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A comprehensive and contemporary “snapshot” of β-lactamases in carbapenem resistant Acinetobacter baumannii

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ABSTRACT

Successful treatment of Acinetobacter baumannii infections require early and appropriate antimicrobial therapy. One of the first steps in this process is understanding which β-lactamase (bla) alleles are present and in what combinations. Thus, we performed WGS on 98 carbapenem-resistant Acinetobacter baumannii (CR Ab). In most isolates, an acquired blaOXA carbapenemase was found in addition to the intrinsic blaOXA allele. The most commonly found allele was blaOXA-A23 (n = 78/98). In some isolates, blaOXA-A23 was found in addition to other carbapenemase alleles: blaOXA-82 (n = 12/78), blaOXA-72 (n = 2/78) and blaOXA-24/40 (n = 1/78). Surprisingly, 20% of isolates carried carbapenemases not routinely assayed for by rapid molecular diagnostic platforms, i.e., blaOXA-A2 and blaOXA-122, all had ISAba1 elements. In 8 CR Ab, blaOXA-82 or blaOXA-122 was the only carbapenemase. Both blaOXA-24/40 and its variant blaOXA-72 were each found in 6/98 isolates. The most prevalent ADC variants were bladADC-30 (21%), bladADC-162 (21%), and bladADC-212 (26%). Complete combinations are reported.

1. Introduction

Multidrug-resistant (MDR) Acinetobacter baumannii (Ab) pose a significant challenge to modern medicine. The World Health Organization categorizes MDR Ab as the “highest priority pathogen” for which antibiotic development is urgently needed (WHO-List, 2020), and studies that aid our understanding of this organism are certain to have significant impact on this crisis. Currently, very few therapeutic agents exist or are in development that can be used to treat MDR Ab infections, including those of the bloodstream. In efforts to address this persistent dilemma, multiple pharmaceutical companies have undertaken different approaches to enhance our current therapeutic arsenal. These efforts include the development of a novel fluoroacycline (eravacycline), siderophore β-lactams (e.g., cefiderocol), and more effective β-lactam β-lactamase inhibitors (e.g.,

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sublactam/ETX2514 and cepfepime/WCK4224) (Choi and McCarthy, 2018; Durand-Reville et al., 2017; Ito et al., 2018; Mushtaq et al., 2017; Papp-Walace et al., 2018; Shapiro et al., 2017; Zhanel et al., 2016). In studies performed to date, these therapeutics are showing significant promise, but the fear is ever present that even these will fall short as universal agents to reliably treat MDR Ab.

β-lactamase inhibition is key in preserving efficacy of our current armamentarium of antibiotics against Ab. However, many times the focus of that inhibition is on a single class of β-lactamase. Clearly, in inhibitor design, we need to take into account all β-lactamases occurring in an individual organism, based upon our understanding of currently circulating alleles in isolates. In addition to OXA carbapenemases that Ab can acquire (e.g., OXA-23 [originally called ARI-1] and OXA-24/40), they possess an intrinsic OXA and AmpC (Acinetobacter Derived Cephalosporinase, ADC) β-lactamase, that can confer resistance to carbapenems and cephalosporins, respectively (Bou et al., 2000; Donald et al., 2000; Hujer et al., 2005). Therefore, class C and D β-lactamases are important targets for intervention as they are the major contributors of β-lactam resistance in Ab.

With these goals in mind, we performed whole genome sequencing (WGS) on 98 carbapenem-resistant Ab isolates (CR Ab, resistant to doripenem, imipenem, and meropenem) (Evans et al., 2017) and assessed the various β-lactamase combinations contained therein. This WGS database, and others like it, will be important in providing context to future work in the area of β-lactamase inhibitor design and provides a comprehensive “snapshot” of recently circulating β-lactamase genes (bla genes) in CR Ab. A better understanding of how to treat infections caused by opportunistic organisms like Ab will potentially be crucial in the fight for patients in the ICU on ventilators that are at an increased risk of hospital-acquired and ventilator-associated pneumonia caused by CR Ab. As CR Ab is a leading cause of hospital-acquired pneumonia, and also reported to be a pathogen of community-acquired Gram-negative pneumonia (Chung et al., 2011; Cilloniz et al., 2019; Lescure et al., 2020; Wong et al., 2017). This could be especially significant during the COVID-19 pandemic (Lescure et al., 2020).

2. Methods and materials

This group of isolates was used in the Primers III study and a description of the isolates can be found there (Evans et al., 2017; Hujer et al., 2006; Perez et al., 2010). In short, the isolates used in this study consisted of 94 carbapenem-resistant Ab collected between 2007 and 2013 from a 6-hospital healthcare system in Northeast Ohio: including 5 community hospitals and a facility serving as both as a long-term care unit and a long-term acute care hospital. Four additional Ab were collected from patients at the Walter Reed Army Medical Center between March 2003 and February 2005. All Ab clinical isolates in this study displayed an MDR phenotype and were resistant to all carbapenem tested.

The genomes of all Ab clinical isolates were sequenced using paired-end NexteraXT libraries by Illumina NextSeq (2 × 150 bp) to ~100-fold coverage. Reads were assembled using SPAdes (Bankevich et al., 2012), annotated using NCBI’s Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016) and deposited in the NCBI SRA and GenBank WGS repositories (BioProjects PRJNA384060 and PRJNA384065). In addition, the DNA sequence for each isolate was analyzed using ResFinder at the Center for Genomic Epidemiology website. β-lactamase genes were manually extracted if not a 100% match with 100% coverage of a known β-lactamase gene.

For PCR analysis of ISAb1 upstream of blaOXA-82 and blaOXA-172, the following primer set was used: 5’ TGAGGATGCATCTCATGTCGA 3’ (OX-51) and 5’ CAGCGAGCTTGGTGTG 3’ (ISAb1) (Segal et al., 2005; Woodford et al., 2006). This combination of primers produces an approximate 1200 bp product when ISAb01 is proximal to the blaOXA.

3. Results

From the WGS data, the combination of β-lactamases found in each isolate was determined, along with the Pasteur and Oxford Multilocus Sequence Types (Bartual et al., 2005; Diancourt et al., 2010). This was done for each of the 98 CR Ab. The predominant Pasteur sequence type was the ST2 group, which is also known as international clonal lineage 2 (IC2). There were 79 CR Ab that were ST2, and 19 that were non-ST2 according to the Pasteur Scheme. Using the Oxford scheme ST1626 and ST1631 accounted for 40 and 23 isolates, respectively. Table 1 summarizes the combination of β-lactamases found in this collection, as well as which STs they were found in.

3.1. blaOXA

In most isolates, an acquired blaOXA carbapenemase was found in addition to the intrinsic or chromosomal blaOXA allele. However, in 18 ST2 isolates, the intrinsic blaOXA acquired a single point mutation that converted blaOXA-66 (the variant associated with IC2 isolates) to blaOXA-82 (Table 1) (Zander et al., 2013). blaOXA-82 was also found as the sole carbapenemase allele in 6% of isolates. In addition, blaOXA-172 was found in two ST1088 isolates. Both OXA-82 (OXA-66 variant with L167V), and OXA-172 (OXA-66 variant with I129V and W222L) are carbapenemases (Mitchell and Leonard, 2014; Schroder et al., 2016; Zander et al., 2013), and were found in combination with ISAb1 upstream of the blaOXA gene as evidenced by PCR analysis.

By far, the most commonly found acquired OXA carbapenemase allele was blaOXA-23 (Table 1) (Donald et al., 2000). This bla gene was detected in 78 of the 98 CR isolates. In 81% (n = 63/78) of the isolates containing blaOXA-23, it was the only carbapenemase present. However, in some isolates it was found in addition to other carbapenemase alleles, i.e., in addition to blaOXA-82 (n = 12/78), blaOXA-72 (n = 2/78) and blaOXA-24/40 (n = 1/78). The carbapenemase genes blaOXA-24/40 (Bou et al., 2000) and its variant blaOXA-72 were each found in 6/98 isolates (both alone and in combination with blaOXA-23), but never found together in a single isolate (Table 1). While the blaOXA-58 allele (Poirel et al., 2005) was found in 2 isolates as the sole carbapenemase gene.

3.2. blaADC

The most prevalent Acinetobacter Derived Cephalosporinase (ADC) variants were blaADC-30 (n = 21/98 isolates), blaADC-162 (ADC-30 variant with an A220E mutation in the Ω loop, n = 21/98), and blaADC-212 (ADC-25 variant with A200D, P219L, and an alanine insertion in the Ω loop, n = 25/98; Tables 1 and 2) (Kuo et al., 2015). Also, of note, blaADC-33 and blaADC-219 (ADC-33 with G222D) were found in 8/98 isolates (Rodríguez-Martínez et al., 2010). Other ADC variants (21/98) were found in all but 2 isolates (Table 1). Numbering of the amino acids in the ADC variants is based on the SANC numbering scheme (Mack et al., 2019).

Many combinations of β-lactamases were found in individual isolates. However, certain combinations were more frequent than others. The 3 most frequently found combinations were: OXA-23/OXA-66/ADC-162 with or without TEM-1 (n = 21); OXA-23/OXA-66/ADC-212 (n = 21); and OXA-23/OXA-66/ADC-30 with or without TEM-1 (n = 40) (Table 1). The high prevalence of OXA-66 reflects the prevalence of ST2 isolates circulating in hospitals (Adams et al., 2019; Wright et al., 2014; Wright et al., 2016).

4. Discussion

The current prevalence of multidrug- and pandrug-resistant strains of Ab, combined with the lack of new antibiotics, underscores
that study, with particular attention being paid to the formed an extensive analysis of the WGS of 98 CR combinations contained within each isolate. Based on these results: New ADC variants in this study.

Building upon our work with PRIMERS III, an evaluation of rapid molecular diagnostics to identify carbapenem susceptibility and resistance in Acinetobacter spp. (Evans et al., 2017), our group performed an extensive analysis of the WGS of 98 CR Ab strains used in that study, with particular attention being paid to the β-lactamase combinations contained within each isolate. Based on these results:

| Pasteur ST - (# of Isolates) | Carbapenemase | Intrinsic OXA | ADC | Other | Oxford ST |
|-----------------------------|---------------|---------------|-----|-------|-----------|
| ST2 - (21)                  | OXA-23        | OXA-66        | ADC-212 |      | ST1631    |
| ST2 - (16)                  | OXA-23        | OXA-66        | ADC-162 | TEM-1 | ST1626 - (13), ST1660 - (1), ST1661 - (1), ST1676 - (1) |
| ST2 - (9)                   | OXA-23        | OXA-66        | ADC-30  |      | ST1626    |
| ST2 - (5)                   | OXA-23        | OXA-66        | ADC-162 |      | ST1626    |
| ST2 - (1)                   | OXA-23        | OXA-66        | ADC-143 | TEM-1 | ST1626    |
| ST2 - (1)                   | OXA-23        | OXA-66        | ADC-213 |      | ST1631    |
| ST2 - (3)                   | OXA-23, OXA-82a | OXA-66    | ADC-56  | TEM-1 | ST1626    |
| ST2 - (3)                   | OXA-23, OXA-82a | OXA-66    | ADC-33  |      | ST1637    |
| ST2 - (3)                   | OXA-23, OXA-82a | OXA-66    | ADC-219 |      | ST1637    |
| ST2 - (3)                   | OXA-23, OXA-82a | OXA-66    | ADC-30  |      | ST1626    |
| ST2 - (1)                   | OXA-82a       | OXA-66        | ADC-30  |      | ST1626    |
| ST2 - (1)                   | OXA-82a       | OXA-66        | ADC-219 |      | ST1637    |
| ST2 - (2)                   | OXA-82a       | OXA-66        | ADC-30  |      | ST1626    |
| ST2 - (1)                   | OXA-82a       | OXA-66        | ADC-33  |      | ST1637    |
| ST2 - (1)                   | OXA-82a       | OXA-66        | ADC-212 |      | ST1631    |
| ST2 - (3)                   | OXA-72        | OXA-66        | ADC-30  |      | ST1626 - (1), ST1628 - (2) |
| ST2 - (1)                   | OXA-72        | OXA-66        | ADC-217 |      | ST1628    |
| ST2 - (2)                   | OXA-23, OXA-72 | OXA-66    | ADC-30  |      | ST1626 - (1), ST1632 - (1) |
| ST2 - (1)                   | OXA-58        | OXA-66        | ADC-30  | TEM-1 | ST1626    |
| ST250 - (3)                 | OXA-23        | OXA-67        | ADC-407 | ADC-216 | ST1646 |
| ST10 - (2)                  | OXA-23        | OXA-68        | ADC-76  |      | ST1647    |
| ST406 - (2)                 | OXA-23        | OXA-71        | ADC-212 |      | ST1635    |
| ST1 - (1)                   | OXA-23        | OXA-69        | ADC-176 | TEM-1 | ST1663    |
| ST25 - (1)                  | OXA-23        | OXA-64        | ADC-30  |      | ST991     |
| ST79 - (1)                  | OXA-23, OXA-24/40 | OXA-65  | ADC-218 |      | ST1629    |
| ST79 - (1), ST93 - (1)      | OXA-24/40     | OXA-65        | ADC-218 | TEM-1 | ST1348 - (1), ST1629 - (1) |
| ST79 - (1)                  | OXA-24/40     | OXA-65        | ADC-218 |      | ST1629    |
| ST406 - (1)                 | OXA-24/40     | OXA-71        | ADC-212 |      | ST1635    |
| ST79 - (1)                  | OXA-24/40     | OXA-100       | ADC-79  | TEM-1 | ST1629    |
| ST32 - (1)                  | OXA-58        | OXA-100       | ADC-79  |      | ST1627    |
| ST406 - (1)                 | OXA-72        | OXA-223       | ADC-220 |      | ST1635    |
| ST1088 - (2)                | OXA-172b      | OXA-172b      | ADC-215 |      | ST1656 - (1), ST1669 - (1) |

Table 1 summarizes β-lactamase alleles present; Table 2 describes the new ADCs in this collection. Table 3 presents what is already known about the key properties and structure/function basis of the OXA carbapenemases and ADC β-lactamases found herein, and Table 4 compares the ability of various rapid molecular diagnostic (RMD) platforms to detect the carbapenemases found in this collection, as determined from the platform’s product literature.

OXA-23 has been shown to be a major driver of carbapenem resistance in Ab, and OXA-24/40, an enzyme similar to OXA-23 in structure and specificity, is also extensively found. Both are acquired high affinity carbapenemases that have a common structural feature thought to be primarily responsible for this property. This structural feature is a hydrophobic bridge of two residues that stretches across the top of the active site (Table 3) (Kaitany et al., 2013; Santillana et al., 2007; Schneider et al., 2011; Smith et al., 2013; Stewart et al., 2019). It is important to note that both of these acquired carbapenemase genes are found on highly transmissible mobile genetic elements, which is particularly worrisome from an infection control standpoint, because not only can horizontal transmission of these common resistance elements occur between Acinetobacter strains and plasmids, but also interspecies plasmid transfer can occur (Grosso et al., 2012).

Of note, we have identified OXA variants in this collection with substitutions that expand their substrate profile. OXA-82 is one such variant, a L167V substitution in OXA-66 greatly enhances the hydrolytic efficiency of that enzyme toward carbapenems (Table 3) (Zander et al., 2013; Mitchell and Leonard, 2014). In most isolates, an acquired blaOXA carbapenemase allele was found in addition to the intrinsic or chromosomal blaOXA allele. However, in 18 isolates it appears that the intrinsic blaOXA-66 was converted by mutation to

| New ADC number | ADC-like | Reference number |
|----------------|----------|------------------|
| ADC-212        | ADC-25 A200D, P219L, Ala219a ins btw P219L and A220 | OTN05897.1 |
| ADC-213        | ADC-25 A200D, ins of Sla that replaces A217 and A220 | OTS51589.1 |
| ADC-214        | ADC-52 G222S, N220T | OTR85897.1 |
| ADC-215        | ADC-170 G211A, P219S, S230T | OTT53070.1 |
| ADC-216        | ADC-25 G75A, R86A, S143P, P169S, N206K, T279P | OTT78301.1 |
| ADC-217        | ADC-30 V262E | OTT60833.1 |
| ADC-218        | ADC-30 A200D, P219L, Ala219a ins btw P219L and A220, K236E | OTU52329.1 |
| ADC-219        | ADC-33 G220D | OTU79650.1 |
| ADC-220        | ADC-25 Q120K, A200D, P219L | OVN97777.1 |

Table 1: β-lactamase combinations found within the 98 carbapenem resistant Acinetobacter baumannii.

Table 2: New ADC variants in this study.
Another such variant in this collection was OXA-172, found in 2 ST1088 isolates. It also appears to have evolved from blaOXA-66, as no other intrinsic blaOXA was found, and it differs from OXA-66 by 2 substitutions, 1129V and W222L. It has been shown that these substitutions result in tighter binding of the enzyme to doripenem and imipenem. In fact, OXA-172 displayed carbapenem gain-of-function, as demonstrated with molecular modeling, OXA 51/66 with a L167V substitution makes room for the rotation of the side-chain of I129; this in turn removes the steric clash of isoleucine with the hydroxyethyl group of carbapenems and affinity is greatly increased.

Table 4
Comparison of RMDs ability to detect carbapenemase genes found in this study.

| Platform                  | OXA-23 | OXA-24/40 | OXA-58 | OXA-72 | OXA-82 | OXA-172 |
|---------------------------|--------|-----------|--------|--------|--------|---------|
| Verigene BC-GN            | Yes    | Yes       | Yes    | Yes    | No     | No      |
| BioFire Film Array        | No     | No        | No     | No     | No     | No      |
| Xpert® Carba-R Assay      | No     | No        | No     | No     | No     | No      |
| Acuitas AMR Gene Panel    | No     | No        | No     | No     | No     | No      |
| ePlex BCID-GN Panel       | Yes    | No        | No     | No     | No     | No      |
| Check-points CT 103XL     | yes    | Yes       | Yes    | Yes    | Yes    | No      |

Data as determined from platform’s product literature.

Predicted to be detected based on in silico analysis, would call it OXA-24/40.
do they detect the presence of ISAb*1 insertion elements upstream of the genes. It is important to keep in mind that without the IS element upstream, these blaOXA, including blaOXA-23, do not confer carbapenem resistance. Even WGS can miss ISAb*1-associated bla genes due to the ISAb*1 insertion sequences being present in multiple locations of the chromosome and plasmids, leading to misassemblies and fragmented contigs when only using short illumina reads. It has been our experience that besides closing genomes by inclusion of long reads, standard PCR amplifications are needed to accurately determine the ISAb positions relative to a gene of interest, such as blaOXA-82 and blaOXA-172 (Zander et al., 2013; Zander et al., 2012).

Table 4 compares the ability of various RMD platforms to detect the carbapenemase genes found in this collection of Ab isolates. As can be seen, none of the currently available platforms are able to detect blaOXA-82 and blaOXA-172. BioFire Film Array, Xpert® Carba-R Assay, and Acuitas AMR Gene Panel do not even detect blaOXA-23, which was by far the most commonly found OXA carbapenemase allele (80% of isolates).

ADCs are chromosomally encoded class C β-lactamases, found in Ab and other Acinetobacter spp., that are responsible for resistance to penicillins, cephalosporins, and βLBI combinations (Hujer et al., 2005). Being among the first laboratories to recognize the importance of this β-lactamide, our early work showed that ADC-7 β-lactamase demonstrates a remarkably high turnover rate for first-generation cephalosporins and relatively low affinity for the commercially available BLIs (Hujer et al., 2005).

The ADC allele most commonly found in this collection was blaADC-212 (n = 25/98 isolates). It encoded an ADC-25-like β-lactamase with the following substitutions: A200D, P219L, and an Ala219a insertion between P219L and A220 (SANC numbering) (Table 2). ADC-33 (ADC-30-like, with a P213R substitution and the same insertion of an Ala residue inside the Ω loop) hydrolyzes ceftazidime, cefepime, and aztreonam, but not carbapenems (Rodriguez-Martinez et al., 2010). We speculate that for ADC-212, the Ala219a (between P219L and A220) might confer an extended-spectrum resistance phenotype similar to ADC-33 (Tables 2 and 3). In efforts to understand the importance of these substitutions in the larger context of structure-activity relationships, we generated preliminary data that suggests the alanine insertion between P219L and A220 in the Ω loop increases ceftazidime MICs from 16 mg/mL to greater than 512 mg/L (unpublished data).

Other studies of ADC variants found within this collection demonstrate that subtle changes in the active site can lead to alterations in substrate turnover. ADC-30 was shown to contribute to sulfactam resistance when overexpressed in Ab (Kuo et al., 2015). Within our collection, blaADC-30 (n = 21/98 isolates) and blaADC-162 (n = 21/98 isolates) were also frequently found. ADC-162 is ADC-30 with an A220E substitution in the Ω loop region. Additionally, ADC-56 present in some of our clinical isolates, is a variant of ADC-30 containing a single mutation at R148Q that confers the ability to hydrolyze cepheime (Table 3) (Tian et al., 2011). Even more worrisome is ADC-68, an ADC that has gained the ability to hydrolyze carbapenems. ADC-68 was not identified in this study, but is worthy of mention given its carbapenemase activity (Jean et al., 2014).

In conclusion, we believe that blaOXA-82 and blaOXA-172 are currently underappreciated as causative agents of, and contributors to, carbapenem resistance in Ab, as they were present in 20% of the CR Ab. Additionally, we anticipate that focusing our efforts of inhibition on targeting the various combinations of β-lactamases found in currently circulating, clinical isolates of Ab will enable us to truly assess whether newly synthesized inhibitors will be effective in the clinic. Our innovative approach considers all the β-lactamases occurring in a single organism based on the WGS combinations observed in this contemporary group of isolates from the US. Inhibitors need to effectively inhibit all of these β-lactamases simultaneously, and it is critical to counter bacterial resistance caused by the expansion and expression of multiple β-lactamases. We believe this a possibility, and that the impact of these studies may have broader implications for other bacterial pathogens.

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Authors Contribution

Andrea M. Hujer: Writing-original and final draft presentations; data analysis; and conceptualization. Kristine M. Hujer: Reviewing and editing of manuscript; data review. David A. Leonard: Conceptualization; reviewing and editing of manuscript. Rachel A. Powers: Conceptualization; reviewing and editing of manuscript. Bradley J. Wallar: Conceptualization; reviewing and editing of manuscript. Andrew R. Mack: Data analysis; reviewing and editing of manuscript. Magdalena A. Taracila: Data analysis. Philip N. Rather: reviewing and editing of manuscript. Paul G. Higgins: Conceptualization; reviewing and editing of manuscript. Steven H. Marshall: Data acquisition, curation, and analysis. Thomas Clarke: Data acquisition, curation, and analysis. Christopher Greco: Data acquisition, curation, and analysis. Pratap Venepally: Data acquisition, curation, and analysis. Lauren Brinkac: Data acquisition, curation, and analysis. Barry N. Kreiswirth: Reviewing and editing of manuscript. Derrick E. Fouts: Supervision; data acquisition and curation; reviewing and editing of manuscript. Robert A. Bonomo: Conceptualization; supervision; reviewing and editing of manuscript.

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