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Insights on carbapenem-resistant *Acinetobacter baumannii*: phenotypic characterization of relevant isolates

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ABSTRACT   *Acinetobacter baumannii* (*A. baumannii*) is an important nosocomial pathogen, which may be a causative agent in a wide-range of human pathologies. Carbapenems are usually considered the last safe and effective choice of drugs for the treatment of Gram-negative infections. The emergence of carbapenem-resistant *A. baumannii* (CRAB) is a critical public health issue as they leave clinicians with limited therapeutic options. In this study, phenotypic methods were used to characterize sixty-two (n = 62) *A. baumannii* isolates, which were included based on their suspected non-susceptibility to meropenem. Minimum inhibitory concentrations (MICs) of meropenem, levofloxacin, gentamicin, sulfamethoxazole/trimethoprim, tigecycline were determined using E-tests, while colistin MICs were determined using broth microdilution. The isolates were subjected to the modified Hodge test (MHT), the modified carbapenem-inactivation method (mCIM) and the imipenem/EDTA combined disk test (CDT). Efflux pump overexpression was studied using agar plates containing phenylalanine-arginine β-naphthylamide (PAβN). Assessment of biofilm-formation was carried out using the crystal violet tube-adherence method. 64.5% of the strains showed meropenem MICs in the resistant range (>8 mg/L), resistance rates were similarly high to the other tested antibiotics. The MHT and mCIM assay were positive in 79.0% and 67.7% of cases, respectively; the presence of an MBL was suggested for 29.0% of isolates. Efflux-pump overexpression was seen in 12.9% of isolates. 54.8% of the isolates were characterized as strong biofilm-producers. Microbiology laboratories have an important role in differentiating the distinct mechanisms by which these pathogens develop the CRAB phenotype, as plasmid-borne carbapenemases are significant from the standpoint of public health microbiology.
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Introduction

The introduction of penicillin G, the first β-lactam antibiotic in the 1940s has been a hallmark in the history of medicine, as this drug (and the subsequent iterations of β-lactam antibiotics that later followed) allowed for the treatment of life-threatening infections, that were previously considered lethal (Lobanovska and Pilla 2017; Erdem et al. 2011). In addition, antibiotics have indirectly pawed the way for the development of many modern medical disciplines, including complex surgery, transplantation medicine, cancer chemotherapy, neonatology, and the treatment of sepsis (Gajdács 2019; van Duin and Paterson 2016). β-lactams are bactericidal drugs that include

penicillin-derivatives, cephalosporins, carbapenems and monobactams; β-lactams are often preferred as first-choice agents in many clinical situations, due to their safety, tolerability, and overall clinical efficacy (Papp-Wallace et al. 2011; El-Gamal et al. 2017). Out of this antimicrobial group, carbapenems (namely imipenem, meropenem, ertapenem and doripenem) have some of the broadest spectrum of activity, being effective in the therapy of infections caused by a plethora of aerobic and anaerobic pathogens (Gajdács et al. 2020). The increasing emergence of antimicrobial resistance (AMR) in bacteria has become one of the most critical public health issues of the 21st century (Medina and Pieper 2016); many trans-national public health organizations have expressed their concerns over the worsening situation, including the European Centers

for Disease Prevention and Control (ECDC), estimating that drug-resistant bacteria are responsible for over 400 000 infections and 25 000 excess deaths annually in the EU alone (ECDC 2009). In a similar report the US Centers for Disease Control (CDC) has projected over two million multidrug-resistant (MDR) infections and 23 000 excess deaths per year (CDC 2020). The phenomenon of AMR may be characterized by two important hallmarks: a) disinterest of pharmaceutical companies towards the development of antimicrobial drugs (due to the lack of returning investments and difficulties in attaining marketing authorization) (Cannas et al. 2015; Chaves-López et al. 2018; Gajdács and Spengler 2019; Usai et al. 2019), and b) the inappropriate use of existing antimicrobials, including their prescription in inappropriate indications, their non-prescription sales (especially from informal healthcare-providers) and their use in self-medication by patients to relieve symptoms (Aslam et al. 2020; Gajdács et al. 2018; Grigoryan et al. 2019). The latter issue is especially critical, as the consumption of antibiotics have been directly linked to the emergence of increasing resistance rates (Johnson 2005; Olesen et al. 2018).

Non-fermenting Gram-negative bacteria (NFGNB) are a heterogenous group of aerobic microorganisms within the Proteobacteria phylum, characterized by the incapacity to ferment sugars (e.g., glucose, maltose) to generate energy for their vital cellular functions (Enoch et al. 2007). From a clinical perspective, the most relevant pathogens among NFGNB include species from the *Acinetobacter baumannii-calcoaceticus* (ABC) complex (consisting of *A. baumannii*, *A. calcoaceticus*, *A. nosocomilalis*, and *A. pitti*), *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex (BCC) and *Stenotrophomonas maltophilia* (Enoch et al. 2007; Gajdács et al. 2019). Due to their adaptability to various ecological niches, NFGNB are often isolated from natural sources, such as aquatic environments, the soil and as plant pathogens (Chawla et al. 2013). *A. baumannii* is one of the most important nosocomial pathogens – possessing the ability to withstand harsh environmental conditions and to persist in healthcare facilities for months in a protective biofilm (often leading to inter- and intra-hospital outbreaks) – which may be a causative agent in a wide-range of pathologies, including respiratory tract infections, bacteraemia/sepsis, meningitis, surgical site and wound infections and urinary tract infections (Sarshar et al. 2021). In addition to being intrinsically resistant to several antibiotics, *A. baumannii* also has the propensity to acquire resistance-determinants against a wide range of antibiotic classes (Bonomo and Szabó 2006). The development of extensively drug resistant (XDR) or even pandrug-resistant (PDR) strains of *A. baumannii* severely limits the therapeutic options of clinicians, often forcing them to turn to antimicrobials with pronounced toxicity

(Rangel et al. 2020); these infections are often characterized by high mortality rates (a recent meta-analysis has reported that 79.9% of *A. baumannii* causing hospital-associated pneumonia (HAP) or ventilation-associated pneumonia (VAP) was MDR, with an overall mortality rate of 42.6% (95% CI, 37.2-48.1%)) (Lim et al. 2019).

Carbapenems have been considered a safe and effective alternative in the therapy of *A. baumannii* infections; however, the rising incidence of carbapenem-resistant *A. baumannii* (CRAB) is a critical concern, which has been facilitated by the sharp increase in the use of carbapenem antibiotics (brought on by the high prevalence of extended-spectrum β-lactamase-producing (ESBL) *Enterobacteriaceae*) and the successful spread of several international clones (Codjoe and Donkor 2018; Frakking et al. 2013; Makharita et al. 2020; Matsui et al. 2018). As CRAB-associated infections often lead to therapeutic failure, clinical microbiology laboratories have pivotal roles in the detection of these isolates, both from a clinical and an infection control perspective (to limit their spread); although molecular techniques (polymerase chain reaction, whole-genome sequencing) are the gold standard in the characterization of suspected CRAB isolates, these techniques are expensive and not always readily attainable by routine laboratories (Bua et al. 2018).

The aim of our present laboratory-based study was to characterize a selection of carbapenem non-susceptible *A. baumannii* isolates using various phenotypic methods – which are available in most routine clinical microbiology laboratories – and to provide insights into the epidemiological features of these pathogens.

Materials and methods

Bacterial strains

A total of sixty-two (n = 62) *A. baumannii* isolates were included in this study, which were kindly provided by various Hungarian and Italian hospitals, originating from different clinical materials. Inclusion of these strains was based on the non-susceptibility criteria to meropenem (MER) used in routine clinical microbiology, defined by EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines v.9.0 (MER disk diameter 23-21 mm: intermediate, <21 mm: resistant) (https://www.eucast.org/clinical_breakpoints/). Identification of the isolates was carried out based on classical phenotypic and biochemical panel-based methods (Leber 2016). All isolates included in the study were re-identified as *A. baumannii* before further assays. For shorter time periods (<1 month), the bacterial strains were maintained on blood agar with continuous passage. For longer periods, the strains were kept in a -80 °C freezer, in a 1:4 mixture of

Table 1. MIC values of meropenem and ancillary antibiotics on the tested bacterial strains.

	Resistant strains (n, %)	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)
Meropenem (MER)	40 (64.5%)	0.5-64	8	32
Levofloxacin (LEV)	42 (67.7%)	0.125-16	2	4
Sulfamethoxazole/trimethoprim (SXT)	33 (53.2%)	0.064-16	2	4
Gentamicin (GEN)	28 (45.2%)	0.5-64	2	16
Tigecycline (TIG)	28 (45.2%)	0.125-8	0.5	2
Colistin (COL)	0 (0%)	0.128-2	0.5	1

85% glycerol and liquid Luria-Bertani medium. During our experiments *A. baumannii* ATCC 19606 was used as a control strain.

Minimum inhibitory concentrations (MICs) of meropenem and ancillary antibiotics

MICs of MER, gentamicin (GEN), levofloxacin (LEV), sulfamethoxazole/trimethoprim (SXT) and tigecycline (TIG) were determined by E-tests (Liofilchem, Roseto degli Abruzzi, Italy) on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). MIC determination for colistin (COL) was carried out using the broth microdilution method in cation-adjusted Mueller-Hinton broth (MERLIN Diagnostika, Berlin, Germany). The interpretation of the results was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v.9.0 (https://www.eucast.org/clinical_breakpoints/). In case of TIG, epidemiological cut-off values were used for interpretation (MIC≤0.5 mg/L as susceptible, MIC>0.5 mg/L as resistant) (Gajdács et al. 2020).

Phenotypic detection of carbapenemase and metallo-β-lactamase production

To establish carbapenemase-production in the isolates included in the study, the isolates were subjected to the modified Hodge test (MHT) and the modified carbapenem-inactivation method (mCIM), optimized for *A. baumannii*, as previously described (Chou et al. 2020; Pitout et al. 2008; Rao et al. 2019). In both assays, MER disks (10 µg; Oxoid, Basingstoke, UK) were utilized and *Escherichia coli* ATCC 25922 was used as an indicator organism.

Metallo-β-lactamase (MBL) production was tested using the imipenem/EDTA combined disk test (CDT), as described previously (Makharita et al. 2020). In preparation to this assay, imipenem/EDTA disks were prepared by adding 750 µg of a sterile 0.5 M EDTA solution to a 10 µg imipenem disk, then disks were dried in a 37 ℃ incubator. The assay was considered positive if the inhibition zone diameter (≥17 mm) of the imipenem/EDTA disk increased compared to the imipenem disk alone (Makharita et al. 2020).

Phenotypic detection of efflux pump overexpression

The effect of phenylalanine-arginine β-naphthylamide (PAβN; a compound with well-known efflux pump inhibitory activity) on the MICs of MER was detected using the agar dilution method described previously (Khalili et al. 2019). During the experiments, the concentration of PAβN was 40 µg/mL in the agar base. A two-fold decrease in MER MICs in the presence of PAβN, compared to the MIC values without the inhibitor, was considered as positivity for efflux pump overexpression (Khalili et al. 2019; Gajdács 2020).

Detection of biofilm-production by the tube-adherence method

Assessment of biofilm-formation was carried out in the tube-adherence method described previously (Dumaru et al. 2019; Behzadi et al. 2020). In short, glass tubes containing 1 mL of sterile trypticase soy broth (bioMérieux, Marcy-l'Étoile, France) were inoculated with 1 µL of the overnight culture of a respective bacterial strains. Respective tubes were then incubated statically for 24 h at 37 ℃. Verification of planktonic growth was observed visually. After the incubation period, the supernatant was then discarded, the adhered cells were rinsed three times with phosphate buffer saline (PBS; Sigma-Aldrich; Budapest, Hungary) and the tubes were patted dry on a paper towel. The contents of the tubes were treated with a 1 mL solution of 0.1% crystal violet (CV; Sigma-Aldrich, Budapest, Hungary) to stain the adhered biomass; the tubes were incubated for 3 h at room temperature with the staining solution. The CV solution was then discarded, the tubes were again rinsed three times with PBS and finally, they were patted dry on a paper towel. Biofilm-formation was observed visually; based on the appearance of visible biofilm lining at the bottom and on wall of the glass tubes, the strains were classified as non-biofilm producers (-), weak biofilm producers (-/+) and strong biofilm producers (+) (Dumaru et al. 2019). All experiments were evaluated by two independent researchers.

Statistical analysis

Descriptive statistical analysis (including means and

percentages to characterize data) was performed using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA).

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and national and institutional ethical standards. Ethical approval for the study protocol was obtained from the Human Institutional and Regional Biomedical Research Ethics Committee, University of Szeged (registration number: 140/2021-SZTE [5019]).

Results

MICs of the tested antibiotics

The MICs of the tested antibiotics, including MIC_{50}, MIC_{90} values, MIC ranges and the percentage of resistant isolates are presented in Table 1. Among the tested ancillary antibiotics, the highest levels of resistance were observed for LEV (n = 42, 67.7%) and SXT (n = 33, 53.2%). All tested isolates were susceptible to COL, with MIC values ranging between 0.128 and 2 mg/L. Based on EUCAST breakpoints, n = 40 (64.5%) of isolates showed MICs above the resistance breakpoint for MER (8 mg/L), with MICs ranging between 0.5 and 64 mg/L.

Phenotypic detection of carbapenemase, MBL production and efflux pump overexpression

Phenotypic detection of carbapenemases was carried out via the use of the modified Hodge test (MHT) and the modified carbapenem-inactivation (mCIM) method. Overall, n = 49 (79.0%) and n = 42 (67.7%) of tested isolates were positive for phenotypic detection of carbapenemases in the MHT and mCIM assays, respectively. If we consider the results of the antibiotic susceptibility testing (MER MIC > 8 mg/L) as a reference in our study, the agreement between the results of the MIC determination and the results of the MHT and mCIM tests were 81.6% and 95.2%. MBL-production was observed in n = 18 (29.0%) using the imipenem/EDTA combined disk test (CDT). Efflux pump-overexpression (based on the PAβN screening agar) was detected in n = 8 (12.9%) of isolates. In the case of n = 3 isolates, efflux pump-overexpression and MHT/mCIM-positivity were detected simultaneously, which was associated with high MICs for MER. Interestingly, for n=3 isolates, high MER MICs were seen with no efflux pump overexpression and negative results in the MHT and mCIM tests.

Biofilm-production in the tested isolates

Out of the sixty-two (n = 62) isolates included in this study, over half (n = 34, 54.8%) was found to be a strong biofilm-producer (+); on the other hand, weak biofilm-producers (-/+) (n = 16; 25.1%) and non-biofilm-positive isolates (-) (n = 12; 20.1%) were seen in similar numbers.

Discussion

AMR is global public health concern, which warrants intersectoral attention, including the public, healthcare-professionals, and government leaders; worsening resistance rates threaten the administration of effective therapy in both humans and animals, in addition to hindering the attainment of Sustainable Development Goals (SDGs) (Gajdács et al. 2021; United Nations 2020). Carbapenems are broad-spectrum agents that are usually considered the last safe and effective choice of drugs for the treatment of MDR Gram-negative infections in many patient populations, especially for the empirical therapy of patients in severe conditions, e.g., in the intensive care unit (cf. fluoroquinolones and aminoglycosides may be contraindicated for many individuals) (Doi 2019). *A. baumannii* can rapidly colonize patients in nosocomial settings, which may be a source of future infections, especially in immunocompromised individuals (Mirzaei et al. 2020). Increased levels of carbapenem-consumption – both locally and globally – has led to the increased prevalence of CRAB (Behzadi and Behzadi 2011; Mózes et al. 2014); based on the data of the ECDC Surveillance Atlas of Infectious Diseases (https://atlas.ecdc.europa.eu/public/index.aspx), the ratio of CRAB isolates in 2014 Hungary and Italy were 64.5% and 89.9%, respectively; this ratio has decreased over a 5-year period (2019), being 51.0% and 79.2% in the same countries. However, the rates of combined resistance (i.e. resistance against fluoroquinolones, aminoglycosides and carbapenems) has increased substantially in Hungary between the 5-year period (2014: Hungary: 38.4%, Italy: 86.3%; 2019: Hungary: 45.6%, Italy: 76.5%). The significance of this was underlined when the World Organization published a list of priority pathogens consisting of MDR bacteria, in which CRAB was categorized as a critical pathogen with highest urgency for the development of novel antimicrobials and alternative antimicrobial treatment strategies (e.g., antimicrobial peptides, photodynamic therapy, phages) (Liu et al. 2020; Stájer et al. 2020; WHO 2017).

In our present study, a collection of *A. baumannii* isolates – suspected of being CRAB – were included and their characterization was carried out using various phenotypic assays. Among the isolates, 64.5% of the strains showed MER MICs in the resistant range, while apart from COL (which retained its susceptibility), resistance rates were similarly high to the other tested antibiotics. Phenotypic carbapenemase detection methods were positive in 79.0% (MHT) and 67.7% (mCIM) of cases, respectively, while the

presence of an MBL was suggested for 29.0% of isolates. Efflux-pump overexpression seemed to be less relevant in the CRAB phenotype, with 12.9% being positive in the plate-based *in vitro* assay. Lastly, over half (54.8%) of the isolates were characterized as strong biofilm-producers.

Carbapenem resistance in *A. baumannii* may be mediated by mutations affecting the penicillin-binding proteins (PBPs), mutations in the porin channels (reducing the transport of antibiotics into the periplasmic space) and over-expression of efflux pumps (e.g., AdeABC) (Makharita et al. 2020; Miljovic et al. 2016); however, the most well-characterized mechanism of resistance in these pathogens is the production of β-lactamase enzymes (carbapenemases), capable of hydrolyzing these last-resort drugs (Bonomo and Szabó 2006; Butler et al. 2019; Makharita et al. 2020). When it comes to *A. baumannii*, Ambler Class D (OXA-type) carbapenemases are the most relevant (Bonomo and Szabó 2006; Butler et al. 2019; Halat and Mourbareck 2020; Makharita et al. 2020); nevertheless, there have been increasing number of reports of resistance mediated by some Class A (KPC) and Class B (VIM, NDM) carbapenemases as well (Halat and Mourbareck 2020; Rodríguez et al. 2018). Most clinical *A. baumannii* isolates harbor a chromosomal blaOXA-51-like carbapenemase; however, presence of this enzyme will only lead to phenotypic carbapenem resistance in conjunction with other resistance determinants (Bonomo and Szabó 2006; Butler et al. 2019; Halat and Mourbareck 2020; Makharita et al. 2020). The carriage of plasmid-borne blaOXA-23-like and blaOXA-58-like carbapenemases is more relevant both for phenotypic resistance and for the potential dissemination in a given healthcare setting/region (Bonomo and Szabó 2006; Halat and Mourbareck 2020; Makharita et al. 2020). In many clinical isolates, the combination of the above-mentioned resistance mechanisms – in addition to the pharmacokinetic barrier provided by the protective biofilm *in vivo* – may result in high MIC values for carbapenems (Cunda et al. 2019; Halat and Mourbareck 2020). Microbiology laboratories have an important role in differentiating the distinct mechanisms by which these pathogens develop the CRAB phenotype, because – as opposed to isolates with chromosomal mutations – isolates carrying plasmid-borne carbapenemases have significance from the standpoint of public health microbiology (Makharita et al. 2020). While there have been renewed interest in the use of tetracycline-type drugs (i.e. tigecycline, eravacycline, omadacycline), in case of carbapenem-resistance, COL is often the only remaining therapeutic option (Butler et al. 2019; Qureshi et al. 2015); this drug is a polycationic peptide, which is given intravenously, leading to the disruption of the outer cell membrane in the relevant pathogens (i.e. displacing bivalent cations), and

subsequent bacterial cell death. Nevertheless, COL has severe adverse events (nephrotoxicity, neurotoxicity) and disadvantageous pharmacokinetic properties, which may limit its usefulness in critically ill patients (Gajdács et al. 2020). In addition, the number reports on COL-resistance are increasingly common around the globe, both regarding the members of the *Enterobacteriaceae* family and for NFGNBs (Butler et al. 2019; Qureshi et al. 2015); for example, in the EuSCAPE Survey (European survey of carbapenemase-producing *Enterobacteriaceae*), COL resistance in carbapenem-resistant *E. coli* and *Klebsiella* spp. was 28.3% (Grundmann et al. 2016). On the other hand, surveillance studies in the US have shown that prevalence of CRAB strains ranged between 33%-58%, which has corresponded to a ~5% resistance to COL (Hidron et al. 2008; Queenan et al. 2012). The present epidemiological situation highlights the important role of antimicrobial resistance surveillance (both on a national and an international level) and stewardship interventions to preserve the efficacy of carbapenem antibiotics for future use.

Acknowledgements

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