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

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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.

INTRODUCTION

Cystic fibrosis (CF) is among the most common recessive genetic disorders globally and affects South Africans at varying frequencies depending on race.[1,2] This disorder occurs at incidences of 1:3 000, 1:10 300 and 1:14 000 in white, mixed-race and black South African population groups, respectively.[3] *Pseudomonas aeruginosa* (*P. aeruginosa*) is recognised as one of the most significant CF lung pathogens in South Africa and worldwide.[4] Persistent lung colonisation of CF patients with *P. aeruginosa* has been associated with poorer patient outcomes such as accelerated decline in lung function and higher rates of mortality.[4-6]

Cystic fibrosis patients are typically infected or colonised with a unique strain of *P. aeruginosa* .[7] However, CF clinics globally have reported multiple instances where CF patients that have shared environments such as CF clinics, or even shared geographical locations carried identical shared *P. aeruginosa* strains.[5,8] These shared *P. aeruginosa* strains, which have also been referred to as epidemic or transmissible strains have been reported in some settings to be more virulent, multidrug resistant (MDR) and associated with increased

mortality rates.[9,10] In 1996, the first epidemic *P. aeruginosa* strain was identified in CF patients from Liverpool, England and was termed the Liverpool Epidemic Strain (LES).[11] Since then, epidemic strains of *P. aeruginosa* have been reported in CF patients from Australia, North America and multiple European countries.[12]

Australia is among the countries with the highest number of reported epidemic *P. aeruginosa* strains in CF patients.[12,13] The *P. aeruginosa* Australian Epidemic Strains (AES) now renamed AUST have been described in CF patients from Tasmania and Australia.[12,14] *Pseudomonas aeruginosa* AUST-01 (ST649), AUST-02 (ST775) and AUST-03 (ST242) are among the most common epidemic strains in these regions.[5,12] 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.[12,15]

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^{o}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.[16] 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^{o}C for 5 min; 28 cycles of 95^{o}C for 30 s, 57^{o}C for 30 s, 72^{o}C for 1 min; and 72^{o}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[17]. 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.[18,19]

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[20,21]. 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[22] and ResFinder[23].

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[24] and were analysed together with genome assemblies from our study strains. Briefly, whole genome alignments were performed using scapper[25] and *P. aeruginosa*Zw92 strain was used as a reference. Recombinant regions were removed using Gubbins v3.2.1[26] and variable sites were obtained using snp-sites v2.5.1[27]. Pairwise SNP distances were calculated using snp-dist v0.8.2[27] and a normalized pairwise SNP distance matrix was used as input for the cluster analysis using the R software environment v4.2.1[28]. 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.[29]

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): $bla_{OXA\text{-}50}$,$bla_{OXA\text{-}1034}$, $bla_{PDC\text{-}3}$,$bla_{PDC\text{-}374}$ 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 colleagues[30], 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. aeruginosas*trains, 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 *P. aeruginosa*AUST-03 strains have also been reported in other studies[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 *P. aeruginosa* AUST-03 strains displaying a MDR phenotype through their antibiograms.[12,15,30] A similar observation was found in this study where the majority (8/11) of the *P. aeruginosa*AUST-03 isolates were MDR or XDR. The study isolates were most frequently resistant to ciprofloxacin [100% (11/11)], cefepime [73% (8/11)], gentamicin [64% (7/11)] and infrequently resistant to tobramycin [18% (2/11)]. Similar findings were reported by Bradbury et al.[30]; however, unlike in the Bradbury et al.[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.[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 *P. aeruginosa*exacerbations.[6] Inhaled gentamycin is recommended by the South African Cystic Fibrosis Consensus Guidelines[6] for the eradication and chronic suppressive treatment of *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 *P. aeruginosa* AUST-03 strains.

Multidrug efflux pumps formed the basis of antibiotic resistance in the genomes of the *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, cefepime, ciprofloxacin, levofloxacin), MexEF-OprN (extrudes ciprofloxacin and imipenem) and MexXY-OprM (extrudes amikacin, cefepime, ciprofloxacin, gentamicin, levofloxacin and tobramycin), which were detected in the 11 isolates.[31,32] Furthermore, mutations in the regulatory genes: *nalC* (S209R and G71E), *nfxB* (Type A),*mexRT* of the 11 study isolates were detected that conferred the overexpression of the MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps, respectively.[31,32] A limited number of clinically relevant acquired ARGs were detected in the study isolates, namely *crpP* (11/11) which confers resistance to ciprofloxacin and*PDC-3* (11/11) which confers resistance to piperacillin/tazobactam.[33-35] Acquired mutations in the DNA gyrase [*gyrA_D87N* (3/11) and *gyrB* (8/11)] and topoisomerase IV [*parE* (1/11)] genes were detected in the*P. aeruginosa* AUST-03 strains, conferring resistance to fluoroquinolones such as ciprofloxacin.[36]

Colistin is considered to be an antibiotic of last resort for the treatment of Gram-negative pathogens such as *P. aeruginosa* in healthcare settings.[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*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 (*pmrAB*) that conferred enhanced colistin resistance.[36] Mutations in these genes were also found in the other 10 *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[38] and the frequent back and forth travel between the two countries may present opportunities for the introduction of *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.[30]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.

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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.

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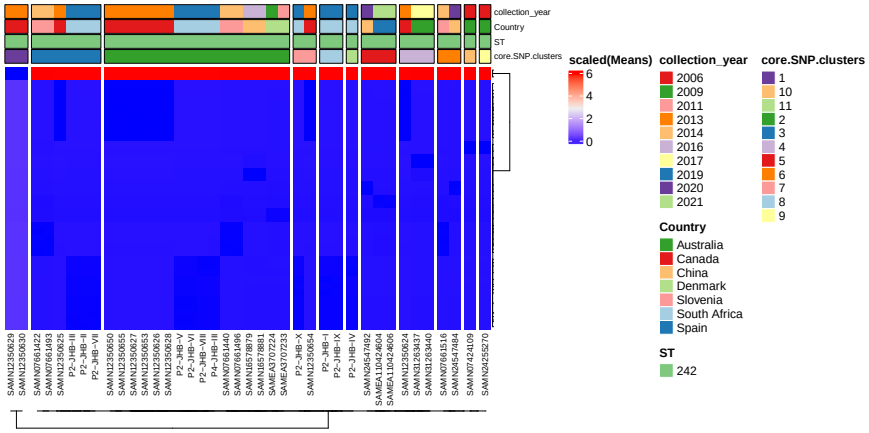
Resistance	Gene	P2 JHB I	P2 JHB II	P2 JHB III	P2 JHB IV	P2 JHB V	P2 JHB VI	P2 JHB VII	P2 JHB VIII	P2 JHB IX	P2 JHB X	P4-JHB-III
Antibiotic Resistance Determinants												
Beta-lactams	bla_{OXA-50}											
	$bla_{OXA-1034}$											
	bla_{PDC-3}											
	$bla_{PDC-374}$											
Ciprofloxacin	*crpP*											
Multidrug Efflux Pumps	*mexAB-OprM*											
	mexCD-OprJ											
	mexEF- OprN											
	mexXY- OprM											
	emrE											
	pmpM											
Acquired Antibiotic Resistance Mutations												
Efflux Pump Regulation	*mexRT*											
	nalC											
	nfxB											
	oprD											
Fluoroquinolones	*gyrA*											
	gyrB											
	parC											
	parE											
Colistin	*pmrAB*											
	phoP											



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