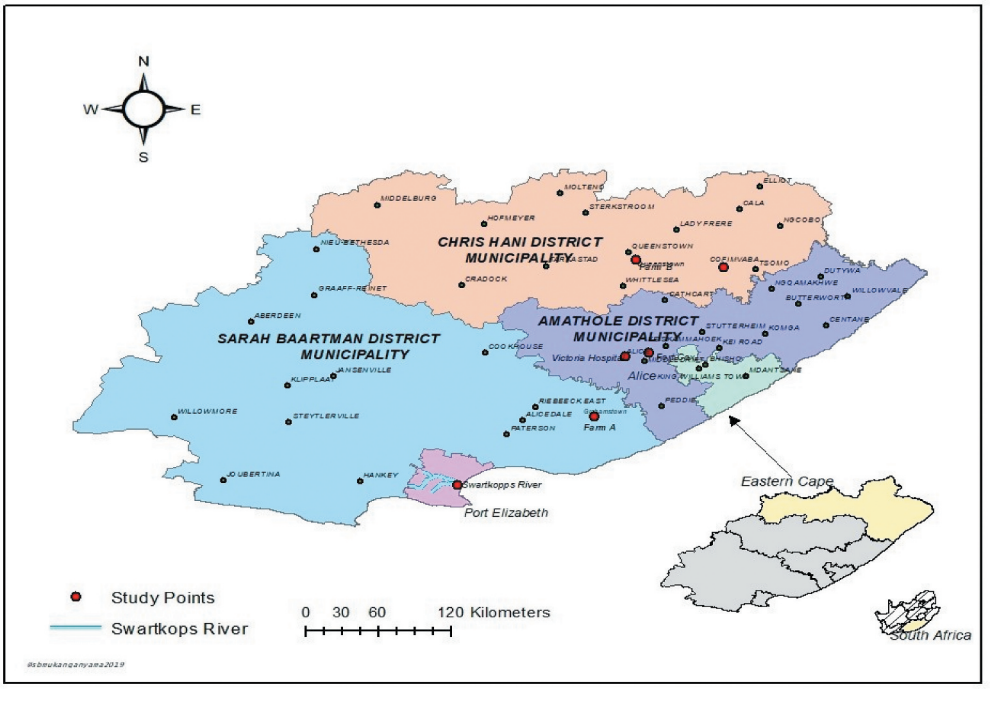

Figure 1. A map showing the study area in the District Municipalities.

Processing of vegetable samples

For the processing of vegetable samples, the method of Du Plessis et al. [23] was adopted. Briefly, 10 g of each vegetable samples were placed in 90 mL of sterile Trypticasein Soy Broth (TSB) in a Stomacher Bag and were macerated with Mixer machine, and 90 mL of the broth were incubated at 37°C for 18–24 h in sterilised bottles. Thereafter, a loopfull of TSB was streaked on the surface of Eosin methylene blue agar (E.M.B) plates, incubated for 24 hours at 37°C. Then single colonies from the overnight culture were streaked on nutrient agar plates, incubated for 24 h at 37°C. Afterwards, glycerol stock was prepared from the nutrient agar plates in nutrient broth.

Processing of soil samples

The method of Da Silva et al. [24] was also adopted for biological analysis of the soil samples. Sixty agricultural soil samples were randomly collected from selected farms in the study areas. A total of 10 grams of each soil sample was suspended in 90 mL of sterile TSB and incubated at 37°C for 18–24 h. Thereafter, a loopfull of TSB was streaked on Eosin methylene blue agar (E.M.B) plates. This was incubated for 24 h at 37°C. Purification process was carried out on nutrient agar as described above.

DNA extraction

Bacterial DNA was extracted by performing the boiling method as described by Jackson et al. [25]. Briefly, 4 mL of nutrient broth (NB) were prepared and presumptive isolates were resuscitated by inoculating a loopful of each isolate into the NB. After incubating for about 18–24 hours at 37°C, 1 mL of the broth cultures was centrifuged at 12,800 rpm for 5 min, the supernatants discarded and then the cells were suspended in 400 µL of sterile distilled water with the use of sterile 1.5 mL Eppendorf tubes. The suspensions were boiled for 10 min at 100°C in a heating block and allowed to cool. Afterwards, the suspensions were centrifuged for 5 min at 12,800 rpm and 3 microlitres of supernatants per reaction were use as DNA templates. The DNA templates were stored at −80°C for further molecular analyses.

Molecular identification of presumptive isolates

The presumptive isolates were confirmed by polymerase chain reaction techniques (PCR). Table 1 summarises the list of primers used and PCR conditions.

Antimicrobial susceptibility test of the confirmed *Enterobacter* species

All confirmed *E. cloacae* isolates were subjected to antimicrobial susceptibility test against 4 panels of carbapenem following the Kirby-Bauer disc diffusion method and results were interpreted according to the Clinical and Laboratory Standards Institute guidelines [27]. The class of carbapenems antimicrobials agents includes doripenem (10 µg), imipenem (10 µg), meropenem (10 µg) and ertapenem (10 µg). Briefly, about 100–200 mL of the bacterial overnight broth was transferred into 5 mL normal saline solution which was adjusted matching 0.5 McFarland standard.

Table 1. Primer sequences of target species and their respective amplicon sizes and PCR cycling conditions [26].

Target strain	Target gene	Primer Sequence (5′→3′)	Amplicon size (bp)	PCR Cycling Condition
Enterobacter genus	*16-SrRNA*	F: CGC GTA CTA TAC GCC ATG AAC GTA R: ACC GTT GAT CAC TTC GGT CAGG	1500	Initial denaturation of 5 min at 95°C followed by 35 cycles, denaturation at 95°C for 30 s, annealing at 60°C for 40 s, extension at 72°C for 2 min and final extension at 72°C for 10 min
Enterobacter cloacae	*fusA*	F: ATT TGA AGA GGT TGC AAA CGAT R: TTC ACT CTG AAG TTT TCT TGT GTTC	341	Initial denaturation of 5 min at 95°C followed by 30 cycles, denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min

Thereafter 100 mL was spread on Muller-Hinton agar plates with the use of sterile glass spreader, and plates were impregnated with the aforementioned carbapenem antimicrobial discs, incubated aerobically at 37°C for 24 h. Then the diameters of zone of inhibition were measured and interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute [27].

Molecular characterisation of the relevant carbapenem resistance genes

All strains of *Enterobacter* spp. that exhibited phenotypic resistance against the 4 test carbapenems were further screened for the relevant carbapenem-resistance genes using the PCR technique and PCR products were viewed using agarose gel electrophoresis. Table 2 shows the list of primers used for the PCR.

Table 2. List of primers for screening of carbapenem resistance genes [28].

Target gene	Primer Sequence (5′→3′)	Amplicon size (bp)	PCR Cycling Condition
bla_{NDM-1}	F: AAA ACG GCA AGA AAA AGC AG R: AAA ACG GCA AGA AAA AGC AG	439	Initial denaturation of 5 min at 95°C followed by 35 cycles, denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 mins
bla_{KPC}	F: AAA ACG GCA AGA AAA AGC AG R: AAA ACG GCA AGA AAA AGC AG	340	Initial denaturation of 5 min at 95°C followed by 35 cycles, denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 mins
$bla_{OXA-48-like}$	F: TTGG TGGC ATCG ATTA TCGG R: GAGC ACTT CTTT TGTG ATGG C	597	Initial denaturation of 5 min at 95°C followed by 35 cycles, denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 mins

Results

Identification and characterisation of recovered *Enterobacter* spp. isolates

A total of 202 presumptive Enterobacter isolates was obtained from all the samples analysed. Results showed that 83% (142/202) of the isolates were Enterobacter genus by PCR. Further characterisation of the isolates revealed that of 142 *Enterobacter* spp., 115 (81%) isolates belonged to the species *E. cloacae*. The number of unidentified species was 27 (19%). Figure 2 shows the agarose gel electrophoresis of the PCR products of the *Enterobacter cloacae*. Table 3 highlights the number of confirmed *Enterobacter* spp. isolated from the different sample types in this study.

Antimicrobial susceptibility patterns of the confirmed *Enterobacter cloacae*

The result of the antibiotic susceptibility testing showed that doripenem had the highest percentage resistance (80%). The following is the percentage resistance exhibited by *Enterobacter cloacae* against the test antibiotics; doripenem (80%), meropenem (75%), imipenem (72%) and ertapenem (66%). It is notable that those isolates showing intermediate susceptibility were considered as resistant. Figure 3 shows the antimicrobial susceptibility patterns of the *E. cloacae* isolates recovered from different sample types in the Eastern Cape Province of South Africa. Figure 4 represents the agarose gel electrophoresis of the PCR products of the amplification of blaNDM-1 gene, while Figure 5 represents the agarose gel electrophoresis of the PCR products of the amplification of blaKPC gene.

Molecular characterisation of the relevant carbapenem resistance genes

Out of 85 *E. cloacae* isolates that showed resistance against the test antibiotics, 41 (48%) isolates were confirmed by PCR to harbour carbapenem-resistant genes (CRGs) and the result is as presented in Table 4. Table 5 highlights distribution of multiple CRGs harboured by *Enterobacter cloacae* isolates.

Percentage occurrence of the antimicrobial resistance genes (ARGs) in *E. cloacae*

All the *Enterobacter cloacae* isolates that exhibited phenotypic resistance against the test carbapenems, isolates from hospital wastewater effluent samples (29%) from the selected

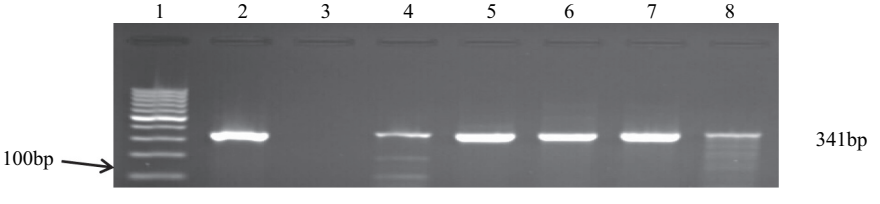

Figure 2. PCR products of the amplification of *fusA* gene. Lane1: 100bp molecular weight marker; Lane 2: positive control (ATCC BAA-2341); Lane 3: negative control; Lanes 4–8: positive *Enterobacter cloacae* isolates.

Table 3. Number of confirmed *Enterobacter* spp. isolated from different sample types.

	Number of confirmed *Enterobacter* species (cfu/ml)		Number of confirmed *E. cloacae* (cfu/ml)			
Sample types	Amathole DM	Chris Hani DM	Sarah Baartman DM	Amathole DM	Chris Hani DM	Sarah Baartman DM
Hospital wastewater effluent	11	11	-	8	9	-
WWTP final effluent	10	14	-	9	9	-
Surface water	18	7	1	18	4	1
Irrigation water	11	5	3	10	5	3
Farm soil	8	14	4	8	14	4
Vegetables	3	20	3	9	10	3

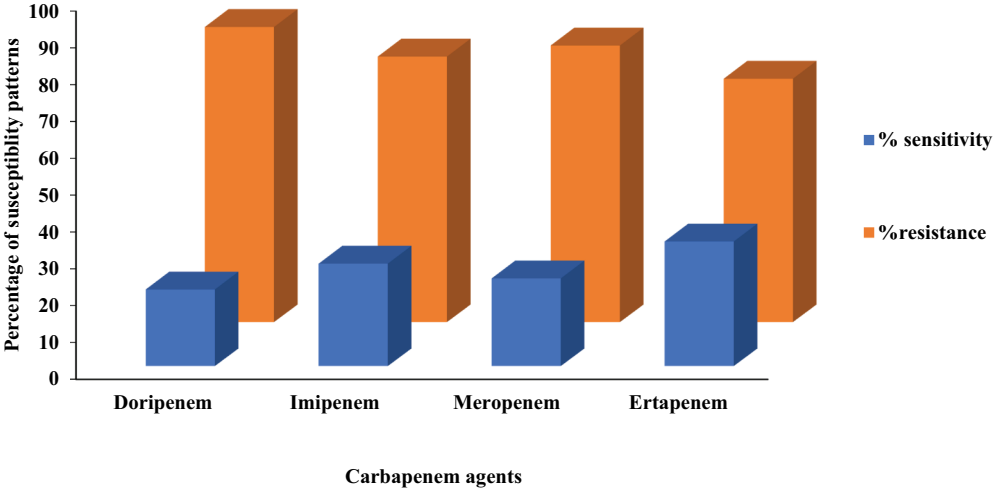

Figure 3. The antimicrobial susceptibility patterns of *E. cloacae* isolates.

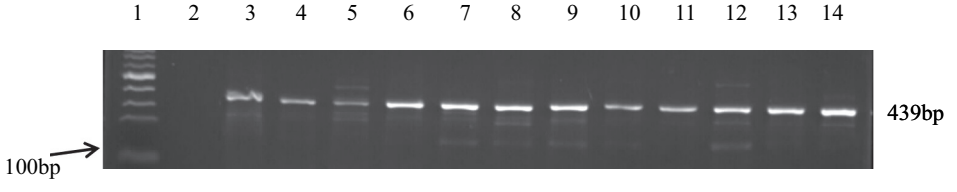

Figure 4. **PCR products of the amplification of bla_{NDM-1} gene**. Lane1: 100bp molecular weight marker; Lane 2: negative control; Lanes 3–14: positive isolates.

hospitals had the highest percentage occurrence of CRGs. Figure 6 shows the proportions of the confirmed *E. cloacae* strains harbouring ARGs in the different environmental samples collected. Results show bla_{NDM-1} had the highest percentage occurrence among the genes and isolates recovered from river water samples that harboured the most CRGs.

Discussion

The advent of carbapenem-resistant *Enterobacter* species poses a serious threat to public health worldwide. The carbapenemase OXA-48-like that hydrolyses antibiotics class D β-lactam are widely disseminated among *Enterobacteriaceae* (including *E. cloacae*) with significant geographical differences [29]. In the present study, different environmental samples collected from selected sampling sites within the Eastern Cape Province, South Africa were analysed in order to detect and characterise the recovered Enterobacter isolates; and the *Enterobacter* spp. isolates that exhibited phenotypic resistance were screened for the relevant CRGs.

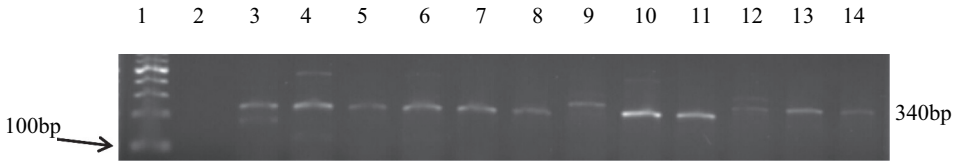

Figure 5. PCR products of the amplification of *bla*$_{KPC}$ gene. Lane1: 100bp molecular weight marker; Lane 2: negative control; Lanes 3–14: positive isolates.

Table 4. Distribution of CRGs identified among carbapenemase-producing *Enterobacter cloacae*.

Carbapenem-resistance genes	Number of isolates	Percentage occurrence (%)
bla$_{NDM-1}$	29/85	34
bla$_{KPC}$	9/85	11
bla$_{OXA-48-like}$	3/85	4
Total	**41**	**100%**

Table 5. Distribution of multiple CRGs identified among the isolates.

		Total		
Patterns of ultiple resistance genes	*E. cloacae* (%)	Unidentified species (%)	Sample types	
bla$_{NDM-1}$, *bla*$_{KPC}$	1	0	Hospital effluent	1
bla$_{NDM-1}$, *bla*$_{OXA-48-like}$	0	1	Hospital effluent	1

In our study, very high incidences of *Enterobacter* species (83%) and *Enterobacter cloacae* (81%) were observed. These findings corroborated a study by Singh-Moodley et al. [30] reporting the detection of carbapenemase-producing *E. cloacae* from clinical isolates, and another work by Gqunta et al. [31] who reported the occurrence of a different species, *Enterobacter asburiae* for the first time in the Eastern Cape Province, South Africa. Jousset et al. [32] also reported weak carbapenemase activity from another species, *Enterobacter kobei*. Since there is a moderately low percentage of unidentified species (19%) from the findings of this present study, there is need for more studies investigating ARGs in *Enterobacter* species.

Among the members of Enterobacteriaceae group, the acquisition of CRGs by uncommon bacterial species is a phenomenon of significant public health concern. Although carbapenem-resistance in *E. coli* strains has been reported [33–35], there is a lack of clinical or epidemiological studies focused on carbapenem resistance among *Enterobacter* species. In the current study, doripenem (80%) had the highest percentage resistance. On the other hand, ertapenem had the highest percentage susceptibility (34%). This makes ertapenem one of the most effective antimicrobial agent against CRE: a finding supported by other studies by Hoban et al. [36] and Karlowsky et al. [37]. [Another study by Guillard et al. [38] reported *E. cloacae* isolates with high percentage susceptibility (>90%) to carbapenems is in contrary to our findings as results in our present study show *E. cloacae* isolates exhibited high percentage resistance with an average of 73%.]

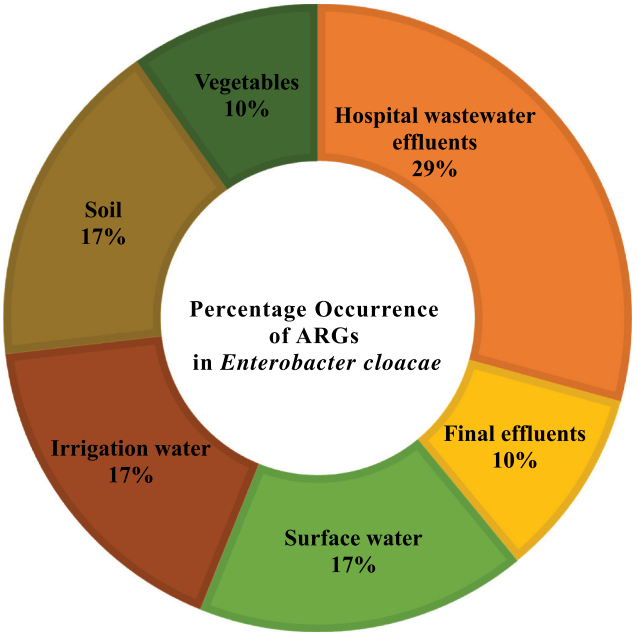

Figure 6. The frequency of occurrence of *Enterobacter cloacae* isolates with ARGs recovered from three DMs in the Eastern Cape Province, South Africa. The following is the order of the percentage occurrence of CRGs in the different environmental samples: hospital wastewater effluents (29%), surface water (17%), irrigation water (17%), soil (17%), final effluents (10%) and vegetables (10%).

Production of carbapenemase typically convenes antibiotic resistance against carbapenems as well as other antibiotics [39]. CRE carry carbapenemase-producing genes on mobile genetic elements (MGE) and these ARGs can be transferred horizontally to other bacteria, hence contributing to the reservoir of AR in both environmental and clinical Enterobacteriaceae [40]. To date, about 11 OXA-48-like variants have been detected, with classical OXA-48 being the most common. These carbapenemases display elevated hydrolytic activity against penicillins and low-level hydrolysis towards carbapenems. Since the first report of the OXA-48-like carbapenemase in Turkey [41], bacterial strains producing the enzyme have been widely investigated in nosocomial and community outbreaks in several parts of the world, particularly in the Mediterranean area and European countries.

To the best of our knowledge this is the first report on potential *Enterobacter* spp. isolates co-harbouring bla_{NDM-1} and $bla_{OXA-48-like}$ carbapenem-resistance genes in the Eastern Cape Province and our findings are in agreement with a study by Nasser [42] reporting the same trend of bacterial AR in Asia. Consequently, future investigations in the Eastern Cape region should consider a broader analysis of ESBLs and CRE determinants using both genotypic and phenotypic methods. Our findings showed that 48% of the phenotypically resistant *E. cloacae* harboured carbapenem resistance genes with bla_{NDM-1} having the highest occurrence (34%). Most recently, there has been a rapid spread of *Enterobacteriaceae* harbouring CRGs in various ecosystems. The highest percentage occurrence of ARGs of 29% was observed in hospital wastewater effluents samples whereas river water, irrigation water and farm soil samples had the same percentage ARGs occurrence of 17% respectively. The lowest percentage occurrence of ARGs was observed in vegetables

and final effluent samples (10%). This may indicate a transfer of ARB from hospital wastewater to the farm via the use of receiving surface water for irrigation purposes.

Furthermore, there is a likelihood of an increase in the prospect of an exchange of genetic material that encodes antimicrobial resistance [43]. In our current study, it was observed that four *Enterobacter* isolates co-harboured at least two different CRGs (three hospital effluents and one farm soil). It is notable that one particular *Enterobacter* isolate belonging to the unidentified species harboured bla_{NDM-1} and $bla_{OXA-48-like}$ genes but exhibited phenotypic susceptibility to imipenem.

Conclusion

The detection of carbapenem-resistant *Enterobacter* harbouring bla_{NDM-1}, bla_{KPC} and $bla_{OXA-48-like}$ genes that were isolated from some environmental samples in South Africa is of great concern to public health. The spread of these ARGs and clones is still not well understood. Carbapenem antibiotics have been established as drugs of last resort. Their obsolescence should be avoided until alternative antibiotics can be devised. The detection of CRE in farm samples such as vegetables and soils may be as a result of the use of wastewater in irrigation or application of activated sludge for soil fertility. These farm practices could lead to the spread of ARGs into the environment. Therefore, effective measures such as ozonation should be carried out during the disinfection stage in order to ensure proper treatment of final effluents before discharge. We also recommend more studies as well as improved techniques (for instance whole genome sequencing) to define the spread of ARGs.

Acknowledgments

The authors are sincerely grateful to the South Africa Medical Research Council (SAMRC) for financial support through grant SAMRC/UFH/P790, and to the University of Fort Hare.

Disclosure statement

The authors declare they have no competing interest.

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