Association between possession of ExoU and antibiotic resistance in *Pseudomonas aeruginosa*

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Abstract

Virulent strains of *Pseudomonas aeruginosa* are often associated with an acquired cytotoxic protein, exoenzyme U (ExoU) that rapidly destroys the cell membranes of host cells by its phospholipase activity. Strains possessing the *exoU* gene are predominant in eye infections and are more resistant to antibiotics. Thus, it is essential to understand treatment options for these strains. Here, we have investigated the resistance profiles and genes associated with resistance for fluoroquinolones and beta-lactams. A total of 22 strains of *P. aeruginosa* from anterior eye infections, microbial keratitis (MK), and the lungs of cystic fibrosis (CF) patients were used. Based on whole genome sequencing, the prevalence of the *exoU* gene was 61.5% in MK isolates whereas none of the CF isolates possessed this gene. Overall, higher antibiotic resistance was observed in the isolates possessing *exoU*. Of the *exoU* strains, all except one were resistant to fluoroquinolones, 100% were resistant to beta-lactams. 75% had mutations in quinolone resistance determining regions (*T81I gyrA* and/or *S87L parC*) which correlated with fluoroquinolone resistance. In addition, *exoU* strains had mutations at *K76Q, A110T, and V126E in ampC*, *Q155I and V356I in ampR* and *E114A, G283E, and M288R in mexR* genes that are associated with higher beta-lactamase and efflux pump activities. In contrast, such mutations were not observed in the strains lacking *exoU*. The expression of the *ampC* gene increased by up to nine-fold in all eight *exoU* strains and the *ampR* was upregulated in seven *exoU* strains compared to PAO1. The expression of *mexR* gene was 1.4 to 3.6 fold lower in 75% of *exoU* strains. This study highlights the association between virulence traits and antibiotic resistance in pathogenic *P. aeruginosa*.

Introduction

*Pseudomonas aeruginosa* infections can be severe in people with a compromised immune system and impaired anatomical structures caused by, for example burns, cystic fibrosis or mechanical abrasions [1]. *P. aeruginosa* is a successful opportunistic pathogen in part due to
its production of a diverse repertoire of pathogenic factors and its innate ability to evade the host immune system [2]. Treatment of P. aeruginosa infections can be challenging due to the inherent antibiotic resistance, where some studies have shown that half of the isolates from clinical infections were resistant to antibiotics [3]. Furthermore, reports on co-selection of antibiotic resistance and pathogenic factors indicate that antibiotic resistance may be a factor for the evolution of more virulent strains of P. aeruginosa or vice versa [4–13].

Many Gram-negative bacteria, including P. aeruginosa, possess type III secretion systems (TTSS), which they utilise to introduce virulence factors directly into host cells [14]. In P. aeruginosa, TTSS transports four secreted factors: ExoU, ExoS, ExoY and ExoT. However, all of these factors may not be common in all P. aeruginosa strains. For example, the exoS gene was present in 58–72%, the exoU gene in 28–42%, the exoY gene in 89% and the exoT gene in 92–100% of isolates from acute infections [15]. Pathogenic strains contain either exoU or exoS, but rarely both [16, 17]. The exoU gene is associated with a genomic island and its acquisition may cause loss of the exoS [18, 19]. The exoU gene encodes a cytotoxic protein that rapidly destroys the cell membranes of mammalian cells by its phospholipase activity [19]. The presence of exoU correlates with phenotypes that are responsible for the severe outcome of many infections including pneumonia [20] and keratitis [21]. Up to two-thirds of ocular isolates of P. aeruginosa possess the exoU gene [22], which is a much higher rate than the isolates from other infections [6, 23, 24].

The frequency of antibiotic resistance of the exoU gene carrying strains is higher than that of exoS-strains; [5, 10] the reason for this higher frequency remains undefined. P. aeruginosa strains with the exoU gene tend to harbour mutations in quinolone resistance determining regions (QRDRs) that lead to fluoroquinolone resistance [5, 9]. Whilst it is known that strains of P. aeruginosa can possess mutations in resistance determining regions affecting beta-lactam susceptibility, such as the chromosomal beta-lactamase gene (ampC), its transcriptional regulator (ampR) [25] and a repressor gene (mexR) that negatively regulates expression of an active efflux pump (MexAB-OprM) [26], the correlation between the exoU carriage and mutations in drug resistance determining regions has not been extensively examined.

We hypothesised that possession of the exoU gene correlates with mutations not only in QRDRs but also in beta-lactam resistance determining regions. The aim of this study was to examine the correlation between the virulent genotypes (exoS vs. exoU) and resistance to beta-lactam and fluoroquinolone antibiotics in P. aeruginosa strains. Furthermore, we examined the relative expression of specific genes to confirm their role in antibiotic resistance.

**Materials and methods**

**Bacterial isolates and antibiotic susceptibility testing**

Twenty-two P. aeruginosa strains isolated from anterior eye infections, microbial keratitis (MK), or lungs of cystic fibrosis patients from India and Australia were used in this study (Table 1). The minimum inhibitory concentrations (MICs) of ceftazidime (Sigma-Aldrich, Inc., St. Louis, MO, USA), cefepime (European Pharmacopoeia, Strasbourg, France) aztreonam (Sigma-Aldrich, Inc), ticarcillin (Sigma-Aldrich, Inc), imipenem (Sigma-Aldrich, Inc), levofloxacin (Sigma-Aldrich, Inc), ciprofloxacin (Sigma-Aldrich, Inc), and moxifloxacin (European Pharmacopoeia) were determined by the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) [27]. The MIC was taken as the lowest concentration of an antibiotic in which no noticeable growth (turbidity) was observed [28] and the break point was established according to published standards [29, 30]. Both resistant and intermediate resistant strains were considered here as resistant.
DNA extraction and sequencing

Bacterial DNA was extracted from overnight cultures grown on Trypticase Soy Agar (TSA; Oxoid Ltd., Basingstoke, UK), using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The extracted DNA was sequenced on MiSeq (Illumina, San Diego, CA, USA) platform generating 300 bp paired-end reads. The paired-end library was prepared using Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). All of the libraries were multiplexed on one MiSeq run. Genome assembly and annotations were performed using SPAdes version 3.11.1 [31] and Prokka version 1.7 [32]. BLAST search was performed to investigate carriage of exoU and exoS genes. All nucleotide sequences were deposited in NCBI GenBank database under Bio-project accession number PRJNA431326.

Sequence analysis and variant calling

The mutations in selected resistance genes (gyrA, gyrB, parC, parE, mexR, ampC, ampD and ampR) of each strain were determined with reference to P. aeruginosa PAO1 (Genbank RefSeq accession no. NC_002516.2). Briefly, the reference genome was mapped to the paired-end reads for each isolate using Bowtie2 version 2.3.2 [33] and the variants were compiled and annotated using SAMtools, version 1.7 [34] and SnpEff version 4.3 [35]. The QRDRs were assigned to amino acid positions 83 to 87 of the GyrA protein, positions 429 to 585 of the GyrB protein, positions 82 to 84 of the ParC protein, and positions 357 to 503 of the ParE protein [36]. For ampC variants, mutations different from common polymorphisms (G27D,
R79Q, T105A, Q156R, L176R, V205L, and G391A), which are present in both susceptible and non-susceptible strains [37] were considered here. Mutations in $mexR$ or $ampR$ were considered as significant for resistance from previous literature [38–40].

**Total RNA extraction and qRT-PCR analysis**

Strains were revived from frozen stocks into 5 mL Trypticase Soy Broth (TSB; Oxoid) and grown to mid-exponential phase (OD$_{660}$ 1.5) and 1 ml was centrifuged at 6000 g for 3 min to harvest the cells. The pellet was mixed with 1 mg/ml lysozyme in Tris-EDTA buffer (TE; 10 mM Tris-hydrochloride and 1.0 mM EDTA pH 8.0) to lyse the cells. RNA extraction was performed using the ISOLATE RNA Mini Kit (Bioline, London, UK) following the manufacturer instructions for RNA isolation from bacteria. The RNA extract was treated with DNase1 (Bioline) to eliminate the DNA contamination and purified by ethanol precipitation [41]. RNA purity and concentration was measured by NanoDrop spectrophotometer (ND-1000, Thermo-Fisher, MA, USA). cDNA was synthesized from 1 $\mu$g of total RNA using SuperScript First-Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) employing random primers and following the manufacturer’s protocol. Quantitative PCR was performed with a PowerUp SYBR Green Master Mix (Applied Biosystems, Austin, TX, USA), using 96 well optical plates (Micro-Amp Fast Optical, Applied Biosystems) following the manufacturer’s instructions and cycle conditions. A 7500 Fast Real-Time PCR System (Applied Biosystems) was used to measure the expression levels of the target DNA sequences using gene specific primers (Table 2). The relative expression levels were quantified using the comparative C$_{T}$ method [42] to obtain the fold change in each gene with reference to the respective genes of $P$. aeruginosa PAO1, which does not carry the $exoU$ gene. A house keeping gene, $rpsL$ encoding the 30S ribosomal protein S12, was used as an internal expression control for normalisation. The experiments were carried out three times in triplicate and the mean and standard deviations were calculated.

**Results**

**Possession of $exoU$/exoS and antibiotic resistance**

BLAST search showed that of the 22 isolates, 8 out of the 13 eye isolates (62%) possessed the $exoU$ gene, while it was absent from the cystic fibrosis (CF) isolates (Fig 1B). Except for a CF strain (PA57) which lacked both $exoU$ and $exoS$ genes, all strains that lacked the $exoU$ gene carried the $exoS$ gene and none of the studied strains harboured both genes. For the $exoS$ strains, 8/13 showed a medium level (2–16 $\mu$g/ml) of resistance to at least one fluoroquinolone and 8/13 were resistant to at least one beta-lactam, mostly ticarcillin (Fig 1A). Six of these eight strains were resistant to

| Genes | Functions | Primers (5–3) | Length (bp) | Nucleotide position in gene | Product length (bp) | References |
|-------|-----------|---------------|-------------|-----------------------------|---------------------|------------|
| $ampC$ | Cephalosporinase | CGGCTCGTTGAGCAAGACCTTC–F | 22 | 264 | 218 | [46] |
| | | AGTCGCGGACTTTGCTGGCTG–R | 22 | 481 | | |
| $mexR$ | Transcriptional regulator | CGCGAGCTGAGGGAAGAAACC–F | 22 | 217 | 150 | [46] |
| | | CGGGGCAAAACACCTGCGATGC–R | 22 | 366 | | |
| $ampR$ | Transcriptional regulator | TGCTGTGATGACTCTCTCAGAC–F | 20 | 215 | 160 | This Study |
| | | AGATCGATGAAAGGGATGCGG–R | 20 | 374 | | |
| $rpsL$ | 30S ribosomal protein S12 (house keeping gene) | GCAACGAGCTGCAGAAGAAAG–F | 21 | 35 | 201 | [46] |
| | | CGGCTGTGCTCTCTCAGAAGATG–R | 23 | 235 | | |

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both a fluoroquinolone and beta-lactam. All except one (PA175) exoU strain were resistant to at least two tested fluoroquinolones with MICs of between 2–128 μg/ml with six strains having 32 for ciprofloxacin. All the exoU strains were resistant to at least three beta-lactams except for PA175, which was only resistant to ticarcillin and imipenem (Fig 1B, 1C and 1D).

Mutations in target genes of QRDRs and possession of exoU

Mutations in four different QRDRs were examined with reference to P. aeruginosa PAO1. Of the strains containing exoU, 6/8 had a T83I mutation in gyrA and 5/8 had combined mutations in both gyrA (T83I) and parC (S87L); none of the exoS strains had either of these mutations. Strains with mutations in gyrA or parC were resistant to all three fluoroquinolones and these mutations correlated with higher MICs for fluoroquinolones (Table 3). None of the exoS strains possessed mutations in gyrA and parC. However, mutations were observed in gyrB and parE in five exoS-strains which were associated with higher MIC to fluoroquinolones (Table 3). Interestingly, no mutations in gyrB or parE were found in the exoU strains. It should be noted that five strains (PA82, PA17, PA40, PA100 and PA171) had no mutations in any of these genes, but were resistant to at least one fluoroquinolone, although resistance tended to be ≤8 μg/ml, except PA82 which had an MIC of 64 μg/ml for ciprofloxacin.

Mutations associated with beta-lactam antibiotics and exoU

This study also examined mutations in cephalosporinase (ampC) and its regulator (ampR) and the efflux pump MexAB-OprM regulator (mexR). A number of mutations were seen in ampC,
but only those mutations that have been previously reported to be significant contributors to resistance were considered here (all observed mutations are shown in S1 Table). Variation in amino acid position V356I was common in six exoU strains, Q155R was found in strain PA82 and no significant mutations were observed in PA34. Such mutations were, however absent in exoS strains. Similarly, all of the exoU strains had a common mutation in the mexR gene at amino acid position 126, changing valine to glutamic acid, in addition to mutations at A110T in PA34 and K76Q in PA175. Such mutations were not present in the exoS strains. Furthermore, all the exoU strains had various mutations (E114A, G283E, and M288R) in the ampR gene, but only one exoS strain (PA171) had mutations in this gene and this at position 244. The susceptibility results showed that possession of such mutations was associated with higher MICs to various beta-lactams, except for strain PA175 which had all these mutations in mexR, ampC, and ampR genes but was sensitive to cefepime and ceftazidime (Table 4).

### Table 3. Mutations in the quinolone resistance determining region of *P. aeruginosa* and the MIC of fluoroquinolones.

| Strain   | Genes | MIC (μg/ml) of Fluoroquinolones |
|----------|-------|--------------------------------|
|          | gyrA  | gyrB  | parC | parE | CIP | LEVO | MOX |
| **exoU strains** |       |       |       |       |     |      |     |
| PA31     | T83I  | S87L  |      |      | 32  | 32   | 64  |
| PA32     | T83I  | S87L  |      |      | 64  | 32   | 128 |
| PA33     | T83I  | S87L  |      |      | 128 | 32   | 64  |
| PA34     |       |       |      |      | 2   | 2    | 8   |
| PA35     | T83I  | S87L  |      |      | 64  | 32   | 128 |
| PA37     | T83I  | S87L  |      |      | 64  | 32   | 128 |
| PA82     |       |       |      |      | 64  | 4    | 4   |
| PA175    | T83I  |       |      |      | 0.25| 0.25 | 1   |
| **exoS strains** |       |       |       |       |     |      |     |
| PA17     |       |       |      |      | 2   | 1    | 8   |
| PA40     |       |       |      |      | 4   | 2    | 2   |
| PA55     |       |       |      |      | 1   | 0.5  | 4   |
| PA59     |       |       |      |      | 0.125| 0.25| 1   |
| PA64     |       |       |      |      |     |      |     |
| PA66     | E468D |       |      |      | 8   | 16   | 16  |
| PA86     | L457-458A* | | | | 0.5 | 1 | 2 |
| PA92     | S466F | A473V | | | 4 | 4 | 16 |
| PA100    |       |       |      |      | 2   | 1    | 8   |
| PA102    |       |       |      |      | 2   | 4    | 8   |
| PA149    |       |       |      |      | 0.5 | 0.5  | 2   |
| PA157    |       |       |      |      | 0.25| 0.5  | 2   |
| PA171    |       |       |      |      | 4   | 2    | 8   |
| PAO1**   |       |       |      |      | 0.25| 0.25 | 1   |

The numbers denote change in amino acid positions when compared with the genome of *P. aeruginosa* PAO1. **Bold** = resistant or intermediate resistant. CIP = ciprofloxacin, LEVO = Levofloxacin, MOX = Moxifloxacin. *Insertion and frameshift variant. **Reference strain

Expression analysis of ampC, ampR and mexR genes

The relative expression of ampC, ampR and mexR genes in all of the exoU strains and three randomly selected exoS strains (PA55, PA86 and PA149) were compared to *P. aeruginosa* PAO1 to analyse the effect of such mutations on expression (Fig 2). The relative expression of
the ampC was two to nine fold higher in all of the exoU strains and was slightly lower in all three exoS strains compared to PAO1. Similarly, the relative expression of the ampR was at least two fold higher for five out of eight exoU strains. For an exoU strain (PA33), the ampR gene was repressed six fold. The expression of mexR gene was repressed in six exoU strains while overexpression of mexR was observed in two exoS strains relative to PAO1.

**Discussion**

The exoU gene is commonly found in *P. aeruginosa* strains isolated from contact lens-related microbial keratitis, at frequencies of 46–54%, [11] whereas it only occurs in 0–14% of non-ocular isolates [6, 22–24]. Similar to a previous report, [10] exoU+ strains in the current study had higher resistance to beta-lactams than exoS+ strains (100% exoU strains vs. 61% exoS strains were resistant to at least one beta-lactam). ExoU secreting *P. aeruginosa* had more mutations in genes that are associated with beta-lactam resistance (*mexR*, *ampC* and *ampR*) than did exoS + strains. Gene expression analysis suggested that such mutations generally lead to antibiotic resistance, as the expression of ampC and ampR generally increased while the expression of mexR was decreased, compared to the sensitive strain PAO1.

Several *in vivo* and *in vitro* studies have shown that the exoU carrying *P. aeruginosa* is associated with severe outcome of diseases [7, 12, 20, 21, 43, 44]. In additions, results of this and

| Strains | Genes | MIC (µg/ml) towards beta-lactams |
|---------|-------|-------------------------------|
|         | mexR  | ampC  | ampR  | CFT | CEF | TIC | IMI | AZT |
| exoU strains |       |       |       |     |     |     |     |     |
| PA31    | V126E | V356I | G283E,M288R | 16  | 16  | 64  | 4  | 16  |
| PA32    | V126E | V356I | G283E,M288R | 16  | 8   | 64  | 4  | 32  |
| PA33    | V126E | V356I | G283E,M288R | 32  | 16  | 128 | 8  | 8   |
| PA34    | A110T, V126E | E114A, G283E,M288R | 4  | 32  | >128 | 16  | 8  |
| PA35    | V126E | V356I | G283E,M288R | 16  | 32  | 128 | 8  | 16  |
| PA37    | V126E | V356I | G283E,M288R | 16  | 8   | 64  | 4  | 8   |
| PA82    | V126E | Q155I | G283E | >128 | >128 | 32  | 1  | 8   |
| PA175   | K76Q, V126E | V356I | G283E,M288R | 2   | 4   | 64  | 4  | 8   |
| exoS strains |       |       |       |     |     |     |     |     |
| PA17    |       |       |       | 4   | 4   | 128 | 1  | 8   |
| PA40    |       |       |       | 1   | 2   | 64  | 4  | 8   |
| PA55    |       |       |       | 32  | 64  | 32  | 4  | 8   |
| PA59    |       |       |       | 2   | 2   | 16  | 1  | 8   |
| PA64    |       |       |       | 16  | 64  | 32  | 1  | 4   |
| PA66    |       |       |       | 16  | 64  | 32  | 0.25 | 8 |
| PA86    |       |       |       | 4   | 8   | 64  | 1  | 8   |
| PA92    |       |       |       | 32  | 64  | 8   | 1  | 1   |
| PA100   |       |       |       | 4   | 8   | 16  | 2  | 0.5 |
| PA102   |       |       |       | 1   | 1   | 16  | 0.5 | 4   |
| PA149   |       |       |       | 2   | 4   | 16  | 1  | 4   |
| PA157   |       |       |       | 2   | 4   | 16  | 1  | 4   |
| PA171   |       |       | R244W | 2   | 1   | 32  | 4  | 8   |
| PAO1**  |       |       |       | 1   | 1   | 16  | 1  | 4   |

The numbers denote change in amino acid positions when compared with the genome of *P. aeruginosa* PAO1.

Bold = resistance or intermediate resistance.
CFT = ceftazidime, CEF = cefepime, TIC = ticarcillin, IMI = imipenem, AZT = Aztreonam.

**Reference strain**

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other studies confirmed that \textit{exoS}+ and \textit{exoU}+ strains have different antibiotic resistance patterns \cite{10, 45}. Therefore, they may require different treatment strategies. Knowing the virulence gene profiles, the clinical outcome of the patients the resistance patterns might be predicted, and this information could be used in deciding appropriate antibiotic treatment.

A mutation at amino acid position 126 (changing valine to glutamic acid) in \textit{MexR} was common in all \textit{exoU}+ strains. Underexpression of \textit{mexR} has been associated with antibiotic resistance in \textit{P. aeruginosa} \cite{46}. Mutations in \textit{mexR} contribute to the over-expression of the MexAB-OprM efflux pump \cite{47, 48} which in turn is specific to increased resistance to beta-lactams \cite{49}. The current study demonstrated that mutation in \textit{mexR} was correlated with lower transcription of this gene in 75\% of \textit{exoU}+ strains. The higher expression of \textit{mexR} in \textit{exoS}+ strains observed here appears to support the hypothesis that possession of the \textit{exoU} gene is associated with beta-lactam resistance.

Various different mutations in \textit{ampC} and \textit{ampR} between \textit{exoU} and \textit{exoS} subpopulations were revealed in the current study. Mutations in \textit{ampC} and \textit{ampR} were more common in \textit{exoU} + strains. Berrazeg \textit{et al} \cite{37} demonstrated that mutations in \textit{ampC} at amino acid positions G27D, R79Q, T105A, Q156R, L176R, V205L, and G391A were not correlated with beta-lactam resistance and hence were excluded here from analysis in the current study. Mutations at amino acid positions Q155I and V356I in \textit{ampC} were observed in \textit{exoU}+ strains, and all these

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Expression of cephalosporinase (\textit{ampC}) its regulator (\textit{ampR}) and the efflux pump MexAB-OprM regulator (\textit{mexR}) in strains. The relative expression levels were compared to \textit{Pseudomonas aeruginosa} PAO1 (widetype, non-\textit{exoU} strain) and are presented as fold change in gene expression. Error bars represent standard deviation of the mean fold change.}
\end{figure}
strains had increased gene expression of \textit{ampC}, suggesting that these mutations may be responsible for this reduced expression. A few \textit{exoS}+ strains and one \textit{exoU}+ strain (PA34) did not have such mutations but were resistant to some beta-lactams. Point mutations in \textit{ampR} (at 114, 182, 283, and 288) can also be responsible for beta-lactam resistance [38] and \textit{exoU}+ strain PA34 carried mutations at E114A and G283E. Mutations at G283E and M288R in \textit{ampR} were exclusive to \textit{exoU}+ strains. These mutations were correlated with over-expression of \textit{ampC} and \textit{ampR} in \textit{exoU}+ strains. The precise mechanism by which acquisition of the \textit{exoU} associated genomic island results in these mutations is not known. For resistance of \textit{exoS}+ strains, it is possible that beta-lactam resistance involves other resistance mechanisms, such as the upregulation of efflux systems MexCD-OprJ, MexEF-OprN, and MexXY-OprM, [50] and hence requires further study for elucidation.

Possession of \textit{exoU} was also associated with higher MICs to fluoroquinolones compared to possession of \textit{exoS}, and this has been shown in previous studies [5, 10, 45–53]. Mutations in the QRDRs of target genes topoisomerase II (\textit{gyrA} and \textit{gyrB}) and topoisomerase IV (\textit{parC} and \textit{parE}) have been previously shown to increase fluoroquinolone resistance in \textit{P. aeruginosa} [54, 55]. Here, it was also observed that fluoroquinolone resistance in \textit{exoU} strains was correlated with a combination of mutations in \textit{gyrA} and \textit{parC}. Sequence analysis indicated that six out of eight \textit{exoU} strains had at least one mutation in either \textit{gyrA} (T83I) or \textit{parC} (S87L). Consistent with other studies, [54, 56] such mutations were responsible for very high MICs to fluoroquinolones. This suggests the possibility that more virulent strains of \textit{P. aeruginosa} that have the \textit{exoU} gene may evolve in the clinical environment where high concentrations of fluoroquinolones are used for treatment; for example, in eye infections [57, 58]. The conditions that favour selection of \textit{exoU}+ strains might result in increased resistance to other antibiotics. In addition, this study also detected several mutations in QRDRs in the \textit{exoS}+ population. Mutations at position E468D of \textit{gyrB} and A473V of \textit{parE} were associated with increase MICs to all tested fluoroquinolones. Such mutations have been previously associated with fluoroquinolone resistance \textit{P. aeruginosa} [59, 60]. It appears that different types of mutations in QRDRs evolved in the \textit{exoU}+ and \textit{exoS}+ strains.

An \textit{exoS}+ strain (PA55) was resistant to all beta lactams except aztreonam but no mutations were observed in the studied genes and expression of genes did not correlate with phenotypic resistance. Furthermore, an \textit{exoU}+ strain (PA82) did not have any mutations in QRDRs but was resistant to ciprofloxacin and levofloxacin. However, mutations in V126E the \textit{mexR} of PA82 has been associated with resistance to fluoroquinolone [26, 61]. The evidence from the current study suggests that mutations A110T (PA34) and K76Q (PA175) in \textit{mexR} may confer susceptibility to ceftazidime even in presence of the V126E mutation. This needs to be confirmed by further study.

In addition, we observed a link between the \textit{exoU} and the origin of the strains in India because only one Australian strain (PA175) possessed the \textit{exoU} gene and the resistance rate was higher in the cohort of Indian strains. However, it should be noted that the possession of \textit{exoU} is highly correlated with antibiotic resistance in \textit{P. aeruginosa} regardless of source and geographical site of isolation [5, 9, 10, 13, 45]. \textit{exoU}+ strains may have an evolutionary advantage by having the potential to be both more resistant and more virulent. This is supported by a study that showed higher prevalence of the \textit{exoU} in isolates collected from the hospital environment [62]. A correlation between the geographic origin and the \textit{exoU} carriage was observed in this study potentially due to the relatively unregulated use of antibiotics in India compared to Australia [63]. However, these observations require confirmation with a larger sample size that should include isolates from various sources and a study of associated epidemiological data.
In conclusion, *exoU* carrying strains, which are common in ocular isolates, showed different antibiotic resistance pattern from isolates with *exoS* genotype. The *exoS*+ strains may be protected from the action of antibiotics due to their ability to cause mammalian cells to ingest them (so-called invasive strains). Their residence inside mammalian cells may offer protection from antibiotics and so diminish selection pressure to convert to antibiotic resistance. The *exoU*+ strains had more mutations in drug resistance determining genes (*gyrA*, *parC*, *mexR*, *ampC* and *ampR*), which was likely to be the cause of higher antibiotic resistance in *exoU*+ strains. Differences in mutational rate in two different virulent genotypes indicate more virulent strains can favourably be evolved in the antibiotic rich environment. Therefore, understanding of both virulence traits and antibiotic resistance is essential for more effective prevention of antibiotic resistance.

Supporting information

S1 Table. Variants of *ampC* gene.
(PDF)

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