Report of epidemic Pseudomonas aeruginosa AUST-03 (ST 242) strains and resistomes in South African cystic fibrosis patients

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October 23, 2023

Abstract

Introduction: Pseudomonas aeruginosa AUST-03 (ST242) has been reported to cause epidemics in cystic fibrosis (CF) patients from Tasmania and Australia and has been associated with multidrug resistance and increased morbidity and mortality. Here, we report epidemic P. aeruginosa (AUST-03) strains in South African CF patients at a public academic hospital detected during a previous study and characterise the resistomes. Methods: The P. aeruginosa AUST-03 (ST242) strains were analysed with whole genome sequencing using the Illumina NextSeq2000 platform. Raw sequencing reads were processed using the Jekesa pipeline and multi-locus sequence typing and resistome characterisation was performed using public databases. Core single nucleotide polymorphism phylogenies were performed on P. aeruginosa ST242 strains from the study and from public databases. Antibiotic susceptibility testing was performed using the disk diffusion and broth microdilution techniques. Results: A total of 11 P. aeruginosa AUST-03 strains were isolated from two children with CF who had pulmonary exacerbations. The majority of the P. aeruginosa AUST-03 strains (8/11) were multidrug resistant (MDR) or extensively drug resistant; and the multidrug efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM were the most clinically relevant antibiotic resistance determinants and were detected in all of the strains. The P. aeruginosa AUST-03 (ST242) study strains were most closely related to strains from Canada, China, Denmark and Slovenia. Conclusion: Epidemic MDR P. aeruginosa strains are present at South African public CF clinics and need to be considered when implementing patient segregation and infection control strategies to prevent further spread and outbreaks.
mortality rates. In 1996, the first epidemic P. aeruginosa strain was identified in CF patients from Liverpool, England and was termed the Liverpool Epidemic Strain (LES). Since then, epidemic strains of P. aeruginosa have been reported in CF patients from Australia, North America and multiple European countries.

Australia is among the countries with the highest number of reported epidemic P. aeruginosa strains in CF patients. The P. aeruginosa Australian Epidemic Strains (AES) now renamed AUST have been described in CF patients from Tasmania and Australia. Pseudomonas aeruginosa AUST-01 (ST649), AUST-02 (ST775) and AUST-03 (ST242) are among the most common epidemic strains in these regions.

The P. aeruginosa AUST-03 (also known as AES-III) epidemic strain was first described in CF patients from Tasmania in 2003 and has caused outbreaks in Tasmania and Australia. The frequent genomic surveillance of CF pathogens in high income countries has enabled the detection of epidemic strains infecting CF patients. However, in low to middle income countries (LMICs) such as South Africa and other African countries where resources are limited, surveillance of CF pathogens is performed infrequently or not at all. The identification of CF lung pathogens that are of increased virulence and are epidemic in nature is of paramount importance for the prevention of outbreaks. To our knowledge, highly transmissible or epidemic strains of P. aeruginosa have not been reported in CF patients from South Africa. Here we report the presence of the Australian/Tasmanian epidemic strain AUST-03 (ST 242) discovered in two CF patients at a public academic hospital in Gauteng, South Africa. The aim of this study was to describe the genomic resistance characteristics of the P. aeruginosa ST 242 (AUST-03) strains discovered at this hospital during a previous study and to make phylogenetic comparisons with P. aeruginosa AUST-03 strains reported in other geographic settings.

2 MATERIALS AND METHODS

2.1 Study setting and ethics statement

The P. aeruginosa AUST-03 (ST 242) strains investigated were isolated from two CF patients attending clinics at a public tertiary academic hospital in Johannesburg, South Africa. The two CF patients (P2 and P4) were part of a study that recruited 22 CF patients at two public academic hospitals in Gauteng, South Africa over the period May 2019 to February 2020. The study was granted ethical approval by the University of the Witwatersrand Human Research Ethics Committee (Reference number: M1811104) and by the University of Pretoria, Faculty of Health Sciences, Research Ethics Committee (Reference number: 466/2018). Sputum specimens were obtained from the CF patients following the provision of written informed assent and consent from the CF patients and their parents, respectively.

2.2 Pseudomonas aeruginosa isolation and analysis

A single sputum specimen was collected from CF patients by means of spontaneous expectoration or after the administration of percussion exercises, with the assistance of the attending physiotherapist at the CF clinic. The sputum specimens were transported on ice to the Department of Medical Microbiology, University of Pretoria and cultured upon arrival on Pseudomonas CN agar (Oxoid, UK) and incubated (Vacutec, South Africa) at 37°C for up to 72 h. Up to 10 presumptive P. aeruginosa colonies of varying size and morphology were selected from the Pseudomonas CN agar (Oxoid, UK) plate of each CF patient sputum and sub-cultured onto 5% sheep blood agar (Diagnostic Media Products, South Africa) and incubated (Vacutec, South Africa) for up to 48 h. Gram staining was performed on the presumptive colonies to confirm purity following incubation and DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). Pseudomonas aeruginosa species confirmation was performed by targeting the species-specific peptidoglycan associated outer membrane lipoprotein (oprL) gene using primers previously described. The PCR reactions were prepared according to the Bioline® mastermix (Bioline®, UK) protocol and amplification of the oprL gene was performed in a Bio-Rad T100 thermocycler (Bio-Rad, USA) using the following cycling conditions: 95°C for 5 min; 28 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min; and 72°C for 10 min. The amplified PCR products were resolved on 1.5% (m/v) SeaKem® LE agarose (Lonza, USA) gel stained with 0.5 μg/μL ethidium bromide and a 100 bp molecular weight marker (ThermoScientific, USA) was used.
as a size reference. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control.

Antibiotic susceptibility testing (AST) was performed on PCR confirmed *P. aeruginosa* isolates using the disk diffusion technique for the following antibiotics: cefepime (30 μg), ceftazidime (30 μg), imipenem (10 μg), meropenem (10 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), ciprofloxacin (5 μg), piperacillin/tazobactam (110 μg) and aztreonam (30 μg) and the broth microdilution technique for colistin according to the Clinical and Laboratory Standards Institute guidelines. Multidrug resistant (MDR) *P. aeruginosa* isolates were defined as those displaying resistance to one or more anti-pseudomonal antibiotics in at least three or more antibiotic classes, while extensively drug resistant (XDR) isolates were those displaying resistance to one or more anti-pseudomonal antibiotics in all but two or less antibiotic classes.

### 2.3 *Pseudomonas aeruginosa* WGS and analysis

Whole genome sequencing (WGS) of the PCR confirmed *P. aeruginosa* study isolates was performed using the Illumina NextSeq 2000 (Illumina Inc., USA) instrument. Multiplexed, paired-end libraries (2 x 150bp) were prepared using the Illumina DNA Prep kit (Illumina, San Diego, USA), followed by sequencing at 100x coverage, according to the manufacturer’s instructions. The raw paired-end *P. aeruginosa* sequencing reads were processed using the Jekesa pipeline v1.0 and using tools and methodology that included species identification and multilocus sequence typing (MLST) as previously outlined. Genomic antibiotic resistance (resistome) determinants in the study strains were characterised using a combination of tools including the Comprehensive Antibiotic Resistance Database (CARD) v3.2.2 and ResFinder.

### 2.4 Phylogenetic comparison of global and study *P. aeruginosa* AUST-03 strains

A total of 61 *P. aeruginosa* ST242 genomes originating from human specimens were retrieved from the National Center for Biotechnology Information (NCBI) using the NCBI datasets tool v15.12.0 and were analysed together with genome assemblies from our study strains. Briefly, whole genome alignments were performed using scapper and *P. aeruginosa* Zw92 strain was used as a reference. Recombinant regions were removed using Gubbins v3.2.1 and variable sites were obtained using snp-sites v2.5.1. Pairwise SNP distances were calculated using snp-dist v0.8.2 and a normalized pairwise SNP distance matrix was used as input for the cluster analysis using the R software environment v4.2.1. Assignment of core single nucleotide polymorphism (SNP) clusters was achieved by a combination of K-means clustering and custom written in R using a silhouette score and SNP cut-off of 0.5 and 20, respectively. Visualisation of cluster heat maps was performed using the ComplexHeatmap package v.14.0.

### 3 RESULTS

#### 3.1 *Pseudomonas aeruginosa* AUST-03 (ST242) colonised patients from the study

The *P. aeruginosa* AUST-03 strain was found in a 16 years old male CF patient (P2) and a 8 years old female patient (P4), who both attended CF clinics at the tertiary academic hospital. Both patients were found by attending clinicians to have been experiencing pulmonary exacerbations, while one of the patients, P4 required oxygen and later passed away. Details on the demographics of the two patients, genetic mutations and comorbidities can be found in Table 1.

#### 3.2 Molecular identification and phenotypic characterisation of *P. aeruginosa* AUST-03 (ST242) isolates from the study

A total of 10 *P. aeruginosa* ST242 and one *P. aeruginosa* ST242 strains as confirmed with PCR and MLST were isolated from the sputum of P2 and P4, respectively. In total, 11 epidemic *P. aeruginosa* ST 242 (AUST-03) strains were recovered from the patients and the results of AST showed that the majority of the strains from P2 [70% (7/10)] were MDR, while the one isolate from P4 was XDR. The following antibiotic resistance rates were recorded among the *P. aeruginosa* AUST-03 strains from the study: ciprofloxacin 100% (11/11), cefepime 73% (8/11), gentamicin 64% (7/11), amikacin 36% (4/11), tobramycin 18% (2/11), ceftazidime 18% (2/11), imipenem 18% (2/11), piperacillin-tazobactam 18% (2/11), meropenem 9% (1/11), aztreonam
9% (1/11) and 9% colistin (1/11). Table 2 details the morphological characteristics and AST profiles of the
P. aeruginosa AUST-03 strains detected in the study.

3.3 Genomic antibiotic resistance and virulence characteristics of the P. aeruginosa AUST-03 isolates

Whole genome sequencing of the 11 P. aeruginosa AUST-03 study strains showed that the main genomic
basis of antibiotic resistance in the P. aeruginosa isolates was efflux pump mediated. Genes conferring
the MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM efflux pumps were detected in all of the
study strains and mutations in the mexR, mexT, nalC and nfxB genes were detected in all of the strains
that conferred the up-regulation of these efflux pumps. Additional efflux pumps emrE and pmpM genes
were also detected in all of the study strains that confer resistance to fluoroquinolones and aminoglycosides,
respectively. The antibiotic resistance genes (ARGs): blaOXA-50, blaOXA-1034, bladbDC-3, bladbDC-374 and
crpP were also found in all of the strains, however, these genes are considered to play a minor role in
P. aeruginosa antibiotic resistance. Acquired mutations in genes were found in the strains that conferred
resistance to fluoroquinolones [gyrA (3/11), gyrB (8/11) and parE (1/11)] and colistin [pmrAB (11/11)].
Details on the major antibiotic resistance determinants of the P. aeruginosa AUST-03 strains from the study
have been illustrated in Figure 1.

3.4 Phylogenetic analysis of P. aeruginosa AUST-03 isolates

A total of 28 P. aeruginosa ST242 genomes from human sources were closely related to the strains from
this study as displayed in Figure 2. The P. aeruginosa ST242 strains from this study formed five distinct
clusters with the global P. aeruginosa ST242 strains at a cut-off of 20 SNPs. The largest of these was
Cluster 2 that consisted of three strains from P2 and the single strain from P4 and also included strains
from Canada, China, Denmark and Slovenia. The remaining P. aeruginosa ST242 from P2 formed Cluster 3
which consisted of three study strains and strains from Canada and Slovenia, while a single P2 strain from
Cluster 7 clustered with a strain from Canada. Clusters 8 and 11 consisted only of P2 study strain/s.

4 DISCUSSION

The CF clinic based at the study setting practices patient segregation based on the segregation of patients
colonised with P. aeruginosa, from those not colonised with P. aeruginosa. This is achieved by scheduling
clinics for each of the patient groups on different weeks. However, due to a lack of frequent genomic
surveillance of P. aeruginosa CF isolates in South Africa, limited knowledge is available on the presence of
epidemic strains being carried by CF patients.

The CF patients from which the P. aeruginosa AUST-03 strains had been detected were children, who were
both experiencing pulmonary exacerbations. Limited data is available on the patient demographics of CF
patients infected or colonised with P. aeruginosa AUST-03; however, the studies that are available have
reported that P. aeruginosa AUST-03 has been associated with increased pulmonary exacerbations.12,30
Only a single study was found in the literature that investigated P. aeruginosa AUST-03 (then referred to
as AES-III) by Bradbury and colleagues30, however, clear comparisons in the age demographics of the CF
patients from that study and the current study could not be made. This was due to Bradbury et al.30 having
only recruited patients during outreach clinics for adult CF patients, while the CF clinics from this study
only attended to children with CF. Bradbury et al.30 did recruit patients as young as 15 years of age in the
study, however, those that were found to harbour P. aeruginosa AUST-03 strains were between the ages of
19 and 34 years old. Furthermore, only a very small number of patients carrying this strain were detected
in the current study.

P2 was found to be exclusively colonised with P. aeruginosa AUST-03, while P4 was found to be colonised
with P. aeruginosa AUST-03 and a second novel strain. Bradbury et al.30 also made a similar observation
in one of their patients and found that the patient was infected with three different P. aeruginosastrains,
including AUST-03. Phenotypic heterogeneity was observed among the ten P. aeruginosa AUST-03 isolated
from P2 with an observed mixture of mucoid (7/10) and non-mucoid (3/10) isolates of varying size. Vari-
ations in the presence of mucoidity among \textit{P. aeruginosa} AUST-03 strains have also been reported in other studies\cite{15,30} and similarly to our study mucoid isolates were no more resistant to antibiotics that non-mucoid isolates.

Multiple studies have reported an increased likelihood of \textit{P. aeruginosa} AUST-03 strains displaying a MDR phenotype through their antibiograms.\cite{12,15,30} A similar observation was found in this study where the majority (8/11) of the \textit{P. aeruginosa} AUST-03 isolates were MDR or XDR. The study isolates were most frequently resistant to ciprofloxacin [100\% (11/11)], ceftazidime [73\% (8/11)], gentamicin [64\% (7/11)] and infrequently resistant to tobramycin [18\% (2/11)]. Similar findings were reported by Bradbury et al.\cite{30}, however, unlike in the Bradbury et al.\cite{30} study, low rates of resistance were observed to amikacin [36\% (4/11)], ceftazidime [18\% (2/11)], imipenem [18\% (2/11)] and aztreonam [9\% (1/11)]. This variation in the two studies may be due to differences in treatment regimens between Australia and South Africa, as well as the differences in time periods (2019 for this study and 2003 for the Bradbury et al.\cite{30} study), which may have affected the availability of certain antibiotics. The high rates of ciprofloxacin resistance may be due to the frequent use of ciprofloxacin in South African settings as this antibiotic is recommended for the treatment of \textit{P. aeruginosa} in CF patients due to the limited availability of inhaled tobramycin. This may explain the high rate of resistance to gentamycin. Additionally, the limited accessibility of tobramycin at public CF clinics in South Africa may also explain the low rates of tobramycin resistance among the \textit{P. aeruginosa} AUST-03 strains.

Multidrug efflux pumps formed the basis of antibiotic resistance in the genomes of the \textit{P. aeruginosa} AUST-03 strains from this study. The most clinically relevant efflux pumps were: MexAB-OprM (extrudes aztreonam, ceftazidime, ciprofloxacin, levofloxacin, meropenem and piperacillin), MexCD-OprJ (extrudes azithromycin, ceftazidime, ciprofloxacin, levofloxacin), MexEF-OprN (extrudes ciprofloxacin and imipenem) and MexXY-OprM (extrudes amikacin, ceftazidime, ciprofloxacin, gentamicin, levofloxacin and tobramycin), which were detected in the 11 isolates.\cite{31,32} Furthermore, mutations in the regulatory genes: \textit{nahC} (S209R and G71E), \textit{nfxB} (Type A), \textit{mexRT} of the 11 study isolates were detected that conferred the overexpression of the MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps, respectively.\cite{31,32} A limited number of clinically relevant acquired ARGs were detected in the study isolates, namely \textit{crpP} (11/11) which confers resistance to ciprofloxacin and \textit{PDC-3} (11/11) which confers resistance to piperacillin/tazobactam.\cite{33-35} Acquired mutations in the DNA gyrase \textit{[gyrA]D87N} (3/11) and \textit{gyrB} (8/11) and topoisomerase IV \textit{[parE]} (1/11) genes were detected in the \textit{P. aeruginosa} AUST-03 strains, conferring resistance to fluoroquinolones such as ciprofloxacin.\cite{36}

Colistin is considered to be an antibiotic of last resort for the treatment of Gram-negative pathogens such as \textit{P. aeruginosa} in healthcare settings.\cite{37} As such, the use of this antibiotic is reserved for MDR isolates, where all other treatment options are ineffective. In this study, only a single \textit{P. aeruginosa} AUST-03 strain (P4) was resistant to colistin and was also found to be XDR, regrettably, this patient passed away. In this patient’s strain, mutations were found in the lipid A regulatory genes \textit{(pmrAB)} that conferred enhanced colistin resistance.\cite{36} Mutations in these genes were also found in the other 10 \textit{P. aeruginosa} AUST-03 strains (P2) from this study, however, these isolates displayed intermediate resistance to colistin. The presence of these mutations could lead to the future development of colistin resistance in the strains.

The three strains from P2 and the strain from P4 clustered together in Cluster 2, which may indicate that these were the initial strains that were acquired by the two patients. This may have been due to strain transfer among the two patients or each patient acquiring this strain at the same time from the same external source. The countries Canada, China, Denmark and Slovenia were also part of this cluster and could have potentially been involved into the introduction of this strain to the study setting, however, further investigation is required. Strains from Canada were a common feature in the three clusters (Clusters 2, 3 and 7) containing strains from this study and global strains. Canada is a popular destination for migration from many African countries including South Africa\cite{38} and the frequent back and forth travel between the two countries may present opportunities for the introduction of \textit{P. aeruginosa} AUST-03 in CF.
clinics. However, introduction from other countries or multiple countries cannot be ruled out. None of the *P. aeruginosa* ST242 (AUST-03) strains from this study clustered with the Australian strains at a cut-off of 20 SNP differences, however, the strains were still closely related and differences may have been due to genomic changes that occurred over time and over the spread of the strain globally. Infection control strategies in South Africa will need to be revised to include the screening of patients that may have travelled or lived in countries where epidemic strains are endemic.

The main limitation from the study was the low numbers of CF patients that were investigated and that adult CF patients from this hospital were not included in the study as *P. aeruginosa* AUST-03 was found mostly in adult CF patients in previous studies. Furthermore, the limited attention given to strain typing of *P. aeruginosa* in the study setting makes it difficult to ascertain when the *P. aeruginosa* AUST-03 first appeared in the study setting and for how long this epidemic strain has been circulating. Future studies investigating *P. aeruginosa* AUST-03 in minor and adult CF patients at other public and private CF clinics across the country are important to establish the spread of this and other epidemic strains.

5 CONCLUSION

The current study presents to our knowledge, the first report of an epidemic strain of *P. aeruginosa* among CF patients from South Africa. The *P. aeruginosa* AUST-03 from the study setting were MDR and similarly to strains from previous outbreaks, were associated with CF patients experiencing pulmonary exacerbations. With the decrease in WGS costs in South Africa, more frequent genomic surveillance of CF pathogens is required. Epidemic strains of *P. aeruginosa* are present at South African CF clinics and it is essential to take them into account when implementing patient segregation and infection control strategies.

AUTHOR CONTRIBUTIONS

Thabo Hamiwe and Marthie M. Ehlers: jointly involved in the conceptualisation of study. Thabo Hamiwe: experimental procedures, results analysis and writing and editing of the original manuscript. Debbie White and Susan Klugman: recruitment of study participants, review and interpretation of clinical records and review and editing of original manuscript. Anthony Smith and Arshad Ismail: funding and facilitation of whole genome sequencing and review and editing of original manuscript. Stanford Kwenda: bioinformatics analysis and review and editing of original manuscript. Lore Van Bruwaene: interpretation of clinical findings and review and editing of original manuscript. Ameena Goga and Marleen M. Kock: analysis of study findings and review and editing of original manuscript. Marthie M. Ehlers: Funding of the study, study supervision, review of study findings and review and editing of original manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank the children, parents and members of staff who were involved or assisted with the study at the Cystic Fibrosis clinic conducted by the hospital. The authors would also like to acknowledge the National Research Foundation and the University of Pretoria for the provision of PhD scholarship funds. and the Fleming Fund for the provision of funds. Sequencing of isolates in this study was made possible by support from the SEQAFRICA project which is funded by the Department of Health and Social Care’s Fleming Fund using UK aid. The views expressed in this publication are those of the authors and not necessarily those of the UK Department of Health and Social Care or its Management Agent, Mott MacDonald.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings made in this study are available from the corresponding author (Dr Thabo Hamiwe) upon request.
REFERENCES


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**Acquired Antibiotic Resistance Mutations**

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