Molecular screening of clinical multidrug-resistant Gram-negative bacteria shows endemicity of carbapenemases, co-existence of multiple carbapenemases, and rarity of *mcr* in South Africa

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Running head: *carbapenemases are endemic in Pretoria*

Tweet: "*molecular screening of patient samples collected at a national reference laboratory in Pretoria shows endemicity of carbapenemases and rarity of mcr colistin resistance genes*"

Impacts:

- Carbapenems and colistin are the last resort antibiotics available for the treatment and eradication of multidrug resistant gram-negative bacteria. Resistance in these antibiotics threaten the public health as it reduces the efficacy of these therapeutics.
- *Klebsiella pneumoniae* was identified to harbour all the identified carbapenemases. This is of concern as *K. pneumoniae* has a history of causing outbreaks and being endemic in healthcare facilities in South Africa.
- It is important to understand how common carbapenem and colistin-resistant GNB are in the healthcare facilities, as it identifies areas that require attention.

Abstract

Background: Extensive use of carbapenems to treat multi-drug resistant (MDR) Gram-negative bacteria (GNB) facilitates the wide dissemination of carbapenemase-producing carbapenem-resistant GNB. Colistin was re-introduced into clinical settings to manage these GNB infections, however there is currently an increase in the dissemination of *mcr*-producing colistin-resistant GNB isolates in clinical settings. The epidemiology of carbapenemases and *mcr* in Pretoria was evaluated

***Methods*:** Clinical MDR GNB were collected and screened for carbapenemase and *mcr* using PCR, their antibiotic susceptibility profiles were elucidated using the Vitek®2 automated system (Biomerieux, France).

Results and Discussion: A total of 306 isolates were collected; majority of these were *Klebsiella pneumoniae* (n=208) and were collected from males (n=158). The isolates were retrieved from a variety of infection sites including urine, blood cultures, and rectal swabs, etc. The Vitek®2 system found that these isolates were largely resistant to β-lactams, where 217 (70.9 %) had reduced susceptibility to at least one carbapenem (ertapenem, meropenem, or imipenem) and 81 isolates (26.5%) were resistant to colistin. PCR screening identified 201 (65.7 %) isolates harbouring carbapenemase genes consisting of $bla_{\text{OXA-48}}$ (170, 84.2 %), bla_{NDM} (31, 15.4 %), bla_{IMP} (5, 2 %), bla_{KPC} (4, 1 %) and bla_{VIM} (5, 2 %). Furthermore, fourteen $bla_{\text{OXA-48}}$-producing isolates were co-harbouring bla_{VIM} (2), bla_{NDM} (9), bla_{KPC} (1) and, bla_{IMP} (2) genes. Only one isolate harboured the *mcr-1* gene, and this is the first report of an *mcr-1* producing *A. baumannii* isolate in South Africa.

***Conclusion*:** There is high endemicity of carbapenemase genes and a low prevalence of *mcr* genes in GNB, particularly in *K. pneumoniae,* in healthcare facilities in Pretoria and surrounding regions of South Africa.

Significance: Healthcare facilities in Pretoria are becoming breeding grounds for MDR infections that threaten public health. Careful use of carbapenems and other antibiotics is necessary to prevent further escalation and outbreak of these MDR strains that can claim several lives.

Keywords: epidemiology; surveillance; carbapenem; polymyxins; Enterobacterales; colistin.

Introduction

Antibiotic resistance is a global public health threat because it decreases therapeutic options for infectious diseases while stalling the progress of modern medicine [1-4]. Resistance to all classes of antibiotics has been observed, including 'last resort' ones such as carbapenems and colistin (polymyxin E) [1, 5]. Carbapenems are β-lactam antibiotics with broad bactericidal activity against both Gram-positive and Gram-negative pathogens in aerobic and anaerobic environments [6, 7]. Carbapenems were initially used in the clinical setting to treat fatal infections caused by *Enterobacterales* that produce extended spectrum β-lactamases (ESBLs) [6, 8]. However, the extensive use of carbapenems resulted in the emergence of carbapenem-resistant Gram-negative bacteria (GNB) [6, 9]. Carbapenem resistance may develop through a variety of cellular mechanisms, which include carbapenemase production, increased activity of efflux pumps, and by porin mutations. These mechanisms may either alone or accompanied by overexpression of AmpC or an ESβL [9-11].

Carbapenemases are a specific group of β-lactamases that hydrolyse carbapenems and render them inactive [6, 8, 9, 12]. Carbapenemase genes have been discovered worldwide, with carbapenem-resistant GNB infections having a 40% mortality rate [13, 14]. A study in South Africa found that 70% of carbapenem-resistant Enterobacteriaceae (CRE) infections were hospital-acquired and the in-hospital mortality rate was 38% [15]. These genes are usually associated with mobile genetic elements such as plasmids, allowing for the wide dissemination of these genes across GNB families.

Colistin was designated as the "highest critically important antimicrobial for human medicine" by the World Health Organization [16, 17]. It is part of the polymyxin antibiotic class of cationic cyclic polypeptide antibiotics that attack the lipopolysaccharide (LPS) layer found on the outer membrane of Gram-negative bacteria. [13, 18]. Colistin was re-introduced into the clinical setting to treat critically ill patients suspected of infections due to multi-drug-resistant bacteria [7, 12, 16, 19]. Acquired resistance to colistin is achieved through various mechanisms, one of which is the modification of the LPS, which reduces the bactericidal activity of colistin [13, 20]. LPS modifications can be achieved through various chromosomal mutations within genes that are within the two-component systems *pmrA/pmrB* and *phoP/phoQ*, i.e., *mgrB. crrb, phoQ*, *pmrB,* etc [10, 11]. Resistance can also develop through the acquisition of mobile colistin resistance (*mcr*) genes that encode MCR enzymes that mediate LPS modifications [11, 21]. *Mcr*-genes have been identified in multiple hospitals across South Africa, mostly in *E. coli* and *K. pneumoniae,* and

have also been identified in livestock and in effluents of wastewater treatment plants [22-24]. Newton-Foot *et al.* (2017) screened for colistin-resistant *E. coli* and *K. pneumoniae* isolates collected between January 2016 and August 2016 by the National Health Laboratory Service at Tygerberg Hospital and found that 83% (15 of 18) of them were *mcr-1* producing [24]. This highlights an increase in *mcr* genes in the Tygerberg Academic hospital and surrounding regional and distinct hospitals [24].

Carbapenemase and *mcr* genes are commonly associated with mobile genetic elements such as plasmids, insertion sequences, and transposons [5, 24-27]. This association allows for the rapid dissemination of these resistance genes worldwide, thus posing a threat to public health [26], because they endanger the efficacy of carbapenems and colistin during treatment [1, 3].

Resistance to the last-resort antibiotics carbapenem and colistin should be carefully monitored to prevent further spread of bacterial resistance and to inform treatment options [13]. Molecular tools such as polymerase chain reaction (PCR) and whole genome sequencing (WGS) have been shown to be useful tools in the identification of resistance genes [28] and for screening of known resistance genes [6]. This study describes the molecular epidemiology of carbapenemase and *mcr* genes in multi-drug resistant (MDR) GNB collected from the medical microbiology laboratory of the Tshwane academic division of the National Health Laboratory Service (Tshwane Academic Division) culture biobank.

Methods

A total of 306 multi-drug resistant clinical Gram-negative isolates were collected from NHLS/UP. All of the clinical isolates were subjected to identification and antibiotic susceptibility testing using the Vitek-2 automated system (BioMérieux, France) according to the manufacturer's instructions. Further, the epidemiological data such as sex, age and sample source was retrieved from the NHLS TrakCare system (Table S1).

Antimicrobial susceptibility test of gram-negative isolates

Identification and antimicrobial susceptibility testing were performed on the isolates using the Vitek® 2 automated system (BioMérieux, France) against seventeen antibiotics. These included: amikacin, gentamicin, ampicillin, amoxicillin-clavulanate, piperacillin tazobactam, cefuroxime, cefotaxime/ceftriaxone, ceftazidime, ertapenem, imipenem, meropenem, colistin, ciprofloxacin, tigecycline, trimethoprim sulphamethoxazole, cefoxitin, and cefepime.

Isolates identified to be colistin-resistant were selected for broth microdilution to determine their exact minimum inhibition concentration (MIC) values (Table S1). The broth microdilution (BMD) was performed according to the CLSI standards [29] using *Escherichia coli* ATCC® 25922™ and/or *Pseudomonas aeruginosa* ATCC® 27853™ for quality control.

PCR-based screening of carbapenemase and mcr genes

All 306 isolates were screened for the presence of carbapenem and colistin resistance genes, regardless of their susceptibility profile. The PCR screening included two multiplex PCRs, one was made up of all five *mcr* primers seen in Table 1, and the second was made up of *bla*$_{VIM}$, *bla*$_{OXA}$ and *bla*$_{NDM}$ primers. The PCR further consisted of two singleplex PCRs for both *bla*$_{KPC}$ and *bla*$_{IMP}$. The PCR reactions were performed according to Table S2. Amplicons and a 100bp DNA ladder (Promega, USA) were viewed using 2% agarose gel electrophoresis.

Table 1. Primer sequences for mcr and carbapenemase PCR screening

Target gene	Primers	Product size	Reference
Mcr -1	F: 5'-AGTCCGTTTGTTCTTGTGGC-3' R: 5'-AGATCCTTGGTCTCGGCTTG-3'	320	[28]
Mcr -2	F: 5'-CAAGTGTGTTGGTCGCAGTT-3' R: 5'-TCTAGCCCGACAAGCATACC-3'	715	[28]
Mcr -3	F: 5'-AAATAAAAATTGTTCCGCTTATG-3' R: 5'-AATGGAGATCCCCGTTTTT-3'	929	[28]
Mcr -4	F: 5'-TCACTTTCATCACTGCGTTG-3' R: 5'-TTGGTCCATGACTACCAATG-3'	1116	[28]
Mcr -5	F: 5'-ATGCGGTTGTCTGCATTTATC-3' R: 5'-TCATTGTGGTTGTCCTTTTCTG-3'	1644	[29]
Mcr -9	F: 5'-TTCCCTTTGTTCTGGTTG-3' F: 5'-GCAGGTAATAAGTCGGTC-3'	1011	[30]
IMP	F:5'- GGAATAGAGTGGCTTAAYTCTC -3' R:5'- GGTTTAAYAAAACAACCACC -3'	232	[31]
KPC	F:5'- TGTCACTGTATCGCCGTC -3' R:5'- CTCAGTGCTCTACAGAAAACC -3'	900	[32]
VIM	F:5'- GATGGTGTTTGGTCGCATA -3' R:5'- CGAATGCGCAGCACCAG -3'	390	[31]
OXA	F:5'- GCGTGGTTAAGGATGAACAC -3' R:5'- CATCAAGTTCAACCCAACCG -3'	438	[31]
NDM	F:5'- GGTTTGGCGATCTGGTTTTC -3' R:5'- CGGAATGGCTCATCACGATC -3'	782	[31]

Briefly, colonies of overnight culture were placed in Eppendorf tubes with one millilitre of 1x phosphate buffer saline and boiled for 10 minutes in a water bath. The tubes were transferred to an ice bath for 5 minutes and then were centrifuged for five minutes at 1000 rpm. Three microliters of supernatant were used for PCR. The PCR pre-mixes were made up of 12.5 µL GoTaq Green Master Mix (Promega, USA), 3 µL of genomic DNA, 1.5 µL for each primer and nuclease-free water to a final volume of 25 µL. The PCRs were conducted according to Table S2.

Ethical approval

Ethics approval was received from the Faculty of Health Sciences: Research Ethics Committee at the University of Pretoria under the reference 581/2020.

Results

A total of 306 multi-drug resistant clinical GNB isolates were collected from NHLS bacterial culture biobank. These isolates were comprised of 208 *Klebsiella pneumoniae*, 29 *Escherichia coli*, 20 *Acinetobacter baumannii*, 16 *Enterobacter cloacae* complex, 10 *Pseudomonas aeruginosa*, nine *Salmonella* Group D, three *Klebsiella oxytoca*, two *Serratia marcescens*, two *Morganella morganii*, and single isolates each of *Citrobacter freundii, Citrobacter koseri, Shigella flexneri, Proteus mirabilis, Proteus vulgaris,* and *Providencia stuartii*. The bacterial strains were isolated from a variety of infection sites, which includes: urine (n = 84), blood culture (n = 68), rectal swab (n = 31) aspirate (fluid/ tracheal, treated; n = 24), Tissue (n = 18), pus (aspirate/swab; n = 15), catheter tip (arterial/intravenous; n = 14), sputum (n = 13), catheter urine (9), central spinal fluid (9), swab (5), stool (4), bronchial alveolar lavage (1) and ventriculoperitoneal shunt (1). Urine was the predominant collected sample, where the majority (96.4 %) of the isolates isolated were of the Enterobacteriaceae family, which are common aetiological agents of urine tract infections. *Klebsiella pneumoniae* was isolated from 21 different infection sites, with urine, blood cultures and rectal swabs being the predominant sites (Figure 1). Thereafter, *A. baumannii* and *E. coli* were isolated from nine and eight sites, respectively.

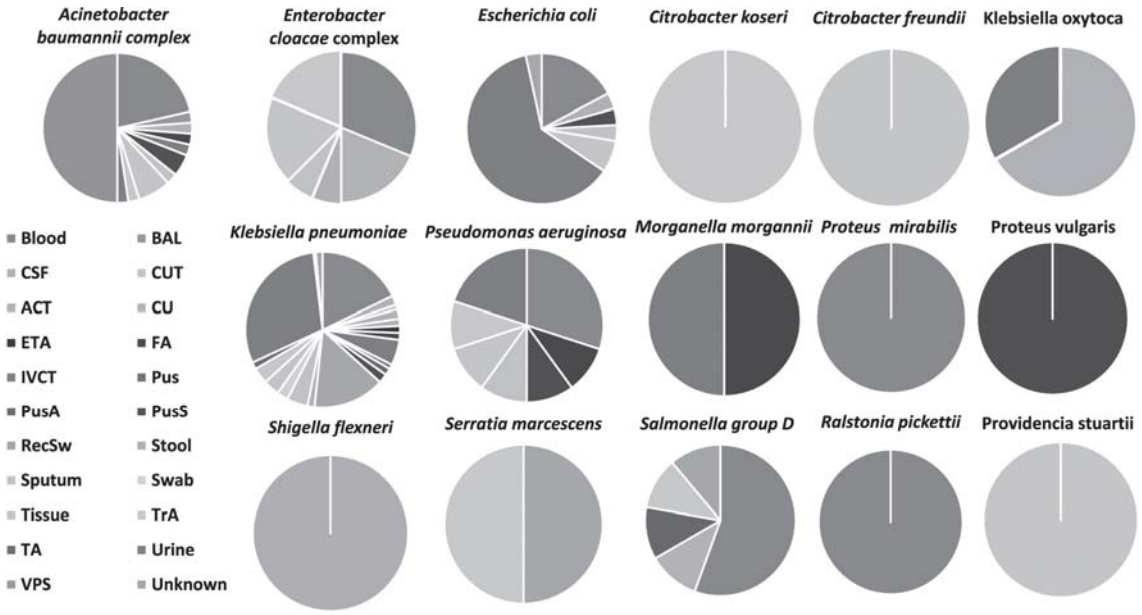

FIG. 1. The distribution of sample sources for each MDR clinical Gram-negative isolate. The sample sources are made up of blood culture, CSF, ACT, ETA, IVCT, PusA, RecSw, sputum, tissue, TA, VPS, BAL, CUT, CU, FA, Pus, PusS, stool, swab, TA, and urine. ACT, arterial catheter tip; BAL, bronchial alveolar lavage; CSF, cerebrospinal fluid; CU, catheter urine; CUT, catheter urine tip; ETA, endotracheal aspirate; FA, fluid aspirate; IVCT, intravenous catheter tip; MDR, multidrug-resistant; PusA, Pus aspirate; PusS, Pus swab; TA, treated aspirate; VPS, ventriculoperitoneal shunt; RecSw, rectal swab.

The epidemiological data of each GNB isolate is seen in Table S1 and summarized in Table S3 (Figure 2). This data shows that majority of the isolates were collected from males (158, 51.63%) and middle-aged people (135, 44.11%), where 100 of the 135 isolates collected from this age group were *K. pneumoniae* (Figure 2b). Neonates, infants younger than 1 years, constituted 12.46% (38) of total isolates; 73.68% (28) of these neonatal isolates were obtained from infants younger than 1 month. Neonates are highly susceptible to bacterial infections due to their immature immune system, thus, neonatal infections lead to a rapid progression of disease [30, 31]. It is therefore, concerning to observe MDR GNB in this age group.

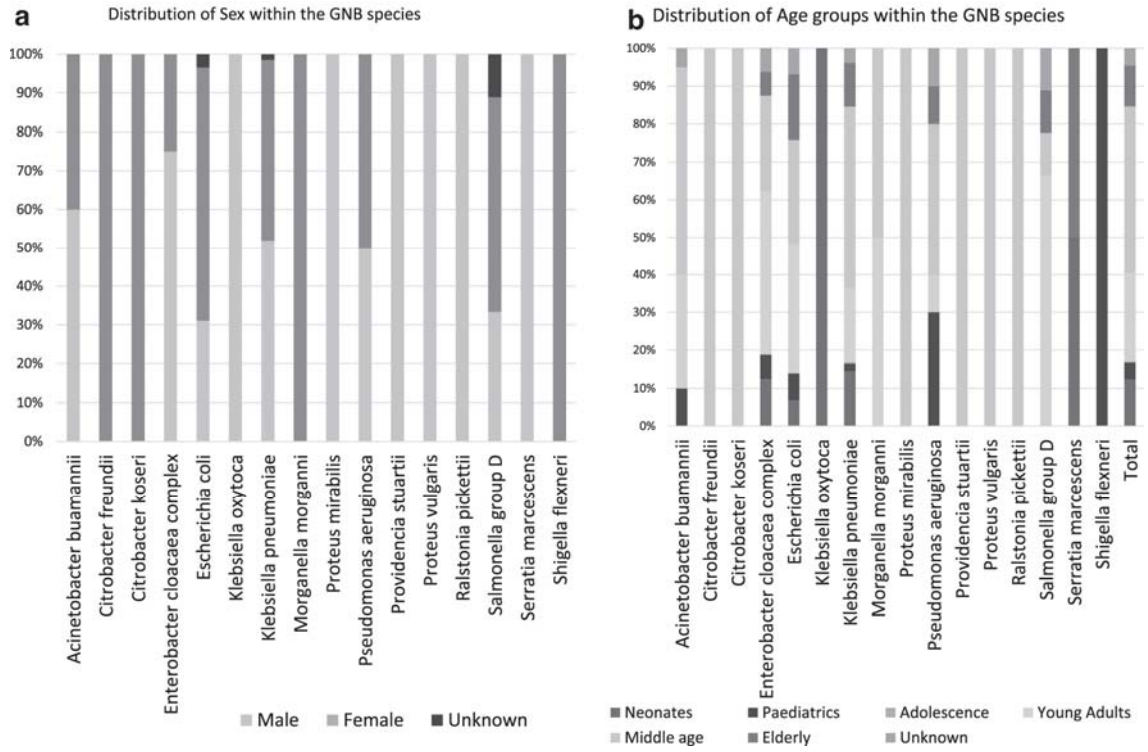

FIG. 2. The demographics of **(a)** sex and **(b)** age groups, of patients from the whom the samples were collected.

Antimicrobial susceptibility profile results

The antimicrobial susceptibility profile seen in Table S1 reveals that more than two thirds of the isolates were highly resistant to β-lactam antibiotics (ampicillin 77.8 %, ceftriaxone/cefotaxime 74.5 %, cefuroxime 71.2 %, cefepime 69.3 %, ceftazidime 69.3 % and cefoxitin 68.5 %). Further, 217 (70.9 %) of isolates were resistant to at least one carbapenem and resistance to each included 57.5% (179) ertapenem, 44.8% (139) imipenem and 41.5% (127) meropenem. The frequency of carbapenem-resistant isolates was calculated to be 64.7% with *K. pneumoniae* isolates being the most predominant. There were however, low levels of colistin resistant isolates (80, 26.1 %) seen amongst the 306 isolates, further, amongst those found to be ertapenem resistant, only 10% (19), were colistin resistant.

Prevalence of carbapenemase genes

All the 306 MDR GNB were subjected to carbapenemase screening regardless of their antibiotic susceptibility profile. The five carbapenemase genes included in the singleplex and multiplex PCR, were KPC (bla_{KPC}), metallo-β-lactamases (MBLs) (bla_{IMP}, bla_{VIM}, bla_{NDM}) and

OXA-48 (*bla*$_{OXA-48}$). Positive controls were included in PCR reactions to ensure PCR validity, and amplicons are seen in Figure 3b.

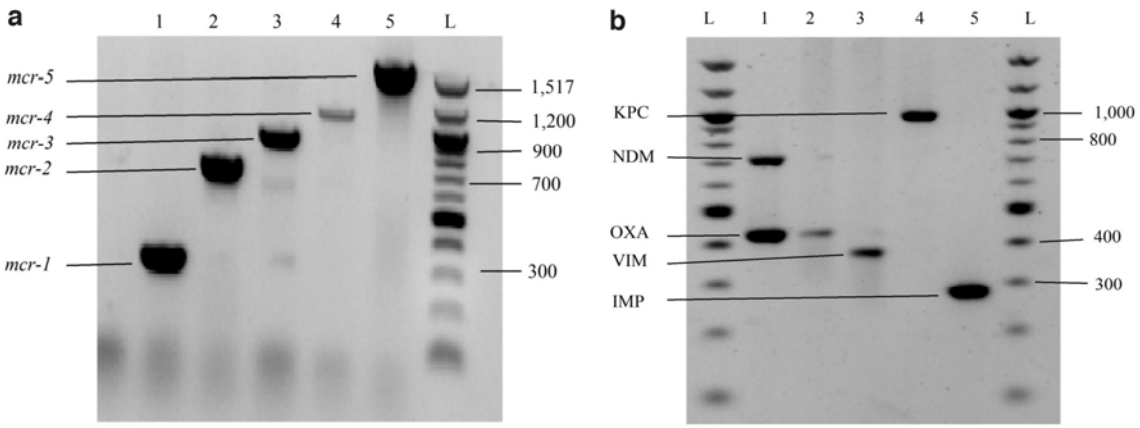

FIG. 3. Gel electrophoresis of PCR amplicons obtained from the isolates. Two percent TBE agarose gel of PCR controls used in the study, ran against a 100 bp ladder **(a)** mcr-1 to mcr-5 controls in lanes 1–5 with base ladder in Lane L. **(b)** Labeled carbapenemase controls, bla$_{KPC}$, bla$_{NDM}$, bla$_{OXA}$, bla$_{VIM}$, and bla$_{IMP}$ ran against 100 bp ladder. mcr, mobile colistin resistance; PCR, polymerase chain reaction; TBE, Tris-Borate-EDTA.

Overall, a total of 201 (65.7 %) of isolates were found to harbour one or more carbapenemase genes and a total of 216 carbapenemase genes were identified. The results, shown in Figure 4, was including 171 (55.6%) OXA-48 producers, where fourteen of these isolates also co-harboured an additional carbapenemase gene (Figure 4). MBL-producers were 41 (13.4%) and were made up of nine NDM-OXA-48 co-producers, 20 NDM producers, two IMP-VIM-OXA-co-producers, two VIM-OXA-48 producers, one IMP-producer, 1 VIM-producer and 1 VIM-NDM producer (Figure 4). Lastly, eight KPC producers were identified, where one isolate co-harboured KPC with OXA-48. The carbapenemase genes were largely identified in *K. pneumoniae* isolates (92%).

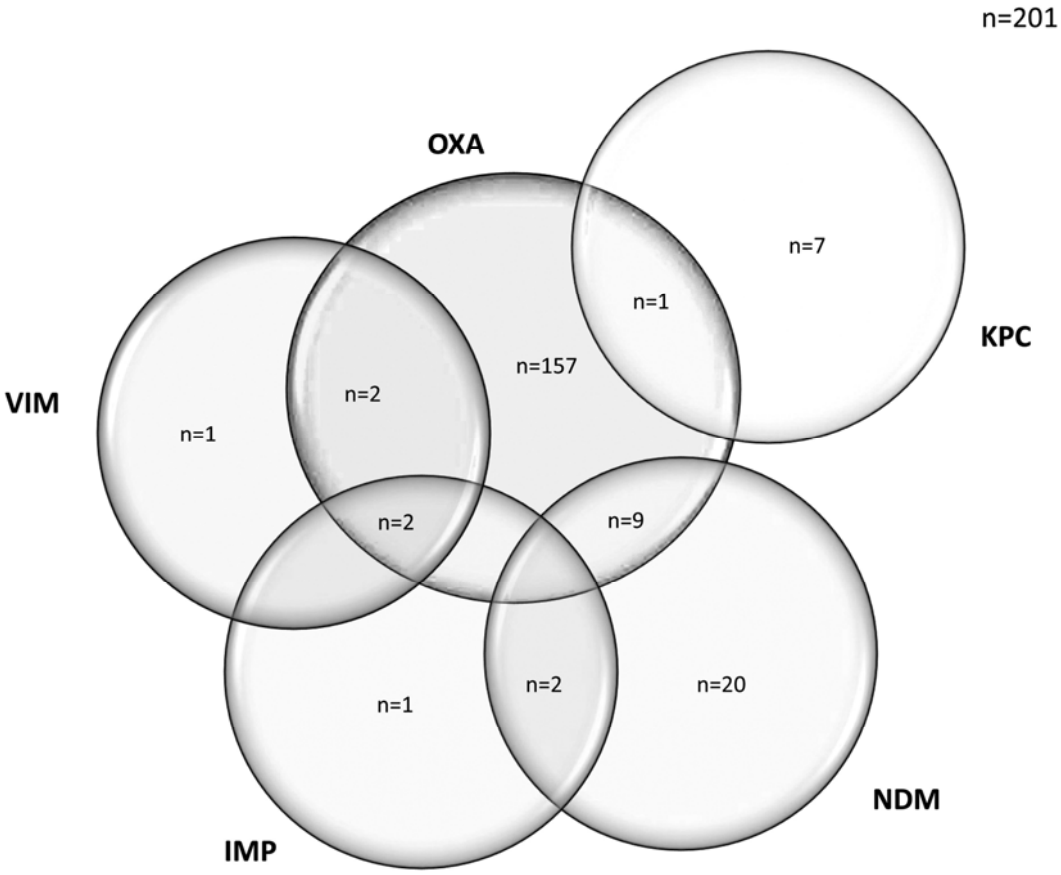

FIG. 4. Distribution of carbapenemases identified in the isolates. Two hundred one isolates were identified to be harboring carbapenemase genes; however, 16 isolates were harboring ≥2 carbapenemases. One hundred fifty-seven isolates were harboring *bla*$_{\text{OXA-48}}$ genes only, thereafter two were coharbored with *bla*$_{\text{VIM}}$, two with *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$, nine with *bla*$_{\text{NDM}}$, and one was coharbored with *bla*$_{\text{KPC}}$. One isolate was harboring *bla*$_{\text{VIM}}$ only, two was coharbored with *bla*$_{\text{IMP}}$, and one was harboring *bla*$_{\text{IMP}}$ only. Twenty isolates were harboring NDM only, and two co-haboured *bla*$_{\text{IMP}}$. Finally, seven isolates were *bla*$_{\text{KPC}}$ producing. NDM, New Delhi Metallo-β-Lactamase.

Prevalence of mcr genes

A total of 80 isolates were identified to be colistin resistant using the Vitek-2 automated system. The MICs of these isolates to colistin was re-evaluated using BMD, which found 76 of those isolates to be colistin resistant. The PCR screening however, only identified one isolate, *A. baumannii* BB2, to harbour an *mcr-1* gene (Table S1). None of the remaining 79 isolates were found to be harbouring *mcr-1, 2,3,4,5* genes. Positive controls were included in PCR reactions to ensure PCR validity, and amplicons are seen in Figure 3a.

Discussion

Using PCR, we screened clinical samples stored at a clinical laboratory, NHLS, in Pretoria, South Africa. This laboratory serves other regions outside of Pretoria. We show from this molecular screening that carbapenemases are pervasive in Pretoria.

Multiple studies have also identified *K. pneumoniae* as the most common organism harbouring carbapenemase genes [32, 33]. In this study, the *K. pneumoniae* isolates harboured all the identified carbapenemases: *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{OXA}$, and *bla*$_{VIM}$. This is concerning because carbapenem-resistant *K. pnuemoniae* (CRKP) has been implicated in outbreaks in South Africa [34, 35], and has previous reports of being endemic in certain hospitals and regions [7]. Xu *et al*. [36] further showed in their meta-analysis that CRKP healthcare-associated infections have a mortality rate of 42%. It is therefore important for healthcare workers and South African health institutes to continually monitor this public health threat, ensuring infection prevention and control measures are implemented to prevent the spread of CRKP.

The isolates were collected from 22 sample sources, with urine (36; 27.45%), blood (68; 22.22%) and rectal swabs (31; 10.13%) being the most prevalent. From blood cultures, *A. baumannii*, *E. cloacae*, *E. coli, K. pneumoniae, P. aeruginosa, Salmonella* Group D, *P. mirabilis,* and *R. picketti* isolates were isolated (Figure 1, Table S1). This is concerning because most of these isolates are part of the ESKAPE group (i.e., *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.), which are commonly MDR, highly virulent, and threaten public health [17, 37]. Furthermore, 13 of these isolates, isolated from blood cultures, were obtained from neonates.

The isolates identified from urine samples were mostly those of *Enterobacteriaceae,* which are common aetiological agents of urinary tract infections (UTIs) [38, 39]. UTIs are commonly identified in women, pregnant women, and immunocompromised patients [40], and in this study, 49 of the 84 (58.33%) urine samples were obtained from women. However, due to incomplete clinical data, the clinical condition of patients is unknown.

The PCR screening showed that there is an overall higher prevalence of carbapenemase genes than *mcr* genes in these isolates. The Vitek® 2 automated system (BioMérieux, France) classified 81 isolates as resistant to colistin, which was confirmed using broth microdilution on 76 of those isolates, representing an colistin resistance sensitivity of 93.83%. Of the 80 isolates, only one (BB2) harboured an *mcr-1* gene. Isolate BB2 was isolated from a fluid aspirate

specimen collected from a 53-year-old female. The Vitek® 2 (BioMérieux, France) identified BB2 to be *A. baumannii* complex. BB2 was also identified to be co-harbouring an OXA-48 gene. In South Africa, *mcr-1* genes are commonly identified in *E. coli* and *K. pneumoniae,* and this is the first report of an *mcr-1* producing *A. baumannii* isolate in South Africa. Colistin resistance is acquired mainly through modification of the Lipid A of the LPS membrane layer through an addition of a 4-amino-4-deoxyl-1-arabinose of phosphoethanolamine [11, 41, 42]. This is achieved through chromosomal mutations in genes encoding the two component systems: PhoPQ and PmrAB, or mutations such as insertions or deletions that result in complete loss of the *mgrB* locus, a regulator of PhoPQ. These mechanisms are commonly identified in Enterobacteriacae species, and *mgrB* is commonly seen in *K. pneumoniae* [41, 42]. In *A. baumannii,* colistin resistance is acquired through complete loss of the Lipid A enzyme, through mutation within its biosynthesis pathway genes, *lpxACD* [43]. Isolates such as *S. marcescens* and *P. mirabilis* are intrinsically resistance to colistin [42]. In this study, the PCR was set up to screen for *mcr-1* to *mcr-5* genes, and thus *mcr-6* to *10* genes would have been missed.

A total of 217 (70.9 %) isolates were found to have reduced susceptibility to at least one carbapenem, i.e., ertapenem, meropenem and imipenem. Carbapenem resistance can be acquired through a combination of different resistance mechanisms that include the loss of major porin proteins, increased activity of efflux pumps and the production of carbapenemases alone or the hyperproduction of ESBLs and AmpCs alongside porin loss or efflux hyperactivity [44-46]. In this study, the prevalence of carbapenemase genes was evaluated and a total of 201 (65.7 %) isolates were identified to harbour a carbapenemase gene. Therefore, of the 217 isolates identified to be resistant to at least one carbapenem, 201 harboured a carbapenemase gene. Thus, sixteen isolates encode other resistance mechanisms that confer carbapenem resistance.

A total of 171 (55.6 %) isolates harboured *bla*$_{\text{OXA-48}}$ genes, 31 (10.1 %) harboured *bla*$_{\text{NDM}}$, five (1.6 %) had *bla*$_{\text{VIM}}$, five (2 %) had *bla*$_{\text{IMP}}$, and four (1 %) harboured *bla*$_{\text{KPC}}$ (seen in Figure 4). It is also seen that 80% of the *bla*$_{\text{VIM}}$, 29% of the *bla*$_{\text{NDM}}$, 12.5% of the *bla*$_{\text{KPC}}$ and 40% of the *bla*$_{\text{IMP}}$ were identified to also co-harbour *bla*$_{\text{OXA-48}}$ genes (Figure 4). This data shows that there is a high prevalence and wide dissemination of oxacillinase carbapenemase in Pretoria, which correlates with the findings by Perovic *et al.* (2020) [15], who found that 52% of carbapenem-resistant Enterobacteriaceae isolates collected in four provinces of South Africa (Gauteng, Western Cape, KwaZulu-Natal and Free State) were *bla*$_{\text{OXA-48}}$-producing.

It is important to understand how common carbapenem and colistin resistant GNB are in hospitals, as these isolates include three of the five ESKAPE nosocomial pathogens. Snyman *et al.* (2021) [47] investigated the gastrointestinal carriage of colistin-resistant microorganisms and *mcr* genes in healthy individuals in Cape Town's communities [47]. The study found no evidence of *mcr* genes and concluded that *mcr* genes are not widely disseminated in the community setting [47]. The low prevalence of *mcr* genes elucidated in this study indicates that in this clinical setting, *mcr* genes are not widely distributed.

The data showed a high presence of carbapenemase genes and a low presence of *mcr* genes in MDR clinical GNB isolates in Pretoria, South Africa. Notably, the co-occurrence of two carbapenemases per isolate is a disturbing finding and suggests the high circulation of carbapenemases among GNB in Pretoria. Evidently, *K. pneumoniae* remains a cardinal MDR pathogen that requires urgent attention. Future studies need to correlate clinical outcomes with isolated resistant organisms to better understand the true implications of the high prevalence of carbapenemases seen in these MDR organisms. Furthermore, the spread of resistant organisms in healthcare settings can be curtailed by effectively practicing appropriate infection prevention and control measures, and continued surveillance helps with the identification of clinical areas that require extra attention.

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Author contributions: MM undertook laboratory work and manuscript drafting; TMSL assisted with collection of isolates and reviewing of the manuscript; LBS assisted with collection of isolates and reviewing of the manuscript; NMM was a co-supervisor to the study and assisted with funding; JOS designed and supervised the study and reviewed the manuscript, as well as assisted with analysis of the data.

References

1. Belguesmia, Y., Hazime, N., Kempf, I., Boukherroub, R. & Drider, D. New bacteriocins from *Lacticaseibacillus paracasei* CNCM I-5369 adsorbed on alginate nanoparticles are very active against *Escherichia coli*. *Int J of Mol Sci* **21**, 8654 (2020).
2. Band, V.I. *et al.* Colistin Heteroresistance Is Largely Undetected among Carbapenem-Resistant Enterobacterales in the United States. *Mbio* **12**, e02881-02820 (2021).
3. Golkar, Z., Bagasra, O. & Pace, D.G. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Cities* **8**, 129-136 (2014).
4. Morrill, H.J., Pogue, J.M., Kaye, K.S. & LaPlante, K.L. in Open forum infectious diseases, Vol. 2 (Oxford University Press, 2015).
5. Poirel, L., Jayol, A. & Nordmann, P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* **30**, 557-596 (2017).
6. Gajdács, M. *et al.* Detection of VIM, NDM and OXA-48 producing carbapenem resistant *Enterobacterales* among clinical isolates in Southern Hungary. *Acta Microbiol Immunol Hung* **67**, 209-215 (2020).
7. Madni, O., Amoako, D.G., Abia, A.L.K., Rout, J. & Essack, S.Y. Genomic Investigation of Carbapenem-Resistant *Klebsiella pneumonia* Colonization in an Intensive Care Unit in South Africa. *Genes (Basel)* **12**, 951 (2021).
8. Codjoe, F.S. & Donkor, E.S. Carbapenem resistance: a review. *Med Sci* **6**, 1 (2018).
9. Meletis, G. Carbapenem resistance: overview of the problem and future perspectives. *Ther Adv Infect Dis* **3**, 15-21 (2016).
10. Di Tella, D. *et al.* Molecular Epidemiological Insights into Colistin-Resistant and Carbapenemases-Producing Clinical Klebsiella pneumoniae Isolates. *Infect Drug Resist* **12**, 3783-3795 (2019).
11. Mmatli, M., Mbelle, N.M., Maningi, N.E. & Osei Sekyere, J. Emerging Transcriptional and Genomic Mechanisms Mediating Carbapenem and Polymyxin Resistance in Enterobacteriaceae: a Systematic Review of Current Reports. *mSystems* **5**, e00783-00720 (2020).
12. Pitout, J.D., Nordmann, P. & Poirel, L. Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. *Antimicrob Agents Chemother* **59**, 5873-5884 (2015).
13. Stefaniuk, E.M. & Tyski, S. Colistin Resistance in Enterobacterales Strains - A Current View. *Pol J Microbiol* **68**, 417-427 (2019).
14. Patel, G., Huprikar, S., Factor, S.H., Jenkins, S.G. & Calfee, D.P. Outcomes of carbapenem-resistant *Klebsiella pneumoniae* infection and the impact of antimicrobial and adjunctive therapies. *Infect Control Hosp Epidemiol* **29**, 1099-1106 (2008).
15. Perovic, O. *et al.* Carbapenem-resistant Enterobacteriaceae in patients with bacteraemia at tertiary hospitals in South Africa, 2015 to 2018. *Eur J Clin Microbiol Infect Dis* **39**, 1287-1294 (2020).
16. Zhang, H., Zhang, J., Kang, Y., Yang, Q. & Xu, Y. Analysis of Susceptibilities of Carbapenem Resistant Enterobacterales to Colistin in Intra-Abdominal, Respiratory and Urinary Tract Infections from 2015 to 2017. *Infect Drug Resist* **13**, 1937-1948 (2020).
17. World Health Organization, Edn. 5th (2017).
18. Olaitan, A.O., Morand, S. & Rolain, J.-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* **5**, 643 (2014).
19. Ramsamy, Y. *et al.* Genomic analysis of carbapenemase-producing extensively drug-resistant *Klebsiella pneumoniae* isolates reveals the horizontal spread of p18-43_01 plasmid encoding blaNDM-1 in South Africa. *Microorganisms* **8**, 137 (2020).

20. Baron, S., Hadjadj, L., Rolain, J.-M. & Olaitan, A.O. Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents* **48**, 583-591 (2016).
21. Liu, Y.-Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* **16**, 161-168 (2016).
22. Snyman, Y. *et al.* Clonal expansion of colistin-resistant *Acinetobacter baumannii* isolates in Cape Town, South Africa. *Int J Infect Dis* **91**, 94-100 (2020).
23. Poirel, L. *et al.* Genetic features of MCR-1-producing colistin-resistant *Escherichia coli* isolates in South Africa. *Antimicrob Agents Chemother* **60**, 4394-4397 (2016).
24. Newton-Foot, M., Snyman, Y., Maloba, M.R.B. & Whitelaw, A.C. Plasmid-mediated *mcr*-1 colistin resistance in *Escherichia coli* and *Klebsiella* spp. clinical isolates from the Western Cape region of South Africa. *Antimicrob Resist Infect Control* **6**, 1-7 (2017).
25. Anyanwu, M.U. *et al.* Is Africa ready for mobile colistin resistance threat? *Infect Ecol Epidemiol* **11**, 1962781 (2021).
26. Kopotsa, K., Sekyere, J.O. & Mbelle, N.M. Plasmid evolution in carbapenemase-producing Enterobacteriaceae: a review. (2019).
27. Ramsamy, Y. *et al.* Mobile genetic elements-mediated Enterobacterales-associated carbapenemase antibiotic resistance genes propagation between the environment and humans: A One Health South African study. *Sci Total Environ* **806**, 150641 (2022).
28. Nordmann, P. *et al.* Identification and screening of carbapenemase-producing Enterobacteriaceae. *Clin Microbiol Infect* **18**, 432-438 (2012).
29. Testing, E.C.o.A.S. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. *EUCAST: Växjö, Sweden* (2016).
30. Govender, K., Vol. PhD (University of KwaZulu-Natal, 2014).
31. Osei Sekyere, J., Reta, M.A. & Bernard Fourie, P. Risk factors for, and molecular epidemiology and clinical outcomes of, carbapenem-and polymyxin-resistant Gram-negative bacterial infections in pregnant women, infants, and toddlers: a systematic review and meta-analyses. *Ann N Y Acad Sci* **1502**, 54-71 (2021).
32. Perovic, O., Britz, E., Chetty, V. & Singh-Moodley, A. Molecular detection of carbapenemase-producing genes in referral Enterobacteriaceae in South Africa: a short report: clinical update. *S Afr Med J* **106**, 975-977 (2016).
33. Nel, P., Roberts, L.A. & Hoffmann, R. Carbapenemase-producing Enterobacteriaceae colonisation in adult inpatients: A point prevalence study. *S Afr J Infect Dis* **34**, 1-5 (2019).
34. Jacobson, R.K. *et al.* Molecular characterisation and epidemiological investigation of an outbreak of blaOXA-181 carbapenemaseproducing isolates of *Klebsiella pneumoniae* in South Africa. *S Afr Med J* **105** (2015).
35. Essel, V., Ntshoe, G., Mphaphuli, E. & Tshabalala, K. A multisectoral investigation of a neonatal unit outbreak of *Klebsiella pneumoniae* bacteraemia at a regional hospital in Gauteng Province, South Africa. *S Afr Med J* **110**, 783-790 (2020).
36. Xu, L., Sun, X. & Ma, X. Systematic review and meta-analysis of mortality of patients infected with carbapenem-resistant *Klebsiella pneumoniae*. *Ann Clin Microbiol Antimicrob* **16**, 1-12 (2017).
37. Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* **18**, 318-327 (2018).
38. Zilberberg, M.D., Nathanson, B.H., Sulham, K., Fan, W. & Shorr, A.F. Carbapenem resistance, inappropriate empiric treatment and outcomes among patients hospitalized with Enterobacteriaceae urinary tract infection, pneumonia and sepsis. *BMC Infect Dis* **17**, 1-13 (2017).

39. Mazzariol, A., Bazaj, A. & Cornaglia, G. Multi-drug-resistant Gram-negative bacteria causing urinary tract infections: a review. *J Chemother* **29**, 2-9 (2017).
40. Geerlings, S.E. Clinical presentations and epidemiology of urinary tract infections. *Microbiol Spectr* **4**, 4.5. 03 (2016).
41. Andrade, F.F., Silva, D., Rodrigues, A. & Pina-Vaz, C. Colistin update on its mechanism of action and resistance, present and future challenges. *Microorganisms* **8**, 1716 (2020).
42. Gogry, F.A., Siddiqui, M.T., Sultan, I. & Haq, Q.M.R. Current Update on Intrinsic and Acquired Colistin Resistance Mechanisms in Bacteria. *Frontiers in Medicine* **8** (2021).
43. Adams, M.D. *et al.* Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother* **53**, 3628-3634 (2009).
44. Ripabelli, G., Sammarco, M.L., Scutellà, M., Felice, V. & Tamburro, M. Carbapenem-Resistant KPC- and TEM-Producing Escherichia coli ST131 Isolated from a Hospitalized Patient with Urinary Tract Infection: First Isolation in Molise Region, Central Italy, July 2018. *Microb Drug Resist* **26**, 38-45 (2020).
45. Hamzaoui, Z. *et al.* Role of association of OmpK35 and OmpK36 alteration and blaESBL and/or blaAmpC genes in conferring carbapenem resistance among non-carbapenemase-producing Klebsiella pneumoniae. *Int J Antimicrob Agents* **52**, 898-905 (2018).
46. Suay-García, B. & Pérez-Gracia, M.T. Present and Future of Carbapenem-resistant Enterobacteriaceae (CRE) Infections. *Antibiotics (Basel)* **8** (2019).
47. Snyman, Y., Whitelaw, A.C., Maloba, M.R.B., Hesseling, A.C. & Newton-Foot, M. Carriage of colistin-resistant Gram-negative bacteria in children from communities in Cape Town (Tuberculosis child multidrug-resistant preventive therapy trial sub-study). *S Afr J Infect Dis* **36**, 241 (2021).