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Emerging Carbapenem-Resistant *Enterobacter cloacae* Producing OXA-48-, VIM- and IMP-Type-*β*-Lactamases in Eastern Cape Hospitals in South Africa

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Abstract

Introduction: *Enterobacter cloacae* strains have been isolated from Eastern Cape hospitalised patients. Methodology: We have molecularly characterised *bla*$_{\text{OXA-48-}}$, *bla*$_{\text{IMP}}$- and *bla*$_{\text{VIM}}$-expressing *E. cloacae* isolates demonstrating resistance to carbapenems from five hospitals by multilocus sequence typing. Organism identification and antimicrobial susceptibility testing was done using automated systems and the isolates were screened for carbapenemases using either conventional or real-time PCR and then typed using multilocus sequence typing. Further characterisation of IMP-type-producing *E. cloacae* isolates, an unusual occurrence in South Africa, was performed by pulsed-field gel electrophoresis. Results and Conclusion: Twenty-five *E. cloacae* isolates from 24 patients were investigated. Eighteen (72%) isolates harboured either one of the following genes: *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$ or *bla*$_{\text{OXA-48}}$. Multilocus sequence typing data and pulsed-field gel electrophoresis showed that several strains from the same geographical region and hospitals were genetically related.

Keywords

***Enterobacter cloacae*, Carbapenem-Producing, Multilocus Sequence Typing**

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1. Introduction

The increase in the rate of antibiotic resistance is a major concern in isolates from the enterobacteriaceae family. Serious infections caused by extended spectrum *β*-lactamase-producing enterobacteriaceae are treated with carbapenems, the broadest spectrum of *β*-lactam antimicrobial agents. Resistance to carbapenems including carbapenemase production is emerging, creating difficulties in the management of life-threatening infections [1]. Amongst the enterobacteriaceae family, *Enterobacter* species cause a number of hospital acquired infections [2] and *Enterobacter cloacae* is the most resistant pathogen [3]. The dominant genes contributing to drug resistance in *E. cloacae* are the plasmid-mediated serine extended spectrum *β*-lactamase (ESBL) $bla_{\text{CTX-M}}$; the serine carbapenemase bla_{KPC} and the metallo-*β*-lacatamases (MBLs) bla_{NDM}, bla_{IMP} and bla_{VIM} [4] [5]. The acquired MBL, $bla_{\text{IMP-1}}$ emerged and spread in Gram-negative bacilli in Japan in the early 1990s [6] and together with its variants have since been detected in other countries worldwide [7]-[12]. $bla_{\text{VIM-1}}$ was first reported in Italy, also in the 1990s [13]. Like bla_{IMP}, variants of this gene have been detected globally [14]-[18]. These genes are located on plasmids that enhance their ability to spread horizontally making treatment of infections more challenging. The production of IMP and VIM *β*-lactamases in the hospital environment has become a serious concern [19] [20]. In South Africa, the emergence of bla_{NDM}, bla_{KPC}, bla_{VIM} and $bla_{\text{OXA-48}}$ and its variants produced by enterobacteriaceae have been reported [21]-[23]. However, to our knowledge there are no studies that document bla_{IMP} genes in *E. cloacae* in South Africa. In this study, we identified *E. cloacae* isolates harbouring IMP, VIM and OXA-48 enzymes and assessed strain clonality to determine whether they were genetically related.

2. Methodology

Carbapenem non-susceptible enterobacteriaceae isolates were submitted to the Antimicrobial Resistance Laboratory at the National Institute for Communicable Diseases for confirmation of carbapenemase-producing enterobacteriaceae (CPE) genes. Upon receipt, organisms were retested for identification and antimicrobial susceptibility testing using automated systems (VITEK® II (bioMèrieux, France) and/or the Microflex MALDI-ToF (BrukerDaltonik, GmbH) and the MicroScan® Walkaway system (Siemens, USA) respectively). The interpretation of susceptibility was done according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. DNA was extracted using a crude boiling method at 95˚C for 25 minutes. The supernatant was harvested and screened for bla_{NDM}, bla_{KPC} and $bla_{\text{OXA-48}}$ and its variants using real-time polymerase chain reaction (PCR) (LightCycler 480 II, Roche Applied Science, LightCycler 480 Probes Master kit, Roche Diagnostics, IN USA) and the Centre for Disease Control and Prevention (CDC) protocol primers for detection of bla_{NDM} and bla_{KPC} and the following primers and probes for $bla_{\text{OXA-48}}$ which was designed for this study: OXA-48Fvariant5'-gCgTggTTAAggATgAACAC-3',OXA-48Svariant5'-CATYTCgggCAATgTAgACAg-3', OXA-48Rvariant 5'-gATgTgggCATATCCATATTCATCgCA-3' and OXA-48probe 5'-CY5-CATTggCTTCggTCAgCATggCT-BBQ-3'. The screening of bla_{GES}, bla_{IMP} and bla_{VIM} was done using conventional PCR (GStorm Thermal Cycler, Somerton Biotechnology Centre, UK and the Qiagen multiplex PCR kit, Qiagen, Germany) and the primers from previous publications [25] [26]. Multilocus sequencing (MLST) was performed on these isolates using previously published primers and conventional typing methods [27]. Conventional PCR was performed for each of the seven reference/house-keeping genes and the products were purified (Qiagen Purification kit; Qiagen, Germany) and sequenced (Inqaba Biotech, South Africa). Sequences were analysed using the online database (http://pubmlst.org/) and analysis tools (http://pubmlst.org/analysis/). For the 15 IMP-producing *E. cloacae* isolates identified by conventional PCR, pulsed-field gel electrophoresis (PFGE) using the *XbaI* restriction enzyme (Thermo Scientific, MA USA) was performed. Electrophoresis was performed on 1% PFGE agarose gel with a CHEF-DR III electrophoresis system (Bio-Rad Laboratories, Richmond, CA, USA). Clustering was done according to Tenover criteria [28], and a cluster was defined as unique PFGE patterns differing by 3 or less bands.

3. Results and Discussion

From January 2013 to April 2014, eighteen of twenty-five *E. cloacae* isolates from twenty-four patients at five hospitals in the Eastern Cape harboured either one of the following genes: bla_{IMP}, bla_{VIM} or $bla_{\text{OXA-48}}$ resulting in further investigation. Isolates were obtained from clinical specimens: urine (n = 8), sputum (n = 1), pus swab (n = 7), catheter (n = 1) and other sterile body fluids (n = 8). Demographic analysis showed that 13 (54%) were male and 11 (46%) were female. The mean age was 40.75 years (including one neonate). Based on antimicrobial

susceptibility testing, majority of the isolates were non-susceptible to ertapenem (96%) and all were susceptible to imipenem and meropenem. The MIC_{50} and MIC_{90} for ertapenem were 2 and ≥4 respectively and ≤1 for imipenem and meropenem for both values. Fifteen isolates harboured bla_{IMP}, two contained bla_{VIM} and one contained bla_{OXA-48}. The remaining seven did not express any of the genes tested. All seven of the PCR-negative isolates were non-susceptible to ertapenem. One isolate positive for bla_{VIM} was fully susceptible to the carbapenem group of antibiotics.

The MLST scheme performed in this study is a new scheme developed and published in 2013 [27] and we were not able to compare the sequence types (ST) obtained in this study to previous sequence types obtained for this organism in South Africa. Five isolates resulted in new sequence types as they have not been located on the database although allelic profiles were generated for all seven reference genes. To our knowledge, this study provides novel information regarding MLST data for *E. cloacae* in South Africa. MLST results show that horizontal transmission of bacteria in the hospital setting is potentially an important factor to consider. **Figure 1** illustrates a timeline describing the isolates from each hospital over a 16 month period and based on the STs observed, horizontal transmission is possible. Phylogenetic analysis revealed that all strains shared a common ancestor and were distantly related as evidenced by the different number of STs detected. Those belonging to the same ST clustered together. Identical STs within the same hospital and among different hospitals could indicate intra- and inter-clonal spread. This information therefore suggests that the isolates are related phylogenetically but does not necessarily infer transmission. The limitation of using a methodology like MLST which investigates a relatively small number of reference genes is that there is substantial genetic diversity within a single species group. Therefore, the use of whole genome sequencing would provide a better understanding of transmission events and genetic relatedness. Another limitation of this study is that we had little epidemiological and clinical data. We were not able to obtain information regarding treatment regimens for the patients and therefore cannot comment on previous exposure to carbapenems.

IMP-type-producing *E. cloacae* have been documented globally, for example in the United Kingdom, the Far East and Australia [8] [11] [29]. To the best of our knowledge, the occurrence of bla_{IMP} genes in *E. cloacae* isolates is an unusual finding in South Africa. Majority (n = 15) of the 18 isolates that were PCR-positive for carbapenemase-producing genes, harboured bla_{IMP}. MLST data for three isolates was not obtained due to sequencing technicalities, four belonged to ST124, four belonged to ST90, two belonged to a new sequence type and displayed identical allelic profiles and, the remaining two belonged to ST93 and ST108. Relatively little is known about IMP-type MBL-producing *E. cloacae* with regards to prevalence, risk factors and clinical effects [8] and although our clinical and epidemiological data is scarce some information can be utilised. The specimen types for these infections were fluid/aspirate (n = 5; 33%), pus swab (n = 5; 33%), urine (n = 3; 20%) and catheter tip and sputum (n = 1; 7% each). Eight (53%) patients had bacteria other than IMP-type-producing *E. cloacae* isolated from the same culture specimen *i.e.* polymicrobial isolation. Susceptibility to antimicrobial agents is presented in **Table 1**.The minimal inhibitory concentration (MIC) breakpoints for imipenem and meropenem were not elevated and was in the susceptible range < 1 for these isolates. Similar findings were observed in the study by Hayakawa *et al.*, in 2014 underscoring the difficulties in identifying metallo-*β*-carbapenemase-producing organisms primarily based on MIC results [8]. This could also be used as a possible explanation for the one bla_{VIM}-positive isolate that was fully susceptible to the carbapenem group of antibiotics in our study.

Further investigation of the IMP-type-producing *E. cloacae* isolates using PFGE for strain comparison was performed using the Bionumerics v 6.5 software (Applied Maths, Belgium). Three major clusters were identified. Clustering was done according to Tenover criteria [28]), and a cluster was defined as PFGE patterns differing by 3 or less bands. The isolates from Cluster A were indistinguishable (no band differences). These included ML0087, ML0279 and ML0207. Cluster B consisted of three isolates that were indistinguishable (ML0197, ML0198 and ML0203). Cluster C consisted of ML0269, ML0280, ML0299 and ML0330. Slight band differences are seen but the patterns were highly similar (more than 90%) (**Figure 2**). The remaining five isolates are related but do not fall within the three major clusters (**Figure 2**).

Moreover, these 15 IMP-producing *E. cloacae* strains were analysed on the Microflex MALDI-ToF instrument and compared using the Flex analysis software (Bruker Daltonik, GmbH). All 15 isolates identified with the same *E. cloacae* reference strain with high confidence. The spectra generated showed that all 15 isolates were similar. Mass peaks of the proteins were comparable and there were no mass protein shifts identified between the isolates. Additionally, the dendograms generated showed that all 15 isolates have the same origin. Two major clusters could be seen with further sub-clustering (**Figure 1** and **Figure 3**).

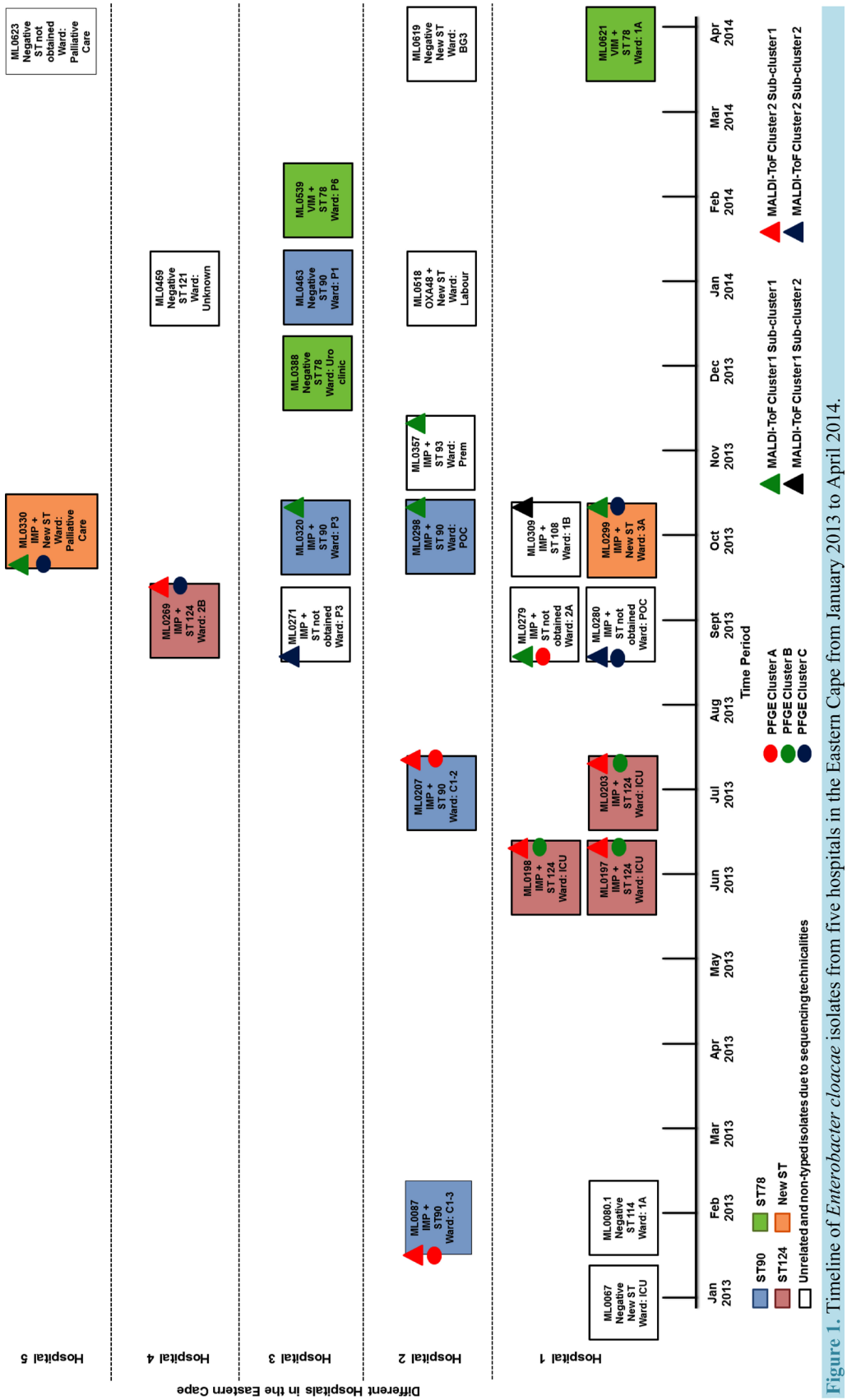

Figure 1. Timeline of *Enterobacter cloacae* isolates from five hospitals in the Eastern Cape from January 2013 to April 2014.

Table 1. Antimicrobial susceptibility testing for 15 IMP-type-producing *Enterobacter cloacae* isolates.

Antibiotics	Susceptible (n, %)	Intermediate (n, %)	Resistant (n, %)
Amikacin	15 (100)	-	-
Amox/K Clav	-	-	15 (100)
Amp/ Sulbactam	-	-	15 (100)
Ampicillin	-	-	15 (100)
Aztreonam	-	-	15 (100)
Cefepime	3 (20)	3 (20)	9 (60)
Cefotaxime	-	-	15 (100)
Cefoxitin	-	-	15 (100)
Ceftazidime	-	-	15 (100)
Cefuroxime	-	-	15 (100)
Chloramphenicol	9 (60)	1 (7)	5 (33)
Ciprofloxacin	5 (33)	-	10 (67)
Colistin	15 (100)	-	-
Ertapenem	-	7 (47)	8 (53)
Fosfomycin	13 (87)	-	2 (13)
Gentamicin	5 (33)	-	10 (67)
Imipenem	15 (100)	-	-
Levofloxacin	10 (67)	-	5 (33)
Meropenem	15 (100)	-	-
Pip/Tazo	-	4 (27)	11 (73)
Piperacillin	-	-	15 (100)
Tetracycline	6 (40)	1 (7)	8 (53)
Tigecycline	9 (60)	1 (7)	5 (33)
Tobramycin	5 (33)	1 (7)	9 (60)
Trimeth/Sulfa	1 (7)	-	14 (93)

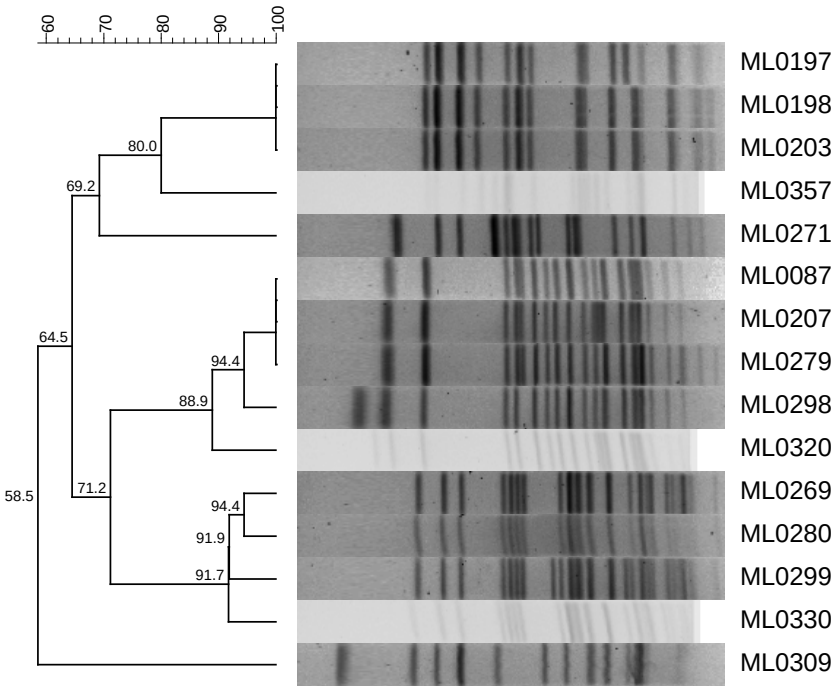

Figure 2. PFGE dendogram and electrophoresis gel pattern for IMP-type-producing *Enterobacter cloacae* isolates.

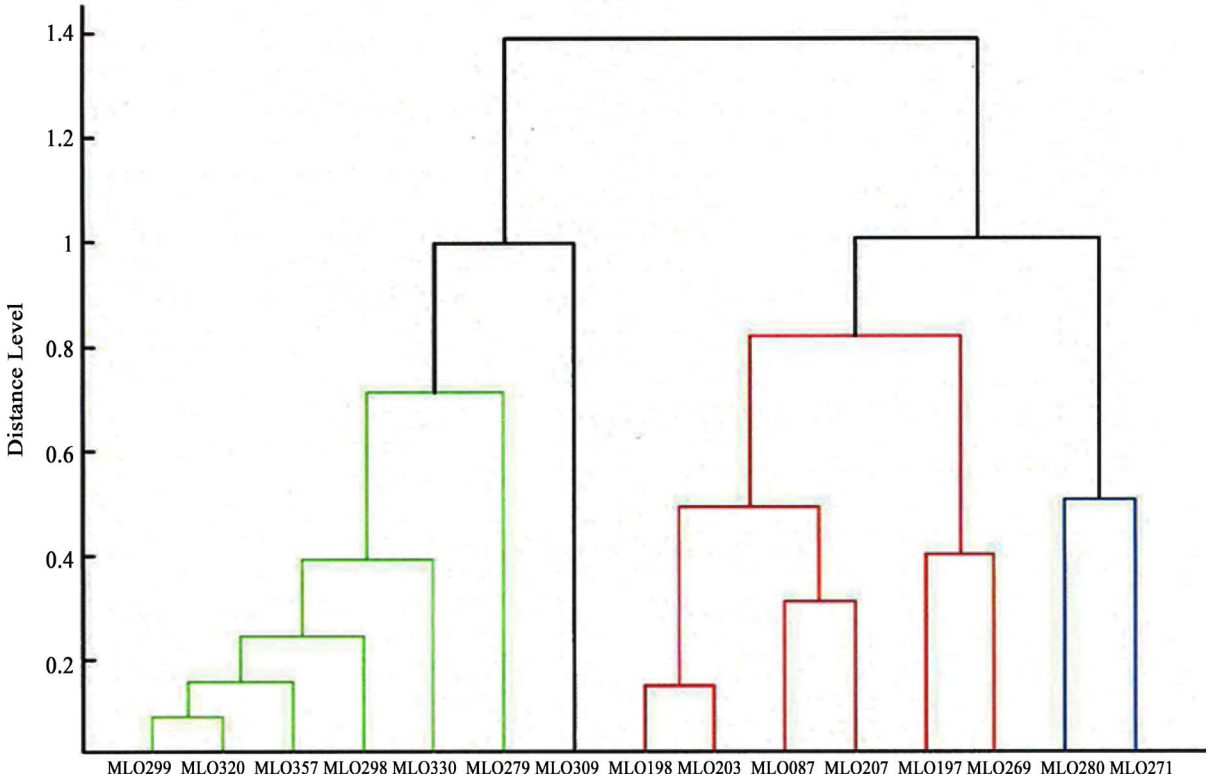

Figure 3. MALDI-ToF dendogram for 15 IMP-type-producing *Enterobacter cloacae* isolates.

The majority of the isolates appear clonally related by MLST. The dendogram generated by mass spectrometry of the 15 IMP-producing isolates indicate that the strains are highly related and PFGE of these 15 isolates identified three major clusters indicating that the strains were related. The possibility that mobile genetic elements may have been transferred among strains possessing the same drug-resistant gene does exist but the exact mechanisms of acquisition are not certain. It is also not known if the strains possessed the same subtype *i.e.* IMP-type genes or variants thereof.

Moreover, it is interesting to note that when the 15 IMP-type-producing *E. cloacae* isolates were screened for bla_{IMP} using real-time PCR, LightCycler 480 II, Roche Applied Science, LightCycler 480 Probes Master kit and the LightMix Modular IMP (ESBL) kit(Roche Diagnostics, IN, USA), bla_{IMP} was not detected in any of these isolates. This may be because the kit is able to detect at least IMP-9, 16, 18, 22 and 25 only. With the conventional PCR used originally in this study, primers from a previous publication were used and were designed from reference sequences downloaded from Genbank to obtain multiple variants [25]. It appears that the latter method is more sensitive to a wider range of IMP variants. Should this prove true, IMP variants are not being detected due to limitations in the methodology resulting in an under-reporting of IMP-type producing *E. cloacae* variants in South Africa.

4. Conclusion

From this study, we can conclude that MBL-producing strains are prevalent in the Eastern Cape hospitals. Further investigation of 15 IMP-producing *E. cloacae* isolates, currently an unusual occurrence in South Africa, revealed that majority of the isolates did appear related within three major clusters.

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