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Draft genome sequences of extensively drug resistant and pandrug resistant *Acinetobacter baumannii* strains isolated from hospital wastewater in South Africa

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ABSTRACT

Objectives: *Acinetobacter baumannii* is a significant opportunistic pathogen causing nosocomial infections. Infections caused by *A. baumannii* are often difficult to treat because this bacterium is often multidrug-resistant and shows high environmental adaptability. Here, we report on the analysis of three *A. baumannii* strains isolated from hospital effluents in South Africa.

Methods: Strains were isolated on Leeds *Acinetobacter* agar and were identified using VITEK®2 platform. Antibiotic susceptibility testing was performed using the Kirby-Bauer Disk diffusion method. Whole-genome sequencing was performed. The assembled contigs were annotated. Multilocus sequence type, antimicrobial resistance, and virulence genes were identified.

Results: The strains showed two multilocus sequence types, ST231 (FA34) and ST1552 (PL448, FG116). Based on their antibiotic susceptibility profiles, PL448 and FG116 were classified as extensively drug-resistant and FA34 as pandrug-resistant. FA34 harbored mutations in *LpxA, LpxC*, and *PmrB*, conferring resistance to colistin, but not *mcr* genes. All three strains encoded virulence genes for immune evasion (capsule, lipopolysaccharide [LPS]), iron uptake, and biofilm formation. FA34 was related to human strains from South Africa; PL448 and FG116 were related to a strain isolated in the United States from a human wound.

Conclusions: The detection of extensively drug- and pandrug-resistant *A. baumannii* strains in hospital effluents is of particular concern. It indicates that wastewater might play a role in the spread of these bacteria. Our data provide insight into the molecular epidemiology, resistance, pathogenicity, and distribution of *A. baumannii* in South Africa.

1. Introduction

Acinetobacter baumannii is a strictly aerobic, non-fermentative, non-motile Gram-negative coccobacillus, and a notorious member of the Gammaproteobacteria class. Worldwide, *A. baumannii* is responsible for a significant number of nosocomial infections, including ventilator-associated pneumonia, surgical site, urinary tract infections, and septicemia [1]. It is an opportunistic and, nowadays, difficult-to-treat bacterial pathogen because it is often resistant to last-line antibiotics and displays a high biofilm formation capacity, leading to increased morbidity and mortality [2]. Numerous studies have demonstrated the ability of *A. baumannii* to produce biofilms on surfaces of biotic and abiotic materials [3].

Here, we report on the analysis of three *A. baumannii* strains isolated in 2017 from hospital effluents of two tertiary hospitals in KwaZulu-Natal Province in South Africa, which belonged to the multilocus sequence types (STs) 231 and ST-1552.

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Table 1
Plasmid type, resistance phenotype, minimum inhibitory concentration, and biofilm formation of *Acinetobacter baumannii* strains in the current study

Isolate	Replicons	Resistance phenotype	MIC (μg/mL)					MBEC* (μg/mL)		Biofilm*
			IMP	CTX	CIP	CAZ	TE	CIP	CAZ	
Appelsbosch Hospital A FA34	5	MEM^R-CIP^R-TIM^R-PRL^R-SAM^R-TZP^R-CAZ^R-FEP^R-CTX^R-CRO^R-DOR^R-IMP^R-CN^R-TOB^R-AK^R-DO^R-MH^R-TE^R-LEV^R-SXT^R-CST^R	64	200	4	128	128	1024	> 8192	strong
Greys Hospital B FG116	2	TIM^R-PRL^R-SAM^R-TZP^R-CAZ^R-FEP^R-CTX^R-DOR^R-IMP^R-CN^R-TOB^R-AK^R-DO^R-MH^R-TE^R-LEV^R-SXT^R	128	200	1	200	64	1024	> 8192	weak
PL448	1	TIM^R-PRL^R-SAM^R-TZP^R-CAZ^R-FEP^R-CTX^R-CRO^R-DOR^R-IMP^R-CN^R-TOB^R-AK^R-DO^R-MH^R-TE^R-LEV^R-SXT^R	128	200	1	200	64	1024	> 8192	weak

AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; DO, doxycycline; DOR, doripenem; FEP, cefepime; IMP, imipenem; LEV, levofloxacin; MBEC, minimum biofilm eradication concentration; MEM, meropenem; MH, minocycline; MIC, minimum inhibitory concentration; PRL, piperacillin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulphamethoxazole; TE, tetracycline; TIM, ticarcillin-clavulanic acid; TOB, tobramycin; TZP, piperacillin-tazobactam.

* Biofilm formation and the minimum biofilm eradication concentrations were determined using the modified microtitre plate assay and the broth microdilution method, respectively, as previously reported [6].

For the isolation of the *A. baumannii* strains PL448, FG116, and FA34, hospital effluent samples were first filtered (0.45μm membrane filter, Millipore, Billerica, MA). The membrane filters were aseptically removed and then incubated overnight on Leeds *Acinetobacter* medium (HiMedia™ Laboratories, India), as previously described [3]. Initial species identification was performed using an oxidase test and growth at 44°C. The species was confirmed using the VITEK®2 system (bioMérieux, Marcy-l'Étoile, France), as previously reported [3].

The antimicrobial susceptibility profiles of PL448, FG116, and FA34 against 20 antibiotics were determined using the Kirby-Bauer disk diffusion method (Oxoid Ltd., UK), as previously reported [3]. The minimum inhibitory concentrations (MICs) of imipenem, ciprofloxacin, cefotaxime, ceftazidime, and tetracycline were determined using the agar dilution method, while the MIC of colistin was determined using the microbroth dilution method, as previously reported [3]. *A. baumannii* ATCC 19606 was used as a reference strain. The results were interpreted according to Clinical and Laboratory Standard Institute guidelines [4] and are shown in Table 1. PL448, FG116, and FA34 differed in their profiles only in their resistance towards colistin, meropenem, ciprofloxacin (for each of these antibiotics: FA34 = R; PL448 and FG116 = S), and ceftriaxone (FA34 and PL448 = R; FG116 = S). Based on the definition of Magiorakos et al. for multidrug-, extensively drug-, and pandrug-resistant bacteria [5], PL448 and FG116 were resistant to agents from eight different antimicrobial categories and, thus, classified as extensively drug-resistant. FA34 was resistant to agents from all antimicrobial categories used to treat *A. baumannii* and, thus, classified as pandrug-resistant.

Genomic DNA was extracted from overnight culture using the cetyltrimethylammonium bromide (CTAB) protocol, as previously reported [6]. Sample preparation and short-read sequencing was performed by Admera Health, LLC (South Plainfield, NY). Genomic sequence data were treated and analyzed using appropriate software programs and tools, as previously reported [7]. Quality control and assembly were performed using the ASA^{3}P pipeline v. 1.4.0 and SPAdes v. 3.13.0 implemented assembly software, available at the Centre for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/SPAdes/). An average sequencing depth of 111 × and an average read length of 147 nt was achieved. The number of assembled contigs ranged between 57 and 104 (Supplementary Table S1). Contigs larger than 200 bp were submitted to National Center for Biotechnology Information (NCBI) for gene annotation using the NCBI Prokaryotic Genome Annotation Pipeline v. 5.1 (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The genome features of the strains are shown in Supplementary Table S1. The genomes had an average size of 4,027,118 bp and an average GC content of 39%. The number of annotated genes ranged between 3919 and 3969, and the number of detected RNAs ranged between 70 and 78.

Multilocus sequence typing (MLST) was performed using the MLST v. 2.0 tool available at the Center for Genomic Epidemiology (https://cge.food.dtu.dk/services/MLST/). The Oxford MLST scheme was used, and analysis revealed that PL448 and FG116 belonged to ST1552, and FA34, to ST231.

The virulence gene analysis was performed using the VFanalyzer tool implemented in the virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFa-nalyzer). FA34 harbored 65 virulence genes, while PL448 and FG116 harbored 53 (Supplementary Table S2). All three isolates harbored two different phospholipases (two copies of phospholipase C and one copy of phospholipase D), two regulatory circuits for quorum sensing (*abaI/abaR*), a two-component regulatory system (*bfmR/S*), a gene known for eukaryotic cell adherence (*ompA*), and a serum resistance gene (*pbpG*).

Several virulence determinants involved in biofilm formation were detected. Among these, the *adeFGH* efflux pumps, Csu pili, and the poly-β-1,6-N-acetylglucosamine (PNAG)-producing operon were common to all three strains. The *bap* gene was present only in PL448 and FG116. In FA34, a protein of only 48% amino acid identity to Bap was detected, thus not being regarded as a true homologue.

Virulence genes involved in immune evasion were also detected. All three strains harbored an identical set of genes required for lipopolysaccharide (LPS) production. The capsule-producing genes differed between FA34 and PL448/FG116 (Supplementary Table S2). All three isolates harbored the acinetobactin operon used for iron uptake, but only FA34 encoded the hem utilization cluster.

Resistome analysis was performed using the Resfinder v. 4.1 tool, as was recently reported [7]. FG116 and PL448 had an identical repertoire of antibiotic resistance genes (n = 7) conferring resistance towards four different antibiotic classes (Table 2): beta-lactams ($bla_{\text{ADC-25}}$-like, $bla_{\text{OXA-23}}$, and $bla_{\text{OXA-51}}$), aminoglycosides (*ant(2'')-Ia*-like and *aph(3')-VIa*), sulphonamides (*sul2*), and tetracyclines (*tet(B)*-like). The beta-lactamases $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ are regarded as intrinsic carbapenemases of *Acinetobacter baumannii* conferring resistance to only ertapenem [8].

The genome of FA34 encoded more antibiotic resistance genes (n = 14) than those of FG116 and PL448, which encoded resistance to nine different antibiotic classes (Table 2). They confer resistance to β-lactams ($bla_{\text{OXA-23}}$, $bla_{\text{ADC-25}}$-like, $bla_{\text{OXA-69}}$, and $bla_{\text{NDM-1}}$), aminoglycosides (*aadA1, aph(3')-Ia, aph(3'')-Ib*-like, *armA, aph(6)-Id*, and *aac(3)-I*-like), macrolides (*mph(E)*), macrolide/lincosamide/streptogramin B (*msr(E)*), phenicols (*cmlA1*-

Table 2
Antimicrobial resistance genes with predicted resistance phenotypes detected in the genomes of *Acinetobacter baumannii* strains in the current study

Strain name	Aminoglycoside	Beta-lactam	Macrolide	Macrolide, Lincosamide and Streptogramin B	Phenicol	Rifampicin	Sulphonamide	Tetracycline	Trimethoprim
FG116	*ant(2'')-Ia, aph(3')-VIa*	bla_{ADC-25}-like, bla_{OXA-23}, bla_{OXA-51}					*sul2*	*tet(B)-like*	
PL448	*ant(2'')-Ia, aph(3')-VIa*	bla_{ADC-25}-like, bla_{OXA-23}, bla_{OXA-51}					*sul2*	*tet(B)-like*	
FA34	*aadA1, aph(3')-Ia, aph(3'')-Ib-like, armA, aph(6)-Id, aac(3)-I-like*	bla_{ADC-25}-like, bla_{NDM-1}, bla_{OXA-69}, bla_{OXA-23}	*mph(E)*	*msr(E)*	*cmlA1-like*	*ARR-2*	*sul1, sul2*	*tet(B)*	*dfrA1*

Table 3
Determination of mutations in proteins involved in colistin resistance in *Acinetobacter baumannii* strains in the current study

Strain	Position of detected mutations							
	LpxA	LpxC		LpxD		PmrB		
	131	120	287	3	199	300	360	363
FG116	Y –> H	C–> R	N –> D	V–>A	A–> S	V–>E	N.D.	Y–>F
FA34	Y –> H	C–> R	N –> D	N.D.	N.D.	N.D.	P–>Q	N.D.
PL448	Y –> H	C–> R	N –> D	V–>A	A–> S	V–>E	N.D.	Y–>F

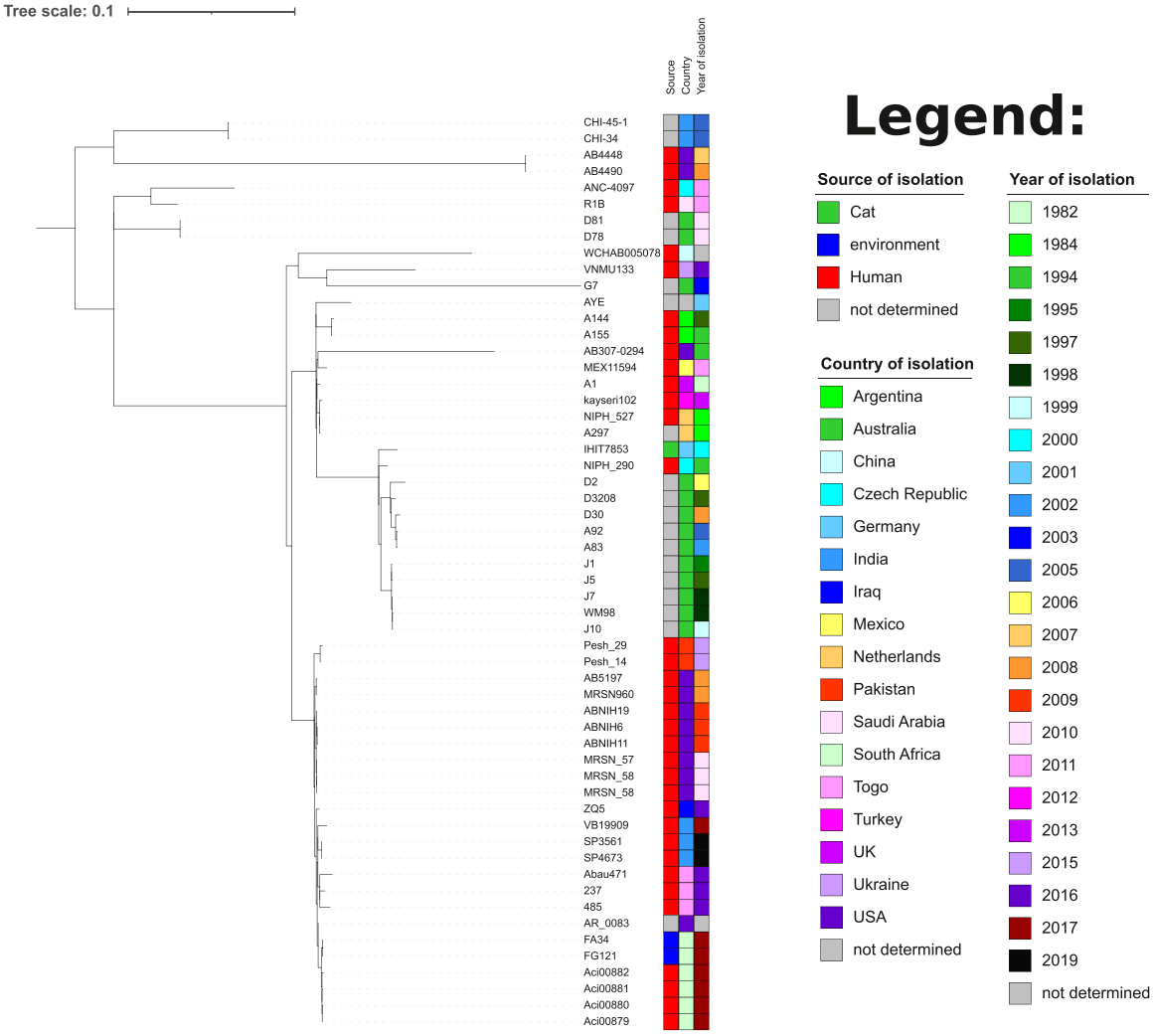

Fig. 1. Core-genome-based phylogenetic tree of closely related *Acinetobacter baumannii* strains to FA34, including FG121 from an earlier study. The relatives of FA34 were identified using BacWGSTdb. Assemblies of these relatives were downloaded from the NCBI database. Core-genome-based phylogeny was produced with ParSNP implemented in the HarvestSuite package. Visualization and annotation of the tree was performed using ITOL v. 6.5.8 with adjustment in Inkscape 0.91 (https://inkscape.org/release/inkscape-0.91/). The metadata of the reference sequences are shown in Supplementary Table S6.

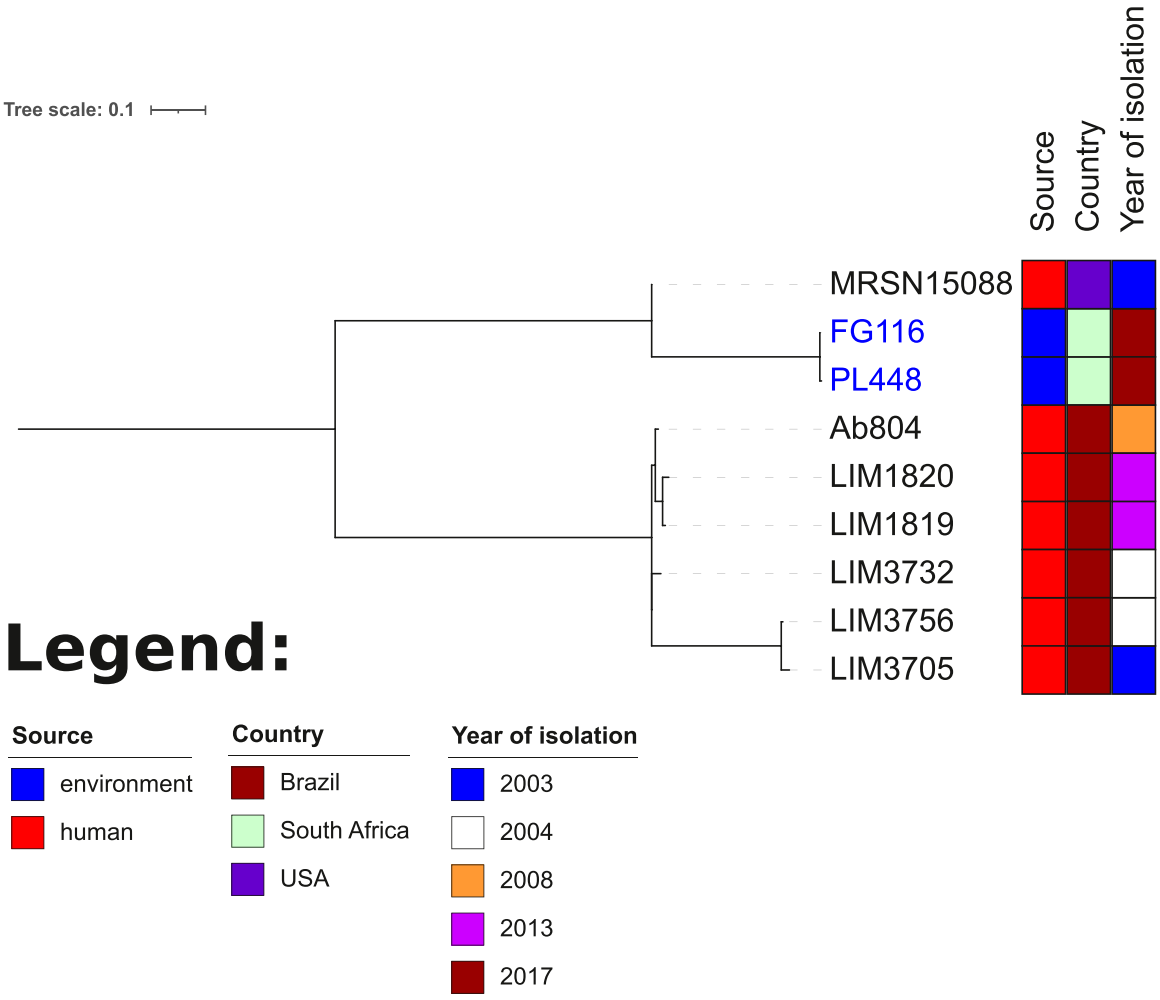

Fig. 2. Core-genome-based phylogenetic tree of closely related *Acinetobacter baumannii* strains to FG116 and PL448. The relatives of FG116 and PL448 were identified using BacWGSTdb. Assemblies of these relatives were downloaded from the NCBI database. Core-genome-based phylogeny was produced with ParSNP implemented in the HarvestSuite package. Visualization and annotation of the tree was performed using ITOL v. 6.5.8 with adjustment in Inkscape 0.91 (https://inkscape.org/release/inkscape-0.91/). The metadata of the reference sequences are shown in Supplementary Table S7.

like), rifampicin (ARR-2), sulphonamides (*sul1* and *sul2*), tetracyclines (*tet(B)*), and trimethoprim (*dfrA1*). The beta-lactamases $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ in FA34 confer resistance to all carbapenems. This is of particular concern, as carbapenems are last-line drugs used to treat multidrug-resistant and extremely drug-resistant bacteria.

FA34 was phenotypically resistant to colistin, but no *mcr* genes were detected. Therefore, a search for mutations in LPS biosynthesis proteins (LpxA, LpxC, LpxD) and the PmrAB two-component system was performed. Mutations in these genes are known to be involved in colistin resistance [9]. The search was performed using tblastN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the respective protein sequences from the colistin-susceptible *A. baumannii* type strain ATCC 17978 (Accession number CP000521.1) towards the genome of FA34. Several mutations in LpxA (Y131H), LpxC (C120R N287D), and PmrB (P360Q) (Table 3) were detected. The LpxA and LpxC mutations identified in FA34 have been described in other previously reported colistin-resistant *A. baumannii* strains [10–13], indicating that these mutations might be responsible for the colistin resistance phenotype.

The antibiotic resistance gene content was consistent with the resistance phenotype for FA34, as shown in Table 1, but not for FG116 and PL448, indicating that FG116 and PL448 might encode other additional resistance mechanisms (e.g. down-regulation or mutation of porins).

Analysis of the plasmid replicons was performed using two approaches. The first search was performed using the tool 'PlasmidFinder', and did not reveal any Enterobacterales plasmid replicons. The second search for the presence of *A. baumannii* replicase proteins, reported by Bertini et al. [14], was performed using tblastN and led to the identification of two different replicon types (GR2 and GR14) in FA34. PL448 and FG116 harbored a different replicon type (GR6).

Analysis of mobile genetic elements (MGEs) was performed using MobileElementFinder, and it was found that ST231 and ST1552 strains contained a different set of MGEs. FA34 harbored one transposon (Tn*6018*) and three insertion sequences (IS*1006*, IS*Ec29*, and IS*Ec28*). FG116 and PL448 harbored the insertion sequences IS*Aba125* (two copies in FG116), IS*Aba13*, and IS*1006*.

The detection of *A. baumannii* isolates genetically closely related to FA34, FG116, and PL448 was performed using the 'single genome analysis' module of the global phylogenetic analysis tool BacWGSTdb (http://bacdb.cn/BacWGSTdb/analysis_single.php). For detection of close relatives, a cgMLST threshold of ≤200 cgMLST allele differences was selected.

For FA34, 55 closely related global *A. baumannii* strains were detected (Supplementary Table S3). The closest relatives to FA34 were four $bla_{\text{NDM-1}}$-positive ST231 *A. baumannii* human isolates from South Africa (Aci00879, Aci00880, Aci00881, and Aci00882; 9–14 cgMLST allele differences; Supplementary Table S3) and one

isolate from the United States (AR_0083, difference of 17 cgMLST alleles). FA34 was highly related to the previously reported *A. baumannii* strain FG121 [7] detected in the same region (Fig. 1).

For FG116 and PL448, only one closely related *A. baumannii* (MRSN15088) was detected within the threshold of ≤200 cgMLST allele differences (Supplementary Table S4,S5). MRSN15088 differed from FG116 by 169 and from PL448 by 168 cgMLST alleles. It was isolated from a human wound in the United States (Fig. 2). Because of the low number of close relatives, we extended the search to strains with a difference of ≤1000 cgMLST alleles to FG116 and PL448. When using this threshold, six additional strains were related to FG116 and PL448 (Ab804, LIM3756, LIM1819, LIM3732, LIM1820, and LIM3705; Supplementary Table S4,S5; Fig. 2). All strains were isolated from human clinical samples in Brazil. All were isolated from bloodstream infections, indicating that ST1552 strains could have a certain virulence potential.

A. baumannii ST231 were described for the first time in 2012 [15] after causing severe infection in a lung transplant recipient. Since this initial report [15], a low number of studies have been reported on this ST (based on a PubMed/NCBI literature search as of 19 July 2022 for '*Acinetobacter baumannii*' and 'ST231' or 'ST-231'). The majority of the reported ST231 *A. baumannii* were carbapenem-resistant (like FA34 from this study) and were isolated from wounds. Three studies reported on the presence of *A. baumannii* ST231in companion animals (dogs and cats) [16,17] and environmental (wastewater) samples [7]. *Acinetobacter baumannii* ST231 depicted an identical carbapenemase-content as FA34 (NDM-1 and OXA-69) and were detected in high prevalence in wound infections in Ghana [18]. These data indicate that carbapenem-resistant *A. baumannii* ST231 are not only capable of causing infection in humans but can also be regarded as a One Health problem because they have been detected in different habitats.

According to a literature search for '*Acinetobacter baumannii*' and 'ST1552' or 'ST-1552', *A. baumannii* ST1552 has been reported in only two studies [19,20] as of 19 July 2022. Like the ST1552 strains reported in the current study, they were carbapenem-resistant (Table 1). They were only detected in human clinical samples. Thus, our study is the first description of *A. baumannii* ST1552 strains isolated from non-human samples.

The present report provides insights into the mechanisms of resistance and virulence of *A. baumannii* from Africa. The detection of extensively drug-resistant (FG116 and PL448) and pandrug-resistant (FA34) *A. baumannii* strains in hospital effluents in South Africa is alarming. Untreated hospital effluents pose a serious public health risk to nearby communities exposed to surface water. Our findings are of environmental, clinical, and public health relevance. They emphasize the significance of surveillance strategies, in particular those performed in a One Health context.

Data availability

This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the bioproject accession numbers PRJNA718726 and PRJNA719017, and Genbank accession numbers JAGISI000000000, JAKUCL000000000, and JAKUDM000000000 for biosamples SAMN18520915, SAMN18520936, and SAMN26149365, respectively. The version described in this manuscript is the first version. The raw sequences have been submitted to the Sequence Read Archive (SRA) under the accession numbers SRR14280111, SRR18114234, and SRR18114233.

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Competing interests

None declared

Ethical approval

This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (UKZN BREC) (Durban, South Africa) [Registration Number of BE063/19].

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.08.024.

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