Diplomarbeit

Susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates to novel cephalosporin antibiotics

Eingereicht von

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Zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

An der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Innere Medizin, Sektion für Infektiologie und Tropenmedizin

Unter der Anleitung von

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Graz, den 29.9.19

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Graz, am 29.9.2019 Philipp Geraldy eh

Danksagung

Mein Dank gilt Frau Assoz. Prof. Zollner-Schwetz für steten Rat und Geduld.

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Abkürzungen

AHL	Acylated homoserine lactone
BHL	N-butyrylhomoserine lactone
BSI	blood stream infection
CF	cystic fibrosis
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
HHQ	2-heptyl-4-quinolone
ICU	intensive care unit
IQS	integrated quorum sensing
KPC	*Klebsiella pneumoniae*-based carbapenemase
MRGN	multidrug-resistant Gram-negative
MRSA	methicillin-resistant S*taphylococcus aureus*
OdDHL	N-(3-oxododecanoyl)-homoserine lactone
OMPTA	outer membrane protein targeting antibiotic
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	penicillin binding protein
PK/PD	pharmacokinetic/pharmacodynamic
PMN	polymorphonuclear neutrophil
PQS	Pseudomonas quinolone signal
QS	quorum sensing
ROS	reactive oxygen species
T2SS	type 2 secretion system

T3SS	type 3 secretion system
VAP	ventilation associated pneumonia
XDR	extensive drug-resistant

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Zusammenfassung

Hintergrund: Carbapeneme sind wichtige Antibiotika in der Therapie von Infekten, welche durch multiresistente *Pseudomonas aeruginosa*-Stämme ausgelöst werden. Sowohl weltweit als auch regional im mikrobiologischen Labor der Universitätsklinik für Innere Medizin an der Medizinischen Universität Graz, werden besorgniserregende Zahlen Carbapenem-resistenter *P. aeruginosa* Isolate vermerkt. Vor kurzem wurden drei weitere Cephalosporine für die Therapie von *P. aeruginosa*-Infektionen zugelassen (Ceftolozan/Tazobactam, Ceftazidim/Avibactam und Ceftobiprol). Wir testeten die Empfindlichkeit Carbapenem-resistenter *P. aeruginosa* Isolate gegenüber diesen neuen Cephalosporine.

Methoden: 60 Carbapenem-resistente *P. aeruginosa*-Isolate wurden verwendet. Wir bestimmten die minimale Hemmkonzentration (MHK) mittels Gradientstreifentests (BioMérieux) entsprechend dem von EUCAST empfohlenen Standardprocedere. Potenzielle Carbapenemase-Aktivität wurde mittels Rapidec® Carba Testkits bestimmt, zur Evaluierung der MBL-Aktivität wurden E-Teststreifen (BioMérieux) verwendet.

Ergebnisse: 80,0% (48/60) der Carbapenem-resistenten *P. aeruginosa*-Isolate waren empfindlich auf Ceftolozan/Tazobactam (MHK50 = 4 mg/l). 71,7% (43/60) zeigten Empfindlichkeit auf Ceftazidim/Avibactam (MHK50 = 4 mg/l). Im Vergleich dazu waren 66,7% (40/60) der Isolate empfindlich auf die Therapie mit Ceftazidim ohne β-Lactamase-Inhibitor. 25,0% (15/60) der Keime waren empfindlich auf Ceftobiprol. Für diese Resistenzbestimmung von Ceftobiprol verwendeten wir PK/PD (pharmakakokinetisch-pharmakodynamische, speziesunabhängige) Grenzwerte (MHK = 32mg/l). 22,0% (13/59) der 59 getesteten *P. aeruginosa*-Isolate zeigten MBL-Aktivität.

Zusammenfassung: Die neuen Cephalosporine Ceftolozan/Tazobactam und Ceftazidim/Avibactam erscheinen vielversprechende Therapieoptionen in der Therapie Carbapenem-resistenter *P. aeruginosa*-Infekte darzustellen.

Abstract

Background: Carbapenems are important antimicrobials for the treatment of infections due to multidrug-resistant *Pseudomonas aeruginosa*. Alarming numbers of carbapenem-resistant Pseudomonas isolates are found worldwide but also locally at the Microbiology Laboratory, Dept. of Internal Medicine, Medical University of Graz. Three novel antimicrobial cephalosporin agents (ceftolozane/tazobactam, ceftazidime/avibactam and ceftobiprole) have been introduced for the treatment of Pseudomonas infections. We tested the susceptibility of carbapenem-resistant *P. aeruginosa* clinical isolates to these antimicrobial agents.

Materials/Methods: 60 carbapenem-resistant *P. aeruginosa* clinical isolates were identified. MIC (minimal inhibitory concentration) was determined using gradient strip tests (BioMérieux) using standard procedures according to EUCAST, carbapenemase-activity was detected by using Rapidec® Carba testing kits and MBL activity was detected by using E-test strips.

Results: 80.0% (48/60) of carbapenem-resistant *P. aeruginosa* isolates were susceptible to ceftolozane/tazobactam (MIC50 = 4 mg/l). 71.7% (43/60) were susceptible to ceftazidime/avibactam (MIC50 = 4 mg/l). In comparison, 66.7% (40/60) were susceptible to ceftazidime alone (MIC50 = 4 mg/l). 25.0% (15/60) were susceptible to ceftobiprole using PK/PD breakpoints (MIC50 = 32 mg/l). 22.0% (13/59) of the tested *P. aeruginosa* isolates expressed MBL-activity.

Conclusions: The novel cephalosporin combinations ceftolozane/tazobactam and ceftazidime/avibactam appear to be a promising treatment option in carbapenem-resistant *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is a bacterium, which is often acquired in a clinical setting and can be extraordinarily hard to treat. It has various possibilities of expressing virulence or resistance to antimicrobial substances and multiple ways of adapting to environmental stresses (Moradali, Ghods et al. 2017). This diversity is reflected by its large genome. The strain PAO1 has been sequenced in 2000 by Stover, Pham et al., counting 6.3 million base pairs (Stover, Pham et al. 2000).

P. aeruginosa is Gram negative and aerobic. It thrives in soil and water, as well as on animal and plant tissue (Green, Schroth et al. 1974, Loveday, Wilson et al. 2014). It is often found in wet environments, such as sinks, toilets, washing machines, bathtubs as well as sewage pipes. Consequentially, *P. aeruginosa* can cause so-called hot tub folliculitis and otitis externa in swimmers (Reid, Porter 1981, Yu, Cheng et al. 2007). Due to its extraordinary perseverance, *P. aeruginosa* may even be found in certain disinfectants, if those contain traces of biological components (Burdon, Whitby 1967).

In a clinical setting, *P. aeruginosa* is a highly relevant pathogen due to its tendency to cause nosocomial infections such as wound infections, infections in the genito-urinary system and severe, nearly ineradicable chronic lung infections in patients suffering from Cystic Fibrosis (Bjarnsholt, Jensen et al. 2009). In Austria, an incidence of 8.0 infections per 100,000 healthy individuals was estimated in 2017 (BMGF 2017). Its ability to form biofilms, the innate resistance to a variety of antibiotics and its ability to rapidly develop resistance to antimicrobial chemotherapy provide notable therapeutic difficulties (Mulcahy, Isabella et al. 2014, Breidenstein, de la Fuente-Núñez et al. 2011). Morphologically, *P. aeruginosa* is a rod-shaped bacterium, expressing multiple pili and a single flagellum, which provides *P. aeruginosa* with unidirectional motility. Depending on multiple factors, such as the density

of the surrounding medium or cell density, *P. aeruginosa* can move by twitching, swarming and flagellum- driven swimming.

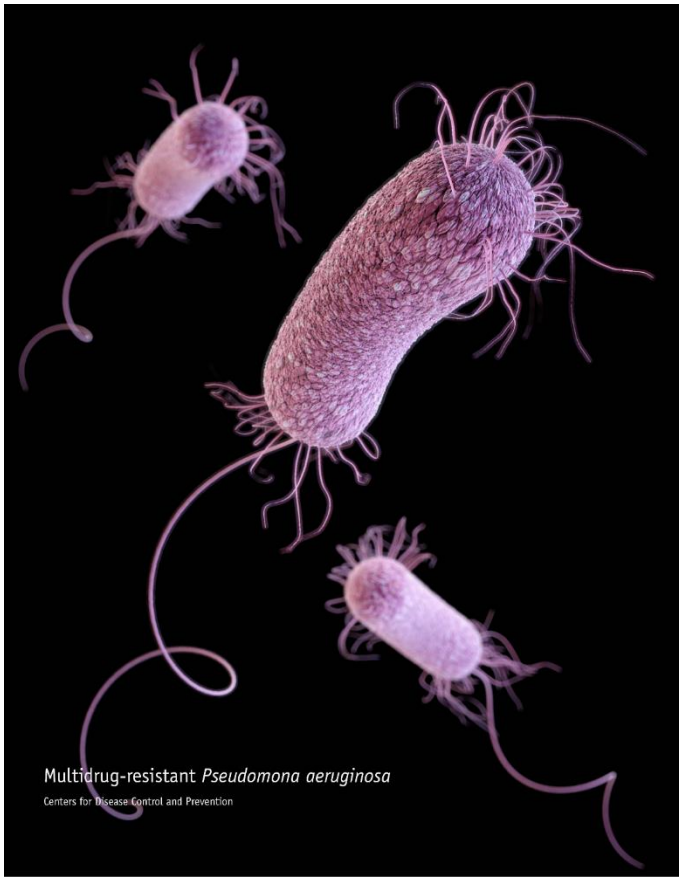

Figure 1: illustration of the bacterial structure of P. aeruginosa, courtesy of the Centre of Disease control/James Archer, source: https://phil.cdc.gov/Details.aspx?pid=16876

1.1 Clinical relevance of *P. aeruginosa*

The AURES, a yearly report provided by the Austrian ministry of health and women (Bundesministerium für Gesundheit und Frauen) shows data on the development and prevalence of different human pathogens. In 2016, there were 697 newly reported isolates of *P. aeruginosa* derived from a blood sample, indicating an incidence of *P. aeruginosa* bloodstream infections (BSI) of 8.0 per 100,000 healthy individuals per year (BMGF 2017). At the university hospital in Graz (LKH Universitätsklinikum Graz), Department of Internal Medicine , *P. aeruginosa* was detected in blood samples (n=46), urine samples derived from midstream urine or from a catheter (n=69 and n=77, respectively), from sputum or tracheal secret, derived from bronchioalveolar lavage (n=55 and n=30, respectively), as well as from material obtained via aspiration (n=4), from the tip of a central venous line (n=1) and from wounds by swabs (n=142) in 2017 (local report on antimicrobial susceptibility). This demonstrates the diversity of infections caused by *P. aeruginosa* as well as its ability to colonize patients. Unfortunately, these infections are often associated with high mortality and morbidity, as Thaden, Park et al. have shown for bloodstream infections: BSI caused by *P. aeruginosa* are linked to the highest mortality, showing a hazard ratio of 1.435 compared to BSI caused by Gram-negative bacteria and *Staphylococcus aureus*, regardless of comorbidities (Thaden, Park et al. 2017). In the USA (United States of America), an overall crude mortality of 29.5% for *P. aeruginosa* septicaemia between 1999 and 2008 has been reported by Ani, Farshidpanah et al. in 2015 by examining data of over 5,000,000 severe sepsis hospitalisations (Ani, Farshidpanah et al. 2015).

1.2 Antimicrobial resistance in *P. aeruginosa*

Antimicrobial resistance is a significant obstacle in the treatment of infections caused by *P. aeruginosa*, as the yearly surveillance report published by the European Centre for Disease Prevention and Control undoubtedly shows (European Centre for Disease Prevention and Control 2017). Approximately one third of *P. aeruginosa* isolates found in cerebrospinal fluid or blood in Europe showed resistance to at least one antibiotic substance commonly used in treatment of such infections (carbapenems, fluoroquinolones, piperacillin with or without tazobactam, aminoglycosides and ceftazidime), 4.9% of which showing resistance to three of the tested antimicrobial groups and 4.3% showing resistance to four tested antimicrobial groups and 4.4% being resistant to five different antimicrobial groups (European Centre for Disease Prevention and Control 2018).

1.2.1 Intrinsic resistance

An intrinsic resistance of a bacterial pathogen describes its innate ability and its genetic reservoir to develop or express resistance to certain antibiotics without acquiring foreign DNA (Alvarez-Ortega, Wiegand et al. 2011). The intrinsic resistance of *P. aeruginosa* is based on multiple different principles which will be described in the following. First of all, it possesses a very low outer membrane permeability (Hancock 1998) which acts as a selective barrier for beta-lactam antibiotics (Nicas, Hancock 1983). Although most Gram-negative bacteria share that common trait of low permeability of the outer membrane, this trait is especially pronounced in *P. aeruginosa*, probably best illustrated by the fact that its overall permeability is ~12-100 times lower than that of *E. coli* (Nicas, Hancock 1983). Though this may already count as old news, *P. aeruginosa*'s membrane permeability is still as much of an issue as it was in 1983. In addition to the fact that only a fraction of the antibiotic substance available in the immediate vicinity passes through the outer membrane, its resistance increases even more through an upregulation of efflux pump systems, the most common being MexAB-OprM. This pump system is responsible for the export of quinolones, penicillins and cephalosporins. Its intrinsic resistance to aminoglycosides, if given, is often mediated by another efflux-pump system, in this case MexXY-OprM (Hocquet, Muller et al. 2008). On the other hand, an induction of AmpC beta-Lactamase and

therefore a decreased efficiency of beta-lactam antibiotics completes the feature of *P. aeruginosa*'s impressive intrinsic resistance (Alvarez-Ortega, Wiegand et al. 2011). The importance in a clinical environment is obvious with *P. aeruginosa* showing intrinsic resistance against penicillin, ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, cefazolin, cefalexin, cefotaxime, ceftriaxone, ertapenem, tetracyclines, tigecycline and moxifloxacin.

1.2.2 Acquired resistance

In addition to the ways of resistance *P. aeruginosa* features innately, there are two pathways to acquire additional resistance mechanisms: horizontal transfer of genetic components and via mutation (Breidenstein, de la Fuente-Núñez et al. 2011). Although horizontal transfer can also be accomplished via resistance islands, transposons, integrons and prophages, in the case of *P. aeruginosa*, it is often plasmid-mediated, with each gene cassette possibly carrying multiple resistance genes (Tanimoto, Tomita et al. 2008). This mechanism mostly mediates aminoglycoside resistance, even though it has been recorded for beta-lactam antibiotics as well.

Mutational resistance describes a phenomenon of antimicrobial resistance, which is caused by spontaneous mutation. Therefore, this can possibly affect every part of the multiple steps involved in developing a reduced susceptibility to antibiotics. It has been shown that *P. aeruginosa* spontaneous mutation frequency varies from $1{:}10^6$ to $1{:}10^9$, but increases if exposed to DNA-damaging substances, for example fluoroquinolones (Breidenstein, de la Fuente-Núñez et al. 2011), and with it the chance of reducing antibacterial susceptibility. Genetic mutation can cause an overexpression of AmpC beta-lactamases, therefore decreasing susceptibility to most beta-lactams (Berrazeg, Jeannot et al. 2015). Another consequence of genetic mutation relevant to antimicrobial resistance are alterations in the oprD-Protein, a porin channel for basic amino acids, small peptides and carbapenems, which, if changed in structure or reduced in number, result in a reduced susceptibility (Kao, Chen et al. 2016). Another mutational feature often observed in clinical resistant *P. aeruginosa* isolates is fluoroquinolone resistance, either mediated by structural changes in GyrA and GyrB (J. K. Lee, Lee et al. 2005), or by greater production of active or inducible efflux pumps (Sun, Deng et al. 2014).

1.2.3 Adaptive resistance

Although first described in 1966 (Barber, Waterworth 1966), the phenomenon of adaptive resistance is in many aspects still not well understood. Especially if one compares it to acquired or intrinsic resistance, knowledge about the mechanisms behind it is still limited (Moradali, Ghods et al. 2017). Still, the fact that approximately 9.4% of *P. aeruginosa* genes fulfil a regulatory function (Stover, Pham et al. 2000) and a plethora of studies in this field emphasize the importance of a thorough understanding of mechanisms of adaption. Adaptive resistance itself describes an inducible mechanism to temporarily increase or create resistance to environmental or antimicrobial stressors, in which the stressors often act as an inductor (Barber, Waterworth 1966, Pagedar, Singh et al. 2011, Barclay, Begg et al. 1992). Once the triggering factor is removed, the bacteria return to their original susceptible state. Several resistance mechanisms to various antimicrobial substances have already been discovered. AmpC, for example, a gene coding for a beta-lactamase, has been shown to be induced by sub-inhibitory concentrations of antibiotics (Zhao, Jiang et al. 2015). Lee, Park et al. showed that *P. aeruginosa* can evolve resistance in the presence of colistin and lose it in the absence. This genetic evolution is likely mediated by amino acid alterations influencing its complex regulatory network and thereby altering the Lipid A, causing a change in the outer membrane (J. Y. Lee, Park et al. 2016). Adaptive polymyxin resistance can also be induced by low Ca2+ or Mg2+ environments by two two-component regulatory systems PmrA-PmrB and PhoP-PhoQ even though they are more likely triggered by polymyxins or antimicrobial peptides (Macfarlane, Kwasnicka et al. 2000, McPhee, Lewenza et al. 2003). Adaptive resistance to aminoglycosides has been described by Hocquet, Vogne et al. in 2003. An overproduction of the transmembrane transporter MexY but not the outer membrane pore OprM was observed during the post-exposure period, depending on the degree of exposure to gentamicin (Hocquet, Vogne et al. 2003). This results in adaptive resistance via increased aminoglycoside efflux, caused by increased expression of the MexXY-OprM pump.

1.3 Biofilm

Another feature contributing to virulence, antibiotic resistance and persistence of *P. aeruginosa* colonisation and infection is the formation of biofilms. It plays a critical role in clinical settings, forming on abiotic surfaces, such as medical equipment, orthopaedic implants or central venous access devices, or biotic surfaces. Especially patients suffering from cystic fibrosis are affected by chronic infections and biofilm formation, which poses significant difficulties in treatment. The development and maturation of *P. aeruginosa* biofilm is a highly sophisticated process, often mediated by cell-to-cell-signalling, e.g. quorum sensing and involving regulatory changes in ~1% of its genes (Whiteley, Bangera et al. 2001). A key component of biofilm formation is the secretion of extracellular polymeric matrix, which can consist of exopolysaccharides (mostly pel, psl and Alginate) (Friedman, Kolter 2004a, Friedman, Kolter 2004b), extracellular DNA, lipids, biosurfactants and proteins (Friedman, Kolter 2004a, Barken, Pamp Sünje J. et al. 2008, Mikkel, Arne et al. 2003). Due to different premises provided by different environments, e.g. a gradient in nutrient availability, being higher at the outer parts and significantly lower at the interior part of a biofilm, bacteria form heterogeneous subpopulations in a mature biofilm. For example, cells at the interior part mature and divide slower than cells at the outer part (Taylor, Yeung et al. 2014). Just like its counterpart in a planktonic state, bacteria in biofilm matrix feature ways of showing resistance, either acquired, adaptive or intrinsic, of comparable complexity. Still, biofilms are considerably more difficult to eradicate than bacteria in a planktonic form, as *P. aeruginosa* in a biofilm possesses inherent properties, impeding successful treatment (de la Fuente-Nunez, Reffuveille et al. 2013).This has been attributed to the broad array of adaptive gene expression changes. Some of these changes influence antibiotic susceptibility by increasing the number of enzymes in the extracellular matrix which are capable of degrading antibiotics. Other adaptive mechanisms attributed to biofilm formation is the slow metabolic state of cells in the basal part of the biofilm, commonly attributed to very limited availability of nutrients (Taylor, Yeung et al. 2014). This creates an anaerobic environment at the inner part of the biofilm, which, in consequence, greatly limits the therapeutic efficiency of aminoglycosides (Kindrachuk, Fernandez et al. 2011, Borriello, Werner et al. 2004). Cells surviving antimicrobial treatment due to non-mutational mechanisms are called “persister” cells and are assumed to be a major contributing factor to persistence of foreign body infections (Lewis 2010). The persistence of biofilm infections is not yet fundamentally

understood, but it is believed that low translational, transcriptional and metabolic activity in some cells are a key component in its recalcitrance (Taylor, Yeung et al. 2014). Furthermore, recent research suggests adaptive mechanisms such as stringent response, a cell's response to a limited supply to nutrients, may influence the development of "persister" cells as well (Maisonneuve, Castro-Camargo et al. 2013).

1.4 Quorum sensing

P. aeruginosa is a complicated and complex organism, capable of performing tasks which require a meticulously orchestrated approach, such as the formation of biofilms or the development from an acute to a chronic infection. The term quorum sensing (QS) was suggested first by a team of three microbiologists in 1994, when they described population density-based regulatory mechanisms, taking place in *P. aeruginosa* amongst others (Fuqua, Winans et al. 1994), and named the process quorum sensing. Different bacteria use different molecules for quorum sensing, nevertheless, the underlying principle is very similar: bacteria produce small, specific molecules, called autoinducers, which diffuse freely through the cell membranes. Accumulation of autoinducers is detected by cognate receptors of the same species of microbes. As soon as a certain threshold is reached, usually representing a certain concentration of bacteria in the immediate vicinity, the bacteria adapt their behaviour in multifarious ways. Most of these autoinducers emanate from three different subgroups of autoinducers; even though the molecular structure is different from genus to genus, they share structural similarities. The subgroups known so far are acylated homoserine lactones (AHL), used by Gram negative bacteria, peptide signals, exclusively used by Gram positive bacteria and a group called autoinducer-2, used by Gram-negative and Gram-positive bacteria (LaSarre, Federle 2013).

For *P. aeruginosa*, quorum sensing is responsible for a vast array of regulatory mechanisms. Up to 10% of the bacterium's entire genome can be affected by means of quorum sensing or adaptive mechanisms (Schuster, Peter Greenberg 2006). There are four different regulatory mechanisms employed in *P. aeruginosa* quorum sensing, interconnected in a hierarchical manner (J. Lee, Zhang 2015). The protein LasI synthesizes the autoinducer N-(3-oxododecanoyl)-homoserine lactone (OdDHL), structure of which was first characterized in 1994 (Pearson, Gray et al. 1994). OdDHL is the cognate signal for the intracellular receptor

LasR. The LasR-OdDHL complex enhances or enables the transcription of multiple virulence factor genes (Schuster, Peter Greenberg 2006, Schuster, Lostroh et al. 2003), stimulating the expression of LasI, thereby establishing a direct positive feedback loop (Kiratisin, Tucker et al. 2002). LasR-OdDHL also activates the expression of *pqsH, pqsR, pqsABCD, rhlI, rhlR and iqsR* (Latifi, Foglino et al. 1996, Déziel, Lépine et al. 2004, Hentzer, Wu et al. 2003, J. Lee, Wu et al. 2013). For a better visualisation of the organisational structure, please refer to figure 2.

RhlI synthesizes the autotransmitter N-butyrylhomoserine lactone (BHL), which forms a complex with RhlR. The compound RhlR-BHL is responsible for the transcription of a vast array of virulence factor genes (Nouwens, Beatson et al. 2003), as well as inducing *rhlI* and its own operon (Winson, Camara et al. 1995) and inhibiting *pqsABCDE and pqsH* (Wade, Calfee et al. 2005).

Pseudomonas quinolone signal (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) both bind to PqsR. Both complexes, PqsR-PQS as well as PqsR-HHQ, induce the transcription of the enzymes pqsABCDE, catalysing the reactions leading to HHQ, pqsH, catalysing the reaction from HHQ to PQS (Déziel, Lépine et al. 2004). PqsR-PQS enhances the transcription of RhlI, thereby forming an extended negative feedback loop (McKnight, Iglewski et al. 2000).

A fourth quorum sensing system has only recently been discovered: the so called IQS-system – to represent its role in integrating quorum sensing and detection of extracellular stress cues (J. Lee, Wu et al. 2013). The synthesis of IQS is regulated by the operons *ambABCDE*. This system has shown to influence the LasI/R and the RhlI/R systems, even playing a vital role in regulating synthesis of BHL, though the exact mechanisms remain unclear (J. Lee, Wu et al. 2013). IQS-synthesis appears to be regulated by the Las-system in a rich medium but also via phosphate depletion stress (J. Lee, Wu et al. 2013).

To this point, several super regulators, controlling various steps of the quorum sensing process, are known and knowledge about genes being expressed by means of QS is constantly growing. Unfortunately, due to the extensive nature of quorum sensing, a more profound approach to this subject would exceed the limitations of this paper.

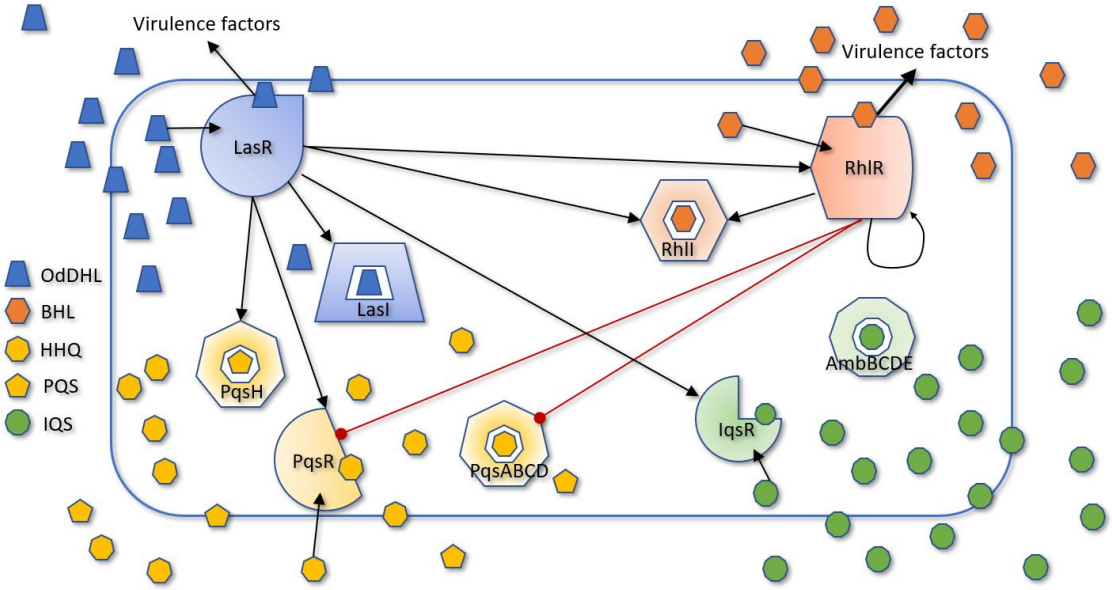

Figure 2: hierarchic structure of QS organisation, (adapted from J. Lee, Zhang 2015)

1.5 Virulence and Pathogenicity

Virulence is defined as a pathogen's ability to infect a host, whereas pathogenicity describes the ability to inflict pathological changes to an organism (Duden 2018a, Duden 2018b). Multiple mechanisms come into play in the establishment and persistence of an infection caused by *P. aeruginosa*. It can infect a remarkable scale of hosts, ranging from mammals, insects, and various plants to single-cell organisms, worms and amoeba. As Siryaporn, Kuchma et al. showed in 2014, virulence in *P. aeruginosa* is not exclusively mediated by chemical stimuli, which would require a very broad set of receptors and pathways, regarding the broad array of possible hosts, but also via mechanosensation and quorum sensing (Siryaporn, Kuchma et al. 2014), therefore supporting the theory of a multifactorial and combinatorial approach to virulence by *P. aeruginosa* (D. G. Lee, Urbach et al. 2006). Most virulence factors are secreted by the type 2 and type 3 secretion system (T2SS, T3SS, respectively), which have shown to be indispensable for virulence and death in a mouse model infection (Jyot, Balloy et al. 2011). T2SS and T3SS seem to have some redundancy in their respective function, as, even though both can be expressed in a single strain, both systems can induce virulence without the other, thereby further enabling *P. aeruginosa* to infect various hosts (Jyot, Balloy et al. 2011). T3SS forms a needle-like structure, injecting

cytotoxins Exo-S, Exo -U, Exo-T and Exo-Y in the cytoplasm of a host cell (Feltman, Schulert et al. 2001).

To avoid clearance by the host's immune system, *P. aeruginosa* employs different strategies. A loss of its flagellum and therefore its swimming motility provides protection in a planktonic state from phagocytosis by macrophages and polymorphonuclear neutrophils (PMN) (Lovewell, Hayes et al. 2014, Floyd, Winn et al. 2016). On the other hand, flagella play an important role in establishing an infection, especially in cystic fibrosis (CF) patients (Rada 2017). Additionally, biofilm formation not only increases antimicrobial resistance, it also reduces immune response: bacteria in a biofilm show a lower activation of the host's complement system and a reduced burst of reactive oxygen species (ROS) when compared to planktonic cells. Furthermore, *P. aeruginosa* in a QS-proficient biofilm formation produce Rhamnolipid B, a QS-dependent metabolite capable of inducing necrosis in PMN (Jensen, Bjarnsholt et al. 2007). Another component of *P. aeruginosa* biofilm is alginate, providing protection various host defence mechanisms, such as uptake by macrophages (Simpson, Smith et al. 1988).

1.6 Treatment options for *P. aeruginosa*

Antibiotics tested in case of a *P. aeruginosa* infection, and therefore representing possible first line therapies, at the LKH Graz are piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin. ciprofloxacin and levofloxacin (local report on antimicrobial susceptibility). The EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing) provide additional MICs for ticarcillin +/- clavulanic acid, ceftazidime/avibactam, ceftobiprole, ceftolozane/tazobactam, doripenem, colistin, amikacin and netilmicin (EUCAST 2018). Especially for patients suffering from cystic fibrosis, a combination therapy of multiple, possibly effective antibiotics may be applied.

Novel antimicrobial substances

In the past decade three new antimicrobial substances or combinations have been introduced which are useful for the treatment of *P. aeruginosa* infections. These are ceftazidime/avibactam, ceftolozane/tazobactam and ceftobiprole.

Ceftazidime/Avibactam

Ceftazidime/Avibactam was approved as Zavicefta® in the European Union in 2016. Indications for the use of Zavicefta® are complicated intra-abdominal infections, complicated urinary tract infections and hospital-acquired pneumonia, including ventilator-associated pneumonia (VAP). Additionally, Zavicefta may be used for treatment of aerobic Gram-negative pathogens for adults if therapeutic options are limited. In vitro, it has shown efficacy especially against Gram-negative microorganisms, amongst others *P. aeruginosa* (European Medicines Agency 2018a). Ceftazidime is a third-generation cephalosporin, whose bactericidal effect is caused by inhibition of the synthesis of the peptidoglycan layer of bacteria currently being in the process of mitosis. Avibactam functions as a non-beta-lactam, beta-lactamase inhibitor, inhibiting Ambler class A, C beta lactamases. In addition, avibactam inhibits some Ambler class D lactamases, including extended spectrum lactamases (ESBL), OXA48-carbapenemase and *Klebsiella pneumoniae*-carbapenemase (KPC), which belongs to the group of serine-based carbapenemases (European Medicines Agency 2018a).

Ceftolozane/Tazobactam

Ceftolozane/Tazobactam was approved for use in the European Union in 2015 under the name Zerbaxa®. Indications for the use of Zerbaxa® are complicated intraabdominal infections and complicated urinary tract infections, including pyelonephritis. In vitro, it has shown to be effective against multiple Gram-negative bacteria, including *Pseudomonas aeruginosa*, as well as certain *Streptococcus* subspecies (European Medicines Agency 2018b). Ceftolozane is a fifth-generation cephalosporin. Its bactericidal effect is – as is generally the case for all cephalosporins - based on binding to certain penicillin binding proteins (PBP), in consequence inhibiting cell wall synthesis, thus causing cell death. Tazobactam is a beta-lactamase-inhibitor, showing efficacy against many, but not all molecular class A beta-lactamases. It shows no effect against MBL, AMP-C enzymes

produced by *Enterobacteriaceae*, serine-based lactamases and Ambler class D lactamases (OXA-carbapenemases) (European Medicines Agency 2018b).

Ceftobiprole

Ceftobiprole is a fifth-generation cephalosporin, which is sold in Austria under the name Zeftera®. Like other cephalosporins, ceftobiprole unfolds its antimicrobial effect by binding to PBP and in consequence inhibits cell wall synthesis, causing cell death in bacteria. Approval for ceftobiprole under the name Zevtera® was not granted by the EU in 2010, due to the conducted studies provided in the application not holding up to the European Medicines Agency's standards (European Medicines Agency 2010). Ceftobiprole has been nationally approved for use in Austria in 2014 under the name Zeftera®, just differing in one letter from the European version Zevtera®. Its intended use is in treatment of pneumonia, acquired in either in a hospital environment or community acquired. Its intended use does not include ventilation acquired pneumonia. Ceftobiprole has shown clinical efficiency against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), *Streptococcus pneumoniae*, and *Enterobacteriaceae*. In vitro, ceftobiprole has been efficient against even more bacteria, including *Pseudomonas* spp. assumed that there are no resistance mechanisms present. The following beta-lactamases bind and degrade ceftobiprole: KPC, ESBL, as well as enzymes belonging to the Ambler A, Ambler B and Ambler D subclass. Ceftobiprole also seems stable to certain AmpC enzymes, although AmpC-expressing *P. aeruginosa* isolates provide high minimal inhibitory concentrations (MIC) (European Medicines Agency 2010).

1.7 Emerging strategies against *P. aeruginosa*

Vaccinations

Emerging strategies currently under investigation tend to take a less generalized approach in terms of applicability to a multitude of pathogens but are much rather tailored to a specific pathogen. Most, if not all mechanisms which have been discovered over the last years are being thoroughly examined to find potential drug targets to eradicate a *P. aeruginosa* population. A reliable approach to preventing infections has been vaccination. Lastly updated on 30.5.2015, the Cochrane database did not find a significant benefit caused by vaccination of CF patients and therefore does not recommend vaccination to prevent *P. aeruginosa* infection in patients with CF yet (Johansen, Gotzsche 2015). Currently, there is one vaccine which has successfully completed phase II trial. It is an OprF/I-based, recombinant vaccine and has shown a significant increase in seroconversion in mechanically ventilated ICU patients compared to a placebo group (Rello, Krenn et al. 2017). Difficulties concerning the development of vaccines are partly due to a high specificity to limited number of serotypes (Doring, Meisner et al. 2007), therefore highly limiting broad applicability.

Phage Therapy

Another approach is reemerging: Phage therapy. Bacteriophages were first described independently by Felix D'Herelle in France and Frederick William Twort in Great Britain at the beginning of the 20th century and were industrially produced in the USA until the rise of antibiotics replacing their therapeutic status in the 1940s. Currently, this approach is still applied in Poland, Russia and Georgia and is receiving international attention due to promising in vitro results. Bacteriophages are viruses which selectively infect bacteria and induce lysis and cell death. In vitro, the findings are indeed promising. Bacteriophages can cause enzymatic degradation of alginate and despite a preexisting biofilm, induce lysis in up to 99% of bacteria, highlighting a possible use in CF patients (Hanlon, Denyer et al. 2001). An engineered bacteriophage, expressing a lactonase, showed to be able to hinder quorum sensing by degrading AHL molecules (Pei, Lamas-Samanamud 2014). Furthermore, phages can express a synergistic effect when combined with antibiotics, sometimes even exceeding the effect both treatments would have if administered separately (Chaudhry, Concepción-Acevedo et al. 2017). In the same paper, Chaudry, Concepción and their colleagues also showed a change in efficacy depending on the concentration of the antibiotic substance and

the time interval between administration of phage and antibiotic influencing the ability to kill *P. aeruginosa*.

Unfortunately, trials in mouse models and in vitro data are not necessarily replicable in a clinical setting. It is difficult to mirror chronic infection models and cystic fibrosis patients in a mouse model, let alone in vitro. Therefore, clinical trials to evaluate usability are very important. Currently, there is one phase I/II study in progress, called Phagoburn (NCT02116010). The aim of this trial is to assess the tolerance and efficacy of local bacteriophage treatment for wound infections caused by *P. aeruginosa* or *E. coli* with a control group treated with the current standard of care, silver (NCT02116010).

Murepavadin

There is one especially promising therapeutic option currently undergoing phase III trials. Murepavadin belongs to a new group of antibiotics called OMPTA (short for: outer membrane protein targeting antibiotics). It inhibits LptD-mediated transport of LPS to the outer membrane (Werneburg, Zerbe et al. 2012), showcasing promising results against XDR (extensive drug-resistant) *P. aeruginosa* in vitro (Sader, Flamm et al. 2018). Murepavadin has proven to be well tolerated even in doses exceeding the expected efficacious levels in phase I and phase II clinical trials and is currently undergoing two phase III clinical trials, investigating the efficacy in hospital acquired pneumonia (NCT03409679, NCT03582007).

1.8 KRINKO classification

For easier clinical classification of Gram negative, multi-resistant bacteria, the commission for hospital hygiene and prevention of infectious diseases (the German translation abbreviated as KRINKO – Komission für Krankenhaushygiene und Infektionsprävention) has developed a simple scheme, called MRGN. MRGN is short for **m**ulti drug-**r**esistant, **G**ram **n**egative, rod shaped bacteria. Consequentially, 3MRGN bacteria show resistance to three of the four tested groups of antimicrobial substances, whereas 4MRGN describes bacterial resistance to all four of the four tested antimicrobials. For each group of antimicrobials, the KRINKO designated one representative antimicrobial substance. The four tested groups are acylureidopenicillins (Piperacillin), third and fourth generation cephalosporins (Cefotaxime and or ceftazidime), carbapenems (imipenem and or meropenem) and fluoroquinolones (Ciprofloxacin). In the case of *P. aeruginosa*, a sample is classified as 3MRGN if it shows susceptibility to only one of the four tested substances and as 4MRGN if testing shows resistance to all four tested groups of antimicrobials. The Robert-Koch-Institute suggests a variety of measures, including isolation and increased standards of hygiene, a hospital ward should take if a case of colonization or infection by a 3MRGN of 4MRGN strain occurs. At the LKH Graz, a modified version of these measures is employed (Institut für Krankenhaushygiene und Mikrobiologie 2017). In other countries, other classifications are in use, such as MDR, XDR and PDR, short for multidrug-resistant, extensively drug-resistant and pandrug-resistant, respectively (Magiorakos, Srinivasan et al. 2012).

1.9 Aim of this paper

The aim of this paper is to evaluate the susceptibility of carbapenem-resistant *P. aeruginosa* isolates against ceftobiprole, ceftazidime/avibactam and ceftolozane/tazobactam *in vitro*.

In addition, we evaluated the prevalence of MBL-production and carbapenemase-activity in carbapenem-resistant *P. aeruginosa* isolates.

2. Methods

Clinical *Pseudomonas aeruginosa* isolates after routine microbiological testing were collected. Each sample of *P. aeruginosa* was stored at – 20 °C until testing. To obtain a bacterial mass sufficient for testing, we bred the isolates on an agar plate for 24 hours at 36°C. To verify the classification "*Pseudomonas aeruginosa*", mass spectrometric analytical methods were applied, using MALDI-TOF MS. Since no patient data was used in this study, a vote by the ethics committee was not necessary.

Susceptibility and MBL testing

The EUCAST-guidelines 2017 were consulted to determine the MIC of the respective antibiotics. The antibiotics used for testing were ceftazidime/avibactam, ceftolozane/tazobactam, ceftobiprole, cefepime, ceftolozane, piperacillin/tazobactam, meropenem, imipenem and colistin.

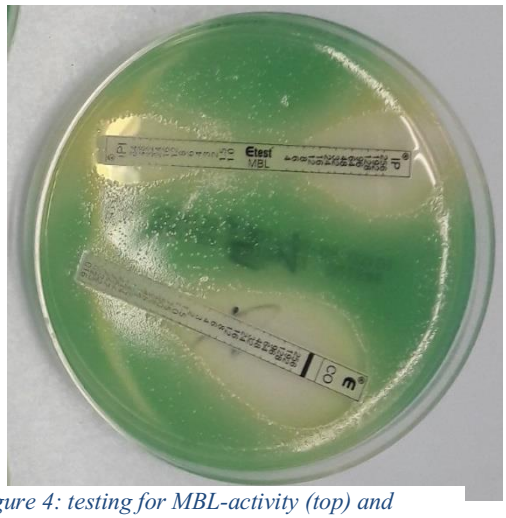

Figure 4: testing for MBL-activity (top) and susceptibility to colistin (bottom)

Gradient strip tests were used to determine the MIC of each antibiotic and to find out possible MBL (Metallo-β-lactamase)-activity. To achieve that, a loop of bacteria dissolved in saline solution 0.9% equating McFarland 0.5 was homogenously spread over a Mueller-Hinton agar plate. Two Gradient strip tests were placed on each agar plate, orientated in a way so the inhibiting areola of each strip would not overlap. After an incubation period of 18 to 24 hours at 35°C, the MIC was determined by putting the agar plate on a black pad and metering the highest value that was still in contact with the bacterial culture. Similar to susceptibility testing, gradient strip tests were used to determine MBL-production. We dissolved loop of bacteria in saline solution 0.9%, equating McFarland 0.5 and spread it homogenously on a Mueller-Hinton agar plate.

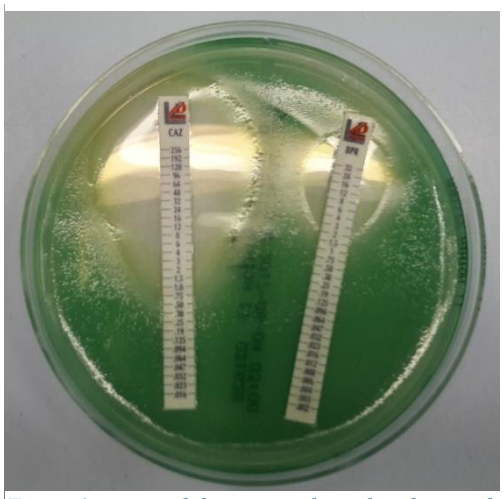

Figure 3: susceptibility testing for ceftazidime and ceftobiprole

We placed a MBL-gradient strip test on the agar plate and incubated it at 35°C for 18 to 24 hours. One half of the strip contained a carbapenem, the other half contained the same carbapenem and EDTA (ethylenediaminetetraacetic acid), an MBL-inhibitor. MIC was determined on both sides and then compared. If the MIC of the carbapenem alone was at least four times the MIC of carbapenem plus EDTA, the test was rated positive.

Testing for carbapenemase activity

Figure 5: testing kit for carbapenemase activity

To specify carbapenemase activity as a possible reason for carbapenem-resistance, we used Rapidec® Carba test kits by BioMérieux. This kit relies on carbapenem hydrolysis by carbapenemase-producing bacteria. The ensuing acidification of the medium changes the colour of indicator phenol red. In order to access carbapenemase enzymes, bacterial lysis was necessary beforehand.

If insufficient bacterial lysis was suspected due to hypermucoid appearance of bacterial isolates, the bacterial colonies and the suspension medium were mixed using a vortex type mixer until the mucoid nature was dissolved.

P. aeruginosa isolates were incubated at 36°C for 24h on Mueller Hinton Agar plates. Bacterial lysis was achieved by adding 10µl of bacterial mass into a prefabricated lysis buffer for 30 minutes at 15-25°C. The lysate was then added to a solution containing phenol red, imipenem and zinc and incubated for 30 minutes at 36°C. If there was no observable colour change after 30 minutes, the incubation period was prolonged for another 90 minutes. A positive test result is shown in figure 5 with the indicator showing a change in colour from “d” to “e”.

3. Results

Origin of isolates

We identified a total of 76 bacterial isolates, using MALDITOF-MS. Amongst those, 92.1% were classified as *Pseudomonas aeruginosa* isolates (70 out of 76). The remaining 6 isolates consisted of 4 *Stenotrophomonas maltophilia* isolates (5%), one *Pseudomonas fluorenscens* and one *Pseudomonas monteilii*.

85.7% (60/70) *P. aeruginosa* isolates tested intermediate or resistant, according to EUCAST guidelines (EUCAST 2018), to imipenem, meropenem or both (86%). In the end, a total of 60 isolates matched the inclusion criteria of mass spectrometric identification as *Pseudomonas aeruginosa* and non-susceptibility to carbapenems.

The 60 included isolates were isolated from the following sites of infection: skin swabs (n=22), respiratory tract (n=17), urinary tract (n=9), bloodstream (n=7), aspirate (n=2), wound swabs (n=2) and one isolate was found on a swab from a PEG (percutaneous endoscopic gastrostomy) feeding tube.

Susceptibility of *P. aeruginosa* isolates to novel cephalosporin antibiotics

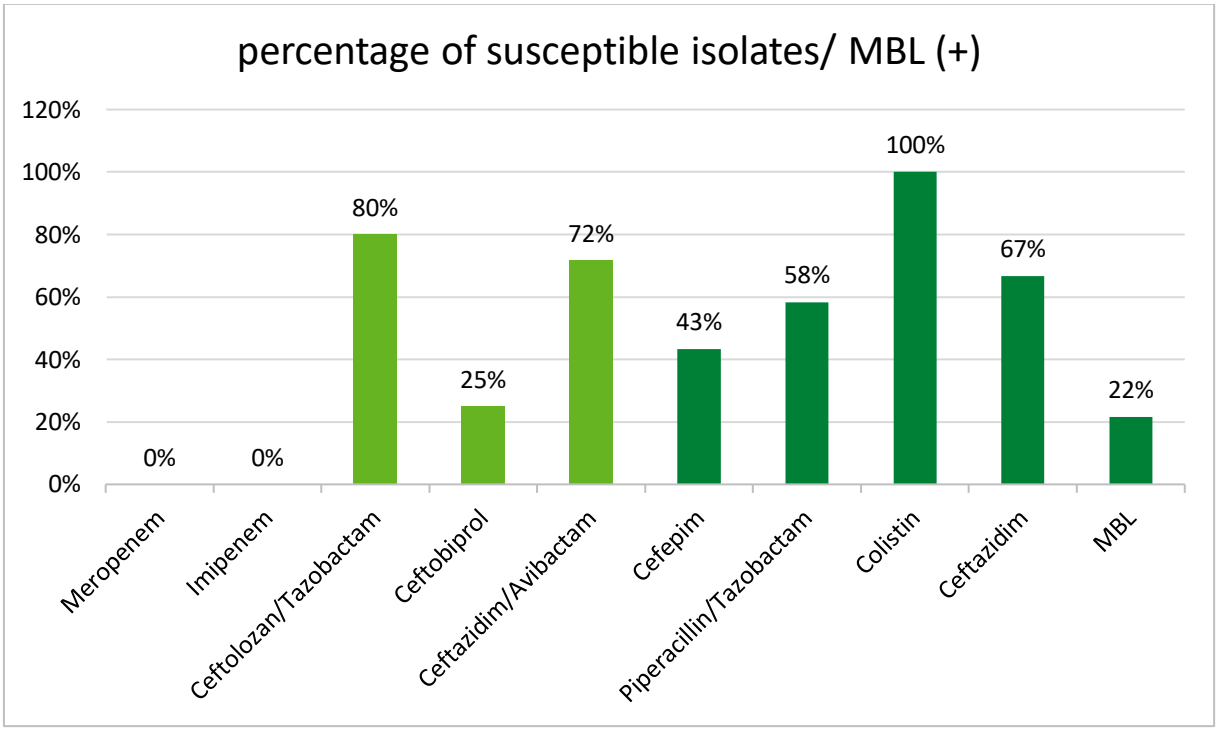

Table 1: percentage of isolates susceptible to each antibiotic and isolates that showed MBL-activity. The bright green columns represent the new antibiotics, the dark green columns represent the antibiotics used for reference and MBL-activity.

80.0% (48/60) of the isolates tested were susceptible to ceftolozane/tazobactam, 25.0% (15/60) of the isolates were susceptible to ceftobiprole and 71.7% (43/60) of the isolates were susceptible to ceftazidime/avibactam. Only colistin showed better success with a susceptibility rate of 100%. 22.0% of the isolates (13/59) showed MBL-activity.

Distribution of MICs

The following figures show the distribution of MICs of meropenem, imipenem, ceftobiprole, ceftazidime/avibactam, ceftolozane/tazobactam, ceftazidime, piperacillin/tazobactam, cefepime and colistin.

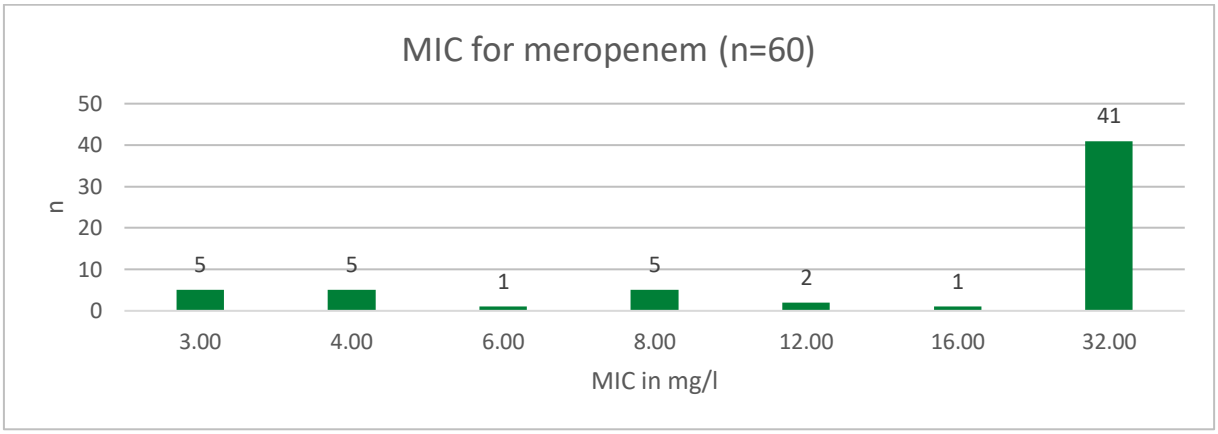

Table 2: frequency of each MIC for meropenem (MIC breakpoint according to EUCAST: 2mg/l), dark green columns represent resistant isolates

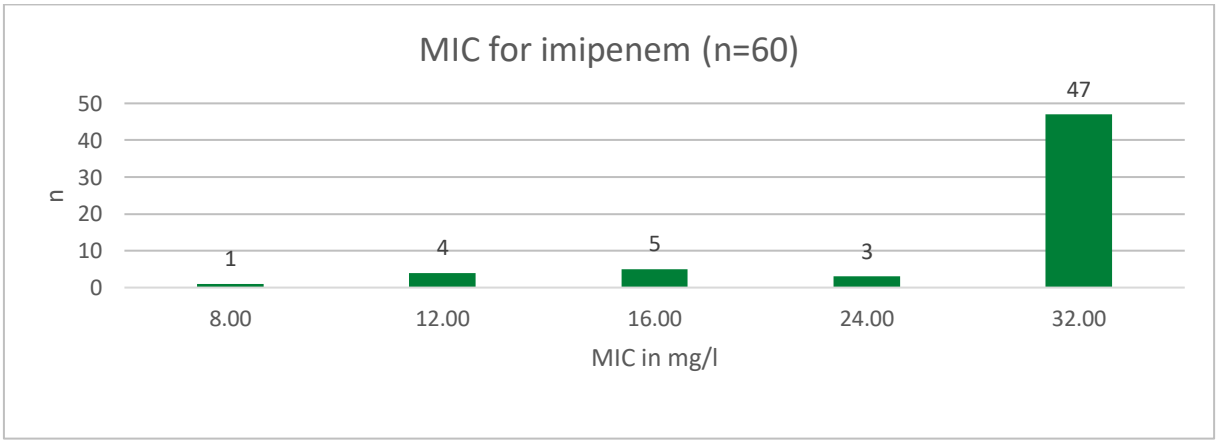

Table 3: frequency of each MIC for imipenem (MIC breakpoint according to EUCAST: 4mg/l), dark green columns represent resistant isolates

68.3% (41/60) of the tested isolates showed a MIC of 32 mg/l or above for meropenem and 78.3% (47/60) of the isolates for imipenem. No isolate which has been included in this study classified as non-resistant to either meropenem or imipenem.

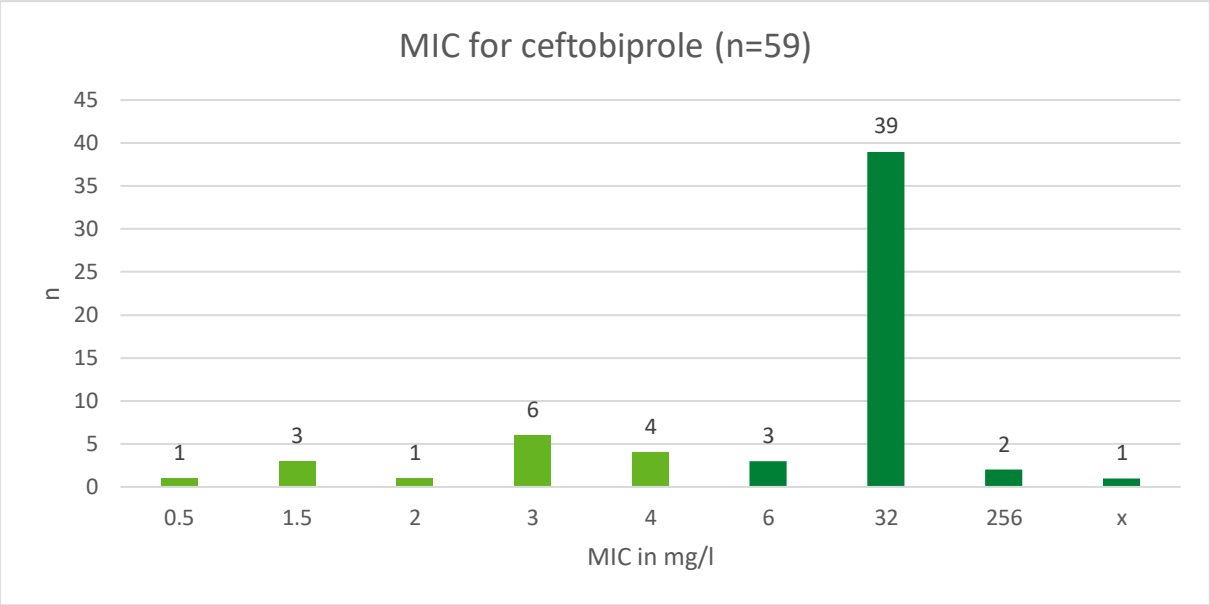

Table 4: frequency of each MIC for ceftobiprole (PK/PD breakpoint according to EUCAST: 4mg/l) bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates

Ceftobiprole showed a susceptibility rate of only 25.4% (15/59). Interestingly, 66.1% (39/59) of the isolates showed the same MIC of 32 mg/l. bright green columns depict susceptible MIC values, dark green columns represent MIC values classified as resistant. One isolate could not be tested (marked "x" on the table).

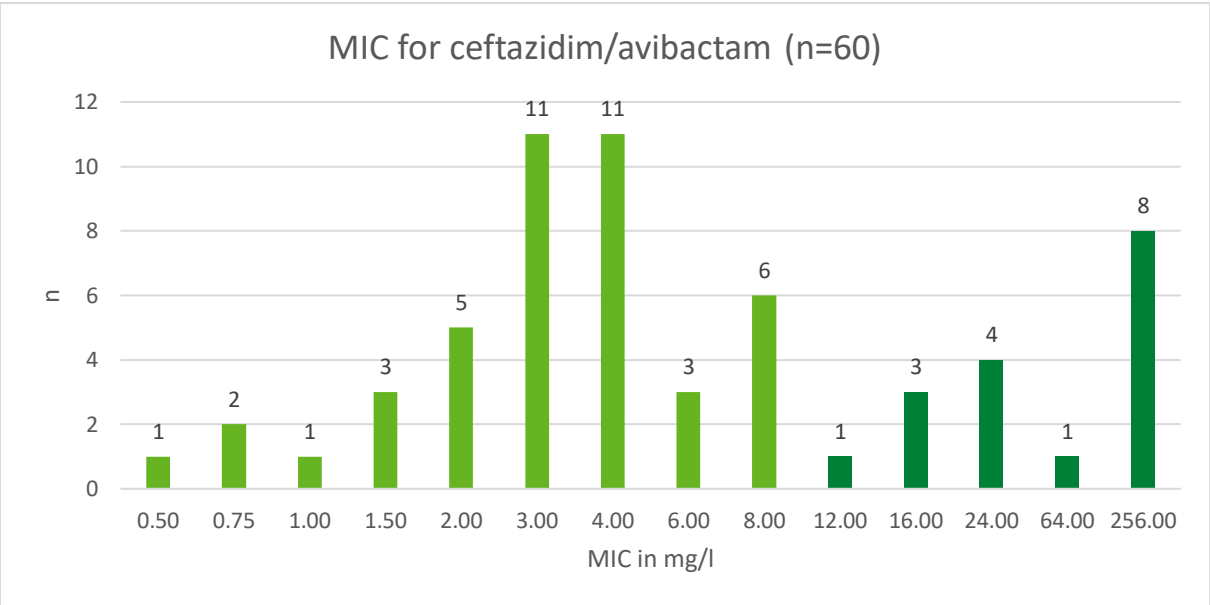

Table 5: frequency of each MIC for ceftazidime/avibactam (MIC breakpoint according to EUCAST: 8mg/l), bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates

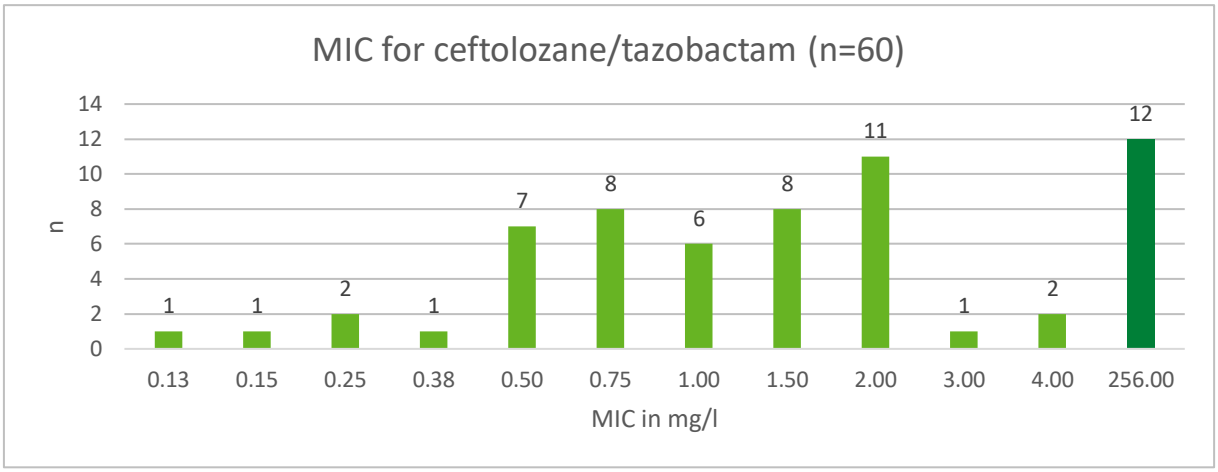

Table 6: frequency of each MIC for ceftolozane/tazobactam (MIC breakpoint according to EUCAST: 4mg/l), bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates

Both ceftolozane/tazobactam and ceftazidime/avibactam showed promising results: 71.6% (43/60) and 80.0% (48/60) of the isolates were susceptible to ceftazidime/avibactam and ceftolozane/tazobactam, respectively. For ceftolozane/tazobactam, all resistant isolates showed a MIC of at least 256 mg/l, whereas for ceftazidime/avibactam, MIC for resistant isolates appeared more homogenously spread, only 8 isolates showed a MIC of 256 mg/l or higher.

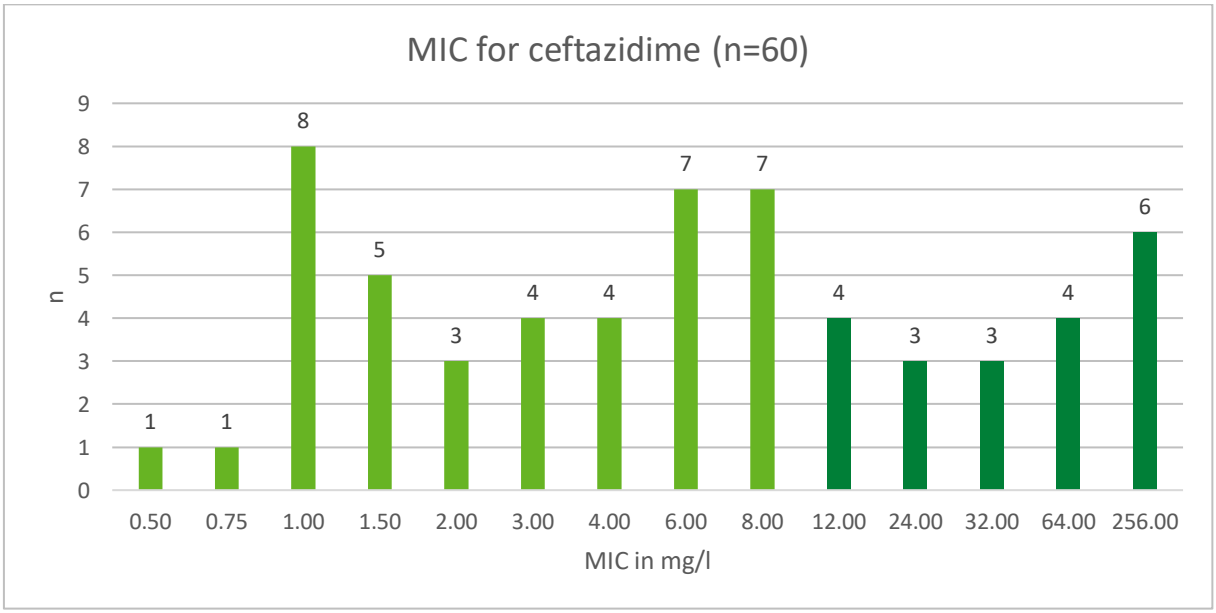

Table 7: frequency of each MIC for ceftazidime (MIC breakpoint according to EUCAST: 8mg/l), bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates

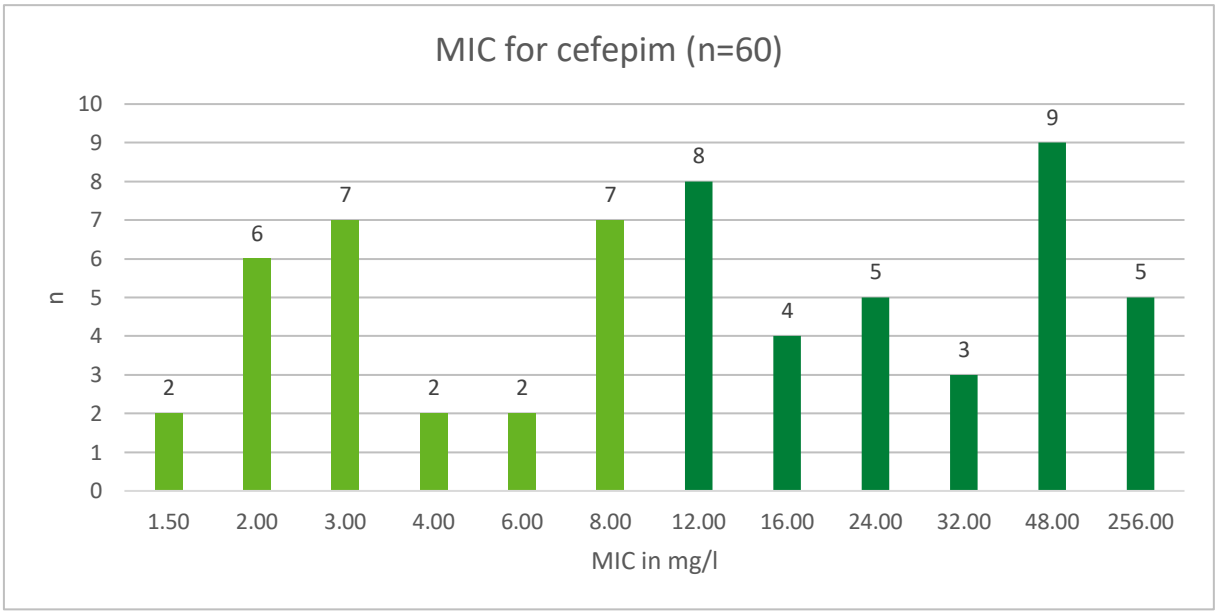

Table 8: frequency of each MIC for cefepime (MIC breakpoint according to EUCAST: 8mg/l) , bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates

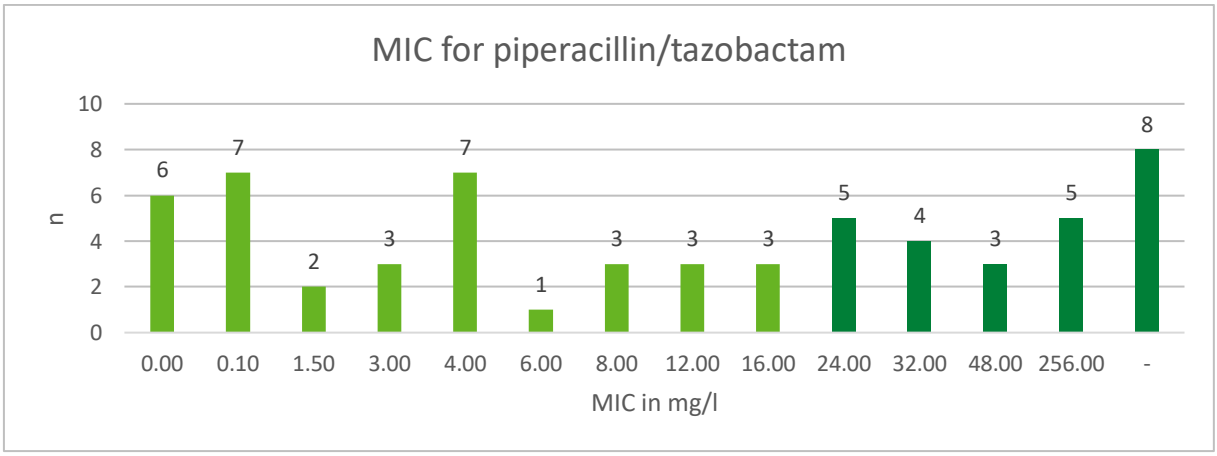

Table 9 frequency of each MIC for piperacillin/tazobactam (MIC breakpoint according to EUCAST: 16 mg/l), bright green columns represent MIC classified as susceptible, dark green columns represent resistant isolates. The column with "-" represents resistant isolates whose MIC was not determined.

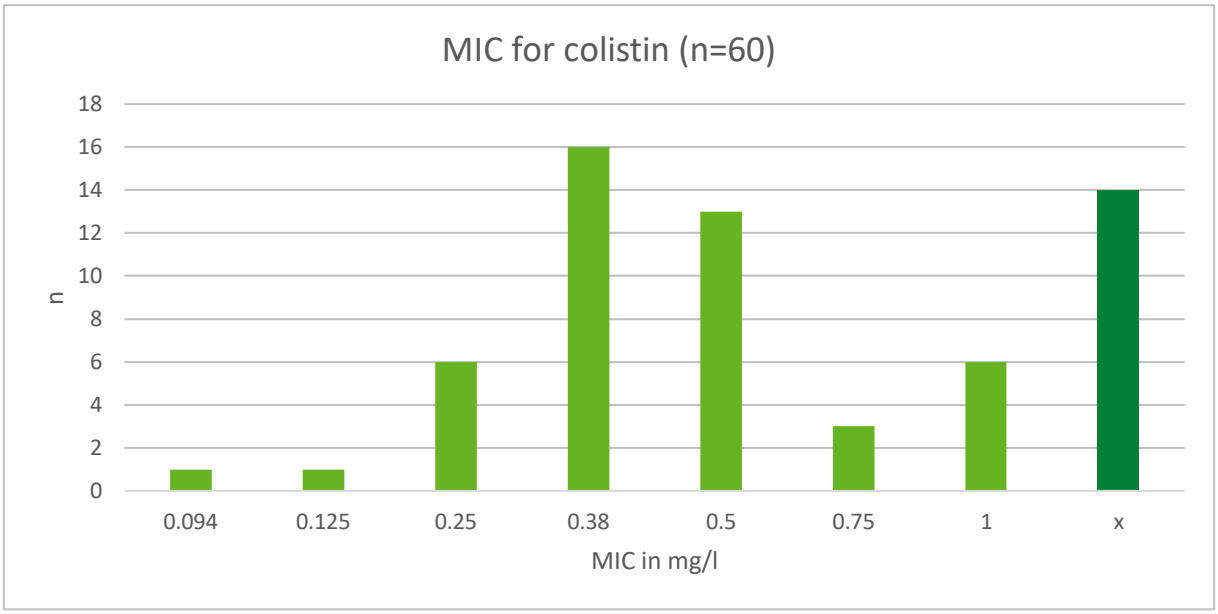

Table 10: frequency of each MIC for colistin (MIC breakpoint according to EUCAST: 2mg/l), bright green columns represent MIC classified as susceptible, the dark green column represents the number of isolates which could not be tested

The antibiotics used for reference showed varying results. While colistin showed 100% efficacy, ceftazidime, cefepime and piperacillin/tazobactam showed varying degrees of success against the tested isolates. 66.7% of the tested isolates were susceptible to ceftazidime (40/60), while only 43.3% of the tested isolates were susceptible to cefepime (26/60). 63.3% of the isolates showed susceptibility to piperacillin/tazobactam (38/60). 14 isolates were not tested for susceptibility to colistin.

Phenotypic testing of Carbapenemase and MBL production

59 isolates were tested for MBL susceptibility, 46 isolates were tested for Carbapenemase production. 22.0% (13/59) of the tested isolates showed MBL activity, whereas 47.8% (22/46) showed Carbapenemase activity. Susceptibility to colistin was unaffected by presence of MBL. Table 11 shows a comparison between the number of isolates expressing MBL-activity and carbapenemase-activity tested by Rapidec Carba test kits. 39.4% (15/38) of MBL-negative samples showed Carbapenemase-activity using the standardized test kit, while 31.8% (7/22) of Carbapenemase-positive samples expressed MBL-activity.

	MBL(+)	MBL(-)	
Carbapenemase (+)	7	15	22
Carbapenemase (-)	1	23	24
	8	38	46

Table 11: comparison of MBL- and carbapenemase-activity (In this table, only isolates tested for both MBL and carbapenemase have been included)

4. Discussion

The 2017 AURES report impressively shows the state of antimicrobial susceptibility of *P. aeruginosa* in Austria in comparison to the rest of the European Union. In 2017, 8.7% of *P. aeruginosa* isolates showed resistance to ceftazidime. Additionally, 13.9% were resistant to carbapenems. While resistance to ceftazidime was the lowest in 2017 compared to resistance levels since 2013, resistance to carbapenems has increased steadily up to its peak in 2017 since 2013 (BMASGK 2018). This alone highlights the importance of antibiotic alternatives in treatment of *P. aeruginosa* infections as well as information about the reliability of current and new therapeutic options in case of resistance to carbapenems or other antimicrobial groups. Comparison of antimicrobial resistance between European countries highlights this even more: for the prevalence of resistance to carbapenems as well as resistance to ceftazidime, more than the half of European countries reported worse results than Austria (BMASGK 2018). Therefore, we tested the recently admitted antibiotics ceftolozane/tazobactam, ceftazidime/avibactam and ceftobiprole for efficacy in clinical carbapenem-resistant *P. aeruginosa* isolates.

Our findings show that ceftolozane/tazobactam and ceftazidime/avibactam deliver promising results in vitro against carbapenem-resistant *P. aeruginosa*-isolates. Ceftolozane/Tazobactam as well as ceftazidime/avibactam showed second and third highest susceptibility rates of all antibiotics included in this study. This indicates valuable utility in treatment of increasingly more resistant *P. aeruginosa*-isolates, as two more antibiotics may be added to standard antimicrobial susceptibility testing. Additionally, direct comparison between a susceptibility rate of 71.6% (43/60) and 66.7% (40/60) for ceftazidime/avibactam and ceftazidime, respectively, suggest a possible benefit in combination of current antibiotics with beta lactamase-inhibitors. In contrast, ceftobiprole only proved to be effective in 25% (15/60) of all isolates. It appears that ceftobiprole does not present a reliable alternative if there is a pre-existing resistance to carbapenems. As we did not conduct molecular or genetic testing, we could not determine molecular mechanisms of antimicrobial resistance. Unfortunately, the precise cause of resistance to ceftobiprole in this study remains unclear, especially as in 2014, Farrell, Flamm et al. found a susceptibility of 64.6% to ceftobiprole, using the same MIC (Farrell, Flamm et al. 2014). It is noteworthy though, that Farrell, Flamm and colleagues did not select *P. aeruginosa* isolates for carbapenem resistance. In 2007, Queenan, Shang et al. found increased lability of ceftobiprole to class A, C and D extended

spectrum beta-lactamases when compared to ceftazidime (Queenan, Shang et al. 2007). This finding may partially explain the subpar results of ceftobiprole in this study.

Between 2009 and 2011, Castanheira, Deshpande et al. collected, compared and screened 529 *P. aeruginosa* isolates which were non-susceptible to doripenem, meropenem and imipenem for carbapenem resistance mechanisms from Mediterranean and European countries. Using EUCAST guidelines, susceptibility for meropenem, imipenem and cefepime was 48.5%, 67.6% and 36.0%, respectively with cefepime being the most effective β-lactam antibiotic included in this study. Using CLSI breakpoints, they found colistin to be effective against 99.3% (Castanheira, Deshpande et al. 2014).

In Brazil, Dias, Diniz et al. screened 28 carbapenem-resistant *P. aeruginosa* isolates for susceptibility to various antimicrobials and phenotypic and genotypic characteristics. They found 100% percent of the isolates to be resistant to imipenem, meropenem, ceftazidime and cefepime. Similar to our findings, 100% of their tested isolates were susceptible to polymyxin B, whereas 100% of our tested isolates were susceptible to colistin (Dias, Diniz et al. 2016).

Wi, Greenwood-Quaintance et al. tested 42 non-carbapenemase producing, carbapenem-resistant *P. aeruginosa* isolates for susceptibility to ceftolozane/tazobactam and referenced their findings by comparing them to susceptibility rates to other antimicrobials. They found ceftolozane/tazobactam to be effective in 95.2%, whereas ceftazidime/avibactam, ceftazidime, cefepime, piperacillin/tazobactam and meropenem showed susceptibility rates of 71.4%, 42.9%, 21.4%, 23.8% and 2.4%, respectively (Wi, Greenwood-Quaintance et al. 2017). The abovementioned findings demonstrate the local variability of *P. aeruginosa* susceptibility to various antibiotics.

In addition, we tested the carbapenem-resistant isolates for carbapenemase- and MBL-production. We found 22.0% (13/59) of our tested isolates to express MBL-activity. In comparison, a recent study conducted by Matzkies, Leitner et al. conducted in south-east Austria found MBL activity in 44.0% (22/50) of the included isolates by retrospectively screening 4MRGN *P. aeruginosa* isolates for MBL activity (Matzkies, Leitner et al. 2019).

The test kits we employed to detect carbapenemase activity showed difficulties in practical use. Interpretation of results was difficult, as it was hard to distinguish a positive result from a negative one. A negative result showed no change in colour, a positive result was supposed to show a change in colour from red to orange or various shades of yellow. Regrettably, the

interpretation of the results was only rarely unquestionable. Most results remained unclear, as shades of red and orange could be influenced by lighting and could only rarely be classified unequivocally. Therefore, we decided to discontinue testing for carbapenemase-activity after 46 samples. Of those tested, 47.8% (22/46) showed carbapenemase activity.

In conclusion, ceftazidime/avibactam and ceftolozane/tazobactam show high efficiency against carbapenem-resistant *P. aeruginosa* in vitro. Further research is needed to evaluate actual clinical benefit by conducting studies in a hospital setting.

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