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# Multidrug Resistant *Pseudomonas aeruginosa* Causing Prosthetic Valve Endocarditis: A Genetic-Based Chronicle of Evolving Antibiotic Resistance

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***Background.*** Successful treatment of infections caused by multidrug-resistant (MDR) *Pseudomonas aeruginosa* is thwarted by the emergence of antibiotic resistance and biofilm formation on prosthetic devices. Our aims were to decipher the molecular basis of resistance in a unique case of prosthetic valve endocarditis (PVE) caused by MDR *P. aeruginosa*.

***Methods.*** Five sequential MDR *P. aeruginosa* blood isolates collected during a 7-month period were recovered from a patient suffering from PVE previously exposed to β-lactam antibiotics. Minimum inhibitory concentrations (MICs) of several classes of antibiotics were used to indicate clinical resistance characteristics; relatedness of the isolates was determined using multilocus sequence typing and repetitive sequence-based polymerase chain reaction. Amplification and sequencing of regulatory and resistance genes was performed.

***Results.*** All isolates belonged to ST 298, possessed *bla*$_{\text{PDC-16}}$, and were resistant to fluoroquinolones and carbapenems. In the course of therapy, we observed a >2-fold increase in cephalosporin resistance (4 μg/mL to >16 μg/mL). Sequencing of the AmpC regulator, *amp*R, revealed a D135N point mutation in cephalosporin-resistant isolates. Common carbapenemase genes were not identified. All isolates demonstrated a premature stop codon at amino acid 79 of the outer membrane protein OprD and mutations in the quinolone resistance-determining regions of *gyr*A and *par*C. Point mutations in *nal*C, an efflux pump regulator, were also observed.

***Conclusions.*** In this analysis, we chart the molecular evolution of β-lactam resistance in a case of PVE. We show that mutations in regulatory genes controlling efflux and cephalosporinase production contributed to the MDR phenotype.

**Keywords.** aortic valve replacement; endocarditis; *Pseudomonas aeruginosa*.

Prosthetic valve endocarditis (PVE) is a potentially life-threatening complication of cardiac valve replacement surgery. The most common pathogens found to cause infective endocarditis (IE) are Gram-positive species, primarily *Staphylococcus aureus*, whereas endocarditis caused by Gram-negative species is less common and often attributed to HACEK organisms, which include the following: *Haemophilus* species, *Aggregatibacter* species, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* species [1]. *Pseudomonas aeruginosa* is an extremely rare cause of PVE; a large international cohort study showed that the incidence is 0.4%, or 11 of 2671 cases [2]. Biofilm formation is also a distinguishing characteristic of *P. aeruginosa* that complicates treatment [3].

Molecular methods are extremely valuable in determining the etiology of IE and assist in clarifying the epidemiology and course of the disease, especially when repeated episodes of infection are caused by the same strain of species and can be defined as a relapse regardless of the length of time between infections [4]. Similarly, molecular analysis serves to uncover the mechanisms of antibiotic resistance that pathogens possess and that impact therapeutic success. Thus, understanding the molecular basis of the disease (applying precision medicine) can explain the clinical course and outcome of IE.

In this report, we sought to investigate the molecular basis of a persistent case of PVE due to *P. aeruginosa*. The case is unique as isolates characterized were collected serially during the course of treatment, occurring throughout 2 aortic valve replacements (AVRs). The different resistance profiles between early and late isolates, in addition to the patient's history of antibiotic therapy, assisted us in the understanding of the complex relationship between medical and surgical interventions and emergent antibiotic resistance.

## Case Presentation

A 58-year-old woman treated with prednisone for Takayasu's arteritis and with a previous AVR (2007) presented to a Houston, Texas tertiary hospital initially in June 2010. She was

hospitalized due to worsening shortness of breath. At that time, a transthoracic echocardiograph (TTE) revealed evidence of a prosthetic valvular incompetence, corroborated by the presence of torn cusps and severe aortic regurgitation. AVR surgery was performed. On postoperative day 24, *P. aeruginosa* bacteremia (isolate 7166; Table 1) was detected, and *Achromobacter* bacteriuria was present.

Initially, a treatment regimen (Figure 1) was started for the bloodstream infection and urinary tract infection with meropenem (Table 1; isolate 1, ID 7166 was meropenem and imipenem resistant) but changed to ceftazidime when laboratory susceptibility data were determined. The patient improved clinically and was discharged but returned to an outside care facility 1 month later with persistent fever. Blood cultures yielded *P. aeruginosa*, and a TTE showed evidence of aortic regurgitation (isolate not available for further study). Meropenem, amikacin, and eventually piperacillin/tazobactam therapy were prescribed, and the patient was then discharged after 2 weeks of treatment.

The patient returned to the tertiary hospital in Houston, Texas, in January 2011 with recurrent fever and dyspnea of 1 month duration. Her cultures again grew *P. aeruginosa* (isolate 2961; Table 1). Piperacillin/tazobactam therapy was started based upon previous susceptibilities. This regimen changed to colistin, rifampin, and high-dose, prolonged infusion meropenem once susceptibility data were determined during admission. The patient's blood cultures cleared, but TTE showed evidence of aortic regurgitation. Subsequently, the patient was found to have dehiscence of the new valve and underwent a third aortic valve replacement with homograft placed 215 days after the second AVR. Despite ongoing antibiotics (including the addition of amikacin), the bacteremia persisted until postoperative day 16 after the addition of doxycycline to meropenem and colistin. Antibiotics were stopped after 8 weeks of PVE treatment, which lead to microbiological resolution.

During the course of treatment, 6 sequential *P. aeruginosa* blood isolates were collected, 1 of which was not included in this analysis because it was obtained while the patient was in an outside care facility and was not available for further analysis. The date of each isolate collection, relevant to the timeline of antibiotic therapy and patient health information, is detailed in Figure 1. The 5 isolates of interest were analyzed to understand the molecular mechanisms behind the observed clinical presentation.

## METHODS AND RESULTS

### Genotypic Analysis
Herein, we analyze a complicated case of PVE caused by MDR *P. aeruginosa*, where resistance to cephalosporins and aztreonam emerged while on therapy. This is one of a few studies where the genetic backgrounds of multiple isolates of *P. aeruginosa* collected from the same patient during a period of 7 months were analyzed to explain the resistance phenotype and correlate with the course of infection.

Susceptibility testing to antibiotics was performed using the MicroScan System (Siemens Healthcare Diagnostics, Tarrytown, NY) and interpreted according to the 2015 Clinical and Laboratory Standards Institute guidelines for *P. aeruginosa*. American Type Culture Collection control strains *P. aeruginosa* 27853 and *E. coli* 25922 were used as control strains. Colistin minimum inhibitory concentrations (MICs) were determined by Etest. All isolates were resistant to carbapenems and fluoroquinolones [ciprofloxacin (MIC >2 μg/mL), meropenem, and imipenem (MICs >8 μg/mL)]. Whereas the initial isolate was susceptible to aztreonam (ATM; MIC of ≤8 mg/mL), cefepime (FEP; MIC of 8 μg/mL), and ceftazidime (CAZ; MIC of 4 μg/mL), subsequent isolates were resistant (MIC >16 μg/mL) (Table 1). There was a clinically important change in this susceptibility (ie, CAZ, ATM, and FEP, from susceptible to resistant) during the 7 months isolates were collected.

β-lactamase genetic amplification and detection was performed using the CT103 kit produced by Check-Points B.V. (Wageningen, The Netherlands) This is a polymerase chain reaction (PCR)–based DNA microarray method for detecting the following β-lactamase (*bla*) genes: *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, and *bla*$_{IMP}$ (carbapenemases). Additionally, this method detects extended-spectrum β-lactamase (ESBL) genes and AmpC genes: *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{CMY}$, *bla*$_{DHA}$, *bla*$_{FOX}$, *bla*$_{MOX}$, *bla*$_{ACC}$, *bla*$_{MIR}$, and *bla*$_{ACT}$. For *bla*$_{TEM}$ and *bla*$_{SHV}$, the assay method allows for distinction between ESBL and non-ESBL variants that exist within an isolate. Briefly, genomic DNA was extracted from bacterial colonies grown overnight using the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Microarray assays were performed generating templates of the target DNA sequence that were amplified and hybridized, detected by the microarray reader, and interpreted using software supplied by the manufacturer. The panel of β-lactamases that are detected by the microarray are optimized for Enterobacteriaceae. The performance of this kit using *P. aeruginosa* is being explored and tested. We emphasize that carbapenemase genes were not detected.

PCR amplifications of genes involved in resistance to cephalosporins (ie, *amp*D, *amp*R, *bla*$_{PDC}$, and *dac*B), resistance to fluoroquinolones (ie, *gyr*A, *gyr*B, *par*C, *par*E), other mechanisms of resistance to carbapenems (*opr*D), and regulation of efflux pumps (ie, *nal*B (*mex*R), *nal*C, *nal*D, *PA5471*, and *mex*Z) were also performed using the primers and methodology described by Cabot et al [5]. Amplicons obtained using primers for the above resistance genes were sequenced at a commercial sequencing facility (MCLAB, South San Francisco, CA). Generated sequences were analyzed using DNAstar SeqMan Pro software (Madison, WI). Generated sequences were then compared with BLAST online software (https://blast.ncbi.nlm.nih.gov), or translated in ExPASy (http://web.expasy.org/translate/), then aligned using ClustalX software (www.clustal.org) to determine DNA mutations that resulted in amino acid mutations.

**Table 1. Antimicrobial Susceptibility Testing (µg/mL)**

| Isolate Number | Isolate ID | Date | AMK | ATM | FEP | CAZ | CIP | IMP | MEM | LVX | GEN | TZP | CST |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7166 | 7/28/10 | ≤16 | ≤8 | ≤8 | 4 | >2 | >8 | >8 | >4 | >8 | ≤16 | 2 |
| 3 | 2961 | 1/14/11 | ≤16 | >16 | >16 | >16 | >2 | >8 | >8 | >4 | >8 | >64 | 1 |
| 4 | 7927 | 1/20/11 | ≤16 | >16 | >16 | >16 | >2 | >8 | >8 | >4 | >8 | >64 | 1 |
| 5 | 8649 | 2/8/11 | ≤16 | >16 | >16 | >16 | >2 | >8 | >8 | >4 | >8 | >64 | 12 |
| 6 | 8096 | 2/19/11 | 32 | >16 | >16 | >16 | >2 | >8 | >8 | >4 | >8 | >64 | 3 |

Antimicrobial susceptibility tests were interpreted according to 2015 Clinical Laboratory Standards Institute (CLSI) criteria for *Pseudomonas aeruginosa*: for aztreonam (ATM), cefepime (FEP), and ceftazidime (CAZ), minimum inhibitory concentration (MIC) ≤ 8 is susceptible (S), MIC = 16 is intermediate (I), and MIC ≥ 32 is resistant (R); for meropenem (MEM), imipenem (IMP), levofloxacin (LVX), and colistin (CST), MIC ≤ 2 is S, MIC = 4 is I, MIC ≥ 8 is R; for amikacin (AMK), MIC ≤ 16 is S, MIC = 32 is I, and MIC ≥ 64 is R; for ciprofloxacin (CIP), MIC ≤ 1 is S, MIC = 2 is I, MIC ≥ 4 is R MIC; for gentamicin (GEN), MIC ≤ 4 is S, MIC = 8 is I, MIC ≥ 16 is R; for piperacillin/tazobactam (TZP), MIC ≤ 16 is S, MIC = 32–64 is I, MIC ≥ 128 is R.

## Cephalosporin Resistance

D135N mutations in *amp*R, which upregulates AmpC expression in *Enterobacter cloacae* [6], were present in all isolates analyzed, with the exception of the first isolate (7166; Table 2). *Pseudomonas*-derived cephalosporinase (PDC) is the chromosomally encoded class C cephalosporinase in *P. aeruginosa* and is often responsible for high-level resistance to advanced generation cephalosporins such as ceftazidime. Expression of the PDC cephalosporinase was assessed in the 5 isolates. Preparation of samples, immunoblotting, and recognition of PDC expression was performed as previously reported [7]. Increased expression of PDC-16 was observed in all isolates with the D135N mutation in the *amp*R gene (Figure 2.) There were also other mutations of note relative to wild-type/reference strain PA01 that are summarized in Table 2.

## Quinolone Resistance

T83I in g*yr*A and S87L in *par*C were found in all isolates. These mutations confer high-level fluoroquinolone resistance [8].

## Carbapenem Resistance

All isolates also had a premature stop codon at amino acid 79 in OprD, resulting in inactivation of the main porin for imipenem uptake [9].

## Mutations in Efflux Pump Regulators

Three point mutations (G71E, A145V, and S209R) were observed in *nal*C; however, their role in an MDR phenotype in *P. aeruginosa* is unclear at the present time. Also of note, isolate 6 developed moderate amikacin resistance (32 µg/mL). In this case, MexXY overexpression might be expected, although specific mutations in *mex*Z were not seen. It could be that
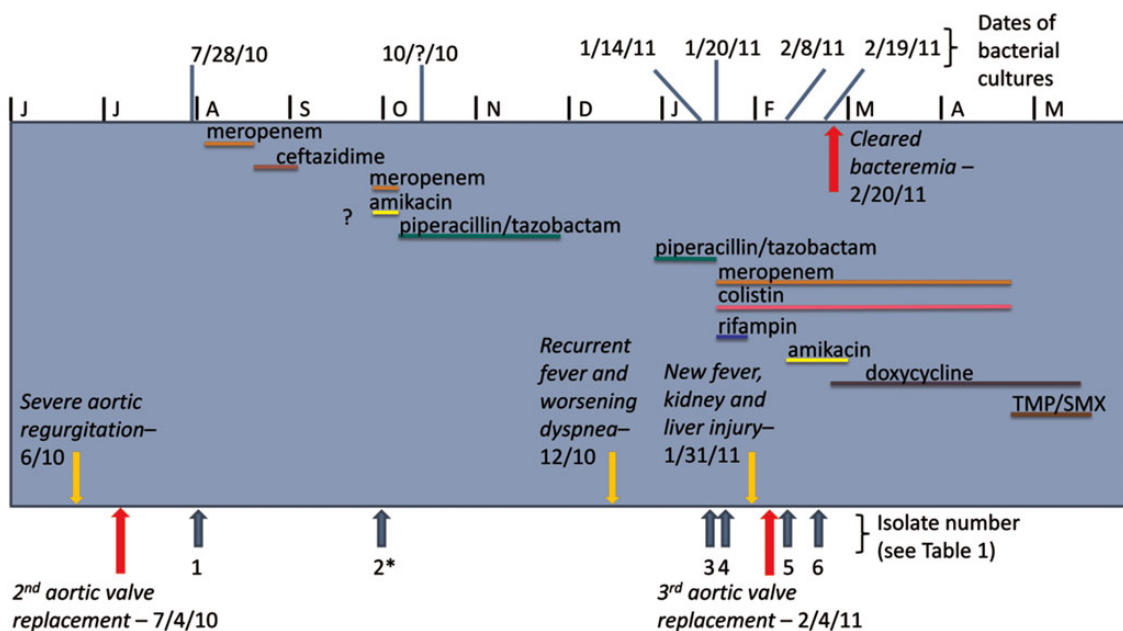


**Figure 1.** Timeline of antibiotic therapy, bacterial cultures, and patient health information. ?, Treated at outside hospital, unclear details; *, Isolate 2 recoved at outside hospital, not included in analysis.

**Table 2. Summary of Mutations**

| Isolate Number | Isolate ID PA01 | ST Type | AmpC PDC-1 | PDC Substitutions WT | AmpC Regulators | | QRDRs | | | |
| | | | | | AmpD and DacB WT | AmpR WT | GyrA WT | GyrB WT | ParC WT | ParE WT |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7166 | 298 | PDC-16 | G27D, T105A, V205L, G391A | WT | M1L, G238E, M288R | T83I | WT | S87L | WT |
| 3 | 2961 | 298 | PDC-16 | G27D, T105A, V205L, G391A | WT | M1L, D135N, G238E, M288R | T83I | WT | S87L | WT |
| 4 | 7927 | 298 | PDC-16 | G27D, T105A, V205L, G391A | WT | M1L, D135N, G238E, M288R | T83I | WT | S87L | WT |
| 5 | 8649 | 298 | PDC-16 | G27D, T105A, V205L, G391A | WT | M1L, D135N, G238E, M288R | T83I | WT | S87L | WT |
| 6 | 8096 | 298 | PDC-16 | G27D, T105A, V205L, G391A | WT | M1L, D135N, G238E, M288R | T83I | WT | S87L | WT |

| Isolate Number | Isolate ID PA01 | OMP | MexAB-OprM regulators | | | MexXY-OprM regulators | |
| | | OprD WT | NalB (MexR) WT | NalC WT | NalD WT | PA5471 WT | MexZ WT |
|---|---|---|---|---|---|---|---|
| 1 | 7166 | Premature stop codon at aa 79 | V126E | G71E, A145V, S209R | WT | Won't amplify | WT |
| 3 | 2961 | Premature stop codon at aa 79 | V126E | G71E, A145V, S209R | WT | Won't amplify | WT |
| 4 | 7927 | Premature stop codon at aa 79 | V126E | G71E, A145V, S209R | WT | Won't amplify | WT |
| 5 | 8649 | Premature stop codon at aa 79 | V126E | G71E, A145V, S209R | WT | Won't amplify | WT |
| 6 | 8096 | Premature stop codon at aa 79 | V126E | G71E, A145V, S209R | WT | Won't amplify | WT |

Reference wild-type comparator amino acid sequences from *Pseudomonas aeruginosa* PA01 isolate listed in Table 2 are as follows:

PDC-1, ACQ82807; AmpD, AAG07910; DacB, NP_251737; AmpR, AAG07496; GyrA, AAG06556; GyrB, AAG03394; ParC, NP_253651; ParE, AAG08352; OprD, NP_249649; NalB (MexR) AAG03813; NalC, AAG07108; NalD, AAG06962; PA5471, AAG08856; MexZ, AF073776.

Abbreviations: PDC, *Pseudomonas*-derived cephalosporinase; QRDR, quinolone resistance-determining region; ST, sequence type; WT, wild-type or no mutation.
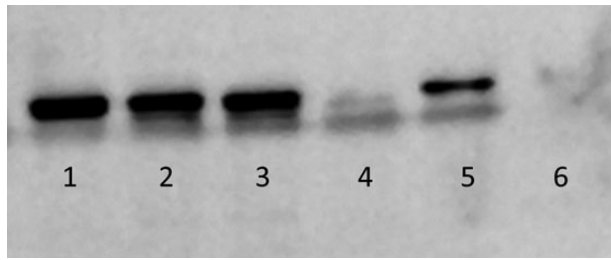
**Figure 2.** Immunoblot expression of *Pseudomonas*-derived cephalosporinase (PDC) β-lactamse, PDC-16. Lanes: 1 – Isolate 2961, 2 – Isolate 7927, 3 – Isolate 8649, 4 – Isolate 7166, 5 – Isolate 8096, 6 – Marker. Probed with 0.1 µg/mL anti-PDC antibody; 1:10 000 dilution of Protein G-HRP conjugate.

additional MexXY regulators were involved, or the presence of an aminoglycoside modifying enzyme, in that resistance phenotype. Additionally, the gene *PA5471* would not amplify in any of the isolates. Deletions of the coding sequence in *PA5471* can lead to MexXY-OprM overexpression [5, 10].

To determine whether recurrent *P. aeruginosa* bacteremia represented relapse of infection or acquisition of a new strain, we performed multilocus sequence typing on all *P. aeruginosa* isolates following the method of Curran et al [11]. All isolates belonged to sequence type 298 and displayed resistance to ciprofloxacin (MIC >2 µg/mL), meropenem, and imipenem (MICs >8 µg/mL) by Microscan; only isolate 5 appeared to be resistant to colistin. The cause of that colistin resistance is undetermined.

Clonality of the isolates was investigated using the DiversiLab Bacterial BarCodes strain typing system (bioMerieux, Durham, NC). Pure genomic DNA was isolated with the UltraClean Microbial DNA Isolation Kit using the protocol for Gram-negative bacteria (MoBio Laboratories, Inc, Carlsbad, CA). The DNA was then used as the template for amplification using the DiversiLab *Pseudomonas* kit, with amplicons analyzed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).
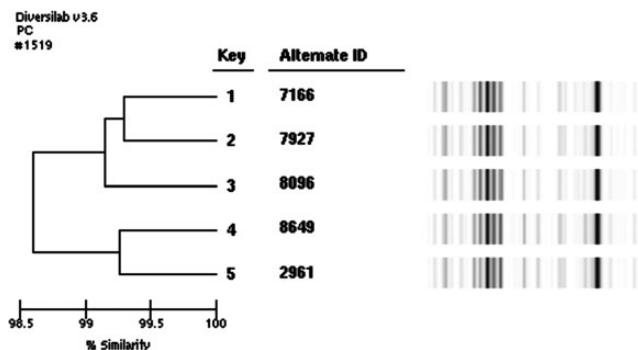


**Figure 3.** Genetic typing with repetitive sequence-based PCR (rep-PCR). Dendrogram of rep-PCR illustrating >98% similarity in band patterns, suggesting persistence of the same *Pseudomonas aeruginosa* strain, rather than re-infection with a different strain.

A similarity dendrogram was generated with the DiversiLab Software (bioMerieux), and isolates with >95% similarities were considered to be of the same clone type (Figure 3). All isolates had >98.5% similarity. This result, together with confirmation of the same sequence type in all isolates, supports the conclusion that the same strain was present throughout the course of infection, despite the evolution of the extended spectrum cephalosporin/aztreonam resistant phenotype after treatment with ceftazidime.

Although much information was uncovered in this investigation it should be noted that certain limitations to this study exist, such as: (1) MICs were reported by breakpoints only (ie, endpoints were not determined), (2) expression of efflux pumps was not examined, and (3) the relevance of *nal*C mutations to efflux pump expression still remains unknown.

## CONCLUSION

We have chronicled the molecular basis of evolving antibiotic resistance in a case of MDR *P. aeruginosa* in a patient with PVE, an infection that is very difficult to eradicate. Serial analysis shows that the occurrence of point mutations contributed to the MDR phenotype. Likely, changes in the efflux pump regulators and reduced porin expression contribute to the carbapenem-resistant phenotype. Our molecular analysis also uncovered point mutations in quinolone resistance-determining regions. However, the key finding during the course of treatment was the mutation in *amp*R leading to the emergence of cephalosporin and aztreonam resistance. This was due to the D135N AmpR amino acid change, which causes a conformational change in the Amp R regulator, leading to overexpression of the PDC-16 β-lactamase that was present in these isolates.

We stress that *ampR* is a global transcriptional regulator also associated with alginate production and biofilm formation. Prolonged exposure to β-lactams before surgical intervention may have contributed to the selection of *ampR* regulator mutants. This should serve as a reminder of the complexity of acquired resistance in *P. aeruginosa* PVE and the therapeutic challenges it entails.

## References

1. Murdoch DR, Corey GR, Hoen B, et al. Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis-Prospective Cohort Study. Arch Intern Med **2009**; 169:463–73.
2. Morpeth S, Murdoch D, Cabell CH, et al. Non-HACEK Gram-negative bacillus endocarditis. Arch Intern Med **2007**; 147:829–35.
3. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother **2001**; 45:999–1007.
4. Chu VH, Sexton DJ, Cabell CH, et al. Repeat infective endocarditis: differentiating relapse from reinfection. Clin Infect Dis **2005**; 41:406–9.
5. Cabot G, Ocampo-Sosa AA, Dominguez MA, et al. Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. Antimicrob Agents Chemother **2012**; 56:6349–57.
6. Kuga A, Okamoto R, Inoue M. ampR gene mutations that greatly increase class C beta-lactamase activity in *Enterobacter cloacae*. Antimicrob Agents Chemother **2000**; 44:561–7.
7. Winkler ML, Papp-Wallace KM, Hujer AM, et al. Unexpected challenges in treating multidrug-resistant Gram-negative bacteria: resistance to ceftazidime-avibactam in archived isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **2015**; 59:1020–9.
8. Vila J, Ruiz J, Goni P, Marcos A, Jimenez de Anta T. Mutation in the *gyr*A gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother **1995**; 39:1201–3.
9. Wolter DJ, Hanson ND, Lister PD. Insertional inactivation of *opr*D in clinical isolates of *Pseudomonas aeruginosa* leading to carbapenem resistance. FEMS Microbiol Lett **2004**; 236:137–43.
10. Muller C, Plesiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **2011**; 55:1211–21.
11. Curran B, Jonas D, Grundmann H, Pitt T, Dowson CG. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J Clin Microbiol **2004**; 42:5644–9.