Extensively Drug-Resistant *Pseudomonas aeruginosa* ST309 Harboring Tandem Guiana Extended Spectrum β-Lactamase Enzymes: A Newly Emerging Threat in the United States

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**Background.** Treatment of serious infections due to multidrug-resistant (MDR) *Pseudomonas aeruginosa* remains a challenge, despite the introduction of novel therapeutics. In this study, we report 2 extensively drug-resistant clinical isolates of sequence type (ST) 309 *P. aeruginosa* resistant to all β-lactams, including the novel combinations ceftolozane/tazobactam, ceftazidime/avibactam, and meropenem/vaborbactam.

**Methods.** Isolates were sequenced using both short-read (Illumina) and long-read technology to identify resistance determinants, polymorphisms (compared with *P. aeruginosa* PAO1), and reconstruct a phylogenetic tree. A pair of β-lactamases, Guiana extended spectrum β-lactamases (GES)-19 and GES-26, were cloned and expressed in a laboratory strain of *Escherichia coli* to examine their relative impact on resistance. Using cell lysates from *E. coli* expressing the GES genes individually and in tandem, we determined relative rates of hydrolysis for nitrocefin and ceftazidime.

**Results.** Two ST309 *P. aeruginosa* clinical isolates were found to harbor the extended spectrum β-lactamases GES-19 and GES-26 clustered in tandem on a chromosomal class 1 integron. The presence of both enzymes in *E. coli* was associated with significantly elevated minimum inhibitory concentrations to aztreonam, cefepime, meropenem, ceftazidime/avibactam, and ceftolozane/tazobactam, compared with those expressed individually. The combination of ceftazidime/avibactam plus aztreonam was active in vitro and used to achieve cure in one patient. Phylogenetic analysis revealed ST309 *P. aeruginosa* are closely related to MDR strains from Mexico also carrying tandem GES.

**Conclusions.** The presence of tandem GES-19 and GES-26 is associated with resistance to all β-lactams, including ceftolozane/tazobactam. Phylogenetic analysis suggests that ST309 *P. aeruginosa* may be an emerging threat in the United States.

**Keywords.** carbapenem-resistant *Pseudomonas aeruginosa*; ceftolozane/tazobactam; combination therapy; GES beta-lactamide.

The Centers for Disease Control and Prevention has identified multidrug-resistant (MDR) *Pseudomonas aeruginosa* as a serious threat, and treatment of such isolates often requires the use of drugs with significant toxicities [1]. Carbapenem resistance in *P. aeruginosa* in the United States is mostly mediated by noncarbapenemase mechanisms [2]. In response, novel therapeutics such as ceftolozane/tazobactam (C/T), which is stable to the pseudomonal AmpC β-lactamase and less susceptible to porin loss and drug efflux, have entered the clinic to combat this threat [3]. Although C/T remains broadly active against most clinical isolates of carbapenem-resistant *P. aeruginosa*, resistance associated with mutations in AmpC or the expression of acquired β-lactamases has been described [4, 5].

The Guiana extended spectrum β-lactamase (GES) enzyme was first isolated from a *Klebsiella pneumoniae* obtained from a rectal swab of an infant in Cayenne, French Guiana [6] and, since then, 32 variants have been identified. In general, these enzymes confer resistance to penicillins, including ureidopenicillins, and oxyimino-cephalosporins, but they show less activity against aztreonam and imipenem [6, 7]. Nonetheless, specific substitutions can significantly alter this susceptibility profile, including G243A, which improves activity against aztreonam.
and G170S, conferring increased carbapenem hydrolyzing activity [8]. These enzymes are found in association with class 1 integrons, a gene cassette acquisition system known to harbor multiple antimicrobial resistance determinants associated with mobile genetic elements [9]. A study of isolates from Mexico City identified ST309 as a potential high-risk clone associated with acquired β-lactamases, and a large percentage of carbapenem-resistant *P aeruginosa* from Mexico have been associated with GES enzymes carried by class 1 integrons [10, 11]. In this study, we report the identification of 2 isolates of extensively drug-resistant *P aeruginosa* ST309 causing bloodstream infections in unrelated patients and carrying simultaneously 2 variants of *bla* 

### METHODS

#### Bacterial Strains and Growth Conditions

Clinical *P aeruginosa* isolates PA_HTX1 and PA_HTX2 were purified on MacConkey agar. Single colonies were tested to ensure they retained the resistance phenotype, and stocks were frozen in Brucella broth plus 15% glycerol and stored at −80°C. *Escherichia coli* TG1 was grown on Lysogeny broth (LB) or LB agar supplemented with 100 µg/mL ampicillin or 25 µg/mL chloramphenicol when needed. All bacteria were grown at 37°C with gentle agitation for liquid media.

#### Genetic Manipulation of *bla* Genes

The genes *bla* 

#### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) of the clinical isolates PA_HTX1 and PA_HTX2 was performed in the clinical laboratory using a Microscan Walk-Away and E-test (for colistin, C/T and ceftazidime/avibactam [CZA]). Synergy testing was performed by applying an aztreonam (ATM) or meropenem (MEM) E-test strip to Mueller-Hinton agar plates containing either ceftazidime plus avibactam (Allergan), at a final concentration of 2.2 µg/mL of the avibactam component, or vaborbactam alone (The Medicines Co.) at 2 µg/mL. This concentration was selected to mimic the serum nadir of avibactam and vaborbactam from human pharmacokinetic/pharmacodynamic data [13–17]. The AST for the *E coli* mutants was performed in triplicate by normalizing strains to an optical density (OD) 

#### Whole-Genome Sequencing and *Pseudomonas aeruginosa* Phylogenetics

Genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN). Genome sequencing of the 2 isolates was performed on a Miseq (Illumina) and with MinION (Oxford Nanopore Technologies) for long reads. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) database (Bioproject: PRJNA415853). Resistance detection, polymorphism analysis, and reconstruction of the phylogenetic tree were performed using a custom pipeline. To study the phylogenetic relationships between the isolates with other *P aeruginosa* genomes, all assembled genomes of *P aeruginosa* available at the NCBI genome database were downloaded, and the MLST was obtained using the mlst tool. Nine additional ST309 genomes were identified, and PAO1 (GCA_000006765.1), PA_D1 (GCA_001721745.1), L10 (GCA_002223805.1), M18 (GCA_000226155.1), and FRD1 (GCA_000829885.1) from STs 539, 1971, 253, 1239, and 111, respectively, were used as references (Supplementary Figure 1). Genome annotation of the 16 genomes was carried out with RAST [18], a core genome was determined with Roary [19], and multiple sequence alignment of the orthogroups belonging to the core genome was done with Muscle [20] and later concatenated to be used as the matrix for phylogenetic reconstruction with RAxML [21]. The best tree of 20 runs was selected with a General Time Reversible evolution model and a Gamma model of rate heterogeneity with 100 bootstrap resampling. The tree was plotted with iTol [22] after rooting at the split between ST309 and the other references, and modification of the distance between the root and the branches of ST309 was changed to a value near zero to allow the visualization of ST309 branching.

#### Expression of Guiana Extended Spectrum β-Lactamase Proteins and β-Lactamase Assay

Overnight cultures of *E coli* cells transformed with GES-19, GES-26, or GES-19/GES-26 in the IPTG-inducible pBA169 expression plasmid were diluted 1:100 in 50 mL LB medium containing 25 µg/mL chloramphenicol (to maintain the plasmid). The diluted cultures were incubated with shaking at 37°C for 0.8. The IPTG was added to a final concentration of 0.5 mM, and cells were incubated with shaking at 20°C for another 20 hours to induce the expression of GES.
proteins. Cells were then pelleted by centrifugation at 6000 rpm for 20 minutes, and the cell pellet was resuspended in 2 mL B-PER (Thermo Fisher) containing 100 µg/mL lysozyme and 20 µg/mL Dnase1 and incubated at room temperature for 15 minutes. Samples were then centrifuged again at 13000 rpm for 5 minutes to collect cell lysate in the supernatant. To ensure the expression of GES enzymes, 50 µM nitrocefin diluted in 50 mM HEPES (pH7.4) was incubated with 1 µL cell lysate, and absorbance at 482 nm (A482) was monitored on Beckman DU800 spectrometer using a 1-cm cuvette. To determine hydrolysis of ceftazidime by the GES enzymes, 50 µM ceftazidime diluted in 50 mM HEPES (pH7.4) was incubated with 1 µL cell lysate, and absorbance at 260 nm (A260) was monitored on Beckman DU800 spectrometer using a 1-cm cuvette.

RESULTS

Two patients admitted to separate hospitals in a large urban hospital network developed bacteremia with MDR P. aeruginosa. In the first case, the patient had 2 weeks of prior exposure to cefepime (FEP), metronidazole, and vancomycin. Subsequently, the patient received MEM for a complicated intra-abdominal infection before isolation of MDR P. aeruginosa (PA_HTX1) (Table 1, Supplementary Table 2) resistant to all β-lactams, including novel β-lactam/β-lactamase inhibitor combinations. Despite therapy with polymyxin B, MEM, and amikacin, the patient’s clinical condition worsened. On the suspicion that the isolate may harbor a metallo-β-lactamase, synergy testing with the combination of CZA plus ATM was performed, with an observed reduction in the ATM MIC from >256 µg/mL alone to 8 µg/mL in the presence of avibactam. The patient was started on 1.5 grams of CZA given after hemodialysis with ATM (2 grams IV daily) and was ultimately cured after 105 days of therapy. In the second case, the patient had bronchoalveolar lavage cultures positive for E.coli, and received 5 days of MEM, before developing MDR P. aeruginosa pneumonia with a similar antimicrobial susceptibility profile to PA_HTX1 (PA_HTX2) (Table 1, Supplementary Table 2). The patient received colistin, MEM, and 3 doses of C/T, but developed bacteremia despite antibiotics, and ultimately died due to the infection. An epidemiologic investigation within the hospital system did not reveal a link between the 2 patients, and there was no shared intensive care unit staff or equipment between the 2 hospitals during the period of patient admissions.

We performed whole-genome sequencing on PA_HTX1 and PA_HTX2. Both isolates were identified as sequence type 309 and carried a chromosomal class 1 integron with multiple resistance determinants, including blagES-19 and blagES-26 (Figure 1A, Supplementary Table 3). To determine whether this tandem carriage of GES β-lactamases was present in other ST309 P. aeruginosa, we performed a search of the assembled P. aeruginosa genomes available in the NCBI database. Eleven ST309 P. aeruginosa (including the 2 patient isolates in this study) were identified. It is interesting to note that a cluster of 5 ST309 isolates of clinical origin harboring tandem blagES genes from the United States and Mexico, isolated from 2006 to 2017, appear to form a distinct group (Figure 1B). An analysis of the integron and surrounding genomic context revealed that the Houston isolates possessed an OXA-2 β-lactamase and aminoglycoside-modifying enzymes not present in the Mexican isolates (Figure 1C). These strains possess a high degree of shared gene content, suggesting a close relationship between the isolates, although several gaps in the genomic alignment of the Houston strains are likely driven by phage and mobile genetic elements (Figure 1D, Supplementary Table 4).

To investigate the genetic bases of the MDR phenotype, we compared genes associated with antimicrobial resistance using P. aeruginosa PAO1 as the reference strain (Supplementary Table 5). The ampC gene for both PA_HTX strains codes for the PDC-19a variant, and polymorphisms in ampR, ampD, and ampG have been previously reported in both sensitive and resistant strains. The sequences of the

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**Table 1. Minimum Inhibitory Concentrations for β-Lactams and β-Lactam/β-Lactam Inhibitor Combinations**

| Strain          | AMP (µg/mL) | ATM (µg/mL) | FEP (µg/mL) | CZA (µg/mL) | C/T (µg/mL) | MEM (µg/mL) | CZA + ATM (µg/mL) | MEM + VAB (µg/mL) | ATM + VAB (µg/mL) |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------------|-------------------|------------------|
| PA_HTX1         | ND          | >256        | >256        | 128         | >256        | >32         | 8                 | >32               | >256             |
| PA_HTX2         | ND          | >256        | >16         | >256        | >256        | >32         | 4                 | >32               | >256             |
| TG1 + pBA169    | 3           | 0.032       | 0.064       | 0.25        | 0.064       | 0.016       | ND                | ND                | ND               |
| TG1 + pBA169::blaGES-19 | >256       | 1.5         | 1           | 4           | 0.125       | 0.012       | ND                | ND                | ND               |
| TG1 + pBA169::blaGES-26 | >256       | 0.25        | 0.5         | 2           | 0.38        | 0.016       | ND                | ND                | ND               |
| TG1 + pBA169::blaGES-19-26 | >256       | 0.094       | 0.5         | 0.125       | 0.125       | 0.064       | ND                | ND                | ND               |
| TG1 + pBA169::blaGES-26 | >256       | 0.094       | 0.5         | 0.125       | 0.125       | 0.064       | ND                | ND                | ND               |

Abbreviations: AMP, ampicillin; ATM, aztreonam; C/T, ceftolozane/tazobactam; CZA, ceftazidime/avibactam; FEP, cefepime; MEM, meropenem; MIC, minimum inhibitory concentration; ND, not done; VAB, vaborbactam.

aPerformed by broth microdilution, all others performed by E-test.

bVaborbactam 2.2 µg/mL, to mimic the mean steady-state nadir in human plasma.
ampD homologs ampDh2 and ampDh3, as well as dacB, which encodes a low molecular weight penicillin-binding protein associated with AmpC expression, were identical to PAO1. The oprD gene was disrupted by insertion of ISPa1328, an IS256 family element that truncated the first 126 base pairs of oprD including the start codon. This is predicted to result in loss of oprD expression and likely contributes to the carbapenem resistance phenotype seen in these isolates. In addition, both HTX isolates possessed a mutation predicted to result in a deletion of 6 amino acids (residues 189–194) near the C-terminal end of MexZ, a repressor of the MexXY efflux pump. This resistance-nodulation-cell division family efflux pump is the primary determinant of aminoglycoside resistance in P. aeruginosa, especially in cystic fibrosis lung isolates [23, 24]. The region with the mutation in the HTX isolates lies at the dimerization interface of MexZ, and we hypothesize that this change may lead to upregulation of the MexXY efflux pump [25]. Mutations in gyrA and parC seen in these isolates have been previously linked to decreased susceptibility to fluoroquinolone antibiotics [2].

The PA_HTX isolates were resistant to all available β-lactams, including the novel combinations of C/T, CZA, and MEM/vaborbactam (Table 1). In the presence of CZA, the MIC of ATM decreased from >256 µg/mL in PA_HTX1 and PA_HTX2 to 8 and 4 µg/mL, respectively. No change in MIC was found with either MEM or ATM in combination with vaborbactam. To evaluate the spectrum of the integron-encoded β-lactamases, blaGES-19 and blaGES-26 were expressed individually, and blaGES-19 and blaGES-26 in tandem, in E. coli TG1 [12] (Table 1). The presence of blaGES-19 and blaGES-26 alone led to modest increases in MICs for ATM, FEP, CZA (GES only), and C/T, but not MEM, compared with TG1 carrying empty pBA169. In contrast, the presence of both blaGES-19 and blaGES-26 resulted in a marked increase of MICs of ATM, FEP, CZA, C/T, and MEM. This effect was reversed by addition of the β-lactamase inhibitors avibactam and vaborbactam. Although
the IPTG-induced levels of enzyme are not physiologic, the results suggest that the presence of both enzymes, rather than one alone, leads to high-level resistance.

To support that the combination of the GES enzymes is responsible for the phenotype, cell lysates of induced E coli TG1 with each of the GES constructs were tested for hydrolysis of nitrocefin and ceftazidime (Figure 2). Nitrocefin hydrolysis by the cell lysate of GES-19, GES-26, and GES-19/GES-26 was 0.34 ± 0.03, 0.19 ± 0.006, and 1.34 ± 0.02 μM s⁻¹ per μL of cell lysate, respectively. It is interesting to note that the cell lysate of the combined GES-19/GES-26 had 4- and 7-fold higher hydrolytic activity than that of GES-19 or GES-26 alone, respectively. Minimal hydrolysis of ceftazidime was seen in the GES-26 cell lysate, whereas hydrolysis by GES-19 was 0.029 ± 0.0015 μM s⁻¹ per μL. With the GES-19/GES-26 lysate, ceftazidime hydrolysis was 0.048 ± 0.0084 μM s⁻¹ per μL, a 1.7-fold increase. Although we were not able to quantify absolute differences in expression of GES enzymes in each lysate, which could account for differences in nitrocefin and ceftazidime hydrolysis, these data suggest that expression of both β-lactamases, as opposed to a single enzyme, provides increased hydrolysis of β-lactams. Thus, the tandem acquisition of GES enzymes in combination with efflux and decreased permeability in P aeruginosa has the potential to further compromise β-lactam activity.

**DISCUSSION**

Infections due to MDR P aeruginosa are associated with increased morbidity, mortality, and higher healthcare costs [26, 27]. This burden of resistance has largely been driven by the success of the epidemic clones ST 111, 175, and 235, which possess a variety of virulence factors and have spread globally [28]. The MDR phenotype in these strains stems from the propensity to develop mutational mechanisms of resistance, such as loss or inactivation of the porin gene oprD (leading to carbapenem resistance) or overexpression of an array of intrinsic efflux pumps [2]. The introduction of newer therapeutics such as C/T, which circumvents these common resistance mechanisms [3, 29], has offered clinicians additional options for treating MDR strains. However, the emergence of resistance to C/T associated with mutations in the intrinsic AmpC cephalosporinase is increasingly reported [4]. In addition, the acquisition of β-lactam resistance arising from exogenous β-lactamase enzymes is another growing concern, especially in the high-risk clones. These resistance determinants are often associated with class 1 integrons in the context of integrative conjugative elements or pathogenicity islands, increasing the potential for dissemination [30]. Most frequently reported are metallo-β-lactamases, specifically VIM and IMP, although a variety of class D OXA enzymes and class A enzymes, including KPC and GES, among others, have been described [5, 31].

The cases reported here offer several important insights into the evolving landscape of MDR P aeruginosa. Both were serious infections due to ST309 P aeruginosa with an extensive antibiotic resistance phenotype. By phylogenetic analysis, these isolates were linked to strains reported from several clusters of ST309 P aeruginosa infections in Mexico City, which also displayed an MDR phenotype [11]. Indeed, GES enzymes seem to be an important contributor to carbapenem resistance in P aeruginosa from Mexico, with a prevalence of 30.6% [10]. Furthermore, these enzymes are also present as plasmid-borne resistance determinants among Enterobacteriaceae from this region, suggesting a potential for spread across species [32]. Neither of our patients had a reported history of travel to Mexico, suggesting that the geographic distribution of ST309, or the mobile genetic element carrying the tandem GES genes, may be more widespread. It is important to understand the circulating resistance mechanisms to detect the potential emergence of new epidemic clones.

Another interesting feature of the study was that high-level β-lactam resistance was associated with the 2 GES variants,

**Figure 2.** Rates of hydrolysis of nitrocefin and ceftazidime from Escherichia coli cell lysates. Escherichia coli lysates containing Guiana extended spectrum β-lactamase (GES-19, GES-26, and GES-19/GES-26) were prepared from cultures grown in the presence of 0.5 mM isopropyl β-D-thiogalactopyranoside for 20 hours to induce protein expression. (A) Hydrolysis of 50 μM nitrocefin in 50 mM HEPES pH7.4 monitored by absorbance at 482 nm. Cell lysate with both enzymes was more efficient than that of GES-19 or GES-26 individually by 4-fold and 7-fold, respectively. (B) Hydrolysis of 50 μM ceftazidime in 50 mM HEPES pH7.4 monitored by absorbance at 260 nm. Hydrolysis of ceftazidime in the presence of cell lysate containing both enzymes was 1.7-fold higher than GES-19 alone.

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present in tandem, in our isolates. In general, the GES enzymes are extended spectrum β-lactamases, although amino acid substitutions leading to increased carbapenemase activity (G170S) are reported. In a recent study, the G170S substitution (in the GES-6 variant) was linked to both increased cefepime hydrolysis and decreased inhibition of the enzyme by clavulanate and tazobactam, although avibactam appears to retain activity [33]. The GES enzymes mimic the phenotype of class B metallo-β-lactamases in that they efficiently hydrolyze all β-lactams with the exception of monobactams, which is likely the basis for the observed efficacy of the CZA plus ATM combination. Avibactam, a novel β-lactamase inhibitor, can bind reversibly and inhibit multiple β-lactamases simultaneously without being susceptible to hydrolysis [34]. It inhibits class A β-lactamases and is effective against AmpC-mediated resistance in P aeruginosa [35]. This effect likely “protects” and allows ATM to be effective against our isolates harboring GES enzymes.

Tandem carriage of GES (GES-1 and GES-5) has been previously reported in a P aeruginosa isolate from Spain [36]. In this report, the entire integron was transferred to a susceptible PAO1, and the individual contributions of each enzyme were not assessed. Guiana extended spectrum β-lactamase-5 is known to have carbapenemase activity due to the presence of serine at position 170. Of note, both GES-19 and GES-26 have glycine at position 170, and we hypothesize that tandem expression, rather than an amino acid change, influences the resistance phenotype. This is supported by the genetic and hydrolysis studies showing a greater than additive effect with tandem versus single expression in E coli. Furthermore, gene dosage has been linked to β-lactam resistance associated with mobile elements in Enterobacteriaceae [37], and this is a potential new mechanism for resistance to C/T in P aeruginosa. It is interesting to note that the P aeruginosa isolates were not susceptible to MEM or ATM in combination with vaborbactam. In the case of MEM, disruption of the OprD porin and the resulting decrease in permeability to MEM would not be overcome with addition of a β-lactamase inhibitor. The lack of efficacy when paired with ATM is likely multifactorial, because vaborbactam appears to be less potent inhibitor of class C β-lactamases, does not inhibit class D enzymes, and may possibly be impacted by porin mutations (vaborbactam uses OmpK36 and OmpK35 to cross the membrane in K pneumoniae), although data regarding vaborbactam entry in Pseudomonas are lacking [38].

CONCLUSIONS

In summary, we report the occurrence of serious infections caused by ST309 P aeruginosa carrying blaGES-19 and blaGES-26 in Houston, Texas. Tandem expression of the 2 GES enzymes under IPTG induction in E coli resulted in resistance to all β-lactams including novel combinations. Ceftazidime/avibactam plus ATM was active in vitro against these 2 isolates and was successfully used in one patient failing polymyxin B-based therapy. The prevalence of GES enzymes reported in Mexico, and the close relation of the strains identified here to P aeruginosa Mexican isolates, suggests that ST309 may be a newly emerging high-risk lineage in the United States.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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