



# The Brazilian Journal of INFECTIOUS DISEASES

[www.elsevier.com/locate/bjid](http://www.elsevier.com/locate/bjid)



## Original article

# Prevalence of antibiotic resistance and virulent factors in nosocomial clinical isolates of *Pseudomonas aeruginosa* from Panamá



Héctor Sambrano<sup>d</sup>, Julio César Castillo<sup>a,b</sup>, Carlos W. Ramos<sup>a</sup>, Brenda de Mayorga<sup>c</sup>, Olga Chen<sup>a</sup>, Ovidio Durán<sup>a</sup>, Carmelo Ciniglio<sup>a</sup>, Criseida Aguilar<sup>a</sup>, Osvaldo Cisterna<sup>e</sup>, Magaly de Chial<sup>ib a,\*</sup>

<sup>a</sup> Universidad de Panamá, Escuela de Biología, Departamento de Genética y Biología Molecular, Panama

<sup>b</sup> Instituto de Investigaciones Científicas y Servicios de Alta Tecnología de Panamá (INDICASAT-AIP), Panama

<sup>c</sup> Universidad de Panamá, Escuela de Biología, Departamento de Microbiología y Parasitología, Panama

<sup>d</sup> Universidad de Panamá, Programa de Maestría en Ciencias Biológicas, Panama

<sup>e</sup> Hospital del Niño, Panama

## ARTICLE INFO

### Article history:

Received 28 June 2020

Accepted 8 November 2020

Available online 4 December 2020

### Keywords:

*Pseudomonas aeruginosa*  
antibiotics resistance  
virulent factors

## ABSTRACT

**Background:** *Pseudomonas aeruginosa* is an important causative agent of nosocomial infections. As pathogen, *P. aeruginosa* is of increasing clinical importance due to its ability to develop high-level multidrug resistance (MDR).

**Methods:** The aim of the present study was to better understand the intrinsic virulence of circulating strains of *Pseudomonas aeruginosa*, by surveying and characterizing the antibiotic resistance profiles and prevalence of virulence factors in 51 clinical isolates of *P. aeruginosa* obtained from children admitted to Hospital del Niño-Panamá during the period of October 2016 until March 2017. Antimicrobial susceptibilities were assessed by determining the minimum inhibitory concentration for 12 antibiotics against *P. aeruginosa* clinical isolates using the VITEK system (<https://www.biomerieux.com>). Additionally, all isolates were examined by Polymerase Chain Reaction (PCR) for the presence of components of the MexAB-OprM efflux pump system (*mexABR*) and pyoverdine receptor genes and betalactamases resistance genes (ESBL) using gene-specific primers.

**Results:** A total of 51 pyoverdine producing clinical isolates were analyzed, all of which expressed resistance genes such as genes of the MexAB-OprM efflux pump system (*mexABR*) and pyoverdine receptor genes (*fpvA*). Out of 51 MDR isolates, 22 were ESBL producers. The most common ESBL gene was *bla*<sub>TEM</sub> expressed by 43% of the isolates. The isolates tested in this study showed increased resistance to antibiotics in the following categories: (i) penicillins (ampicillin (69%), piperacillin (22%); (ii) pyrimethamines (trimethoprim, 65%); (iii) nitrofurans (nitrofurantoin, 63%), and (iv) third-generation cephalosporin cefotaxime (53%). These results underscore a high prevalence of MDR amongst clinical isolates from Panama.

\* Corresponding author.

E-mail address: [mdechial@yahoo.com.mx](mailto:mdechial@yahoo.com.mx) (M. de Chial).

<https://doi.org/10.1016/j.bjid.2020.11.003>

1413-8670/© 2020 Sociedade Brasileira de Infectologia. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Conclusions: The present study indicates that prevalence of blaTEM-carrying strains is increasing with subsequent multidrug resistance in Panamá and as well reported worldwide. The virulent factors identified in this study provide valuable information regarding the prevalence of resistance genes and their potential impact on treatments that exploit the unique physiology of the pathogen. To prevent further spread of MDR, the proportions of resistant strains of *Pseudomonas aeruginosa* should be constantly evaluated on healthcare institutions of Panamá. More importantly, this information can be used to better understand the evolution and dissemination of strains hoping to prevent the development of resistance in *Pseudomonas aeruginosa*. Future studies quantifying the expression of these virulent genes will emphasize on the acquisition of multidrug resistance.

© 2020 Sociedade Brasileira de Infectologia. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

*Pseudomonas aeruginosa* is an important causative agent of nosocomial infections. This bacterium is considered an opportunistic pathogen that affects the health of immunocompromised individuals such as those with diabetes, cancer, cystic fibrosis, advanced HIV infections (acquired immunodeficiency syndrome, AIDS), severely burn patients and those that underwent major surgeries.<sup>1</sup>

As pathogen, *Pseudomonas aeruginosa* is of increasing clinical importance because of their innate resistance to multiple agents and their ability to develop high-level multidrug resistance (MDR) due to the presence of several virulence factors encoded in its genome (<-- -->Poole, 2016). Several studies have shown that this innate resistance is directly associated to the expression of bacterial efflux pumps and porins such as the Resistance-Nodulation-cell-Division (RND)-type efflux pump, MexAB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexXY-OprM.<sup>2</sup> These important RND-type efflux pumps are constitutively expressed in wild-type cells and are responsible for the intrinsic resistance to most antimicrobial agents.<sup>3-7</sup>

The MexAB-OprM system was initially characterized from an isolate of *P. aeruginosa* cultured under iron-depleted conditions in the presence of the iron chelator 2,2'-dipyridyl (Dipy).<sup>8,9</sup> Under this condition, protein levels for the MexAB-OprM members were increased, suggesting that the efflux pump plays an essential role in its survival under iron deficiency conditions<sup>8,10,11</sup> (Oglesby-Sherrouse et al., 2009)<-- -->.

The MexAB-OprM system includes the proteins MexA, MexB, OprM, and MexR. MexB is the molecular subunit that selectively recognizes specific antibiotics and mediates their expulsion; MexA functions as the interconnecting bridge between OprM and MexB. OprM is a lipoprotein localized in the outer membrane whose role is mediating the final expulsion of the antibiotic across the bacterial outer membrane. MexR acts as repressor of the MexAB-OprM system and autoregulates MexR.<sup>12</sup>

In addition to the MexAB-OprM efflux pump system, *Pseudomonas aeruginosa* produce small molecules known as siderophores, which are iron-chelating molecules that contributes to their virulence; siderophores have also been connected to mechanisms of antibiotic resistance (<-- --

>Djapgne et al., 2014). Under iron-limiting conditions, *P. aeruginosa* produces two main siderophores, pyoverdine and pyochelin, which scavenge iron from host proteins, contributing to its virulence.<sup>13-15</sup> The ferri-pyoverdine complex uptake is carried out by TonB-dependent receptors with the help of the transporter FpvB,<sup>16</sup> resulting in its internalization into the periplasm.<sup>17</sup> In addition to its molecular function, the pyoverdine receptor genes have also been used to genotype several strains of *P. aeruginosa*.<sup>18</sup>

Another important resistance mechanism in Gram-negative bacteria such as *P. aeruginosa* is the production of  $\beta$ -lactamases, hydrolytic enzymes involved in the degradation of antibiotics ( $\beta$ -lactams). Among this group, the extended-spectrum  $\beta$ -lactamases (ESBLs) represent an important sub-class of enzymes that confer resistance to oxymino-cephalosporins, such as cefotaxime (CTX), ceftriaxone, and ceftazidime (CAZ), and the monobactam aztreonam (ATM). Because of this enhanced resistance phenotype against some of the most effective antibiotics, strains carrying ESBLs genes can become a serious clinical problem.<sup>19</sup>

Several genes coding  $\beta$ -lactamases have been identified at low frequency in *P. aeruginosa*.<sup>20</sup> blaTEM-1 was the first plasmid-encoded  $\beta$ -lactamase gene isolated from *E. coli*; this isolate came from a blood culture of a patient named Temoniera in Greece, hence the acronym TEM.<sup>21</sup> blaTEM-1-like  $\beta$ -lactamases have also been found in other species of the family Enterobacteriaceae and other bacterial species such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*.<sup>22</sup> Among the ESBL sub-class, SulfHydriyl Variable-2 (SHV-2) ESBLs were first identified in a German strain of *Klebsiella ozaenae*; this novel enzyme is capable of hydrolyzing cefotaxime and to a lesser extent ceftazidime.<sup>23</sup> Several other studies have reported the existence of non-blaTEM and non-blaSHV hydrolytic enzymes that do not share the evolutionary and functional origin of typical ESBLs.<sup>24</sup> These non-blaTEM and non-blaSHV plasmid-encoded  $\beta$ -lactamases and cefotaximases (blaCTX-M) are able to hydrolyze cephalothin and cefotaxime, and to lesser extent benzylpenicillin and ceftazidime.<sup>23</sup> Therefore, the characterization of  $\beta$ -lactamases from drug-resistant *P. aeruginosa* can greatly contribute to the understanding of mechanisms of molecular pathogenesis, antibiotic resistance and virulence in bacteria<-- -->.<sup>25,26</sup>

To better understand the intrinsic virulence of circulating strains of *Pseudomonas aeruginosa* from Panamá, we surveyed and characterized the antibiotic resistance profiles and the prevalence of virulence factors in 51 clinical isolates of *P. aeruginosa* obtained from children admitted to Hospital del Niño-Panamá.

## Materials and methods

### Bacterial Isolates and culture methods

Fifty-one strains of *P. aeruginosa* were isolated from various sites of infections (blood, tracheal secretions, wound, eye and ear discharge, and ulcer) from patients admitted to Hospital del Niño, a Children's Hospital located in Panama City, Panamá (Table 1). These biological samples were collected by the Hospital's Microbiology Laboratory between October 2016 and March 2017. In brief, bacterial isolates were grown in Amies transport media with charcoal (Thermo Fisher Scientific, United States) for initial pathogen collection and growth and Bacto Casamino Acids medium (CAA) (Becton-Dickinson, United States) to detect pyoverdine secretion (green-fluorescent pigment). For pyoverdine production, samples were incubated at 37 °C for 24 hours as previously described.<sup>27</sup> All strains were store in 50% glycerol at -80 °C. The strain *P. aeruginosa* ATCC 27853 was used as reference for this study.

### Antimicrobial Susceptibility Test

Minimum Inhibitory Concentrations (MICs) of selected antibiotics against *P. aeruginosa* clinical isolates were evaluated using the VITEK system (bioMérieux) according to manufacturer's instructions. Susceptibility testing results were based on guidelines set by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) ([http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Disk\\_test\\_documents/2020\\_manuals/Manual\\_v\\_8.0\\_EUCAST\\_Disk\\_Test\\_2020.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Manual_v_8.0_EUCAST_Disk_Test_2020.pdf)). As main criteria of MDR, a *P. aeruginosa* isolate was considered as non-susceptible if it displayed resistance to  $\geq 1$  antimicrobial agent in  $\geq 3$  antimicrobial assays. The antimicrobial agents assayed in this study included (i) aminoglycosides (amikacin - AMI) and gentamicin - GENTA), (ii) fluoroquinolones (ciprofloxacin - CIPRO), (iii)  $\beta$ -lactam-cephalosporins (cefazidime - CEFTA), cefepime - CEFE), cefotaxime - CEFO), (iv)  $\beta$ -lactam-penicillins (extended spectrum) (piperacillin - PIPE), ampicillin - AMPI), pyrimethamines (trimethoprim - TRI), (iv) nitrofurans (nitrofurantoin - NITRO) and (v) carbapenems (meropenem - MERO) and imipenem - IMI).

### DNA Extraction and Molecular Detection of Resistance Genes

Isolates were grown in CAA liquid medium overnight at 37 °C and DNA was extracted from 3 ml of culture following the method for Gram-negative bacteria as described previously (<-->Chen and Kuo, 1993). All PCR reactions were prepared using Omega Bio-Tek's, 2x Taq Master Mix, 0.5 pmol of each primer in 25  $\mu$ l reactions. The *mexABR* genes were detected

by multiplex PCR using primers listed in Table 2. Cycling conditions consisted of an initial denaturation step at 94 °C for 3 minutes, followed by 32 cycles of 94 °C for 30 seconds, 57 °C for 45 seconds, and 72 °C for 1 minute as described in Auda Al-Grawi et al.<sup>12</sup> Pyoverdine receptor genes (*fpvA*) were amplified simultaneously by multiplex PCR listed in Table 2. The PCR conditions were first a denaturation at 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and terminating with a last cycle at 72 °C for 10 min.<sup>16</sup> To detect the  $\beta$ -lactamase genes *blaTEM*, *blaSHV*, *blaCTX-M* we used the primers listed in Table 2. PCR included 1 min denaturation (95 °C followed by 30 cycles of 96 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s and final extension of 72 °C for 10 min. Conditions were identical for other assays except the annealing temperatures which were 55 °C and 44 °C for *blaCTX-M* and *blaTEM*, respectively, as described in Refs. 28,29. Amplicons were resolved in 1% agarose gels; for determination of DNA band sizes were used a 100 bp ladder (VWR, Cat No. K180). *blaTEM* amplicons were purified and sequenced to verify sequence identity (Macrogen Inc, Maryland USA).

## RESULTS

### Microbial and Antibiotic Susceptibility

To characterize the prevalence of virulence factors and genes involved in antimicrobial resistance, we collected clinical isolates from various types of wounds. All clinical isolates (n = 51) of *P. aeruginosa* were pyoverdine producers, as measured by the production of a green fluorescent pigment on CAA agar (Supplementary Fig. 1). Resistance rates (number of resistant isolates) and prevalence (%) of all *Pseudomonas aeruginosa* isolates against seven antibiotic classes (12 antibiotics) tested are summarized in Fig. 1. In our susceptibility tests, one strain showed resistance to AMI, 0 to GENTA (0%), two to CIPRO (4%), 11 to PIPE (22%), 35 to AMPI (69%), six to CEFTA (12 %), three to CEFE (6%), 27 to CEFO (53%), 33 to TRI (65%), 32 to NITRO(63%), 13 to MERO (25%), 17 to IMI (33%) and, two to CIPRO (12%), three to GENTA (6%), two to AMPI (4%), and 33% to TRI.

### Molecular Detection of Resistance Markers

To detect the presence of components of the *MexAB-OprM* system, we used PCR to amplify gene products for *mexA*, *mexB*, *mexR*. Amplicons for *mexA* (product size: 503 bp), *mexR* (product size: 411 bp) and *mexB* (product size: 280 bp) were detected in all 51 isolates as shown in Fig. 2A.

Next, we tested for the presence of pyoverdine receptors genes, *fpvAI*, *fpvAII*, *fpvAIII* in all of our isolates and found positive amplification for all of the receptor genes in all 51 strains. Amplicons of 326 bp for *fpvAI*, 897 bp for *fpvAII* were detected and a PCR fragment size of 506 bp for the *fpvAIII* receptor gene as shown in Fig. 2B.

To better understand the virulence of our isolates, we proceeded to test for the presence of *blaTEM*-like, *blaSHV*-like, *blaCTX-M*-like  $\beta$ -lactamase genes that may confer resistance against antibiotics. Among the  $\beta$ -lactamase genes tested, PCR products for a *blaTEM*-like  $\beta$ -lactamase were detected in only

**Table 1 – Date of isolation, source, patient sex and hospital unit where the strains were identified.**

Sample ID	Collection date	Sex	Source	Unit
144650	06-10-16	F	Catheter smear	
145202	09-10-16	M	ND	Intensive care
145472	13-10-16	F	Endotracheal aspirate	Intensive care
144748	03-10-16	F	Blood culture catheter	Intensive care
145008	30-09-16	F	Blood culture catheter	Intensive care
143930	22-09-16	M	Blood culture catheter	Intensive care
145805	18-10-16	F	Soft tissue (right ear)	Emergency room
146218	24-10-16	F	Uroculture	Intensive care
146836	02-11-16	M	Uroculture	Neonatology room
146968	04-11-16	F	Bronchial Secretion	Intensive care
147052	06-11-16	M	Blood culture	Neonatology room
147148	07-11-16	F	Endotracheal aspirate	Intensive care
147314	09-11-16	M	Soft tissue (right eye)	Medicine room 2
147052	06-11-16	M	Blood culture	Neonatology room
147284	09-11-16	F	Uroculture	Outpatient room
147427	11-11-16	M	Soft tissue	Neonatology room
147563	14-11-16	F	Bronchial Secretion	Intensive care
147988	15-11-16	M	Sputum	Intensive care
147999	17-11-16	M	Bronchial Secretion	Intensive care
148088	21-11-16	M	Soft tissue	Medicine room 1
148207	22-11-16	F	Soft tissue	Medicine room 1
148205	22-11-16	M	Bronchial Secretion	Intensive care
148204	22-11-16	M	Endotracheal aspirate	Intensive care
148461	25-11-16	F	Soft tissue	Neonatology room
149895	13-12-16	U	Endotracheal aspirate	Neonatology room
149856	13-12-16	M	Uroculture	Short stay respiratory room
150080	15-12-16	F	Soft tissue	Neonatology minimal care room
150434	20-12-16	M	Endotracheal aspirate	Medicine room 1
150544	21-12-16	M	Bronchial Secretion	Medicine room 6
150603	22-12-16	M	Blood culture smear	Intensive care
151402	03-01-17	F	Blood culture	Medicine room 5
151662	07-01-17	M	Endotracheal aspirate	Intensive care
151936	11-01-17	F	Soft tissue	Medicine room 4
152486	17-01-17	M	Soft tissue	Medicine room 3
152785	22-01-17	M	Bronchial Secretion	Intensive care
152847	23-01-17	M	Endotracheal aspirate	Medicine room 6
152953	24-01-17	M	Soft tissue	Intensive care
153773	03-02-17	F	Cerebrospinal fluid	Neonatology room
153839	04-02-17	F	Soft tissue	Emergency room
154034	07-02-17	F	Uroculture	Emergency room
154265	09-02-17	F	Bronchial Secretion	Medicine room 5
154462	12-02-17	F	Anal tissue	Recovery room
154691	15-02-17	M	Surgical wound	Neonatology room
154898	17-02-17	F	Soft tissue (left ear)	Short stay respiratory room
156103	06-03-17	F	Blood culture	Hemato-oncology room
156045	06-03-17	F	Catheter	Intensive care
156365	09-03-17	M	Soft tissue	Neonatology room
156539	11-03-17	M	Surgical Wound	Medicine room 5
156577	12-03-17	M	Pharyngeal tissue	Medicine room 5
156663	13-03-17	M	Uroculture	Medicine room 5
156727	14-03-17	M	Bronchial Secretion	Intensive care

43% of our isolates (22/51). A PCR product of 425 bp for blaTEM-like gene was detected as shown in Fig. 2C, which was confirmed by DNA sequencing for a select number of isolates (Supplementary Fig. 2). Amplicons for blaCTX-M-like or blaSHV-like  $\beta$ -lactamases were not detected in our isolates.

## DISCUSSION

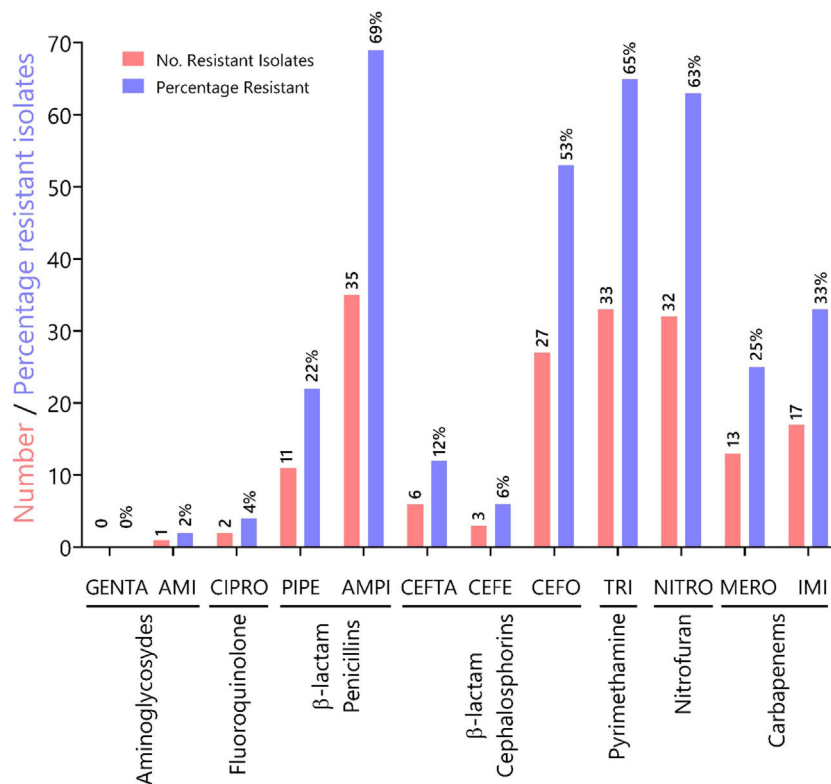
*Pseudomonas aeruginosa* is a Gram-negative bacterium with a capacity to produce a wide range of virulence factors which

makes it a MDR pathogen for which there are limited therapeutic options. Therefore, the understanding of resistance mechanisms can facilitate the development of new therapeutics.

In this study, we tested the resistance pattern of *P. aeruginosa* using antibiotics of the following groups: aminoglycosides (amikacin and gentamicin), fluoroquinolones (ciprofloxacin), other  $\beta$ -lactams (cephalosporins, ampicillins) (ceftazidime, cefepime, cefotaxime, piperacillin, ampicillin), pyrimethamine (trimethoprim), nitrofurantoin (Nitrofurantoin) and carbapenems (meropenem and imipenem).

**Table 2 – Primers used to amplify MexABR genes, pyoverdine receptor genes (*fpvA*) genes and  $\beta$ -lactamase genes blaTEM, blaSHV, blaCTX-M.**

Gene	Forward primer sequence	Reverse primer sequence	Product size (bp)
<i>mexA</i>	CTCGACCC GATCTACGTC	GTCTTCACCTCGACACCC	503
<i>mexR</i>	GAACTACCCCGTGAA TCC	CACTGGTCGAGGAGATGC	411
<i>mexB</i>	TGTCGAAGTTTTCATTGATAG	AAGGTCACGGTGATGGT	280
<i>fpvAI</i>	CGAAGGCCAGAACTACGAGA	TGTAGCTGGTGTAGAGGCTCAA	326
<i>fpvAII</i>	TACTCGACGGCCTGCACAT	GAAGGTGAATGGCTTGCCGT	897
<i>fpvAIII</i>	ACTGGGACAAGATCCAAGAGAC	CTGGTAGGACGAAATGCGAG	506
<i>blaSHV</i>	GCGAAAGCCAGCTGTCGGGC	GATTGGCGGGCTGTTATCGC	538
<i>blaCTX-M</i>	GTGCAGTACCAGTAAAGTTATGG	CGCAATATCATTGGTGGTGCC	538
<i>blaTEM</i>	AAAGATGCTGAAGATCA	TTTGGTATGGCTTCATTC	425

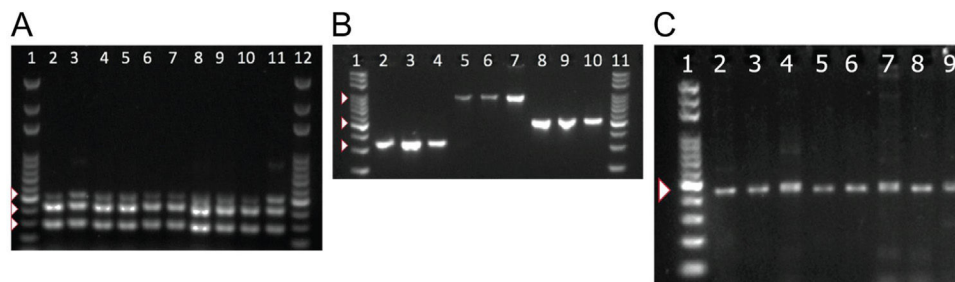


**Fig. 1 – Characterization of antibiotic resistance phenotypes in *Pseudomonas aeruginosa* isolates from Panamá (n = 51). Data shown indicates the number of resistant isolates (red) and prevalence for each isolate (blue) against seven antibiotic classes. Antibiotic key: amikacin (AMI), gentamicin (GENTA), ciprofloxacin (CIPRO), ceftazidime (CEFTA), cefepime (CEFE), cefotaxime (CEFO) piperacillin (PIPE), ampicillin (AMPI), trimethoprim (TRI), nitrofurantoin (NITRO), meropenem (MERO) and imipenem (IMI).**

In Panama, the Central Reference Public Health Laboratory (LCRSP) of the Gorgas Memorial Institute for Health Studies (ICGES) reviewed the National Antibiotic Resistance Database from 2007 until 2013.<sup>30</sup> For the purpose of our study, we chose the Gram-negative bacterium *P. aeruginosa* because of its medical importance and innate ability to develop antibiotic resistance. In our study, this bacterium was shown to be more susceptible to two aminoglycosides gentamicin (100%) and amikacin (98%), fluoroquinolones (ciprofloxacin, 96%) and two third generation cephalosporins, ceftazidime (88%) and cefepime (94%), as shown in Fig. 1. By contrast, the isolates tested in this study showed increased resistance to antibiotics in the following categories: (i) penicillins (ampicillin

69%, piperacillin 22%); (ii) pyrimethamines (trimethoprim 65%); (iii) nitrofurans (nitrofurantoin 63%), and (iv) third-generation cephalosporin cefotaxime (53%), in contrast to previous reports.<sup>30</sup> Resistance values for carbapenems oscillated between 25 and 33% for meropenem and imipenem, respectively.

In the United States, the National Healthcare Safety Network reported that *Pseudomonas aeruginosa* is the sixth most common nosocomial pathogen and the second most common in ventilator-associated pneumonia in hospitals.<sup>31</sup> Nguyen et al.<sup>31</sup> reported 9.7% resistance rate to at least one aminoglycoside and 19.3% to at least one carbapenem compared to 2% and 25-33%, respectively, in this study. The reported resistance



**Fig. 2** – PCR amplification of *MexAB-OprM* system, pyoverdine receptors genes, and the  $\beta$ -lactamase gene *blaTEM*. Detection of *mexA*, *mexB* and *mexR* genes of *P. aeruginosa* isolates by PCR (A). Lane 1 and 12 corresponds to the molecular weight marker (100 bp DNA ladder). Amplicon sizes: *mexA* (503 bp), *mexB* (280 bp) and *MexR* (411 bp). Positive bands are indicated by white arrowheads. Molecular detection of pyoverdine receptor genes *fpvAI*, *fpvAII* and *fpvAIII* genes from *P. aeruginosa* isolates by PCR (B). Lane 1 and 11 corresponds to the 100 bp DNA Ladder. Lanes 2-4: *fpvA* type I (326 bp); Lanes 5-7: *fpvA* type II (897 bp) and 8-10: *fpvA* type III amplicons (506 bp). Positive bands are indicated by white arrowheads. PCR Amplification and sequencing of *blaTEM*-like  $\beta$ -lactamase genes in eight positive isolates of *P. aeruginosa* (C). PCR detection of the  $\beta$ -lactamase gene *blaTEM* (425 bp). Lane 1 on panel A corresponds to the 100 bp molecular weight DNA ladder. Positive bands are indicated by a white arrowhead. Multiple sequence alignments showing homology to *blaTEM*-like genes from *E. coli* and *P. aeruginosa* are shown in Supplementary Fig. 2.

rate for cephalosporins was 10.3% (cefepime or ceftazidime) compared to 6%, 12% and 53% for cefepime, ceftazidime and cefotaxime, respectively, in this study. Resistance for one fluoroquinolone was reported in 21.6% of isolates compared to 4% in our study. Overall, the MDR index was reported to be 14.2% (previous study), much lower than the 59% observed in the present study. The difference between the two studies could be explained by the presence of intrinsic genetic differences between circulating isolates, which is corroborated by the presence of various resistance mechanisms and/or genes that contribute to virulence and resistance to various drugs representatives of seven antibiotic classes.

The *MexAB-OprM* efflux system is responsible for the development of resistance to a number of antimicrobials including quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, and  $\beta$ -lactams.<sup>3,6,7,32,33</sup> In our study we detected the presence of genes of the *MexAB-OprM* efflux system under iron-limiting conditions, as evidenced by the growth of *P. aeruginosa* under these conditions (CAA medium) and the presence of amplicons for *mexA*, *mexB* and *mexR* by multiplexed standard PCR (Fig. 2A). These results are in line with two previous reports where all components were encoded in the genome and induced by the iron-limiting conditions and their detections indicate that they confer or allow the development of resistance to different antibiotics.<sup>12,33-36</sup> The functional expression of each system components and its individual contribution to the resistance phenotype measured is the topic of a follow up study.

Given that iron is key for the development of antibiotic resistance in *Pseudomonas aeruginosa*, in this bacterium the mechanism of iron uptake is mediated by siderophores, pyochelone and pyoverdine, making them all crucial for the establishment of infection processes.<sup>15,37-39</sup> Several studies have shown that increasing concentrations of iron decreased resistance to antibiotics.<sup>40,41</sup> Under iron-repleted conditions, *P. aeruginosa* shows enhanced resistance to the antibiotics tigecycline and tobramycin; the production of pyoverdine

was shown to be an important factor for resistance against tigecycline.<sup>41,42</sup> In the present study, all 51 clinical isolates were pyoverdine producers (Table 1) and were PCR-positive for all pyoverdine receptor genes, being *fpvAI*-type the most prevalent type (44%), followed by *fpvAIII*-type (31%) and *fpvAII*-type (31%) (Fig. 2B). Our results agree with a previous study where the clinical isolates studied showed a similar prevalence<sup>43</sup> meaning that production of pyoverdine increases virulence of the bacteria.<sup>38</sup> A previous study associated *P. aeruginosa* antibiotic resistance with the presence of *fpvA* genes; the reported prevalence for the receptor genes *fpvAI*, *fpvAII*, and *fpvAIII* were 30%, 20%, and 23%, respectively.<sup>44</sup> Recently, a whole genome sequence of two MDR strains revealed as well that gene encoding putative proteins for iron uptake and other virulent factors were detected in both strains. The two siderophores pyochelin and pyoverdine were observed in both MDR strains and their corresponding receptors genes.<sup>45</sup> These results agree with those found in this study for all MDR.

Pyoverdine pigment production is ubiquitous amongst  $\beta$ -lactamase-producing strains of *P. aeruginosa*. Because both pyoverdine and  $\beta$ -lactamase production are necessary for the acquisition of drug resistance capabilities,<sup>25,26</sup> we decided to screen our isolates for the presence of ESBLs genes such as *blaTEM*, *blaCTX-M* and *blaSHV* using PCR. Twenty-two strains were PCR positive for the presence of the ESBL *blaTEM*-like genes (43%) (Fig. 2C); no PCR products were obtained for *blaCTX-M* and *blaSHV* genes. All *blaTEM*-positive isolates were highly similar to the *blaTEM* gene of *Pseudomonas aeruginosa* strain SJP2. An earlier study in *P. aeruginosa* by Girlich et al.<sup>46</sup> reported a 0% prevalence of *CTX-M*-like and *SHV*-like genes, and the presence of a gene that coded for a  $\beta$ -lactamase<sup>VEB-1</sup> (*blaTEM*-like) inserted in an integron; these results are in line with the present study. By contrast, a study by reported a similar prevalence for *blaTEM*-like genes (26.7%), as well as low prevalences for low-frequency  $\beta$ -lactamases such as *blaCTX* (17.3%) and *blaSHV* (10.7%).<sup>47</sup> Similarly, a study by Bokaeian et al.<sup>48</sup> reported prevalences of 30% for

blaTEM-like, 6.6% for blaVHS-like and 0% for blaCTX-like  $\beta$ -lactamases. Furthermore, certain strains of *Pseudomonas* and *Acinobacter spp.* exhibit profound differences in the prevalence of  $\beta$ -lactamase-like genes; in these strains the most prevalent type is blaVHS-like (75%), followed by blaCTX-like (57.5%) and finally blaTEM (15%).<sup>23</sup> Together the presence of  $\beta$ -lactamases and the MexAB-OprM efflux pump system greatly contributes to resistance against many classes of  $\beta$ -lactams in *P. aeruginosa*.<sup>7</sup> Our initial screening provided important clues regarding the potential pathogenicity and geographic occurrence of circulating nosocomial strains in Panama.

Different approaches have been used to fight infections caused by *P. aeruginosa*, from the design of new drugs to metagenomics projects aimed at leading to the discovery of new antibiotics; however, despite such advances, together they have been of limited success<sup>41</sup> due to the diversity of resistance mechanisms that allows the bacterium to evade a wide variety of antibiotics. New therapeutic strategies have been developed, i.e. by combining iron-chelators with antibiotics, given the preponderant physiological role of iron in the physiology of this pathogen.<sup>42,49–52</sup> The virulence factors identified in our study provides valuable information regarding the prevalence of resistance genes and their potential impact on treatments that exploit the unique physiology of the pathogen. More importantly, this information can be used to better guide future control strategies, understand the evolution and dissemination of strains hoping to prevent the development of resistance in *Pseudomonas aeruginosa*.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bjid.2020.11.003>.

## REFERENCES

- Pirnay J-P, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, et al. *Pseudomonas aeruginosa* Population Structure Revisited. *PLoS One*. 2009;4:e7740.
- Livermore DM. Of *Pseudomonas*, Porins, Pumps and Carbapenems. *Journal of Antimicrobial Chemother*. 2001;47:247–50.
- Strateva T, Yordanov D. *Pseudomonas aeruginosa* - a phenomenon of bacterial resistance. *Journal of Medical Microbiology*. 2009;58:1133–48.
- Goli H, Nahaei M, Ahangarzadeh Rezaee M, Hasani A, Kafil H, Aghazadeh M, Nikbakht M, Khalili Y. Role of MexAB-OprM and MexXY-OprM Efflux Pumps and Class 1 Integrins in Resistance to Antibiotics in Burn and Intensive Care Unit Isolates of *Pseudomonas aeruginosa*. *Journal of Infection and Public Health*. 2018;11:364–72. <http://dx.doi.org/10.1016/j.jiph.2017.09.016>.
- Ozer B, Duran N, Onlen Y, Savas L. Efflux Pump Genes and Antimicrobial Resistance of *Pseudomonas aeruginosa* Strains Isolated from Lower Respiratory Tract Infections Acquired in an Intensive Care Unit. *The Journal of antibiotics*. 2011;65:9–13.
- Nikaido H, Pagès JM. Broad-specificity Efflux Pumps and their Role in Multidrug Resistance of Gram-negative Bacteria. *FEMS microbiology reviews*. 2011;36:340–63.
- Wi YM, Greenwood-Quaintance KE, Schuetz AN, Ko KS, Peck KR, Song JH, et al. Activity of Ceftolozane-Tazobactam against Carbapenem-Resistant, Non-Carbapenemase-Producing *Pseudomonas aeruginosa* and Associated Resistance Mechanisms. *Antimicrobial agents and chemotherapy*. 2017;62(1):e01970–17.
- Poole K, Krebs K, McNally C, Neshat S. Multiple Antibiotic Resistance in *Pseudomonas aeruginosa*: Evidence for Involvement of an Efflux Operon. *Journal of Bacteriology*. 1993;175:7363–72.
- Poole K. Multidrug Efflux Pumps and Antimicrobial Resistance in *Pseudomonas aeruginosa* and Related Organisms. *J Mol Microbiol Biotechnol*. 2001;2:255–64.
- Liu Y, Yang L, Molin S. Synergistic Activities of an Efflux Pump Inhibitor and Iron Chelators against *Pseudomonas aeruginosa* Growth and Biofilm Formation. *Antimicrobial Agents and Chemotherapy*. 2010;2020:3960–3.
- Pesingi PV, Singh BR, Pesingi PK, Bhardwaj M, Singh SV, Kumawat M, Sinha DK, Gandham RK. MexAB-OprM Efflux Pump of *Pseudomonas aeruginosa* Offers Resistance to Carvacrol: A Herbal Antimicrobial Agent. *Frontiers in microbiology*. 2019;10:2664.
- Auda Al-Grawi Ibtesam Ghadban, khdir Al-Absali Amara, Hashim Kareem Noor, Abdul-Latif Belal Sabah. Occurrence of MexAB-OprM Efflux Pump Operon on Septicemic *Pseudomonas aeruginosa* Chromosome. *Iraqi Postgraduate Med Journal*. 2012;11:97–102.
- Meyer JM, Abdallah MA. The Fluorescent Pigment of *Pseudomonas fluorescens*: Biosynthesis, Purification and Physicochemical Properties. *Journal of General Microbiology*. 1978;107:319–28.
- Cornelis P, Hohnadel D, Meyer JM. Evidence for Different Pyoverdine-Mediated Iron Uptake Systems among *Pseudomonas aeruginosa* Strains. *Infection and Immunity*. 1989;57:3491–7.
- Meyer J, Neely A, Stintzi A, Georges C, Holder I. Pyoverdine is Essential for Virulence of *Pseudomonas aeruginosa*. *Infection and Immunity*. 1996;64:518–23.
- Chial M de, Ghysels B, Beatson SA, Geoffroy V, Meyer JM, Pattery T, et al. Identification of Type II and Type III Pyoverdine Receptors from *Pseudomonas aeruginosa*. *Microbiology*. 2003;149:821–31.
- Ghysels B, Dieu BT, Beatson SA, Pirnay J, Ochsner UA, Vasil ML, et al. FpvB, an Alternative Type I Ferripyoverdine Receptor of *Pseudomonas aeruginosa*. *Microbiology*. 2004;150:1671–80.
- Winstanley C, Kaye SB, Neal TJ, Chilton HJ, Miksch S, Hart C. Genotypic and Phenotypic Characteristics of *Pseudomonas aeruginosa* Isolates Associated with Ulcerative Keratitis. *Journal of medical microbiology*. 2005;54:519–26.
- Chroma M, Kolar M. Genetic Methods for Detection of Antibiotic Resistance: Focus on Extended-Spectrum  $\beta$ -Lactamases. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2010;154:289–96.
- Jiang X, Zhang Z, Li M, Zhou D, Ruan F, Lu Y. Detection of Extended-Spectrum  $\beta$ -Lactamases in Clinical Isolates of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 2006;50:2990–5.
- Bradford P. Extended-Spectrum  $\beta$ -Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of this Important Resistance Threat. *Clinical microbiology reviews*. 2001;14:933–51.
- Livermore DM. Beta-Lactamases in Laboratory and Clinical Resistance. *Clin Microbiol Rev*. 1995;8:557–84.
- Jamali S, Shahid M, Sobia F, Singh A, Khan HM. Phenotypic and Molecular Characterization of Cefotaximases, Temoniera, and Sulfhydryl Variable  $\beta$ -Lactamases in *Pseudomonas* and *Acinetobacter* Isolates in an Indian Tertiary Healthcare Center. *Indian J Pathol Microbiol*. 2017;60:196–201.

24. Bauernfeind A, Grimm H, Schweighart S. A New Plasmidic Cefotaximase in a Clinical Isolate of *Escherichia coli*. *Infection*. 1990;18:294–8.
25. Ullah W, Qasim M, Rahman H, Jie Y, Muhammad N. B-lactamase-Producing *Pseudomonas aeruginosa*: Phenotypic Characteristics and Molecular Identification of Virulence Genes. *Journal of the Chinese Medical Association*. 2017;80:173e177.
26. Ullah W, Qasim M, Rahman H, Bari F, Khan S, Dworeck T, et al. Molecular Identification of TEM-116 B-Lactamase Gene in Isolates of Pathogenic *Pseudomonas aeruginosa*: A First Report from Pakistan. *Tropical Journal of Pharmaceutical Research*. 2017;16:149–54.
27. De Vos D, Chial M de, Cochez C, Jansen S, Tümmler B, Meyer JP, et al. Study of Pyoverdine Type and Production by *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients: Prevalence of Type II Pyoverdine Isolates and Accumulation of Pyoverdine-Negative Mutations. *Archives of Microbiology*. 2001;175:384–8.
28. Adesoji AT, Ogunjobi AA, Olatoye IO. Molecular characterization of selected multidrug resistant *Pseudomonas* from water distribution systems in southwestern Nigeria. *Annals of Clinical Microbiology and Antimicrobials*. 2015;14:39, <http://dx.doi.org/10.1186/s12941-015-0102-4>.
29. Adesoji Ayodele T, Ogunjobi Adeniyi A. Detection of Extended Spectrum Beta-Lactamases Resistance Genes among Bacteria Isolated from Selected Drinking Water Distribution Channels in Southwestern Nigeria. *BioMed research international*. 2016;2016:7149295.
30. Moreno J, Conte E, Tribaldos, et al. Antibiotic Resistance Profiles in Panama: Trends from 2007 to 2013. *Pharm Pharmacol Int J*. 2018;6:350–5.
31. Nguyen L, Garcia J, Gruenberg K, et al. Multidrug-Resistant *Pseudomonas aeruginosa* Infections: Hard to Treat, But Hope on the Horizon? *Curr Infect Dis Rep*. 2018;20:23, <http://dx.doi.org/10.1007/s11908-018-0629-6>.
32. Horna G, López M, Guerra H, et al. Interplay between MexAB-OprM and MexEF-OprN in clinical isolates of *Pseudomonas aeruginosa*. *Sci Rep*. 2018;8(16463).
33. Kishk RM, Abdalla MO, Hashish AA, Nemr NA, El Nahhas N, Alkahtani S, Abdel-Daim MM, Kishk SM. Efflux MexAB-Mediated Resistance in *P. aeruginosa* Isolated from Patients with Healthcare Associated Infections. *Pathogens (Basel, Switzerland)*. 2020;9:471.
34. Li XZ, Nikaido H, Poole K. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 1995;39:1948–53.
35. Abbas HA, El-Ganiny AM, Kamel HA. Phenotypic and Genotypic Detection of Antibiotic Resistance of *Pseudomonas aeruginosa* Isolated from Urinary Tract Infections. *African health sciences*. 2018;18:11–21.
36. Poole K. Efflux-mediated antimicrobial resistance. In: *Antibiotic Discovery and Development*. Berlin/Heidelberg, Germany: Springer; 2012. p. 349–95.
37. Cornelis P, Dingemans J. *Pseudomonas aeruginosa* Adapts its Iron Uptake Strategies in Function of the Type of Infections. *Frontiers in cell and infection microbiology*. 2013;3:75, <http://dx.doi.org/10.3389/fcimb.2013.00075>.
38. Kang D, Revtovich AV, Chen Q, Shah KN, Cannon CL, Kirienko NV. Pyoverdine-Dependent Virulence of *Pseudomonas aeruginosa* Isolates From Cystic Fibrosis Patients. *Frontiers in microbiology*. 2019;10:2048, <http://dx.doi.org/10.3389/fmicb.2019.02048>.
39. Bonneau A, Roche B, Schalk IJ. Iron acquisition in *Pseudomonas aeruginosa* by the siderophore pyoverdine: an intricate interacting network including periplasmic and membrane proteins. *Sci Rep*. 2020;10:120, <http://dx.doi.org/10.1038/s41598-019-56913>.
40. Kreamer NN, Costa F, Newman K. The ferrous iron-responsive BqsRS two-component system. *MBio*. 2015;6:1–12.
41. Ezraty B, Barras F. The 'Liaisons Dangereuses' Between Iron and Antibiotics. *FEMS microbiology reviews*. 2016;40:418–35.
42. Oglesby-Sherrouse AG, Djapgne L, Nguyen AT, Vasil AI, Vasil ML. The Complex Interplay of Iron, Biofilm Formation, and Mucoidy Affecting Antimicrobial Resistance of *Pseudomonas aeruginosa*. *Pathogens and disease*. 2014;70:307–20.
43. Díaz Y, Núñez J, Mayorga B de, Jaramillo F, Chen O, Paredes M de, et al. Epidemiología Molecular de Aislados Clínicos de *Pseudomonas aeruginosa* en el Complejo Metropolitano Harmodio Arias Madrid. *Tecnociencia*. 2012;14.
44. Kashina A, Brown P. Diversity of Antimicrobial Resistance and Virulence Determinants in *Pseudomonas aeruginosa* Associated with Fresh Vegetables. *International Journal of Microbiology*. 2012, <http://dx.doi.org/10.1155/2012/426241>.
45. Madaha EL, Mienie C, Gonsu HK, Bughe RN, Fonkoua MC, Mbacham WF, Alayande KA, Bezuidenhout CC, Ateba CN. Whole-genome sequence of multi-drug resistant *Pseudomonas aeruginosa* strains UY1PSABAL and UY1PSABAL2 isolated from human broncho-alveolar lavage, Yaoundé, Cameroon. *PloS one*. 2020;15:e0238390.
46. Girlich D, Naas T, Leelaporn A, Poirel L, Fennewald M, Nordmann PL. Nosocomial Spread of the Integron-located veb-1-like Cassette Encoding an Extended-Spectrum B-Lactamase in *Pseudomonas aeruginosa* in Thailand. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*. 2002;34:603–11.
47. Peymani A, Naserpour-Farivar T, Zare E, Azarhoosh KH. Distribution of *bla*TEM, *bla*SHV, and *bla*CTX-M genes among ESBL-producing *P. aeruginosa* isolated from Qazvin and Tehran Hospitals, Iran. *Journal of preventive medicine and hygiene*. 2017;58:E155–60.
48. Bokaeian M, Shahraki Zahedani S, Soltanian Bajgiran M, Ansari Moghaddam A. Frequency of PER, VEB, SHV, TEM and CTX-M Genes in Resistant Strains of *Pseudomonas aeruginosa* Producing Extended Spectrum β-Lactamases. *Jundishapur journal of microbiology*. 2014;8:e13783, <http://dx.doi.org/10.5812/jjm.13783>.
49. Mislin GL, Schalk IJ. Siderophore-dependent Iron Uptake systems as Gates for Antibiotic Trojan Horse Strategies against *Pseudomonas aeruginosa*. *Metallomics: Integrated Biometal Science*. 2014;6:408–20.
50. Smith DJ, Lamont IL, Anderson GJ, Reid DW. Targeting Iron Uptake to Control *Pseudomonas aeruginosa* Infections in Cystic Fibrosis. *The European respiratory journal*. 2013;42:1723–36.
51. Rampioni G, Pillai CR, Longo F, Bondi R, Baldelli V, Messina M, Imperi F, Visca P, Leoni L. Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence. *Scientific reports*. 2017;7:11392.
52. Liu Jun, Hou Jin-Song, Li Yi-Bin, Miao Zhi-Ying, Sun Ping-Hua, Lin Jing, et al. Novel 2-Substituted 3-Hydroxy-1,6-dimethylpyridin-4(1H)-ones as Dual-Acting Biofilm Inhibitors of *Pseudomonas aeruginosa*. *Journal of Medicinal Chemistry*. 2020, <http://dx.doi.org/10.1021/acs.jmedchem.0c00763>.