Uncovering changes in the phenotype and transcriptome of *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device

Dissertation
der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Mumina Javed
aus London

Tübingen 2021

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	06.05.2021
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	Prof. Dr. med. Matthias Willmann
2. Berichterstatter:	Prof. Dr. Andreas Peschel

Declaration

I hereby declare that the thesis I submit for my doctorate with the title:

"Uncovering changes in the phenotype and transcriptome of *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device"

is my own independent work, that I used only the sources and resources cited and have clearly indicated all content adopted either word-for-word or in substance. I declare that the University of Tübingen's guidelines to ensure good academic practice (Senate decision of 25.5.2000) have been observed. I solemnly swear that this information is true and that I have not concealed any relevant information. I am aware that making a false declaration is punishable by a fine or by a prison term of up to three years.

Tübingen, 18.01.2021

Acknowledgements

A great number of individuals have been pivotal in the progress of my PhD, for whom I am extremely grateful. First and foremost, I would like to express my thanks to AG Peschel and the Institute of Medical Microbiology and Hygiene for their guidance and support throughout my project. Being a part of your team was a wonderful introduction to academic life in Tübingen: the summer outings, Christmas gatherings and celebrations of each other's successes. I learned from you all about the importance of team work.

I was extremely lucky to have been surrounded by great research groups, making every day entertaining. I would like to give special thanks to Anne, Taiyeb, Nermin and Cordula from AG Maier for all the advice, fun and chocolate covered snacks. I was lucky to move in to our new lab with you all.

Viola and Max: we laughed so much every day we worked together. I was far from home but I never felt it with you both. In a way I am glad that our group does not go on; these past four years as a student in your team will be forever mine and mine only. A heartfelt thank you to my supervisor, Matthias Willmann. It has been a joy to work under your supervision: the freedom you gave me for my research, along with your patience and understanding means that I grew up a lot in four years. You had high standards for me and constantly moved the goalposts, a result of which I became the scientist I am today.

I have so much gratitude for my siblings, for restorative tea sessions with Nilima, for the friends I've made in Tübingen. A special thank you to Sarah Ushurhe for reminding me how far I've come since our days together at KP: I am so excited to watch you bloom too. To my partner Rakhal: you believed in me way before I believed in myself. This thesis is a labour of love and I dedicate it to you. Thank you for being you and for your endless patience and support.

Lastly, my parents: Abba, Amma and Mama. Thank you for carrying me high.

Table of Contents

Abstract

Multi-drug resistant (MDR) *Pseudomonas aeruginosa* strains are increasingly becoming a cause for global concern, with the World Health Organisation (WHO) listing *P. aeruginosa* as one of the critical pathogens in urgent need of new antibiotics. Colistin is a last resort drug for Gram negative infections; however the nephrotoxicity of colistin and emerging resistance to it are a deterrent for treatment. Resistance to colistin by *P. aeruginosa* can be mediated via chromosomal mutations: including modification of lipid A and loss of lipopolysaccharides (LPS). One method to prevent the spread of resistance is to employ reliable and practical antimicrobial susceptibility testing (AST) methods for colistin. Currently, the broth microdilution method (BMD) is considered the gold standard for AST, however this method is relatively labour intensive, slow and impractical. Several commercial products are now available, which work to the same principle as the BMD, but in a more convenient and user-friendly format. There are a number of difficulties encountered specifically in colistin susceptibility testing: such as the use of different susceptibility breakpoints applied in different studies, the cationic nature of colistin, its large molecular size, and heteroresistance.

Another method of slowing the development of antimicrobial resistance (AMR) is to consider compartments of infection in clinical situations where the bacteria are receiving sub-inhibitory levels of colistin. The effects of such exposure on virulence and related traits such as biofilm formation and serum resistance after exposure are underexplored. Biofilm formation is one of the antibiotic-resistance mechanisms in *P. aeruginosa*, enabling persistence and survival, and low penetration of antibiotics into the bacterial community. Evolution experiments in combination with next generation sequencing (NGS) can uncover molecular mechanisms behind development of colistin resistance, and novel devices such as morbidostats can considerably speed up the process of generating resistant isolates.

The aims of this study were two fold. In order to accurately measure the level of resistance to colistin developing within the morbidostat, it was necessary to select an AST method, which can account for difficult isolates straddling the MIC breakpoint, and results in higher number of errors. Thus, we aimed to generate a comprehensive collection of colistin resistant *P. aeruginosa* strains with a range of MICs using the morbidostat, and to compare and evaluate five colistin susceptibility testing methods: three commercial BMD products, the gradient Etest, and the colorimetric reaction based Rapid Polymyxin Pseudomonas test (Rapid PP) against the reference BMD method, using these morbidostat generated isolates.

The second aim of the study was to generate *P. aeruginosa* isolates cultivated with exposure to colistin, metronidazole and a combination of the two antibiotics for 21 days, and complete RNA-Seq to uncover the transcriptional changes over time. The changes in the virulence related phenotypes were measured with isolates at four key time points.

For the first aim, a total of 131 *P. aeruginosa* isolates were used for colistin susceptibility test evaluation (100 colistin susceptible, 31 colistin resistant). The 31 colistin resistant isolates are

derived from 18 colistin susceptible *P. aeruginosa* strains that evolved resistance in the morbidostat at different MIC ranges. The categorical agreement (CA) rates for MICRONAUT-S, SensiTest and Rapid Polymxyin Pseudomonas were 94.7%, 93.9%, 92.4%, respectively. The Sensititre achieved the highest CA score (96.9%), while the Etests had the lowest CA score (84%). The very major discrepancies (VMD) rates for all tests were between 3.2-67.4%. We concluded that the commercial BMD methods are a suitable alternative to the gold standard BMD method, with the Micronaut MIC strip tests providing a wide range of MICs for testing.

To investigate effects of antibiotic pressure, we cultivated *P. aeruginosa* in a semi-automated morbidostat device with colistin, metronidazole and a combination of the two antibiotics for 21 days, and completed RNA-Seq to compare differential gene expression over time. The susceptibility of isolates to colistin was measured using the commercial BMD method Micronaut MIC strip. Within 21 days, strains developed resistance to colistin.

After seven days of colistin exposure, strains developed an ability to grow in serum, indicating that colistin is a driver of bacterial modifications which protect against serum complement factors. At Day 21, we saw the biggest increase in serum resistance, with no significant changes in susceptibility to serum found in isolates exposed to only metronidazole or in the control condition, with LB medium. Colistin-resistant strains also show significantly increased biofilm formation: the cell density in biofilm increases under exposure to colistin, while metronidazole modulates this effect. Notably, strains exposed to colistin showed a decrease in virulence, when measured using the *Galleria mellonella* infection model. After 21 days there was a significant attenuation of virulence potential compared to the baseline strain. This effect was not seen in the combination drug condition, with metronidazole seemingly having a modulatory effect on the impact of colistin exposure on virulence.

These phenotypic changes were underlined by a series of differential gene expression changes, in particular LPS modifications, spermidine synthesis (via *speH* and *speE*) and the major stress response regulator *rpoS.*

Our results suggest a clinically important bacterial evolution under sub-lethal antibiotic concentration leading to potential for significant changes in the clinical course of infection.

.

Zussamenfassung

Multiresistente *Pseudomonas aeruginosa* Stämme werden zunehmend zu einer Quelle globaler Besorgnis. Die Weltgesundheitsorganisation (WHO) listet *P. aeruginosa* als ein kritisches Pathogen, für das dringend neue Antibiotika benötigt werden. Colistin ist ein Reserveantibiotikum für Gram negative Bakterien. Seine Nierenschädlichkeit und aufkommende Resistenzen halten jedoch oft von einer Behandlung ab. Colistinersistenz wird bei *P. aeruginosa* durch chromosomale Mutationen vermittelt: Einschließlich Veränderungen von Lipid A und einem Verlust von Lipopolysacchariden (LPS). Eine Methode die Verbreitung von Resistenzen aufzuhalten sind zuverlässige und praktikable Antibiotika Resistenztests für Colistin. Aktuell ist die Mikrodilutionsmethode (MDM) der Goldstandard. Diese ist jedoch arbeitsintensiv, langwierig und unpraktikabel. Mittlerweile gibt es einige kommerziell verfügbare Produkte die nach dem selben Prinzip wie die MDM funktionieren, aber praktischer und benutzerfreundlicher sind. Die Schwierigkeiten bei der Resistenztestung sind unterschiedliche Grenzwerte welche in verschiedenen Studien verwendet werden, die kationischen Eigenschaften von Colistin, sein Molekulargewicht sowie Heteroresistenzen.

Eine andere Möglichkeit der Resistenzentwicklung entgegenzutreten ist, klinische Sachverhalte, in denen Bakterien sub-inhibitorischen Colistinkonzentrationen ausgesetzt sind, zu betrachten. Die Auswirkung dieser Exposition auf die Virulenz und verwandte Eigenschaften wie Biofilmbildung und Serumresistenz ist nicht ausreichend erforscht. Biofilmbildung, einer der Resistenzmechanismen von *P. aeruginosa*, ermöglicht Persistenz und verhindert, dass das Antibiotikum zur Bakteriengemeinschaft durchdringt. Experimente zur Evolution in Kombination mit Next Generation Sequencing (NGS) können molekulare Mechanismen, die der Resistenzentwicklung zu Grunde liegen, aufdecken. Neuartige Geräte wie der Morbidostat können die Erzeugung resistenter Isolate beträchtlich beschleunigen.

Die Ziele dieser Studie sind zweierlei. Um den Grad der Colistinresistenz innerhalb des Morbidostats zuverlässig messen zu können, war es notwendig eine Testmethode zu wählen, die die minimale Hemmkonzentration (MHK) schwieriger Isolate bestimmen kann. Diese können wegen uneindeutiger Ergebnisse zu einer höheren Fehlerquote führen. Daher war es unser Ziel mittels des Morbidostat eine umfassende Sammlung an Isolaten mit einer großen Bandbreite an MHK zu erzeugen, anhand derer wir sechs Colistinresistenz Testmethoden vergleichen und auswerten konnten: Drei kommerziell verfügbare MDM, einen Epsilometertest, den auf einer kolorimetrischen Reaktion basierenden Rapid Polymyxin Pseudomonas Test (Rapid PP), sowie die MDM als Referenzmethode.

Das zweite Ziel der Studie ist *P. aeruginosa* Isolate unter Einfluss von Colistin, Metronidazol und einer Kombination der beiden über 21 Tage zu kultivieren und RNA Sequenzierungen durchzuführen um graduelle Veränderungen im Transkriptom zu entdecken. Die Veränderungen in virulenzrelevanten Phänotypen werden zu vier Zeitpunkten gemessen.

Insgesamt 131 *P. aeruginosa* Isolate wurden zur Testevaluation herangezogen (100 sensitiv, 31 resistent). Die 31 resistenten Isolate wurden im Morbidostat aus 18 sensitiven Stämmen auf verschieden hohe MHK gezüchtet. Das *categorial agreement* (CA) der Sensitivität für MICRONAUT-S, SensiTest uns Rapid PP waren respektiv 94,7%, 93,9% und 92,4%. Der Sensititre erzielte die höchste CA-Wertung (96,6%), während der E-Test die niedrigste Wertung (84%) erreichte. Die *very major discrepancies* (VMD) rates lagen bei allen Tests zwischen 3,2% und 67,4%.

Wir kultivierten *P. Aeruginosa* in einem Morbidostat unter Einfluss von Colistin, Metronidazol und einer Kombination der beiden über 21 Tage zu kultivieren und führten RNA Sequenzierungen durch um differentielle Genexpression über den Beobachtungszeitraum zu vergleichen. Innerhalb von 21 Tagen entwickelten die Stämme Resistenzen gegen Colistin.

Nach sieben Tagen Colistinexposition, fangen die Stämme an auch in menschlichem Serum zu wachsen, was darauf hinweist, das Colistin bakterielle Veränderungen vorantreibt, die gegen Komplementfaktoren schützen. Nach 21 Tagen sehen wir die höchste Serumresistenz. Im Vergleich dazu gab es keine signifikanten Veränderungen in Isolaten die nur mit Metronidazol behandelt wurden oder der Kontrollgruppe, die in LB Medium wuchs. Colistinresistente Stämme zeigten auch eine signifikant stärkere Biofilmbildung: Die Zelldichte im Biofilm war nach Colistinexposition erhöht, während Metronidazol diesen Effekt modulierte. Bemerkenswert ist, dass Stämme die mit Colistin behandelt wurden eine Abschwächung der Virulenz zeigten, gemessen im Infektionsmodell mit *Galleria mellonella*. Nach 21 Tagen gab es einen deutlichen Abfall virulenten Potentials im Vergleich mit dem Ausgangsstamm. Dieser Effekt wurde in der Kombinationstherapie nicht beobachtet, womit Metronidazol scheinbar einen Einfluss auf die Wirkung Colistins auf die Virulenz hat.

Diese phänotypischen Veränderungen wurden unterstrichen/betont durch eine Reihe von Veränderungen in der differentiellen Genexpression, besonders LPS Modifikationen, Spermidinsynthese (via *speH* und *speE*) sowie im *major stress response regulator rpoS.*

Abbreviations:

AST	Antimicrobial susceptibility testing
AMR	Antimicrobial resistance
BMD	Broth microdilution
bp	Base pairs
CA	Categorical agreement
CD	Coefficient difference
CFU	Colony forming units
EA	Essential Agreement
HIS	Heat inactivated serum
HR	Hazard ratio
ISO	International Organization for Standardization
LB	Lysogeny broth
LPS	Lipopolysaccharide
Mbp	Mega base pair
MD	Major discrepancy
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
NHS	Normal human serum
nm	Nanometre
OD	Optical density
OM	Outer membrane
PBS	Phosphate-buffered saline
PDR	Pan drug resistant
VMD	Very major discrepancy
XDR	Extensively drug resistant

List of publications:

M Javed, V Ueltzhoeffer, M Heinrich, H Siegrist, R Wildermuth, F Lorenz, R Neher, M Willmann (2018): Colistin susceptibility test evaluation of multiple-resistance-level *Pseudomonas aeruginosa* isolates generated in a morbidostat device. *Journal of Antimicrobial Chemotherapy*, Volume 73, Issue 12, December 2018, Pages 3368–3374, https://doi.org/10.1093/jac/dky337

M Javed, B Jentzsch, M Heinrich, V Ueltzhoeffer, S Peter, U Schoppmeier, A Angelov, S Schwarz, M Willmann: Transcriptomic basis of serum resistance and virulence related traits in XDR *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device. *Frontiers in Microbiology*, accepted December 2020

Contributions to publications:

M Javed, V Ueltzhoeffer, M Heinrich, H Siegrist, R Wildermuth, F Lorenz, R Neher, M Willmann (2018):
Colistin susceptibility test evaluation of multiple-resistance-level *Pseudomonas aeruginosa* isolates generated in a morbidostat device.

MJ and MW contributed to the study design. MJ completed 30% of the assays and analysed the resulting data from all assays. VU, MH, HS, RW and FL completed the remainder of assays. RN provided technical support. MJ wrote the manuscript, which was then edited by MW.

M Javed, B Jentzsch, M Heinrich, V Ueltzhoeffer, S Peter, U Schoppmeier, A Angelov, S Schwarz, M Willmann, Transcriptomic basis of serum resistance and virulence related traits in XDR *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device.

MJ contributed to the study design, completed majority of experiments and analysed all results. BJ, MH and VU completed some assays for this study. MJ, BJ, MH, VU, SP, SS and MW selected the strains for the study and contributed to designing and writing the protocols used. AA assisted with completing RNA-Seq. MJ wrote the manuscript, which was then edited by MW.

1. Introduction

1.1 Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod shaped bacterium that is found ubiquitously within the environment with a distinctive blue-green colouration (Brown, 1954). Generally, *P. aeruginosa* lives as a relatively harmless organism, inhabiting diverse environments such as soil, plants, to nematodes and animals. They are also commonly found to inhabit healthy humans, without causing infection. However, in cases with immunocompromised individuals or those receiving immunosuppressive medication, the opportunistic pathogen *P. aeruginosa* takes advantage of a repressed immune response and the body's reduced ability to clear an infection. Apart from its limited requirements for growth and remarkable ability to tolerate varied physical conditions, *P. aeruginosa* forms biofilms which serve primarily as a defence mechanism against antimicrobial treatment. Biofilms also enables survival in community and nosocomial settings, such as on catheters, stents and ventilators, with samples even being isolated from soaps and hydrotherapy pools. Its capability of persisting in such environmental niches and the level of morbidity and mortality it can cause has catapulted it to one of the top priority pathogens for which new and effective antibiotic treatments are urgently needed, as listed by the World Health Organisation (WHO)[1].

1.2 Clinical importance and relevance of *P. aeruginosa*

The ability of *P. aeruginosa* to persist in varied environments can be attributed to its large genome, which consists of 6.3 Mbp, with 5,570 predicted open reading frames (ORF)[2]. The complexity of its genome allows this microbe to have an adaptable metabolism and little in way of nutritional requirements, which enables it to colonise burn wounds, respiratory, gastrointestinal and urinary tracts.

Burn wounds provide complex microenvironments whereby colonisation of bacterial pathogens such as *P. aeruginosa* can occur and proliferate. Bacterial infection of severe burn wounds is currently a major challenge, with those involving P. *aeruginosa* being amongst the most severe, causing delays in burn patient recovery. There is also a significant risk of bacteraemia and septic shock as the infection spreads into the circulatory system. *P. aeruginosa* is often responsible for cases of otitis externa, a bacterial infection of the external ear canal, causing inflammation and temporary loss of hearing[3]. It also plays a major role in cases of corneal infection, using contact lenses, the accompanying solution and the epithelial cell damage on the eye as habitats[4]. The most worrying cases of infection with *P. aeruginosa* include ventilator-associated pneumonia or cystic fibrosis (CF), particularly in immunocompromised patients, where it can cause irreversible damage to lung tissue[5,6].

1.3 Development of antibiotic resistance

The term antibiotic refers to natural or synthetic compounds that in particular concentrations restrict the growth of bacterial cells, or cause their death. The discovery of penicillin in 1928 heralded a new era for medical innovation[7]. However, within a few decades it became clear

that antibiotic resistance was a significant threat, with infections that were once easily treated, becoming a threat once more. It is thought that by 2050, ten million people worldwide will lose their life due to infections with antibiotic-resistant microorganisms, a significant trajectory with 700,000 deaths caused by infections with AMR in 2014[8]. This emergence of antibiotic resistance is driven by the overprescribing of antibiotics in medicine and overuse in the farming industry[9]. In 2014 the WHO classified antibiotics resistance as a severe threat to public health[1], with an annual cost of billions of euros in healthcare related costs[10,11].

The war against antimicrobial resistance (AMR) is fought on two fronts: the discovery of novel antimicrobial products and combating emergence of resistance through antimicrobial stewardship. There have been very little promising novel antibiotics in the last 30 years, with the discovery and development being slow, costly and labour intensive. Novel compounds also run the risk of eventual resistance developed to it. Antimicrobial stewardship includes a set of practices to avoid the excessive use and misuse of antimicrobial drugs, better infection control practices, improving sanitary conditions and food-handling and improving diagnostic tests. Fortunately, the development of automated methods of testing and faster, more accurate susceptibility tests look set to slow the trajectory of the number of deaths by AMR.

AMR can occur in bacterial populations via mobile genetic elements or through mutations of genes encoding resistance mechanisms. *P. aeruginosa* has a high level of intrinsic resistance due to the production of enzymes with diverse targets that render the antibiotic ineffective, such as inactivating β-lactamases. Modifications include phosphorylation, acetylation and adenylation. In addition to these enzymes, *P. aeruginosa* has several multi-drug resistance efflux pumps that translocate antibiotic products out of the cell. Four key efflux pump systems have been characterised so far: MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM[1213]

Antibiotics with synergistic interactions can inhibit multiple targets, delaying and limiting the pathogen's ability to accumulate concurrent mutations that affect the multiple targets. Antagonistic interactions can reduce the selective advantage of single-drug resistant mutants, causing selection against resistance. Collateral sensitivity, whereby the development of resistance to one drug induces sensitivity to another, has recently been found to be an effective method in combating AMR, through cycling doses and various drug pairs[13,14].

1.4 Virulence determinants in *P. aeruginosa*

Virulence potential in pathogenic bacteria is essential for the transmission and development of infectious diseases and yet remains poorly understood[15]. Genes related to virulence control motility, cytoxicity and production of virulence factors as a means to manoeuvre host physiology and overcome host defences[16]. Various cell mechanisms mediate bacterial virulence: *P. aeruginosa* uses its type IV pili for twitching motility and increase adhesion in the environment and during infection, and its flagella for chemotaxis and attachment of bacteria

to host cells, and can also activate of the host inflammatory response via toll-like receptors. The production of virulence factors and biofilm formation is enacted through quorum sensing, a cell density–dependent signalling system. Virulence factors are secreted into the extracellular environment and aid the movement of bacteria through epithelial and endothelial cell junctions. Elastases such as LasB cleave endothelial cadherin and other extracellular matrix components, which disrupts vascular endothelial junctions. The type III secretion system (T3SS) effectors ExoS and ExoY stimulate cell death resulting in defects in the epithelial cell barrier[17]. These factors enable *P. aeruginosa* to move from the initial colonisation site to the bloodstream[18]. Once established, biofilm can resist antibiotics and the host immune response.

Successful results from several studies have now established the wax moth *Galleria mellonella* as an alternative model host for exploring virulence factors of pathogens such as *P. aeruginosa*[19–22]. In 1971, one of the first studies to investigate mechanisms of pathogenicity with *G. mellonella* larvae, reported that infection with *P. aeruginosa* resulted in degeneration in the cellular and humoral immune response in larvae[21]. Another study with high impact found that mutant strains with defective lipopolysaccharides showed a 10,000 fold decrease in virulence when compared to the wild type[19]. *G. mellonella* serves as a useful and convenient model for analysis of virulence factors and their connection to overcoming the host immune response.

Biofilm are a complex bacterial community, and are composed of a rich matrix of extracellular polymeric substance (EPS), including polysaccharides, proteins, and extracellular DNA. Biofilm form on indwelling devices such as catheters, heart valves and orthopaedic prostheses making infections difficult to manage for clinicians, but are also found in mucus or tissues. Previous studies have found that biofilm affects the pharmacokinetics and pharmacodynamics of antimicrobial substances, with drug tolerance increasing with maturation of biofilm: bacterial killing with a arrange of antibiotics was reduced >4-fold in old biofilms compared with young biofilm in *P. aeruginosa*[23,24]. The poor penetration of antibiotics in biofilm has been attributed to low uptake of antibiotics by oxygen-deprived organisms[25].

Biofilm formation follows a successive process of attachment, growth, maturation and dispersal. It begins when motile planktonic cells adhere to a surface. These cells begin to undergo key changes such as downregulation of motility and virulence factors, whilst ramping up formation of an extracellular matrix made of exopolysaccharides. The communication and social collaboration between cells leads to a community forming. Under inducing conditions, the cells become motile once more and begin to distribute themselves to new locations. *P. aeruginosa* produces three key polysaccharides that are crucial for biofilm development[26]: alginate, Pel and Psl. Alginate is overproduced in mucoid variants of *P. aeruginosa,* and is the dominant polysaccharide in the extracellular matrix in biofilm formed with this variant[27]. Non-mucoid strains are commonly isolated from the lungs of CF patients, and are associated with higher morbidity and mortality. The biofilm produced by the mucoid variants, and the overproduction of alginate has been shown to reduce antibiotic penetration and to inhibit phagocytosis of bacteria[28]. Non-mucoid strains employ Pel and Psl polysaccharides for

formation of biofilm[29]. The loss of biofilm formation is specifically attributed to the ability of Pel to initiate and maintain cell-cell interactions[26,30]. One study established that Pel maintains cell-to-cell interactions in biofilm from strain PA14, with Pel polysaccharides providing a major structural scaffold for the community. Deletion of *pelB* resulted in loss of biofilm structure. However, these results were strain-specific. Loss of Pel production in strain PAO1 show little difference in attachment or biofilm development. In this strain, Psl proved to be the key polysaccharide for biofilm maturity. In their study, Pel also played a key role in developing resistance to the antibiotic aminoglycoside[30].

Resistance to the bactericidal effect of serum is one of the major virulence aspects of *P. aeruginosa*[31]. The host innate immune system uses serum components, such as antibodies and proteins of the complement system. There is a higher occurrence of serum resistance amongst *P. aeruginosa* strains isolated from blood, wounds and urine, than in strains isolated from cystic fibrosis infection[32], possibly due to loss of O-antigens and poor diffusion of complement factors as a result of protective biofilm. Therefore, susceptibility to serum and resistance may be a key phenotype to allow differentiation between invasive and non-invasive strains.

1.5 Using novel automated methods to fight drug resistance

In the fight against AMR, recent developments in technology have made it easier to monitor the evolution of bacterial populations over time and in detail, particularly using sequencing. Bacteria are well suited to this style of experimentation, due to their short generation time and relatively simple physiology, allowing for up to tens of generations of evolution to take place in a day. Toprak *et al* used a custom device called a morbidostat to examine antibiotic resistance evolution in *Escherichia coli* under constant selection pressure using chloramphenicol, doxycycline, and trimethoprim[33]. The morbidostat uses culture vials, an optical detection system and assembly of a computer-controlled array of peristaltic pumps used for transfer of liquid. The use of 15 vials for culturing enables a number of experiments to be completed in parallel. Each culture vial contains a magnetic stirrer bar to maintain a constant stirring of the culture and avoid excessive biofilm formation. The semi-automated morbidostat continuously adjusts the concentration of antibiotics to maintain a constant growth rate of bacteria in culture. The drug concentration is carefully calculated for each population, in order to avoid population failure. The bacteria are under strong pressure to evolve resistance to exposure to sub-inhibitory exposure of the drug. Using next generation sequencing (NGS), this study showed how mutations accumulated and affected antibiotic resistance. Mutations in membrane proteins and transcription/translation genes lead to adaptation to the protein synthesis inhibiting drugs chloramphenicol and doxycycline. Interesting, the appearance and fixation of mutations were mostly sequential, although they observed two different mutations that appeared simultaneously in competing clones in the population[33]. An experimental evolution approach, combined with new sequencing technology are particularly valuable to explore and examine the genetic pathways along which resistance evolves, as well as the nature and order of mutations that arise, and the

trajectory of resistance. Such methods can help develop novel approaches to using antibiotics efficiently. The morbidostat provides several key advantages over sequential batch culture methods that have been previously used[33,34].

One study that achieved interesting results with automated methods also used a morbidostat to evolve 88 isogenic *E. coli* populations against 22 antibiotics for 3 weeks[35]. For every drug, two populations were evolved under strong selection and two under mild selection. They quantified the evolved populations' resistances against all 22 drugs, and constructed two separate cross-resistance networks for strongly and mildly selected populations. Bacterial populations that evolved resistance against antibiotics under strong selection acquired high levels of cross-resistance against several antibiotics. The bacterial populations evolved under milder selection acquired relatively weaker cross-resistance[35]. The results demonstrated selection strength is a significant factor in contributing to the complexity of AMR. They concluded that use of high doses of antibiotics to clear infections has the potential to promote increase of cross-resistance in nosocomial settings.

Another prominent example of automated methods used a feedback-controlled robotic platform for high-throughput lab evolution[36], controlling both population size and selection pressure for drug resistance. Experiments with approximately 100 *E. coli* K-12 gene-deletion strains led to the discovery of targeted genetic perturbations that significantly affect spontaneous antibiotic resistance evolution, due to strong epistatic interactions with resistance mutations. Strains that were initially sensitive to tetracycline evolved resistance faster and converge to the same limit of resistance as strains that were initially resistant.

1.6 Aims of this thesis:

The use of a novel device such as the morbidostat is not yet fully established in research. Thus, our first aim was to generate a large collection of colistin resistant *P. aeruginosa* strains with a range of MICs using this convenient and semi-automated device, cultivating 31 clinical strains over a period of 58 days and taking samples of isolates every two to three days in order to measure the development of resistance to colistin. We selected a number of strains, both susceptible and resistant to colistin, so that we can compare and evaluate five colistin susceptibility testing methods: three commercial BMD products, the gradient Etest, and the colorimetric reaction based Rapid Polymyxin Pseudomonas test (Rapid PP) against the reference BMD method, using these morbidostat generated isolates. The resulting MICs from each test were organized into four categories, based on the recommendations from the CLSI and calculated as a percentage relative to the reference BMD methods. The AST methods were also scored on ease of use, MIC range that can be tested, and time taken until a result. In order to accurately measure the level of resistance to colistin developing within the morbidostat, it was necessary to select an AST method that can account for difficult isolates straddling the MIC breakpoint, which often results in higher number of errors. The test selected after investigation was applied to the second aim of this thesis, as it was comparable to the gold standard BMD method.

The second aim of the study is to generate *P. aeruginosa* isolates cultivated with exposure to colistin, metronidazole and a combination of the two antibiotics for 21 days, and complete RNA-Seq to uncover the transcriptional changes over time. We used a strain derived from bloodstream infections and cultured it in our morbidostat for 21 days, under three experimental conditions: no antibiotic, single drug (colistin or metronidazole), and a combination of the two antibiotics. At four key time-points we measured the impact of antibiotic exposure on clinically relevant phenotypes such as antibiotic resistance, biofilm formation, serum resistance and *in vivo* virulence. This is the first study to examine the evolution of an extensively drug resistant (XDR) *P. aeruginosa* strain in a morbidostat device under strong antibiotic pressure, and to demonstrate that the acquisition of colistin tolerance can affect phenotypic traits generally associated with virulence.

Chapter 2

Results and Discussion

2.1 Evaluation of AST methods using morbidostat generated strains

From the 87 colistin-susceptible strains, we chose 18 for cultivation in the morbidostat in order to generate isolates resistant to colistin with a range of MICs. These strains were genetically diverse and susceptible to colistin. Within 58 days, all 18 strains developed resistance to colistin, indicating a 100% resistance generation efficacy by the morbidostat.
We selected 31 colistin-resistant isolates. To test the ability of AST methods to accurately differentiate between susceptibility and resistance, we additionally chose 13 isolates cultivated in the morbidostat that had increased in MIC value, but were still susceptible to colistin. These isolates indicated changes towards resistance, but had not developed resistance according to the EUCAST guidelines. Ultimately, we used 131 *P. aeruginosa* isolates for AST testing (87 clinical strains and 44 morbidostat-generated strains).

The BMD method was used to determine the range of MICs for all 131 isolates. Colistin MICs were determined to be between 0.25 and 512 mg/. One hundred isolates (76%) were within the susceptible range and 31 strains (24%) were categorized as resistant. The MIC_{50} for the 87 colistin-susceptible strains was calculated as 1 mg/L and as 8 mg/L for the morbidostat-derived strains. The MIC_{90} for colistin-susceptible and morbidostat-derived strains were 2 mg/L and 64 mg/L, respectively. The MIC_{50} for all 131 strains was 2 mg/L and the MIC_{90} was 32 mg/L. We tested the validity of our in-house BMD method: we used the raw values from a representative sample of *P. aeruginosa* strains, including both colistin-susceptible and -resistant strains. Two independent observers measured the MICs of eight strains on three different instances, with six replicates per strain. Comparing the results of both observers, an intraclass correlation coefficient (ICC) of 0.99 ($P<0.001$) was observed, indicating a high level of agreement between the observers. We compared the MIC results of each observer from three different days as a measure of repeatability. An ICC of 0.89 ($P<0.001$) was calculated for the first observer and an ICC of 0.93 ($P<0.001$) for the second observer. These values demonstrate a high repeatability of the test results. The mean CoV for both observers was 11.05%, demonstrating a high degree of repeatability and precision for each experiment.

The minimum requirements for test results according to the CLSI were used to measure the results (CA ≥90%, EA ≥90%, MD ≤3.0% and VMD ≤1.5%). The correlations between Etest at both 24 and 48 h timepoints and the BMD are significantly lower than between other AST methods and BMD. Etests also failed to achieve the ≥90% standard required for CA, with 84.0% at 24 h and 84.7% at 48 h. The EA rate was 70.5%–72.0% for both 24 and 48 h timepoints. The VMD rate was 67.7% and 61.3%, at 24 and 48 h respectively, indicating that Etests are unable to recognize resistant isolates. There were no MD errors for the Etest at 24 h, and at 48 h the MD rate was 1.0%. The Etest had one of the lowest hands-on times, with 25 min taken on average to test five isolates.

Of the commercial BMD methods, the Sensititre had the strongest results, achieving the CLSI requirements for three out of four categories: CA, EA and MD. The results of this method correlated well with the BMD. The MICRONAUT-S achieved 94.7% CA. However, it failed to

meet the CLSI requirements in other areas, particularly EA (86.7%) and VMD (6.5%). The SensiTest had an EA rate of 88%, with 102/116 isolates within a 2-fold dilution of the BMD results The VMD rate was 6.5%.

The Rapid PP achieved a CA rate of 92.4%, categorizing 121/131 isolates correctly. Although it recorded the highest number of false-positive results (MD of 9.0%), it only showed one VMD. The hands-on time for this test was 25 min to test five isolates, including preparation time. Most notably, test results were available to read after 4 h, compared with 18–24 h for all other tests.

To further understand the subtleties between results of the testing methods, we divided the MICs of resistant isolates into three resistance levels: low (4–8 mg/L), medium (16–64 mg/L) and high (128–512 mg/L). The Etest (24 h) only categorized 2/10 isolates correctly in the low-resistance division. The CA rate for the other tests in the low-resistance division fell to 80% (also below the CLSI minimum), except for Rapid PP, which achieved 90%. The EA rate dropped for the SensiTest in the low-resistance division compared with the total isolates, achieving 50%, compared with 88.0% for all isolates combined. This pattern was replicated for the two other commercial BMD products: the MICRONAUT-S and Sensititre achieved EA rates of 86.7% and 90.7% for all isolates, which decreased to 50% and 70%, respectively, in the low-resistance division. The VMD rate increased significantly when isolates with low resistance were tested: the MICRONAUT-S, SensiTest and Sensititre yielded a 20% VMD rate. The Rapid PP achieved the lowest VMD rate, at 10% (1/10). There were no VMD errors, and a 100% CA rate, for commercial BMD methods using medium-resistance isolates.

The isolates were also divided into susceptible and resistant isolates. In the susceptible category, the Etest achieved a CA rate of 100% agreement with the BMD at 24 h. Etests at 48 h had a CA rate of 99%. With the resistant isolates, however, the CA rate decreased to 38.7%, further supporting the view that Etests are unable to discriminate between resistant and susceptible isolates. The commercial BMD tests achieved similar results with susceptible and resistant isolates, with a 91%–98% CA rate, with the Sensititre reporting the highest value (98%) when evaluated with susceptible isolates

2.2 Investigating transcriptomic and phenotypic changes after antibiotic pressure

PA77 strains continuously exposed to colistin reached a colistin MIC >64 mg/L at Day 21. This is at least 16-fold higher than the clinical EUCAST breakpoint of colistin for *P. aeruginosa* at ≥2mg/L. The metronidazole-only and LB medium control strains did not develop colistin resistance.

We examined whether the ability to form biofilms was altered under antibiotic exposure. Looking at the number of viable cells in biofilm, by far the biggest increase in development of biofilm occurred in the colistin only condition at Day 21, with strains producing 30-fold more viable cells in biofilm than the baseline strain ($p < 0.001$). All three time-points in this

condition showed significantly increased biofilm production relative to the baseline. Looking deeper between drug conditions, we saw an increase in the number of viable cells in biofilm for isolates exposed to colistin for seven days, with 14.5 x 10^7 viable cells in biofilm compared to isolates exposed to metronidazole for the same time period with 6.7 x 10^7 viable cells in biofilm ($p < 0.001$), and in the combination drug condition (6.1 x 10^7 viable cells in biofilm) ($p < 0.05$)

Within 14 days of exposure to colistin as a single drug, there was a faster trajectory of increased biofilm formation than for isolates exposed to metronidazole only ($p < 0.05$) or in the combination of the two drugs ($p < 0.05$) (Fig 4B), which may indicate that metronidazole begins to affect the biofilm forming capabilities of the isolates. When compared to the baseline, there were a higher number of viable cells in biofilm in metronidazole-only isolates (17.5 x 10^7) ($p < 0.001$) at Day 14 than the other conditions at this time-point. After 21 days of exposure, isolates from the combination drug condition had 5-fold more viable cells in biofilm than the baseline ($p < 0.01$), while the biofilm formed by metronidazole only isolates did not differ significantly. Between drug conditions, the metronidazole-only condition isolates showed 17-fold decrease in viable cells in biofilm after 21 days of exposure (14.7 x 10^7 viable cells in biofilm) compared to colistin-only isolates at Day 21 (254.1 x 10^7) ($p < 0.001$). The combination drug condition isolates also showed a slower trajectory of increased biofilm formation, with 80.5% less biofilm produced at Day 21 compared to the colistin-only condition ($p < 0.001$).

The metronidazole-only isolates showed a general increase in biomass in biofilm when compared to both the baseline strain and the control LB medium isolates. The values adjusted relative to the baseline and LB medium isolates showed isolates sampled at Day 7 in all three drug conditions have a decrease in biomass produced in biofilm compared to control isolates, with colistin-only isolates producing 76.2% of biomass in biofilm, and combination isolates producing 66.9% of biomass in biofilm. However, isolates cultivated in metronidazole only show an increase in biomass in biofilm compared to the two other drug conditions. Isolates exposed to metronidazole for 14 days produced 26% more biomass in biofilm than isolates exposed to colistin and metronidazole at the same time point ($p < 0.05$). Isolates exposed to colistin for 14 days produced 36% less biomass in biofilm than isolates cultivated in metronidazole only ($p < 0.01$), and 10% less biomass in biofilm compared to combination drug isolates ($p < 0.05$). The isolates exposed to colistin and metronidazole for 21 days showed the highest increase in biomass in biofilm compared to the isolates cultured in the two other drug conditions, with 159% biomass measured, compared to 128.3% for colistin isolates ($p < 0.01$), and 124.6% biomass measured for metronidazole only isolates ($p < 0.01$).

Next, we tested resistance of the evolved isolates to complement factors, with resistance being defined from the ability of these isolates to grow in human serum. The coefficient difference (CD) for the baseline strain indicates a killing effect: the bacteria are not able to grow in 50% serum. However, after seven days of exposure to colistin in the morbidostat, the isolates have a CD which indicates development of resistance to complement factors in the

same serum. At Day 14 of colistin exposure, isolates were still resistant to serum complement, but less than at Day 7. At Day 21, we see the biggest increase in serum resistance.
In the combination drug condition, there was also a notable development to serum resistance by Day 7. At Day 14 and Day 21, strains became again slightly more susceptible to serum. The metronidazole-only and control condition do not show a strong deviation from the baseline susceptibility to serum.

We also investigated the virulence of the evolved isolates in a *G. mellonella* model of infection. Seven days of single exposure to colistin did not lead to a significant change in virulence (HR = 0.75). At Day 14 we still did not observe a significant deviation from baseline. However, at Day 21 we noted a significant attenuation of virulence potential compared to the baseline (HR = 0.36). This effect was not seen in the combination drug condition, with metronidazole seemingly having a modulatory effect on the impact of colistin exposure on virulence. Isolates cultured with metronidazole as a single-drug condition also demonstrated no statistically significant changes in virulence. This was the same for isolates in the control condition (LB medium only).

To examine the transcriptional profile of the isolates that had been evolved under different drug pressures, we performed RNA-Seq analysis on *P. aeruginosa* PA77 at the baseline and on strains grown for 7, 14 and 21 days in presence of single or a combination of antibiotics. Due to the drastic changes in the phenotype of the morbidostat-generated strains, namely increased biofilm formation, decrease in virulence and loss of susceptibility to serum, we wanted to investigate general changes in the differentially expressed genes between the three time-points and four conditions. For differential gene expression, a threshold of log2 fold change (FC) of +/-4 was applied.

Among the genes that were upregulated include the *arnBCADTEF* operon (also known as *pmrHFIJKLME*), which has a crucial role in colistin resistance in *P. aeruginosa* through the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A. The *arnBCADTEF* operon is upregulated in colistin within 21 days but not differentially expressed at a significant level in isolates exposed to LB medium and metronidazole only.

Two other gene transcripts, *speH* and *speE*, were significantly upregulated in the colistin only and combination drug conditions, as part of the operon *speEH-pmrAB*. The highest positive log fold change for *speE* occurred in colistin Day 21 isolates with 7.25, followed by colistin and combination isolates after seven days of drug exposure: 6.83 and 5.95 respectively. In contrast, *speE* was downregulated in the medium-control isolates, with a log FC of -0.29 at Day 7, -0.08 at Day 14 and a log fold change of 1.18 at Day 21.

One gene of interest that was downregulated is *rpoS*. The highest fold change difference for this gene transcript in particular occurs after seven days of exposure to metronidazole, with a downregulation in expression at -5.74, closely followed by isolates cultured in colistin for 14 days (log2 FC: -5.25) and isolates cultivated in a mix of colistin and metronidazole for seven days (log2 FC: -5.22). Isolates in the medium-control condition fall

below the threshold of ≤-4 log2 ratio FC, but are clearly downregulated none the less, ranging from log2 FC of -0.46 (LB medium Day 14) to -2.42 (LB medium Day 21).

2.3 Discussion

The automated nature of the morbidostat enabled us to generate hundreds of colistin resistant strains with relatively low effort compared to traditional batch sequential culture methods. Within this collection of morbidostat-generated strains, we had a wide range of MIC values indicating low to high resistance to colistin, generally in line with exposure to colistin over time. This variety of values allows us to evaluate the effect of sub-inhibitory concentrations of antibiotics in a bacterial population, and test the accuracy of AST methods with strains that have been found to be problematic in clinical situations, leading to false results. Such a strain collection can take reference centres and large laboratories years to achieve, whereas in this case we were able to do this with low effort and within 58 days.

Accurate colistin susceptibility testing methods ensure optimal patient outcome and combat evolution of drug resistance. Worryingly, our study demonstrated poor results with Etests, which are commonly used in diagnostic laboratories even today. The Etest failed to achieve the required CLSI minimum in three out of four categories. Previous studies have attributed this to poor diffusion of colistin through agar[43,44]. The remainder of the commercial AST methods achieved strong results in CA values (> 90%), and yet none of the tests met the CLSI recommendations in all categories, particularly in the VMD criterion. The performance of all test methods decreased significantly in particular for low-level colistin resistant isolates.

An exciting result from the comparison of AST methods is a test with a drastically lower hands-on time than other commercial tests. The Rapid PP took 25mins to test one isolate, and only 4hrs to produce a result. Other AST methods require 18-24hrs to produce a result, which means that the Rapid PP test can allow results to be read on the same day of testing. The panel tests between 2 and 8mg/L, which serve the requirements of most diagnostic laboratories. The Rapid PP test is easy to perform, specific and sensitive, with a simple visual observation of results. The employment of this test may contribute to prudent use of colistin, by distinguishing between infections caused by colistin-susceptible or colistin-resistant *P. aeruginosa* isolates.

The morbidostat simulates a clinical situation where compartments of infection have not been eradicated by antibiotic treatment. Bacterial populations in such compartments receive sub-lethal doses of antibiotics, which leads to tolerance of the drug. The setup of this device enabled us to test a combination of drugs: colistin and metronidazole. Metronidazole has no bactericidal effect on *P. aeruginosa* but triggers the SOS response which results in mutagenesis. We hypothesised that as a result of changes in the genome of the isolate exposed to metronidazole, it would lead to an alternative profile after exposure to colistin, other than the changes that have been observed previously[34,74,89]. As such, we found that sub-inhibitory exposure of colistin has a clear effect in many of the factors that we have

looked at: after 21 days there was increased biofilm formation, a loss in susceptibility to serum and a decrease in virulence. Metronidazole contributes a modulatory effect on two of these factors: with less viable cells in biofilm and less susceptibility to serum complement factors in combination and metronidazole only condition isolates. Analysis of transcriptional changes show reduced expression of *rpoS* in isolates exposed to colistin and metronidazole over 21 days, relative to the baseline strain PA77 might have been an adaptation mechanism to increase resistance to colistin. Exposure to increasing concentrations of colistin led to significant increased expression of *speH* and *speE* (*speE/H*). The upregulation of these genes may be a mechanism to confer tolerance to colistin under antibiotic pressure in the morbidostat, particularly as it is not highly differentially expressed in isolates exposed to metronidazole only and is found upregulated in isolates that are colistin-resistant.

The relatively high-throughput nature of the morbidostat allowed us to investigate many clinical strains in parallel, with a variable number of replicates. Such large scale investigations in future studies may uncover common pathways and transcriptomic changes that take place, predisposing a strain to develop tolerance to a drug. Isolating such pathways and markers would enable better decisions to be made regarding antibiotic treatment. The results of evolutionary experiments such as these contribute to the fight against AMR, improving patient care and saving lives.

References

1. Tacconelli E, Carrara E, Savoldi A, *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* 2018; **18**: 318–27.

2. Stover CK, Pham XQ, Erwin AL, *et al.* Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* 2000; **406**: 959–64.

3. Reid TM, Porter IA. An outbreak of otitis externa in competitive swimmers due to Pseudomonas aeruginosa. *J Hyg (Lond)* 1981; **86**: 357–62.

4. Stapleton F, Carnt N. Contact lens-related microbial keratitis: how have epidemiology and genetics helped us with pathogenesis and prophylaxis. *Eye* 2012; **26**: 185–93.

5. Wilson R, Aksamit T, Aliberti S, *et al.* Challenges in managing Pseudomonas aeruginosa in non-cystic fibrosis bronchiectasis. *Respir Med* 2016; **117**: 179–89.

6. Pang Z, Raudonis R, Glick BR, Lin T-J, Cheng Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol Adv* 2019; **37**: 177–92.

7. Roy J. Thirty Years of Penicillin Therapy. : 29.

8. de Kraker MEA, Stewardson AJ, Harbarth S. Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLoS Med* 2016; **13**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5127510/. Accessed December 7, 2020.

9. Martin MJ, Thottathil SE, Newman TB. Antibiotics Overuse in Animal Agriculture: A Call to Action for Health Care Providers. *Am J Public Health* 2015; **105**: 2409–10.

10. Lister PD, Wolter DJ, Hanson ND. Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clin Microbiol Rev* 2009; **22**: 582–610.

11. Jit M, Ng DHL, Luangasanatip N, *et al.* Quantifying the economic cost of antibiotic resistance and the impact of related interventions: rapid methodological review, conceptual framework and recommendations for future studies. *BMC Med* 2020; **18**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7059710/. Accessed December 7, 2020.

12. Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* 2013; **4**: 223–9.

13. Udekwu KI, Weiss H. Pharmacodynamic considerations of collateral sensitivity in design of antibiotic treatment regimen. *Drug Des Devel Ther* 2018; **12**: 2249–57.

14. Barbosa C, Römhild R, Rosenstiel P, Schulenburg H. Evolutionary stability of collateral sensitivity to antibiotics in the model pathogen Pseudomonas aeruginosa. *eLife* **8**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6881144/. Accessed December 16, 2020.

15. Hickey C, Schaible B, Nguyen S, *et al.* Increased Virulence of Bloodstream Over Peripheral Isolates of P. aeruginosa Identified Through Post-transcriptional Regulation of Virulence Factors. *Front Cell Infect Microbiol* 2018; **8**. Available at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00357/full. Accessed December 6, 2020.

16. McIsaac SM, Stadnyk AW, Lin T-J. Toll-like receptors in the host defense against Pseudomonas aeruginosa respiratory infection and cystic fibrosis. *J Leukoc Biol* 2012; **92**: 977–85.

17. Golovkine G, Faudry E, Bouillot S, Voulhoux R, Attrée I, Huber P. VE-Cadherin Cleavage by LasB Protease from Pseudomonas aeruginosa Facilitates Type III Secretion System Toxicity in Endothelial Cells. *PLOS Pathog* 2014; **10**: e1003939.

18. Golovkine G, Reboud E, Huber P. Pseudomonas aeruginosa Takes a Multi-Target Approach to Achieve Junction Breach. *Front Cell Infect Microbiol* 2018; **7**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5770805/. Accessed December 7, 2020.

19. Jarrell' KF, Kropinski AM. The Virulence of Protease and Cell Surface Mutants of Pseudomonas aeruginosa for the Larvae of Galleria me/lone/la. : 6.

20. Tsai CJ-Y, Loh JMS, Proft T. Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 2016; **7**: 214–29.

21. Madziara-Borusiewicz K, Lysenko O. The mechanism of pathogenicity of Pseudomonas aeruginosa: VII. The influence of toxic proteinase on hemocytes of Galleria mellonella. *J Invertebr Pathol* 1971; **17**: 138–40.

22. Raneri M, Pinatel E, Peano C, *et al.* Pseudomonas aeruginosa mutants defective in glucose uptake have pleiotropic phenotype and altered virulence in non-mammal infection models. *Sci Rep* 2018; **8**: 1–15.

23. Cao B, Christophersen L, Thomsen K, *et al.* Antibiotic penetration and bacterial killing in a Pseudomonas aeruginosa biofilm model. *J Antimicrob Chemother* 2015; **70**: 2057–63.

24. Hengzhuang W, Wu H, Ciofu O, Song Z, Høiby N. In Vivo Pharmacokinetics/Pharmacodynamics of Colistin and Imipenem in Pseudomonas aeruginosa Biofilm Infection. *Antimicrob Agents Chemother* 2012; **56**: 2683–90.

25. Coquet L, Junter GA, Jouenne T. Resistance of artificial biofilms of Pseudomonas aeruginosa to imipenem and tobramycin. *J Antimicrob Chemother* 1998; **42**: 755–60.

26. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Curr Opin Microbiol* 2007; **10**: 644–8.

27. Hentzer M, Teitzel GM, Balzer GJ, *et al.* Alginate Overproduction Affects Pseudomonas aeruginosa Biofilm Structure and Function. *J Bacteriol* 2001; **183**: 5395–401.

28. McCaslin CA, Petrusca DN, Poirier C, Serban KA, Anderson GG, Petrache I. Impact of alginate-producing Pseudomonas aeruginosa on alveolar macrophage apoptotic cell clearance. *J Cyst Fibros* 2015; **14**: 70–7.

29. Wozniak DJ, Wyckoff TJO, Starkey M, *et al.* Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms. *Proc Natl Acad Sci* 2003; **100**: 7907–12.

30. Colvin KM, Irie Y, Tart CS, *et al.* The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix: Polysaccharides of the P. aeruginosa biofilm matrix. *Environ Microbiol* 2012; **14**: 1913–28.

31. Mikucionyte G, Dambrauskiene A, Skrodeniene E, Vitkauskiene A. Biofilm formation and serum susceptibility in Pseudomonas aeruginosa. *Cent Eur J Med* 2014; **9**: 187–92.

32. Schiller NL, Millard RL. Pseudomonas -Infected Cystic Fibrosis Patient Sputum Inhibits the Bactericidal Activity of Normal Human Serum. *Pediatr Res* 1983; **17**: 747–52.

33. Toprak E, Veres A, Michel J-B, Chait R, Hartl DL, Kishony R. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet* 2012; **44**: 101–5.

34. Dößelmann B, Willmann M, Steglich M, *et al.* Rapid and Consistent Evolution of Colistin Resistance in Extensively Drug-Resistant Pseudomonas aeruginosa during Morbidostat Culture. *Antimicrob Agents Chemother* 2017; **61**. Available at: https://aac.asm.org/content/61/9/e00043-17. Accessed May 15, 2020.

35. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. *Front Microbiol* 2017; **8**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5425606/. Accessed December 7, 2020.

36. Lukačišinová M, Fernando B, Bollenbach T. Highly parallel lab evolution reveals that epistasis can curb the evolution of antibiotic resistance. *Nat Commun* 2020; **11**: 1–14.

37. Tumbarello M, Repetto E, Trecarichi EM, *et al.* Multidrug-resistant Pseudomonas aeruginosa bloodstream infections: risk factors and mortality. *Epidemiol Infect* 2011; **139**: 1740–9.

38. Li J, Nation RL, Turnidge JD, *et al.* Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 2006; **6**: 589–601.

39. Kang C-I, Kim S-H, Kim H-B, *et al.* Pseudomonas aeruginosa Bacteremia: Risk Factors for Mortality and Influence of Delayed Receipt of Effective Antimicrobial Therapy on Clinical Outcome. *Clin Infect Dis* 2003; **37**: 745–51.

40. Fiaccadori E, Antonucci E, Morabito S, d'Avolio A, Maggiore U, Regolisti G. Colistin Use in Patients With Reduced Kidney Function. *Am J Kidney Dis* 2016; **68**: 296–306.

41. Anon. EUCAST: Warnings! Available at: https://eucast.org/ast_of_bacteria/warnings/. Accessed November 20, 2020.

42. Clinical and Laboratory Standards Institute ed. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: M07-A10 ; approved standard*. 10. ed. Wayne, PA: Committee for Clinical Laboratory Standards; 2015.

43. Goldstein FW, Ly A, Kitzis MD. Comparison of Etest with agar dilution for testing the susceptibility of Pseudomonas aeruginosa and other multidrug-resistant bacteria to colistin. *J Antimicrob Chemother* 2007; **59**: 1039–40.

44. Galani I, Kontopidou F, Souli M, *et al.* Colistin susceptibility testing by Etest and disk diffusion methods. *Int J Antimicrob Agents* 2008; **31**: 434–9.

45. Hawley JS, Murray CK, Jorgensen JH. Colistin Heteroresistance in Acinetobacter and Its Association with Previous Colistin Therapy. *Antimicrob Agents Chemother* 2008; **52**: 351–2.

46. Magiorakos A-P, Srinivasan A, Carey RB, *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

47. Bankevich A, Nurk S, Antipov D, *et al.* SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J Comput Biol* 2012; **19**: 455–77.

48. Ozer EA, Allen JP, Hauser AR. Characterization of the core and accessory genomes of Pseudomonas aeruginosa using bioinformatic tools Spine and AGEnt. *BMC Genomics* 2014; **15**: 737.

49. Anon. Sequence Alignment/Map format and SAMtools | Bioinformatics | Oxford Academic. Available at: https://academic.oup.com/bioinformatics/article/25/16/2078/204688. Accessed November 20, 2020.

50. Anon. MEGA - Molecular Evolutionary Genetics Analysis. Available at: https://www.megasoftware.net/mega4/. Accessed November 20, 2020.

51. Anon. EUCAST: Clinical breakpoints and dosing of antibiotics. Available at: https://www.eucast.org/clinical_breakpoints/. Accessed May 16, 2020.

52. Giani T, Morosini MI, D'Andrea MM, García-Castillo M, Rossolini GM, Cantón R. Assessment of the Phoenix™ automated system and EUCAST breakpoints for antimicrobial susceptibility testing against isolates expressing clinically relevant resistance mechanisms. *Clin Microbiol Infect* 2012; **18**: E452–8.

53. Dafopoulou K, Zarkotou O, Dimitroulia E, *et al.* Comparative Evaluation of Colistin Susceptibility Testing Methods among Carbapenem-Nonsusceptible Klebsiella pneumoniae and Acinetobacter baumannii Clinical Isolates. *Antimicrob Agents Chemother* 2015; **59**: 4625–30.

54. Chew KL, La M-V, Lin RTP, Teo JWP. Colistin and Polymyxin B Susceptibility Testing for Carbapenem-Resistant and mcr-Positive Enterobacteriaceae: Comparison of Sensititre, MicroScan, Vitek 2, and Etest with Broth Microdilution. *J Clin Microbiol* 2017; **55**: 2609–16.

55. Hindler JA, Humphries RM. Colistin MIC Variability by Method for Contemporary Clinical Isolates of Multidrug-Resistant Gram-Negative Bacilli. *J Clin Microbiol* 2013; **51**: 1678–84.

56. Jayol A, Nordmann P, André C, Poirel L, Dubois V. Evaluation of three broth microdilution systems to determine colistin susceptibility of Gram-negative bacilli. *J Antimicrob Chemother* 2018; **73**: 1272–8.

57. Matuschek E, Åhman J, Webster C, Kahlmeter G. Antimicrobial susceptibility testing of colistin – evaluation of seven commercial MIC products against standard broth microdilution for Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. *Clin Microbiol Infect* 2018; **24**: 865–70.

58. Carretto E, Brovarone F, Russello G, *et al.* Clinical Validation of SensiTest Colistin, a Broth Microdilution-Based Method To Evaluate Colistin MICs. *J Clin Microbiol* 2018; **56**. Available at: https://jcm.asm.org/content/56/4/e01523-17. Accessed November 20, 2020.

59. Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *J Infect Dis* 2008; **197**: 1079–81.

60. Anon. Antimicrobial resistance and healthcare-associated infections - Annual epidemiological report 2014 [2012 data]. Available at: https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-and-healthcare-associated-infections-annual. Accessed June 19, 2020.

61. Falagas ME, Kasiakou SK, Saravolatz LD. Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clin Infect Dis* 2005; **40**: 1333–41.

62. Lee J-Y, Park YK, Chung ES, Na IY, Ko KS. Evolved resistance to colistin and its loss due to genetic reversion in Pseudomonas aeruginosa. *Sci Rep* 2016; **6**: 1–13.

63. Löfmark S, Edlund C, Nord CE. Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. *Clin Infect Dis* 2010; **50**: S16–23.

64. Land KM, Johnson PJ. Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa. *Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother* 1999; **2**: 289–94.

65. Hocquet D, Bertrand X. Metronidazole increases the emergence of ciprofloxacin- and amikacin-resistant Pseudomonas aeruginosa by inducing the SOS response. *J Antimicrob Chemother* 2014; **69**: 852–4.

66. Hood MI, Becker KW, Roux CM, Dunman PM, Skaar EP. Genetic Determinants of Intrinsic Colistin Tolerance in Acinetobacter baumannii McCormick BA, ed. *Infect Immun* 2013; **81**: 542–51.

67. McPhee JB, Lewenza S, Hancock REW. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. *Mol Microbiol* 2003; **50**: 205–17.

68. Mulcahy LR, Isabella VM, Lewis K. Pseudomonas aeruginosa biofilms in disease. *Microb Ecol* 2014; **68**: 1–12.

69. Ghadaksaz A, Imani Fooladi AA, Hosseini HM, Amin M. The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. *J Appl Biomed* 2015; **13**: 61–8.

70. Maurice NM, Bedi B, Sadikot RT. Pseudomonas aeruginosa Biofilms: Host Response and Clinical Implications in Lung Infections. *Am J Respir Cell Mol Biol* 2018; **58**: 428–39.

71. Ballok AE, O'Toole GA. Pouring Salt on a Wound: Pseudomonas aeruginosa Virulence Factors Alter Na+ and Cl− Flux in the Lung. *J Bacteriol* 2013; **195**: 4013–9.

72. Miajlovic H, Smith SG. Bacterial self-defence: how Escherichia coli evades serum killing. *FEMS Microbiol Lett* 2014; **354**: 1–9.

73. Kunert A, Losse J, Gruszin C, *et al.* Immune Evasion of the Human Pathogen Pseudomonas aeruginosa: Elongation Factor Tuf Is a Factor H and Plasminogen Binding Protein. *J Immunol* 2007; **179**: 2979–88.

74. Willmann M, Klimek AM, Vogel W, *et al.* Clinical and treatment-related risk factors for nosocomial colonisation with extensively drug-resistant Pseudomonas aeruginosa in a haematological patient population: a matched case control study. *BMC Infect Dis* 2014; **14**: 650.

75. Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome assemblies with multiplex MinION sequencing. *Microb Genomics* 2017; **3**. Available at: https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000132. Accessed June 25, 2020.

76. Javed M, Ueltzhoeffer V, Heinrich M, *et al.* Colistin susceptibility test evaluation of multiple-resistance-level Pseudomonas aeruginosa isolates generated in a morbidostat device. *J Antimicrob Chemother* 2018; **73**: 3368–74.

77. Necchi F, Saul A, Rondini S. Development of a high-throughput method to evaluate serum bactericidal activity using bacterial ATP measurement as survival readout. *PLOS ONE* 2017; **12**: e0172163.

78. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**: 2114–20.

79. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 2012; **9**: 357–9.

80. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; **15**: 550.

81. Lo Sciuto A, Cervoni M, Stefanelli R, Mancone C, Imperi F. Effect of lipid A aminoarabinosylation on Pseudomonas aeruginosa colistin resistance and fitness. *Int J Antimicrob Agents* 2020: 105957.

82. Chou HT, Kwon D-H, Hegazy M, Lu C-D. Transcriptome Analysis of Agmatine and Putrescine Catabolism in Pseudomonas aeruginosa PAO1. *J Bacteriol* 2008; **190**: 1966–75.

83. Qu L, She P, Wang Y, *et al.* Effects of norspermidine on Pseudomonas aeruginosa biofilm formation and eradication. *MicrobiologyOpen* 2016; **5**: 402–12.

84. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-Localized Spermidine Protects the Pseudomonas aeruginosa Outer Membrane from Antibiotic Treatment and Oxidative Stress. *J Bacteriol* 2012; **194**: 813–26.

85. Lee J-S, Heo Y-J, Lee JK, Cho Y-H. KatA, the Major Catalase, Is Critical for Osmoprotection and Virulence in Pseudomonas aeruginosa PA14. *Infect Immun* 2005; **73**: 4399–403.

86. Bricio-Moreno L, Sheridan VH, Goodhead I, *et al.* Evolutionary trade-offs associated with loss of PmrB function in host-adapted Pseudomonas aeruginosa. *Nat Commun* 2018; **9**: 1–12.

87. Bolard A, Schniederjans M, Haüssler S, *et al.* Production of Norspermidine Contributes to Aminoglycoside Resistance in pmrAB Mutants of Pseudomonas aeruginosa. *Antimicrob Agents Chemother* 2019; **63**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6761568/. Accessed May 9, 2020.

88. Nowicki EM, O'Brien JP, Brodbelt JS, Trent MS. Characterization of Pseudomonas aeruginosa LpxT reveals dual positional lipid A kinase activity and coordinated control of outer membrane modification. *Mol Microbiol* 2014; **94**: 728–41.

89. Romano KP, Warrier T, Poulsen BE, *et al.* Mutations in pmrB Confer Cross-Resistance between the LptD Inhibitor POL7080 and Colistin in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* 2019; **63**. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6709506/. Accessed July 17, 2020.

90. Burns SM, Hull SI. Comparison of Loss of Serum Resistance by Defined Lipopolysaccharide Mutants and an Acapsular Mutant of Uropathogenic Escherichia coli O75:K5. *Infect Immun* 1998; **66**: 4244–53.

91. Hong M, Payne SM. Effect of mutations in Shigella flexneri chromosomal and plasmid-encoded lipopolysaccharide genes on invasion and serum resistance. *Mol Microbiol* 1997; **24**: 779–91.

92. Bravo D, Silva C, Carter JA, *et al.* Growth-phase regulation of lipopolysaccharide O-antigen chain length influences serum resistance in serovars of Salmonella. *J Med Microbiol* 2008; **57**: 938–46.

93. Ohno A, Isii Y, Tateda K, *et al.* Role of LPS length in clearance rate of bacteria from the bloodstream in mice. *Microbiology* 1995; **141**: 2749–56.

94. Short FL, Sario GD, Reichmann NT, Kleanthous C, Parkhill J, Taylor PW. Genomic Profiling Reveals Distinct Routes To Complement Resistance in Klebsiella pneumoniae. *Infect Immun* 2020; **88**. Available at: https://iai.asm.org/content/88/8/e00043-20. Accessed September 16, 2020.

95. Esposito EP, Cervoni M, Bernardo M, *et al.* Molecular Epidemiology and Virulence Profiles of Colistin-Resistant Klebsiella pneumoniae Blood Isolates From the Hospital Agency "Ospedale dei Colli," Naples, Italy. *Front Microbiol* 2018; 9. Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01463/full. Accessed November 25, 2020.

96. Kojic M, Venturi V. Regulation of rpoS Gene Expression in Pseudomonas: Involvement of a TetR Family Regulator. *J Bacteriol* 2001; 183: 3712–20.

97. Whiteley M, Bangera MG, Bumgarner RE, *et al.* Gene expression in Pseudomonas aeruginosa biofilms. *Nature* 2001; 413: 860–4.

98. Ciofu O, Tolker-Nielsen T. Tolerance and Resistance of Pseudomonas aeruginosa Biofilms to Antimicrobial Agents—How P. aeruginosa Can Escape Antibiotics. *Front Microbiol* 2019; 10. Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00913/full#h3. Accessed July 17, 2020.

99. Wei Q, Ma LZ. Biofilm Matrix and Its Regulation in Pseudomonas aeruginosa. *Int J Mol Sci* 2013; 14: 20983–1005.

100. Lombardo M-J, Aponyi I, Rosenberg SM. General Stress Response Regulator RpoS in Adaptive Mutation and Amplification in Escherichia coli. *Genetics* 2004; 166: 669–80.

101. Schuster M, Hawkins AC, Harwood CS, Greenberg EP. The Pseudomonas aeruginosa RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 2004; 51: 973–85.

Chapter 3

Appendix

Accepted manuscripts

Colistin susceptibility test evaluation of multiple-resistance-level *Pseudomonas aeruginosa* isolates generated in a morbidostat device

Mumina Javed[1,2], Viola Ueltzhoeffer[1], Maximilian Heinrich[1,2], Hans Justus Siegrist[1], Ronja Wildermuth[1], Freia-Raphaella Lorenz[1], Richard Neher[3], Matthias Willmann[1,2]

[1]Interfaculty Institute of Microbiology and Infection Medicine Tübingen, Institute of Medical Microbiology and Hygiene, Tübingen, Germany.

[2]German Center for Infection Research (DZIF), partner site Tübingen, Tübingen, Germany

[3]Biozentrum, University of Basel, Basel, Switzerland

Journal of Antimicrobial Chemotherapy, Volume 73, Issue 12, December 2018, Pages 3368–3374, https://doi.org/10.1093/jac/dky337

Abstract

The rise of extensively drug resistant (XDR) strains of *Pseudomonas aeruginosa* is alarming, in particular because colistin often remains the only treatment option and because resistance to colistin is increasingly observed. Thus, reliable and practical antimicrobial susceptibility methods for colistin are severely needed. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has advised against gradient and disk diffusion tests, but recommends the broth microdilution method (BMD). A number of commercial products based on the principle of the recommended BMD are now available, but data regarding their diagnostic value are lacking for *P. aeruginosa*, mostly because it is difficult to collect a sufficient number of resistant isolates, even if multiple centers work together. We have used a morbidostat device to generate colistin resistant strains and have evaluated three commercial BMD products.

Colistin resistant strains were generated in a morbidostat device. Resistant strains were categorized in a low resistant (MIC 4 - 8 mg/L), medium resistant (MIC 16 - 64 mg/L) and high resistance group (MIC ≥ 128 mg/L). BMD was performed according to ISO standard 20776-1 and was used as gold standard. MICRONAUT-S (MERLIN Diagnostika GmbH, Bornheim, Germany), SensiTest Colistin (Liofilchem, Roseto degli Abruzzi, Italy), and the Sensititre (Thermo Fisher Scientific, Waltham, USA) were carried out according to the manufacturers' instructions and compared to the gold standard method BMD.

A total of 100 clinical colistin susceptible *P. aeruginosa* strains were included in the study. Of these, 17 were processed in the morbidostat, and all of them became colistin resistant (100% resistance generation efficacy), resulting in 31 resistant strains with different MIC values (32% low resistance, 58% medium resistance, and 10% high resistance). For all 131 study strains (n = 100 colistin susceptible, n = 31 colistin resistant) categorical agreement (CA) was 91.7% (MICRONAUT-S), 94.6% (SensiTest), and 96.9% (Sensititre), while essential agreement was 86.9% (MICRONAUT-S), 91.5% (SensiTest), and 90.8% (Sensititre). Of note, CA was low in the low resistance group (80% Sensitest, 70% both MICRONAUT-S and Sensititre). The Very Major Discrepancy (VMD) rate was 12.9% (MICRONAUT-S), 6.5% (Sensitest), 9.6% (Sensititre) in all isolates, with up to a 30% VMD rate in the low resistance group.

Using a morbidostat device enables to generate a sufficient number of colistin resistant isolates for antimicrobial susceptibility test evaluation, even in a setting where colistin resistance has not been seen in clinical *P. aeruginosa* isolates. The three commercial tests did not differ significantly in their diagnostic performance. None of them met the minimum requirements in order to be recommended for use by CLSI, mostly due to a high rate of VMD. Performance was generally lower in the low resistance group, demonstrating that improvement is needed particular for *P. aeruginosa* strains with MIC values around the breakpoint.

Introduction:

The World Health Organisation (WHO) has listed *P. aeruginosa* as one of the critical pathogens in urgent need of new antibiotics[1]. Infections with *P. aeruginosa* are associated with high mortality and morbidity, in particular in immunocompromised patients[37]. Extensively drug resistant (XDR) strains of this bacterium are often only susceptible to colistin, which is now established as a last resort drug[38]. Colistin is a broad range antibiotic that is part of the polymyxin family. It is effective against Gram-negative organisms like *P. aeruginosa*, but has no activity against Gram-positive bacteria. The rising emergence of resistance against colistin, and concerns of toxicity in therapy, makes it crucial to use a fast and reliable antimicrobial susceptibility method to categorise isolates in case treatment becomes necessary[39,40].

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) advised against the use of gradient and disk diffusion tests[41]. In 2016, the Clinical Laboratory Standards Institute (CLSI) and EUCAST recommended ISO-20776 standard broth microdilution method for MIC determination of colistin, with freshly prepared or frozen antibiotic[42]. However, BMD methods are impractical for diagnostic laboratories due to the workload required. Several commercial products are now on the market, which work to the same principle as the BMD, but in a more convenient and user-friendly format.
Colistin susceptibility testing has varied in previous studies with Gram-negative bacteria, likely due to a number of difficulties encountered in antimicrobial susceptibility testing (AST) of colistin: namely the use of different susceptibility breakpoints, the cationic nature of colistin, its large molecular size, and heteroresistance[43–45].

Another important reason for the observed heterogeneity could be the MIC range of the study strains. Selecting isolates at extreme ends of the minimum inhibitory concentration (MIC) scale will reduce the rate of errors, whereas a selection of isolates straddling the breakpoint would result in higher errors. Based on the current literature, it is likely that the rate of significant errors are being underreported, due to the small number of resistant isolates and those around the susceptibility breakpoint being included in comparing AST methods. One reason for this is the fact that colistin-resistant isolates of *P. aeruginosa* have low prevalence in the clinics or remain undetected due to the pitfalls of susceptibility testing. Thus, investigation of colistin AST methods is commonly outside the scope of a single laboratory. Such studies are usually only feasible for national reference laboratories which hold comprehensive biobanks of strains that have been collected over many years from various laboratories. In order to overcome these limitations and to enable evaluation of AST methods even years before low frequency resistance becomes more prevalent and a highly relevant clinical issue, we used a device called morbidostat to acquire *P. aeruginosa* strains on distinct levels of colistin resistance. The morbidostat is a continuous culture automated device that can grow bacteria under constant selection pressure and forces them to develop resistance in cases where resistance is mediated by chromosomal alterations. Our group has previously reported that colistin resistance conferring mutations acquired in the morbidostat

are very similar to those observed in colistin resistant clinical isolates of *P. aeruginosa*[34]. For these reasons, the morbidostat can be used to transform colistin susceptible clinical isolates into resistant ones, thereby resembling a "natural" acquisition of resistance. With this methodology, a sufficiently large strain collection can be constructed, comprised of isolates with different levels of resistance.

The objective of this study was to generate a comprehensive collection of colistin resistant *P. aeruginosa* strains, and to compare and evaluate three commercial BMD products, the gradient Etest, and the colorimetric reaction based Rapid Polymyxin Pseudomonas test (Rapid PP) against the reference BMD method.

Methods:

Genomic and phenotypic characterization of the colistin susceptible clinical strains

A starting total of 87 colistin susceptible strains from a collection of *P. aeruginosa* strains isolated from patients with bloodstream infection were chosen for the study. Species identification was performed using MALDI-TOF mass spectrometry and the Vitek 2 system (bioMérieux, Marcy lÉtoile, France). Antibiotic susceptibility for 15 antibiotics was assessed by Etest (MIC TestStrip, Liofilchem, Italy) and strains categorised according to their resistance profile[46]. In order to determine genetic relatedness, all strains were whole genome sequenced on a HiSeq2500 platform (Illumina, San Diego, USA) using a 2 x 125 bp approach. SPAdes (version 3.7.0)[47] was applied as *de novo* assembly tool, and a core genome was constructed by Spine (version 0.1.2)[48]. SNPs were subsequently called using SAMtools (version 0.1.19)[49], and pairwise SNP distance matrix was constructed by MEGA (version 7.0.26)[50].

Generation of colistin resistant isolates in the morbidostat

The protocols for building and using a morbidostat are described elsewhere in great detail[33,34]. Eighteen of the above mentioned strains were continuously cultivated for up to 58 days in the morbidostat with colistin. Figure 1 demonstrates a break-down of the isolates in our study. Samples of the cultures were taken every 2-3 days. To ensure replicability of AST methods, each isolate was grown on a range of blood agar plates containing up to 64mg/L of colistin. This was to ensure a truly resistant population, devoid of persister cells or dormant bacteria. One isolate from the colistin containing blood agar plate was taken and grown overnight on plain blood agar plates at 37°C. These colonies were frozen via Microbanks (Pro-Lab Diagnostics Inc, Texas, USA). All AST methods were performed with one isolate taken from these frozen stocks and grown overnight on plain blood agar plates. The ability of the morbidostat to pressure susceptible isolates into developing resistance is termed the generation efficacy rate, and is calculated by measuring the number of strains that became resistant, from the number of colistin susceptible strains used. The overall approach of generating resistant isolates was approved by our local ethical review committee (489/2017BO2).

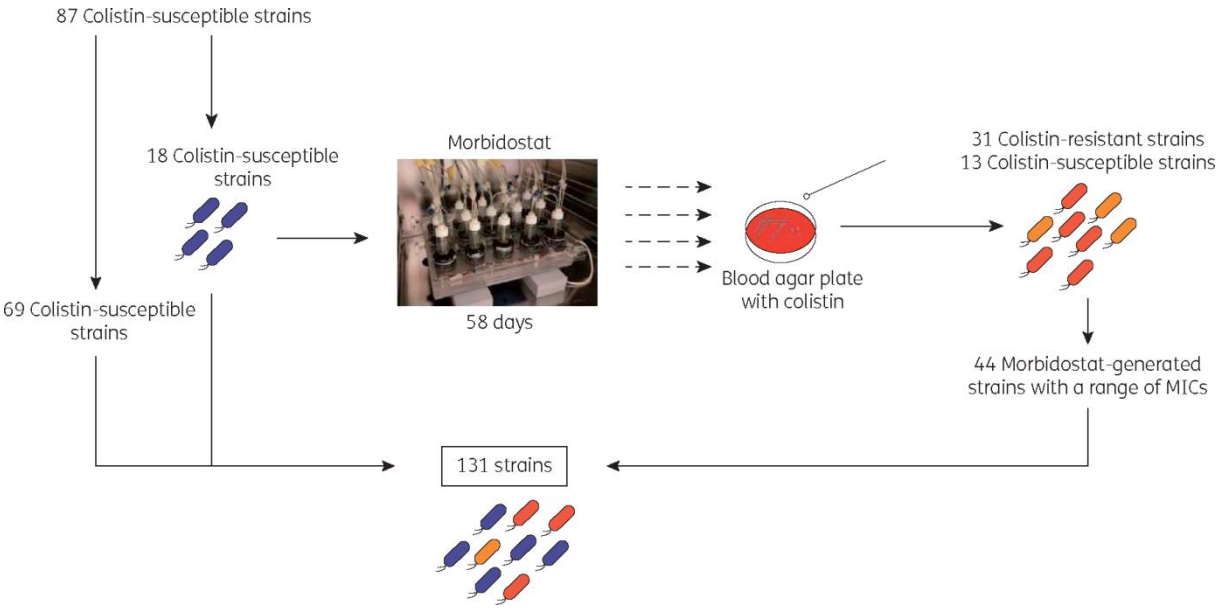


Figure 1: MIC: Minimum inhibitory concentration. Susceptibility to colistin is measured at ≤2mg/L, and resistance to colistin is >2 mg/L. The rod shapes represent *Pseudomonas aeruginosa* strains. Blue strains: clinical isolates susceptible to colistin. Orange strains: strains derived from the morbidostat with an increased MIC value but still susceptible to colistin. Red strains: strains derived from the morbidostat being resistant to colistin. The broken arrows represent strains which have been taken from the morbidostat every 2-3 days and plated on blood agar plates supplemented with colistin. Cultivation in the morbidostat took up to 58 days.

Broth microdilution

All isolates were tested for colistin susceptibility using broth microdilution (BMD), performed according to ISO standard 20776-1[42]. Briefly, colistin sulfate was dissolved in cation-adjusted BBL™ Mueller Hinton II Broth (CAMHB) (BD, Franklin Lakes, USA) and BMD carried out in 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany). *P. aeruginosa* ATCC 27853 was used as the colistin susceptible quality control strain, and the MIC determined at 2mg/L by the reference BMD method. BMD was performed on each isolate in triplicates, and the median was applied as the final MIC value.

Antimicrobial test comparison and data interpretation

MICRONAUT-S (MERLIN, Diagnostika Gmbh, Bornheim, Germany), SensiTest Colistin (Liofilchem, Roseto degli Abruzzi, Italy), Sensititre (colistin only panel) (Thermo Fisher Scientific), Rapid Polymyxin Pseudomonas (ELITech Group, Paris, France) tests were carried out according to the manufacturers' instructions. Etests (bioMérieux, Marcy l'Etoile, France) were also carried out according to the manufacturer's instructions; however, they were read at two time points: 24hrs and 48hrs. The hands-on time was calculated as the average time taken to test one strain, including preparation time.

Colistin breakpoints for *P. aeruginosa* were applied, as proposed by EUCAST (Susceptible ≤2mg/L, Resistant >2 mg/L)[51]. The range of MICs for colistin available within each testing

method are as listed: BMD: 0.25 – 512mg/L, Etest: 0.016 – 256mg/L, MICRONAUT-S: 0.25 – 64mg/L, SensiTest: 0.25 – 16mg/L, Sensititre: 0.25 – 128mg/L, Rapid PP: 2 – 8mg/L.

The resulting MICs from each test were organised into four categories, calculated as a percentage relative to the reference BMD method[52]. Categorical Agreement (CA) is defined as the qualitative interpretation of MIC from the testing method agreeing with the reference BMD method; the bacteria are categorised as either susceptible or resistant. Essential Agreement (EA) is achieved when the MIC result from the testing method is within ± one dilution step of the reference BMD method. For all tests, the calculation for the EA rate was adjusted due to the MIC range that each testing method provides, by changing the total number of isolates in the denominator. A Major Discrepancy (MD) occurred when the isolate was categorised as resistant, and the reference BMD method indicates it is susceptible. This was calculated with the number of susceptible isolates as the denominator. The Very Major Discrepancy (VMD) category is defined as the MIC result from the testing method being categorised as susceptible to colistin, when the reference BMD method indicates that it is resistant, calculated with the number of resistant isolates used as the denominator. The Rapid PP was not included in EA categories due to the limited range of MICs that can be measured.

All colistin resistant isolates were separated into three resistance levels: low, medium and high level of colistin resistance: 4-8mg/L, 16-64mg/L, and 128 – 512mg/L, respectively.

Statistical analysis

All statistical analyses were completed using GraphPad Prism, software version 6 (GraphPad Software, San Diego, USA). A Spearman's rank correlation was applied to measure the association between the MIC results of each AST method and the BMD. A *p*-value of ≤0.05 was considered significant.

Results:

Genomic structure and antibiotic susceptibility of the study isolates

A representative sample of strains was required for the purposes of this study. Eighty-seven clinical strains that were susceptible to colistin were selected. Each strain was genetically distinct from each other (Table S1 Supplementary data). The minimum pairwise distance between all isolates was 183 SNPs and the maximum was 72,478 SNPs. The median genetic distance was 23,162 SNPs. The selected strains did not contain any *mcr* genes. A resistance profile was set up for each strain by categorising them according to the results of Etests using 15 antibiotics (Table S2): 53% non-MDR, 41% MDR, 6% XDR and 0% pandrug resistant (PDR).

Resistance generation efficacy of the morbidostat

From the 87 colistin-susceptible strains, we chose 18 for cultivation in the morbidostat in order to generate isolates resistant to colistin with a range of MICs (Figure 1). These strains were genetically diverse and susceptible to colistin. Within 58 days, all 18 strains developed resistance to colistin, indicating a 100% resistance generation efficacy by the morbidostat.

From this pool of strains cultivated in the morbidostat, we selected 31 colistin-resistant isolates. To test the ability of AST methods to accurately differentiate between susceptibility and resistance, we additionally chose 13 isolates cultivated in the morbidostat that had increased in MIC value, but were still susceptible to colistin. These isolates indicated changes towards resistance, but had not developed resistance according to the EUCAST guidelines ($n=1$ with MIC=1 mg/L, $n=12$ with MIC=2 mg/L). Ultimately, we used 131 *P. aeruginosa* isolates for AST testing (87 clinical strains and 44 morbidostat-generated strains).

Colistin susceptibility of the study isolates

The range of colistin MICs, according to the BMD method, for all 131 isolates is presented in Figure 2 and Table S3(a). Colistin MICs were determined to be between 0.25 and 512 mg/L (MIC 0.25 mg/L, $n=2$; MIC 0.5 mg/L, $n=8$; MIC 1 mg/L, $n=36$; MIC 2 mg/L, $n=54$; MIC 4 mg/L, $n=3$; MIC 8 mg/L, $n=7$; MIC 16 mg/L, $n=6$; MIC 32 mg/L, $n=4$; MIC 64 mg/L, $n=8$; MIC 128 mg/L, $n=1$; and MIC 512 mg/L, $n=2$). One hundred isolates (76%) were within the susceptible range and 31 strains (24%) were categorized as resistant. The MIC_{50} for the 87 colistin-susceptible strains was calculated as 1 mg/L and as 8 mg/L for the morbidostat-derived strains. The MIC_{90} for colistin-susceptible and morbidostat-derived strains were 2 mg/L and 64 mg/L, respectively. The MIC_{50} for all 131 strains was 2 mg/L and the MIC_{90} was 32 mg/L. Further details on the origin and morphology of the 31 colistin-resistant strains are provided in Table S3(b).

Inter- and intra-assay repeatability and precision for the in-house BMD method

The gold standard of AST is the BMD. We tested the validity of our in-house BMD method used in this study. We used the raw values from a representative sample of *P. aeruginosa* strains, including both colistin-susceptible and -resistant strains, shown in Table S3(c). Two

independent observers measured the MICs of eight strains on three different instances, with six replicates per strain. Comparing the results of both observers, an intraclass correlation coefficient (ICC) of 0.99 (P<0.001) was observed, indicating a high level of agreement between the observers. We compared the MIC results of each observer from three different days as a measure of repeatability. An ICC of 0.89 (P<0.001) was calculated for the first observer and an ICC of 0.93 (P<0.001) for the second observer. These values demonstrate a high repeatability of the test results. The mean CoV for both observers was 11.05% (median CoV=0%), demonstrating a high degree of repeatability and precision for each experiment.

Performance of commercial AST methods

The performance of each method relative to the reference BMD method is presented in Table 1 and the MIC values resulting from each AST method in Table S3(a). The minimum requirements that each testing method must meet in order to be recommended for diagnostic use according to the CLSI have been used to measure the results (CA ≥90%, EA ≥90%, MD ≤3.0% and VMD ≤1.5%).

Table 1: The performance values of each AST method compared to the reference BMD method.

AST Method	CA	EA[1]	MD[2]	VMD[3]	Spearman's *r*
	% (n)	% (n)	% (n)	% (n)	
Etest (24hrs)	84.0 (110/131)	70.5 (91/129)	0.0 (0/100)	67.7 (21/31)	0.478
Etest (48hrs)	84.7 (111/131)	72.0 (93/129)	1.0 (1/100)	61.3 (19/31)	0.470
MICRONAUT-S	94.7 (124/131)	86.7 (111/128)	5.0 (5/100)	6.5 (2/31)	0.731
SensiTest	93.9 (123/131)	88.0(102/116)	6.0 (6/100)	6.5 (2/31)	0.692
Sensititre	96.9 (127/131)	90.7 (117/129)	2.0 (2/100)	6.5 (2/31)	0.728
Rapid Polymyxin Pseudomonas[4]	92.4 (121/131)	N/A	9.0 (9/100)	3.2 (1/31)	N/A

Table 1: AST: Antimicrobial susceptibility testing. BMD: Broth microdilution. CA: Categorical Agreement, EA: Essential Agreement, VMD: Very Major Discrepancy.

[1]EA rate for each testing method was adjusted to account for the MIC range that is available on the testing panel. For example, the MICRONAUT-S test is able to evaluate MICs between 0.025 and 64mg/L, and so only isolates in this range are included in the evaluation for this category. [2]The MD is calculated as a percentage relative to the BMD testing results, with the number of susceptible isolates as the denominator. [3]The VMD is calculated as a percentage relative to the BMD testing results, with the number of resistant isolates as the denominator. [4]The Rapid Polymyxin Pseudomonas was not included in evaluation of EA and Spearman's rank correlation due to limitations in its testing range (2-8mg/L). All Spearman values had a p value < 0.0001.

The correlations between Etest at both 24 and 48 h timepoints and the BMD are significantly lower than between other AST methods and BMD (Spearman's r=0.478 and 0.470, respectively). Etests also failed to achieve the ≥90% standard required for CA, with 84.0% at 24 h and 84.7% at 48 h. The EA rate was 70.5%–72.0% for both 24 and 48 h timepoints. The VMD rate was 67.7% and 61.3%, at 24 and 48 h respectively, indicating that Etests are unable to recognize resistant isolates. There were no MD errors for the Etest at 24 h, and at 48 h the MD rate was 1.0%. The Etest had one of the lowest hands-on times, with 25 min taken on average to test five isolates (Table S4).

Of the commercial BMD methods, the Sensititre had the strongest results, achieving the CLSI requirements for three out of four categories: CA, EA and MD. The results of this method correlated well with the BMD (Spearman's r=0.728). The MICRONAUT-S achieved 94.7% CA. However, it failed to meet the CLSI requirements in other areas, particularly EA (86.7%) and VMD (6.5%) (Spearman's r=0.731). The SensiTest had an EA rate of 88%, with 102/116 isolates within a 2-fold dilution of the BMD results (Spearman's r=0.692). The VMD rate was 6.5%.

The Rapid PP achieved a CA rate of 92.4%, categorizing 121/131 isolates correctly. Although it recorded the highest number of false-positive results (MD of 9.0%), it only showed one VMD. The hands-on time for this test was 25 min to test five isolates, including preparation time (Table S4). Most notably, test results were available to read after 4 h, compared with 18–24 h for all other tests.

Table 2: The performance values of AST methods compared to reference BMD method using different resistance levels.

AST Method	Category								
	CA (%)			EA (%)			VMD (%)		
	Resistance level			Resistance Level			Resistance Level		
	Low (4 - 8mg/L) *n* = 10	Medium (16 - 64mg/L) *n* = 18	High (128 - 512mg/L) *n* = 3	Low (4 - 8mg/L) *n* = 10	Medium (16 - 64mg/L) *n* = 18	High (128 - 512mg/L) *n* = 3	Low (4 - 8mg/L) *n* = 10	Medium (16 - 64mg/L) *n* = 18	High (128 - 512mg/L) *n* = 3
Etest (24hrs)*	20.0 (2)	27.8 (5)	100 (3)	10.0 (1)	5.6 (1)	N/A	80.0 (8)	72.2 (13)	0.0 (0)
Etest (48hrs)*	30.0 (3)	33.3 (6)	100 (3)	10.0 (1)	5.6 (1)	N/A	70.0 (7)	66.7 (12)	0.0 (0)
MICRONAUT - S*	80.0 (8)	100 (18)	100 (3)	50.0 (5)	72.2 (13)	N/A	20.0 (2)	0.0 (0)	0.0 (0)
SensiTest**	80.0 (8)	100 (18)	100 (3)	50.0 (5)	N/A	N/A	20.0 (2)	0.0 (0)	0.0 (0)
Sensititre*	80.0 (8)	100 (18)	100 (3)	70.0 (7)	72.2 (13)	N/A	20.0 (2)	0.0 (0)	0.0 (0)
Rapid Polymyxin Pseudomonas	90.0 (9)	100 (18)	100 (3)	N/A	N/A	N/A	10.0 (1)	0.0 (0)	0.0 (0)

Table 2: BMD: Broth microdilution. AST: Antimicrobial susceptibility testing. CA: Categorical Agreement, EA: Essential Agreement, VMD: Very Major Discrepancy. N/A: not applicable. *The Etest, MICRONAUT-S and Sensititre tests were excluded from the high resistance division, due to the maximum MIC measurement being 256mg/L, 64mg/L and 128mg/L respectively. **The SensiTest was excluded from the medium and high resistance divisions for EA, as the highest MIC that can be measured is 16mg/L. ***The Rapid Polymyxin Pseudomonas was excluded from EA calculations due to the limited MIC range that it measures (2-8mg/L).
n = total number of isolates within the range of resistance being measured. The concordance between AST methods and the BMD in each category is presented as a percentage. The values in brackets represent number of isolates that agree with the BMD.

Evaluation of AST methods with different levels of resistance

To further understand the subtleties between results of the testing methods, we divided the MICs of resistant isolates into three resistance levels: low (4–8 mg/L), medium (16–64 mg/L) and high (128–512 mg/L), as presented in Table 2. The Etest (24 h) only categorized 2/10 isolates correctly in the low-resistance division. The CA rate for the other tests in the low-resistance division fell to 80% (also below the CLSI minimum), except for Rapid PP, which achieved 90%. The EA rate dropped for the SensiTest in the low-resistance division compared with the total isolates, achieving 50%, compared with 88.0% for all isolates combined. This pattern was replicated for the two other commercial BMD products: the MICRONAUT-S and Sensititre achieved EA rates of 86.7% and 90.7% for all isolates, which decreased to 50% and 70%, respectively, in the low-resistance division. The VMD rate increased significantly when isolates with low resistance were tested: the MICRONAUT-S, SensiTest and Sensititre yielded a 20% VMD rate. The Rapid PP achieved the lowest VMD rate, at 10% (1/10). There were no VMD errors, and a 100% CA rate, for commercial BMD methods using medium-resistance isolates.

The isolates were also divided into susceptible and resistant isolates (Table S5). In the susceptible category, the Etest achieved a CA rate of 100% agreement with the BMD at 24 h. Etests at 48 h had a CA rate of 99%. With the resistant isolates, however, the CA rate decreased to 38.7%, further supporting the view that Etests are unable to discriminate between resistant and susceptible isolates. The commercial BMD tests achieved similar results with susceptible and resistant isolates, with a 91%–98% CA rate, with the Sensititre reporting the highest value (98%) when evaluated with susceptible isolates.

Discussion

Due to the semi-automated cultivation procedure of the morbidostat, we were able to acquire 31 colistin-resistant strains with low effort. This is a collection that might take even reference centres and large laboratories years to achieve, whereas in our case all strains achieved various levels of resistance within 58 days. Apart from colistin-resistant strains at various levels of resistance, we were also able to select strains with MICs in the upper range of susceptibility. This enabled us to measure the performance of AST methods in terms of categorization of isolates for all relevant MIC ranges.

One crucial factor to ensure optimal patient outcome and to reduce the spread of resistance is accurate colistin susceptibility testing methods. Despite all of the commercial AST methods except the Etest gaining CA values >90%, none of them met the CLSI recommendations in all categories[52] most notably for the critical VMD criteria. This was particularly the case for low-level colistin-resistant isolates, for which generally the test performance of all methods decreased significantly.

The Etest performed poorly compared with other AST methods, failing to reach the required CLSI minimum in three out of four categories (CA, EA and VMD), which may be owing to reduced diffusion of colistin through the agar. This pattern is replicated in other studies: in 2015, Dafopoulou *et al*[53] found colistin Etests to have a VMD rate of 39.3% using *Klebsiella pneumoniae* and *Acinetobacter baumannii* strains. A more recent study by Chew *et al*[54] in 2017 found a VMD of 12%, measured with Enterobacteriaceae. In this study, the CA and EA of the Etest at 24 h were 84% and 70.5%, respectively, with a VMD rate of 67.7%.

Commercial BMD methods show stronger correlation with the reference BMD method compared with Etests. The Sensititre achieved the best results in our study, meeting and exceeding the CLSI requirements in three out of four categories (CA, EA and MD). However, the VMD rate exceeded the limit indicated by the CLSI (6.5%). The Sensititre has been reported to have strong results in other studies with Gram-negative bacilli[55–57] with Matuschek *et al*[57] in particular reporting a 100% EA rate for both Sensititre and MICRONAUT-S tests; although their study included only 21 isolates of *P. aeruginosa*. Another study compared Sensititre with the reference BMD and found that it had a CA of 90.1% and EA of 89.5%, using

strains of Enterobacteriaceae[54]. Their VMD rate was 4%, with one isolate out of 30 categorized incorrectly as susceptible.

Within diagnostics, it is important to note the role of testing capacity. Of the commercial BMD methods, the hands-on time for the Rapid PP was one of the shortest at 25 min to test one isolate with the shortest time to result (4 h), compared with 18–24 h for other AST methods, meaning that test results can be read on the same day of testing. However, the MICRONAUT-S has an MIC evaluation panel of up to 64 mg/L, making it appropriate for many laboratories and clinics, with a similar hands-on time.

Reviewing the numerous studies that have compared AST methods, it is difficult to generate any conclusions, as the reference methods used have varied, as well as the calculation methods for the categories[55–58]. A warning against gradient tests was issued by EUCAST in 2016[41], and so only studies that use the recommended BMD method as a reference should be applied to clinical outcomes. This study uses the highest number to date of both susceptible and resistant *P. aeruginosa* isolates, a pathogen of critical status, in combination with colistin, a last-resort antibiotic. The results of the study shed light on the problem areas of AST methods, namely with isolates around the susceptibility breakpoint and at the lower end of the resistance scale.

Acknowledgements

We thank Bianca Do¨ßelmann and Nadine Hoffmann for their contribution to our strain collection. We also extend our gratitude to Prof. Andreas Peschel and his team for their continuous support and guidance.

Funding

The work was supported by the German Center for Infection Research (grant number: TTU 08.702).

Transparency declarations

None to declare.

Supplementary data

Tables S1 to S5 appear as Supplementary data at JAC Online.

Transcriptomic basis of serum resistance and virulence related traits in XDR *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device

Mumina Javed[1,2], Benedikt Jentzsch[1,2], Maximilian Heinrich[1,2], Viola Ueltzhoeffer[1], Silke Peter[1,2], Ulrich Schoppmeier[1], Angel Angelov[3], Sandra Schwarz[1], Matthias Willmann,[1,2,4]

[1]Interfaculty Institute of Microbiology and Infection Medicine Tübingen, Institute of Medical Microbiology and Hygiene, Tübingen, Germany.

[2]German Center for Infection Research (DZIF), partner site Tübingen, Tübingen, Germany

[3]NGS Competence Center Tübingen (NCCT), Tübingen, Germany

[4] Eurofins MVZ Medizinisches Labor Gelsenkirchen, Gelsenkirchen, Germany

Frontiers in Microbiology, accepted for publication in December 2020

Abstract

Colistin is a last resort antibiotic against the critical status pathogen *Pseudomonas aeruginosa*. Virulence and related traits such as biofilm formation and serum resistance after exposure to sub-inhibitory levels of colistin have been underexplored.

We cultivated *P. aeruginosa* in a semi-automated morbidostat device with colistin, metronidazole and a combination of the two antibiotics for 21 days, and completed RNA-Seq to uncover the transcriptional changes over time. Strains became resistant to colistin within this time period. Colistin-resistant strains show significantly increased biofilm formation: the cell density in biofilm increases under exposure to colistin, while the addition of metronidazole can remove this effect.

After seven days of colistin exposure, strains develop an ability to grow in serum, suggesting that colistin drives bacterial modifications conferring a protective effect from serum complement factors. Of note, strains exposed to colistin showed a decrease in virulence, when measured using the *Galleria mellonella* infection model. These phenotypic changes were characterised by a series of differential gene expression changes, particularly those related to LPS modifications, spermidine synthesis (via *speH* and *speE*) and the major stress response regulator *rpoS*.

Our results suggest a clinically important bacterial evolution under sub-lethal antibiotic concentration leading to potential for significant changes in the clinical course of infection.

Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic bacterium and a frequent cause of healthcare acquired infections (HAIs). It belongs to the group of ESKAPE pathogens, which consist of six microorganisms, namely *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp*,* with a high tendency for causing challenging, drug-resistant, nosocomial infections[59]. *P. aeruginosa* accounts for up to 15.4% of bloodstream infections (BSIs) in intensive care units (ICUs) in Western Europe [60], causing high mortality rates.

One of the drugs of last resort against multi drug resistant (MDR) strains of *P. aeruginosa* is colistin. Colistin has a bactericidal effect: as a cationic cyclic peptide, it is able to bind to anionic lipopolysaccharide (LPS) modules and displace Ca2+ and Mg2+ from the outer cell membrane of *P. aeruginosa*, leading to disruption in the permeability of the membrane, leaking of cell contents, and cell death [61]. Although the spread of colistin resistance is not critical to date, resistance has emerged in some instances worldwide, particularly with the increased reliance on colistin for treating multidrug-resistant Gram-negative bacterial infections [62]. It is crucial that new measures are taken to prevent the development of resistance against this last resort drug, including using microbial evolution experiments to uncover the molecular basis of adaptive evolution. Antibiotics are often given in combination in standard therapeutic regimens, with metronidazole being a common drug partner for the treatment of infections by obligate and facultative anaerobic bacteria. It is effective for the management of intra-abdominal infections, gynaecological infections, septicaemia, endocarditis, bone and joint infections, amongst several other types of infections[63]. Metronidazole in treatment inhibits DNA synthesis and DNA damage by oxidation, causing single-strand and double-strand breaks that lead to DNA degradation and cell death. Metronidazole is activated when reduced, with molecules binding non-specifically to bacterial DNA, inactivating the DNA and key enzymes of the pathogen; leading to a high level of DNA breakage [64]. An interesting study found that despite metronidazole having no bactericidal effect on *P. aeruginosa*, *in vitro* exposure to a therapeutic concentration of metronidazole increased the number of mutations through induction of the SOS response, thus leading to emergence of antibiotic resistant bacteria [65]

Advances in next-generation sequencing of RNA have enabled the analysis of transcriptional changes that occur in bacteria when continuously grown *in vitro* in presence of sub-lethal doses of antibiotics[66,67]. These evolutionary studies have been extremely valuable in identifying genes and pathways that confer antibiotic resistance, in cases where this is mediated by chromosomal mutations. However, there is a significant lack of mass or high throughput evolutionary studies. In addition, how the transcriptional changes under antibiotic pressure and combination regimens affect other clinically relevant phenotypes remains poorly explored. Such changes may alter the manifestation and severity of the infection as well as the response to treatment and involve factors such as biofilm formation, immune response evasion through serum resistance and virulence.

The ability of *P. aeruginosa* to form biofilms is associated with severe infections and significant morbidity and mortality[68]. Biofilms provide *P. aeruginosa* an enormous advantage by promoting survival on medical devices such as catheters, evasion from the immune system, and tolerance to antimicrobial therapy. Increased biofilm formation has also been found to contribute to increased virulence[69,70]. Other virulence factors of *P. aeruginosa* include, amongst others, toxins, exoproteases, phospholipases, the presence of type IV pili and flagella[71]. Serum contains more than thirty proteins of the complement system, and is a crucial component of the host innate immune response which can also initiate the adaptive response. The ability to inhibit complement activation is also considered a virulence trait. However, besides few examples of pathogenic bacteria that carry "*ad hoc*" molecules to block the activity of the complement[72,73], very little is known about the molecular basis of increased serum resistance and the clinical ramifications overall in *P. aeruginosa*.

We performed the present work using a morbidostat. A morbidostat is a semi-automated culturing device that continuously monitors bacterial growth and adjusts antibiotic concentration to induce bacterial resistance against the drug. In combination with 'omic' approaches, experiments using a morbidostat can shed light on the different evolutionary trajectories of antibiotic resistance development as well as further phenotypic modification of clinical relevance. Our previous work with this device concluded that selection for colistin tolerance results in a rise of mutations in *pmrB* and *pmrE* in the clinical strain *P. aeruginosa* PA77[34], which are common in other clinical isolates. Here we used the same clinical strain, derived from bloodstream infections and cultured it in our morbidostat for 21 days, under three experimental conditions: no antibiotic, single drug (colistin or metronidazole), and a combination of the two antibiotics. At four key time-points we measured the impact of antibiotic exposure on clinically relevant phenotypes such as antibiotic resistance, biofilm formation, serum resistance and virulence. This is the first study to examine the evolution of an extensively drug resistant (XDR) *P. aeruginosa* strain in a morbidostat device under strong antibiotic pressure, and to demonstrate that the acquisition of colistin tolerance can affect phenotypic traits generally associated with virulence.

Methods

Strain selection: characteristics of PA77

The *P. aeruginosa* clinical strain PA77 was isolated at the University Hospital Tübingen, Germany [74]. PA77 is extensively drug resistant (XDR)[46], being non-susceptible to all antibiotics except colistin and fosfomycin. Multilocus sequence typing analysis show PA77 belongs to the high-risk sequence type (ST) ST308[74].

Study design and experimental conditions

The morbidostat system was built following the detailed instructions by Toprak et al[33]. with the modifications outlined in previous work[34]. As part of our hygiene protocol, we ran 80%

ethanol through the tubing system and 3% sodium hypochlorite for 30mins each, and distilled water was used to rinse the tubing after each solution. The biological waste from the morbidostat was fed into a container with a neutralising solution.

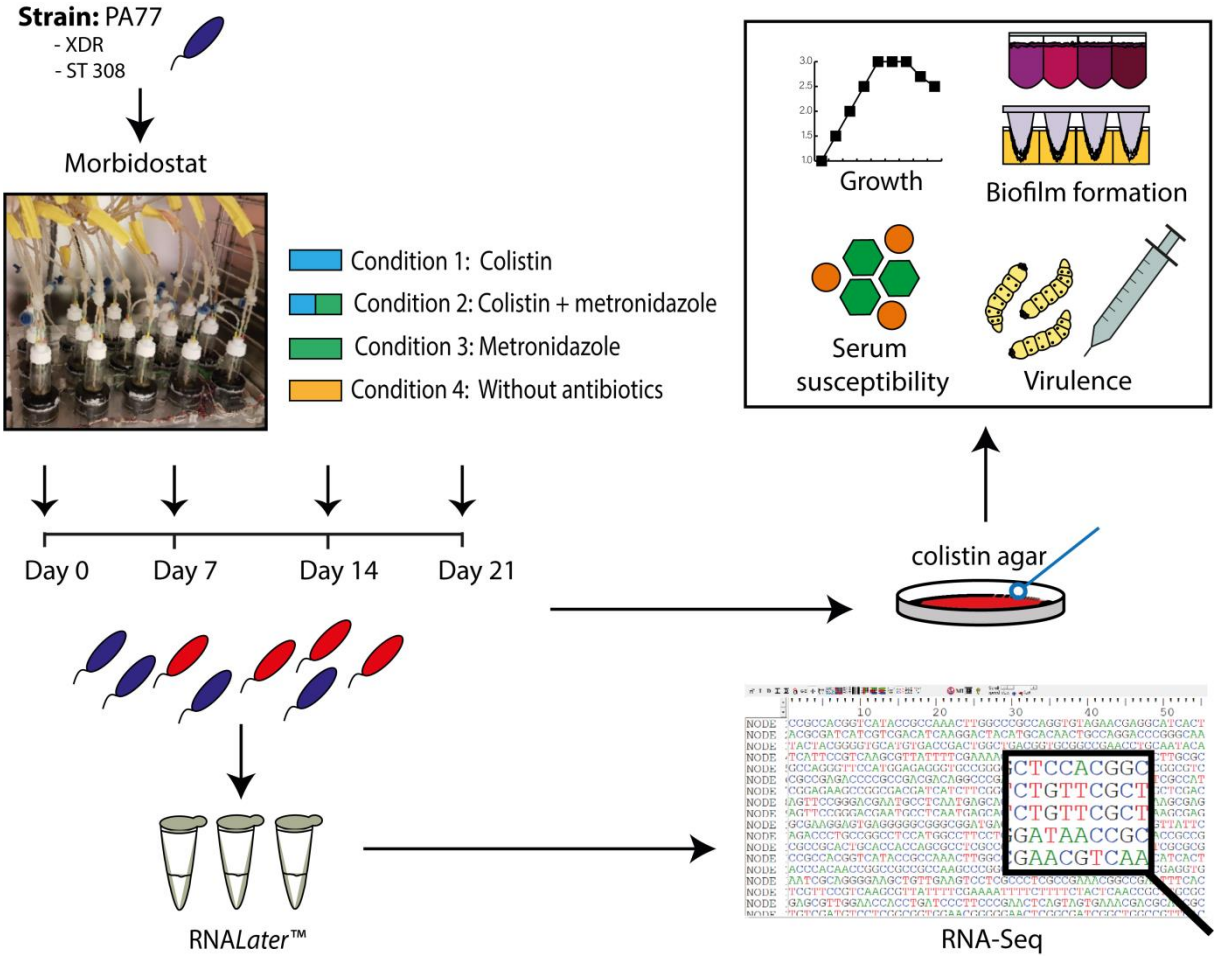


Figure 1: Experimental set up presented as a flow chart. The XDR strain PA77, ST 308, was cultivated in the morbidostat in 20 ml LB medium under four drug conditions: 2-500mg/L colistin only (Condition 1), a combination of 2-500mg/L colistin and 50 mg/L metronidazole (Condition 2) and 50 mg/L metronidazole only (Condition 3). A control condition without antibiotics was also completed (Condition 4). Samples of the isolates were taken every 2-3 days, with Day 0, 7, 14 and 21 selected as the key time-points of interest for our study. Isolates generated from the morbidostat were stored in RNA*Later* Stabilization Reagent (Qiagen, Hilden, Germany) to preserve the integrity and genetic profile of the bacteria. Finally, 36 isolates were generated in culture: from four different drug conditions, with three isolates per condition and three experimental time-points. The baseline strain PA77 is considered the Day 0 isolate. XDR: extensively drug resistant. ST: sequencing type. Blue isolates: susceptible to colistin (≤ 2 mg/L). Red isolates: colistin-resistant (> 2 mg/L).

Figure 1 demonstrates an overview of the study design. Strain PA77 was continuously cultivated for 21 days in the morbidostat in four conditions. The first condition was with colistin as a single drug starting with 2 mg/L and with a final concentration of 500 mg/L. The second single drug condition is with 50mg/L of metronidazole, and the third condition is with a combination of increasing colistin and 50mg/L metronidazole. The fourth condition was completed as a control, with plain LB medium and no antibiotics. For every condition we ran three replicates in different vials in the morbidostat to investigate whether evolutionary trajectories were stable in all strains. We took samples of the culture at 7, 14 and 21 days of drug exposure, which means collectively there were three replicates over three time points, in four conditions. This totals 37 strains including the baseline strain of PA77 being the original clinical strain not evolved in the morbidostat. All strains were assessed in phenotypic and transcriptomics assays. These samples were processed with RNA*Later* Stabilization Reagent (Qiagen, Hilden, Germany) and ultimately frozen via the Microbank system (Pro-Lab Diagnostics Inc, Texas, USA) at -80°C. All phenotypic assays were performed with one bead taken from these frozen stocks and grown overnight on blood agar plates containing 2mg/L or 16mg/L colistin. This was to ensure a truly resistant population, devoid of persister cells or dormant bacteria.

DNA extraction, library preparation and genome sequencing for baseline strain PA77

The genome sequencing was performed at the NGS Competence Center Tübingen (NCCT) using both short (Illumina) and long Oxford Nanopore Technology (ONT) reads for the baseline strain PA77. For ONT, genomic DNA was isolated using Qiagen Genomic Tip G/20, the integrity and quality of the preparations were evaluated on a Femto Pulse capillary electrophoresis device (Agilent, Santa Clara, United States). Library preparation was performed with Ligation Sequencing Kit (SQK-LSK109) with Native Barcoding Expansion (NBD104). The pooled libraries were sequenced on a MinION flow cell (FLO-MIN106D) for 48 hours. For short read sequencing, the DNA was extracted with the DNeasy UltraClean Microbial Kit (Qiagen). Illumina sequencing libraries were prepared using Nextera DNA Flex library prep kit (Illumina), using combinatorial dual indexing. The Illumina libraries were pooled and sequenced on a NextSeq 500 High-Output flow cell with 2 x 150 bp.

De novo *genome assembly and annotation for baseline strain PA77*

Whole genome assembly of the baseline strain was performed using the hybrid mode of Unicycler v.048[75]. Standard Unicycler parameters were used.

RNA*Later storage*

Samples were taken from the morbidostat every 2-3 days and stored in *RNA*Later RNA Stabilization Reagent (Qiagen, Hilden, Germany) to preserve the integrity and genetic profile of the isolates. Up to 10^8 cells were centrifuged at 12,000 x g for 2 min. The supernatant was removed and the pellet resuspended in 1 ml of *RNA*Later solution and incubated for 1 hr at

room temperature. The pellet was centrifuged once more at 12,000 x g for 5 min, the supernatant removed and the remaining pellet was frozen at -80°C.

RNA sequencing

Total RNA was extracted with the Quick RNA Fungal/Bacterial Kit (Zymo Research), using lysozyme for cell lysis (0.4 mg/ml). The integrity and quality of the RNA preparations was evaluated with agarose gel electrophoresis and Agilent Bioanalyzer 2100 assays. Total RNAseq Library Kit (Zymo Research) was used for both ribosomal RNA depletion and RNAseq library preparation, using 500ng total RNA as input. We constructed three libraries per RNA sample. The libraries were sequenced on a NextSeq 500 High-Output flow cell (75 cycles, single reads).

Colistin susceptibility of PA77 and isolates cultivated in morbidostat

After comparison against broth microdilution (BMD) and other commercial tests with positive results[76], antibiotic susceptibility testing was determined using MICRONAUT MIC-Strip (MERLIN Diagnostika Gmbh, Bornheim, Germany): a commercial broth microdilution system using the international reference methodology (ISO 20776-1) and completed according to the manufacturer's instructions. Briefly, isolates were taken from -80°C stocks and grown on plain agar plates overnight. An inoculum of 0.5 McFarland was prepared in 5ml NaCl, and then further diluted 1:200 in Mueller Hinton broth. 100µl of this dilution was pipetted into MIC-Strips which contained freeze dried colistin ranging from 0.0625 to 64mg/L. The strips were incubated for 18-24hrs at 37°C, and results read visually.

Growth assays

Isolates were grown overnight on selective colistin blood agar plates containing 2 mg/L or 16 mg/L colistin. A subculture was made to optical density (OD_{600nm}) 0.1 in LB medium. 200 µl of this subculture was transferred to sterile, flat-bottomed, polystyrene 96-well microtiter plates. The growth rate of bacteria was determined by incubating the plate at 37°C, measuring the OD of the culture in each well at 600 nm at 30 min intervals for 24 hrs using a microtiter plate reader (SPECTRAmaxPLUS384 Molecular Devices Inc., USA), and growth curves generated.

Biofilm formation assay with peg lid device

Isolates from −80°C storage were grown overnight on sheep blood agar plates containing 2 mg/L or 16 mg/L of colistin. After dilution of this culture to 0.5 McFarland in LB medium, 200µl was transferred to all but the negative control wells of a flat-bottom 96-well microtiter plate (Nalgene Nunc International, Rochester, USA) Polystyrene microtiter lids were immersed into this subculture (Nalgene Nunc International, Rochester, USA) and incubated aerobically at 37°C for 2 hrs, followed by incubation at 37°C for 20-22 hrs anaerobically with Anaerocult tabs (Merck & Co, N.J, USA). The peg lids were dipped in 0.9% NaCl three times for 10sec each to remove planktonic bacteria, and sonicated in an ultrasonic bath (Sonorex™ RK100) for 10 mins. The peg lid is then placed in a mixture of 75 µl CHAPS and 75 µl EDTA solution on a

rocking table (20 Hz) for one hour, and the number of viable CFU per ml was determined by serial dilution plating, and counting colonies after 20-24 hrs incubation at 37°C.

Biofilm density quantification with crystal violet staining

Isolates from −80°C storage were grown overnight on sheep blood agar plates containing 2mg/L or 16mg/L of colistin. After dilution of this culture to 0.5 McFarland in LB medium, 200µl was transferred to all but the negative control wells of a flat-bottom 96-well microtiter plate (Nalgene Nunc International, Rochester, USA) and incubated aerobically at 37°C for 24hrs. Planktonic cells were stained with an aqueous solution of 0.1% crystal violet for 30 min. The excess crystal violet was discarded, and wells were rinsed with distilled water. Stained biofilms were resuspended in 5% acetic acid for 30mins, and absorbance was measured at 590 nm by a microtiter plate reader. Assays were performed in triplicate in three independent experiments, with standard deviations indicated.

Serum selection

Normal human serum (NHS) from five healthy donors (Department of Transfusion Medicine, University Hospital Tübingen) was stored in aliquots at −80°C. The optimum serum dilution was determined for the baseline strain PA77. Normal human serum (NHS) from healthy donors (Department of Transfusion Medicine, University Hospital Tübingen) was stored in aliquots at −80°C. Strain PA77 was incubated with a percentage of serum, ranging from 10% to 90%. Serum was diluted with PBS (Gibco, Gaithersburg, USA). They were then incubated with a luciferase compound at 37°C for 0, 2 and 4 hrs and the luminescence of ATP produced in culture measured as an indication of growth. It was determined that 50% was the optimum concentration of serum to use for this study.

Serum killing assay

Serum killing assays were completed with BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, USA) as described [77]. Normal human serum (NHS) from healthy donors (Department of Transfusion Medicine, University Hospital Tübingen) was stored in aliquots at −80°C. Heat inactivated serum (HIS) was generated by incubating the serum at 56°C for 30 min.

Overnight culture of bacteria was diluted to OD 600nm 0.1 and subcultured for 1hr. Strains were incubated at 37°C in 100µl HIS- or NHS-PBS in a 96 well V-bottom microtiter plate (Greiner bio-one, Frickenhausen, Germany) in triplicates for 0, 2, 4, and 6hrs. After incubation, plates were centrifuged at 3,500 × g for 5 min and the pelleted bacteria were resuspended in 100 µl PBS. To determine the number of viable bacterial cells, 50µl bacterial suspension and 50 µl BacTiter-Glo™ reagent were transferred to a white LUMITRAC™ 96 well F-bottom microtiter plate (Greiner bio-one, Frickenhausen, Germany) and the adenosine triphosphate (ATP) levels produced by the bacteria were quantified with a Tecan Infinite® 200 PRO. Resulting luminescence values were log transformed (natural logarithm) and the linear regression coefficients of the resulting growth curves in serum (log luminescence over time)

were used to calculate a coefficient difference (CD). Here, the regression coefficient of the growth in HIS (αHIS) was subtracted from the regression coefficient of growth in NHS (αNHS).

CD = αNHS - αHIS

Growth in HIS was considered a growth control, hence the coefficient needed to be positive to be considered an adequate experimental setup. A CD value below 0 indicates either effective killing of bacteria by the serum or growth inhibition by the serum. Serum killing happened when the NHS coefficient was negative. Higher negative values would generally indicate a stronger impact of the serum on the bacteria; lower negative values indicate the development of resistance to serum. CD values ≥ 0 indicates no effect of the serum on growth, meaning a resistance to serum.

In vivo *virulence assays*

Galleria mellonella larvae of the same size and weight range were purchased from Biosystems Technology (TruLarv™). Subcultured bacteria were serially diluted in PBS to 8-10 CFU. Each *G. mellonella* larvae was injected with 10µl of 8-10 CFU bacterial dilution using a 30 gauge syringe (BD Biosciences, Allschwil, Switzerland). The larvae were then incubated at 37°C and monitored for 36hrs after infection, and the death events were recorded every 2hrs. Death was defined as when the larvae stopped responding to touch. Ten microliter aliquots of the bacterial dilutions injected into the larvae were plated in triplicates on LB agar plate and the CFU was determined after overnight incubation to ensure that the injected inoculum was in the 8-10 CFU range. A hazard ratio (HR) was calculated with a Cox proportional hazard model (using Stata version 12.1), factoring in the CFU injected into each larvae.

Identification of differentially expressed genes with Geneious Prime

We used Geneious Prime (version 2020.0.5) to analyse and visualise the transcriptomic sequencing data. Reads from morbidostat-generated isolates were pre-processed with Trimmomatics[78] aligned to the reference sequence PA77 using Bowtie2[79]. We used six replicates per isolate, with at least 10 mio high quality reads. Expression levels of each isolate were compared, and volcano plots generated with the plugin DESeq2[80]. The genes of interest were filtered by selecting the ratio of significance to 25 and a log2 fold expression of +/-4.

Statistical analysis

GraphPad PRISM 5 (GraphPad Software, San Diego California USA) and Stata version 12.1 (Stat Corp., College Station, USA) were used to perform null hypothesis testing. All error bars represent standard deviation. For each figure, the number of replicates and other information relevant for assessing the accuracy and precision of the measurements are included in the corresponding legend.

Results:

Characteristics of PA77 and morbidostat generated isolates from NGS

Hybrid assembly of the baseline strain PA77 resulted in two contigs with total length of 6.9 Mbp. The baseline strain was used as the template for differential gene expression analysis.

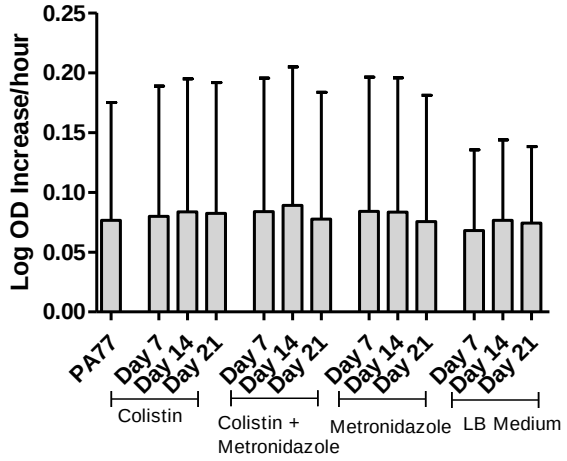


Figure 2: No changes in the growth rates of baseline strain and evolved isolates. Isolates were inoculated in plain LB medium and the optical density measured every 30mins at OD 600nm, during incubation at 37°C for 24hrs, with three replicates per isolate. The results are presented at Log OD increase per hour, with error bars representing the 95% confidence intervals

No changes in growth rates between baseline, control and experimental isolates

Isolates from each condition and key time points were cultured in plain LB medium and growth measured every 30mins via OD reader. The results are displayed as Log-OD increase per hour (Figure 2) and as growth curves in Figure S1. There was no difference in the log increase in OD between isolates, indicating that the growth kinetics of the evolved isolates were similar to those of the baseline strain.

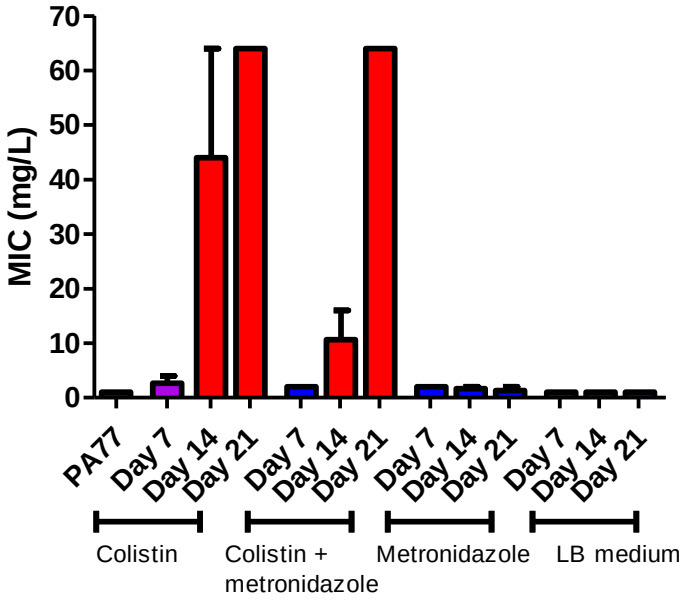


Figure 3: Measure of colistin sensitivity in baseline strain PA77 and evolved isolates. Distribution of the minimum inhibitory concentrations (MICs, mg/L) for colistin (n=37) with isolates cultivated in the morbidostat across four different conditions and samples at three time-points: seven, 14 and 21 days of cultivation. The results are presented as the mean of three biological replicates and range. Blue: indicates susceptibility to colistin (MIC ≤ 2mg/L). Red: indicates resistance to colistin (MIC > 2mg/L). Purple: indicates a mix of replicates that are both susceptible and resistance to colistin.

Resistance to colistin increases as exposure in morbidostat continues for up to 21 days

PA77 strains continuously exposed to colistin reached a colistin MIC >64 mg/L at Day 21. This is at least 16-fold higher than the clinical EUCAST breakpoint of colistin for *P. aeruginosa* at ≥ 2mg/L (EUCAST: Clinical breakpoints and dosing of antibiotics) (Figure 3). The metronidazole-only and LB medium control strains did not develop colistin resistance. The full MIC values are presented in Supplementary Table 1.

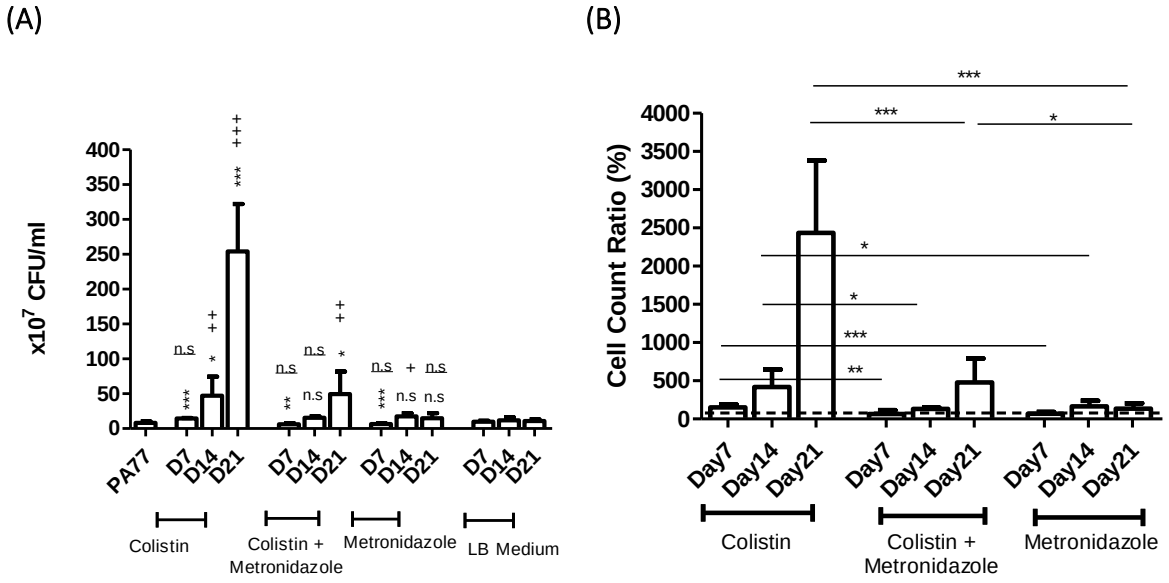


Figure 4: Altered number of viable cells in biofilms of the evolved isolates. (A): Measure of viable cells in biofilm produced by isolates cultivated in the morbidostat for 21 days in four different conditions: colistin only, a combination of colistin and metronidazole, metronidazole only and no antibiotic as a control condition. Six replicates per isolate were taken from the morbidostat and grown in LB medium for 24hrs and the resulting biofilm detached chemically and mechanically, serially diluted and plated on agar. The CFU was counted and recorded as values from 10^7. The statistical analysis shown are averages of at least three independent experiments ± sd. Comparison to the baseline strain PA77 is indicated by * : n.s, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Comparison to LB medium isolates is denoted using +. n.s, not significant; + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$. (B) The values in panel A were adjusted to a ratio relative to LB medium isolates and displayed here as a bar chart, with a *t*-test used to calculate the difference between the three experimental conditions at each time point. * $p < 0.05$, *** $p < 0.001$. The dashed line represents the values for the control isolates, which is set to 100%.

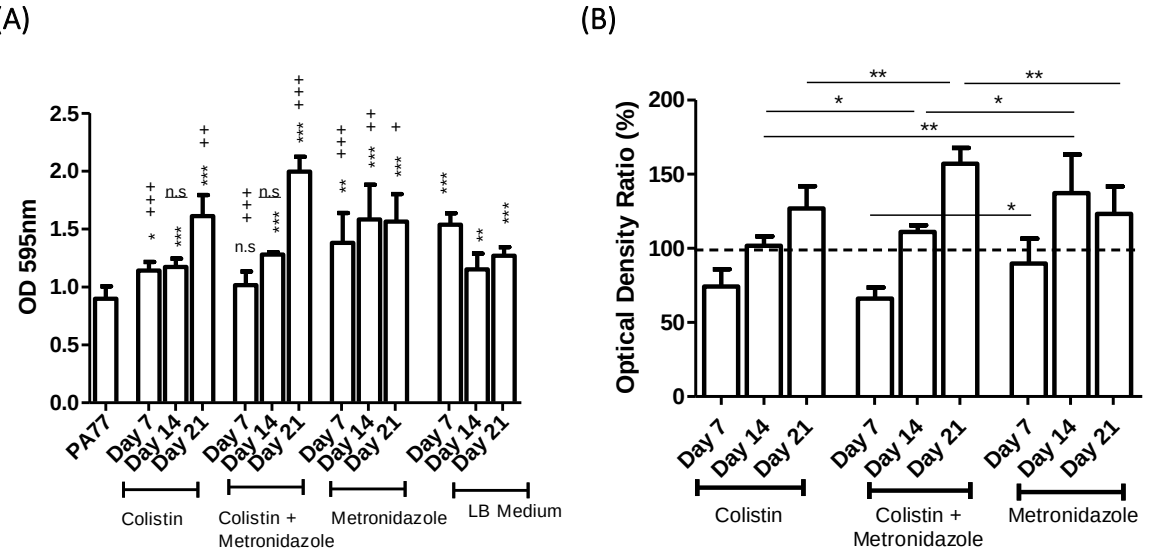


Figure 5: Changes in biomass in biofilms of the evolved isolates. (A): The OD values of biofilm staining assays using six replicates per isolate and the standard deviation are presented for strains cultivated in the morbidostat for 21 days in four different conditions: colistin only, a combination of colistin and metronidazole, metronidazole only and in LB medium as a control. The baseline strain PA77 was used as a Day 0 comparison. A *t*-test was used to compare the time-points to each other. Comparison to the baseline strain PA77 is indicated by *: n.s, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Comparison to LB medium isolates is denoted using +. n.s, not significant; + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$. (B) The values of the experiments in panel A have been adjusted as a ratio, relative to the values of the LB medium isolates and displayed in a bar chart as percentages. The three experimental conditions have been compared according to the time point, and a *t*-test was used to calculate the significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The dashed line represents the values for the control isolates, and is set to 100%.

Increase in number of viable cells, density and biomass in biofilm after colistin exposure

Next, we assessed whether the ability to form biofilms was altered under antibiotic exposure. Looking at the number of viable cells in biofilm, by far the biggest increase in development of biofilm occurred in the colistin only condition at Day 21, with strains producing 30-fold more viable cells in biofilm than the baseline strain ($p < 0.001$) (Fig 4A). The growth rate of the evolved isolates had not changed compared to the baseline (Fig 2), which indicates that the changes in biofilm are independent of the growth rate. All three time-points in this condition showed significantly increased biofilm production relative to the baseline. Looking deeper between drug conditions, we saw an increase in the number of viable cells in biofilm for isolates exposed to colistin for seven days, with 14.5 x 10^7 viable cells in biofilm compared to isolates exposed to metronidazole for the same time period with 6.7 x 10^7 viable cells in biofilm ($p < 0.001$), and in the combination drug condition (6.1 x 10^7 viable cells in biofilm) ($p < 0.05$) (Fig 4B).

Within 14 days of exposure to colistin as a single drug, there was a faster trajectory of increased biofilm formation than for isolates exposed to metronidazole only ($p < 0.05$) or in the combination of the two drugs ($p < 0.05$) (Fig 4B), which may indicate that metronidazole begins to affect the biofilm forming capabilities of the isolates. When compared to the baseline, there were a higher number of viable cells in biofilm in metronidazole-only isolates (17.5×10^7) ($p < 0.001$) at Day 14 than the other conditions at this time-point (Fig 4A). After 21 days of exposure, isolates from the combination drug condition had 5-fold more viable cells in biofilm than the baseline ($p < 0.01$), while the biofilm formed by metronidazole only isolates did not differ significantly. Between drug conditions, the metronidazole-only condition isolates showed 17-fold decrease in viable cells in biofilm after 21 days of exposure (14.7×10^7 viable cells in biofilm) compared to colistin-only isolates at Day 21 (254.1×10^7) ($p < 0.001$) (Fig 4B). The combination drug condition isolates also showed a slower trajectory of increased biofilm formation, with 80.5% less biofilm produced at Day 21 compared to the colistin-only condition ($p < 0.001$) (Fig.4B).

The metronidazole-only isolates showed a general increase in biomass in biofilm when compared to both the baseline strain and the control LB medium isolates (Fig 5A). The adjusted values showed isolates sampled at Day 7 in all three drug conditions show a decrease in biomass produced in biofilm compared to control isolates, with colistin-only isolates producing 76.2% of biomass in biofilm, and combination isolates producing 66.9% of biomass in biofilm. However, isolates cultivated in metronidazole only show an increase in biomass in biofilm compared to the two other drug conditions (Fig 5B). Isolates exposed to metronidazole for 14 days produced 26% more biomass in biofilm than isolates exposed to colistin and metronidazole at the same time point ($p < 0.05$) (Fig 5B). Isolates exposed to colistin for 14 days produced 36% less biomass in biofilm than isolates cultivated in metronidazole only ($p < 0.01$), and 10% less biomass in biofilm compared to combination drug isolates ($p < 0.05$). The isolates exposed to colistin and metronidazole for 21 days showed the highest increase in biomass in biofilm compared to the isolates cultured in the two other drug conditions, with 159% biomass measured, compared to 128.3% for colistin isolates ($p < 0.01$), and 124.6% biomass measured for metronidazole only isolates ($p < 0.01$).

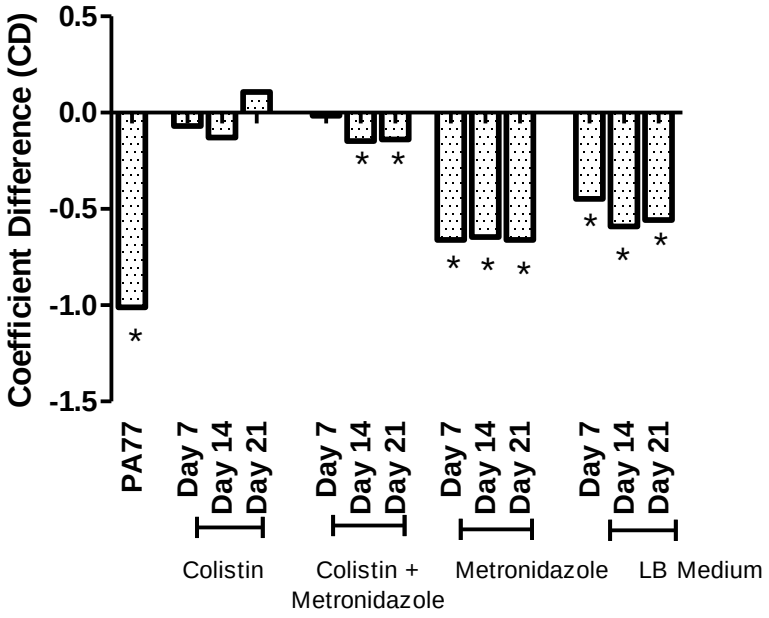


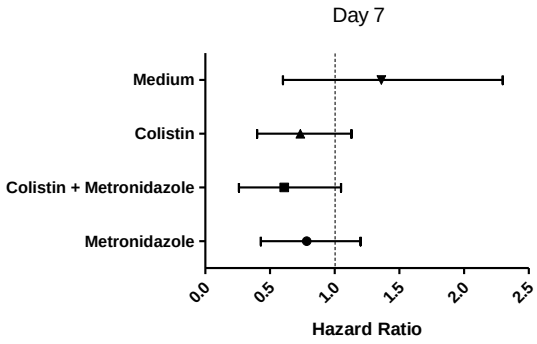
Figure 6: Transient decrease in susceptibility to serum in strains evolved in presence of colistin. Susceptibility to serum was measured by incubating *P. aeruginosa* isolates with 50% human serum and a luciferase compound, and measuring luminescence of ATP produced over 6hrs. The results are presented as a bar graph with coefficient difference values calculated using log regression. Isolates with a negative αNHS value, indicating that they are killed in serum, are marked with an asterisk (*). PA77: baseline strain. Colistin: colistin only. Colistin + metronidazole: a combination of colistin and 50mg/L metronidazole. Metronidazole: 50mg/L metronidazole. LB medium: control condition isolates where isolates were not exposed to antibiotics and only cultured in LB medium.

Isolates become resistant to complement factors in serum within seven days of colistin exposure

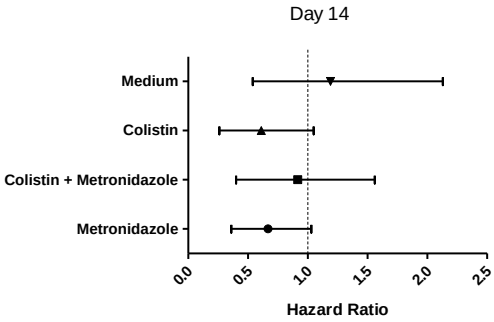
Next, we tested resistance of the evolved isolates to complement factors (Figure 6, Figure S1 A-M), with resistance being defined from the ability of these isolates to grow in human serum. The coefficient difference (CD) for the baseline strain is -1.01, which indicates a killing effect: the bacteria are not able to grow in 50% serum. However, after seven days of exposure to colistin in the morbidostat, the isolates have a CD of -0.07, which indicates development of resistance to complement factors in the same serum. At Day 14 of colistin exposure, isolates were still resistant to serum complement, but less than at Day 7 (CD = -0.129). At Day 21, we see the biggest increase in serum resistance, with a CD of 0.108.

In the combination drug condition, there was also a notable development to serum resistance by Day 7 with a CD of -0.01. At Day 14 and Day 21, strains became again slightly more susceptible to serum (CD -0.148 and -0.139 respectively). The metronidazole-only and control condition do not show a strong deviation from the baseline susceptibility to serum.

(A)



(B)



(C)

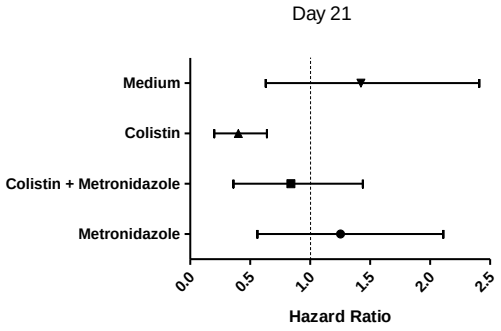


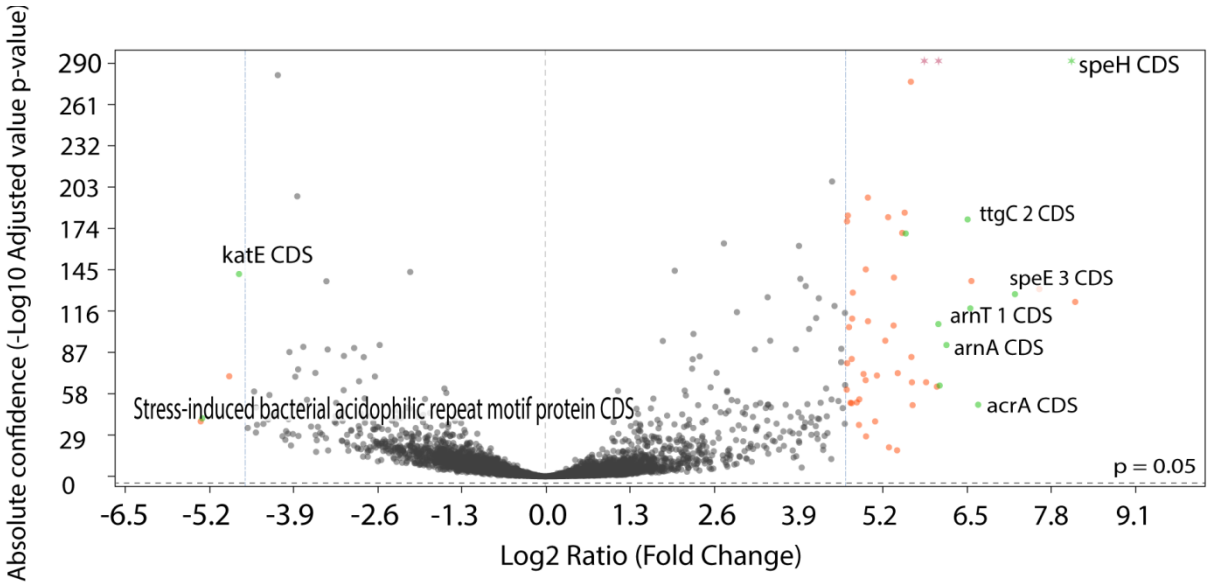
Figure 7: Prolonged exposure to colistin decreases virulence of *P. aeruginosa* PA77 in a *Galleria mellonella* model of infection. (A), (B), (C): The virulence potential of isolates was measured with *G. mellonella* larvae. The results are displayed as forest plots per time-point. The hazard ratio is calculated relative to the baseline condition strain PA77
Colistin (▲): colistin only. Colistin + metronidazole (■): a combination of colistin and 50mg/L metronidazole. Metronidazole (●): 50mg/L metronidazole. LB medium (▼): control condition isolates not exposed to antibiotics and only cultured in LB medium.
(A) Day 7: Compared to the baseline strain, there is no significant change in virulence potential of these strains. (B) Day 14: There is no significant effect on virulence potential in any of these conditions overall, relative to the baseline strain. (C) Day 21: The isolates exposed to colistin in the single-drug condition show a decrease in virulence relative to the baseline strain.

Decreased virulence in* G. mellonella *after infection with isolates exposed to colistin for 21 days

We examined the virulence of the evolved isolates in a *G. mellonella* model of infection [20] (Fig7 A-C, Figure S3 A-C). Seven days of single exposure to colistin did not lead to a significant change in virulence (HR = 0.75, lower 95% CI: 0.40, upper 95% CI: 1.13, p = 0.13). At Day 14 we still did not observe a significant deviation from baseline. However, at Day 21 we noted a significant attenuation of virulence potential compared to the baseline (HR = 0.36, lower 95%

CI: 0.20, upper 95% CI: 0.64, p = 0.01). This effect was not seen in the combination drug condition, with metronidazole seemingly having a modulatory effect on the impact of colistin exposure on virulence. Isolates cultured with metronidazole as a single-drug condition also demonstrated no statistically significant changes in virulence. This was the same for isolates in the control condition (LB medium only).

A)



B)

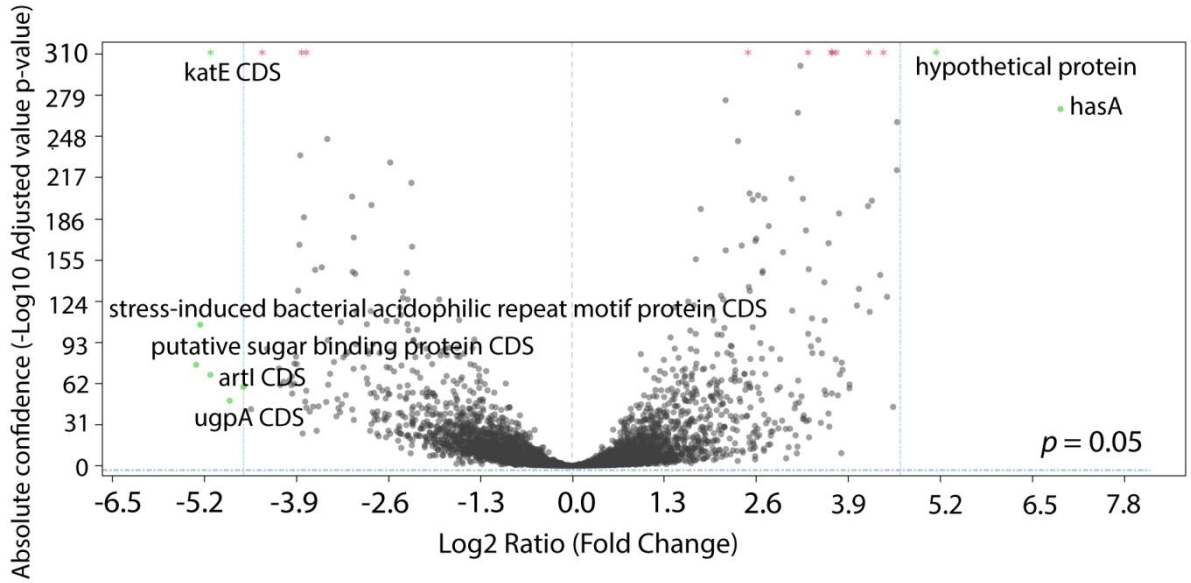


Figure 8: Comparison of differentially expressed genes between conditions. (A) and (B): The statistical significance and magnitude of change for the differentially expressed genes are depicted as volcano plots. Day 21 isolates are shown here, cultivated two conditions: (A) comparison between colistin only and baseline, and (B) comparison between control isolates grown in LB medium and baseline. The log2 fold change is plotted against the significance (-Log10 adjusted *p* value). The horizontal dashed line represents a ratio of significance, set at 0.05. Each point represents a gene, with genes of interest are highlighted in green and labelled. Genes that are within the significant interest threshold but unlabelled are orange. Genes that do not meet the criteria are coloured in black.

Significant variation in the transcriptomic profile of the evolved isolates compared to control isolates

To examine the transcriptional profile of the isolates that had been evolved under different drug pressures, we performed RNA-Seq analysis on *P. aeruginosa* PA77 at the baseline and on strains grown for 7, 14 and 21 days in presence of single or a combination of antibiotics. Due to the drastic changes in the phenotype of the morbidostat-generated strains, namely increased biofilm formation, decrease in virulence and loss of susceptibility to serum, we wanted to investigate general changes in the differentially expressed genes between the three time-points and four conditions (Figure 8 A and B and Figure S2 A-J). For differential gene expression, a threshold of log2 fold change (FC) of +/-4 was applied.

Among the genes that were upregulated include the *arnBCADTEF* operon (also known as *pmrHFIJKLME*), which has a crucial role in colistin resistance in *P. aeruginosa* through the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A[81]. The *arnBCADTEF* operon is upregulated in colistin within 21 days (Fig 8A), but not differentially expressed at a significant level in isolates exposed to LB medium (Fig 8B) and metronidazole only (Fig S2.J).

Two other gene transcripts, *speH* and *speE*, were significantly upregulated in the colistin only and combination drug conditions, as part of the operon *speEH-pmrAB*. The highest positive log fold change for *speE* occurred in colistin Day 21 isolates with 7.25 (differential absolute confidence 128.25, $p < 0.001$) (Fig 8A) followed by colistin and combination isolates after seven days of drug exposure: 6.83 (differential absolute confidence: 59.5, $p < 0.001$) and 5.95 (differential absolute confidence: 56.43, $p < 0.001$) respectively (Table S1). In contrast, *speE* was downregulated in the medium-control isolates, with a log FC of -0.29 at Day 7 (differential absolute confidence: -1.96, p value: 0.006), -0.08 at Day 14 (differential absolute confidence: -0.06, $p < 0.001$) and a log fold change of 1.18 at Day 21 (differential absolute confidence: 5.9, $p < 0.001$) (Table S1).

One gene of interest that was downregulated is *rpoS*. The highest fold change difference for this gene transcript in particular occurs after seven days of exposure to metronidazole, with a downregulation in expression at -5.74 (differential absolute confidence -95.99, $p < 0.001$) (Table S1), closely followed by isolates cultured in colistin for 14 days (log2 FC: -5.25,

differential absolute confidence: -129.11, $p < 0.001$) and isolates cultivated in a mix of colistin and metronidazole for seven days (log2 FC: -5.22, differential absolute confidence: -156.4, $p < 0.001$). Isolates in the medium-control condition fall below the threshold of ≤-4 log2 ratio FC, but are clearly downregulated none the less, ranging from log2 FC of -0.46 (LB medium Day 14) to -2.42 (LB medium Day 21) (Table S1).

Discussion

In this work, we used one XDR clinical strain of *P. aeruginosa* and cultured them in a morbidostat device with two antibiotics for 21 days, in order to discover the many ways the strain can evolve resistance in a simulated clinical setting. The aim of this work was to investigate the specific contribution of metronidazole alongside the development of colistin resistance in *P. aeruginosa* and evaluate the effect of antibiotic stress and on bacterial fitness, geno- and phenotype.

A number of assays were performed to elucidate the changes in the bacterial phenotype under distinct antibiotic pressure. Subinhibitory exposure of colistin has a clear effect in many of the factors that we have looked at: by the end of 21 days there was increased biofilm formation, a loss in susceptibility to serum and a decrease in virulence. Metronidazole seemingly contributes a modulatory effect on two of these factors: with less viable cells in biofilm and less susceptibility to serum complement factors in combination and metronidazole only condition isolates, as measured by quantifying the concentration of ATP in serum over time. Interestingly, isolates cultured in colistin, both as a single drug and in combination are unable to grow in either heat-inactivated serum or active serum at 6hrs, unlike isolates cultivated in metronidazole, which continue to grow in heat-inactivated serum. This indicates a developed sensitivity to heat-stable factors in serum as a result of exposure to sub-inhibitory concentrations of colistin.

Analysis of the transcriptomic profile uncovered a series of differential gene expression patterns. One gene of interest is *speE*, encoding a spermidine synthase, an enzyme that catalyzes the irreversible transfer of a propylamine group from the amino donor S-adenosylmethioninamine (decarboxy-AdoMet) to putrescine (1,4-diaminobutane) to yield spermidine. Polyamines such as spermidine are involved in several biological processes, including binding to nucleic acids, stabilizing membranes, and protecting against host immune responses[82]. Spermidine may be of clinical importance as a biofilm inhibitor: catheters immersed with norspermidine were effective in disrupting mature biofilm[83]. Exogenous spermidine also protected *P. aeruginosa* against polymyxin B through stabilisation of lipopolysaccharides (LPS), while endogenously synthesized spermidine conferred a protective effect against the host immune response to clinical strains of *P. aeruginosa* that produce high amounts of it[84]. Its upregulation as part of the operon *speEH-pmrAB* is interesting, as *pmrAB* genes are well established as mediating resistance to colistin and other antibiotics[85,86] with one study finding that the PmrAB regulon activates *speE* in the presence of antimicrobial peptides such as colistin[87].

Our transcriptomics results indicated that exposure to increasing concentrations of colistin led to significant increased expression of *speH* and *speE* (*speE/H*). The upregulation of these genes may be a mechanism to confer tolerance to colistin under antibiotic pressure in the morbidostat, particularly as it is not highly differentially expressed in isolates exposed to metronidazole only and is found upregulated in isolates that are colistin-resistant. The efficacy of colistin is directly related to the LPS structure, specifically through binding to anionic LPS components of the outer membrane, causing increase of cell permeability and cell death by cell lysis. *P. aeruginosa* defends against colistin by the addition of l-4-aminoarabinose (l-Ara4N) to lipid A phosphates[88]. The proteins for the synthesis and transfer of l-Ara4N are encoded by the *arnBCADTEF* operon and are regulated by the PmrAB and PhoPQ two-component regulatory systems. *In-vitro* evolution studies confirm this effect: high-level colistin resistance does not evolve in the absence of a functional *arnBCADTEF* operon[81].

Upregulation of the *arnBCADTEF* operon – as we have observed it under colistin exposure – results in decreased polymyxins binding to the cell surface and the development of cross-resistance to colistin and other antibiotics[89]. The LPS is also an inducer of the complement system, activated via its components lipid A, core, and O-antigen. Bacteria expressing long O-antigen chains are usually more resistant to serum than their O antigen-deficient isogenic mutants [90,91,92] and are particularly necessary for serum resistance in *P. aeruginosa*[93]. Naturally, there was no resistance to colistin for isolates exposed to only metronidazole; these isolates also did not become resistant to serum and the gene transcripts *speE/H* and the *arnBCADTEF* operon were not upregulated significantly (Table S1). Resistance to complement is strongly associated with the capability of systemic survival, multiplication, and spread of a wide range of Gram-negative pathogens[94]. Therefore we speculate that modifications in the LPS via upregulation of the *speE/H* genes and *arnBCADTEF* operon, regulated by pmrAB, may contribute to colistin resistance and loss of susceptibility to serum in our colistin-resistant isolates generated in the morbidostat.

The results of the virulence assays completed using *G. mellonella* show a significant decrease in virulence for one type of isolate: those exposed to colistin for 21 days (Fig 7C, Fig S3C). The remaining isolates over the four conditions and three time-points show no significant difference in virulence potential when compared to the baseline strain PA77. This effect has been found in another recent study using Gram negative bacteria[95]. This study assessed the virulence profile of 16 colistin resistant *K. pneumoniae* isolates with different levels of colistin resistance, and found that the colistin MIC of *K. pneumoniae* isolates is predictive of their lethality (LD_{50} and LD_{90} values) in *G. mellonella*. High colistin MIC values were predictive of lower virulence of the isolates, indicating that genetic adaption to high levels of colistin resistance could somehow impair *K. pneumoniae* infectivity, although they did not observe any significant correlation between colistin-resistance mechanisms and virulence. In other research, *P. aeruginosa* with LPS deficiencies display attenuation in virulence in *G. mellonella*[19,20] and so it is likely that isolates exposed to colistin for 21 days may have undergone transcriptional changes related to LPS modifications that make them lose their

virulence potential. Metronidazole alone as a single drug treatment did not have any effect on virulence. Isolates exposed to metronidazole in combination with colistin, tend to be relatively virulent when compared to the single drug isolates. This indicates that metronidazole slows down the trajectory of decreasing virulence for strains evolved in combination with colistin.

The downregulation of expression of the RNA polymerase-encoding gene *rpoS* in response to the drugs colistin and metronidazole is surprising, especially considering its role as a major stress-response regulator: it was expected that this gene would be upregulated in isolates cultured with antibiotics in the morbidostat. It is well documented that *rpoS* is expressed mainly during the stationary growth phase[96], and it may be that the continuous changes in growth kinetics in the morbidostat due to the addition of colistin at regular intervals may have led to an alternative transcriptomic profile. Alternatively, the reduced expression of *rpoS* in isolates exposed to colistin and metronidazole over 21 days, relative to the baseline strain PA77 might have been an adaptation mechanism to increase resistance to colistin. Indeed, previous studies have reported that *rpoS* mutants of *P. aeruginosa* produced more biofilm, and biofilms produced were much more resistant to being killed by tobramycin than wild-type *P. aeruginosa* biofilms[97]. They also found enhanced flagellar motility exhibited by the *rpoS* mutants, which led to increase in biomass in biofilm, similar to what we have seen in our study. Increased and better developed biofilm protect against penetration of colistin[98] with extracellular polymeric substances reported to be the greatest barrier to diffusion for drug penetration into various bacteria[99]. We see more biofilm produced in isolates exposed to colistin, either as a single treatment or in combination, than with metronidazole only. As deletion of this transcription factor has several important downstream effects[100], its reduced expression is likely to contribute to the altered transcriptional profile observed in the evolved isolates. *RpoS* controls close to 800 genes[101] including several virulence factors such as pyocyanin, exotoxin A, LasA and LasB elastases, and exoenzyme S, and affects the expression of genes related to quorum sensing. Some of these genes are downregulated in our morbidostat-generated isolates, such as heat shock protein *dnaK*, and chaperonins *groES* and *groEL*, although not to a significant level (Table S1).

The semi-automated morbidostat provides several advantages over traditional serial transfer evolution experiments. It enables us to simulate a clinical situation, as it represents compartments of infection within the human body that have not been fully eradicated by antibiotic treatment. These compartments of infection are exposed to sub-lethal concentrations of antibiotic, contributing to general tolerance. Isolates cultured in the morbidostat received sub-inhibitory doses of colistin, which creates a constant level of antibiotic pressure. The addition of metronidazole, which has no bactericidal effect on *P. aeruginosa*, but is a documented trigger for mutagenesis, leads to a significantly varied phenotype. In cases where colistin resistance develops, virulence potential decreases. However, isolates are able to survive and grow in human serum longer than their colistin-sensitive counterparts. Alongside the ability to maintain a higher number of viable cells in

biofilm, the results of our work indicate the potential of a change in the course of infection due to significant evolutionary events under antibiotic treatment.

Acknowledgments

We thank Libera Lo Presti, Michael Sonnabend and Erwin Bohn for their contributions to the manuscript. We would also like to thank Richard Neher for his support with the morbidostat. We also extend our gratitude to our colleagues at AG Peschel for their advice and guidance regarding the experiments in this work.

Ethics

The overall approach of generating resistant isolates was approved by our local ethics review committee.

Funding

The work was supported by the German Center for Infection Research (grant number: TTU 08.702).

Supplementary data

Supplementary Figures:

Colistin susceptibility test evaluation of multiple-resistance-level *Pseudomonas aeruginosa* isolates generated in a morbidostat device. *Journal of Antimicrobial Chemotherapy*, Volume 73, Issue 12, December 2018, Pages 3368–3374, https://doi.org/10.1093/jac/dky337

Table S1: Results of SNP matrix determining genetic distance between clinical colistin susceptible isolates
Table S2: Antibiotic susceptibility results from 15 antibiotics for 87 clinical *Pseudomonas aeruginosa* strains using Etests
Table S3: MIC values of 131 *Pseudomonas aeruginosa* strains determined by BMD and five commercial AST methods
Table S4: Comparison of preparation, testing and incubation time for AST methods against the reference BMD
Table S5: Comparison of AST methods against reference BMD with isolates divided into susceptible and resistant categories

Transcriptomic basis of serum resistance and virulence related traits in XDR *P. aeruginosa* evolved under antibiotic pressure in a morbidostat device. *Frontiers in Microbiology*, accepted December 2020

Table S1: MIC values for morbidostat generated strains
Table S2: Transcriptomic data from 37 isolates

Figure S1: Growth curves of PA77 and evolved isolates in LB medium over 24hrs
Figure S2: Growth curves of PA77 and evolved isolates in 50% human serum
Figure S3: Kaplan-Meier survival curves of PA77 and evolved isolates using *G. melonella*
Figure S4: Comparison of differentially expressed genes between drug conditions