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Insertion Sequences And Plasmids Are Involved In Increased Carbapenem-Resistant Acinetobacter Baumannii Infections In Two South African Hospitals

N van Heerden[1], PM Makhoahle[2], A van der Spoel van Dijk[1,3*]
[1]Department of Medical Microbiology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
[2]Department of Health Sciences, Faculty of Health and Environmental Sciences Central University of Technology – Free State, South Africa
[3*]National Health Laboratory Service, Universitas, Bloemfontein, South Africa.

Abstract

Background: *Acinetobacter baumannii is a serious carbapenem-resistant nosocomial pathogen. Carbapenemase genes such as bla$_{OXA-23}$ and bla$_{NDM}$ and insertion sequences (ISs) near these genes, which can be located on plasmids, primarily contribute to carbapenem resistance in A. baumannii. However, South African studies have not investigated the prevalence of A. baumannii infections and the effect of the carbapenemase genes, ISs, and plasmids on carbapenem resistance. Therefore, this study determined the prevalence of carbapenemase genes, ISs, and plasmids in carbapenem-resistant A. baumannii (CRAB) isolates from patients in two hospitals in the Free State Province, South Africa, and elucidated the effect of ISs and plasmids on carbapenem resistance.*

Methods: *A total of 1697 CRAB isolates were analysed from patients in the two hospitals from January 2018 to September 2020. Isolates (n = 162/1697) were screened for carbapenemase genes, ISs, and plasmids using the BD MAX Check-Points CPO Assay and a multiplex PCR. Results were compared to the antibiotic susceptibility profiles of the original isolates. Some isolates (n = 30) were Sanger sequenced for gene confirmation and to determine the location of the ISs to the carbapenemase genes and on plasmids.*

Results: *Most isolates from Universitas (90.5%) and Pelonomi (84.9%) were carbapenem-resistant. The bla$_{OXA-23}$ was the most prevalent gene; ISAba1 was present in 144 bla$_{OXA-23}$-positive isolates, and ISAba2 was detected in three of these isolates. The bla$_{NDM}$ was the second most prevalent gene; 42 bla$_{NDM}$-containing isolates had ISAba125. All but one ISAba125-positive isolate was carbapenem-resistant. Nine isolates with only the intrinsic bla$_{OXA-51-like}$ co-harboured different combinations of the ISs. Most isolates co-harbouring ISAba1 and bla$_{OXA-23}$ or only with bla$_{OXA-51-like}$ were carbapenem-resistant. ISAba2 or ISAba3 did not increase carbapenem resistance. ISAba1 was upstream of bla$_{OXA-23}$ in total- and plasmid DNA and upstream of bla$_{OXA-51-like}$ in total DNA, where they may have assisted in inducing resistance. Forty-two of the plasmid-containing isolates were carbapenem-resistant.*

Conclusion: *The co-existence of ISs and carbapenemase genes upstream of bla$_{OXA-51-like}$, bla$_{OXA-23}$, or bla$_{NDM}$ on plasmids may contribute to carbapenem resistance. Elucidating the effect of ISs on carbapenem resistance can lead to the development of a novel therapeutic agent for CRAB infections.*

Keywords: *Acinetobacter baumannii, carbapenem resistance, nosocomial infection, carbapenemase genes, insertion sequences, plasmids, transposons*

1 INTRODUCTION

Hospitalised patients can acquire carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections. Imipenem, meropenem, and doripenem are combined with colistin or tigecycline to serve as last-resort antibiotics to treat CRAB. However, carbapenem resistance is increasing worldwide (Vivo et al., 2022). In a 2015 Philippines cohort, carbapenem resistance increased from 27.2% in 2006 and 22.1% in 2010 to 54.1% in 2015 (Hsu et al., 2017). In 2021, 95% of 42 CRAB isolates were collected between 2017 and 2018 from four Khartoum, Sudan, hospitals and were multidrug-resistant (MDR) (Al-Hassan et al., 2021). In South Africa, 53–60% of patients with sepsis had CRAB infections in a Durban hospital, KwaZulu Natal province. In the Tshwane region, Gauteng province, meropenem-resistant *A. baumannii* increased by 24%, and imipenem-resistant *A. baumannii* increased by 30% from 2008 to 2014 in 95% of investigated isolates (Lowe et al., 2018; Nogbou et al., 2021). However, no published studies exist for the Free State province (FS).

Carbapenem resistance is caused by genes encoding carbapenemases and oxacillinases (OXA) from the Ambler class D and, less so, the metallo-β-lactamases of the Ambler class B. The class B genes include the beta-lactamase gene NDM (bla_{NDM}), encoding metallo-β-lactamases, and class D OXA. The most prominent genes in *A. baumannii* include bla_{OXA-23} (Peleg et al., 2008; Poirel et al., 2008; Boo and Crowley, 2009; Mendes et al., 2009; Gordon and Wareham, 2010; Poirel and Nordmann, 2015). The $bla_{OXA-51-like}$ is intrinsically present in *A. baumannii*, serving as a species identifier without contributing to multidrug resistance (Rafei et al., 2014). Moreover, insertion sequences (ISs) contribute to resistance by increasing or activating the expression of neighbouring genes (Villalón et al., 2013).

Insertion sequences can spread resistance genes because a composite transposon has a resistance gene with an IS on either side (Mahillon and Chandler, 1998), such as Tn*2006*, which has two inverted copies of IS*Aba*1 flanking bla_{OXA-23} (Nigro and Hall, 2016). *Acinetobacter baumannii* possesses over 30 ISs; however, only a few are characterised, including IS*Aba*1, IS*Aba*2, and IS*Aba*3 situated mainly near $bla_{OXA,}$ and IS*Aba*125 near bla_{NDM} (Pagano et al. 2016; Wright et al., 2017). These ISs often contribute to antimicrobial resistance by adding or creating a promoter upstream or close to a resistance gene like bla_{OXA-23}, bla_{NDM}, and even $bla_{OXA-51-like}$ (Wu et al., 2015). The IS*Aba*1 is necessary upstream of $bla_{OXA-51-like}$ and bla_{OXA-23} to confer carbapenem resistance (Turton et al., 2006; Nigro and Hall, 2016). An IS*Aba*2 can occur at the 5′ end of bla_{OXA-58} and bla_{OXA-23}, whereas IS*Aba*3 is near the 3′ end or upstream of bla_{OXA-58} (Poirel and Nordmann, 2006). Furthermore, IS*Aba*125 can bracket bla_{NDM}, thereby forming the Tn*125* transposon, which can aid in spreading and disseminating bla_{NDM}. IS*Aba*125, 1,087 bp long, provides the -35-hybrid promoter, thereby enabling the expression of bla_{NDM} (Poirel et al., 2011; Nordmann et al., 2016).

Carbapenemase genes can occasionally cause resistance without ISs, primarily when located on a plasmid, owing to the higher gene dosage of the higher copy number associated with plasmids. Although chromosomal, $bla_{OXA-51-like}$ and IS*Aba*1 can be transferred via plasmids (Chen et al., 2010). Plasmids pABTJ1 (Zhu et al., 2013) or pAZJ221 (Liu et al., 2015) are the sources of Tn*2009* dissemination; however, these plasmids were only observed in China. Transposons Tn*2006*, Tn*2008*, Tn*2009*, and *Aba*R4 were also present in conjugative plasmids and, therefore, helped facilitate the dissemination of bla_{OXA-23} (Nigro and Hall, 2016). In *A. baumannii*, bla_{NDM} is primarily carried in Tn*125* on plasmids belonging to the pNDM-BJ01-like family (Hu et al., 2012).

A study done in Pretoria, South Africa, in 2018 in two tertiary hospitals reported that 29% of isolates from one hospital and 42% from the other had the IS*Aba*1 element upstream of the $bla_{OXA-51-like}$ gene. However, no increased resistance was observed, and IS*Aba*1 was absent from the coding strand of bla_{OXA-23} and bla_{OXA-58} in all the isolates (Lowe et al., 2018). Furthermore, isolates with IS*Aba*1 and a novel IS, IS*Aba*10, inserted in IS*Aba*1 preceding bla_{OXA-23}, were more resistant than isolates with only an IS*Aba*1 upstream of bla_{OXA-23} (Lee et al., 2011). However, ISs in CRAB in the FS have not been investigated, and their location near the carbapenemase genes and on plasmids is unknown. Therefore, this study determined the prevalence of multidrug-resistant *A. baumannii* isolates from patients in two hospitals, as well as the carbapenemase genes present in these isolates. Furthermore, the presence, contribution, and location of the IS*Aba*1, IS*Aba*2, IS*Aba*3, and IS*Aba*125 elements on the chromosome or plasmids of CRAB strains were elucidated. This is the first study in South Africa to determine the presence and role of ISs on carbapenem resistance in *A. baumannii*.

2 MATERIALS AND METHODS

2.1Data extraction and analysis

Antibiotic susceptibility testing results for all *A. baumannii* isolates analysed by the laboratories of the two hospitals (Universitas and Pelonomi) from 01 January 2018 to 30 September 2020 were extracted from the laboratory TrakCare system, and Vitek result forms to obtain the antibiotic resistance profiles. Extracted data were pseudo-anonymised and cleaned using a Microsoft Excel spreadsheet behind a firewall on a secure computer to prevent duplication of samples and to determine the body sites where *A. baumannii* was collected in the hospital wards where patients stayed during hospitalisation. Personal information was omitted to maintain patients' anonymity. Informed consent was waived as this was a retrospective study that included routinely collected isolates.

2.2Study setting, isolate collection, and ethics statement

This retrospective study was conducted from January 2018 to June 2022 and approved by the Health Sciences Research Ethics Committee of the University of the Free State (approval number: UFS-HSD2019/0569/2805-0001). Routinely detected and identified *A. baumannii* isolates (n = 162) from January 2018 to September 2020 were collected from two FS hospitals and sent to the National Health Laboratory Service bacteriology laboratory at Universitas in Bloemfontein, FS province. Specimens included blood cultures, aspirates, tissue, and catheter tips. The strains were isolated by the routine laboratory technologists using MacConkey agar and identified using Gram staining, subculturing, and the automated Vitek 2 AutoMicrobic System (BioMerieux, Marcy-l'Étoile, France). The Vitek 2 AutoMicrobic System was used to test antibiotic susceptibility according to the manufacturer's instructions and the local laboratory standard operating procedures. All collected isolates were stored in duplicate; short-term isolates were stored in Tris-ethylenediaminetetraacetic acid buffer at -4°C, whereas isolates for long-term storage were inoculated in bacterial preservatives (Pro-lab Diagnostics, Ontario, Canada) at -80°C.

2.3Molecular carbapenemase gene detection and characterisation

DNA was extracted via heat lysis. Briefly, heat lysis of a loopful of bacterial culture was performed at 95°C in 100 µL of nuclease-free water in a microcentrifuge tube for 30 min. After heating, freezing at -80°C for 30 min followed. Centrifugation at 16 000 × *g* for 20 min allowed pelleting of cell debris. The supernatant that contained the DNA was removed and stored at -20°C until further use in PCR assays. Nanodrop readings (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) were performed for all extracted DNA for quantification and purity.

An in-house multiplex PCR (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA) was performed to detect $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-23}}$, and bla_{KPC} using MyTaq™ HS Mix (Bioline Reagents, London, UK). Cycling conditions started with denaturation at 95°C for 5 min, followed by 30 cycles that included denaturation at 95°C for 30 s, annealing at 56°C for 90 s, and elongation at 72°C for 90 s. Finally, an elongation step at 72°C for 10 min was performed. A singleplex PCR was performed to identify bla_{NDM}. Cycling conditions were as mentioned previously, except for an annealing temperature of 55°C. Primers used for the PCRs are listed in Table 1. National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) analysis was performed on all primers.

The BD MAX Check-Points CPO Assay was performed on the automated BD MAX System (Check-Points, Wageningen, The Netherlands) to detect bla_{KPC}, bla_{NDM}, bla_{VIM}/bla_{IMP}, and $bla_{\text{OXA-48}}$ per the manufacturer's protocol at the University of Johannesburg in isolates collected from both hospitals in 2019.

PCR products were separated using a 2% agarose gel (SeaKem® LE Agarose, Lonza, Bioscience, Walkersville, MD, USA) in a 1 × Tris-acetic acid-EDTA (TAE) buffer at 100 V for 60 min. Samples were loaded with GelRed™ Nucleic Acid Gel Stain, 10 000 × (GelRed® Nucleic Acid Gel Stain, Biotium, San Francisco Bay Area, CA, USA) in water to allow visualisation. All PCR results were analysed using the GelDoc system (GelDoc™ XR+ with ImageLab™ software, Bio-Rad). Since *A. baumannii* contains the intrinsic $bla_{\text{OXA-51-like}}$, the presence of a band representing this gene was used to confirm DNA and amplification.

2.4Insertion sequences IS*Aba*1, -2, -3, and -125 detection

Insertion sequences IS*Aba*2 and IS*Aba*3 were detected in $bla_{\text{OXA-23}}$- and $bla_{\text{OXA-51-like}}$-positive isolates using a multiplex PCR and the primers listed in Table 1. Cycling conditions were as described before, except for an annealing temperature of 54°C. A singleplex PCR was used to detect IS*Aba*1 in $bla_{\text{OXA-23}}$-containing isolates with an annealing temperature of 52°C. bla_{NDM}-positive isolates were subjected to a singleplex PCR to detect IS*Aba*125 using the primers listed in Table 1 under the conditions described before, except with an annealing temperature of 58°C. As previously described, all PCR products were separated in a 2% agarose SeaKem® LE (Lonza Bioscience) gel using 1 × TAE buffer at 100 V for 60 min.

2.5Plasmid analysis

Isolates with only one of the specific *bla* genes or ISs and different combinations of the genes and ISs were randomly selected for plasmid analysis. Plasmid DNA was extracted from 50 isolates collected from 2018 to 2020 using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Plasmid DNA was stored at -20°C until further use. The presence of plasmids was determined using 0.6% agarose gel (SeaKem® LE agarose, Lonza Bioscience) and electrophoresis, run at 100 V for 45–60 min (PowerPac Basic, Bio-Rad) with a supercoiled plasmid ladder (New England Biolabs, Ipswich, MA, USA) to

determine the plasmid size and visualised using GelRed staining (Biotium). A pGEM.HPV (Promega, Madison, WI, USA) plasmid of 3 050 bp was used as a control.

A single- or multiplex PCR was performed to determine whether the same genes and ISs in the total DNA of the isolate were on plasmids (T100™ Thermal Cycler, Bio-Rad). For example, if $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23}}$, and IS*Aba*1 were amplified in the PCR of total genomic DNA, the plasmid DNA underwent a PCR to detect if the same genes were present in a plasmid, establishing whether the genes and ISs are chromosomal or plasmid-located.

2.6Sanger sequencing of genes and ISs on the chromosome and plasmids

Thirty strains with a typical clean PCR band for the ISs and their respective genes were selected. PCR products were cleaned using a Wizard® PCR Clean-up protocol (Promega) and a sequencing PCR using the Big Dye Terminator V 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Sanger sequencing was performed to confirm the correct IS. Furthermore, IS*Aba*1, -2, and -3 combined with $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51-like}}$ and IS*Aba*125 combined with bla_{NDM} were sequenced. Sequencing was performed to confirm the presence of the gene and determine the location and orientation of the ISs on the plasmid or chromosome concerning the carbapenemase genes. The forward primer of the IS and the reverse of the gene were used to determine the location of the IS relative to the gene on the chromosome or plasmid using the primers in Table 1 (Figure 1). Analysis was performed using UniPro UGENE v.33 and NCBI BLAST.

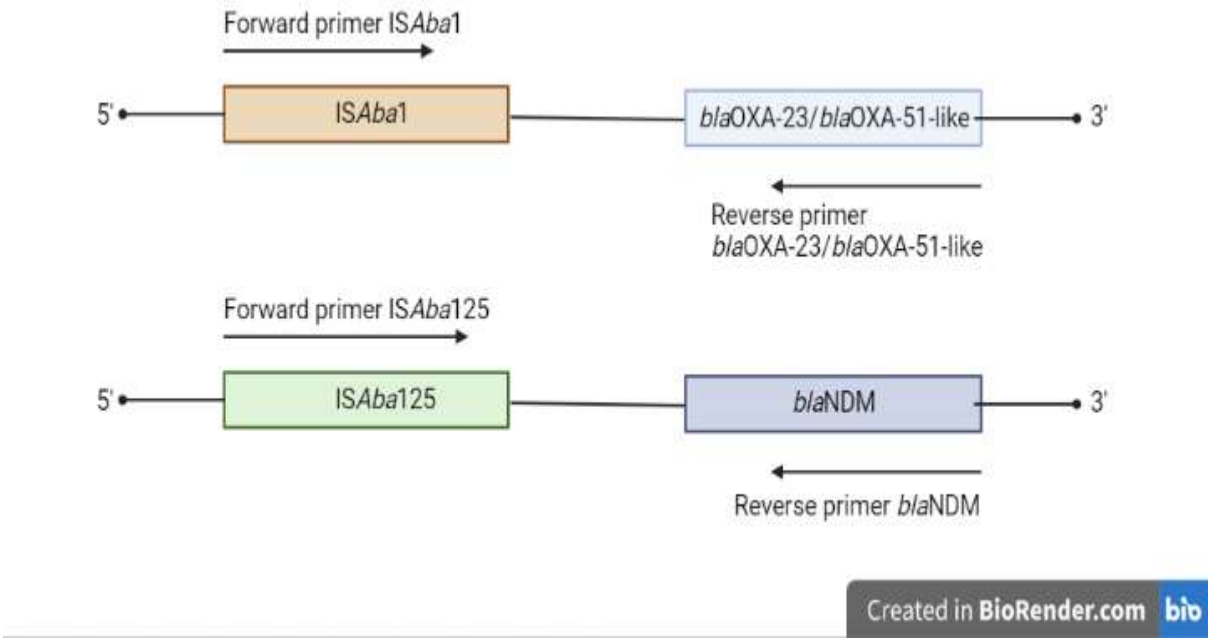

Figure 1. An example of the forward and reverse primers used during Sanger sequencing of the insertion sequence (IS)/gene element to determine the upstream or downstream location of the IS to the gene in plasmid or genomic DNA (Figure not drawn to scale).

2.7Sequencing data analysis

Each target was sequenced bidirectionally. First, sequence data were analysed with UniPro UGENE v.33 and the NCBI BLAST. Briefly, the forward and reverse read chromatograms were mapped to a reference strain obtained from GenBank. Next, the ends of the reads were trimmed until most of the noise was erased and only quality peaks were retained. After that, if gaps or mismatches were present in one of the strands, the reference- and other strands were used to correct errors in nucleotide-calling faults owing to ambiguous peaks. If the base call differed in the two strands, the one with the most substantial peak was used. The consensus was then exported and copied onto a text document. Finally, reference strains for the genes and ISs were compared to the consensus to determine if the correct gene and ISs were present and the location and orientation of the ISs.

3 RESULTS

3.1Data analysis

Data were extracted for 750 samples from patients in Universitas and 947 in Pelonomi from 01 January 2018 to 30 September 2020 (two years and nine months). Most *A. baumannii* isolates from Universitas (90.5%, n = 679/750) and Pelonomi (86.3%, n = 817/947) were carbapenem-resistant (Table 2).

Patient and isolate data are presented in Supplementary Table 1. Briefly, patients from Universitas were mainly male (51.9%, n = 389), 47.6% (n = 357) female, and 0.5% (n = 4) of unknown sex. From Pelonomi, 60.7% (n = 575) of patients were male, 39% (n = 369) were female, and the sex of 0.3% (n = 3) was unknown. Isolates were primarily collected from both hospitals' multidisciplinary- and neonatal intensive care units. A total of 17.5% (n = 131) and 15.9% (n = 119) of isolates from Universitas were obtained from the multidisciplinary- and neonatal intensive care units, respectively, and 20.7% (n = 196) and 19.3% (n = 183) of isolates from Pelonomi from these wards, respectively. Specimen types primarily included blood culture (Universitas: 27.1%; Pelonomi: 25.1%), tracheal aspirate (Universitas: 24.8%; Pelonomi: 16.5), and superficial swabs (Universitas: 4.9%; Pelonomi: 22.5%).

3.2The prevalence of carbapenemase genes in *A. baumannii* isolates

The $bla_{\text{OXA-23}}$ gene is the most prevalent carbapenemase gene in *A. baumannii* isolates. The intrinsic $bla_{\text{OXA-51-like}}$ gene was present in 100% (n = 162/162) of isolates, confirming species identity. Of the isolates, this gene was the only *bla* type in 5.6% (n = 9/162) of the genes tested. Most isolates contained the $bla_{\text{OXA-23}}$ gene (90.7%; n = 147/162), and bla_{NDM} was detected in 38.9% (n = 63/162) of isolates. The $bla_{\text{VIM/IMP}}$ and $bla_{\text{OXA-48}}$ genes were detected in one isolate each, and no $bla_{\text{OXA-58}}$ or bla_{VIM} were detected. Most isolates containing carbapenemase genes were carbapenem-resistant (Table 3).

3.3ISs associated with carbapenemase genes

Carbapenemase genes and ISs were co-harboured in most *A. baumannii* isolates. The intrinsic $bla_{\text{OXA-51-like}}$ was the only gene detected in nine isolates; these isolates co-harboured no ISs. All isolates with $bla_{\text{OXA-23}}$ (n = 147) had IS*Aba*1, as did a strain with $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-48}}$. Two $bla_{\text{OXA-23}}$-containing isolates with IS*Aba*1 had IS*Aba*125, and three $bla_{\text{OXA-23}}$-containing isolates had IS*Aba*2 and IS*Aba*125. Of the 57 bla_{NDM}- and $bla_{\text{OXA-23}}$-containing isolates, 22 (39%) had IS*Aba*1, and 35 (61%) had IS*Aba*1 and IS*Aba*125. An isolate with $bla_{\text{OXA-23}}$, bla_{NDM}, and $bla_{\text{VIM/IMP}}$ only had IS*Aba*1 (Table 4).

3.4The contribution of ISs to carbapenem resistance in *A. baumannii* isolates

The IS*Aba*1 element was detected in 157 isolates. Five of the six $bla_{\text{OXA-51-like}}$ and IS*Aba1*-positive isolates (83.3%) were imipenem- and meropenem-resistant. All 90 isolates that co-harboured $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23}}$, and IS*Aba*1 were carbapenem-resistant. Isolates that co-harboured bla_{NDM}, IS*Aba*1, and IS*Aba*125 were mostly carbapenem-resistant (96.7%, n = 59/61); one isolate had intermediate resistance and another one was susceptible. Isolates that only had IS*Aba*2 or IS*Aba*3 or without any ISs were carbapenem-susceptible (Table 5). These results indicate that IS*Aba*1 possibly contributes to carbapenem resistance in *A. baumannii* isolates when co-harboured with carbapenemase genes.

3.5Carbapenemase genes and ISs located on plasmids

The plasmid DNA of 50 isolates with singular- or different combinations of carbapenemase genes or ISs was extracted to determine whether they contained plasmids with carbapenemase genes and ISs. Of these, 47 isolates contained plasmids. The plasmids underwent PCR, and 62% (n = 29/47) of isolates had the same genes in the total DNA and plasmids, and 4% (n = 2/47) had a carbapenem-resistance gene or IS present in plasmid DNA that was not in the total genomic DNA. Furthermore, 32% (n = 15/47) of the isolates had carbapenem-resistance genes and ISs in the total DNA but not on plasmids, and 2% (n = 1/47) had an IS and carbapenemase gene both present and absent in plasmid DNA compared to within the total DNA. Two of the three isolates with different genes or ISs in the plasmid and total DNA had IS*Aba*2, and one had $bla_{\text{OXA-23}}$ in a plasmid. One of these, isolate P8, also had bla_{NDM} absent in the plasmid DNA (Supplementary Table X). Furthermore, genes and ISs were co-harboured on plasmids. Most isolates co-harboured $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23}}$, and IS_{Aba1} (43%; n = 20/47) on plasmids (Figure 2).

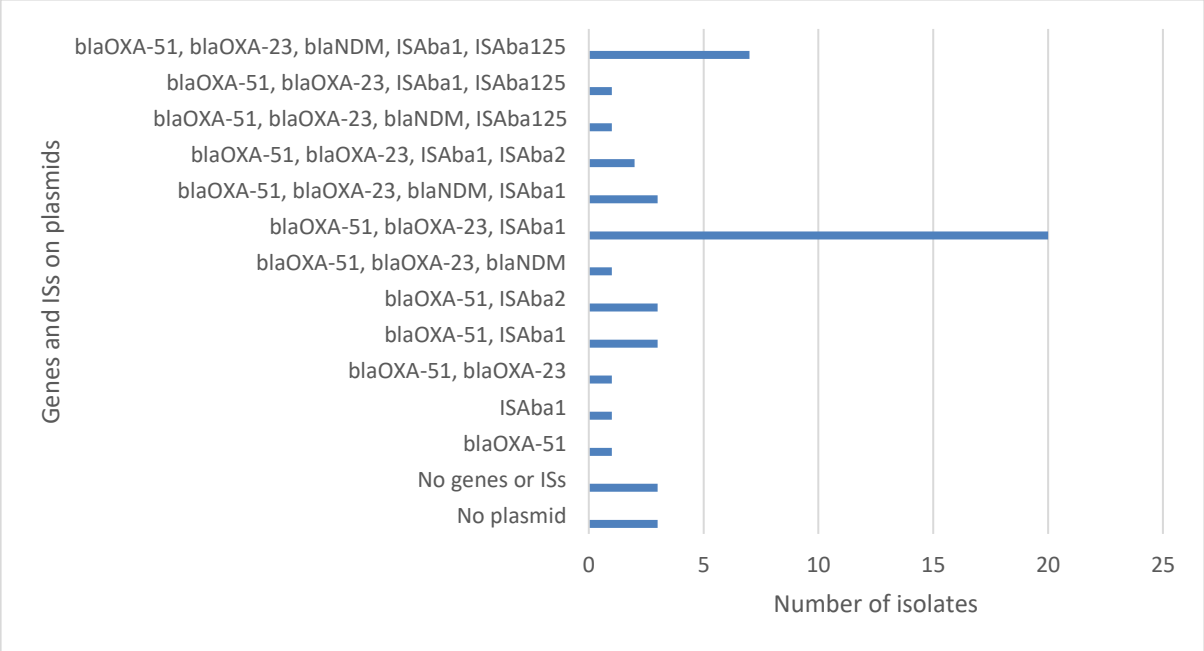

Figure 2. Genes (*bla*) and insertion sequences co-harboured in 50 *Acinetobacter baumannii* isolate plasmid DNA.

Of the isolates with *bla* genes and ISs only on the chromosome, bla_{NDM} was the most prevalent gene (n = 8), and $bla_{OXA\text{-}51\text{-}like}$, IS*Aba*1, and IS*Aba*125 were on the chromosome alone in four isolates. Other genes and ISs like $bla_{OXA\text{-}23}$, $bla_{VIM/IMP}$, $bla_{OXA\text{-}48}$, IS*Aba*2, and IS*Aba*3 were only on the chromosome in ≤ 2 isolates (Figure 3).

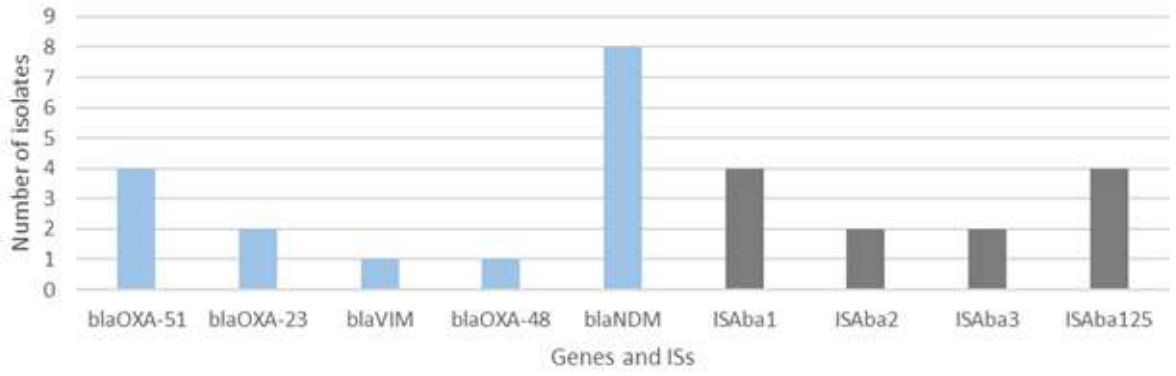

Figure 1. The number of *Acinetobacter baumannii* isolates with *bla* genes and insertion sequences absent in plasmid DNA and only present in total DNA.

3.6Susceptibility profiles of plasmid-containing isolates

Of the 50 isolates analysed, seven (14%) were susceptible to carbapenems, one (2%) had intermediate resistance, and 42 (84%) were carbapenem-resistant. No plasmids were present in two of the susceptible isolates. In two of the remaining susceptible isolates, IS*Aba*2 was present in plasmid DNA but absent from genomic DNA. In three susceptible isolates, genes and ISs were in the genomic DNA and absent from the plasmid DNA. The *bla* genes and ISs in the resistant and intermediate isolates are presented in (Supplementary Table X2).

3.7The location of IS*Aba*1 upstream of $bla_{OXA\text{-}23}$ in plasmid and total genomic DNA

Two isolates per IS with typical clear bands were selected for Sanger sequencing. Using the forward primer of IS*Aba*1 and the reverse of the OXA-gene, no isolates with IS*Aba*1/$bla_{OXA\text{-}51\text{-}like}$, IS*Aba*2 or IS*Aba*3/$bla_{OXA\text{-}23}$ or $bla_{OXA\text{-}51\text{-}like}$ were successfully sequenced from the genomic DNA. However, IS*Aba*1 was located upstream of $bla_{OXA\text{-}23}$ in

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the 5′ to 3′ direction in isolate U10 from Universitas. The correct ISs were confirmed after analysis through UGene and NCBI BLAST (Figure 4).

5′-
CTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGNAAATTTGA
CGATAATCAAAATACTGACCTGCNAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAA
GCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACA
AGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAG
ATATAACTCATTGAGATGTGTCATAGTATTCGTCGTTAGAAAACAATTATTATGACATTATTTCAA
TGAGTTATCTATTTTTGTCGTGTACAGAGCTCTTTT**TTATTTTCTATTGATCTGGTGTTTAAAATG**
AATAAATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTTCAGCATAA
TTTAATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATTCATCAATACTTT
GATGAAAAAAACACCTCAGGTGTGCTGGTTATTCAAACAGATAAAAAAATTAATCTATATGGTA
ATGCTCTAAGCCGCGCAAATACAGAATATGTGCCAGCCTCTACATTTAAAATGTTGAATGCCCT
GATCGGATTGGAGAACCAGAAAACGGATATTAATGAAATATTTAAATGGAAGGGCGAGAAAAG
GTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGCCATGAAGCTTTCTGCAGTCCCA
GTCTATCAGGAACTTGCGCGACGTATCGGTCTTGATCTCATGCAAAAAGAAGTAAAACGTATTG
GTTTTAAAAAACCGGTAATGCTGAAATTGGACAGCAGGTTGATA**-3′**

Figure 4. Sanger sequence of IS*Aba*1 (blue) located upstream of *bla*$_{OXA-23}$ (pink) in the 5′ to 3′ direction in genomic DNA from isolate U10, referenced against *Acinetobacter baumannii* strain CGAB09 from GenBank (GenBank accession nr EU604835.1). The red nucleotides indicate where the sequence of IS*Aba*1 differed in isolate U10 from the reference *A. baumannii* strain CGAB09.

Plasmid DNA from isolate P23 was sequenced for IS*Aba*1 and *bla*$_{OXA-23}$. The forward and reverse strands were mapped to *A. baumannii* reference strain SCM52/08 (GenBank accession no FJ628170.1). Insertion sequence *Aba*1 was located upstream of *bla*$_{OXA-23}$ in the 5′ to 3′ direction. A gap of 356 bp was observed between IS*Aba*1 and *bla*$_{OXA-23}$ from positions 535 to 891 (Figure 5).

5′--------------------------------
TCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCGTTGAATCA
AAGCAATACGCTCTTTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGGATAGGTAT
CGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTTTTCCCCATTTCCAAT
TGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAACTGAGAAAT
TTGACGATAATCAAAATACTGACCTGCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGT
AAGCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCA
CAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGAT
AAGATATAACTCATTGAGATGTGTCATAGTA--
--

ATCGGATTGGAGAACCAGAAAACGGATATTAATGAAATATTTAAATGGAAGGGCGAGAAAAGG
TCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGCCATGAAGCTTTCTGCAGTCCCAG
TCTATCAGGAACTTGCGCGACGTATCGGTCTTGATCTCATGCAAAAAGAAGTAAAACGTATTGG
TTTCGGTAATGCTGAAATTGGACAGCAGGTTGATAATTTCTGGTTGGTAGGACCATTAAAGGTT
ACGCCTATTCAAGAGGTAGAGTTTGTTTCCCAATTAGCACATACACAGCTTCCATTTAGTGAAA
AAGTGCAGGCTAATGTAAAAAATATGCTTCTTTTAGAAGAGAGTAATGGCTACAAAATTTTTGG
AAAGACTGGTTGGGCAATGGATATAAAACCACAAGTGGGCTGGTTGACCG------ -3′

Figure 5. Sanger sequencing results of plasmid DNA of isolate P23 sequenced with the forward primer of IS*Aba*1 and reverse primer of *bla*$_{OXA-23}$. IS*Aba*1 (blue) was located upstream of bla$_{OXA-23}$ (pink) in the 5′ to 3′ direction.

IS*Aba*1 was upstream of *bla*$_{OXA-51-like}$ and *bla*$_{OXA-23}$ in plasmid DNA. Two strains were sequenced to determine the location and orientation of IS*Aba*1 to *bla*$_{OXA-51-like}$ and *bla*$_{OXA-23}$ using the forward primer of the IS and the reverse

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of the respective gene. Isolate U24 from Universitas was sequenced for IS*Aba*1/*bla*$_{OXA-51-like}$. The forward and reverse strands were mapped to *A. baumannii* reference strain AbSK-17 plasmid pAbSK-OXA-82 (GenBank accession nr GQ352402.1). The IS*Aba*1 element was located upstream of *bla*$_{OXA-51-like}$ in the 5′ to 3′ direction. A gap of 331 bp was observed between IS*Aba*1 and *bla*$_{OXA-51-like}$ from position 8,697 to 9,028 (Figure 6).

5′- --

**CGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAAATGGCTATAAAGCG
TTGAATCAAAGCAATACGCTCTTTCGTATCTGAATTTCCACGTTTATTAAGCAATGTCCAAAGG
ATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTTCGTTTTCCCC
ATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAACATATTGAAAATCAA
CTGAGAAATTTGACGATAATCAAAATACTGACCTGCAAAGAAGCGCTGCATACGTCGATAAAAT
GATTGTGGTAAGCACTTGATGGGCAAGGCTTTAGATGCAGAAGAAAGATTACATGTTTGCTTTA
AAATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACTTGTTCCATTTTAGATATTT
GTTTAAGATAAGATATAACTCATTGAGATGTGTCATA**------------------------------

**TTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAAGTATTTAAGTGGGATGG
TAAAAAAAGGTTATTCCCAGAATGGGAAAAGGACATGACCCTAGGCGATGCCATGAAAGCTTC
CGCTATTCCAGTTTATCAAGATTTAGCTCGTCGTATTGGACTTGAGCTCATGTCTAAGGAAGTG
AAGCGTGTTGGTTATGGCAATGCAGATATCGGTACCCAAGTCGATAATTTTTGGGTGGTGGGT
CCTTTAAAAATTACTCCTCAGCAAGAGGCACAGTTTGCTTACAAGC**------------------------------

-- **-3′**

Figure 6. Sanger sequencing results of plasmid DNA of isolate U24 sequenced with the forward primer of IS*Aba*1 and reverse primer of *bla*$_{OXA-51-like}$. IS*Aba*1 (blue) was located upstream of *bla*$_{OXA-51-like}$ (green) in the 5′ to 3′ direction. The red nucleotide differed in the sequencing results from the reference strain *Acinetobacter baumannii* AbSK-17 (GenBank accession nr GQ352402.1.).

IS*Aba*125/*bla*$_{NDM}$ sequencing was unsuccessful. However, IS*Aba*125 and *bla*$_{NDM}$ were successfully sequenced separately. The sequences of IS*Aba*125 and *bla*$_{NDM}$ were searched for with an *A. baumannii* reference strain on GenBank (accession nr LC032101.1). Two copies of IS*Aba*125 bracketed *bla*$_{NDM}$ in the 5′ to 3′ direction, with the first copy of IS*Aba*125 445 bp upstream of *bla*$_{NDM}$ and the second 8,042 bp downstream of *bla*$_{NDM}$.

4 Discussion

This study confirmed global and local reports that multidrug-resistant *A. baumannii* is becoming more resistant to carbapenems, which ISs exacerbate. Most study isolates were carbapenem-resistant. Furthermore, isolates were primarily from multidisciplinary and neonatal intensive care unit patients. These patients are most vulnerable to infection and prolonged hospital stay, a considerable risk factor for contracting multidrug or carbapenemase-resistant *A. baumannii* (Montefour et al., 2008).

This study detected several carbapenemase genes in *A. baumannii* isolates from the two hospitals from 2018–2020. The class D bla_{OXA-23} gene was the most prevalent in CRAB isolates, in agreement with global and South African reports (Wang et al., 2007; Liu et al., 2015; Anane et al., 2020; Ishtiaq et al., 2021; Hassan et al., 2021; Nogbou et al., 2021). bla_{OXA-23} is often carried on plasmids, which can aid in the dissemination of this gene (Mugnier et al., 2010). The high genetic plasticity of *A. baumannii* allows for the accumulation of resistance determinants and could explain the high prevalence of bla_{OXA-23} in isolates from Universitas and Pelonomi. Furthermore, four transposons facilitate the spread of bla_{OXA-23} when associated with IS*Aba*1: Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009*, with Tn*2006* being the most prominent (Nigro and Hall, 2016). The presence of Tn*2006* on a plasmid can contribute to the high prevalence of bla_{OXA-23} in the Universitas and Pelonomi hospitals. Furthermore, bla_{NDM}, less prevalent but with a more potent carbapenemase activity than the OXA genes (Pagano et al., 2016), was detected in 38.9% of the isolates in these FS hospitals. Contrastingly, this gene is less reported globally and in other South African provinces (Lowings et al., 2015; Chatterjee et al., 2016; Santimaleeworagun et al., 2016; Agoba et al., 2018; Hassan et al., 2021). The difference in the prevalence of bla_{NDM} between the provinces could be due to different *A. baumannii* strains or the dissemination of the gene via transposons. These results support the consensus that class B metallo-β-lactamases are less reported than class D oxacillinases.

At least one OXA/NDM gene and its associated ISs are needed to cause carbapenemase resistance in *A. baumannii* (Villalón et al., 2013). ISs carry a promotor within them, and acquisition near carbapenemase-encoding genes causes increased gene expression (Mahillon and Chandler, 1998; Turton et al., 2006; Wu et al., 2015). Four ISs were investigated in this study—IS*Aba*1, IS*Aba*2, IS*Aba*3, and IS*Aba*125—following reports of expected association with the detected carbapenemase genes (Corvec et al., 2007; Bogaerts et al., 2008; Mugnier et al., 2009; Villalón et al., 2013; Pagano et al., 2016). All four ISs were present in FS isolates, two of which appear to influence carbapenem resistance. Except for one isolate, isolates with IS*Aba*1 and IS*Aba*125 were resistant to carbapenems. IS*Aba*1 was present in all bla_{OXA-23}-harbouring isolates, either as the only IS or in different combinations with IS*Aba*2, IS*Aba*3, and IS*Aba*125. Another isolate with only bla_{NDM} was carbapenem susceptible, suggesting that IS*Aba*1 associated with bla_{OXA-23} and IS*Aba*125 near bla_{NDM} could render isolates carbapenem-resistant (Turton et al., 2006; Pagano et al., 2016). In this study, IS*Aba*1 was upstream of the bla_{OXA-23} gene in the total genomic and plasmid DNA of two carbapenem-resistant isolates, possibly enhancing carbapenem resistance by adding a promoter. Similarly, Anane et al. (2020) indicated that IS*Aba*1/$bla_{OXA-51-like}$ and IS*Aba*1/bla_{OXA-23} significantly influenced the carbapenem minimum inhibitory concentrations (MICs) in *A. baumannii* isolates. The carbapenem-susceptible isolate with IS*Aba*1/bla_{OXA-23} was not sequenced, and the reason for the susceptibility is unclear, requiring further investigation.

The intrinsic $bla_{OXA-51-like}$ generally does not contribute to carbapenem resistance (Rafei et al., 2014). However, previous studies have indicated that IS*Aba*1 can be upstream of the $bla_{OXA-51-like}$ gene in carbapenem-resistant and susceptible isolates; however, IS*Aba*1 upstream of $bla_{OXA-51-like}$ was insufficient to cause carbapenem resistance in *A. baumannii* isolates (Bratu et al., 2008; Pagano et al., 2013). In contrast, our study indicated that isolates co-harbouring IS*Aba*1 with $bla_{OXA-51-like}$ were carbapenem-resistant, concurring with the findings of Turton et al. (2006) who also reported that IS*Aba*1 overexpressed $bla_{OXA-51-like}$, thereby causing carbapenem resistance when 7 bp upstream of $bla_{OXA-51-like}$ in the 5′ to 3′ direction. However, in our study, IS*Aba*1 was 331 bp upstream of $bla_{OXA-51-like}$ in a plasmid, which could provide a higher copy number of the gene, leading to resistance. Sequencing of IS*Aba*1/$bla_{OXA-51-like}$ in total genomic DNA was unsuccessful, which could be due to the IS being too far from the gene to detect using Sanger sequencing or mismatches, resulting in unsuccessful primer binding.

The bla_{NDM} is a chimeric combination of aminoglycoside phosphotransferase fusion and a pre-existing metallo-β-lactamase gene. The promoter of IS*Aba*125 is upstream of bla_{NDM} and possibly drives the overexpression of this gene (Toleman et al., 2012). This study is the first report in SA of the presence of IS*Aba*125 in nosocomial CRAB isolates with the bla_{NDM}. IS*Aba*125 was detected in most bla_{NDM}-positive isolates and co-harboured with the IS*Aba*1 element, consistent with previous reports (Mussi et al., 2005; Khatun et al., 2015). Of the isolates that contained IS*Aba*1 and IS*Aba*125, 94.9% were carbapenem-resistant. However, when combining the forward primer of IS*Aba*125 and the reverse primer of bla_{NDM}, only the forward primer was sequenced during Sanger sequencing, which could be due to orientation differences between the gene and IS. The sequence of IS*Aba*125 was then compared to the reference strain with GenBank accession nr LC032101.1 and two copies of IS*Aba*125 bracketed

bla$_{NDM}$ in the 5′ to 3′ direction. Both copies of IS*Aba*125 had the same orientation. A difference of 445 bp was observed between the upstream copy of *bla*$_{NDM}$ and 8,042 bp between the *bla*$_{NDM}$ and the downstream copy, possibly forming the *Tn*125 transposon. However, Mishra et al. (2013) indicated that the *bla*$_{NDM}$ could also be linked to IS*CR1* and IS*CR16*, which could then aid in the mobilisation of the gene, and not only IS*Aba*125. Resolving these findings will need further investigation. Furthermore, since IS*Aba*1 and *bla*$_{OXA-23}$ were also present in these resistant isolates, it cannot be deduced with certainty that IS*Aba*125 causes resistance when associated with *bla*$_{NDM}$ and not IS*Aba*1 influencing *bla*$_{OXA-23}$. Furthermore, carbapenemase genes and ISs are not the only cause of carbapenem resistance. Other factors, such as efflux pumps and defects in permeability, could also have influenced resistance, not only IS*Aba*1 or IS*Aba*125. In addition, IS*Aba*1 and IS*Aba*125 can also disrupt the *CarO* gene (Mussi et al., 2005; Poirel and Nordmann, 2006). Therefore, ISs in the current study could cause resistance by interrupting the *CarO* gene and outer membrane proteins pump expression (Mussi et al., 2005; Bedenić and Sardelić, 2016).

Insertion sequences *Aba*2 and IS*Aba*3 are primarily associated with *bla*$_{OXA-58}$ and *bla*$_{OXA-23}$ (Corvec et al., 2007; Villalón et al., 2013; Pagano et al., 2016). In this study, seven isolates had IS*Aba*2, of which three had *bla*$_{OXA-23}$. None of the isolates had *bla*$_{OXA-58}$. Three of the IS*Aba2*-positive isolates were carbapenem-susceptible, and four were resistant. However, all four resistant isolates also had IS*Aba*1 and IS*Aba*125, possibly contributing to the resistant phenotype instead of IS*Aba*2. The three carbapenem-susceptible isolates did not have *bla*$_{OXA-23}$ nor *bla*$_{OXA-58}$; therefore, this IS potentially cannot overexpress these genes to confer resistance. These findings imply that IS*Aba*2 and IS*Aba*3 may not play principal roles in carbapenem resistance like IS*Aba*1 and IS*Aba*125.

One of the primary mechanisms for disseminating resistance genes and ISs between different *A. baumannii strains* and other pathogenic bacteria can be attributed to plasmids through conjugation (Partridge et al., 2018). In this study, 47 of the 50 isolates harboured plasmids of ~8.5 kb, concurring with studies that reported different combinations of genes and ISs in plasmids of ~8.5 kb (Mishra et al., 2013; Liu et al., 2014). Therefore, the plasmids in this study are possibly similar to pAB0057. However, pAB0057 does not harbour any resistance genes (Liu et al., 2014), whereas all the plasmids in this study had carbapenemase genes and ISs. Saranathan et al. (2014) investigated the presence of plasmids in 55 *A. baumannii* isolates, and 95% of isolates contained at least one plasmid. They reported plasmids of 0.5 kb to >25 kb in size and concluded that antimicrobial resistance could be mediated through multiple plasmids in *A. baumannii* (Saranathan et al., 2014). However, the isolates in the current study were not typed. Therefore, more studies are needed to determine which plasmids are present in *A. baumannii* isolates from these two hospitals and in South Africa.

In this study, the same carbapenemase genes and ISs were present on plasmids and chromosomes in 62% of isolates, suggesting carriage of these genes and ISs between the chromosome and plasmids. In contrast, 32% of isolates had genes and ISs absent in the plasmid DNA, implying that these elements are only chromosomal. Interestingly, *bla*$_{OXA-23}$ and IS*Aba*2 were observed in a plasmid in three isolates but not in the total genomic DNA. The plasmids could have been fragmented during crude DNA extraction; therefore, the total DNA PCR did not detect this gene and IS. In the same isolate, IS*Aba*2 was only found in the plasmid DNA. The PCR of the total DNA may have missed the IS*Aba*2, or it has yet to transposase.

In this study, the *bla*$_{OXA-51-like}$, *bla*$_{OXA-23}$, and IS*Aba*1 were the most commonly plasmid-mediated elements, in agreement with previous studies (Salgado-Camargo et al., 2020; Saranathan et al., 2014). Similarly, the *bla*$_{OXA}$-type genes were the most prevalent carbapenemase type on plasmids in a recent study (Lam and Hamidian, 2024). However, Douraghi et al. (2020) reported no plasmids with *bla*$_{OXA-23}$, whereas in this study, 95.6% of plasmid-harbouring isolates had *bla*$_{OXA-23}$. The presence of *bla*$_{OXA-51-like}$ on a plasmid may be problematic. The *bla*$_{OXA-51-like}$ was originally intrinsic to *A. baumannii*; however, *bla*$_{OXA-51-like}$ with the IS*Aba*1 upstream of this gene was transferred from *A. baumannii* to *Acinetobacter nosocomialis* and one clone of *Acinetobacter* genomic species "Close to 13TU", conferring resistance to these strains (Lee et al., 2012). IS*Aba*1 can be inserted into plasmids via transposons such as Tn*2006*, Tn*2008*, Tn*2009*, and AbaR4 (Lee et al., 2012; Liu et al., 2015; Hamidian and Nigro, 2019; Fedrigo et al., 2022), which explains the high prevalence of *bla*$_{OXA-23}$ and *ISAba*1 on plasmids in the investigated isolates. The *repAci6* plasmid in the Hamidian et al. (2014) study was responsible for *bla*$_{OXA-23}$ dissemination in global clones 1 and 2 strains. Furthermore, Tn*2006* was reported in a *repAci1* plasmid, suggesting that this transposon

was mobilised by *repAci6* (Blackwell and Hall, 2019). However, transposons were not investigated in the current study and should be included in future research.

During the current study, plasmid analysis was performed on 21 bla_{NDM}-harbouring isolates, of which twelve had bla_{NDM} on a plasmid, while ten also harboured IS*Aba*125. This finding is consistent with a recent study, which reported that bla_{NDM} was plasmid-mediated; however, the study did not screen for IS*Aba*125 (Lam and Hamidian, 2024). In contrast, Mishra et al. (2013) indicated that bla_{NDM} was chromosomally mediated and not transferable by plasmids. The authors also speculated that bla_{NDM} could only undergo horizontal transfer from *Acinetobacter* to *Enterobacteriales* through IS*Aba*125. In this study, IS*Aba*1 and IS*Aba*125 were observed in plasmid DNA; however, except for four isolates with IS*Aba*1 and IS*Aba*125, respectively, presumptively only the chromosome, all other positive isolates had these ISs in total and plasmid DNA. Therefore, whether IS*Aba*1 and IS*Aba*125 are only located on a plasmid in these isolates is unclear. IS*Aba*3 was not observed on any plasmids, and IS*Aba*2 was present in only three isolates with plasmids; therefore, IS*Aba*2 and IS*Aba*3 are likely not plasmid-mediated.

Insertion sequences contribute a promoter upstream of carbapenemes genes, which regulates the overexpression of the resistance gene when plasmid-mediated (Villalón et al., 2013). In this study, IS*Aba*1 was upstream of $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}23}$ on a plasmid of a carbapenem-resistant *A. baumannii* isolates. Similarly, Saranathan et al. (2014) sequenced IS*Aba*1 with the OXA genes, confirming the presence and location of the IS upstream of the carbapenemase genes. In 117 *A. baumannii* isolates collected from ten hospitals in Taiwan, 49.6% carried IS*Aba*1/$bla_{OXA\text{-}51\text{-}like}$ on a plasmid. Four isolates had an additional copy of IS*Aba*1/$bla_{OXA\text{-}51\text{-}like}$ on the chromosome. After analysing these four isolates, the authors speculated that the plasmid-located copy of IS*Aba*1/$bla_{OXA\text{-}51\text{-}like}$ was acquired via one-ended transposition facilitated by Tn*6080*. Furthermore, isolates with $bla_{OXA\text{-}51\text{-}like}$ on the plasmids had higher MICs to carbapenems than isolates with $bla_{OXA\text{-}51\text{-}like}$ only on the chromosome (Saranathan et al., 2014). The higher MIC could be due to the higher copy number of the plasmids providing increased gene dosage. Notably, the proximity of resistance genes to ISs does not necessarily mean that the IS is responsible for disseminating the genes. For example, bla_{KPC} is typically surrounded by ISKpn6 and IKSpn7, and these ISs are embedded in a Tn*4401* transposon, which is the cause of the transposition events (Cuzon et al., 2011). However, transposons were not investigated in the current study.

Most *A. baumannii* isolates containing plasmids in this study were carbapenem-resistant. Tn*2006* carries an AbaR4 resistance island in a *repAci6* plasmid, transferring imipenem, meropenem, and ticarcillin/clavulanate resistance into a susceptible recipient. However, this plasmid had a size of 86.3 kb, whereas the plasmids found in this study were only ~8.5 kb. The same authors also reported two small cryptic plasmids of 2.7 and 8.7 kb (Hamidian et al., 2014), which could align with the results of the current study. The most frequently carried genes on plasmids are responsible for aminoglycoside resistance (60.6%), and the second most frequent are β-lactam genes (Salgado-Camargo et al., 2020), similar to our findings. The acquisition of bla_{NDM} via plasmids increases imipenem resistance in *A. baumannii* isolates (Abouelfetouh et al., 2020). Two of the carbapenem-susceptible isolates in this study did not harbour any plasmids, and the lack of plasmids in these isolates could explain the susceptibility. Apart from $bla_{OXA\text{-}51\text{-}like}$, IS*Aba*2 was the only element in the two isolates. IS*Aba*2 and IS*Aba*3 are primarily associated with $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}23}$ (Corvec et al., 2007; Villalón et al., 2013; Pagano et al., 2016), and the absence of $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}23}$ in these isolates may indicate that IS*Aba*2 does not overexpress these genes to cause resistance. Although IS*Aba*1 and IS*Aba*125 are suspected of contributing to carbapenem resistance (Lopes and Amyes, 2012), these two ISs were on the chromosome of two carbapenem-susceptible isolates in this study. Therefore, the susceptibility in our study could be due to these ISs being inverted or downstream of $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}51\text{-}like}$, and bla_{NDM}, thereby not providing the gene with an upstream promoter. The isolate with intermediate carbapenem resistance lacked bla_{NDM} and IS*Aba*125 in the plasmid DNA; however, these elements were present in the total DNA, and IS*Aba*125 upregulating bla_{NDM} could lead to intermediate resistance.

The current study had some limitations, including a small sample size and a different number of isolates collected from each hospital each year. Therefore, the increased prevalence of the resistance genes from 2018 to 2020 could not be determined. Furthermore, isolates were only collected from two hospitals in the FS and may not accurately represent the genes and ISs present in the province or South Africa. IS*Aba*125/bla_{NDM} sequencing was unsuccessful in the plasmid DNA, and total DNA and IS*Aba*1/$bla_{OXA\text{-}51\text{-}like}$ in total genomic DNA failed.

Furthermore, whether IS*Aba*1 or IS*Aba*125 is the main contributor to resistance when associated with their respective carbapenemase genes must be investigated. *Acinetobacter baumannii* has many antimicrobial resistance mechanisms, and it cannot be concluded that these mechanisms do not also contribute to the carbapenem resistance observed in these isolates. Lastly, statistical analysis was not performed to determine whether the genes and ISs causing carbapenem resistance are significant.

5 CONCLUSION

This is the first study to report the prevalence of CRAB and carbapenemase genes in two FS hospitals and the first to elucidate that IS*Aba*125 may contribute to carbapenem resistance when upstream of *bla*NDM in *A. baumannii* isolates in South Africa. Carbapenem resistance in *A. baumannii* is of growing concern, as these antibiotics are currently used as a last-resort treatment for *A. baumannii* infections. Urgent attention to antibiotic stewardship is needed to prevent the spread of these resistant *A. baumannii* isolates in South Africa, specifically in the Free State. IS*Aba*1, and possibly IS*Aba*125, could be the leading ISs causing resistance to carbapenems in *A. baumannii* isolates from Universitas and Pelonomi. Resistance genes and ISs were also chromosomal and plasmid-mediated in *A. baumannii* isolates. Furthermore, resistance genes with upstream ISs in the chromosome and plasmids possibly enhance gene expression and, thus, carbapenem resistance. A possible therapeutic target for these ISs can be presented with this knowledge, leading to the development of a novel antimicrobial to combat *A. baumannii* infections. Future studies should include a larger sample group from more hospitals nationwide to investigate IS*Aba*1 and IS*Aba*125 as the primary contributors to carbapenem resistance through knockout studies and next-generation sequencing. Furthermore, whether these ISs could be possible targets for antimicrobial agents needs investigation, and whether other resistance mechanisms are associated with ISs and contribute to their resistance activity must also be elucidated.

6 Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could potentially create a conflict of interest.

7 Author Contributions

NvH and AvdSvD created the study concept and design. NvH performed data collection and analysis. NvH drafted the first manuscript. AvdSvD reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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12 Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Figure legends

Tables

Table 1. Primers for detecting carbapenemase genes and insertion sequences in *Acinetobacter baumannii*

Target	Primer name	Primer sequence 5′ to 3′	Amplicon size (bp)	Annealing temperature (°C)	Reference
IS*Aba1*	IS*Aba*1-F	CACGAATGCAGAAGTTG	549	52	Turton et al., 2006
	IS*Aba*1-R	CGACGAATACTATGACAC			
IS*Aba*2	IS*Aba*2-F	AATCCGAGATAGAGCGGTTC	1100	56	Poirel and Nordmann, 2006
	IS*Aba*2-R	TGACACATAACCTAGTGCAC			
IS*Aba*3	IS*Aba*3-F	CAATCAAATGTCCAACCTGC	403	55	Poirel and Nordmann, 2006
	IS*Aba*3-R	CGTTTACCCCAAACATAAGC			
IS*Aba*125	IS*Aba*125-F	TGTATATTTCTGTGACCCA	255	58	Nordmann et al., 2016
	IS*Aba*125-R	GAAGGCGAATTCAAACATGAGGTGC			
$bla_{\text{OXA-23}}$	$bla_{\text{OXA-23}}$-F	GATCGGATTGGAGAACCAGA	501	56	Turton et al., 2006
	$bla_{\text{OXA-23}}$-R	ATTTCTGACCGCATTTCCAT			
$bla_{\text{OXA-48}}$	$bla_{\text{OXA-48}}$-F	GCGTGGTTAAGGATGAACAC	438	55	Zowawi et al., 2014
	$bla_{\text{OXA-48}}$-R	CATCAAGTTCAACCCAACCG			
$bla_{\text{OXA-51-like}}$	$bla_{\text{OXA-51-like}}$-F	TAATGCTTTGATCGGCCTTG	353	60	Turton et al., 2006
	$bla_{\text{OXA-51-like}}$-R	TGGATTGCACTTCATCTTGG			
bla_{KPC}	bla_{KPC}-F	ATCTGACAACAGGCATGACG	452	55	Zowawi et al., 2014
	bla_{KPC}-R	GACGGCCAACACAATAGGTG			
bla_{NDM}	bla_{NDM}-F	GCAGGTTGATCTCCTGCTTG	203	55	Zowawi et al., 2014
	bla_{NDM}-R	ACGGTTTGGCGATCTGGT			

Table 2. Carbapenem resistance profiles of *A. baumannii* isolates collected from two hospitals (Universitas and Pelonomi) in the Free State province, South Africa

Susceptibility profile	Universitas (%, n)	Pelonomi (%, n)
Resistant	90.5% (679/750)	86% (817/947)
Intermediate	0.4% (3/750)	0.3% (3/947)
Susceptible	8.1% (61/750)	13.1% (124/947)
No data available	0.9% (7/750)	0.6% (6/947)

Table 3. Carbapenemase genes identified in 162 *Acinetobacter baumannii* isolates and their carbapenem susceptibility profiles

		Susceptibility profiles		
Carbapenemase genes	No. of isolates (%)	R	I	S
None	9 (5.6)	5	0	4
$bla_{\text{OXA-23}}$	88 (54.3)	88	0	0
bla_{NDM}	5 (3.1)	3	0	2
$bla_{\text{OXA-48}}$	1 (0.6)	0	0	1
$bla_{\text{OXA-23}}$ and bla_{NDM}	57 (35.2)	56	1	0
$bla_{\text{OXA-23}}$ and $bla_{\text{OXA-48}}$	1 (0.6)	1	0	0
$bla_{\text{OXA-23}}$, bla_{NDM}, and $bla_{\text{VIM/IMP}}$	1 (0.6)	1	0	0
Total	162	154	1	7

R, resistant; I, intermediate resistance; S, susceptible

Table 4. Carbapenemase genes and insertion sequences (ISs) in 162 *Acinetobacter baumannii* isolates from two hospitals in the Free State province, South Africa.

Carbapenemase genes	No of isolates	IS*Aba*1	IS*Aba*1, -2	IS*Aba*1, -2, -3	IS*Aba*1, -125	IS*Aba*2	IS*Aba*2, -3	No ISs
No genes	9	4	1	1	N/A	1	1	1
$bla_{\text{OXA-23}}$	88	85	3	0	N/A	0	0	0
bla_{NDM}	5	0	0	0	4	N/A	N/A	1
$bla_{\text{OXA-48}}$	1	0	0	0	N/A	0	0	1
$bla_{\text{OXA-23}}$ and bla_{NDM}	57	19	0	0	38	N/A	N/A	0
$bla_{\text{OXA-23}}$ and $bla_{\text{OXA-48}}$	1	1	0	0	N/A	0	0	0
$bla_{\text{OXA-23}}$, bla_{NDM}, and $bla_{\text{VIM/IMP}}$	1	1	0	0	0	0	0	0
Total	162	110	4	1	42	1	1	3

N/A, not applicable – isolates were not screened for the particular IS as, according to the literature, it is not associated with the gene

Table 5. Carbapenemase genes, insertion sequences, and carbapenem resistance profiles of 162 *Acinetobacter baumannii* isolates.

Carbapenemase genes and insertion sequences	**Nr of isolates**	**Resistance profile**		
		R	**I**	**S**
$bla_{\text{OXA-51-like}}$, IS*Aba*1	6	5	0	1
$bla_{\text{OXA-23}}$, IS*Aba*1	90	90	0	0
bla_{NDM}, IS*Aba*1, IS*Aba*125	61	59	1	1
$bla_{\text{OXA-51-like}}$, IS*Aba*2, IS*Aba*3	2	0	0	2
No insertion sequences	3	0	0	3
Total	162	154	1	7

R, resistant; I, intermediate resistance; S, susceptible