EXPERT REVIEWS

Carbapenem resistance in *Acinetobacter baumannii*: laboratory challenges, mechanistic insights and therapeutic strategies

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Unprecedented levels of antimicrobial resistance in bacterial isolates have prompted great concerns globally. In 2012 the WHO released a publication outlining the evolving threat of antimicrobial resistance in order to raise awareness and to stimulate coordinated international efforts. The carbapenem class of antibiotics is largely considered as an antibiotic of last-resort when treating infections. Now carbapenem resistance further limits treatment options. In this article the authors discuss carbapenem resistance in *Acinetobacter baumannii*, a bacterial isolate often implicated in nosocomial infections. Virulence factors, intrinsic and acquired resistance mechanisms, together with laboratory challenges in the detection and antibiotic susceptibility testing of *A. baumannii* make this a truly problematic isolate. Therapeutic options are exceedingly limited, relying on polymyxins in combinations with other antibiotics, with few, if any, new active agents in the pipeline.

Acinetobacter baumannii is an aerobic, nonlactose fermenting, oxidase-negative, Gram-negative coccobacillus that is most commonly found associated with healthcare environments. Although initially considered of low pathogenic potential in healthy individuals, *A. baumannii* is now largely considered as an important pathogen implicated in nosocomial infections [1]. *A. baumannii* can survive on moist and dry surfaces and may be present in foodstuffs [2] and on healthy skin [3,4]. These factors, together with both intrinsic and acquired antibiotic resistance, account for the success of *A. baumannii* as a significant cause of outbreaks and endemic spread of resistant clones throughout the world [5–8]. Significant cost, morbidity and mortality have been reported with outbreaks [9] and even criminal charges have been directed against hospitals where outbreaks have occurred [10].

Classification & epidemiology
Originating from the family Moraxellaceae, the genus *Acinetobacter* contains at least 21 named

species, of which *A. baumannii* is the most important in human infections. *A. baumannii* is part of the *Acinetobacter calcoaceticus–A. baumannii* complex, which includes *A. calcoaceticus* (genomic species 1; an environmental species of limited clinical significance), *A. baumannii* (genomic species 2), *Acinetobacter pittii* (genomic species 3) and *Acinetobacter nosocomialis* (genomic species 13TU), which are all highly genetically related and difficult to distinguish phenotypically [11,12]. *A. baumannii* has been found to be associated with greater resistance to antibiotics and higher mortality among bacteremic patients compared with other genomic species [13].

European surveillance data from 2009 showed that the *Acinetobacter* spp. was implicated in intensive care unit (ICU)-acquired pneumonia up to 21.8% of the time, in ICU-acquired bloodstream infections up to 17.1% and in ICU-acquired urinary tract infections up to 11.9% [14]. Highest rates were seen in Italy, Lithuania and Slovakia. Six countries (Belgium, Italy, Malta, Portugal,

Table 1. Intrinsic resistance in *Acinetobacter baumannii*.

Antibiotics	Resistance
Ampicillin	R
Amoxycillin-clavulanate	R
Ticarcillin	–
Ticarcillin-clavulanate	–
Piperacillin	–
Piperacillin-tazobactam	–
Cefazolin	R
Cefotaxime	R
Ceftriaxone	R
Ceftazidime	–
Ertapenem	R
Imipenem	–
Meropenem	–
Ciprofloxacin	–
Chloramphenicol	–
Aminoglycosides	–
Trimethoprim	R
Trimethoprim-sulphamethoxazole	–
Fosfomycin	R
Tetracyclines/tigecycline	–
Polymyxin B/colistin	–

Non-fermentative Gram-negative bacteria are also intrinsically resistant to benzylpenicillin, cefoxitin, cefamandole, cefuroxime, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin and linezolid. EUCAST [202] provide clinical breakpoints (Version 3.1, 2013) for *Acinetobacter* spp. for carbapenems (doripenem, imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (amikacin, gentamicin, netilmicin, tobramycin), colistin and trimethoprim-sulfamethoxazole. CLSI [109] provide addition clinical breakpoints for penicillins (ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, ticarcillin-clavulanic acid), cephalosporins (ceftazidime, cefepime, cefotaxime, ceftriaxone) and tetracyclines (tetracycline, doxycycline, minocycline).
–: Antibiotics could still be tested for and used if the organism tests susceptible; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility; R: Resistance.
Data taken from [123].

Slovakia and Spain) that collected detailed resistance data from microorganisms associated with ICU-acquired infections reported carbapenem-resistant isolates in *A. baumannii* to be up to 80% [14]. The Comparative Activity of Carbapenem Testing study included 274 *A. baumannii* isolates from 80 centers in 16 countries, including 14 countries from Europe during 2008–2009, and reported the overall rate of imipenem-resistant strains to be 47.1%, with higher rates in Turkey, Greece, Italy, Spain and England compared with France, Germany and Sweden [15]. Carbapenamase oxacillinase (OXA)-58-type was most frequently found in Europe during reported *A. baumannii* outbreaks, followed by OXA-23-type [1].

In a recent study from the USA examining *Acinetobacter* spp. nosocomial bloodstream infections, 63% were found to be due to *A. baumannii*, with the remainder due to *A. pittii* and *A. nosocomialis* [16]. In their study, 93.0% of *A. baumannii* bloodstream isolates, collected between 1995 and 2003, were susceptible to imipenem. *A. pittii* and *A. nosocomialis*, as shown in other studies, tended to be more susceptible to carbapenems together with aminoglycosides and fluoroquinolones [17–20]. US-wide surveillance data, however, demonstrates that *A. baumannii* resistant to carbapenems has increased by nearly eight-times, going from 5.2% in 1999 to 40.8% in 2010, and increasing in all but 1 year during the period [201]. The largest and most consistent increase came from the Midwest (East North and West South Central states) which saw the largest increase followed by the south Atlantic and Pacific states. It has been suggested that some degree of overestimation of carbapenem resistance may have contributed to by epidemic spread and hospital outbreaks of resistant clones [16]. Molecular epidemiology has shown that carbapenamse OXA-23 and OXA-51 are the most common, based on 65 carbapenem nonsusceptible isolates from New York, Pennsylvania, Florida, Missouri, Nevada and California between 2008 and 2009 [21].

During 2006–2007, the SENTRY Antimicrobial Surveillance Program assessed 544 *Acinetobacter* spp. from 41 medical centers located in ten countries in the Asia-Pacific region [22]. A total of 42.3% of the isolates were nonsusceptible to imipenem or meropenem, and this resistance phenotype was most common in isolates recovered from Singapore (95.2%), Korea (87.0%) and Taiwan (62.5%). The distribution of OXA-type genes among *Acinetobacter* spp. in Asia-Pacific nations was comprised mainly of carbapenamase OXA-23, while OXA-24/40 and OXA-58 were less common [22].

A worldwide collection of 5127 *Acientobacter* spp. collected between 2005 and 2009 from 140 hospitals in 32 countries in North America (17.1%), Europe (22.9%), Latin America (25.2%) and the Asia-Pacific region (34.8%), showed the overall nonsusceptibility rate to imipenem and meropenem to be 45.9 and 48.2%, respectively. However, the nonsusceptibility percentage had increased from 27.8 and 37.5% in 2005 when compared with 2009, with nonsusceptibility rates of 62.4 and 64.4% for imipenem and meropenem, respectively [23]. Data collected over a similar time period for the Tigecycline Evaluation and Surveillance Trial showed that carbapenem-resistant *Acinetobacter* spp. were more prevalent in the Middle East, Latin America and the Asia-Pacific rim than in Europe or North America [24]. In Japan, clinical isolates of *Acinetobacter* spp. from 176 medical facilities in all geographical regions were tested and it was found that 18% (out of total of 305 clinical isolates) had MICs of imipenem ≥4 mg/l. The OXA-51-like carbapenemase gene was detected in 52 out of these 55 isolates [25].

Carbapenem resistance has also been reported in animal husbandry. In a surveillance study in 2010 at a dairy farm in Paris, France, OXA-23-producing *Acinetobacter* spp. were identified from the rectal swabs from nine out of 50 cows. All isolates belonged to the *Acinetobacter* genomospecies (DNA group) 15TU, which is known to be phylogenetically related to *Acinetobacter lwoffii*. The dairy farmer indicated that most animals from which OXA-23 producers had been identified had received antimicrobial drugs in the previous weeks [26].

Mechanisms of carbapenem resistance

Acinetobacter spp. are noted for their extensive antimicrobial resistance and capability to acquire antimicrobial-resistance genes extremely rapidly [27,28]. Whole-genome analyses demonstrate a large repertoire of resistance genes [29–31]. Intrinsic resistance to β-lactams is exemplified by a chromosomally encoded AmpC cephalosporinase, to which cefepime and carbapenems appear to be stable. The European Committee on Antimicrobial Susceptibility (EUCAST) provide laboratory guidelines for reporting antimicrobial susceptibility for *A. baumannii* isolates (see TABLE 1). To date, *A. baumannii* has become resistant to almost all antimicrobial agents that are currently available [32]. Resistance mechanisms often work synergistically, including antimicrobial-degrading enzymes, efflux pumps, target modification and porin deficiency.

Carbapenemases

The most prevalent mechanism of carbapenem resistance in *A. baumannii* is enzymatic degradation by carbapenemases, namely OXA-type and metallo-β-lactamases (MBL). Several types occur, some with close geographic associations. More specifically, acquired OXA-type carbapenem-hydrolyzing class D β-lactamases of the OXA-23, OXA-24/40 and OXA-58 subfamilies, and the intrinsic OXA-51-type are common among *A. baumannii* isolates [33]. Endemic spread of class B MBL (mostly IMP and VIM) expressing *A. baumannii* has also been widely reported [28]. Reported carbapenemases in *A. baumannii* isolates are summarized in TABLE 2.

Efflux pumps

There are five families of efflux-pump proteins that are associated with multidrug resistance in Gram-positive and Gram-negative organisms: the ATP-binding cassette superfamily, the major facilitator superfamily, the multidrug and toxic-compound extrusion family, the small multidrug resistance family and the resistance nodulation division (RND) family [34]. Three RND systems, AdeABC, AdeIJK and AdeFGH, have been characterized and reported to be responsible for multidrug-resistant (MDR) in *A. baumannii* [35].

The major efflux mechanism associated with carbapenem resistance in *A. baumannii* is the chromosomally encoded tripartite efflux pump, AdeABC, present in approximately 80% of clinical isolates. Overexpression of this pump, tightly regulated by *adeRS* genes encoding a two-component regulatory system [36], confers resistance to aminoglycosides and decreased susceptibility to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, trimethoprim and ethidium bromide, as well as to netilmicin and meropenem [37,38]. Other regulatory genes are also likely to be important given reports of overexpression of AdeABC in the absence of mutations in *adeRS* genes [39,40]. Synergy between acquired oxacillinases and the AdeABC pump has been reported and implicated in higher levels of resistance to β-lactams, including carbapenems [41].

The other recognized efflux systems, such as RND pumps AdeIJK and AdeFGH, and non-RND efflux systems, such as CraA and AmvA (major facilitator superfamily pumps), AbeM (member of the multidrug and toxic-compound extrusion family) and AbeS (small multidrug resistance efflux pump), have not been implicated in carbapenem resistance [35,42–44].

Porin loss

Porins are outer membrane proteins (OMPs) able to form channels allowing the transport of molecules across lipid bilayer membranes. Variations in their structure or regulation of porin expression can provide a mechanism to escape from antibacterial pressure [45]. *A. baumannii* intrinsically have a smaller number

Table 2. Carbapenemases reported in *Acinetobacter baumannii*.

Carbapenemase class	Enzyme	Characteristics	Ref.
Metallo-β-lactamases (ambler class B; zinc ion at active site)	IMP-like (IMP-1, -2, -4, -5, -6, -8, -10, -11, -19) VIM-like (VIM-1, -2, -3, -4, -11) SIM-1	Class 1 integrons	[1,28,33,154–156]
	NDM-1, NDM-2	Most likely linked to a transposon, Tn*125*, bracketed by two copies of insertion sequence IS*Aba125*, and not plasmid related, in contrast to what is observed in the Enterobacteriaceae	[1,155,157–160]
Oxacillinases (ambler class D; serine residue at active site)	OXA-23 cluster (OXA-23, -27 and -49)	Acquired; found either on the chromosome or on plasmids, in association with IS*Aba1* within Tn*2006* and Tn*2008* transposons or with IS*Aba4* in Tn*2007*	[1,28,33,156]
	OXA-24/40 cluster (OXA-25, -26, -40 and -72)	Acquired; chromosomal or plasmid, no associated IS elements	
	OXA-58	Acquired; found mostly on plasmids in association with insertional sequences IS*ABa1*, IS*AB3* and IS*18*	
	OXA-51 cluster (OXA-51, -64, -65, -66, -68, -69, -70, -71, -78, -79, -80, -82 and -143	Intrinsic chromosomally and/or plasmid-located; confers carbapenem resistance when the insertion sequence IS*Aba1* element is inserted upstream of the gene	

OXA: Oxacillinase.

and size of porins compared with other Gram-negative organisms, contributing to the intrinsic outer membrane impermeability. To date, three porins have been implicated in carbapenem resistance when their expression is reduced; CarO, Omp 33–36 and OprD homolog [45], although more recent work suggests the OprD homolog may in fact not be involved [46].

The carbapenem-associated OMP, also called CarO (a 29 kDa protein), is the most characterized porin in *A. baumannii*. The analysis of strains with high MICs to imipenem (up to 16 mg/l) showed disruptions in the *carO* gene by the various insertion elements and thus loss of expression [47,48]. Alteration of the expression of CarO in the outer membrane reduces the penetration of imipenem into the cell, therefore contributing to drug resistance [49]. Meropenem resistance, however, may be mediated by another porin-mediated pathway, given the absence of a meropenem-binding site on CarO [49]. Another such example is Omp33-36 (a 33–36 kDa protein) [50].

As previously mentioned, despite the OprD homolog (a 43 kDa protein) displaying similarities with the carbapenem-specific channel in *Pseudomonas aeruginosa*, recent evidence suggests that it is in fact not involved in carbapenem resistance, but acts as a OprQ-like protein allowing for specific binding sites for iron and magnesium ions and allowing *A. baumannii* to adapt to stress conditions [46].

Another important OMP found in *A. baumannii* includes the heat-modifiable protein HMP-AB, belonging to the OmpA family, but it does not appear to play a significant role in antibiotic resistance. However, it remains an important virulence factor in its role as a secreted emulsifier for adhesion to surfaces and formation of biofilms [51,52].

Host–pathogen interactions

Despite the availability of 16 *A. baumannii* genome sequences and the advancements in genetic manipulation, the knowledge about host–pathogen interactions involving *A. baumannii* is still in its infancy. Whole-genome sequencing studies demonstrate that *A. baumannii* contains a vast array of antibiotic drug resistance determinants, some of which are located in pathogenicity islands [29,53]. Random mutagenesis of the *A. baumannii* ATCC 17978 strain led to identification of several mutants in six different pathogenicity islands, which presented attenuated virulence toward the non-mammalian models *Caenorhabditis elegans* and *Dictyostelium discoideum*. The relevant mutated genes encoded transcription factors, multidrug efflux transport systems, and a urease operon; however, the role of these genes in mammalian virulence were not assessed [53].

A list of currently reported virulence factors in *A. baumannii* is shown in TABLE 3. Siderophores are iron uptake mechanisms composed of low molecular-mass compounds with high affinity for iron. *A. baumannii* makes use of an acinetobactin siderophore to sequestrate iron from human cells in order to survive in the human body [54]. Interestingly, these elements have structural and functional similarities to a siderophore produced by the fish pathogen *Vibrio anguillarum* [55], suggesting their acquisition via horizontal transfer, or the existence of a common ancestor between these two species. In *A. baumannii*, the expression of siderophores occurs in seven different operons [55–57] and requires the participation of an *entA* ortholog, located outside the acinetobactin cluster [58]. The expression of such genes can vary greatly among clinical strains [59] and were shown to be important for virulence toward eukaryotic cells and the *Galleria mellonella* invertebrate model [58].

Biofilm formation is one of the best characterized virulence factors in *A. baumannii* [54,60,61]. This may explain its success at causing hospital-acquired infections and its ability to persist in the hospital environment. Tomaras *et al.* demonstrated that the

Table 3. Summary of *Acinetobacter baumannii* virulence factors.

Designation	Function	Ref.
csuA/BABCDE	Chaperone–usher pili assembly system, attachment, biofilm formation	[62]
bar, *bas* and *bau* gene cluster	Synthesis of the acinetobactin siderophore, iron uptake	[56]
ompA	Attachment, adherence, invasion, motility, biofilm formation, growth in human serum, eukaryotic cell apoptosis	[69]
abaI	Quorum-sensing molecule, autoinducer synthase	[78]
bap	Maintenance of mature biofilm structure	[67]
bfmS and *bfmR*	Regulation of the csu operon, biofilm formation	[65]
pbpG (penicillin-binding protein 7/8)	Resistance to complement-mediated bactericidal activity, possible influence on the structure of peptidoglycan	[73]
pgaABCD	Biofilm formation	[66]
ptk and *epsA*	Capsule polymerization and assembly, growth in human ascites, survival in human serum	[76]
lpsB	Lipopolysaccharides biosynthesis, resistance to human serum	[75]
pld	Survival and proliferation in human serum, invasion of eukaryotic cells	[74]
plc1	Cytotoxicity	[81]
entA	Biosynthesis of the acinetobactin precursor 2,3-dihydroxybenzoic acid, iron uptake	[58]
ata	Biofilm formation, adhesion and virulence	[68]
pglL	*en bloc* O-glycosylation mechanism, biofilm formation, virulence	[61]
nfuA	Intracellular iron utilization, protection from oxidative stress	[79]
Fibronectin binding proteins (tonB, ompA, 34 kDa omp)	Adherence	[80]

csu operon is responsible for the synthesis of a chaperone–usher pili assembly system, which is essential for biofilm formation on abiotic surfaces [62]. Interestingly, this operon was not important for adherence to bronchial epithelial cells [63]. It has also been shown that biofilm formation and adherence to airway epithelial cells varies widely between clinical strains, with no differences between outbreak and non-outbreak strains [64]. Equally important for biofilm formation is the BfmR response regulator, whose absence was previously shown to negatively impact on the expression of the *csu* operon. On the other hand, the *bfmS* gene encoding the sensor kinase counterpart of this two-component system plays a less relevant role in biofilm formation [65]. Additionally, other molecules and proteins seem to have a role in the biogenesis and/or the establishment and maintenance of biofilm, including the *pgaABCD* operon, which encodes the synthesis of a poly-β-1-6-*N*-acetylglucosamine (PNAG polysaccharide) that was previously demonstrated to be critical for biofilm formation under dynamic conditions [66], the large surface adhesin Bap, which participates in the initial attachment to eukaryotic cells or abiotic surfaces [67], the recently characterized *Acinetobacter* trimeric autotransporter, the Ata surface protein adhesion [68] and an *en bloc O*-glycosylation mechanism [61].

After adherence to human cells, it appears that *A. baumannii* can induce apoptosis via an OMP (Omp38 or OmpA). However, OmpA is not the only factor involved, as an OmpA mutant caused incomplete attenuation of cell death [69]. It also contributes to biofilm development on plastics [52], and persistence and growth in human serum [70], which supports its suggested contribution to *A. baumannii* dissemination during infection [71,72]. Beside this, the *pbpG* gene, which encodes the putative low-molecular-mass penicillin-binding protein 7/8 [73], two phospholipase D genes [74], a glycosyltransferase that participates in lipopolysaccharide (LPS) synthesis [75] and the genes *ptk* and *epsA* that are required for capsule polymerization and assembly [76] also contribute to *A. baumannii*'s ability to thrive in human serum or ascites. Quorum sensing has been shown to regulate a wide array of virulence mechanisms in many Gram-negative organisms, particularly *P. aeruginosa*. Up to four different quorum-sensing signal molecules have been identified in *Acinetobacter*, indicating that this may be a central mechanism for autoinduction of multiple virulence factors [77,78].

Besides the aforementioned virulence factors, others have recently been identified, including the NfuA Fe-S scaffold protein [79], three fibronectin binding proteins [80], and a phospholipase C protein [81]. Recently, our group demonstrated, using a *C. elegans* model, that the GacS sensor kinase controls *A. baumannii* virulence toward *Candida albicans* filaments [82]. Cerqueira and Peleg have found that two in-frame deletion mutants (*gacA* and *gacS*) were attenuated in virulence toward *C. albicans* filaments and mammals (mice), and demonstrated defects in pili synthesis, motility, biofilm formation and structure, resistance to human serum and reduced ability to metabolize aromatic compounds [Cerqueira GM, Peleg AY, Unpublished data].

Among the currently characterized virulence factors of *A. baumannii*, exopolysaccharide production is the best characterized regarding interaction with the immune system. LPS not only protects bacteria from host defences [83], but it was also identified as the major immunostimulatory factor [84], which is recognized by the immune system through interaction with and signaling by the Toll-like receptors (TLR) [84,85]. Interestingly, in mice, LPS was found to stimulate the immune system through binding to TLR4 [84], while in humans both TLR2 and TLR4 appear to be important for the signaling that leads to the release of the pro-inflammatory cytokines IL-8 and TNF-α [85]. However, human airway epithelial cells were used to demonstrate the induction of TLR-dependent IL-8 and β-defensin 2 protein, upon *A. baumannii* infection. This is in agreement with the observation that pathogen-associated molecular patterns of *A. baumannii* activate NF-κB and mitogen-activated protein kinase pathways stimulate the release of cytokines and chemokines (i.e., macrophage inflammatory protein 2, monocyte chemoattractant protein 1, IL-8, keratinocyte-derived chemokine, TNF-α, IL-1B and IL-6) [84,86,87], although *A. baumannii* induces the production of significantly less inflammatory cytokines than less clinically relevant species [64]. This finding suggests the existence or a stronger expression of a mechanism associated with immune evasion in *A. baumannii*.

In terms of cell-mediated immune response, Breslow *et al.* showed recently that, during systemic infection of mice with *A. baumannii*, neutrophils are the predominant immune cells [88]. With regard to the classical, antibodies-based immune response, previous studies reported that iron-regulated OMPs and the *O*-polysaccharide component of LPS are the main targets during *Acinetobacter* infection [89,90], and that passive immunity directed to siderophores is able to control the pathogen proliferation *in vitro* [89]. More recently, active and passive immunizations demonstrated the robustness and immunological properties of the antibody response induced against different *A. baumannii* surface determinants [91–96].

Laboratory methods
Organism identification
Microscopy reveals *A. baumannii* to be a nonmotile, Gram-negative coccobacillary rod. In clinical practice, *A. baumannii* may be difficult to decolorize on Gram staining and can be initially falsely reported as Gram-positive cocci from direct smears from blood culture bottles. *A. baumannii* will grow on standard, non-selective agar. Appearance on horse blood agar is that of smooth, opaque (or white), mucoid colonies that are nonhemolytic and are smaller than that of Enterobacteriacea. Growth on MacConkey agar appears as a nonlactose fermenter. Other laboratory aspects include oxidase, indole and esculin negativity, catalase positivity and that they are able to oxidize glucose.

Most automated systems perform poorly when differentiating between different *Acinetobacter* spp. Vitek®2, API20NE (bioMérieux, Marcy l'Etoile, France) and Phoenix (Becton Dickinson, NJ, USA) systems will identify down to *A. calcoaceticus–A. baumannii* complex, leading to *A. pittii* and *A. nosocomialis* often erroneously identified as *A. baumannii*. Given that *A. baumannii* is associated with higher mortality and greater antibiotic resistance,

being able to differentiate between the different species with the *A. calcoaceticus–A. baumannii* complex is important [13].

MALDI-time of flight (TOF) mass spectrometry (MS) systems appear to perform better at species differentiation than phenotypic systems [97]. Importantly, MALDI-TOF MS has been demonstrated to differentiate *A. baumannii* from *A. pittii* and *A. nosocomialis* [98], although when compared with the molecular technique of sequencing the *rpoB* gene, MALDI-TOF MS was useful in identifying *A. baumannii* but not other species in the genus [99]. Of particular interest is the use of MALDI-TOF MS to rapidly identify carbapenamase-producers by incubating bacteria with imipenem for up to 4-h prior to analyzing the mixture. The presence and absence of peaks representing imipenem and its natural metabolite was able to differentiate between isolates with and without a carbapenamase [100]. The 16S–23S rRNA gene intergenic spacer sequence-based identification is another reliable method and a helpful tool for elucidation of the clinical significance of the different species of the *A. calcoaceticus–A. baumannii* complex [11].

The role of the clinical laboratory is critical in the early identification, assessment and management of outbreaks due to *A. baumannii*. Pulsed-field gel electrophoresis (PFGE) has historically been the molecular gold standard for identifying clonality, but it is time consuming and requires considerable technical expertise to perform and interpret, and can be influenced by DNA degradation that can prevent typing of some strains, an issue particularly true for *Acinetobacter* spp. [101]. Molecular typing systems that provide quick and accurate results would therefore be useful in evaluating outbreaks. Examples of these newer technologies include repetitive sequence-based PCR and broad-range PCR/electrospray ionization mass spectrometry, both semi-automated systems that generate rapid results with a turnaround time of one working day (~6 h). In a comparison study with PFGE, both methods offered a rapid generation of results and demonstrated generally good concordance for *A. baumannii* genotyping, with PCR/electrospray ionization MS demonstrating the best concordance with PFGE [102].

Antibiotic susceptibility testing

Accurate antibiotic susceptibility testing is paramount for providing therapeutic options to the treating clinicians. Most clinical microbiology laboratories rely heavily on automated systems as the primary method of susceptibility testing because of their

efficiency and convenience [103,104]. Several studies, however, have reported significant error rates in determining the carbapenem susceptibility of *Acinetobacter* isolates by such methods [105–108]. Similar problems have been reported when using automated systems to test the susceptibility to other antimicrobial agents, such as amikacin, gentamicin and tobramycin [106]. When compared with broth microdilution, significant rates of 'very major errors' (VME; i.e., reported susceptible when resistant) were seen with the MicroScan (Dade Behring Inc., CA, USA), BD Phoenix (Becton Dickinson Diagnostic Systems, MD, USA) and Vitek 2 systems (bioMérieux) [107]. When EUCAST breakpoints were applied [202], testing for imipenem had a VME rate of 25.2% across all three systems, with some improvement in testing for meropenem, with VME rates of 17.8–18.7%. In comparison, disk diffusion and Etest® (AB Biodisk, Solna, Sweden) performed better, with VME rates between 0.9 and 5.6%, except for imipenem testing by disk diffusion, where the VME rate was observed to be 17.8% [107]. Kulah *et al.* recommend that clinical laboratories using the MicroScan system should consider using a second independent antimicrobial susceptibility testing method (e.g., Etest) to validate imipenem susceptibility [105]. Markelz *et al.* also advise specific caution in interpreting the results of antimicrobial susceptibility testing methods for doripenem, and highlight the lack of Clinical and Laboratory Standards Institute (CLSI) breakpoints to guide reporting [107].

The lack of an international consensus regarding carbapenem susceptibility breakpoints for *Acinetobacter* spp. can also dramatically affect the results of testing. The imipenem and meropenem breakpoints for *Acinetobacter* spp. established by EUCAST are sensitive at ≤2 µg/ml and resistant at >8 µg/ml [202], compared with the current CLSI breakpoints of sensitive at ≤4 µg/ml and resistant at ≥16 µg/ml (see Table 4) [109]. EUCAST also report that *A. baumannii* has intrinsic resistance to ertapenem, and should always be reported as resistant (see Table 1). Despite CLSI reducing breakpoints for *Pseudomonas* spp. for meropenem and imipenem and providing new breakpoints for doripenem in the latest update, *Acinetobacter* spp. breakpoints remain unchanged since 2009 [110].

Carbapenamase detection

The presence of a carbapenemase can be detected by a number of methods in the clinical laboratory, including 'flagging' by automated systems, selective agar, modified Hodge test, synergy tests (e.g., Etests or double disc tests) and molecular methods.

Table 4. Comparison of clinical breakpoints for *Acinetobacter baumannii*.

	EUCAST (2013) [202]				CLSI (2012, M100-S22) [109]			
	MIC breakpoint (mg/l)		Zone diameter (mm), disc content 10 g		MIC breakpoint (mg/l)		Zone diameter (mm), disc content 10 g	
	S	R	S	R	S	R	S	R
Doripenem	≤1	>4	≥21	<15				
Imipenem	≤2	>8	≥23	<17	≤4	≥16	≥16	≤13
Meropenem	≤2	>8	≥21	<15	≤4	≥16	≥16	≤13

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility; R: Resistant; S: Susceptible.

Automated systems have shown variability in their ability to predict carbapenemase production as the underlying mechanism of carbapenem resistance, although much of this work has been documented with *Enterobacteriaceae* [103,111].

Selective and chromogenic agar preparations have been used in the detection of MDR *A. baumannii* from surveillance cultures. CHROMagar™ *Acinetobacter* (CHROMagar; Paris, France) is an example of a selective agar that includes a chromogenic substrate and agents that inhibit growth of other Gram-negative, Gram-positive and yeast isolates. Originally evaluated in the screening of ICU patients [112], the media has now been reformulated such that *Acinetobacter* spp. appears as bright salmon red colonies. CHROMagar *Acinetobacter* alone has been reported to not differentiate MDR from non-MDR-*Acinetobacter* [113,114]. More recently, the addition of '*Klebsiella pneumoniae* carbapenemase supplement' was able to select out carbapenem-resistant *A. baumannii* [115]. CHROMagar *Acinetobacter* now supplies an optional supplement, CR102, to specifically select for strains resistant to carbapenems.

The modified Hodge test has been extensively used as a phenotypic technique for detecting carbapenemase activity since it is routinely available in the clinical laboratory and recommended by the CLSI. For the detection of MBL in *Acinetobacter* spp., the performance of the Hodge test is improved by addition of zinc sulfate (140 μg/disk) to the imipenem disk [116,117]. Reduced sensitivity has been reported for the detection specifically of NDM-1, although this was also improved with the addition of zinc sulphate (100 μg/ml) to the culture medium [118]. In a recent study of 19 carbapenemase-producing *A. baumannii* isolates, the modified Hodge test gave negative results for all tested NDM-producing strains and only weak positive results for VIM-, IMP- and OXA-type producers [119].

For Double-Disk Synergy Test, Lee *et al.* [116] found that although EDTA (~1900 μg) disks were better at detecting MBL-producing strains among *Pseudomonas* spp., 2-mercaptopropionic acid (3 μl) and sodium mercaptoacetic acid (3 mg) disks performed better for *Acinetobacter* spp., while ceftazidime-sodium mercaptoacetic acid double-disk synergy tests failed to detect 22 out of 80 (28%) MBL-producing *Acinetobacter* spp. They also observed an important practical caveat with total or partial loss of MBL-producing cells during room temperature storage of the isolates, indicating the importance of testing imipenem susceptibility and MBL production at the same time. Loss of bla_{IMP-1} from *A. baumannii* isolates stored at room temperature has also been reported by other investigators [120].

Molecular techniques have become the mainstay and gold standard for carbapenemase detection. Gene-specific PCR-based techniques and, more recently, multiplex PCR and microarray techniques for detecting several carbapenemase genes in a single test, have been produced, but are mostly focused on the detection of genes in Enterobacteriaceae [119,121,122]. The limit of molecular techniques, ultimately, remains the failure to detect carbapenemase producers due to unknown carbapenemase genes.

There is an ongoing debate in the literature about when is the correct time to do further testing for the presence of a carbapenemase, or to rely on the MIC alone. The question remains as to whether the detection of a carbapenemase, either by phenotypic or molecular methods, should prompt the susceptible or intermediate antibiotic susceptibility categories to be reinterpreted as resistant on the assumption that the breakpoints are inadequate. However, with MIC breakpoints now being set at lower values, the laboratory can detect and report the presence of 'clinically significant' resistance without the need to screen for resistance mechanisms, such as extended-spectrum β-lactamases and carbapenemases. Animal models, pharmacokinetic (PK)/pharmacodynamic (PD) analysis, Monte Carlo simulation and the new lower EUCAST breakpoints support this approach; however, much of the literature relates to Enterobacteriaceae. From 2011 onwards, both EUCAST and CLSI recommend to not routinely test for the presence of an extended-spectrum β-lactamase or carbapenemase, and report the susceptibilities to β-lactam antibiotics as found [109,123]. EUCAST suggest in their recent publication from the Expert Rules Working Group that if an isolate has a low MIC to a carbapenem, based on susceptible clinical breakpoints, then success with the use of a carbapenem can be predicted, regardless of the presence of a carbapenemase [123].

However, we caution against this approach for several reasons. First, there is a lack of clinical evidence to support this. It is not clear whether these carbapenemase-producing, carbapenem-susceptible organisms are more likely to rapidly evolve to become resistant in the setting of complicated infection, where deep abscesses or infection of foreign material is present. Suboptimal antibiotic levels may be the perfect trigger for upregulation of carbapenemase production. In fact, the published literature reports not only of success, but also of failures of therapy in the presence of a carbapenemase despite a low MIC [124,125], although much of this data relates to Enterobacteriaceae. Another concern is that detection of outbreaks may be delayed if the authors just rely on MICs, which would impact infection control efforts. These carbapenemases reside on mobile genetic elements that can move between Gram-negative genera, some of which will more often appear as carbapenem-susceptible, such as Enterobacteriaceae, and others will more often appear resistant, such as *Pseudomonas* and *Acinetobacter*. However, the ongoing challenge of testing for carbapenemases is which isolates to test? This should really be based on the background prevalence at your institution. For institutions with low prevalence, testing all carbapenem-susceptible strains will be too laborious with very low yield. This may not be the case in institutions with high prevalence or during an outbreak setting.

Therapeutic options

The carbapenem antibiotics (imipenem, meropenem and doripenem) are considered the agents of choice for *A. baumannii* infections. The presence of resistance to these agents constrains therapeutic options. Despite the relative limitation in current evidence to guide therapy in these cases, combination therapy with colistin, and a carbapenem ± rifampin seems to hold the most promise.

Polymyxins

Polymyxin E (colistin) and polymyxin B are the two commercially available polymyxins used in the treatment of MDR *Acinetobacter* infections and are consistently reported with the highest rates of susceptibility compared with other classes of antibiotics [126]. Colistin is administered parenterally as colistin methanesulfonate, which is hydrolyzed *in vivo* to colistin A (polymyxin E1) and colistin B (polymyxin E2). Similar to the aminoglycosides, colistin has concentration-dependent bacterial killing activity with rapid bactericidal activity and a post-antibiotic effect (defined as the persistent suppression of bacterial growth after a brief exposure to an antibacterial) against Gram-negative organisms [127]. Pharmacodynamically, the free drug area under the concentration–time curve (AUC):MIC ratio is the parameter best associated with its efficacy [128]. Recent studies have independently suggested that higher than standard dosing should be used to decrease the time to achievement of therapeutic concentrations [129,130]. In a recent study by Dalfino *et al.* [131], ICU patients received a loading dose of 9 million units followed by a dose of 4.5 million units 12-h (there are ~12,500 units per mg of colistin methanesulfonate) [132]. This high-dose, extended interval regimen had high efficacy (82.1% clinical cure rate), without significant renal toxicity (17.8%, which subsided within 10 days after cessation for treatment) and without the development of colistin-resistance. Monotherapy with colistin, however, has been reported to be associated with rapid regrowth *in vitro*, thought to be due to the existence of subpopulations with higher MIC values or heteroresistance in some clinical strains [133–135]. Therefore, combination therapy is recommended, for which synergy with carbapenems and rifampin have been most often reported in the published literature [126]. Favorable *in vitro* triple synergistic combination has also been reported [136].

Tigecycline

A semisynthetic derivative of minocycline that inhibits the 30S ribosomal subunit, which is stable against tetracycline-specific resistance mechanisms, such as Tet(A–E) and Tet(K) efflux pumps and the Tet(O) and Tet(M) determinants that provide ribosomal protection. Similar to polymyxins, the PD target for efficacy was shown to be free drug AUC:MIC in the treatment of *A. baumannii* pneumonia in a murine model [137]. The authors in this study extrapolate that tigecycline doses of up to 200 mg/day may be required to provide adequate exposure for *A. baumannii* infections. Traditional dosing would also likely fail in bacteremia due to tigecycline's wide volume of distribution, resulting in low serum concentrations [138]. The absence of clinical breakpoints for tigecycline to define nonsusceptibility for *A. baumannii* further complicates any recommendations for its use. More worrisome still have been the reports of high resistance rates among *A. baumannii* isolates [139,140] and the ability for isolates to upregulate efflux-pump mechanisms when exposed to tigecycline to rapidly develop resistance [40,138]. Clinical studies have now also been published describing the emergence of resistance and clinical failures [141,142].

β-lactam antibiotics

Carbapenems, as with all β-lactam antibiotics, exhibit a concentration-independent killing effect, which is maximized when serum concentrations are four to eight-times the MIC of the infecting organism. The most important PK/PD index is the percentage of time that serum concentrations remain above the MIC (i.e., %T > MIC). To increase the likelihood of attaining the appropriate T > MIC, and thereby effect, when used in either combination or monotherapy, the employment of different strategies, such as shortening the dosing interval, administering by continuous infusion or administering by a prolonged intermittent infusion time, may be beneficial [143–145]. In the setting of a carbapenemase-producing *A. baumannii* that tests susceptible to a carbapenem (i.e., the MIC is less than the clinical breakpoint), the use of a carbapenem as monotherapy should be cautiously employed.

Sulbactam, a β-lactamase inhibitor, available in combination with ampicillin in the USA, has bactericidal activity against *A. baumannii* and reported activity against carbapenem-resistant isolates [146,147]. In a clinical study supporting the use of ampicillin/sulbactam in infections with carbapenem-resistant *A. baumannii*, the authors only report a cure rate of 29%, which they report as superior to the comparator group who received polymyxins (cure rate: 18%) [148]. Further caution with the use of sulbactam as monotherapy is supported by a recent *in vitro* study, where ampicillin–sulbactam activity was only 6% of the carbapenem-resistant *A. baumannii* in their collection of clinical isolates from Bangkok, Thailand [149]. Therapy with sulbactam needs to be based on susceptibility testing results.

Aminoglycosides & fluoroquinolones

Resistance to both classes is common, and their use should be guided by laboratory susceptibility testing. Of the aminoglycosides, amikacin appears to be the more active than gentamicin or tobramycin. It is recommended that aminoglycosides be used only in combination with other antimicrobials due to their poor tissue penetration, safety issues, and inferior clinical outcomes in patients with Gram-negative bacteremia. Aminoglycosides exhibit concentration-dependent killing. The PK/PD index predictive of successful treatment is C_{max}:MIC, which is maximized with once-daily dosing for a C_{max}:MIC ratio of ≥10 [150]. Aminoglycosides should be dosed based on an adjusted body weight in obese patients due to their preferential distribution into lean body mass compared with adipose tissue. Suboptimal dosing of aminoglycosides occurs due to an increased volume of distribution (i.e., ascites, pregnancy, obesity and volume-resuscitated patients) and in those patients with significant changes in drug clearance (i.e., burns, cystic fibrosis and renal insufficiency) [151].

Fluoroquinolones are known to exert a concentration-dependent killing effect, with maximal killing when the peak:MIC ratio is between 8 and 12. More recent data suggest that the a high peak concentration, along with moderate to prolonged postantibiotic effect, leads to improved clinical outcomes with these agents [152]. The PK/PD principle that guides this is the AUC:MIC ratio, with a ratio of AUC:MIC of 125 or higher required for

treating Gram-negative infections [150]. Unfortunately, due to the emergence of resistance, the fluoroquinolone class has consistently shown limited activity against *Acinetobacter* spp. Even when applying PK/PD principles with maximal dosing in a Monte Carlo simulation, target attainment rates were still low [153]. Hence, fluoroquinolones are only recommended in combination therapy.

Expert commentary

Infections due to *Acinetobacter* spp. have continued to challenge clinicians and infection-control practitioners throughout the world. The organism appears perfectly suited to thrive in the harsh environments of our ICU under major antibiotic and disinfectant selection pressure. Defined sources of hospital outbreaks have been numerous and exemplify the organism's ability to survive under a range of hospital environment conditions. The ability to distinguish between the more common *Acinetobacter* species has remained a challenge in the laboratory; however, newer molecular technologies may change this in the future. Other laboratory challenges include standard susceptibility testing, for which certain automated systems for certain antibiotics may perform poorly. At this stage, our understanding of the host–pathogen interactions associated with *A. baumannii* are limited, but with the advent of whole-genome sequencing and genetic manipulation technologies, this area is now moving forward. Treatment of carbapenem-resistant *A. baumannii* remains a continual challenge. The polymyxins and tigecycline are options; however, they each have their unique limitations. Emerging clinical data suggest that colistin combination therapy, preferably with a carbapenem, may be more effective than colistin alone; however, larger studies are still required.

Five-year view

We must remain optimistic about the future of our efforts to prevent, treat and control infections caused by MDR Gram-negative bacteria such as *A. baumannii*. Efforts to identify novel drug targets or alternative strategies that hinder or abolish the organism's ability to cause disease should be a priority. Furthermore, we must strive to translate research outcomes into practice so that the wealth of laboratory findings can make a difference at the bedside. In the meantime, deepening our understanding of the role of PK/PD relationships for currently available antibiotics, such as the polymyxins, β-lactams and combination therapies, will help advance our treatment strategies to optimize patient outcomes and prevent the emergence of further resistance.

Financial & competing interests disclosure

Key issues

- Carbapenem resistance among *Acinetobacter baumannii* isolates is increasing on a global scale and has been widely implicated in nosocomial infections and hospital outbreaks.

- With an ability to survive in a broad range of environments and a wide spectrum of intrinsic and acquired antibiotic resistance mechanisms, *A. baumannii* is a very challenging pathogen to treat.

- Carbapenemases, the most prevalent mechanism of carbapenem resistance, further limit therapeutic options, and given their location on mobile genetic elements, can spread widely to other organisms and other patients.

- Laboratory identification and detection of carbapenem resistance is problematic; however, gains have been made with the increasing availability of modern methods, including mass spectrometry and molecular techniques.

- The most promising treatment option for carbapenem-resistant *A. baumannii* appears to be the combination of colistin, a carbapenem ± rifampin; however, more studies are required.

- New drug targets and improved drug exposure by harnessing the pharmacokinetic/pharmacodynamic parameters of the currently available antibiotics will help improve clinical outcomes for patients affected with life-threatening infections with carbapenem-resistant *A. baumannii*.

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