Phenotypic and Genomic Characterisation of Serial *Pseudomonas Aeruginosa* Isolates from Pre and Post Lung Transplantation Patients with Cystic Fibrosis

Dissertation

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Abbreviations

µl	:	Microliter
16S rRNA	:	16S ribosomal RNA
AMR	:	AntiMicrobial Resistance
AST	:	Antimicrobial Susceptibility Testing
BLAST	:	Basic Local Alignment Search Tool
BTx	:	Before Lung Transplantation
C/T	:	Ceftolozane/ Tazobactam
CARD	:	Comprehensive Antibiotic Resistance Database
CDC	:	Centers for Disease Control and Prevention
CDS	:	Coding DNA Sequences
CF	:	Cystic Fibrosis
CFTR	:	Cystic Fibrosis Transmembrane Conductance Regulator
DNA	:	Deoxyribonucleic Acid
ESBL	:	Extended-Spectrum Beta-Lactamase
g/mol	:	Gram per mole
HAI	:	Hospital-Acquired Infection
HGT	:	Horizontal Gene Transfer
IS	:	Insertion sequence
LTx	:	Lung Transplantation
MEGA	:	Molecular Evolutionary Genetics Analysis
MDx	:	Molecular Diagnostics
MDR	:	Multi-Drug Resistant
MGE	:	Mobile Genetic Element

MIC	:	Minimum Inhibitory Concentration
mg/l	:	Milligram per Liter
MLST	:	Multi-Locus Sequence Typing
NCBI	:	National Center for Biotechnology Information
NGS	:	Next Generation Sequencing
PATRIC	:	Pathosystems Resource Integration Center
PCR	:	Polymerase Chain Reaction
PTx	:	Post Lung Transplantation
RGI	:	Resistance Gene Identifier
RNA	:	Ribonucleic Acid
SNP	:	Single Nucleotide Polymorphism
SPLT	:	Serial Peri-Lung Transplantation
VFDB	:	Virulence Factor DataBase
WGS	:	Whole Genome Sequencing
WHO	:	World Health Organization

Zusammenfassung

Fortschritte in der Entwicklung antimikrobieller Empfindlichkeitstestsystemen (AST) haben zu neuen Testtechnologien/ Auslesesysteme geführt. Die Genauigkeit wurde so verbessert und die Zeit bis zum Erhalten des Ergebnisses verkürzen. Dies bietet eine bessere Alternative zu den traditionellen, kulturbasierten Tests, die eine visuelle Inspektion erfordern. Mit molekulardiagnostischen Geräten kann man den Erreger der Krankheit bestimmen und zusätzlich bestimmte Resistenzmarker/Gene identifizieren. Whole Genome Sequencing (WGS) hat das Gebiet der Infektionsbiologie revolutioniert, jedoch hat diese Technologie hat noch gewisse Einschränkungen und die Ergebnisse müssen durch kulturbasierte AST-Techniken validiert werden. Darüber hinaus spiegelt die in-silico-Analyse möglicherweise nicht den genauen Bakterienstatus wider. Daher ist die Kopplung sowohl phänotypischer als auch der genotypischer Charakterisierungsmethoden wichtig, um Informationen über das bakterielle Resistom zu erhalten. Der Einsatz von Transkriptomik in Genotyp-Phänotyp-Korrelationsstudien kann zu einem besseren Verständnis des Resistoms führen, da es dem Echtzeit-Nachweis der antimikrobiellen Resistenz in zellulo näher kommt. Bei lebensverändernden Erkrankungen wie Mukoviszidose (CF) ist eine personalisierte Therapie unerlässlich. *P. aeruginosa*, das wichtigste Bakterium in der Lunge von Patienten mit CF, ist ein komplexer Organismus mit phänotypischer Plastizität und einem Arsenal an Resistenzfaktoren. Um eine Infektion in der CF-Lunge durch *P. aeruginosa* zu stoppen, muss sein vollständiges Resistom in Verbindung mit phänotypischen Besonderheiten untersucht werden.

Abstract

Advancement in antimicrobial susceptibility testing (AST) systems has resulted in development of sophisticated testing technologies and readout systems that improved the accuracy and reduced the time to result. Such rapid AST technologies, thus, are a better alternative to the traditional culture based assay that demands visual inspection to obtain the results. By using the molecular diagnostic devices, one can determine the pathogen causing the disease and can also identify certain resistance markers and genes for a particular antimicrobial agent. Whole genome sequencing (WGS) has revolutionized the field of infection biology but the technology still has limitations and the results need to be validated by culture-based AST techniques. Further *in silico* analysis may not mirror the exact bacterial status. Therefore, coupling both phenotypic as well as genotypic characterization to achieve information on the bacterial resistome is important. Use of transcriptomics in genotype- phenotype correlation studies can lead to better understanding of the resistome as it is closer to real-time detection of genuine *in cellulo* antimicrobial resistance. In the case of life altering disease such as cystic fibrosis (CF), personalized therapy is vital. *P. aeruginosa* which is the most prominent bacterial species dwelling in the lungs of patients with CF is a complex organism with phenotypic plasticity and an arsenal of genetic resistance factors. In order to arrest infection in the CF lungs by *P. aeruginosa*, it's complete resistome coupled with phenotypic peculiarities needs to be studied.

List of publications

van Belkum, A., Pelegrin, A. C., **Datar, R.**, Goyal, M., Palmieri, M., Mirande, C., Chalansonnet, V., & Orenga, S. (2020). Differences and overlaps between Phd studies in diagnostic microbiology in industrial and academic settings. *Medical Microbiology and Immunology*, *209*(3), 217-223.

Datar, R., Pelegrin, A. C., Orenga, S., Chalansonnet, V., Mirande, C., Dombrecht, J., Perry, J. D., Perry, A., Goossens, H., & van Belkum, A. (2021). Phenotypic and genomic variability of serial peri-lung transplantation *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Frontiers in Microbiology*, *12*.

van Belkum, A., Almeida, C., Bardiaux, B., Barrass, S. V., Butcher, S. J., Çaykara, T., Chowdhury, S., **Datar, R.**, Eastwood, I., Goldman, A., Goyal, M., Happonen, L., Izadi-Pruneyre, N., Jacobsen, T., Johnson, PH., Kempf, VAJ., Kiessling, A., Bueno, JL., Malik, A., Malmström, J., Meuskens, I., Milner, PA., Nilges, M., Pamme, N., Peyman, SA., Rodrigues, LR., Rodriguez-Mateos, P., Sande, MG., Silva, CJ., Stasiak, AC., Stehle, T., Thibau, A., Vaca, DJ., & Linke, D. (2021). Host-pathogen adhesion as the basis of innovative diagnostics for emerging pathogens. *Diagnostics*, 11(7), 1259.

Datar, R., Perrin, G., Chalansonnet, V., Perry, A., Perry, J. D., van Belkum, A., & Orenga, S. (2021). Automated antimicrobial susceptibility testing of slow-growing *Pseudomonas aeruginosa* strains in the presence of tetrazolium salt WST-1. *Journal of Microbiological Methods*, 106252.

Datar, R., Orenga, S., Pogorelcnik, R., Rochas, O., Simner, P. J., & Van Belkum, A. (2021). Recent Advances in Rapid Antimicrobial Susceptibility Testing. *Clinical Chemistry*, 68(1), 91–98.

Personal contribution

Mentioned as per the order of the chapters in the PhD thesis.

Chapter 1.1 (introduction): Advances in susceptibility testing

Chapter 1 is an introductory chapter. It contains a mini review that was published in the journal Clinical Chemistry (impact factor 8.3) in January 2022. Please find it here: doi.org/10.1093/clinchem/hvab207. It is a sub-section of this chapter. In this review we discuss the recent developments in the automated susceptibility testing (AST) market and briefly describe what is still needed for implementation in day-to-day management of the treatment of infectious diseases. We talk about several novel techniques which revolve around AST by considering phenotypic profiles, multi-omics-based approaches, imaging-based AST, microfluidics etc. We also talk about both classical testing methods as well as technologies that are based on next generation sequencing (NGS). I have contributed to this publication by writing the sections on phenotypic AST specifically imaging based AST and direct microscopy. I have also introduced microfluidics as a novel emerging technology that can be useful for AST.

Chapter 1.2: Scope of the thesis

This sub-section of chapter 1 describes the objectives of the thesis and was written by me personally.

Chapter 1.3 (introduction): *Pseudomonas aeruginosa* strains from patients with cystic fibrosis.

This sub-section of chapter 2 describes the problem area and why there is a need for correct characterization and diagnosis of slow-growing *P. aeruginosa* isolates especially in the case of immune-compromised patients. This section was written by me personally.

Chapter 2: VITEK® EVO Imaging technology for phenotypic characterization of slow growing and mucoid *P. aeruginosa* from patients with CF.

AST provides data that can be used to help decide on antibiotic dosage and treatment solutions, which is important for improving the infected patient's healthcare. Rapid phenotypic AST is still broadly needed to define appropriate antibiotic therapy for critically ill patients. In this short report, we discuss one of the rapidly emerging fields of diagnostics based on phenotypic and imaging-based AST. Not only do we introduce a new AST technique which is presently

just a prototype, but we also shed light on how this technique can possibly be an improvement as compared to the conventional culture-based assays used for AST. Finally, the limitations of this new imaging-based technology will be discussed. I planned and performed the experiments with the help of my colleagues at bioMérieux, Marcy Etoile and I wrote the first version of the manuscript.

Chapter 3: Use of water soluble, chromogenic tetrazolium salts to improve susceptibility testing of slow growing strains

In this study we aimed to determine the AST profiles of the strains (n=12) obtained from the sputum samples of six patients with CF that underwent lung transplantation (LTx) using automated AST technology VITEK 2 (bioMérieux, Marcy). However, we could not obtain the results as the bacteria failed to show sufficient growth in the VITEK cards during the 18h run time. In order to improve the growth monitoring of these strains, we added WST1, a substrate used for detecting cell proliferation. As a positive control we tested a panel of 13 additional clinical strains that were from patients without CF (non-CF). We tested these strains (12 CF + 13 non-CF) using VITEK 2. We also checked and compared the efficiency of the substrate with CF as well as non-CF strains. I did all of the microbiological experiments, tabulated and calculated the outcomes of the experiments and wrote the first version of the manuscript. I submitted the paper and managed the entire publication process.

The paper was published in Journal of Microbiological Methods (impact factor 2.3) in May 2021. Please find it here: https://doi.org/10.1016/j.mimet.2021.106252

Chapter 4: Epidemiology and genotype-phenotype correlation

The aim of this study was to analyze the epidemiology, evolution and overall resistome of *P. aeruginosa* strains serially obtained from CF patients, and a non-CF bronchiectasis patient, who all underwent lung transplantation (LTx). Impaired lung function and chronic infections due to colonization and infection by complex microbiota elevates the health risk of patients with CF which ultimately leads to full respiratory insufficiency and death. For patients with end-stage CF, LTx is the only remaining therapeutic option. *P. aeruginosa* is one of the key pathogens involved in persistent lung infections and treating infections by multidrug resistant *P. aeruginosa* strains poses increased difficulties as after antibiotic treatment it frequently persists and produces a chronic infection. Such strains display different phenotypic and genomic characteristics compared to their wild-type ancestors. I have looked at the genomic

resistance profile and have tried to find correlation between the phenotype and genotype of the strains.

Multi-locus sequence typing (MLST) and construction of a core genome based phylogenetic tree using bioinformatics tools was employed to define the genomic relatedness between the 36 serially obtained *P. aeruginosa* strains (Newcastle upon Tyne, UK) and a panel of 672 *P. aeruginosa* strains from different niches and 4 different strain collections. I did all of the microbiological experiments as well as data analyses. I calculated and interpreted the outcomes of the experiments and wrote the first version of the manuscript. I submitted the paper and managed the entire publication process.

The paper was published in Frontiers Microbiology (impact factor 5.6) in March 2021. Please find it here: https://doi.org/10.3389/fmicb.2021.604555

Chapter 5: Use of transcriptomics to elucidate genotype-phenotype correlation in multidrug resistant *P. aeruginosa* obtained from patients with CF

The aim of the project was to define the level of expression of various genes that are known to play an important role in the drug resistance of *P. aeruginosa* from patients with CF. The expression of genes from *P. aeruginosa* strains from CF patients was compared with wild type control strains. This would support our understanding as to which genes could have an impact on the increase in resistance especially in case of strains from patients with CF. Further, I also tried to correlate the level of expression of biofilm genes with the wet lab crystal violet assay data. This may help to decipher the relationship between the genotype and the phenotype in biofilm production. The data analysis part is still ongoing.

This study was carried out as a secondment and a collaborative effort at Uniklinikum Tübingen. I conceived the study with the help of my supervisors and setup the preliminary experiments to study growth curves followed by RNA extraction, purification and sequencing. Dr. Michael Sonnabend, Dr. Angel Angelov and I interpreted the sequence data and I have written the first version of a manuscript with the data I currently have.

Chapter 6: Common discussion section covering the aforementioned topics.

In this chapter I discuss the current approaches used for AST and the drawbacks of these techniques. I have also mentioned the new technologies and how they can overcome the drawbacks of conventional testing methods (time consuming, chance of making errors as most

of the methods are based on visual monitoring, not automated hence needs an expert to check and validate the results obtained etc.). Most importantly I describe the set of strains I dealt with and how challenging it is to find a susceptibility testing method for slow growing strains as standard protocols do not work well. Further I have introduced the use of NGS based technology and multi-omics-based technology that are improving day by day and can give us promising results in less time. I have also mentioned that personalized therapy is needed for patients suffering from chronic illnesses and the need for antimicrobial stewardship. Genotype-phenotype correlation studies can help the doctors decide on the correct and personalized treatment options. This section was written by me personally.

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Mini-Review

Recent Advances in Rapid Antimicrobial Susceptibility Testing

Rucha Datar,[a] Sylvain Orenga,[a] Romain Pogorelcnik,[b] Olivier Rochas,[c] Patricia J. Simner,[d] and Alex van Belkum [e,*]

BACKGROUND: Antimicrobial susceptibility testing (AST) is classically performed using growth-based techniques that essentially require viable bacterial matter to become visible to the naked eye or a sophisticated densitometer.

CONTENT: Technologies based on the measurement of bacterial density in suspension have evolved marginally in accuracy and rapidity over the 20th century, but assays expanded for new combinations of bacteria and antimicrobials have been automated, and made amenable to high-throughput turn-around. Over the past 25 years, elevated AST rapidity has been provided by nucleic acid-mediated amplification technologies, proteomic and other "omic" methodologies, and the use of next-generation sequencing. In rare cases, AST at the level of single-cell visualization was developed. This has not yet led to major changes in routine high-throughput clinical microbiological detection of antimicrobial resistance.

SUMMARY: We here present a review of the new generation of methods and describe what is still urgently needed for their implementation in day-to-day management of the treatment of infectious diseases.

Rapid Antimicrobial Susceptibility Testing

Rapid identification of an infectious pathogen and accelerated determination of its antimicrobial susceptibility are basic elements in the design of (adjusted) therapy for bacterial infections (1). Initially focusing on infectious agents deriving from hospitalized patients, antimicrobial susceptibility testing (AST) is becoming increasingly important to individuals in the community as well because of the emergence and spread of multidrug-resistant organisms and their immediate public health consequences. Classically, with the aid of relatively simple and straightforward microbial cultivation systems, viable bacterial cells are amplified into large numbers from diverse types of clinical specimens, which can be used for immediate identification at the microbial species level and for AST. Current AST technologies do not include precise assessment of the within-host activity of the antimicrobials already used empirically for treatment. The effect of the interactions of drugs with the normal microbiota of the patient is largely ignored as well (2). It is generally advised that experimental inclusion of aspects of the host environment (e.g., by doing AST in the presence of serum or blood), the pathogen under investigation, and the antimicrobial to be applied is accounted for when performing AST (3). Unfortunately, the current gold standard AST methodology for routine medical microbiology laboratories is broth micro-dilution (BMD) testing, a methodology which allows little experimental flexibility (4). BMD is a method with technical and conceptual shortcomings (5). Shortcomings include the need for visual and non-automated reading, all-manual procedures, and the inhomogeneous growth of certain bacterial species. Still, all new AST methods must demonstrate compatibility with BMD for mostly historical and regulatory reasons and this may obstruct rapid introduction of otherwise useful new methods. This requires reconsideration since proof of concept and proof of principle data for new AST systems are frequently published, especially systems that suggest increased rapidity in combination with equal accuracy in comparison with existing routine AST platforms.

Dozens of different and often innovative technology platforms have been developed for AST over the past decades (6). These platforms are promising, and among the most recent and innovative platforms is one based on the quantification of the changes in dissolved oxygen levels during bacterial growth phases (7). The platform contains a large number of culture chambers pre-filled with hydrogel containing oxygen-sensing nanoprobes and

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different concentrations of antimicrobials. The antimicrobial resistance levels thus defined agree with values determined in reference BMD systems. Alternatively, the use of vibrational spectra for distinguishing between bacteria that are either dead or alive is progressing (8). Using surface-enhanced Raman scattering sensors in combination with nano-gap chemistry and machine learning algorithms for analysis of complex spectral data, bacterial metabolic profiles post-antimicrobial exposure can be successfully correlated with antimicrobial susceptibility or resistance. Deep neural network models are able to discriminate cellular responses in the presence of antimicrobials from untreated controls in 10 min with >99% accuracy (8). These are some examples in a large flux of new rapid AST (RAST) methods that have been developed over the past 5 to 10 years (6).

Through industrial development processes, some of the above technologies have recently been rendered compatible with high-quality and high-throughput RAST application in the routine diagnostic laboratory (6, 9–11). After initial academic research, the tedious examination of the dozens of clinically relevant microbial species in combination with hundreds of antimicrobials that must be tested in various concentrations remains a substantial hurdle for application of new RAST systems in routine diagnostics. Overcoming these developmental hurdles and some of the remaining biological obstructions (Table 1) is a major issue blocking the successful introduction of new RAST systems in clinical use (12). The purpose of this mini-review is to define the AST state of affairs, focusing on both pheno- and genotypic technologies that are either close to commercial launching or for which there are already first-generation products on the market.

Table 1. Technological and (micro-)biological barriers to antimicrobial susceptibility testing system development.

Microbiology	Technology
• Small number of cells	• Need for ID and AST at the same time (?)
• Slow growth	• Speed–phenotypic AST in less than 4 h is a challenge
• Lag time	• Mandatory daily QC testing (irrespective of method used)
• Heterogeneity of antibiotic resistance	• Recurring issues and development delays in semiautomated AST (Microscan, Phoenix, Vitek2)
• Induction of resistance	• Poor quality of assays from some manufacturers
• Low level resistance	• Recurring issues with gradient test quality
• Cidal vs static antibiotics	• Influx of new phenotypic methods–difficult to asses
• Detection of new mechanisms	• Clinical Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing

Phenotypic RAST Technologies

A phenotype is a set of observable characteristics of a bacterial cell resulting from the interaction of its genome with the environment. The term covers the organism's morphology or physical form and structure, its developmental genetics, its biochemical and physiological properties, its behavior, and the overall products of that behavior. When a phenotype is considered in the presence vs absence of antimicrobials, clear differences are usually observed that will help define whether or not the bacterial isolate under investigation is susceptible or resistant to the antimicrobial applied. Such phenotypic principles are at the core of current automated and high-throughput, routinely applied RAST technologies. Current and future methods are discussed below.

COMMERCIAL PHENOTYPIC TECHNOLOGIES

The commercial automated AST market is dominated by a limited number of manufacturers. These are bioMérieux with the VITEK®2 system, Becton-Dickinson with the Phoenix™, Beckman Coulter with the MicroScan, and ThermoFisher with the Sensititre AIM™ and ARIS™ systems. While the standard BMD methods and most of these systems monitor turbidity increases to evaluate cell proliferation, some systems rely on the measurement of metabolic activities. This is achieved with fluorogenic substrates in the MicroScan (13) or with a colorimetric redox indicator such as in the Biolog Omnilog, an alternative system for exhaustive bacterial phenotyping used in research settings (14). The metabolic indicators can be very useful in the case of slow-growing bacterial isolates (15), especially mycobacteria (16). Another means to improve the performance of these systems would be via intelligent software that could improve the reliability of data usage or accelerated data analysis that would shorten runtime of a test (17). Hardware improvement has been targeted as well and the frequency of use of microelectrochemical systems is increasing (1, 9). It is

interesting to note that the level of customer satisfaction is relatively high with all of the automated systems. Consequently, innovation for all of these systems is mostly incremental: new cards or cassettes for new drugs, additional dilutions of drugs, novel combinations of drugs and, quite frequently, introduction of updated clinical breakpoints and interpretation rules. A recent new player in the field, Accelerate Diagnostics, launched their Pheno® System, which combines fluorescence in situ hybridization for bacterial identification and automated microscopy to monitor growth rates for AST. Although initially aimed at filling niches in the overall AST market, the system has attractive features including more rapid turnaround time (18).

Besides the automated BMD approaches, disk diffusion (DD) is also widely used on a global scale. Easy to perform and relatively cheap, DD is a much-appreciated central tool in many laboratories. Also, for DD there are (semi-)automated systems available (19). These systems provide plate reading and automated translation of inhibition zones to susceptibility categories as defined by the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (20). This technology is robust, simple, and much appreciated by many laboratories in low- and middle-income countries. Standardized rapid DD methods from positive blood culture broths have been recently introduced by both the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute (21). The combination of these methods with total laboratory automation will provide an affordable and viable alternative method for many laboratories. The use of antimicrobial gradient strips (e.g., ETEST® by bioMérieux or MIC Test Strip by Liofilchem is similar to DD albeit that a direct minimum inhibitory concentration (MIC) determination is included, which potentially increases the value of this methodology (22). If strip reading is executed with care and expertise, gradient tests offer semiquantitative information where DD does not provide an estimate for a MIC.

Emerging phenotypic RAST methods are many but 2 are close to clinical launch. Systems developed by QuantaMatrix and Q-Linea have passed clinical studies and are in advanced stages of regulatory evaluation (23, 24). QuantaMatrix technology immobilizes bacteria in an agar plug and individual bacteria multiplication in the absence or presence of antibiotics can then be followed by microscopy. Q-Linea uses high-speed, high-resolution optical detection and an image analysis algorithm to continuously evaluate the collected images to quantify accumulating bacterial biomass. For all new technologies, of which there are many, full-scale clinical studies combining all relevant clinical specimens and microbial species with all frequently used antimicrobials are being designed or are in progress.

INNOVATION IN CLASSICAL TECHNOLOGIES

Recent innovative platforms may rely on resazurin as a fluorogenic redox indicator facilitating more rapid detection of bacterial viability (25), but they have not yet been adapted for routine AST. There is a continuous battle between product optimization, regulatory requirements, and the costs of such innovations. In all, new or extended versions of existing assays require revalidation studies and these are very costly; hence, the accepted technologies improve only incrementally. So, for the classical test formats only the most recent addition, the Accelerate Pheno system, which combines in situ hybridization for microbial species identification and direct microscopy of single cells to micro-colonies for AST, is a clear technological advancement. The technology has been validated in numerous studies and is now being accepted into the workflow of clinical laboratories, although real high-throughput application may still be cumbersome (26, 27). QMAC-dRAST™ by QuantaMatrix is an automated system that can determine the susceptibility profile of bacteria from a positive blood culture in 6 h. The 206 clinical isolates, including both gram-negative and gram-positive species, that were tested showed concordance with the results from a standard BMD test with an accuracy rate of 91.1% (28).

An interesting new trend is the increasing use of commercially available matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS or MALDI) systems for susceptibility testing (29). Although these systems have only been cleared by the United States Food and Drug Administration (FDA) for bacterial species identification at this stage, a handful of different strategies for MALDI-mediated AST have already been published and several research use only assays are available. Interestingly, MALDI can also be used to track and trace plasmids and, consequently, the antimicrobial resistance markers harbored by such plasmids (30). This application of a proteomic method to define a resistance profile needs further exploration. The use of neural network analyses will help to better use peak profiles and intensities and may guarantee reduced variability and better interlaboratory reproducibility (31). Given the fact that several of these bioinformatics methods can be used with the standard spectra as developed during species identification and with rapidity, this is an interesting domain. There are first examples on how a combination of MALDI spectra and classical phenotypic susceptibility patterns, either for individual isolates of microorganisms or averaged among longitudinal collections of microorganisms, can be used to develop systems that in the end predict a susceptibility profile based on MALDI data only (32). Another obvious approach meriting deeper investigation is the one using high-end mass spectrometers, for instance those in which highly multiplexed peptide mapping for bacterial

speciation, AST, and even virulence profiling can be performed (33).

IMAGING-BASED AST

Advancement in imaging-based AST was largely supported by the development of novel optical imaging sensors that have enhanced sensitivity and resolution for the analysis of microbes in clinical samples, as well as by progress in image processing technology. Such imaging-based technologies are generally used to detect growth and study changes in the morphology of bacteria. The imaging systems can monitor data in real time, have low culture dependency, and may reduce the time to results as compared to conventional techniques used to detect MIC (34). It has been shown how optical sensor-based nanofluidic devices can help determine MICs in 30 min or less of processing time (35). Imaging-based technologies include novel sample preparation steps but time-consuming steps such as loading of cells and cell identification based on counting have been minimized (34). Gradientech's QuickMIC technology is an amalgamation of microfluidics and photomicrography to detect inhibition of growth in the presence of a drug gradient. This technology that is currently seeking FDA clearance for AST in blood samples gives AST results in 2 to 5 h (36). Given the often profound changes of bacterial morphology under the influence of antimicrobials, it is expected that imaging will become progressively more important in automated AST.

AST based on direct microscopic examination works on the principle that cellular or morphological changes can generate information on the visible effect antimicrobials have in real time. The commercially available system oCelloscope™ (BioSense Solutions) identifies bacteria from clinical samples and then uses digital time-lapse angled field microscopy and image analysis in standard 96-well plates. The resulting images are analyzed using an algorithm and growth curves that depict the MICs of antimicrobials are plotted within 3 hours. However, the performance of this system has not been formally defined (37). Single-cell morphological analysis makes use of bright-field microscopy for determination of changes that are induced by antimicrobials in cells immobilized on an agarose channel chip (28). The MultiPath™ technology employs nonmagnified digital imaging to detect biomolecules tagged with antibody-coated fluorescent nanoparticles. Antibody-coated magnetic particles are used to bind to target cells and to pull them down to the camera surface, thus eliminating background signal and enabling a wash-free assay for clinical samples. The system counts individual targets in large areas and performs growth monitoring for multiple targets cells and enables the determination of MIC values. The producers of the MultiPath system are currently seeking FDA clearance (36). Imaging is a very rich domain in the research and development of new AST systems.

USE OF MICROFLUIDICS IN RAST

Rapid advancement in the field of microfluidics have yielded novel AST approaches (36). Innovations including digital microfluidics, agarose micro-channeling, electro-kinetics, and microfluidic confinement have substantially promoted this field over the past decade. The design of the microfluidic devices makes them easy to use and helps to alter the time lost in pre-processing steps such as sample mixing or creating antimicrobial gradients which normally take time when done manually for many samples. Further, microfluidics makes the handling of the clinical samples easier since smaller volumes are needed, which leads to reduction in the cost of reagents needed for the assays. These devices allow one to make changes in their design in order to incorporate assays that use colorimetric substrates or even a mesh of neutrophils for detection of sepsis (38). That said, the ease of use of microfluidics will be essential for its further acceptation in the AST field. In the recent past, microfluidics has emerged as a simple, cost-effective, miniaturized, and portable solution for diagnostic applications and this technology is being further explored to suit the clinical AST settings (39).

Genotypic RAST Technologies

NUCLEIC ACID AMPLIFICATION-BASED TESTING

The development of PCR-mediated detection of antimicrobial resistance genes dates back more than 30 years (40). Over the past 3 decades, PCR tests for essentially all annotated antimicrobial resistance (AMR) genes have been presented and, in principle, a comprehensive catalogue for all such genes present in a single bacterial isolate can be developed. However, this is extremely labor intensive and expensive, so not practical. Although multiplex AMR PCR tests have been developed, their use in routine clinical microbiology is still relatively restricted and niche applications are more frequent than broad application (41). The maximum capacity of multiplexing seems to be in the order of about 50 targets, which may be enough to cover the top priority AMR markers. There are alternative nucleic acid amplification technologies besides PCR such as loop-mediated isothermal amplification assays and the use of DNA arrays, but they all suffer from the same limitations when multiplexing is involved. However, broad targeted next-generation sequencing panels may be able to overcome this limitation.

GENOME TESTING

Bacterial whole genome sequences (WGS) provide the most comprehensive review possible of antimicrobial resistance markers present and the use of next-generation

sequencing (NGS) has brought this close to clinical application over the past decade. The quality and accessibility of NGS is rapidly increasing (42). WGS can now be determined for acceptable prices and the software for push-button detection of AMR markers is becoming available. This allows for the complete definition of a strain's so-called resistome and correlation of the resistome with (phenotypic) antimicrobial susceptibility patterns (Fig. 1). The first routine applications of this type of resistance testing are being developed (44). Still, a pure bacterial culture is generally needed prior to strain-specific resistome analysis. For clonal organisms such as *Mycobacterium tuberculosis*, the analytical sensitivity and specificity of AMR detection against the mostly applied drugs are close to 100% and Public Health England has already adopted routine genomic AST for *M. tuberculosis* (45). Full application will depend on further development in the realm of NGS and establishment of more comprehensive reference databases for deduction of real-life bacterial resistance levels from WGS. The comparative analyses of software suites suited for optimal translation of a nucleotide sequence into a phenotype are a constant priority (46). It is important to realize that NGS also allows for sequencing nucleic acids directly from clinical specimens without the time-consuming microbial cultivation procedure. These so-called metagenomic or non-targeted sequencing approaches may substantially accelerate NGS-based testing as well (47). It has already been shown that the analytical sensitivity and specificity of metagenomic NGS tests are competitive and may approach 100% for some pathogens or clinical syndromes (48). However, a targeted NGS approach may be more viable for detecting AMR due to increased analytical sensitivity. Still, a transparent, performance-based evaluation of flexibility in the bioinformatics tools of choice, while demonstrating proficiency in meeting common performance standards, is urgently required for AST by NGS (49). A newly proposed approach involves a community-driven effort to establish and maintain benchmarking platforms to assess performance in a qualitative manner (50). This should be based on different AST use cases. Further, these authors suggest that agreed and well-defined data sets should help define the pipelines' implementation, validation, and quality control. Benchmarking should include the selection and the characteristics of the data sets with respect to quality, choice of pathogens and AMR markers, evaluation criteria of the pipelines, and the way these resources should be deployed in the users' community. Efforts to attain this level of pragmatic standardization require our full attention. It must be emphasized that AST by NGS will not be a panacea. Not all resistance mechanisms will be detectable from the genome (heterogeneous resistance may be hard to detect for instance) and not all mechanisms detected will be phenotypically expressed. Multifactorial resistance mechanisms may be expressed in a manner that may be very dependent on the overall genome context whereas some mechanisms are only expressed when the microbial population is mixed. Of note, taking an adequate clinical sample, transporting and storing it, as well as its transition into the routine diagnostic process, may still provide substantial bottlenecks for downstream genomic analyses. Finally, the AST by NGS domain will become very competitive, with at current about 150 institutes that submitted around 250 patent applications over the past years. This may delay or even obstruct product development.

Transcriptomic testing is closer than comparative genomics to real-time detection of genuine in cellulo antimicrobial resistance. Consequently, RNAseq assays have been developed (51). Rapid RNAseq followed by bioinformatics was applied to explore potential markers in the transcriptome profile of *Neisseria gonorrhoeae* upon azithromycin exposure (52). Validation of candidate markers using PCR showed that 2 markers could generate accurate AST results. Further validation of RNAseq is needed to provide further evidence of the quality of RNA markers.

Concluding Remarks

New technologies will continue to improve the quality and usefulness of AST. The integration of phenotypic susceptibility data with real-life patient data and high-density information based on omics studies will lead to predictive models for optimized treatment of patients with infections (32, 53). Also, the simultaneous detection of phenotypic and genotypic markers, for which tests that take <5 h have already been developed (54), provides an avenue that combines the positive aspects of both the phenotypic and genotypic methods and neutralizes most of the negative characteristics. It is frustrating that AST for combinations of antimicrobials is still in its infancy, while combination antibiotic therapy is frequent (55).

Systems for sharing genome data need to be developed, as well as systems that safeguard the quality of the data in the genome databases. It also must be realized that genome data do not only provide information as to antimicrobial resistance. The same data can be used to detect organisms, define their species nature, perform epidemiological tracing, and define virulence profiles (56). Definition of variants of concern, a concept developed in virology, will also play an important role in the surveillance of bacterial infections. Implementation of such fully integrated "cover-all" diagnostic approaches will take time but the first clinical studies are commencing. With the help of self-learning systems, artificial intelligence (57), and further optimization of automation,

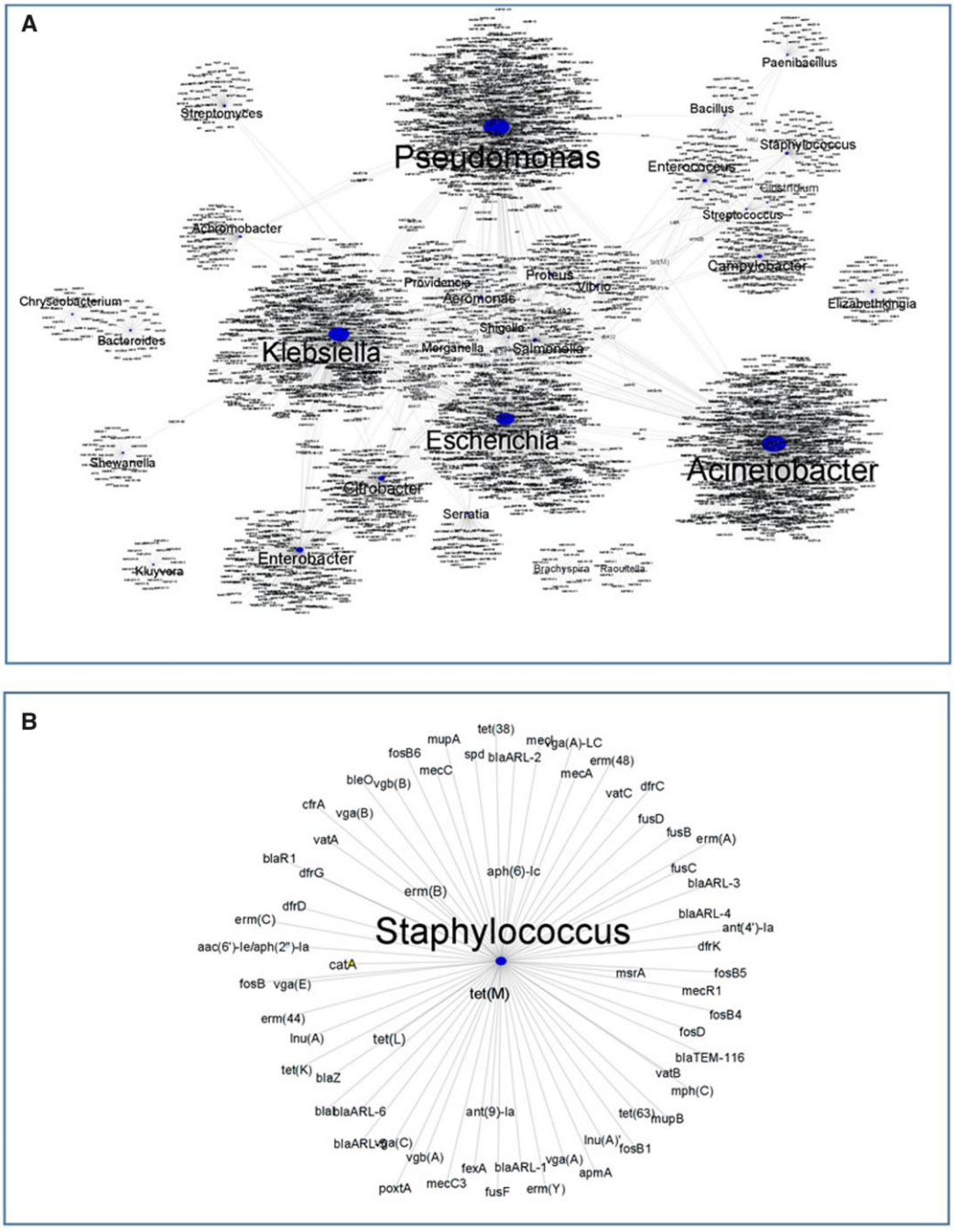

Fig. 1. Resistome representations for 30 clinically relevant bacterial genera (A) and a detailed example of the cumulative resistome for the genus *Staphylococcus* spp. (B).

Blue nodes represent a genus and genes are represented by their labels. A genus and a gene are connected by an edge if there exists one known resistance phenotype induced by this gene for at least one species in this genus. Graph layout was performed using Cytoscape (43). All genes involved in resistance to all clinically used antimicrobials are identified in a cloud around a genus-specific nucleus. When genus-specific clouds are connected, this hints at functionally similar genes shared by both genera. For *Pseudomonas* spp., *Acinetobacter* spp. and *Klebsiella* spp., the largest number of antimicrobial resistance-associated genes have been identified to date.

the clinical microbiology laboratory will continue to play an essential role in the control and prevention of antimicrobial resistance development.

Nonstandard Abbreviations: AST, antimicrobial susceptibility testing; BMD, broth micro-dilution; RAST, rapid antimicrobial susceptibility testing; DD, disk diffusion; MIC, minimum inhibitory concentration; AMR, antimicrobial resistance; NGS, next-generation sequencing

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Chapter 1.2: Scope of the thesis

This thesis describes phenotypic and genotypic characterization of slow growing, mucoid, multi-drug resistant and biofilm forming *P. aeruginosa* isolates obtained from patients with CF before, during and after lung transplantation LTx.

I have been using various AST approaches including conventional culture-based assays such as broth micro-dilution tests, gradient diffusion and disc diffusion testing. I supplemented these manual methods with automated AST using VITEK 2. I also incorporated metabolic substrates to improve test quality where needed. I have tried to develop imaging-based AST and I included genotypic AST approaches based on WGS and resistome typing. *P. aeruginosa* strains were collected from six patients serially before, during and after LTx. I attempted to elucidate genotype-to-phenotype correlations through transcriptomic approaches and compared the expression patterns of crucial antimicrobial resistance (AMR) genes with antibiotic susceptibility profiles of these isolates *in-vitro*. Additionally, we catalogued the AMR and virulence genes found in the strains while comparing intra- and inter-patient strain variability. In the end I have been able to compare the efficacy of a large variety of AST methods when applied to the collection of slow-growing isolates. This provides insight in the quality of the methods and defines where improvement is still needed. The inclusion of genomic and transcriptomic data generates a future vision for further optimization of AST for fastidious and mucoid strains of *P. aeruginosa*.

Chapter 1.3 (Introduction): *Pseudomonas aeruginosa* strains from patients with cystic fibrosis

Cystic fibrosis

Cystic fibrosis (CF) is one of the most widely recognized and life altering autosomal recessive diseases among inhabitants of Western countries. It affects as many as 1 in 3,400 infants all of whom are of Caucasian descent (Cutting, 2015). The cause of CF is dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) caused by mutations in the *cftr* gene. Among the large number of mutations documented in this gene, the mutation F508del is widely reported as it affects 90% of the patients with CF. The dysfunction of CFTR from mutated *cftr* leads to inefficiency in chloride and water transfer on the tissue surface of the lungs thus causing increased production of slimy and viscous mucous secretion. Subsequent lung diseases, mostly infections, are the fundamental drivers of elevated morbidity and mortality among patients with CF. Patients with pre-existing conditions such as respiratory failure caused by extensive lung damage including bronchiectasis or chronic endobronchial infections are even more severely affected (Sanders & Fink, 2016). For patients with end-stage CF, lung transplantation (LTx) is the only remaining effective and feasible therapy, as there is 50% probability of survival 10-year post-LTx (Snell, 2017).

Not only does CF worsen lung health by physically blocking the respiratory pathways, it also has severe repercussions in the functioning of multiple organs. Sweat glands, biliary ducts of the liver, and the pancreas can be affected where the last leads to insulin deficiency. The occurrence of CF-induced diabetes is proportional to the age of the patient, chronic lung damage, acute pulmonary exacerbations (APEs), poor nutritional status etc. The main goal while looking after patients with CF is to reduce the APEs as they are linked to the progression of the disease (Bennett *et al*., 2014).

The respiratory tract microbiota of CF patients that is identified as inflammatory includes pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Haemophilus influenzae*, *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and nontuberculous mycobacteria (NTM). The pathogenic role of the most prevalent pathogen, *P. aeruginosa*, will be discussed below.

Pseudomonas aeruginosa

Among the opportunistic pathogens that dwell in the respiratory tract of patients with Cystic Fibrosis (CF), *P. aeruginosa* is the most frequent. Its prevalence, especially for strains with a mucoid phenotype, indicates advancement in the deterioration of lung functioning and colonization or infection reduces host' chances of survival (Cutting, 2015).

P. aeruginosa, belonging to the phylum *Proteobacteria,* is a ubiquitously distributed microorganism that thrives in water and soil as well as in human and animal faecal material (Neves *et al*., 2014; Crone *et al*., 2020; Rossi *et al*.,2021). *P. aeruginosa* is a Gram negative, facultative anaerobic non-spore forming bacterial species that is also an opportunistic pathogen (Neves *et al*., 2014). Owing to the large genome size (approximately 5.5–7 Mb) with one or more copies of possibly different plasmids present, *P. aeruginosa* can resist environmental changes. The genome encodes for an arsenal of regulatory genes, transporters and virulence factors which helps the bacteria to overcome nutritional or environmental stress (Silby *et al*., 2011). Additionally, Turner *et al*., (2015) have reported that the core genome of *P. aeruginosa* comprises of 665 "essential" genes, that are vital for *P. aeruginosa* growth in CF lungs. However, the *P. aeruginosa* pan-genome consists of 54,272 genes and the crucial genes account for only about 1% of the pan-genome (Freschi *et al*., 2018). This shows for extensive genomic diversity within *P. aeruginosa spp*. (Klockgether *et al*., 2011). *P. aeruginosa* strains PAO1, PA14 and PA7 are widely spread and thus are considered as species representatives (Stewart *et al*., 2013).

P. aeruginosa is known to cause infections especially in immunocompromised patients, such as for example those with CF and other obstructive pulmonary diseases (Eklöf *et al*. 2020). It is also known to cause infections in patients who are intubated or in patients with permanent (bladder) catheters (Barbier *et al*., 2013). Furthermore, *P. aeruginosa* is an important pathogen in diabetic foot ulcers, burn wounds, as well as otitis media and keratitis which may even happen in otherwise healthy subjects (Silby *et al*., 2011; Kerr *et al*. 2009). Infections caused by *P. aeruginosa* pose a challenge in terms of adequacy of treatment.

The intrinsic resistance to different antibiotics by *P. aeruginosa* is determined by a mix of physical, physiological and genetic determinants (Ciofu *et al*., 2019). The ability to tolerate high antibiotic doses leads to the need for extended therapy that may lead to more rapid selection of resistant mutants (Horcajada *et al*., 2019). *P. aeruginosa* is considered as a member of the *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, and *Enterobacter spp.* (ESKAPE) group of pathogens that are considered as important targets for the development of novel therapeutics.

Biofilm lifestyle of *P. aeruginosa*

The lifestyle of *P. aeruginosa* in the lungs of patients with CF is strongly influenced by the micro-environment. The lung environment impacts the development of the bacteria and oftentimes leads to growth in the form of small aggregates or communities representing starting biofilms (Rossi *et al*., 2021).

Reversion of regulation from the chronic to the acute pattern has been observed in the mutational patterns of different chronically present isolates (Bartell *et al*., 2019). During chronic infection of the lungs and long-term *P. aeruginosa* colonization of the airways in CF, *P. aeruginosa* persists in the form of biofilms (Singh *et al*., 2000, Bjarnsholt *et al*., 2013). This finding of a lung-related biofilm lifestyle aligns with studies showing that *P. aeruginosa* has elevated expression of genes coding for production of alginate and *psl*-encoded exopolysaccharides along with other biofilm determinants (Rossi *et al*., 2018, Kordes *et al*., 2019, Wu *et al*., 2019). Over the course of time and drug therapy, the lung environment alters. The bacteria dwelling in the airways adapt over time to the environment and this can be traced by observing the genomic changes as well as changes in the phenotype of the resident bacteria (Rossi *et al*., 2021). Additionally, *in vivo* studies show that expression of proteases and type III secretion system (T3SS) genes is continued in the planktonic cells as well as in later chronic stages of infection.

The driving forces that lead to bacterial adaptation and evolution in the airways of the patients are the innate presence of various factors of the host immune system and the continued antimicrobial drug use during treatment. More than most other pathogenic bacterial species *P. aeruginosa* has evolved mechanisms such as biofilm formation to resist and tolerate these threats (Rossi *et al*., 2021).

The *in vitro* analysis of biofilms produced by *P. aeruginosa* has provided substantial information about the developmental stages involved in biofilm production starting from attachment to a substrate to building of a structured consortium. In CF clinics, a poor prognosis is defined upon overproduction of alginate in *P. aeruginosa* strains. The alginate hyper-production is linked to mutations in *mucA* or regulatory genes that control expression of the alginate biosynthesis operon (Pedersen *et al*., 1992). When the biofilm concept was still in its

infancy, alginate production was considered as a marker to explain chronic *P. aeruginosa* infections. However, now it has become clear that long-term *P. aeruginosa* infection in CF lungs has a complex multifactorial basis, which makes early predictions of chronicity very difficult.

Furthermore, polymicrobial biofilm also plays a significant role in defining the MDR status of *P. aeruginosa* as the bacteria involved serve as a pool of genes that are transferred through horizontal gene transfer (HGT) between the bacterial species (Redfern *et al*., 2021). To share the genetic information between the bacteria, the bacteria in biofilms communicate with each other via cell signalling/ quorum sensing. *LasR* is one of the essential quorum sensing-related genes in *P. aeruginosa* (Ahmed *et al*., 2019; Lee *et al*., 2015). The purpose of cell signalling is to also offer mutual protection and for gathering nutrients (Chen *et al*., 2019). This type of communication can take place through a specialised secretion system namely the type VI secretion system (T6SS). This system has gathered attention in recent years due to its occurrence in various gram-negative bacteria including *P. aeruginosa* (Gao *et al*., 2017; Chen *et al*., 2019).

The ability to attach to surfaces and motility are important virtues in the process of biofilm formation and growth. Flagella play an important role in cell surface adherence in case of *P. aeruginosa*. It is observed that in *P. aeruginosa* lacking type IV pili, only a single layer of biofilm is formed but the mature mushroom-shaped structure cannot be developed (Chen *et al*., 2019; Barken *et al*., 2008). *Pil*A, which is a major subunit of pilin, is important for pilus assembly and is involved in attachment and motility. *Fli*C is essential for flagellum assembly and motility in *P. aeruginosa* (Nguyen *et al*., 2018).

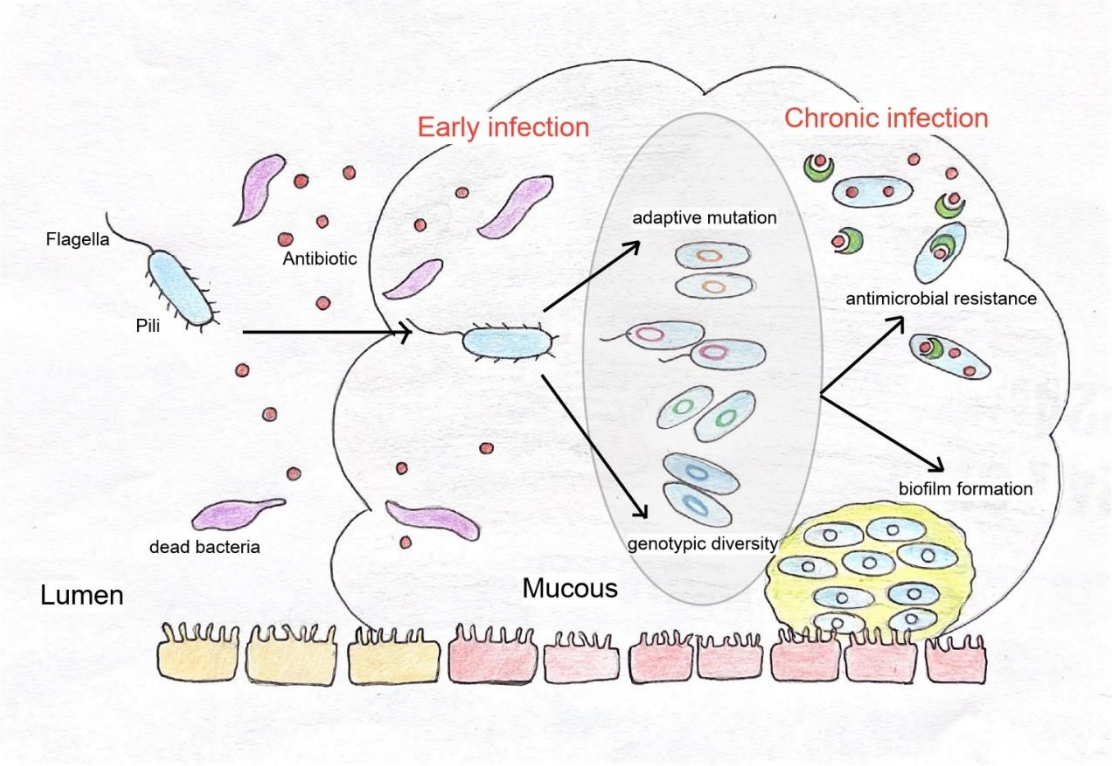

Figure 1: The figure which is adapted from Rossi *et al*., 2021, depicts from left to right, the time course and adaptation of *P. aeruginosa* in the lungs of patients with CF. During early infection, *P. aeruginosa* has a wild-type phenotype and it slowly starts adapting to the environmental conditions in the CF lungs. In the chronic infection stage, *P. aeruginosa* is completely adjusted to the CF lung environment and shows high genotypic and/or phenotypic variation and biofilm development.

Antibiotic resistance development

One of the most important reasons for the improved life expectancy of patients with CF and why patients can now live into their 50s is the improvement in antimicrobial therapy. Hence, in CF airways the colonizing *P. aeruginosa* should be able to deal with the continued presence of one or more antibacterial agents. Even more aggressive antibiotic treatment is applied during pulmonary exacerbations which is the prime cause of resistance selection in *P. aeruginosa* from CF patients (Rossi *et al*., 2021). In an ideal scenario, however, the treatment is started when *P. aeruginosa* is identified in the clinical samples from patients. After *P. aeruginosa* is found in the samples, the antimicrobial susceptibility testing (AST) profile is checked. A combination

of treatment history and the results from microbiological analysis are the guiding factors to decide on further treatment strategies. The use of whole genome sequencing (WGS) has demonstrated that clinical surveillance often underestimates bacterial persistence and changes in antibiotic susceptibility. This results in treatment failure without identifying factors involved in resistance development (Marvig *et al*., 2015). Hence, in order to better define treatment regimens for infections in CF airways, it is necessary to recognize the persistence mechanisms along with identifying the antimicrobial resistance factors (Waters *et al*., 2019).

P. aeruginosa routinely obtained from chronic infections are often multi-drug resistant since they show resistance to at least two or more classes of antibiotics as resistance development is related to the long-lasting treatment of persistent infections (Rossi *et al*., 2021). Apart from the antibiotic resistance genes, the acquisition of mobile genetic elements and gene mutations also play an important role (Botelho *et al*., 2019) in elevated resistance in *P. aeruginosa* infections.

Mutational resistance could be multifactorial due the accumulation of multiple mutations over time as in the case of aztreonam and colistin resistance (Jochumsen *et al*., 2016, Jorth *et al*., 2017). The resistance could also be due to different mutations in antibiotic targets as in the case of fluoroquinolone resistance by mutations in DNA gyrase genes (Hooper *et al*., 2015). Resistance could be, like in the case of tobramycin resistance, caused by mutations in ribosomal genes (Halfon *et al*., 2019).

Some mechanisms of resistance impart low levels of resistance to antibiotics. One example is the mutational inactivation of MexZ (a negative regulator) which leads to overexpression of efflux pump MexXY. This leads to a slight increase in resistance to aminoglycosides while having no observable change on the metabolism in *P. aeruginosa*. The low-level resistance conferred by the bacteria increases chances of survival and ultimately helps in evolving a highly resistant phenotype (Frimodt-Møller *et al*., 2018).

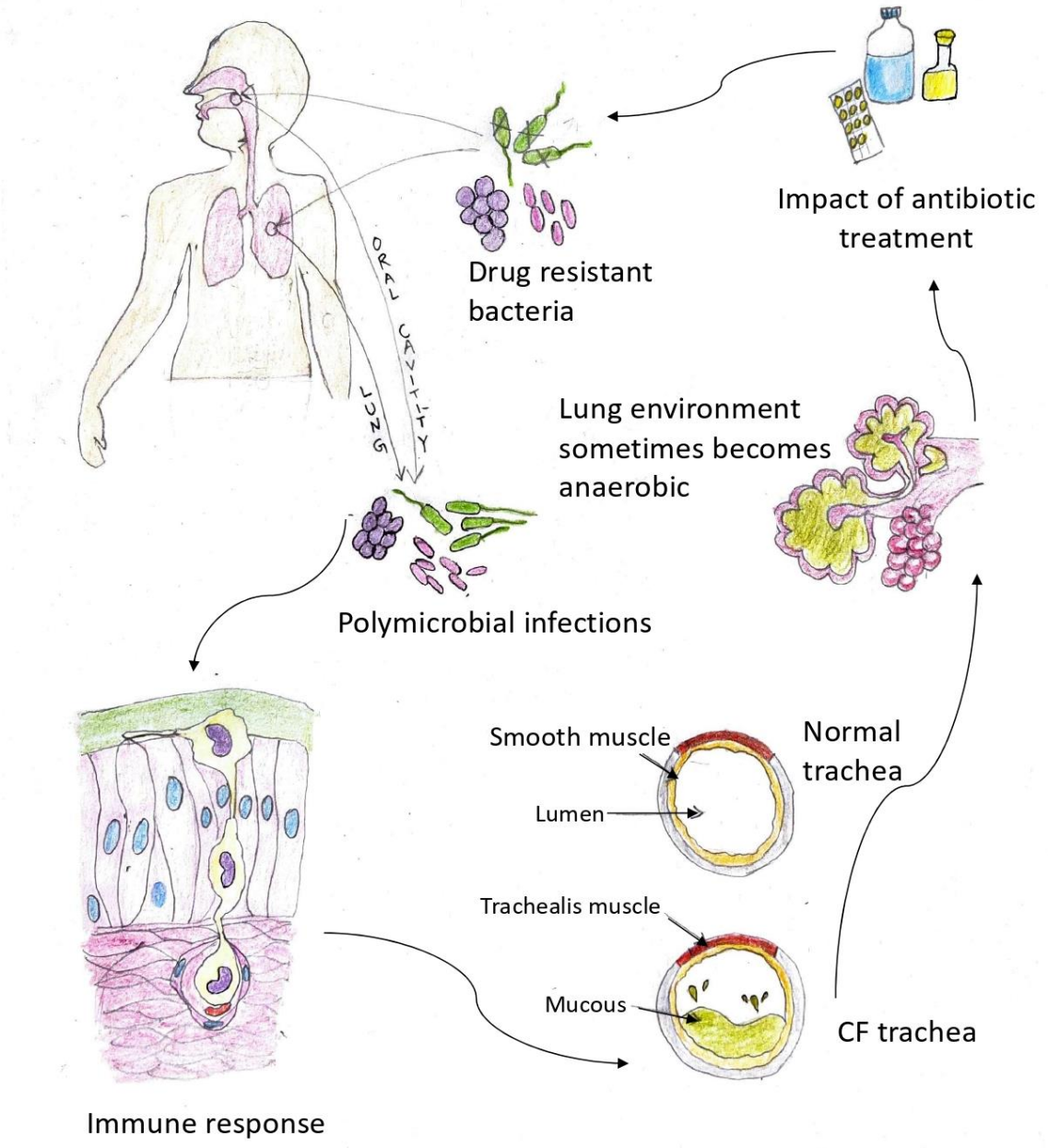

Figure 2: The figure depicts an overview of how lung environment changes over time when drug resistant bacteria like *P. aeruginosa* dwell and multiply in the lungs of patients with CF.

Clinical challenges and opportunities

In vitro phenotypic strain characterization has helped to gather important information about underlying mechanisms of microbial adaptation (Moradali *et al*., 2017). Through this, several characteristic phenotypic traits have been frequently observed in clinical isolates of *P. aeruginosa*, especially if the *P. aeruginosa* strains were obtained from patients with CF and chronic infection. Some of these phenotypic traits that are repeatedly observed include multi-drug resistant profiles of the strains, decrease of catabolic functions, augmented adherence and formation of aggregates, and a lower rate of growth when grown in laboratory setup. Bacterial aggregation or biofilm formation, changes in metabolism, and production of signal molecules monitored by sensors with high sensitivity should be used as diagnostic methods in clinical settings for determining early stages of chronic infection (Rossi *et al*., 2021). Imaging based AST technologies can shed light on not only the resistance status of the bacteria but can also give indications on biofilm formation and individual bacterial morphology (Khan *et al*., 2019).

Genomic data of the pathogen and their subsequent analysis have improved the surveillance of CF-associated lung infections. This has created a need for improved diagnostics and therapeutics based on genotypic profiles of the bacteria. WGS has revolutionized the field of epidemiological typing and is replacing older methods such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and several multiplex PCR arrays to determine identity of the isolates (Van Belkum *et al*., 2020). The biggest merit of WGS is accuracy in genotyping that is sought for in clinical research and for thorough investigations of clonal dispersal and inter-patient transmissions. Important information about adaptive processes in bacteria in individual patients can be obtained from bacterial genome sequences from multiple isolates collected longitudinally. Further, as the genomic size and complexity of *P. aeruginosa* is an intrinsic problem, the search for single genetic determinants responsible for complex phenotypes is challenging (Rossi *et al*., 2021).

Identification of genetic markers or virulence factors is the key to predict if bacteria have the potential to establish chronic infections in healthcare settings. Studying genes in which accumulation of mutations is in higher frequency than expected can lead to better understanding of pathogenic abilities of the bacteria involved (Pelegrin *et al*., 2021; Rossi *et al*., 2021).

This been said, not all mutations play a role in imparting resistance to *P. aeruginosa* in severe infections. The presence of genes with multiple simultaneous mutations possibly increases the adaptive success of the *P. aeruginosa* strains from chronic infections. Diagnostics based on

identification of these diverse mutational profiles should be developed as they can aid in predicting the cause of chronicity of the infection as well as give information on the persistent nature of infecting bacteria and the response of strains to different antibiotics.

Rapid advancement in the field of WGS and development of robust genome sequence databases can be helpful in studying the existing but also novel resistance patterns and mechanisms that bacteria employ to withstand the effect of antibiotics. Machine learning approaches can enable one to convert the observed genomic variations into consistent adaptive patterns that can help one to predict resistance to antimicrobials (Khaledi *et al*., 2020). Predictions like these are important in evolving healthcare and research settings.

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Chapter 2: VITEK® EVO Imaging technology for phenotypic characterization of slow growing and mucoid *P. aeruginosa* from patients with CF

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Introduction

Development and deployment of new technologies has led to advancement in antibiotic susceptibility testing (AST) protocols that helps in estimating the minimum inhibitory concentrations (MICs) of antibiotics that are required to inhibit the growth of or even kill bacteria. The classical MIC defines the lowest concentration of antibiotic which inhibits visible growth of a bacterial strain when incubated for 16-20 hours at 35°C+/-2°C in an ambient air incubator (Weinstein, 2018).

Imaging-based phenotypic AST methods usually rely on photography and report the morphological changes or different growth patterns of bacteria through images obtained (Maugeri *et al*., 2019; Leonard *et al*., 2018). OCelloscope (BioSense Solutions ApS, Denmark) is a low image resolution technology that manages to analyse as many as 96 conditions simultaneously using time-lapse microscopy for AST determination (Fredborg *et al*., 2013). Another commercially available technology is the Accelerate Pheno System (Accelerate Diagnostics, USA) that helps in bacterial identification as well as AST of bacteria and yeasts directly from positive blood cultures while dismissing the overnight sub-culturing step. This saves a significant amount of time. This system is capable of evaluating the risk of ventilator-associated pneumonia (VAP) in patients before the clinical symptoms were seen (Douglas *et al*., 2015). The optical and imaging-based AST technologies enable one to perform real-time analysis of bacteria more or less directly from clinical samples or including a brief enrichment procedure. One can study the effect of antibiotics and the concentration effects over time directly on the individual bacteria (Datar *et al*., 2021; Khan *et al*., 2019). Another imaging-based tool (which is not commercially available yet) called Bacterial Cytological Profiling (BCP; Division of Biological Sciences, University of California, San Diego, La Jolla, California, USA) allows one to measure the impact of antibiotic treatment by measuring changes in cell size, shape, length, permeability and also the chromosome number in eukaryotes. This is

achieved by using microscopy and fluorescent dyes. This tool is very useful for characterising multi-drug resistant (MDR) organisms as it promises accelerated throughput (Quach *et al*., 2016).

The VITEK® EVO Imaging system (VITEK EVO) is still a proof-of-principle prototype and concerns an evolution of the phenotypic VITEK® 2 system aiming to increase its current capabilities by expanding its result acquisition method. This new technology relies on direct holographic imaging of bacteria in the wells of the VITEK® 2 cards associated with advance image and kinetics analysis to generate clinically relevant results.

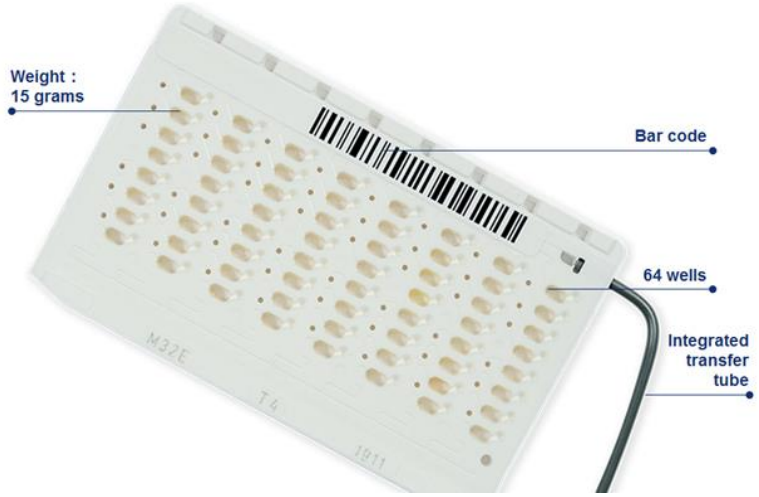

Figure 1: A schematic representation of a VITEK® 2 card. Each VITEK® 2 card contains at least one positive control well with no antibiotic (growth-promoting broth only) and multiple wells with increasing concentrations of various antibiotics in the broth.

The primary objective of implementing this new technology is to improve the sensitivity and specificity of the VITEK® 2 testing system and the robustness of the results obtained. Additionally, the aim was to try to gain more insights on the biofilm producing capability of mucoid bacterial isolates. It was considered especially interesting to see the morphological and/or growth changes in some mucoid bacteria and organisms that are otherwise fastidious (atypical and/or slow-growing) and that fail to give results with the VITEK® 2 protocol due to an extended time to result. Here we tested 12 *P. aeruginosa* isolates from patients with cystic fibrosis (CF) that were morphologically and phenotypically different from the *P. aeruginosa* ATCC 27853 (wild-type control strain, non-biofilm producer) and API:1401109 (native control strain from the bioMérieux collection; biofilm producer) using VITEK EVO. During earlier

efforts we were unable to reliably define MICs of some antibiotics for some of these *P. aeruginosa* isolates using the VITEK® 2 system because of their unique, mucoid and slow-growing nature (Datar *et al*., 2021).

Materials and methods

1. Strain selection

Twelve *P. aeruginosa* isolates from patients with CF were selected for the study. These isolates were obtained at the Freeman hospital in Newcastle upon Tyne (UK) from 6 chronically ill patients (2 serial isolates per patient were used in the study: one before lung transplantation (BTx) and one post lung transplantation (PTx). All of the isolates from an individual patient were phenotypically very similar and shared ≥98.7% average nucleotide identity (ANI) at the genome level (Datar *et al*., 2021). Apart from the 12 *P. aeruginosa* isolates that were obtained from the patients with CF, two control strains (API: 1401109 and ATCC: 27853) whose AST profiles were known served as a positive and negative controls for biofilm production and were included in the study.

Table 1: The 12 *P. aeruginosa* isolates identified on the basis of patient number, time of isolation and specimen type. The 2 non-cystic fibrosis *P. aeruginosa* control strains were used for comparison (because of their distinct time to result using VITEK EVO and their ability or not to form biofilm) with the isolates from patients who underwent lung transplantation (LTx).

Isolate number	Origin	Patient number	Time	Specimen type
API:1801131	UK	1	4m BTx	BAL
API:1801134	UK	1	10m PTx	BAL
API:1801135	UK	2	1Y BTx	BAL
API:1801139	UK	2	6m PTx	BAL
API:1801140	UK	3	11m BTx	BAL
API:1801146	UK	3	10m PTx	BAL
API:1801147	UK	4	3m BTx	BAL
API:1801155	UK	4	3.5m PTx	BAL
API:1801156	UK	5	1Y BTx	BAL
API:1801160	UK	5	5m PTx	BAL
API:1801161	UK	6	2m BTx	BAL
API:1801165	UK	6	12m PTx	BAL
API: 1401109	bioMérieux strain collection (control strain)	NA	NA	NA
ATCC: 27853	bioMérieux strain collection (control strain)	NA	NA	NA

BAL, Bronchoalveolar lavage; NA, Not applicable; BTx, Before Lung transplantation; PTx, Post lung transplantation; m= month, Y= year; API, bioMérieux culture collection number, ATCC, American Type Culture Collection.

2. VITEK EVO Imaging

The 14 *P. aeruginosa* isolates were studied using VITEK® 2 cards AST-N240 which are specific for aerobic Gram-negative bacilli of clinical interest. In this study we focused on image analysis of wells that contained aztreonam (ATM) and imipenem (IMP) as we could not get

adequate results with VITEK® 2 for these two antibiotics on those isolates. The AST- N240 card well map showing the positions of ATM and IMP is shown below:

Table 2: AST- N240 card well map showing the positions of ATM and IMP analysed by VITEK EVO imaging

Wells	Antibiotic	Concentration ($mg.l^{-1}$)
1	Positive control	No antibiotic
2	Positive control	No antibiotic
6-8	Aztreonam	2, 8, 32
26-29	Imipenem	1, 2, 6, 12

3. Imaging overview of bacterial growth in the wells

For this study, the VITEK EVO imaging prototype was programmed to capture images of the wells on the AST cards individually every twenty-six minutes during 26 hours of incubation. The images were taken using an inbuilt camera. Light targeted towards the wells is partly dispersed by the bacteria present, while a portion of the incident light remains unchanged. The phase shift of the two wave fronts creates holograms which are recorded by a sensor.

Detailed imaging was done for all of the 14 strains. Per well fourteen images, corresponding to 14 positions inside the well (Figure 2) were taken every 26 minutes. This ensured a complete overview of the planktonic growth and biofilm distribution between the two sides of the well. In order to further overcome the limitations of the VITEK® 2, the run time was manually extended from 18 hours (the maximum run time for commercially available VITEK® 2) to 26 hours (as the isolates were slow-growing and showed visible growth on the media plates only after 24 hours of incubation at 37 °C).

The sample preparation and handling protocol was identical to that of the standard VITEK® 2 protocol mentioned in Datar *et al.*, (2021).

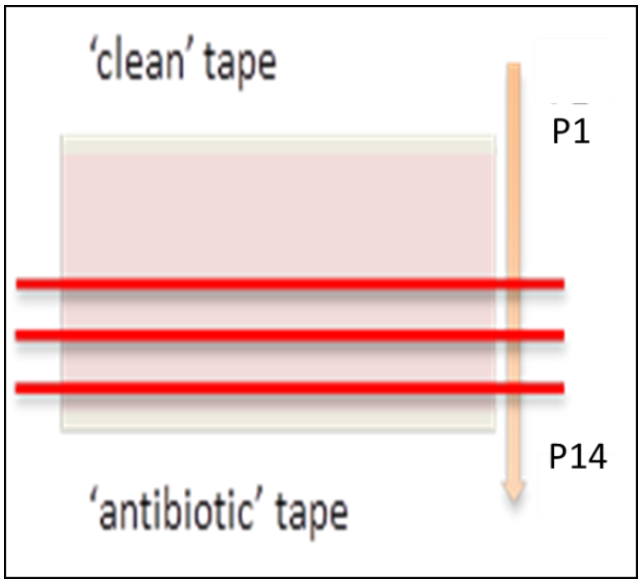

Figure 2: Representative image displaying the imaging positions in individual well in the VITEK 2 card. In order to have a complete overview the growth pattern of the *P. aeruginosa* isolates in the VITEK® 2 wells, Position 1 (P1) and Position 14 (P14) were selected to see the growth near the tapes. The red lines show the distance between two imaging positions. The antibiotic tape contains antibiotic on it while the clean tape is without antibiotic.

4. Image analysis

First, the images were analysed by a script to extract the holographic data corresponding to each focal plane. In order to suppress the artefacts, present in the images (film folds, residual crystals from culture media components and/or antibiotics, etc.), a Gaussian filter allowing to smoothen the images was applied. The script allowed subtraction of background signal to generate images without artificial disturbances. To conceal elements that originate from bubbles or the top or bottom plastic films sealing the wells of the cards, pixels which intensity was not between 20 and 240 pixels were removed from the image by using a mask. Finally, the median of the "masked" images obtained was calculated and the images were saved in Tag(ged) Image File Format (TIFF).

The second script allowed the previously acquired data to be plotted in three dimensions. This made it possible to follow growth according to the position in the well and over time. The curves thus obtained show the time on the abscissa, the position on the ordinate as well as the magnitude of the median of the grey value of each image. The latter is represented by a colour scale. The lowest level (0.00) corresponds to blue, while the highest level (0.20) is symbolized in red.

5. Prediction of MICs

As the standard VITEK® 2 run could not define the MIC values of ATM and IMP, ETEST® (bioMérieux, Marcy L'Etoile, France) was used according to the manufacturer's instructions to determine reference MICs for the 14 strains tested with VITEK EVO.

5.1 Determination of reference MIC by ETEST®

To determine the MICs of ATM and IMP, ETEST® strips were placed on Mueller Hinton E (bioMérieux) agar plates according to the manufacturer's guidelines. The plates were incubated for 20-24 hours at 37° C after which an elliptical zone of clearance was observed. The point of inhibition of growth that intersected with the strip was recorded as the MIC for that antibiotic. However, when an asymmetrical zone of inhibition was observed, the highest MIC value was recorded. ATCC 27853 was used as an experimental control.

5.2 Prediction of MIC by VITEK EVO

The images obtained through the VITEK EVO run were carefully inspected visually. Additionally, the 3D plots helped to better understand the growth dynamics of the *P. aeruginosa* strains. As each well in the VITEK® 2 card contains a specific concentration of antibiotic, we observed the graphs showing reduction in growth in the wells with increasing antibiotic concentration. The observations were then cross checked with the reference MIC values to see if the image analysis results coincide with ETEST® findings.

6. Biofilm production

As *P. aeruginosa* is capable of producing biofilm, we verified if the isolates from patients with CF were biofilm producers or not and if the growth on the sides of the well or directly on the films covering the wells correlated to biofilm production. A two-step biofilm assay using crystal violet was performed (O'Toole *et al*., 2011). Isolates from patients with CF were cultured in a 96-well round-bottom plate and incubated at 37° C without shaking for 26 hours. The second step consisted of successive washing of the plate using an 0.8% saline solution and then adding dye (crystal violet) to the wells. The plate was incubated at room temperature before being washed again, to allow coloration of biofilm (Doll *et al*., 2016). Crystal violet is a basic dye that binds to negatively charged molecules, thereby staining the bacteria as well as the biofilm matrix.

Results & Discussion

By using VITEK EVO imaging technology, it is possible to visualize the bacterial growth occurring within VITEK® 2 AST cards progressively as incubation time passes. The images show a progressive darkening of grey levels indicating increased bacterial growth which is directly proportional to the exponential growth during incubation (Figure 3).

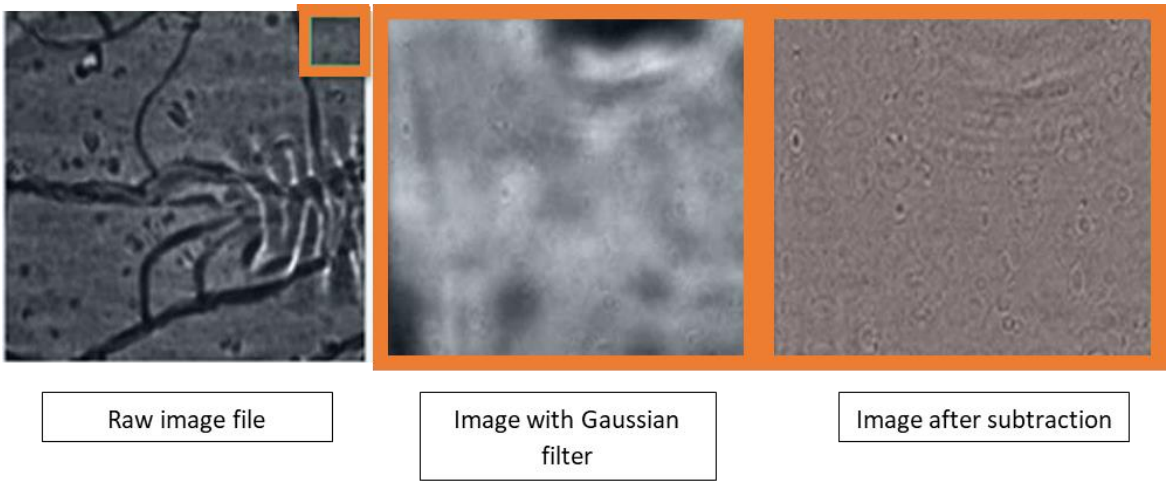

Figure 3: The image below shows an example of how a raw image is processed in order to eliminate artefacts by applying the Gaussian filter. The bold black lines seen in the raw image file is due to the folding of the film and the artefacts. In this figure a part of the image is magnified and the Gaussian filter enables smoothening of the raw images. A second image processing script further subtracts background disturbance and generates a final image. As the incubation time increases, the grey level in the image increases indicating bacterial growth.

As VITEK EVO is a prototype incorporating an optical module in the VITEK®2 instrument, it was possible to make necessary modifications in the system such as increasing the incubation time and ascertaining parameters such as the number of images taken per well, the camera position for imaging, the antibiotic wells that need to be focussed on etc. VITEK EVO allowed us to have 2D as well as 3D graphs after image processing which gave a complete overview of growth of fastidious *P. aeruginosa* in the wells (Figure 4).

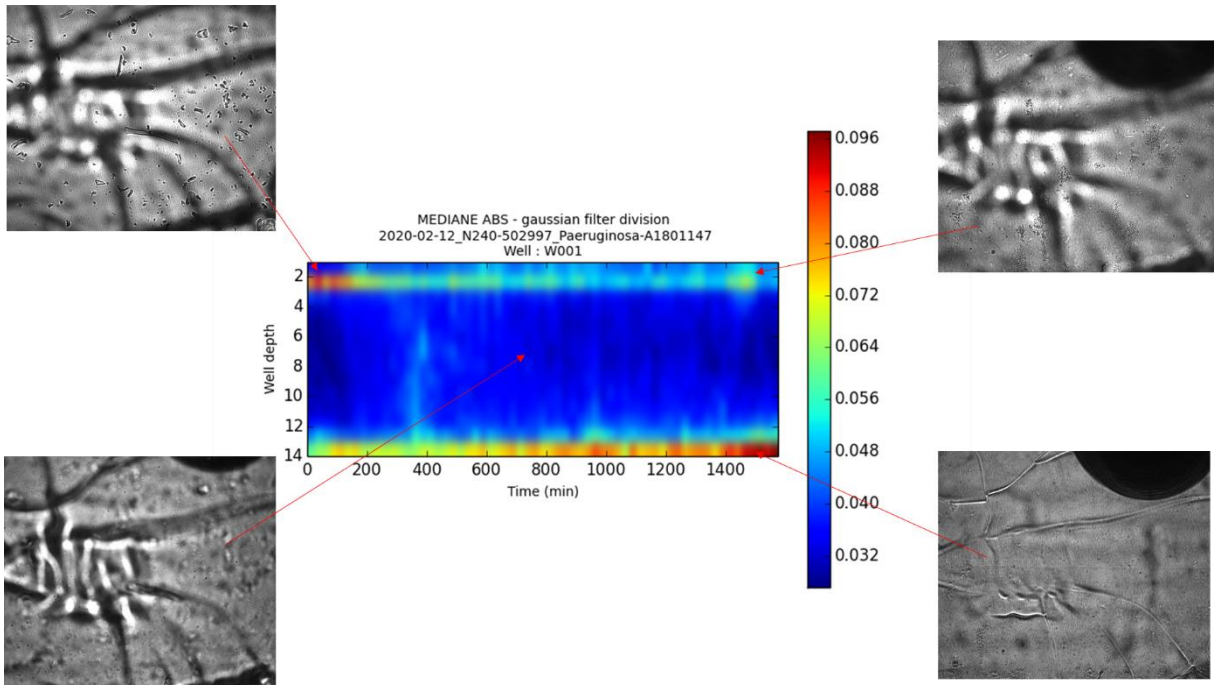

Figure 4: The figure illustrates a representative 2D graph/representation obtained for isolate API: 1801147. The graph shows bacterial growth dynamics within the control well number 1of the AST N-240 card. The images depict the bacterial growth at different positions in the well (positions shown by red arrows) and different time points during the course of the run. These images enable us to see an increase in the level of grey in the images over time until the image finally darkens indicating dense bacterial growth.

VITEK EVO Imaging also allows the observation of different growth behaviours of bacteria occurring within the wells of the cards. It was thus, for example, possible to obtain images showing planktonic growth and/or growth occurring in even or inhomogeneous clusters on the sealing films. Through previous analyses, we recorded that the isolates obtained from patients with CF were not only slow growing but were also mucoid, which pointed towards the possibility that the isolates were biofilm producers. The biofilm assay with crystal violet revealed that all of the isolates from the patients produced biofilm as compared to that of the (negative) quality control strain ATCC: 27853 (API: 1402224) (Figure 5).

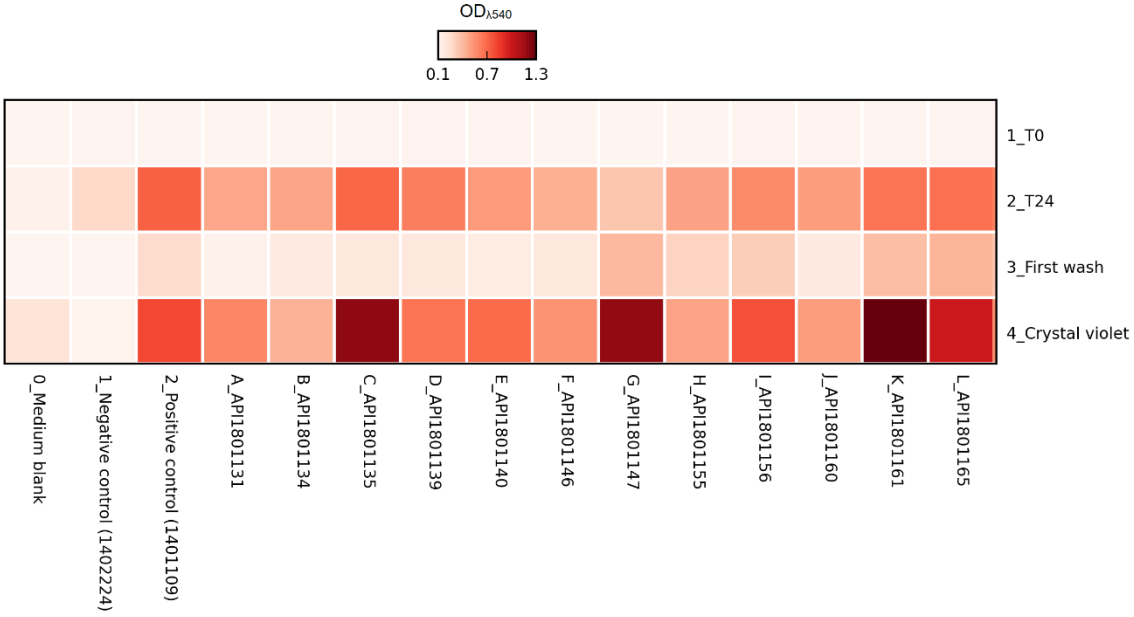

Figure 5: Heatmap showing the results of the crystal violet assay. All isolates from patients with CF included in the current study were capable of producing biofilm. API:1402224 (*P. aeruginosa* strain not capable of forming biofilm) and API: 1401109 (*P. aeruginosa* strain capable of forming biofilm) were used as negative and positive biofilm controls respectively. Readings were taken using TECAN Infinite M200 (Austria) microplate reader at different time points in order to monitor the growth changes during the various time points. At T0 the growth medium was inoculated with *P. aeruginosa* strains (row 1_T0) and at T24 the OD readings were also noted (row 2_T24). After the first wash using 0.8 g% saline (row 3_First wash), all the planktonic cells were removed and only biofilm was left in the wells. Dyeing with crystal violet allowed us to visualize biofilm which was adhered to the walls of the wells (row 4_Crystal violet).

The VITEK EVO technology helped us to visualize the effect of antibiotics on the growth of the strains and also showed bacterial growth near the sides of the wells which could be biofilm (as shown in Figure 6).

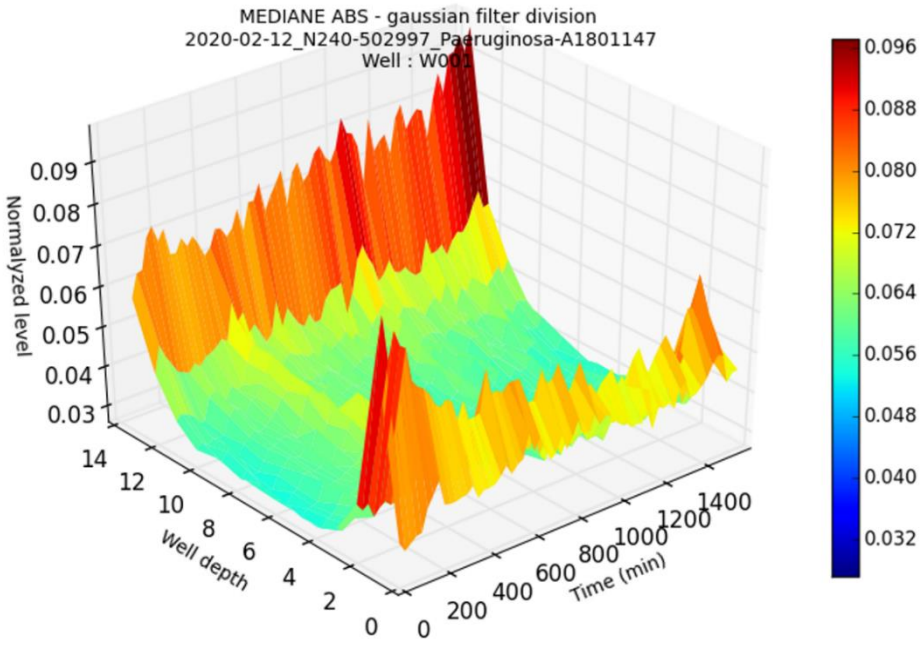

Figure 6: Example of a 3D graph built using two scripts. The 3D curve shows: the time on the abscissa, the position on the ordinate as well as the magnitude of the median light intensity. The latter is represented by a colour scale. The lowest level (0.00) corresponds to blue, while the highest level (0.20) is symbolized in red. Similar graphs were constructed for all the tested isolates.

Finally, the imaging technology of the VITEK EVO allows direct visualization of the impact of antibiotics on bacterial growth. It is thus possible to observe the impact of antibiotics on bacteria, not just under the action of ATM and IMI for example, but change in bacterial growth pattern in the presence of antibiotics of other families can also be observed. An absence of growth, where bacteria can no longer divide in the presence of bactericidal molecules or bacteriostatic molecules, is also observed.

Isolate number	VITEK EVO	
	Aztreonam	Imipenem
API:1801131		
API:1801134		
API:1801135		
API:1801139		
API:1801140		
API:1801146		
API:1801147		
API:1801155		
API:1801156		
API:1801160		
API:1801161		
API:1801165		
API:1401109		
ATCC:27853		

Figure 7: The figure represents the capability of VITEK EVO to predict the effect of the antibiotics ATM and IMI on the bacterial growth in the VITEK® 2 card wells as compared to the results recorded by the ETEST® assay (used for determination of reference MICs). The white cells represent that the imaging analysis did not give results that were concordant with the MICs obtained through ETEST. The blue cells represent that the image analysis predicts the MICs of antibiotics which is concordant with the results obtained through ETEST. The red circles indicate the strains being intermediate or resistant category according to the CLSI guidelines while the yellow circles indicate that the strains are susceptible to the studied antibiotic/s when checked with ETEST.

VITEK EVO Imaging technology can be used in addition to transmittance measurements normally performed in the VITEK® 2 instrument. The analysis of holographic images will ensure that AST results are obtained adequately, thus allowing optimization and performance expansion of VITEK® 2. It is possible to visualize the growth through images within the wells of the cards and identify certain growth behaviours that may be specific to a species or isolate of bacteria or the presence of a particular antibiotic (Figure 8A and 8B at the end of manuscript). Lack of biofilm destruction for instance could be included as a counterargument for using the antibiotic involved for treatment of the patient from whom the strain originated. As VITEK EVO is a proof of principle prototype, it allows to change the parameters like incubation time, number of images taken, the duration between each image taken and the antibiotics that need

to be focused on. Extensive optimization is still warranted, with more microbial species, larger numbers of strains and additional antimicrobials to be included. The system could be an interesting option for analysing the efficacy of new anti-biofilm compounds. VITEK EVO uses the same instrument as VITEK 2 which is already in the market but with an added camera feature which makes the instrument novel and interesting. The camera allows one to take multiple images on per well basis at different depths of the wells. The data are well interpretable and aligned with alternative testing formats (including ETEST). Additionally, VITEK EVO can help to predict the growth patterns, biofilm producing capabilities and resistance profiles of the test bacteria.

VITEK EVO is still in its infancy and has a long way to go before it serves as a state-of-the-art imaging technology system. It is not yet a user-friendly technology as it uses complicated image processing and data analysis steps which demand technical expertise. Moreover, a limited panel capacity and the high cost of instruments and consumables as compared to conventional testing methods/ assays, are all significant issues that restrict this system from rapid development (Khan *et al*., 2019; Syal et al., 2017). This been said, we acknowledge the fact that we tested a limited number of strains, which were atypical clinical isolates and were multi-drug resistant. As we chose to take 14 images per well, it restricted the possibility of testing more strains in one run. If one/ two images per well are taken, it can enable the user to test more than 6 strains per run and panel capacity can be increased.

Atypical biofilm producing strains further complicate our understanding with respect to result acquisition and reliability of the results obtained and hence it would be unfair to demerit this system due to sample size limitation. The fact that this prototype can be used to estimate resistance status and biofilm production of atypical nature of the bacterial isolates makes it a useful and novel technology.

Acknowledgement

The authors wish to thank Master's thesis intern Jade Loiseau for her preliminary experiments with the VITEK EVO prototype which served as a base and lead to functional development in the prototype.

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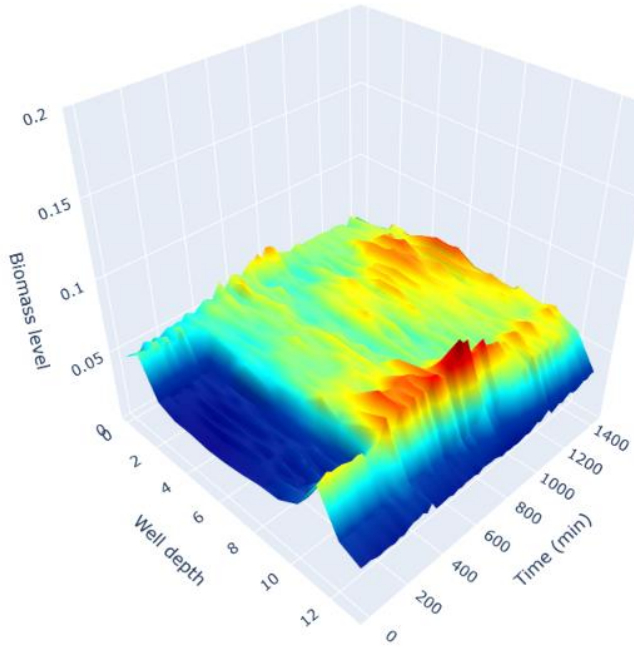
MEDIANE ABS - gaussian filter division
2020-02-26_N240-502978_Paeruginosa-A1801134
W006

W006



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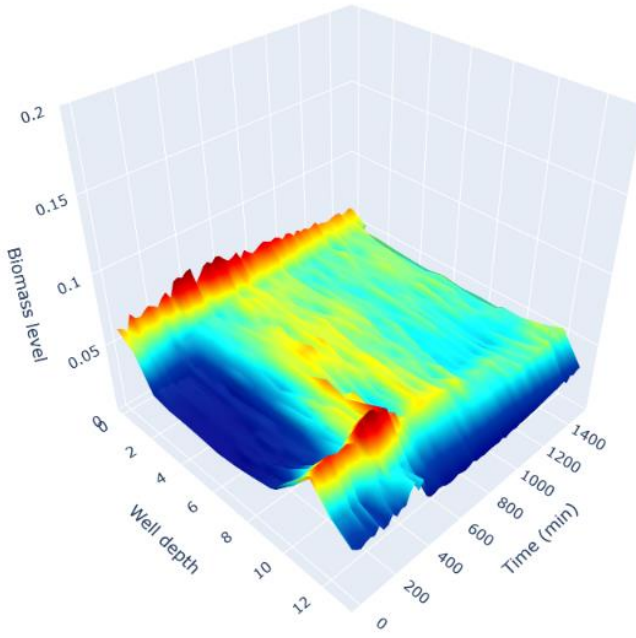
2020-02-26_N240-502978_Paeruginosa-A1801134
W007

W007



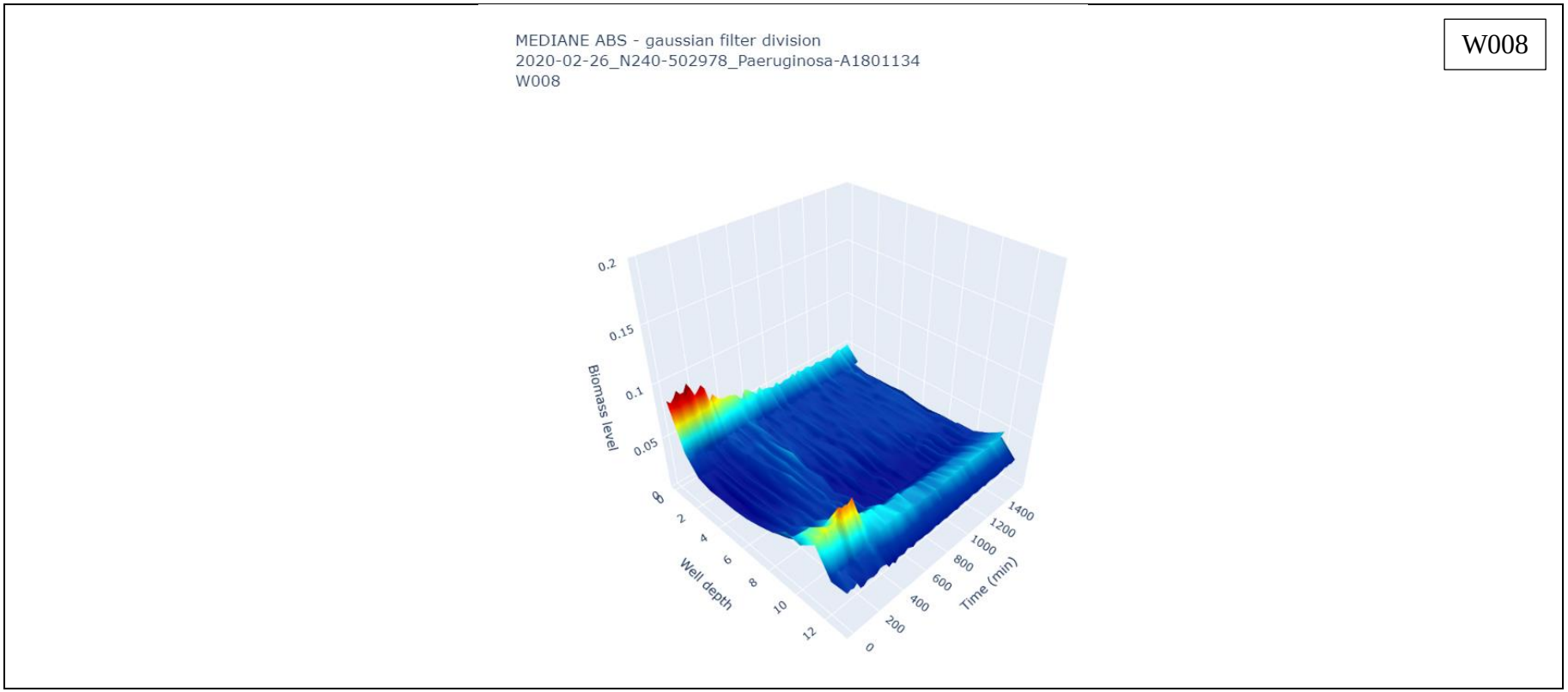


Figure 8A: The figure depicts the 3D graphs of each well containing aztreonam and the bacterial growth evolution over time in the well for API: 1801134 which was obtained from patient with CF. The reference MIC was obtained using ETEST and was 32 $mg.l^{-1}$ which corresponds to less growth as seen in the third 3D curve (W008) which contains the same concentration of antibiotic.

Wells	antibiotic	Concentration ($mg.l^{-1}$)
W006, W007, W008	aztreonam	2, 8, 32

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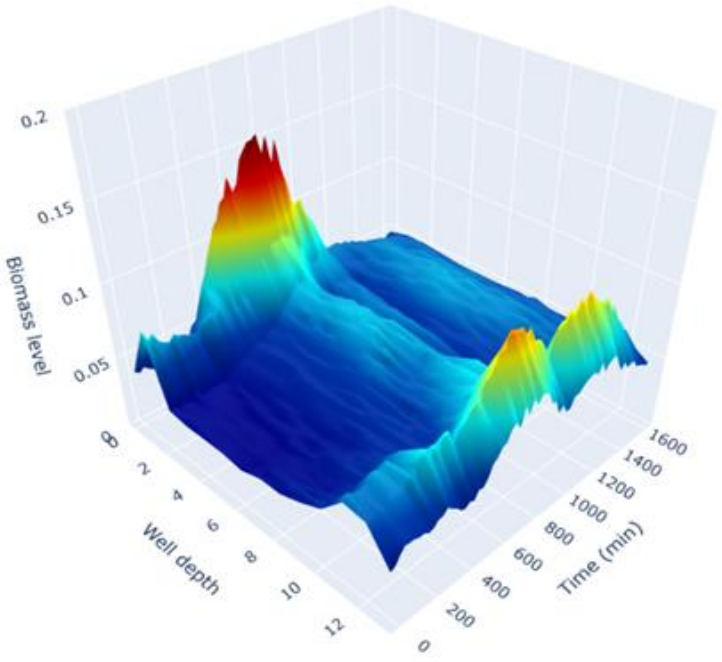
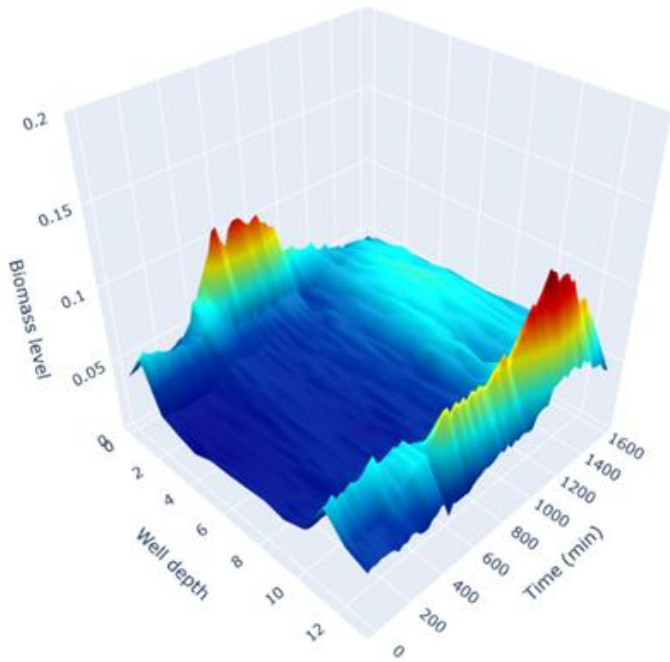
W026

W026



MEDIANE ABS - gaussian filter division
2020-03-04_N240-503183_Paeruginosa-A1801131
W027

W027



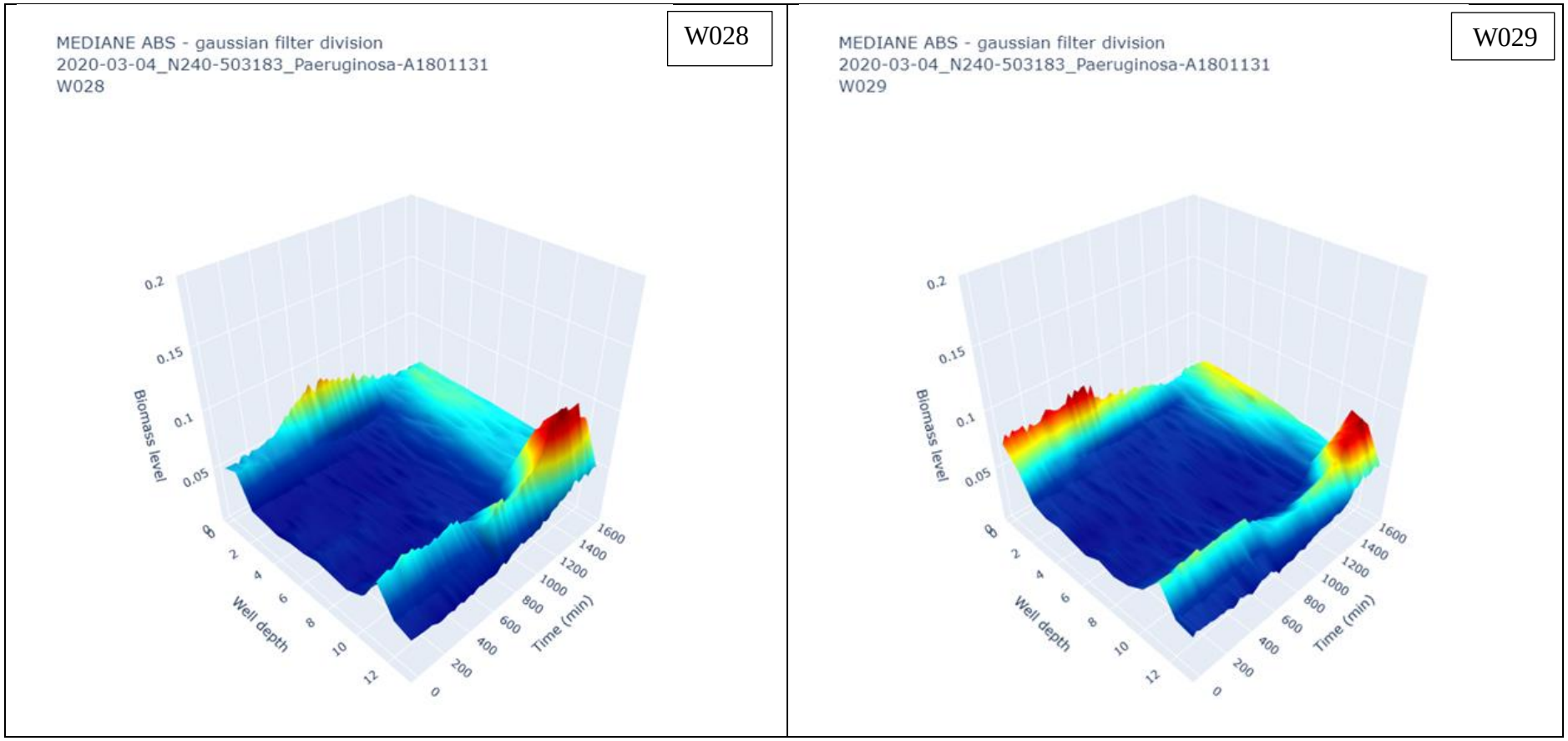


Figure 8B: The figure depicts the 3D graphs of each imipenem well and the bacterial growth evolution over time in the well for API: 1801131 which was obtained from patient with CF. The reference MIC was obtained using ETEST and was >32 mg.l^{-1}. The bacterial growth in the well does not look reduced as the maximum concentration of imipenem in the wells is 12 mg.l^{-1}.

Wells	antibiotic	Concentration (mg.l^{-1})
W26, W27, W28, W29	imipenem	1, 2, 6, 12

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Automated antimicrobial susceptibility testing of slow-growing *Pseudomonas aeruginosa* strains in the presence of tetrazolium salt WST-1

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ABSTRACT

Slow growing, mucoid isolates of *Pseudomonas aeruginosa* require adaptation of the protocol used for automated antimicrobial susceptibility testing (AST). In the present study we used a water soluble tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) in combination with menadione for possibly improving AST of slow growing and biofilm-forming *P. aeruginosa* isolates from cystic fibrosis (CF) patients. WST-1 and menadione addition ensures sensitive detection of microbial growth increase in the presence of antibiotics that may remain undetected with the automated VITEK® 2 method. We observed that 32.8% of *P. aeruginosa* isolates from CF and bronchiectasis patients produced an elevated absorbance signal intensity thereby increasing the sensitivity while maintaining the accuracy of VITEK 2. Our study merits future investigation with other slow growing pathogenic bacterial species.

1. Introduction

Cystic fibrosis (CF) is a life altering disease caused by an inherited genetic disorder mainly affecting Caucasians. In patients with CF, an increased amount of mucus is secreted that potentially blocks the airways. This modified environment promotes infections by opportunistic bacterial species, especially *Pseudomonas aeruginosa*. Further, chronicity of CF lung infection due to *P. aeruginosa* co-depends on its ability to form biofilms that can effectively coat mucosal surfaces. The biofilms provide conditions for bacterial persistence, as embedded sessile bacteria are inherently more difficult to eradicate than planktonic bacteria. Moreover, the molecular mechanisms underlying the *P. aeruginosa* biofilm-associated pathogenesis and the host response to *P. aeruginosa* biofilms remain underexplored (Maurice et al., 2018). Lung transplantation (LTx) remains as a last resort for patients with advanced lung infections due to the limited efficacy of conventional antibiotic therapies. The total number of adult CF patients undergoing LTx has increased by 23.8% within a time-span of 5 years (Benden et al., 2019). These care measures contribute to a significant increase in the costs of CF-related hospitalization (Van Gool et al., 2013; Perkins et al., 2021).

During the course of lung transplantation (i.e., before, during and after LTx), the patients are exposed to numerous drugs. For infection management, it is necessary to select the type of antibiotics and their dosage on the basis of phenotypic antimicrobial susceptibility testing (AST) of recognized pathogens. Currently, there are different automated AST systems available in the market namely VITEK® 2 (bioMérieux SA, Marcy L'Etoile, France), Phoenix (Becton Dickinson Diagnostics, Sparks, MD, USA), MicroScan (Beckman Coulter, Brea, CA, USA) and Accelerate Pheno system (Accelerate Diagnostics, USA) to assess the response of microorganisms against a variety of antibiotics (Zhou et al., 2018) with potential limitations in case of atypical bacteria such as *P. aeruginosa* from patients with CF (Burns et al., 2001).

When automated AST is performed, major errors in minimum inhibitory concentration (MIC) prediction are observed in the case of slow growing bacteria (Denton et al., 2010). The errors can be identified by comparing the MICs obtained by testing using automated technology such as VITEK® 2 and the results obtained from the traditional culture-based susceptibility testing assays such as ETEST or broth microdilution. According to the report of a European Quality Assessment survey conducted in 2007, the discrepancies in the results obtained from the various AST methods are more frequent when testing mucoid *P. aeruginosa* strains (Denton et al., 2010). Hence, it is important to improve and validate the current AST systems to obtain more reliable data that are in agreement with the AST results from the conventional

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assays. It is necessary to standardise AST protocols especially for slow growing, mucoid *P. aeruginosa* strains from CF patients as that impacts optimal selection of therapy.

In this study we aimed to determine the AST profiles of slow growing, biofilm forming *P. aeruginosa* isolates obtained from patients with CF who underwent LTx. We examine the response to the antibiotics used in automated AST technology, VITEK 2. Further, we assessed the AST profiles of these *P. aeruginosa* strains in the presence of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), a chromogenic tetrazolium salt substrate for growth monitoring, that might assist the detection of cell growth using the VITEK 2 system (Hashimoto et al., 1964, Ishiyama et al., 1993, Meletiadis et al., 2001, Singh et al., 2011, Cen et al., 2018). WST-1 was specifically chosen as this is a commercially available tetrazolium salts. Its biological reduction generates a water soluble coloured compound, well adapted for kinetic follow-up in culture broth.

2. Materials and methods

2.1. Sample collection and identification of isolates

Thirty six slow growing *P. aeruginosa* isolates were obtained at different time points from six patients who underwent LTx at Freeman hospital in Newcastle upon Tyne, United Kingdom. The isolates were retrieved during the course of LTx (before LTx, time of LTx and post LTx) in the years 2006–2017. During each of these time intervals, sputum samples and/or broncho-alveolar lavage (BAL) samples were collected and *P. aeruginosa* isolates were obtained after plating on Columbia agar +5% sheep blood (COS agar) where visible colonies were observed on the plates after more than 26 h of incubation at 37 °C and they showed a clearly mucoid phenotype (Datar et al., 2021). These thirty six isolates constitute Panel 1 which looked atypical when compared to the other panel of strains (Table 1). In addition to this, a panel of *P. aeruginosa* reference strains (Panel 2) was selected from the culture collection at bioMérieux ($n = 13$). Panel 2 consisted of strains that were acquired from clinical settings but not from patients with pulmonary infections nor CF. The strains were isolated from blood, urine or pus samples (Table 1). The strains in Panel 2 served as comparatively faster growing isolates as visible colonies on COS agar plates were seen after incubation at 37 °C within 20 to 24 h. The strains from Panel 2 were also non-mucoid controls and their AST profiles were previously determined using VITEK® 2 (as indicated in supplementary Table 1). Two strains viz. API:1402224 (corresponding to ATCC 27853) and API:1401109 were used as process controls throughout the course of all experiments. The former is a weak biofilm-forming CLSI / EUCAST quality control strain while the latter strain was able to form biofilm.

All strains from glycerol stocks were revived by sub-culturing them on COS agar at 37 °C for 24 h. The species identity of the strains was confirmed by MALDI-ToF MS (Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry) on VITEK® MS (bioMérieux).

2.2. Determination of MICs using VITEK 2

The 36 isolates from Panel 1 were sub-cultured on COS agar plates and incubated at 37 °C for 24 h prior to the analysis using VITEK 2 (v7.01). Bacterial suspensions equivalent to a 0.5 McFarland turbidity standard were prepared using saline. Three ml suspensions equivalent to 10^7 CFU ml^{-1} were loaded into the VITEK 2. AST 240 cards were used for testing the strains against an array of antibiotics. VITEK 2 AST cards comprise 64 wells, each well contains growth medium and various concentrations of different antibiotics, except for the PC well, which contains only growth medium. The AST 240 card allows for testing of non-fermenting, Gram-negative bacteria including *P. aeruginosa*. In this study we have focussed on six antibiotics viz. amikacin (AN), aztreonam (ATM), ciprofloxacin (CIP), imipenem (IPM), levofloxacin (LEV) and tobramycin (TM), belonging to three different antibiotic families that

Table 1
The 36 *P. aeruginosa* isolates (Panel 1) represented on the basis of the patient number, time of isolation and specimen type. The 13 non cystic fibrosis *P. aeruginosa* strains (Panel 2) were clinical isolates obtained from pus, urine or hemoculture for comparison (on the grounds of time to result using VITEK 2 and their ability to form biofilm) with the isolates from Panel 1.

Panel	Isolate number	Country	Patient number	Time	Specimen type
Panel 1	API:1801131	UK	1	4 m BTx	BAL
	API:1801132	UK	1	TTx	BAL
	API:1801133	UK	1	6 m PTx	BAL
	API:1801134	UK	1	10 m PTx	BAL
	API:1801135	UK	2	1Y BTx	BAL
	API:1801136	UK	2	4 m BTx	BAL
	API:1801137	UK	2	TTx	BAL
	API:1801138	UK	2	TTx	BAL
	API:1801139	UK	2	6 m PTx	BAL
	API:1801140	UK	3	11 m BTx	BAL
	API:1801141	UK	3	11 m BTx	BAL
	API:1801142	UK	3	TTx	BAL
	API:1801143	UK	3	TTx	BAL
	API:1801144	UK	3	7 m PTx	SPUTUM
	API:1801145	UK	3	7 m PTx	SPUTUM
	API:1801146	UK	3	10 m PTx	BAL
	API:1801147	UK	4	3 m BTx	BAL
	API:1801148	UK	4	3 m BTx	BAL
	API:1801149	UK	4	3 m BTx	BAL
	API:1801150	UK	4	3 m BTx	BAL
	API:1801151	UK	4	3 m BTx	BAL
	API:1801152	UK	4	TTx	BAL
	API:1801153	UK	4	TTx	BAL
	API:1801154	UK	4	TTx	BAL
	API:1801155	UK	4	3.5 m PTx	BAL
	API:1801156	UK	5	1Y BTx	BAL
	API:1801157	UK	5	1Y BTx	BAL
	API:1801158	UK	5	8 m BTx	BAL
	API:1801159	UK	5	TTx	BAL
	API:1801160	UK	5	5 m PTx	BAL
	API:1801161	UK	6	2 m BTx	BAL
	API:1801162	UK	6	TTx	BAL
	API:1801163	UK	6	6 m PTx	BAL
	API:1801164	UK	6	6 m PTx	BAL
	API:1801165	UK	6	12 m PTx	BAL
	API:1801166	UK	6	12 m PTx	BAL
Panel 2	API:1302348	Senegal	NA	NA	PUS
	API:1302344	Senegal	NA	NA	URINE
	API:1302334	Senegal	NA	NA	PUS
	API:1301111	Senegal	NA	NA	PUS
	API:1302279	France	NA	NA	HEMOCULTURE
	API:1302278	France	NA	NA	HEMOCULTURE
	API:0804057	Unknown	NA	NA	HEMOCULTURE
	API:0603105	Unknown	NA	NA	URINE
	API:0409124	Unknown	NA	NA	URINE

(continued on next page)

Table 1 (*continued*)

Panel	Isolate number	Country	Patient number	Time	Specimen type
	API:9704002	Unknown	NA	NA	URINE
	API:9001094	France	NA	NA	URINE
	API:7906033	USA	NA	NA	HEMOCULTURE
	API:0212022	Unknown	NA	NA	HEMOCULTURE

BAL, Bronchoalveolar lavage; NA, Not applicable; BTx, Before Lung transplantation; TTx, Time of lung transplantation; PTx, Post lung transplantation; m = month, Y = year; API, bioMérieux culture collection number.

are used frequently for treatment of *P. aeruginosa* infections. CLSI guidelines were followed to interpret the susceptibility profiles of the strains from both panels (Supplementary table 1). The strains were classified as resistant, intermediate or susceptible based on the CLSI breakpoints (CLSI, 2017). Growth curves of these strains were determined at 600 nm, as per the usual VITEK 2 protocol.

2.3. Determination of reference MIC by ETEST®

ETEST® (bioMérieux, Marcy L'Etoile) strips were applied on Mueller Hinton E agar plates according to manufacturer's guidelines to test the six antibiotics used in the study for MIC determination. The plates were incubated at 37 °C for 20–24 h and the elliptical zone of clearance was observed. The point of inhibition of growth that intersected with the strip was recorded as MIC of that antibiotic. However, when an asymmetrical zone of inhibition was observed, the highest MIC value was recorded. ATCC 27853 was used as an experimental control.

2.4. Addition of substrate WST-1

WST-1 and menadione (Sigma Aldrich) were used at concentrations of 125 μM and 5 μM, respectively (Supplementary table 2). Menadione promotes electron transfer to enhance the reduction of WST-1 (Stapelfeldt et al., 2017). Both compounds were added to the 10^7 CFU ml^{-1} bacterial suspension in order to increase absorbance level and thereby facilitate detection of bacterial growth using the VITEK 2 system. Bacterial growth was monitored at 430 nm (absorbance peak of product of WST-1 reduction) using VITEK 2, to assess the effect of the substrate on the growth detection of strains from both the panels. The growth curves depicting the effect of WST-1 were plotted and compared to the growth curves from VITEK 2 runs without WST-1 and menadione at 600 nm.

2.5. Bioinformatic analysis

Growth curve graphics were generated in Python (v3.5.4) using the Matplot library (v3.0.3). Each graph gathered depicts the VITEK® 2 growth curves of the strains of interest for specific antibiotics. Each VITEK 2 well contains a growth medium and a specific concentration of this antibiotic, except for the reference control well that contains growth medium only. The legend of each well, following XX_YYY_ZZ pattern, means that the well is at position XX in the VITEK 2 64 well cards, contains YYY antibiotic (or PC for Positive Control well), at ZZ (concentration in mg l^{-1}).

3. Results and discussion

3.1. Identification and AST

The identification of the bacterial strains from Panel 1 and 2 using VITEK MS analysis confirmed that the strains were *P. aeruginosa*. The investigation revealed that the isolates from Panel 1 were slow growing (colonies were observed after 26 h of incubation at 37 °C on COS agar plates) and mucoid in nature. The full AST profile of some *P. aeruginosa* isolates belonging to Panel 1 could not be determined due to insufficient growth during the run time of ~18 h of the VITEK 2 analysis indicated as terminated results (TRM). However, in case of Panel 2 the AST profiles of all the strains could be obtained at the end of the routine VITEK 2 analysis (Supplementary table 1). This indicates that for the VITEK 2 system to detect the inhibition of some isolates from Panel 1 by antimicrobials, the achieved readings in changes of absorbance were not significant enough to assess antibiotics sensitivity. As we could not obtain MIC values of the six antibiotics used in the study using VITEK 2, we used ETEST for determining MICs that served as a reference (Supplementary table 3). The unusual behaviour of Panel 1 isolates, with a high proportion of "no results" with the standard VITEK 2 AST protocol lead us to use this set of isolates, with Panel 2 as a control set, to test an alternative protocol. Even though the isolates were obtained at different time points, they had similar phenotypes and morphotypes. Hence, we did not relate the results to the timing of isolation and focussed only on improving the possibility of obtaining the MICs within the duration of the standard protocol.

3.2. Addition of substrate

Addition of tetrazolium salts for improving AST has been long reported; Meletiadis et al., 2020, Mahmoud et al., 2019, Aguiar et al., 2017, van de Sande et al., 2005, Ahmed et al., 2004 etc. have incorporated tetrazolium salts in various colorimetric assays to improve the prediction of viable cell counts. These tetrazolium salts can be rapidly reduced by the NAD(*P*)H in living cells and turn into orange/ yellow colour to enhance the absorbance signal (Zhang et al., 2016).

In this study in order to improve the AST profiling of slow growing, biofilm forming *P. aeruginosa* strains using VITEK 2, WST-1 and menadione were added to the suspension of the bacterial strains from both Panel 1 and Panel 2. Those compounds had no impact on growth, evaluated by kinetic measurement of OD 600 nm (Supplementary Fig. 1). Further, this combination of substrates improves the sensitivity and leads to detection of growth with a stronger signal and hence, improves AST profiling as for the isolate API:1801150 (test isolate from Panel 1). For this isolate, the VITEK 2 run without substrate was terminated without MIC values for the antibiotics in the panel. However, after addition of WST-1 and menadione, an increased absorbance was noted. The OD at 430 nm shows elevation that indicates that the bacterial growth detection was better with addition of the substrate. API:1801150 isolate, initially terminated during the VITEK 2 run, could emit more signal with addition of substrate (Fig. 1).

3.2.1. Effect of WST-1 + menadione on determination of susceptibility to aminoglycosides

In case of amikacin, we observed that the signal intensity for most initially terminated isolates i.e. API:1801132, API:1801133, API:1801134, API:1801150, API:1801156, API:1801157 and API:1801158 was enhanced by reduction of WST-1 + menadione at sub-inhibitory concentrations of the antibiotic. However, API:1801131, API:1801148, API:1801151, API:1801154, API:1801159 and API:1801161 failed to further grow significantly even in the Positive Control (PC) well and hence for these isolates the addition of substrate does not improve MIC prediction (representative graphs in Fig. 2). In the case of amikacin the signal intensity improved in 31.8% isolates (Fig. 3). When the isolates were tested against the other aminoglycoside in the panel (tobramycin), the signal intensity could not be enhanced further for as many isolates except for API: 1801132, API: 1801150 and API: 1801157 (Fig. 3). The isolates were found to undergo a 0.12 fold rise in the signal intensity on addition of WST-1.

3.2.2. Effect of WST-1 + menadione on determination of susceptibility to β-lactam antibiotics

When the isolates were tested against imipenem in the presence of WST-1, for 55.5% of the isolates from Panel 1 higher signal intensity could be recorded by VITEK 2. WST-1 addition was found to be effective

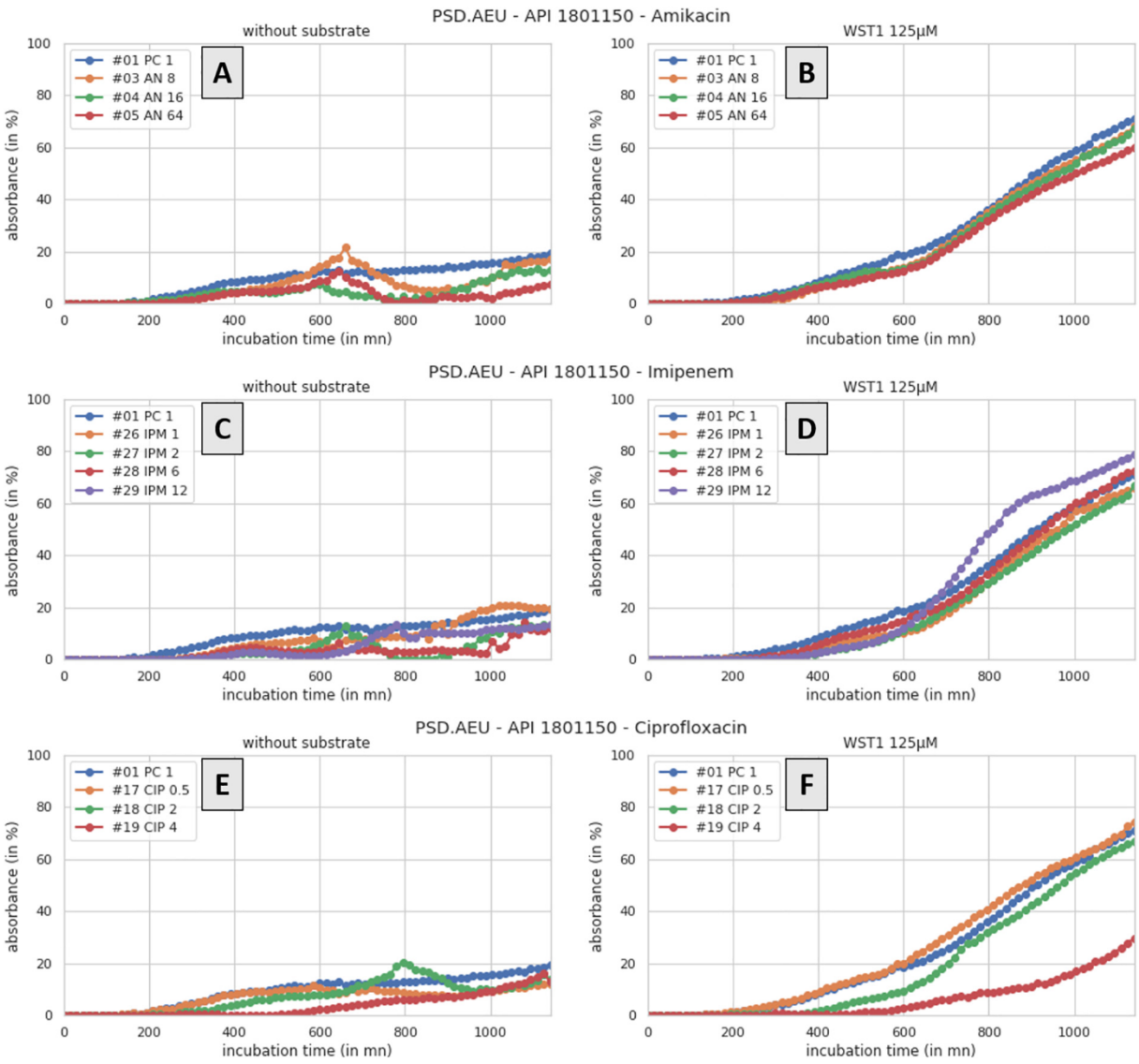

Fig. 1. Effect of addition of WST-1 + menadione on the terminated (TRM) *P. aeruginosa* isolate from CF patient when tested with antibiotics from three different drug families. Growth kinetics *P. aeruginosa* strain API:1801150 assessed in VITEK 2, in the presence tetrazolium salt (WST-1) in combination with menadione (B), (D) and (F) at 430 nm and in their absence (A), (C) and (E) at 600 nm.

in 10 out of 36 isolates while API:1801131, API:1801148, API:1801149, API:1801151, API:1801152, API:1801154, API:1801161, API:1801162 were terminated in both cases (Fig. 3). Without substrate and after addition of WST-1, their growth curves remained comparable. .

Sixteen out of thirty-six isolates were terminated initially when tested against aztreonam using VITEK 2. The addition of WST-1 while analysing the TRM isolates could improve the signal in 11 out of these 16 isolates but failed to add any value to the five other TRM isolates in Panel 1 (Fig. 3).

3.2.3. Effect of WST-1 + menadione on determination of susceptibility to fluoroquinolones

When the isolates were tested with the fluoroquinolones (ciprofloxacin and levofloxacin), in the presence of WST-1 and menadione, then the signal showed substantially increased intensity. The isolates API:1801132, API: 1801133, API: 1801134, API: 1801145 and API: 1801150 emitted higher signals in the presence of WST-1 with ciprofloxacin whereas 9 out of 36 isolates showed elevation in signal with levofloxacin (Fig. 3). API:1801131 along with some other terminated isolates did not improve with or without substrate addition (Fig. 4). The result (MIC) interpretation was not altered in the case of both API: 1801156 and API:1801159 as the growth curves corresponding to LEV 8 and CIP 2 respectively did not change when recorded at 430 nm (Fig. 4). The reference MIC obtained by ETEST of these isolates for the respective antibiotics were 6 $mg.l^{-1}$ and 2 $mg.l^{-1}$ respectively.

After addition of substrates to the isolates in Panel 1, the isolates which were "terminated" under standard VITEK 2 conditions showed increase in signal intensity. As much as 32.82% of the total isolates from Panel 1 responded positively and elevated signal intensity was recorded (Fig. 3). 3.2.4 Effect of WST-1 + menadione on the strains from Panel 2.

In case of Panel 2, the signal that was detectable due to sufficient growth during the 18 h incubation time in VITEK 2 was further enhanced (Fig. 5). Further, it supports the successful detection of growth of non-mucoid and faster growing strains (colonies were observed after 20 h of incubation at 37 °C on COS agar plates) upon addition of WST-1.

In summary, the cell proliferation signal in the presence of WST-1 was more effective in case of strains from Panel 2 in comparison to the Panel 1.

We recorded that the isolates from Panel 1 were not only slow growing but also mucoid, which suggests that the isolates were biofilm

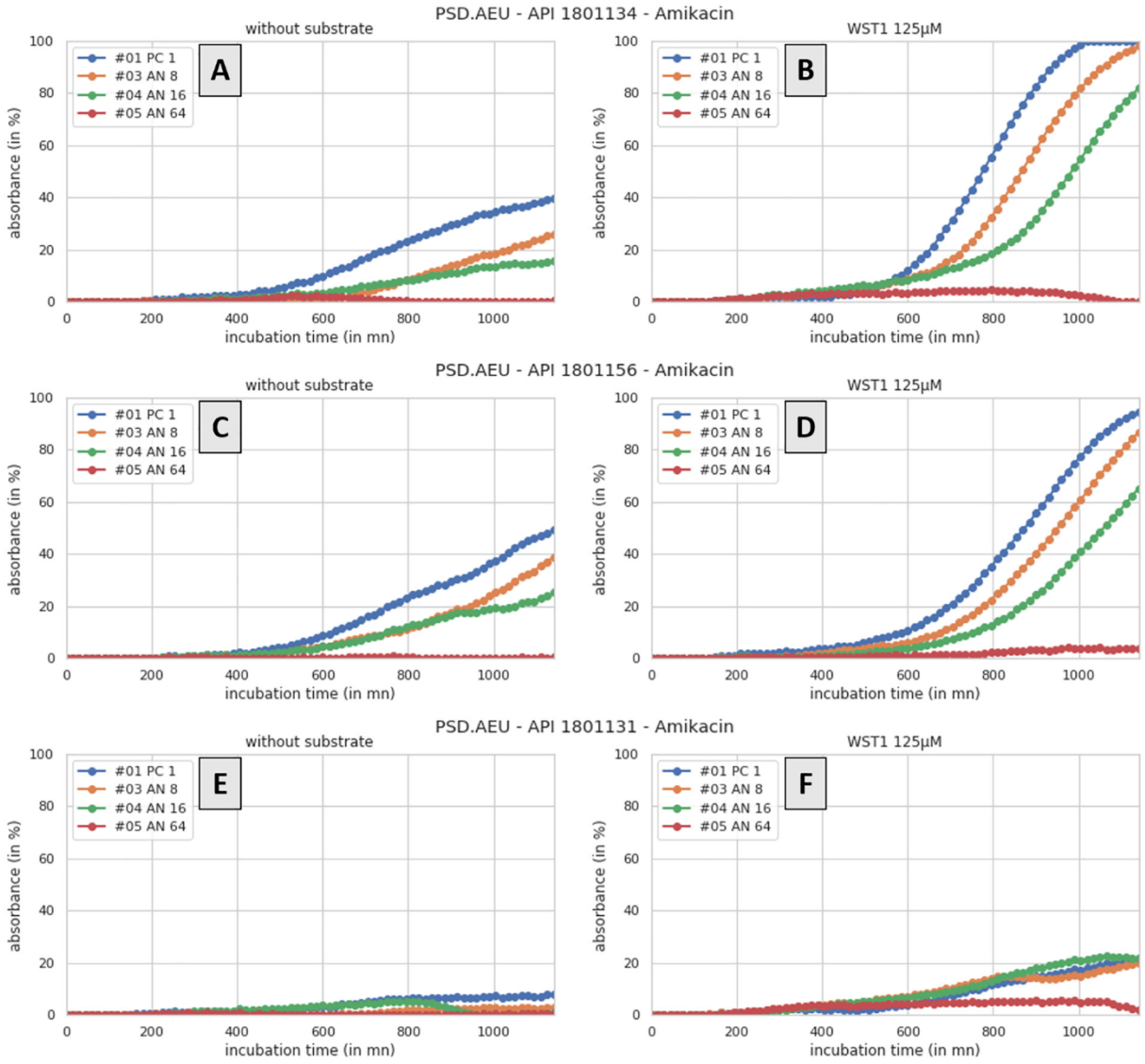

Fig. 2. Influence of WST-1 + menadione on the growth response of *P. aeruginosa* isolates from CF patients when tested with amikacin. The left panel i.e. (A), (C) and (E) represents growth in the absence of the substrate whereas right panel i.e. (B), (D) and (F) indicates growth determination in the presence of tetrazolium salt. Graph (B) and (D) shows how WST-1+ menadione can increase the signal intensity as compared to Graph (A) and (C). The graph corresponding to amikacin concentration (AN64) does not increase in Graph (B) which shows that the result interpretation is not compromised.

producers. This was supported by genomic analysis (Datar et al., 2021) and separate phenotypic analyses, which also showed that the substrate combination has no effect on the amount of biofilm produced (data not shown). Hence, the improvement in the susceptibility testing in VITEK 2, with addition of WST 1 + menadione, should not be related to an alteration of the biofilm formation.

Moreover, as automated AST fails to give results for some slow growing strains, this necessitates reliance on the traditional culture-based methods such as broth micro-dilution (which is the reference method), disk diffusion (Kirby Bauer test), antibiotic gradient diffusion (ETEST) etc. These methods provide results but as these methods are based on visual inspection, the accuracy of the results could be compromised due to error in visual interpretation. Using ETEST with biofilm forming isolates can be tricky as usually formation of the inhibition zone during agar diffusion susceptibility testing is due to a switch from planktonically-growing bacteria to biofilm-growing bacteria with increased tolerance to antibiotics (Høiby et al., 2019). More recently, a report by Kidd et al. (2018), suggested some possible shortcomings of the current culture-based AST approach. Most systems fail to replicate the in situ condition resulting in inadequate results. Feng et al. (2018) have developed a SYBR Green I/PI assay providing promising results for AST improvement. Thus, incorporation of various derivatives of tetrazolium salt in the traditional systems has been found to improve the sensitivity of AST by improving the detection of cellular proliferation (Taneja and Tyagi, 2007; Singh et al., 2011). Through their recent work, Xie et al. (2021) have emphasized the need for accurate testing of multi-drug resistant mycobacteria. Their group has recently developed another tetrazolium salt based assay for determining precise growth rate of slow growing mycobacteria in the presence of antimycobacterial drugs. As the application of tetrazolium salts is well known for testing *M. tuberculosis* strains, similar use of derivatives of tetrazolium salt (WST-1) could improve the assessment of slow growing *P. aeruginosa* strains.

4. Conclusion

In summary, the present study attempts to delineate the role of WST-1 + menadione, an indicator to help record cell growth more sensitively,

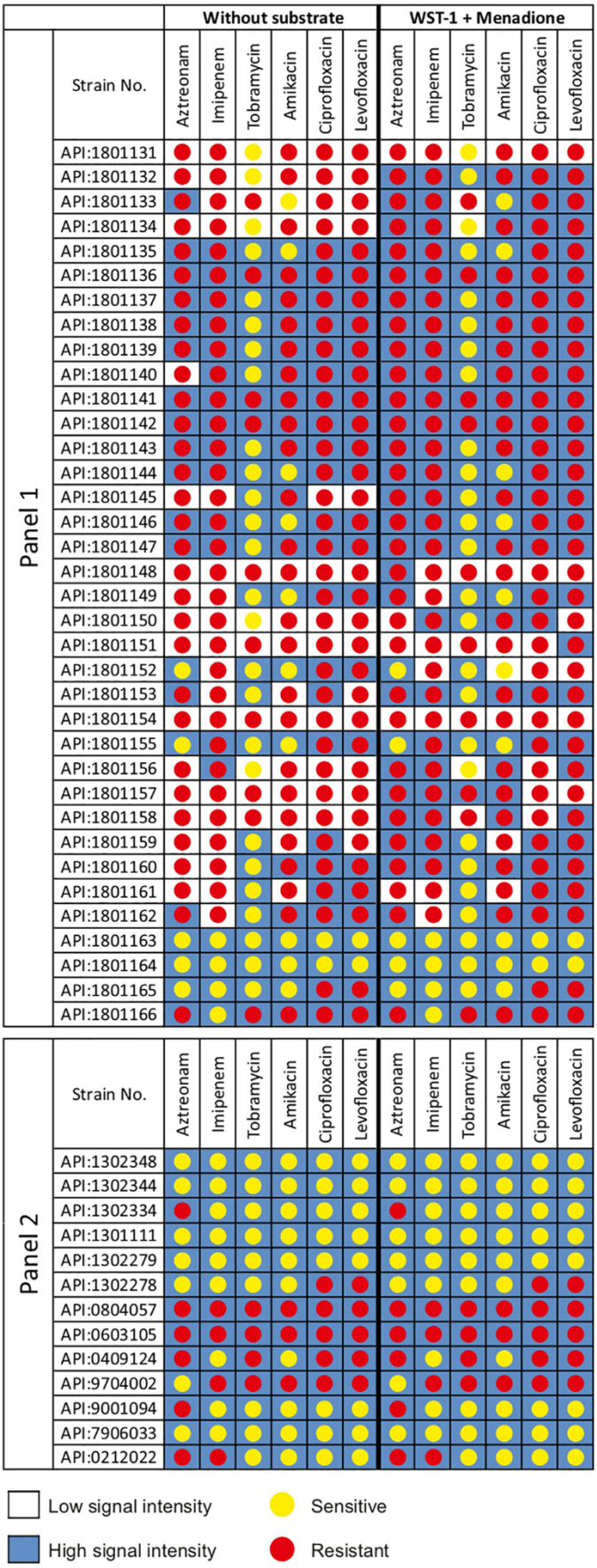

Fig. 3. Effect of addition of WST-1 + menadione on the signal absorbance detected by VITEK 2. The figure represents the effect of addition of tetrazolium salt (WST-1) in combination with menadione on the signal intensities recorded by the VITEK 2 system at 430 nm and compares it to the signal intensities recorded at 600 nm by VITEK 2 system prior to the addition of substrates. The white cells represent low signal intensity (terminated, no MIC result) while the blue cells represent increase in the signal intensity which is required for predicting the MICs of antibiotics. The red circles indicates the strains being intermediate or resistant category according to the CLSI guidelines while the yellow circles indicate that the strains are susceptible to the studied antibiotic/s. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

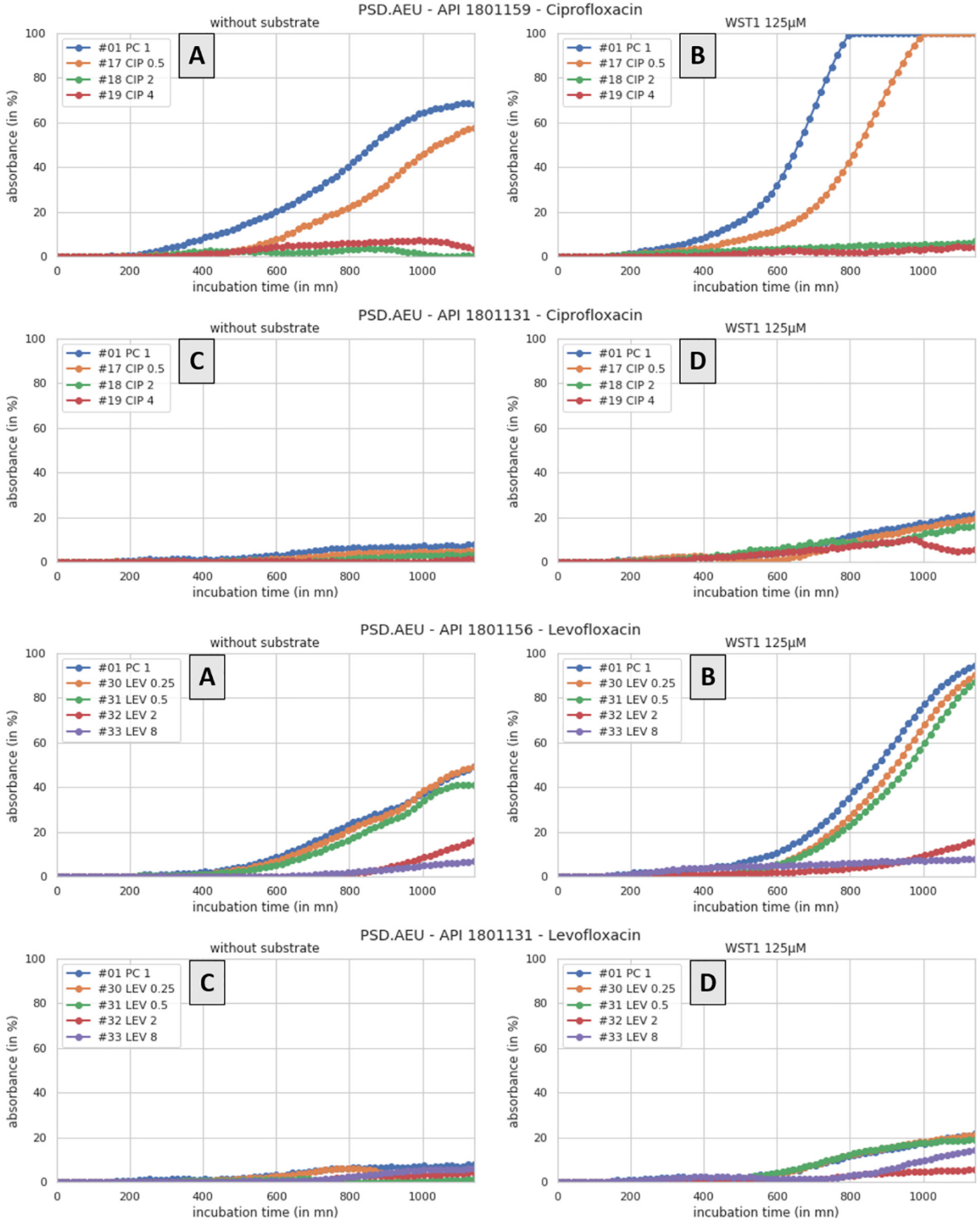

Fig. 4. A: Influence of WST-1 + menadione on the growth detection of *P. aeruginosa* isolates from CF patients when tested with ciprofloxacin. Graph (B) shows how there is improvement in growth detection by VITEK 2 system on addition on WST-1+ menadione. However, graph(D) shows how there was no additional impact on growth detection after addition on WST-1 in presence of ciprofloxacin. B: Influence of WST-1 + menadione on the growth detection of *P. aeruginosa* isolates from CF patients when tested with levofloxacin. Graph (B) shows how there is improvement in growth detection by VITEK 2 system on addition on WST-1+ menadione. However, graph (D) shows how there was no additional impact on growth detection after addition on WST-1 in presence of levofloxacin.

in improving the AST testing for slow growing *P. aeruginosa* strains. The subtle improvement in AST profiling represents a challenge due to the nature of the strains involved in the study and it further highlights the complexities associated with establishing the antibiotic susceptibility of slow growing, mucoid *P. aeruginosa* isolates. Further, results based on the traditional culture-based techniques (BMD and ETEST) could provide greater insight into the response of *P. aeruginosa* strains against the array of antibiotics but suffers the constraint of high cost, time for detection and level of expertise. Thus, innovation in the area of diagnostics could be achieved by continuously improving the existing

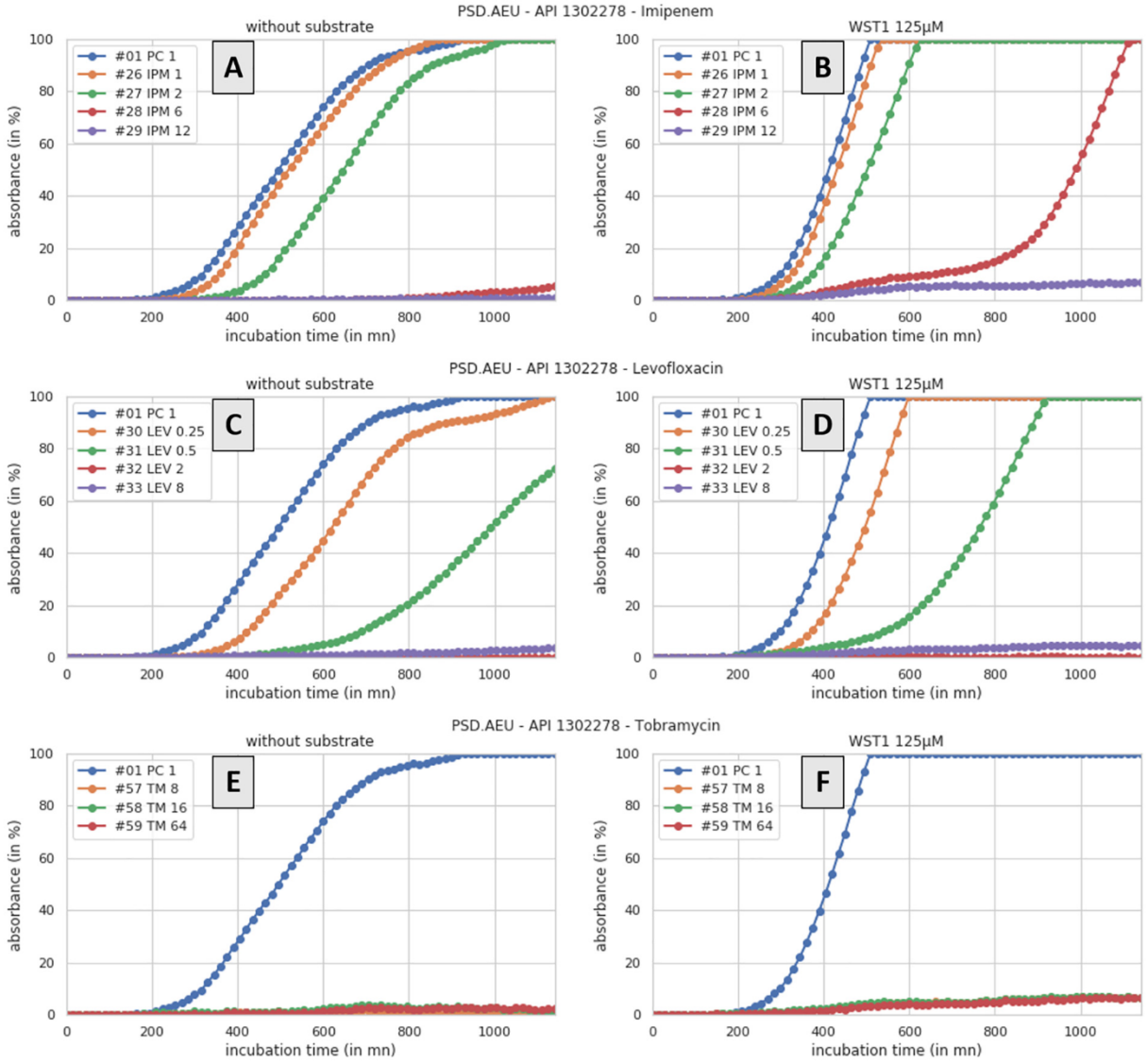

Fig. 5. Influence of WST-1 + menadione on the growth detection of non-CF *P. aeruginosa* strains. The graphs in the right column show the impact of addition of WST-1+ menadione on growth detection at 430 nm using VITEK 2.

assay models. Use of WST-1 AST profiling is a step towards characterizing the slow growers. Its transposition in routine testing demands future validations with an extensive panel of slow growing *P. aeruginosa* strains.

Author contributions

AvB, SO and VC conceived the study. JDP and AP selected *P. aeruginosa* isolates previously serially collected and gathered the metadata *P. aeruginosa* isolates from the patients serially and gathered the metadata. RD conducted the microbiological experimentation for *P. aeruginosa isolates* and analysed the data. GP assisted in the analysis of data obtained from VITEK 2. RD interpreted the results obtained and wrote the first version of the manuscript. All authors discussed the results and edited the manuscript.

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Ethical approval

All isolates examined in this study were recovered as a result of routine diagnostic procedures. No human samples were collected specifically for the purposes of this study. The researchers were supplied with samples of *Pseudomonas* from an archived frozen collection. Researchers at bioMérieux were not given access to any data that would allow identification of a particular patient. As the project did not require the use of any human material or any personal data, approval of the local ethics committee was not required.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106252.

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Phenotypic and Genomic Variability of Serial Peri-Lung Transplantation *Pseudomonas aeruginosa* Isolates From Cystic Fibrosis Patients

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Cystic fibrosis (CF) represents one of the major genetic and chronic lung diseases affecting Caucasians of European descent. Patients with CF suffer from recurring infections that lead to further damage of the lungs. Pulmonary infection due to *Pseudomonas aeruginosa* is most prevalent, further increasing CF-related mortality. The present study describes the phenotypic and genotypic variations among 36 *P. aeruginosa* isolates obtained serially from a non-CF and five CF patients before, during and after lung transplantation (LTx). The classical and genomic investigation of these isolates revealed a common mucoid phenotype and only subtle differences in the genomes. Isolates originating from an individual patient shared ≥98.7% average nucleotide identity (ANI). However, when considering isolates from different patients, substantial variations in terms of sequence type (ST), virulence factors and antimicrobial resistance (AMR) genes were observed. Whole genome multi-locus sequence typing (MLST) confirmed the presence of unique STs per patient regardless of the time from LTx. It was supported by the monophyletic clustering found in the genome-wide phylogeny. The antibiogram shows that ≥91.6% of the isolates were susceptible to amikacin, colistin and tobramycin. For other antibiotics from the panel, isolates frequently showed resistance. Alternatively, a comparative analysis of the 36 *P. aeruginosa* isolates with 672 strains isolated from diverse ecologies demonstrated clustering of the CF isolates according to the LTx patients from whom they were isolated. We observed that despite LTx and associated measures, all patients remained persistently colonized with similar isolates. The present study shows how whole genome sequencing (WGS) along with phenotypic analysis can help us understand the evolution of *P. aeruginosa* over time especially its antibiotic resistance.

Keywords: *Pseudomonas aeruginosa*, lung transplantation, antimicrobial resistance, cystic fibrosis, whole genome sequencing

INTRODUCTION

Cystic fibrosis (CF) is one of the most common and ultimately lethal autosomal recessive diseases, which primarily affects Caucasians of European descent (Cutting, 2015). Lung diseases are the main cause of morbidity and mortality among CF patients, especially those leading to respiratory failure caused by extensive lung damage, such as bronchiectasis or chronic endobronchial infections (Sanders and Fink, 2016). For patients with end-stage CF, lung transplantation (LTx) is the only viable option, as there is a 50% survival rate 10-year post-LTx (Snell et al., 2017).

The respiratory tract microbiota of CF is diverse and the most prevalent pathogens include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and non-tuberculous mycobacteria (NTM). The exact incidence for each bacterial species has changed over the past years and continues to change, sometimes according to the geographic locale of the patients (Salsgiver et al., 2016). Frequent antibiotic therapies for patients with CF tend to select for antibiotic resistant variants. Multidrug-resistant (MDR) *P. aeruginosa* is one of the major end-stage bacterial species among CF patients, as even after antibiotic treatment it frequently recurs and establishes chronic infection. Such strains also display evolution of phenotypic and genomic characteristics over time (Winstanley et al., 2016; Stefani et al., 2017).

As *P. aeruginosa* is highly versatile in its pathogenicity and has significant genomic plasticity, assessing the whole genome sequence and identifying antimicrobial resistance (AMR) genes could be very useful to gain insights into the emergence of resistance against currently used antimicrobials. Population-level analysis and comparative genomics can help us to identify the co-existence of diverse strains during chronic illnesses and how they differ genotypically (Chung et al., 2012; Celli et al., 2015). High resolution genomics can enable us to better understand the high-risk clones (HRC) and segregate them as MDR or extensively-drug resistant or pan-drug resistant (Magiorakos et al., 2012). Amongst the many *P. aeruginosa* HRC studied, sequence type (ST) 235 is the most widespread lineage across the world.

The aim of this study is to evaluate how whole genome sequencing (WGS) and bio-informatic data analysis could assist in evaluating the impact of LTx on the diversity and antibiotic resistance profile of resident *P. aeruginosa* isolates. We studied culture based phenotypes of the isolates in combination with WGS to analyze the local epidemiology, evolution and resistome of *P. aeruginosa* isolates. They were serially obtained from five CF patients and a non-CF bronchiectasis patient, who all underwent LTx. Genomic data were analyzed versus a large control panel of *P. aeruginosa* strains (n = 672) from different ecological and clinical niches.

MATERIALS AND METHODS

Sample Collection and Isolation of *Pseudomonas aeruginosa*

For the investigation of isolates from the same LTx patients, obtained at different time points, before, during and after LTx, serial peri-lung transplantation (SPLT) *P. aeruginosa* isolates, data from the six patients who underwent LTx during the years 2006–2017 were retrieved from the Freeman Hospital in Newcastle upon Tyne (United Kingdom). These patients were in the age group of 24–42 years at the time of LTx. For each patient, sampling time range from 6 to 22 months. At various intervals (i.e., before, during and after LTx) either broncho-alveolar lavage (BAL) or sputum specimens were collected for microbiological assessment at the Freeman Hospital. We selected all colony variants (mucoid, non-mucoid, large, small, etc.) isolated at a particular time interval. If only one isolate was selected for testing, then it means that all colonies of *P. aeruginosa* showed a consistent appearance. We cannot of course discount the possibility that colonies that look identical may have different phenotypes in terms of, for example, antimicrobial susceptibility. In total, 36 *P. aeruginosa* isolates belonging to five CF patients and a non-CF bronchiectasis patient, were isolated and preserved at −80°C until further analysis (**Table 1**). A sub-selection of 12 isolates, one pre- and one post-LTx from each of the six patients was used for full antibiotic susceptibility characterization (star marked in **Table 1**). Three isolates were used as quality controls throughout the study: one CLSI/EUCAST quality control strain (ATCC: 27853) and 2 isolates resistant to carbapenems by impermeability (BioMérieux collection numbers: API: 1508289 and API: 9208013).

Phenotypic Characterization of Serial Peri-Lung Transplantation *Pseudomonas aeruginosa* Isolates

Identification of SPLT *Pseudomonas aeruginosa* Isolates

The 36 *P. aeruginosa* isolates obtained were revived from glycerol stocks by streaking on Columbia agar + 5% sheep blood (COS agar) and incubated at 37°C for 24 h. The identity of the isolates was validated using mass spectrometry (Matrix Assisted Laser Desorption Ionization–Time of Flight MS) using VITEK® MS (BioMérieux).

Antibiotic Susceptibility Testing Profiles of SPLT *Pseudomonas aeruginosa* Isolates

The 36 isolates were tested for their resistance/susceptibility profile against an array of antimicrobials by disk diffusion assay (Oxoid) according to CLSI (2017) guidelines. Additionally, minimum inhibitory concentrations (MIC) of a sub-selection of 12 isolates were defined by broth micro-dilution (BMD) assays to validate the results from disk diffusion assays. The antibiotic susceptibility testing (AST) profiling by BMD involved assessment using eight antibiotics viz. amikacin (AK), ciprofloxacin (CIP), levofloxacin (LEV), tobramycin (TOB), colistin (COL), imipenem (IMP), aztreonam (ATM), and ceftolozane-tazobactam (CZT). For BMD, the antibiotics were diluted using Mueller Hinton Broth (MHB) forming a concentration gradient in 96 well plates. Concentrations were chosen based on the upper limit of each antibiotic defined by the CLSI. The bacterial suspension prepared as per CLSI guidelines was incubated at 37°C for 24 h. The MICs were recorded after 24-h incubation by visual examination. For both methods, the

TABLE 1 | The 36 *Pseudomonas aeruginosa* isolates represented on the basis of the patient number.

Isolate ID	Specimen type	Collection date[a]	Patient ID	Time to LTx (months)[b]
5627*	BAL	6/2/2015	Patient 1	4 Pre-LTx
5852	BAL	10/7/2015	Patient 1	0
6182	BAL	4/18/2016	Patient 1	6 Post-LTx
6984*	BAL	8/1/2017	Patient 1	10 Post-LTx
5892*	BAL	11/3/2015	Patient 2	12 Pre-LTx
6291	BAL	7/7/2016	Patient 2	4 Pre-LTx.
6543	BAL	11/5/2016	Patient 2	0
6544	BAL	11/5/2016	Patient 2	0
6820*	BAL	5/17/2017	Patient 2	6 Post-LTx
2755*	BAL	7/14/2011	Patient 3	11 Pre-LTx
2756	BAL	7/14/2011	Patient 3	11 Pre-LTx
3472	BAL	6/29/2012	Patient 3	0
3473	BAL	6/29/2012	Patient 3	0
3856	Sputum	1/7/2013	Patient 3	7 Post-LTx
3857	Sputum	1/7/2013	Patient 3	7 Post-LTx
4007*	BAL	4/9/2013	Patient 3	10 Post-LTx
3731*	BAL	11/2/2012	Patient 4	3 Pre-LTx
3732	BAL	11/2/2012	Patient 4	3 Pre-LTx
3734	BAL	11/2/2012	Patient 4	3 Pre-LTx
3735	BAL	11/2/2012	Patient 4	3 Pre-LTx
3736	BAL	11/2/2012	Patient 4	3 Pre-LTx
3940	BAL	2/22/2013	Patient 4	0
3941	BAL	2/22/2013	Patient 4	0
3942	BAL	2/22/2013	Patient 4	0
4139*	BAL	6/3/2013	Patient 4	3,5 Post-LTx
4836*	BAL	4/25/2014	Patient 5	12 Pre-LTx
4837	BAL	4/25/2014	Patient 5	12 Pre-LTx
3312	BAL	9/3/2014	Patient 5	8 Pre-LTx
5579	BAL	5/14/2015	Patient 5	0
3927*	BAL	10/10/2015	Patient 5	5 Post-LTx
160*	BAL	7/26/2006	Patient 6	2 Pre-LTx
179	BAL	9/3/2006	Patient 6	0
810	BAL	4/11/2008	Patient 6	6 Post-LTx
811	BAL	4/11/2008	Patient 6	6 Post-LTx
1118*	BAL	11/7/2008	Patient 6	12 Post-LTx
1119	BAL	11/7/2008	Patient 6	12 Post-LTx

*Two strains (star marked) per patient were selected and a panel of 12 P. aeruginosa isolates was created as a representative subset of the 36 strains. BAL, Bronchoalveolar lavage; NA, Not available; LTx, Lung transplantation. *Samples selected as a representative subset of the P. aeruginosa panel. [a]Month/day/year format. [b]Zero (0) represent samples obtained during the lung transplantation operation.*

isolates were classified as resistant, intermediate or susceptible based on CLSI breakpoints. The antibiotics chosen in this study are commonly used for treating *P. aeruginosa* infections.

Genomic Characterization of SPLT Isolates *Pseudomonas aeruginosa*

Multi-locus sequence typing (MLST) and construction of a core genome-based phylogenetic tree using bio-informatic tools was employed to define the genomic relatedness between the 36 serial *P. aeruginosa* isolates and a control panel of 672 *P. aeruginosa* strains.

Genomic DNA from all 36 isolates was extracted using the UltraClean® microbial DNA isolation kit (Qiagen, Netherlands) as per the manufacturer's instructions. The qualitative and quantitative estimation of the obtained DNA was carried out using the LabChip® GX Touch™ nucleic acid analyzer (PerkinElmer, United States) and Qubit dsDNA BR assay kits (Thermo Fisher Scientific, Waltham, MA, United States). The high-quality DNA was used for whole genome library preparation using S4 Reagent Kit v1.5 by following manufacturer's instructions. The resultant libraries were sequenced on the NovaSeq 6000 platform (Illumina Inc.) using 2 × 150 bp chemistry at CeGaT (Tübingen, Germany).

Bio-Informatic Data Processing and Analysis

Raw reads were quality filtered and assembled using the A5-MiSeq pipeline. The assembled contigs were used for further downstream analysis viz. annotation and phylogenetic analysis. The potential for AMR was inferred by annotating the genomes using the $PATRIC_{3.6.2}$ server. The virulome and resistome were analyzed using the Virulence Factors Database (VFDB) and Comprehensive Antibiotic Resistance Database (CARD) database on March 12, 2020 and September 9, 2019, respectively (Chen et al., 2005; Alcock et al., 2020). The genomic antibiogram was validated using the BioMérieux EPISEQ® CS tool (v1.0.2)[1]. The genomic antibiogram depicted gene abundance. Gene abundance is the number of times the genes involved in the particular function (e.g., efflux pump conferring resistance) appears in the whole genome sequence. As these genes encoding resistance are usually part of a group, presence or absence of one gene from the group could impact the level of resistance. A core genome-based phylogenetic tree using the FAST tree method was constructed to quantify the genomic relatedness level between the SPLT isolates. This tree was constructed using the parsnp tool (v1.2; Treangen et al., 2014) and was curated using iTOL (v5; Letunic and Bork, 2016). Alternatively, the 7-loci MLST of the 36 *P. aeruginosa* isolates was performed using PubMLST[2] and BioMérieux EPISEQ® CS to decipher STs and to define relatedness among the isolates (Jolley et al., 2018).

In case of disparity in results between PubMLST and BioMérieux EPISEQ® CS, sequence reads were aligned with the housekeeping gene reference alleles using the BWA MEM algorithm from the Burrows-Wheeler Alignment Tool (BWA; v0.7.17). We used SAMtools (v1.7) to obtain BAM files of the alignments, which were later visualized using the integrative genomics viewer (IGV; *v2.4.1; Li et al., 2009; Li and Durbin, 2010; Robinson et al., 2011).

Comparative Genome Analysis of SPLT *Pseudomonas aeruginosa* Isolates

A core genome single nucleotide polymorphism (SNP)-based approach using the parsnp tool was used to compare the

[1]www.biomerieux-episeq.com

[2]https://pubmlst.org

sequence of SPLT *P. aeruginosa* isolates to diverse strains of *P. aeruginosa* (n = 672) to define the genomic variation between *P. aeruginosa* strains from different niches and geographies. The 672 *P. aeruginosa* strains were obtained from three collections: the private BioMérieux collection (n = 219; van Belkum et al., 2015); the Kos collection (n = 390; Kos et al., 2015); and the Pirnay collection (n = 63; Pirnay et al., 2009). Further, the phylogeny was reconstructed based on the core genomes.

RESULTS

Identification and MLST Profiling of the SPLT *Pseudomonas aeruginosa* Isolates

On COS agar plates, the isolates were mucoid and slow growing. Despite the aberrant growth patterns for the strains, the verification of the identification of bacterial isolates using VITEK® MS (BioMérieux) confirmed that all isolates were *P. aeruginosa*. The WGS of the CF isolates generated an average of 50,043,100 ± 7,423,658 (average ± SD) pair-end reads per genome (**Supplementary Table 1**). *De novo* assembly revealed a mean genome size of 6.36 ± 0.17 mb (mean ± SD) with average coverage of 591× (±91×). Both MLST profiling methods traced four STs covering 18 genomes. However, the remaining 18 genomes (the majority belonging to patients 1, 2, 5, and 6) could not be assigned to any known ST and, hence, represent novel STs. LTx had no impact on the changes in the ST of the isolates. For patient 3, all of the isolates belonged to the same known ST (146), while for patients 1 and 5 they all belonged to novel STs. For patients 2 and 6, some of the isolates pre- and post-transplantation belonged to the same known ST (ST1029 and ST1567, respectively) while others belonged to novel STs. Finally for patient 4, some of the isolates at the time of transplant belonged to the same known sequence type (ST882) as one of the earlier isolates, while others belong to a novel ST identical to the post-LTx isolate (**Supplementary Table 1**).

Through the BioMérieux EPISEQ® CS analysis, ST235 was assigned to isolates 810, 1,118, and 1,119 which were isolated from patient 6 after LTx. MLST performed by aligning the reads to the reference sequence analysis showed mutation in the *acs*A allele in isolate 1,118. However, no mutation was found in the isolates 810 and 1,119. For our specific set of *P. aeruginosa* isolates, BioMérieux EPISEQ® CS proved to be a more robust MLST tool as it could assign STs to a larger number of isolates.

Comparison of *Pseudomonas aeruginosa* From Different Collections of Isolates

The genomic comparison of the isolates under investigation with the genomes of 672 isolates depicted unique clustering of the SPLT isolates in seven different clades spanning across the range of isolates isolated from diverse niches (**Figure 1**). However, the SPLT isolates were found to be conserved at the individual patient level, forming a monophyletic clade per patient, except for the case of patient 6 where isolates formed two distinct clades. Clade I contained the isolates 160, 179, and 811 with ST1567 whilst the other clade (clade II) was comprised of isolates 810, 1,118, and 1,119, with ST235 according to BioMérieux EPISEQ CS tool.

Genomic Analysis and Antibiotic Resistance

The genomic investigation of the 36 *P. aeruginosa* isolates using WGS revealed differences in the genome architecture sharing ≥98.7% average nucleotide identity (ANI; **Supplementary Table 1**). Further, a core genome phylogeny developed using the FAST tree method suggested formation of six different clades representing isolates from six different patients. We found intergroup variability with respect to the origin of the isolates. However, clustering did not correlate with time of isolation of the isolates around LTx (**Figure 2**).

The nucleotide sequences of the small subunit (SSU) 16S rRNA gene of all the isolates were extracted and taxonomic identity was reconfirmed using a BLAST search. Further, we noted the presence of 6,150 ± 193 (mean ± SD) coding sequences (CDS) per genome. A subset of the CDSs accounting for conferring antibiotic resistance was derived using CARD, which was in the range of 55 ± 1 resistance CDS per isolate. The genes retrieved from the CARD analysis were mostly classified as beta-lactam resistance genes (n = 6), efflux pumps (n = 37), an aminoglycoside resistance gene (n = 1), other antibiotic inactivation enzymes (n = 8), antibiotic resistance gene variants or mutants (n = 4), a gene modulating bacterial permeability to antibiotics (n = 1), genes altering cell wall charge conferring antibiotic resistance (n = 2) and an antibiotic target protection protein gene (n = 1; **Figure 3**).

BioMérieux EPISEQ® CS analysis showed the presence of various antibiotic resistance genes (ARGs) in *P. aeruginosa* isolates. We noted different frequencies of genes conferring resistance against drugs or drug families such as aminocoumarin, aminoglycoside (100%), polymyxin B (100%), chloramphenicol (100%), elfamycin (88.89%), sulfonamide, sulfone (100%), fosfomycin (100%), azithromycin, ciprofloxacin, erythromycin, novobiocin, tetracycline (*oprM* gene, 2.78%), beta-lactam, carbapenem, cephalosporin, cephamycin, penicillin (from 97.22% for OXA-50 to 8.33% for PDC-2 or PDC-3), and fluoroquinolone (100%). For strain 3,473 (belonging to patient 3), we noted the prevalence of genes responsible for resistance against the antibiotics azithromycin, chloramphenicol, ciprofloxacin, erythromycin, novobiocin and tetracycline. Interestingly, in contrast to all other isolates, isolate 4,837 (deriving from patient 5) lacked the presence of genes providing resistance against the drug family "beta-lactam, carbapenem, cephalosporin, cephamycin, and penicillin" (**Figure 4**).

Virulence Genes

The investigation of virulence factors in *P. aeruginosa* isolates using VFDB revealed isolates containing various virulence genes ranging from 236 to 266 in number. Genes with no known functionality in the VFDB database were eliminated. The remaining 28 genes were found to be differentially abundant across the genomes off all isolates in the study. The major functional attributes of these 28 genes were adherence (17.8%,

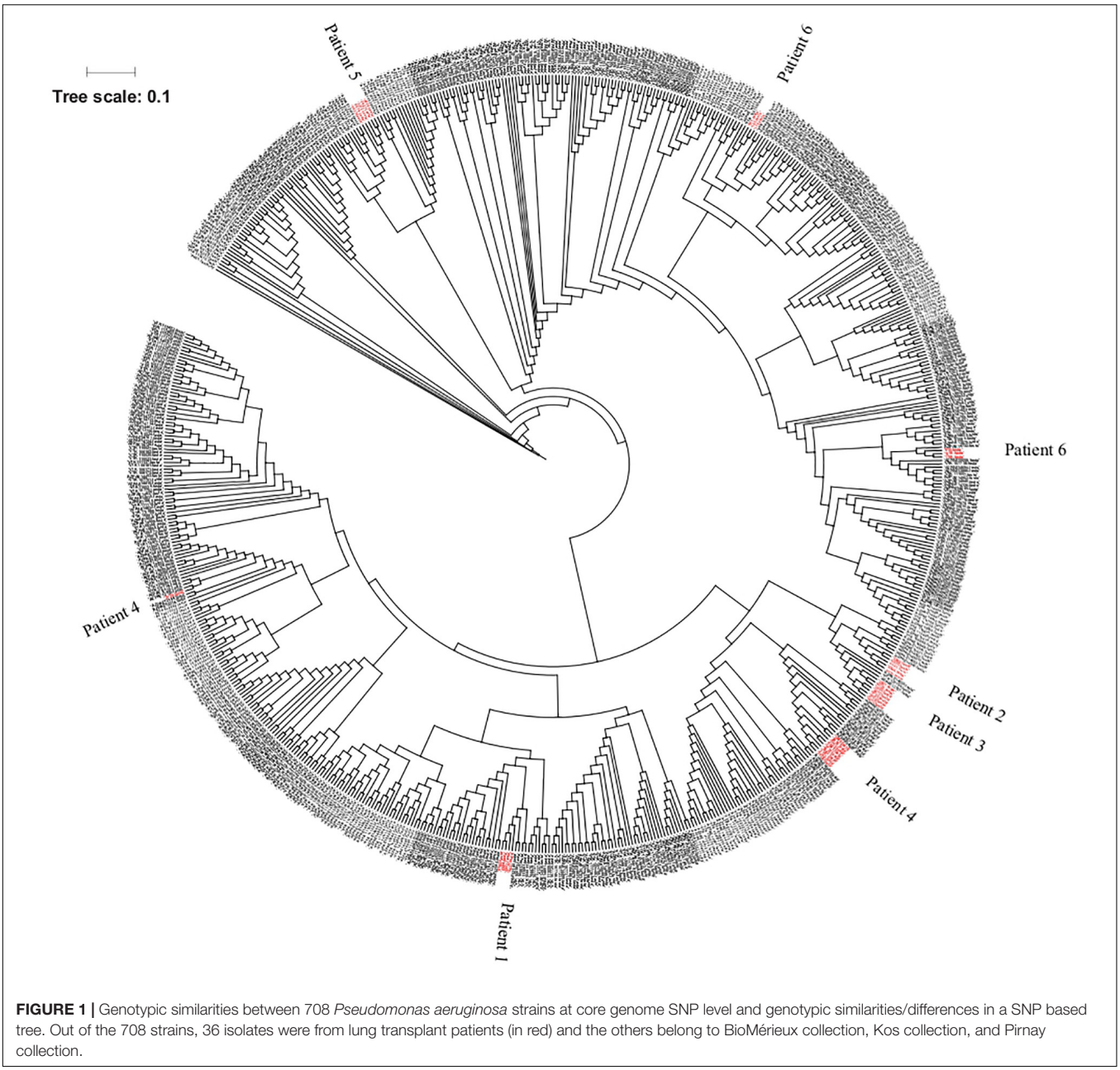

FIGURE 1 | Genotypic similarities between 708 *Pseudomonas aeruginosa* strains at core genome SNP level and genotypic similarities/differences in a SNP based tree. Out of the 708 strains, 36 isolates were from lung transplant patients (in red) and the others belong to BioMérieux collection, Kos collection, and Pirnay collection.

relative abundance) and Type VI secretion (14.2%, relative abundance; **Figure 5**).

Antibiotic Susceptibility Testing

More than 75% of the isolates were found to be resistant to at least half of the antibiotics under study (**Table 2**). Isolates 810 (patient 6) and 4,139 (patient 4) were found to be the most susceptible. Tobramycin was found to be effective against 64% of the isolates. Additionally, isolates 3,735 and 3,736 (patient 4), 6,291 and 6,543 (patient 2), 5,852 (patient 1), and 3,473, 3,472, 2,755 and 2,756 (patient 3) were found to be resistant to all the antibiotics tested. Interestingly, all the isolates of an ST belonging to one patient did not always show the same susceptibility pattern (**Figure 3**).

We tried to correlate the phenotypic antibiograms to the AMR gene patterns obtained using the CARD analysis, but there were inconsistencies in the results. In patient 3, isolates 4,007 and 3,856 were susceptible to tobramycin, piperacillin and amikacin in contrast to other genotypically similar isolates from the same patient (**Figure 3**). We noted the unique presence of a β-lactam resistance gene bla_{PDC-2} for isolates 810, 1,118, and 1,119, isolated post-transplant from patient 6. Similarly strain 3,312 (8 months before LTx) was the only strain containing a gene for the antibiotic target protection protein *rpsJ*. Interestingly,

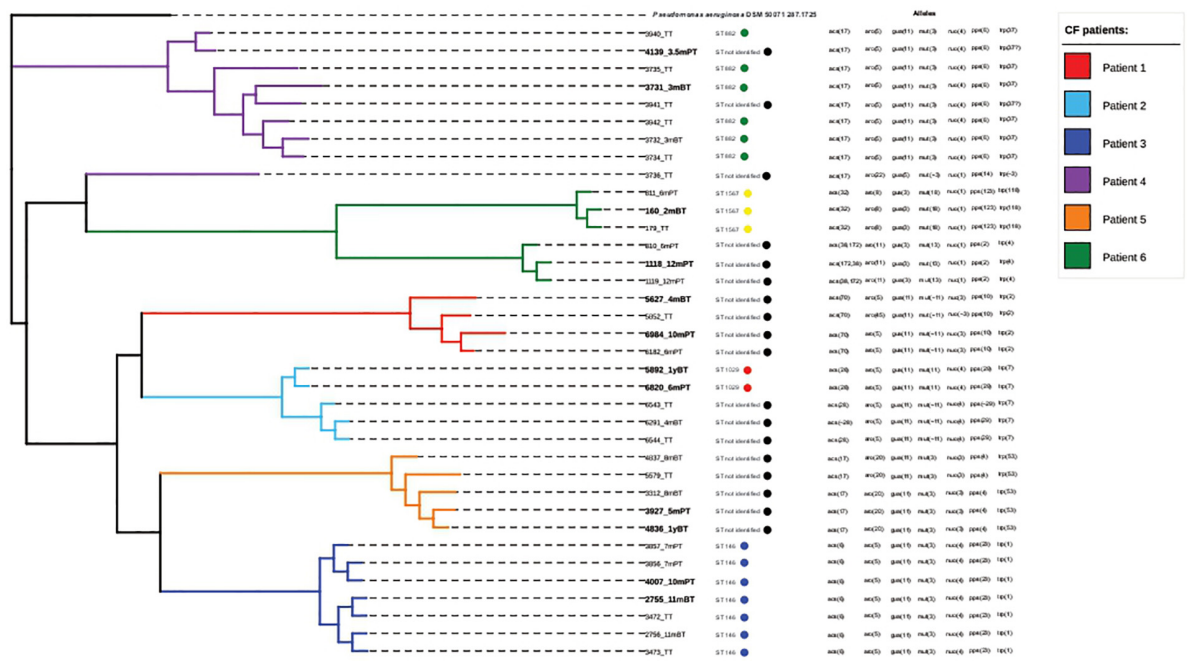

FIGURE 2 | The phylogenetic tree constructed using the whole genome sequence (WGS) of 36 clinical *Pseudomonas aeruginosa* isolates demonstrates the variability between the isolates. Each clade represents one patient. The isolates selected for phenotypic analysis using broth micro-dilution (BMD) assay (in bold) are from two different time points: before and after transplant. The figure also depicts the sequence types and allelic differences among the 36 *P. aeruginosa* isolates from lung transplantation (LTx) patients.

5 months after the LTx this gene seemed to be lost from isolate 3,927. Unlike others, isolates 6,291, 6,543, and 6,544 (4 months before LTx and during LTx, respectively) isolated from patient 2 lacked the gene modulating permeability to antibiotics. The isolates from different time points from patient 4 lacked the presence of *mexZ* gene, which codes for a regulator of an important efflux pump responsible for imparting resistance to aminoglycosides (**Figure 3**).

DISCUSSION

Our analysis shows that *P. aeruginosa* isolates from different time points, but from the same patient, contained identical STs suggesting lack of cross infection in the patients under study. These isolates showed resistance to antibiotics in comparison to the susceptible reference isolates. Correct treatment depends on continuous and serial assessment of resistance features. Moreover, in the majority of recurrent infections patients tend to harbor genotypically similar *P. aeruginosa* isolates (Workentine et al., 2013). *P. aeruginosa* is a tough bug to kill and it persists even after prolonged antibiotic treatment as well as lung transplantation (Walter et al., 1997; Mainz et al., 2012; Beaume et al., 2017; Parkins et al., 2018). Therefore, a more frequent follow-up of infection after LTx is necessary to see how the same strain evolves over the course of time.

We determined the taxonomic identity and genomic relatedness of the *P. aeruginosa* isolates. We observed significant genomic homology for the isolates from the same CF patients. Furthermore the MLST typing of the 36 SPLT isolates demonstrated characteristic patient specific unique STs. This observation is supported by the difference seen in the Canadian MLST typing of *P. aeruginosa* (Jeukens et al., 2019). They noted a significant heterogeneity and a need of personalized patient care in the context of CF Core genome phylogeny of the 36 SPLT isolates depicted six clades, one per patient, establishing the fact that patient specific genotypic diversity exists for the *P. aeruginosa* populations during chronic CF infection (Fothergill et al., 2007; Mena et al., 2008; Bragonzi et al., 2009; Wilder et al., 2009; Huse et al., 2010; Mowat et al., 2011; Chung et al., 2012; Workentine et al., 2013; Jiricny et al., 2014). The genotypic and phenotypic divergence among the isolates from different patients could be a result of antibiotic treatment driven evolution of the *P. aeruginosa* populations. Alternative reasons for such evolution could be selective stress due to nutrient and oxygen deficiency, impaired immune response and barrier function of the mucosal linings (Feliziani et al., 2014; Lozano et al., 2018). In CF patients, *P. aeruginosa* causes infections through two ways, i.e., using both adaptive and mutational mechanisms (Lister et al., 2009). The study highlights how isolates from the same patients evolve over time and how it is difficult to correlate the increased MIC with the

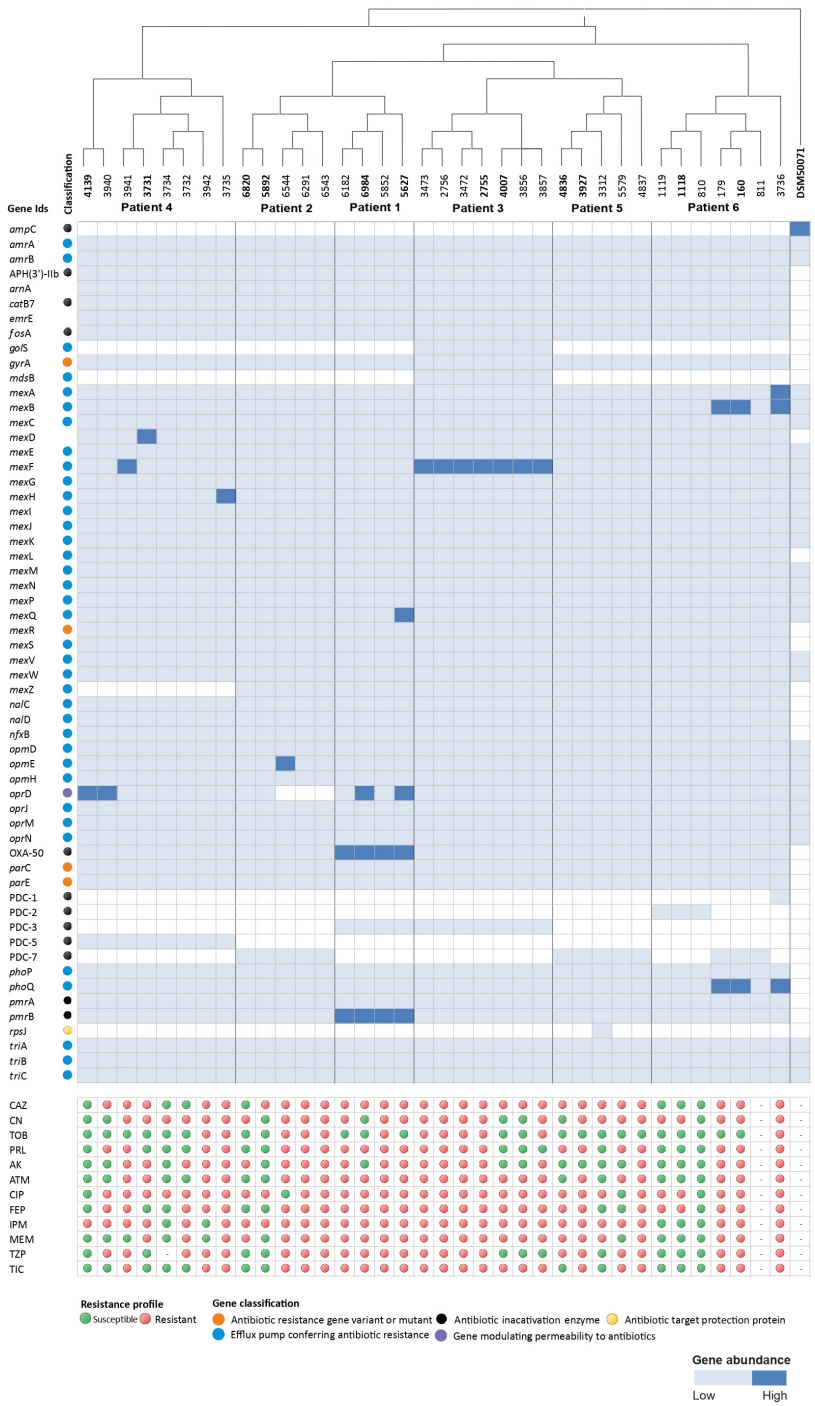

FIGURE 3 | Heatmap-dendrogram analysis of 36 *Pseudomonas aeruginosa* isolates and reference stains depicting the taxonomic relatedness of the isolates isolated across three time points: before LTx, at the time of LTx and after LTx. Furthermore, the qualitative estimates of antibiotic resistance genes (ARGs) were compared against the observed antimicrobial resistance (AMR) phenotypes. AST results are shown by color code red (resistant) and green (susceptible).

genotype as there tends to be no clear differences at the genomic level. The resistant nature of the strains could also be related to mutations. Thus, identification of the mutations along with the resistance genes could aid our understanding of increased antibiotic resistance (Khaledi et al., 2019).

Likewise, the assessment of genomic similarity between the SPLT isolates from the other clinical and environmental *P. aeruginosa* strains does not hinder the branching pattern of the SPLT isolates under study except for strains from patients 4 and 6. It is supported by a previous report focusing on

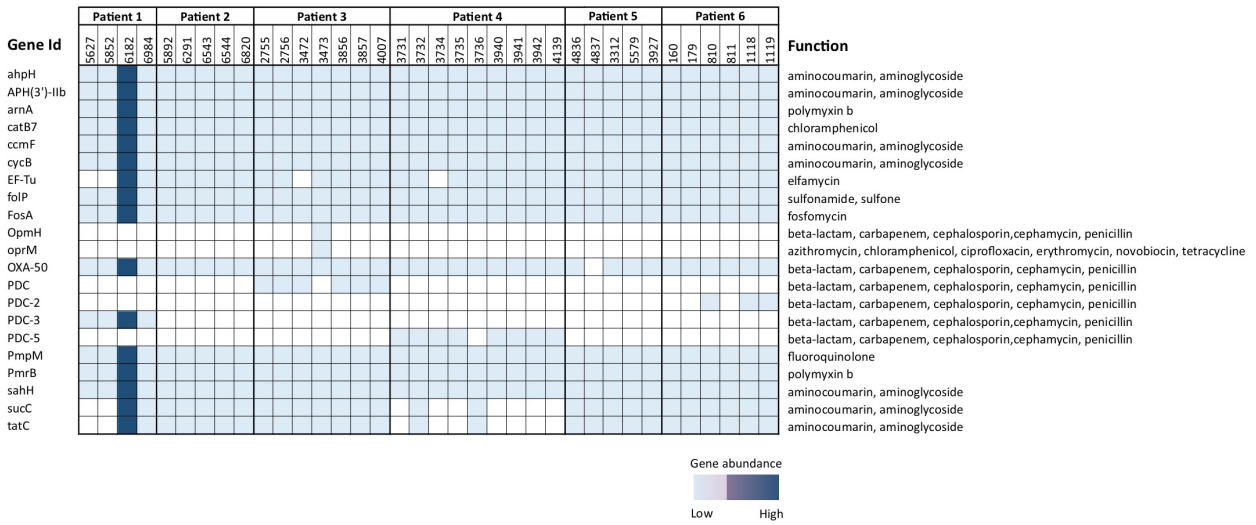

FIGURE 4 | Presence and absence of antimicrobial resistance genes in 36 *Pseudomonas aeruginosa* isolates. Varied degree of presence of genes conferring resistance against drug families was noted. The analysis was done using EPISEQ.

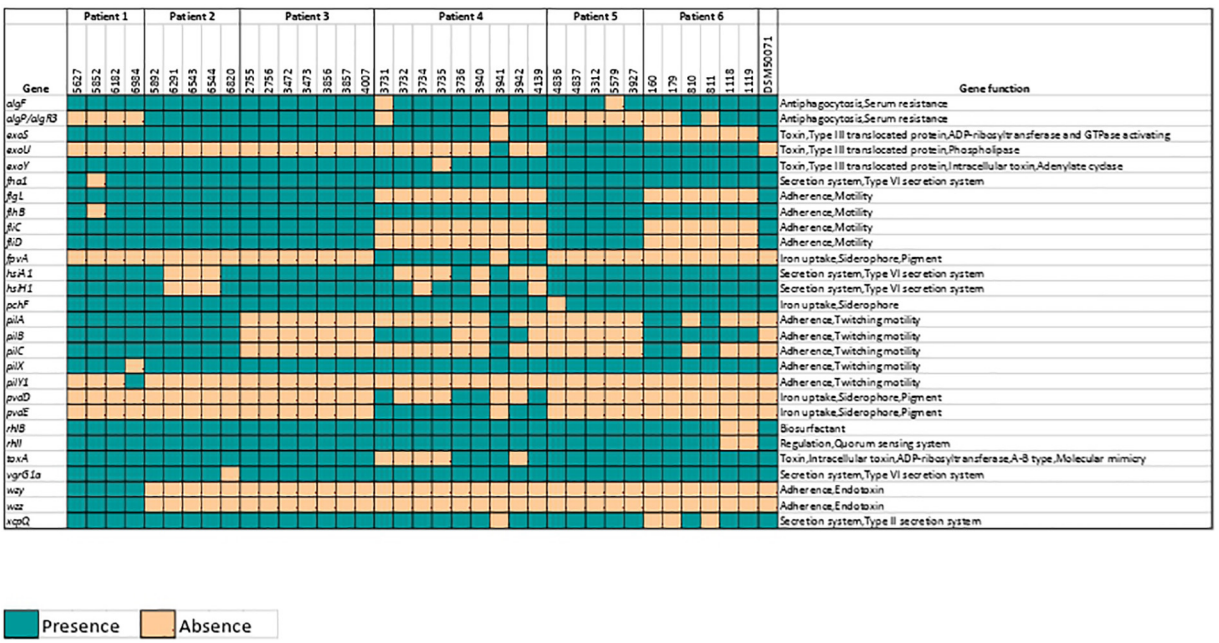

FIGURE 5 | Presence of various virulence factors among 36 *Pseudomonas aeruginosa* isolates. Yellow square indicates absence while green square indicates presence of virulence genes identified using Virulence Factors Database (VFDB).

indels, recombination or SNPs for definition of inter-patient variability and intra-patient similarity (Huse et al., 2010). Interestingly in patient 6, two clades were formed. One clade consisted of strains before LTx while the other consisted of strains after LTx, which shows how the genotype can alter overtime. Additionally, we saw the spread of the SPLT isolates throughout the phylogenetic tree based on core genes, still showing patient specific clustering. This observation was found to be coherent with the noted difficulties in discriminating CF isolates based on their geography and origin (Jeukens et al., 2019; Pincus et al., 2020).The latter demands further understanding of the emergence of resistance and virulence among these *P. aeruginosa* to avoid or manage lung infections (Cho et al., 2014; Jaillard et al., 2017).

TABLE 2 | The MICs (mg/L) of the eight antibiotics against the panel of 12 *P. aeruginosa* isolates by broth micro-dilution (BMD).

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
Antibiotics	**Strain Ids**											
	5,627	**6,984**	**5,892**	**6820**	**2,755**	**4,007**	**3,731**	**4,139**	**4,836**	**3,927**	**160**	**1,118**
Aztreonam	64	256	128	128	256	256	64	4	32	256	256	8
Imipenem	64	32	16	32	8	32	32	16	32	32	32	0.5
Colistin	<0.5	<0.5	<0.5	<0.5	2	1	<0.5	1	1	<0.5	32	<0.5
Tobramycin	4	4	4	4	4	4	4	4	4	4	4	4
Amikacin	32	8	16	16	64	8	32	4	16	16	16	4
Ciprofloxacin	4	4	2	4	4	4	8	0.5	4	8	8	2
Levofloxacin	16	16	8	16	16	32	32	16	8	16	32	16
Ceftolozane + Tazobactam	4	4	32	32	32	4	4	0.5	8	32	16	4

The strains were categorized as susceptible (white cells) and resistant (red cells) according to the CLSI guidelines on MIC breakpoints.

Our investigation of genomic features revealed various AMR and virulence genes. The main characteristics of the virulence genes were related to adhesion and secretion thereby suggesting that these isolates could be biofilm producers. Similarly, the isolates displayed a varied response to the array of antibiotics even though the isolates have homologous MLST types found during the analysis. This might mean that the resistance profile might also be related to host-related factors, which are largely unknown. There have been extensive studies on increased antibiotic resistance in *P. aeruginosa*. Also, the microbes have an ability to integrate exogenous DNA to withstand the selective pressure of antibiotics. Thus, in order to understand these dynamics, there have been various efforts to understand the correlation of genotype and phenotype (e.g., response to antibiotics) by several researchers (Mena et al., 2008; Hazan et al., 2014; Macia et al., 2014; Sousa and Pereira, 2014; Kos et al., 2015; Jaillard et al., 2017; Lozano et al., 2018; Garcia-Nuñez et al., 2020). Furthermore, the MLST profiles derived from two different tools-PubMLST and EPISEQ and the phenotypic antibiotic susceptibility profiles shows disparity. This finding highlights the limitations of the tools and databases and recognizes the impact this could have on predicting resistance and treating infections (Ng et al., 2021). As this study has focused on a very specific set of isolates, from respiratory tract specimens of a few patients from the same hospital, those observed advantages for each tool may be different with a different set of strains.

Pseudomonas aeruginosa is a key organism in cases of bronchiectasis leading to increased mortality, as documented in Europe (Ringshausen et al., 2013). The observation that the isolates are genetically alike but show AMR heterogeneity is evident through the recent increase in cases of bronchiectasis by *P. aeruginosa*. Moreover, studies focusing on the United Kingdom population suggested approximately 29% mortality in the bronchiectasis patients (Loebinger et al., 2009). Thus, the British Thoracic Society emphasizes microbiological assessment and personalized treatment to mitigate drug resistance during bronchiectasis due to the multi-drug resistance profile of *P. aeruginosa* (Pasteur et al., 2010).

CONCLUSION

Our study sheds light on the lack of intra-patient diversity and phenotypic plasticity of *P. aeruginosa* isolates associated with CF patients undergoing LTx. Most patients with CF carry similar *P. aeruginosa* isolates before, during and after LTx. Genomic similarity between these isolates is observed but they show phenotypic variation. Thus, despite continuous *P. aeruginosa* colonization and infection by a single genotype, these isolates still show varied responses to antibiotics. Our study equally endorses careful assessment for antibiotic susceptibility and high-throughput genomic-level monitoring of antibiotic therapy against *P. aeruginosa* in patients with CF.

DATA AVAILABILITY STATEMENT

Whole genome sequences for all samples used in this study have been deposited in National Centre for Biotechnology Information (NCBI) and are available under BioProject ID: PRJNA630383 here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA630383. The isolates for which sequence type were not assigned have been submitted to PubMLST database under the ID: BIGSdb_20200507140502_147974_63511.

ETHICS STATEMENT

All isolates examined in this study were recovered as a result of routine diagnostic procedures. No additional human samples were collected for the purposes of this study. The researchers were supplied with samples of *Pseudomonas* from an archived frozen collection. Researchers were not given access to any data that would allow identification of a particular patient. As the project did not require the use of any human material or any personal data, approval of the local ethics committee was not required.

AUTHOR CONTRIBUTIONS

AB, SO, and JP conceived the study. JP and AP collected *P. aeruginosa* isolates from the patients serially and gathered the metadata. RD conducted the microbiological experimentation for *P. aeruginosa* isolates, interpreted the sequence data and wrote the first version of the manuscript. AC, RD, and HG carried out the whole genome sequencing studies. JD assisted in the WGS analysis. All authors discussed the results and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.604555/full#supplementary-material

Supplementary Figure 1 | Genomic similarity among the strain of *P. aeruginosa* isolated from LTx patients, represented by the average nucleotide identity (ANI) as present inter-genomic distances.

Supplementary Table 1 | Genomic characteristics of *P. aeruginosa* isolates isolated from LTx patients. It includes the attributes of sequencing, MLST types and genomic features viz. ARGs and virulence genes.

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Conflict of Interest: During this study AB, SO, VC, RD, AP, CM, and JD were employes of BioMérieux, a company designing, developing, and marketing tests in the domain of infectious diseases. The company was not involved in the design of the current study and the opinions expressed are those of the authors and may be different from formal company opinions and policies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 5: Use of transcriptomics to elucidate genotype-phenotype correlation in multidrug resistant *P. aeruginosa* obtained from patients with CF

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Introduction

Pseudomonas aeruginosa is one of the bacterial species belonging to the *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp.* (ESKAPE) pathogen group. ESKAPE pathogens are frequently multi-drug resistant (Rice *et al*., 2008). In the context of cystic fibrosis (CF), *P. aeruginosa* ultimately colonises the lungs of most patients suffering from CF. In such chronic CF infections, *P. aeruginosa* dwells in the mucosal linings of the lungs and creates a favourable environment for the bacteria to proliferate. To combat such bacterial infections, CF patients are subjected to aggressive antibiotic treatment and frequent hospital visits for treatment to help improve their life expectancy (Rossi *et al*., 2021). The prolonged course of infection urges *P. aeruginosa* to frequently adapt to the unfavourable environment created by the antibiotics present. *P. aeruginosa* undergoes genetic evolution and it can also add additional antimicrobial resistance (AMR) genes through horizontal gene transfer to its already huge intrinsic arsenal of resistance genes (Khaledi *et al*., 2016). These adaptive mechanisms help transform environmental clones into lineages capable of persisting in the CF airways. Overall, CF-associated isolates of *P. aeruginosa* display frequent resistance to various broad-spectrum antibiotics which leads to therapeutic challenges. As finding novel antibiotics is a current clinical and therapeutic bottleneck, it is essential to optimize the current diagnostic modalities, treatment options and ways of prevention of spread of *P. aeruginosa* infections in and between patients with CF (Khaledi *et al*., 2016).

Current strategies involving whole genome sequencing (WGS) cover the comprehensive identification of AMR genes and the correlation between gene presence and the phenotypic resistance profiles of the bacteria (Datar *et al*., 2021). The developments in next generation sequencing (NGS) technologies have generated a positive impact on the field of microbiological diagnostics and in phenotypic and epidemiological monitoring of pathogens (Khaledi *et al*., 2020, Rossi *et al*., 2021). However, defining infectious disease treatment based on the resistance profiling obtained using WGS in clinical settings is still challenging. Moreover, microbial survival strategies based on a continual rate of mutations may lead to modification in drug targets or metabolism thereby reducing the therapeutic impact of antibiotics (Drlica *et al*., 2009). Thus, additional genotype-to-phenotype association studies are still important. Extrapolating the treatment response from the resistome of an especially complex group of organisms such as *P. aeruginosa* represents a daunting task (Rossi *et al*., 2021). In *P. aeruginosa*, gene modifications are not only limited to the coding region of antimicrobial drug targets but often mutations in the genes encoding efflux pumps and diverse regulatory genes are also observed (Khaledi *et al*., 2016).

Recently, transcriptomic analysis has been used for identifying and further detailing of genotype-phenotype relationships (Khaledi *et al*., 2016). In the present study we apply transcriptome analysis to improve our understanding of the active resistome of *P. aeruginosa* strains serially isolated from six CF/ bronchiectasis patients who underwent lung transplantation (LTx), those isolates being genetically and morphologically similar but with distinct antibiotic susceptibility profiles (Datar *et al*., 2021).

Materials and Methods

Sample collection and isolates

Thirty-six *P. aeruginosa* isolates were obtained from sputum samples and broncho-alveolar lavage fluids from five chronically ill patients with CF and one patient suffering from bronchiectasis whom all underwent LTx (Datar *et al*., 2021). The patients were followed for a long period of time (~1 to 4 years) and the *P. aeruginosa* strains were isolated at Freeman hospital, Newcastle upon Tyne. For each patient, the *P. aeruginosa* morphotype that was most abundant was collected before transplantation (BTx), at the time of transplantation (TTx) and post transplantation (PTx) to study *in vivo* evolution of the isolates during the course of infection (Datar *et al*., 2021). The 36 isolates were transported to bioMérieux and were

preserved at -80°C until further analysis. Two wild type strains (ATCC: 27853 and PAO1) were used for quality and process control throughout the study (Table 1).

Table 1: The 36 *P. aeruginosa* isolates represented on the basis of the patient number, moment of isolation and specimen type.

BAL, Bronchoalveolar lavage; NA, Not applicable; BTx, Before Lung transplantation; TTx, Time of lung transplantation; PTx, Post lung transplantation; m= month, Y= year; API, bioMérieux culture collection number.

Panel	Isolate number	Country	Patient number	Isolation moment	Specimen type
Panel 1	API:1801131	UK	1	4m BTx	BAL
	API:1801132	UK	1	TTx	BAL
	API:1801133	UK	1	6m PTx	BAL
	API:1801134	UK	1	10m PTx	BAL
	API:1801135	UK	2	1Y BTx	BAL
	API:1801136	UK	2	4m BTx	BAL
	API:1801137	UK	2	TTx	BAL
	API:1801138	UK	2	TTx	BAL
	API:1801139	UK	2	6m PTx	BAL
	API:1801140	UK	3	11m BTx	BAL
	API:1801141	UK	3	11m BTx	BAL
	API:1801142	UK	3	TTx	BAL
	API:1801143	UK	3	TTx	BAL
	API:1801144	UK	3	7m PTx	SPUTUM
	API:1801145	UK	3	7m PTx	SPUTUM
	API:1801146	UK	3	10m PTx	BAL
	API:1801147	UK	4	3m BTx	BAL
	API:1801148	UK	4	3m BTx	BAL
	API:1801149	UK	4	3m BTx	BAL
	API:1801150	UK	4	3m BTx	BAL
	API:1801151	UK	4	3m BTx	BAL
	API:1801152	UK	4	TTx	BAL

	API:1801153	UK	4	TTx	BAL
	API:1801154	UK	4	TTx	BAL
	API:1801155	UK	4	3.5m PTx	BAL
	API:1801156	UK	5	1Y BTx	BAL
	API:1801157	UK	5	1Y BTx	BAL
	API:1801158	UK	5	8m BTx	BAL
	API:1801159	UK	5	TTx	BAL
	API:1801160	UK	5	5m PTx	BAL
	API:1801161	UK	6	2m BTx	BAL
	API:1801162	UK	6	TTx	BAL
	API:1801163	UK	6	6m PTx	BAL
	API:1801164	UK	6	6m PTx	BAL
	API:1801165	UK	6	12m PTx	BAL
	API:1801166	UK	6	12m PTx	BAL

The isolates were revived on COS agar medium by incubating at 37 °C and it takes ~26 hours for visible colonies to develop. The isolates were not only growing slowly as compared to the control strains but also showed mucoid consistency (Datar *et al*., 2021).

All the isolates from patients that underwent LTx were categorized as multidrug resistant (resistant to three or more antimicrobial classes) (Table 2). The antibiotic susceptibility data were either hospital or institution derived or determined in house using the VITEK® 2 system (bioMérieux) or ETEST® strips (bioMérieux) (Datar *et al*., 2021). The classification of MIC breakpoints was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein *et al*., 2018).

As a reference strain for differential gene expression calculation and sequence variation analysis, we chose the PAO1 strain.

Table 2: Reference susceptibility testing results obtained by ETEST. Aztreonam (Susceptible, ≤8; Intermediate, 16; Resistant, ≥32); Imipenem (Susceptible, ≤2; Intermediate, 4; Resistant, ≥8); Tobramycin (Susceptible, ≤4; Intermediate, 8; Resistant, ≥16); Amikacin (Susceptible, ≤16; Intermediate, 32; Resistant, ≥64); Ciprofloxacin (Susceptible, ≤0.5; Intermediate, 1; Resistant, ≥2); Levofloxacin (Susceptible, ≤1; Intermediate, 2; Resistant, ≥4). The red cells stand for resistance, yellow cells stand for intermediate and green cells stand for susceptible isolates as per the CLSI guidelines for MIC breakpoints.

API strain number	Aztreonam	Imipenem	Tobramycin	Amikacin	Ciprofloxacin	Levofloxacin
1801131	>256	>32	3	>256	6	>32
1801132	32	>32	2	162	4	12
1801133	64	>32	8	16	3	16
1801134	32	>32	1	64	4	12
1801135	>256	>32	2	24	1	4
1801136	64	16	8	64	1	2
1801137	64	16	4	32	1	4
1801138	64	16	4	64	1	4
1801139	>256	>32	2	48	6	>32
1801140	>256	>32	6	192	4	16
1801141	32	16	8	64	4	4
1801142	64	16	8	64	4	8
1801143	64	16	4	64	4	8
1801144	64	8	1	16	4	8
1801145	48	16	4	64	4	12
1801146	>256	8	2	24	4	>32
1801147	192	>32	3	32	8	16
1801148	32	>32	16	>256	8	24
1801149	48	4	2	16	4	8
1801150	64	>32	3	>256	8	32
1801151	64	>32	16	>256	12	32
1801152	1	>32	1	8	4	8
1801153	64	>32	1	64	6	12
1801154	>256	24	8	>256	8	32
1801155	0.75	>32	1	3	1	4
1801156	>256	>32	3	48	1.5	6
1801157	48	24	16	64	12	24
1801158	32	>32	16	64	4	4
1801159	64	>32	1	64	2	2
1801160	>256	>32	3	48	3	16
1801161	>256	>32	1.5	32	12	>32
1801162	64	>32	2	32	4	4
1801163	4	1	1	8	0.25	0.75
1801164	4	0.5	1	8	0.25	1
1801165	6	0.75	1.5	16	1.5	8
1801166	64	0.75	8	64	4	24

Growth and RNA extraction

The thirty-six *P. aeruginosa* isolates and the two control strains were grown in LB medium in triplicate in flasks at 37°C while shaking (180 rpm) to ensure full aeration. As the slow growing strains had a significantly different growth rate when compared to the control strains, we harvested the bacterial cells when OD 2.0 was recorded irrespective of the time each strain took to reach OD 2.0 when checked at 600 nm optical density.

The cells were harvested individually (36 isolates + 2 control strains) in RNA Protect (Qiagen, The Netherlands). The sample pellets of biological replicates were preserved at -80° C before proceeding with RNA extraction with RNeasy Protect Bacteria Mini Kit (Qiagen, The Netherlands). Lysis of the bacterial cells was obtained by removing the supernatant, re-suspending the frozen sample pellets in 200 µl freshly prepared TE buffer (pH 8.0) containing 2 $mg.ml^{-1}$ Proteinase K (Thermo Fisher Scientific, Massachusetts, U.S) and 15 $mg.ml^{-1}$ lysozyme (Thermo Fisher Scientific) and by incubating at 37° C for 10 minutes. RLT buffer (Qiagen, The Netherlands) containing beta-mercaptoethanol was added and the solution was centrifuged at 5000 x g for 10 minutes. Ethanol was added to the supernatant and column purification of total RNA was conducted according to the manufacturers' instructions. Approximately 5-10 µg of crude mRNA thus obtained was subjected to DNase digestion using recombinant DNAse I from Sigma Aldrich, United States and the reactions were purified using the columns provided in the RNA Clean & Concentrator-5 assay (Zymo Research, United States) and manufacturer's instructions were followed. The resultant purified RNA was checked for quality and integrity using the Agilent Bioanalyzer 2100 instrument. Samples that had RNA integrity number (RIN) higher than 9 were used for preparing sequencing libraries. This protocol was followed for all the three batches with biological replicates of the *P. aeruginosa* strains used in the study.

Library preparation and mRNA sequencing

Libraries for RNAseq were prepared using the Illumina Stranded Total RNA Prep and ligation was carried out with Ribo-Zero Plus protocol (Illumina, California, US). Barcode ligation was performed by using Illumina RNA UD Indexes Set B. The resultant libraries were quantified and pooled in equimolar concentrations. Sequencing was performed on a NovaSeq 6000 using 100bp chemistry cycles using the SP Reagent kit v1 with single end mode (101 cycles) (Illumina).

Reads mapping and quality assessment

The raw reads were filtered and trimmed using the (angelovangel/nxf-fastqc pipeline), which uses the (fastp) (Chen *et al*., 2018) program with default filter and trimming parameters. The filtered reads were aligned against the genomes of PAO1 using the (Rsubread) (Liao *et al*., 2019) package in R. For visual exploration of RNA-seq count data, transformations were applied in order to make the data more "homoscedastic" and having approximately the same range of variance at different ranges of the mean count values. Here, the variance stabilizing transformation (*vst*) from DESeq2 were applied and a heatmap was constructed to visualize the results.

Differential gene expression analysis

The count data obtained with featureCounts (an efficient general-purpose program for assigning sequence reads to genomic features) were used in differential gene expression analysis with DESeq2 (Liao *et al*., 2014; Love *et al*., 2014). The design formula used was: ~ patient + transplantation, where patient was a factor with 7 levels (control, Patient1…Patient6) and transplantation was a factor with 3 levels (before, at and after). Moreover, interactive Glimma plots (Su *et al*., 2017) were generated for comparison of expression patterns of genes from isolates BTx + TTx and PTx .

Results and Discussion

Clinicians are left with few antimicrobial drugs that can be prescribed to patients with CF owing to the emergence and prevalence of multidrug resistance profiles of the bacteria causing infections in the lungs of these patients. Due to the inability of the antibiotics to kill or to arrest the growth of these bacterial species, higher doses of broad-spectrum antibiotics are often prescribed leading to further resistance development (López-Causapé *et al*., 2018). For accurate prediction of resistance profiles of complex bacteria like *P. aeruginosa*, it becomes important to not only look at the phenotypic profiles of the bacteria but also to delve deeper into the genotypic and transcriptomic profiling of *P. aeruginosa* (Dötsch *et al*, 2015 and Moradigaravand *et al*, 2018). Identification of molecular markers is crucial for justifying antibiotic stewardship using molecular diagnostics especially in the case of opportunistic pathogens with phenotypic plasticity (Gordon *et al*, 2014; Bradley *et al*, 2015).

In this study, we attempted to understand the genotype-phenotype correlation through transcriptomics. Transcriptomic testing is close to real-time detection of genuine *in cellulo*

antimicrobial resistance (Datar *et al*., 2021). In the recent past, efforts in the direction of genotype-phenotype correlation by using transcriptomics have been made. Unlike our approach of looking at the up- or down-regulation of genes involved in antibiotic resistance and finding a correlation with the resistance phenotype, Khaledi *et al*., 2016 have used machine learning approaches in a blinded study to correlate the phenotypic profiles to the molecular markers that make *P. aeruginosa* highly antibiotic resistant. But even machine learning techniques demand cataloguing of the different molecular markers that can play a pivotal role in determining the resistance status of the bacteria (Khaledi *et al*., 2016). Therefore, creating a repertoire of genes coding for resistance, biofilm formation and toxins is essential.

We studied *P. aeruginosa* isolates from patients with CF that underwent lung transplantation. These isolates grew equally slowly and showed a mucoid consistency on both LB and BHI plates. We therefore decided to continue using LB as a medium for bacterial cell growth and extraction.

The preliminary quality assessment of the transcripts revealed that the transcripts from all biological replicates showed higher homology to each other than from transcripts from other isolates. Further, by plotting a heatmap we could verify that all the transcripts belonging to the isogenic strains from one patient grouped together and therefore were very similar whereas the transcripts were different when compared to those obtained from groups of strains that were derived from other patients (Figure 1). This observation was in line with a previous study in which we had noticed inter-patient genome differences and intra-patient genome similarities based on core genome phylogeny (Datar *et al*., 2021).

Figure 1

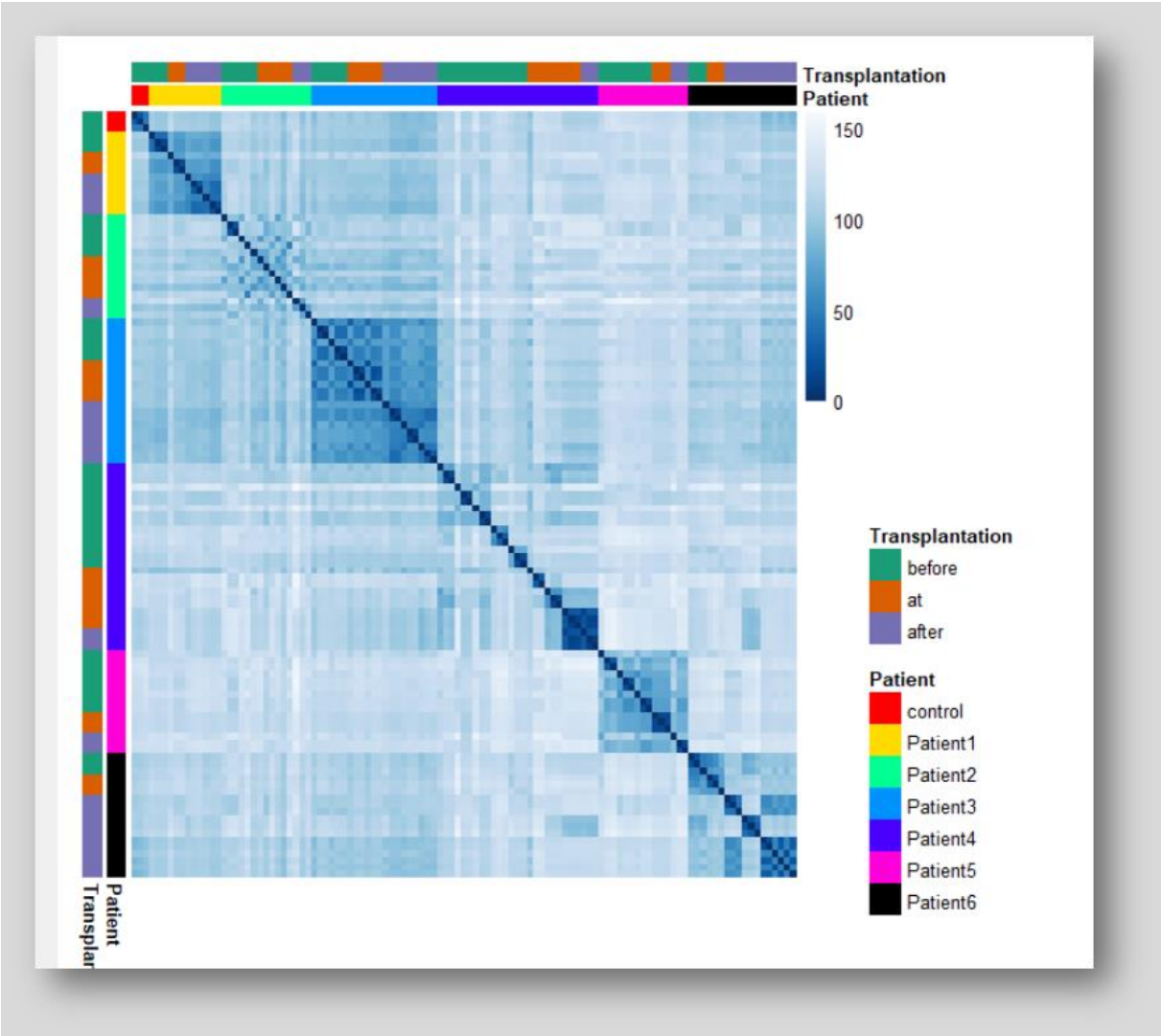

Figure 1: For visual exploration of RNA-seq data, transformations were applied in order to make the data more "homoscedastic" and having approximately the same range of variance at different ranges of the mean count values. Here, the variance stabilizing transformation (*vst*) from DESeq2 was applied. Isolates from each patient showed similarity when their transcripts were compared and showed differences when isolates from two different patients were compared.

Interactive Glimma plots (Figure 2) helped to observe the changes in gene expression that happened over the course of LTx. We also studied inter-patient differences in gene expression levels. The genes that were significantly upregulated only PTx are mentioned in Table 1. Apart from this, we observed that genes *phz*M and *phz*S were downregulated as compared to other genes in the isolates that were obtained PTx. Both genes convert phenazine precursors to

pyocyanin which is one of the most studied *P. aeruginosa* toxins and is known for imparting the characteristic blue-green pigment to *P. aeruginosa*. Mavrodi *et al*., (2001) through their study have underlined the involvement of pyocyanin in toxic activities. Recent studies have demonstrated a number of additional physiological roles of pyocyanin, such as serving as a signalling compound, facilitating biofilm development, promoting iron acquisition, and influencing colony formation. It is also known to confer a broad-spectrum antibiotic activity against other microorganisms (Schiessl *et al*., 2019 and Zhu *et al*., 2019). Pyocyanin and similar other genes play a key role in biofilm production. Therefore, it is crucial to study and catalogue genes responsible for biofilm production as they impact antibiotic resistance especially in case of *P. aeruginosa*. Figure 2 shows up/down regulations of genes *phz*M and *phz*S from isolates obtained serially over the course of treatment and LTx.

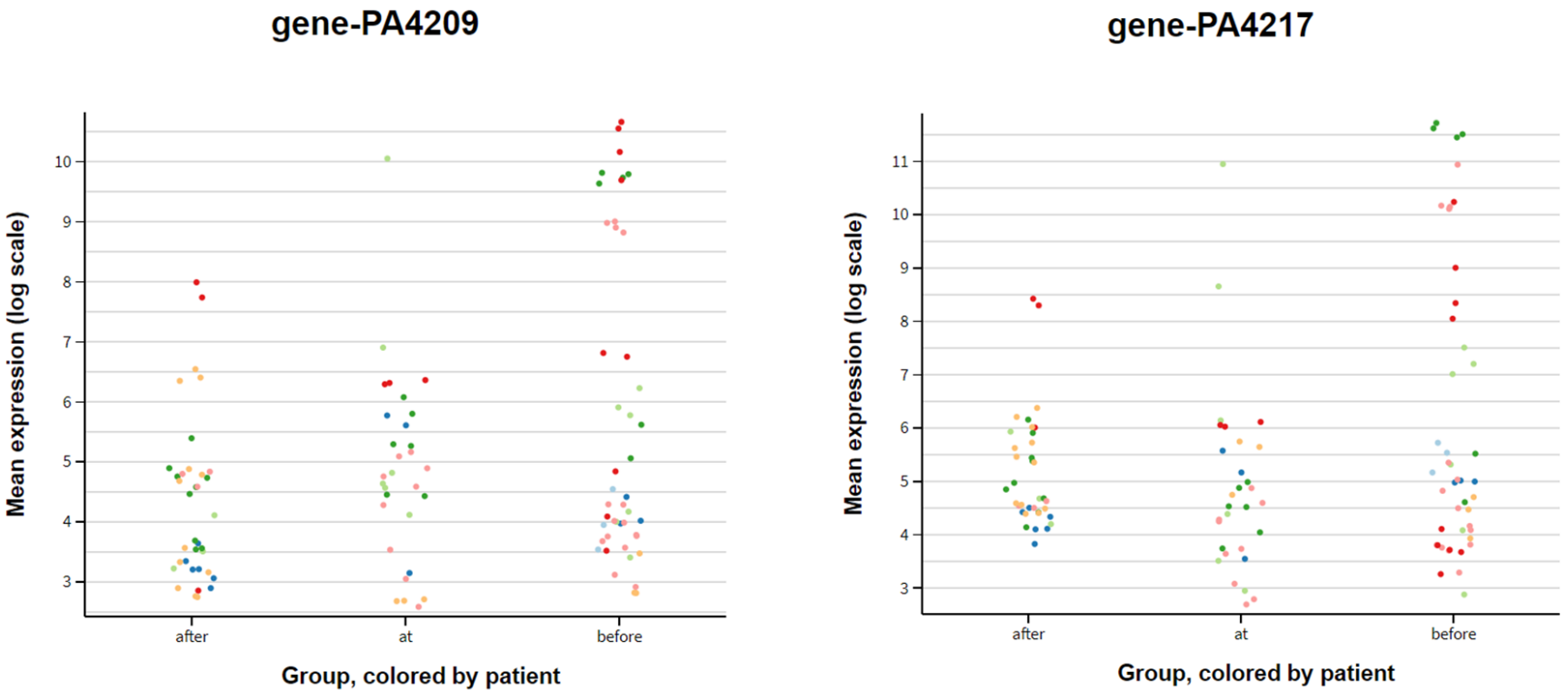

Figure 2: Interactive Glimma plots depicting the up/down regulations of genes *phz*M and *phz*S from isolates obtained serially over the course of treatment and LTx. Transcript PA4209 is *phz*M while transcript ID PA4217 is *phz*S. Each coloured dot represents one isolate from a patient and the same coloured dots represent isolates from the same patient and the biological replicates.

Table 3: All genes that were significantly upregulated only PTx are mentioned in the table below:

Gene ID	Gene product
gene-PA4209	phenazine-specific methyltransferase
gene-PA1151	pyocin-S2 immunity protein
gene-PA1927	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
gene-PA4217	Hypothetical protein
gene-PA4141	Hypothetical protein
gene-PA0820	Hypothetical protein
gene-PA1887	Hypothetical protein
gene-PA1888	Hypothetical protein
gene-PA2818	aminoglycoside response regulator

Biofilm development is also highly problematic as higher concentrations of antibiotics are required for treating infections involving biofilms, for some antibiotics up to 1,000 times higher than that applied for planktonic cells (Høiby *et al*., 2015). Providing high antibiotic concentrations through topical administration, application of combined antimicrobials and/or sequential therapies or the use of adjuvants to improve the efficacy of antibiotics are therapeutic strategies that are employed or have been proposed to treat infections due to biofilms (Ciofu *et al*., 2019). Redfern *et al*., (2021) have discovered genes and accessory genes that play an important role in biofilm production. Not only that, SNPs in core genome that enhance the production of biofilm have been identified and a correlation between the biofilm production and the SNPs has been studied (Redfern *et al*., 2021). As biofilm production is linked to elevated antibiotic resistance, an effort in the direction of elucidating the regulation of genes important in biofilm production is therefore crucial to decide if administration of drugs in higher concentrations is needed and for choosing appropriate drug treatment.

We also observed that *amp*C (which is an important beta-lactamase gene) is upregulated in the isolates derived PTx. a*mp*C expression was low in the control strain ATCC 27853 which is a wild type strain. This confirms our understanding that if patients are exposed to an array of antibiotics for a prolonged period of time, during the treatment and even before the LTx, the bacteria become antibiotic resistant. Interestingly, in case of Patient 6, the strains were clustered into two groups as seen on the Glimma plot. The isolates from Patient 1 also were

divided in small groups on the Glimma plot based on the time of isolation (Figure 3). Thus, depending on the time of isolation, the strains showed various gene expression levels.

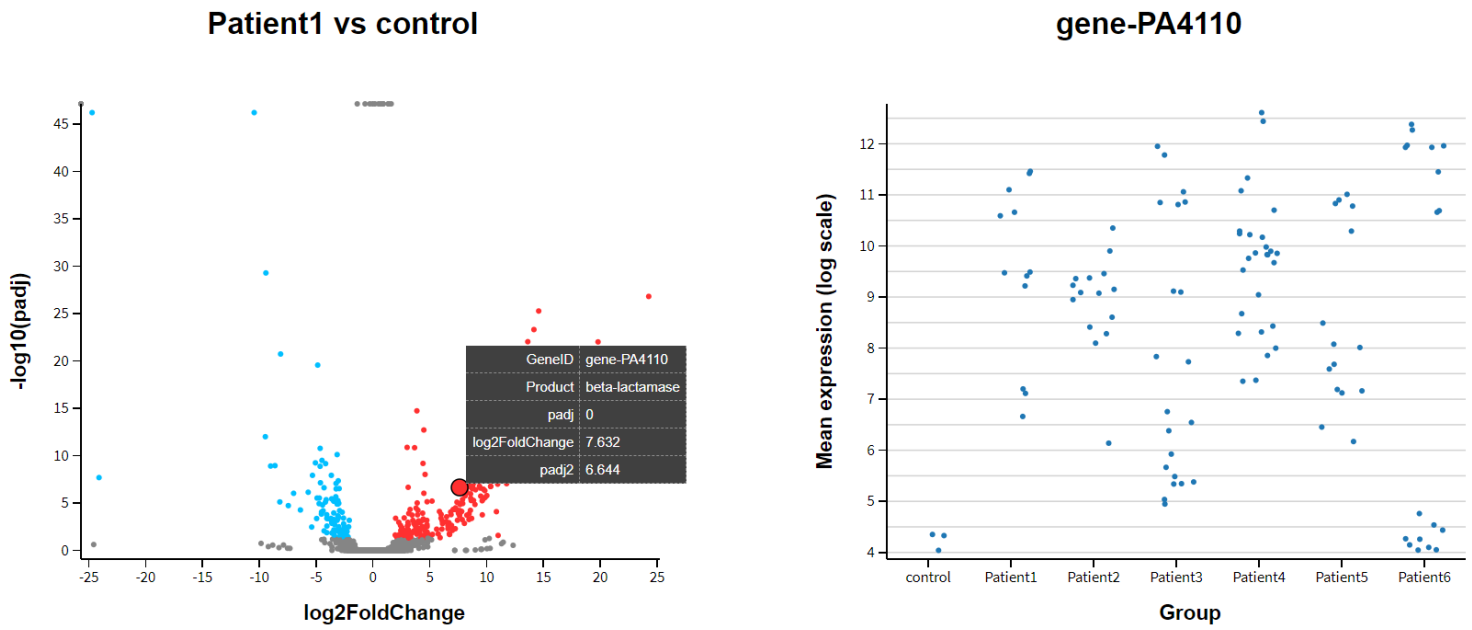

Figure 3: The interactive Glimma plot depicts up and down regulation of several genes that were identified by mapping the RNA transcripts with the reference strain PAO1. Each red dot represents one gene that was upregulated while each blue dot stands for one gene that was downregulated. A threshold of a log2-fold change of **1 (2-fold change)** and a false discovery rate of **0.05** was used (indicated in grey). The figure in the right shows *ampC* regulation in the biological replicates from the six Patients. In the wild type control strain ATCC 27853, low expression of *ampC* is observed whereas the test strains show different, mostly higher levels of expression.

Our study attempts to shed light on how the use of transcriptomics in genotype-phenotype correlation studies could lead to better understanding of *P. aeruginosa* resistome dynamics. We would like to acknowledge the fact that in this study the limited number of included strains with a susceptible phenotype represented a bottleneck as such a panel might have served as a control group to compare with the multi resistant strains that we studied in this study. Further, we studied only a small panel of *P. aeruginosa* isolates from patients with CF which were all obtained from one hospital. The up and downregulation of the resistance genes was not based on the stimulus that could have been created if antibiotics were added to the nutrient medium during incubation and before RNA extraction. Since this was an exploratory study to initially explore the transcriptional changes during prolonged infection, we put more emphasis in finding the biofilm forming capabilities of these mucoid and slow growing isolates and also tried to elucidate the regulation of the molecular markers (AMR genes and genes encoding for toxins) that are responsible for the multi-drug resistant status.

However, precise prediction of antibiotic resistance profiles solely based on genotypes is still challenging due to the complexity of some resistance mechanisms (Khaledi *et al*., 2016). Although recent studies have demonstrated the potential of NGS as a tool for antibiotic resistance prediction (Zankari *et al*., 2013, Gordan *et al*., 2014, Kos *et al*., 2015, Jaillard *et al*., 2018), they were mainly restricted to the investigation of a limited set of well-known resistance-associated loci. Furthermore, the impact of multifactorial transcriptional regulation on the resistance phenotype needs to be studied in addition to data acquired through genome sequencing. Nevertheless, the application of next-generation sequencing (NGS) technologies has the potential to significantly accelerate bacterial species identification and resistance profiling and could provide information, not only on the current drug susceptibility of a pathogen, but also on its potential to evolve as resistant phenotype (Khaledi *et al*., 2016).

Conclusion

Our results suggest that transcriptomic approaches could contribute to unravelling of the comprehensive resistome of *P. aeruginosa*. It might also help to uncover how different treatment regimens over the course of LTx affect the phenotypic and genotypic evolutionary paths in the clinical setting, especially in case of chronically ill patients. Cataloguing of genes involved in imparting resistance is essential to have an overview of the complete resistome of complex pathogen such as *P. aeruginosa*. Of note, for a variety of reasons the current bio-

informatic analysis is limited in scope despite having a large amount of transcriptomics data. A deeper analysis still needs to be performed.

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Ethical approval

All isolates examined in this study were recovered as a result of routine diagnostic procedures. No human samples were collected specifically for the purposes of this study. The researchers were supplied with samples of *Pseudomonas* from an archived frozen collection. Researchers at bioMérieux and Uniklinikum Tuebingen were not given access to any data that would allow identification of a particular patient. As the project did not require the use of any human material or any personal data, approval of the local ethics committee was not required.

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Chapter 6: Common discussion and conclusion

The present thesis describes the longitudinal investigation of serially isolated *P. aeruginosa* isolates obtained from patients suffering from cystic fibrosis (CF). All the test isolates analysed and studied in the various chapters in this thesis originate from patients with CF in the Freeman hospital, Newcastle upon Tyne, UK. The study employs different methods combined with microbial genomics to better understand the epidemiology, phenotypic and genotypic variability of these strains. In an attempt to elucidate the phenotypic complexity of *P. aeruginosa*, we employed different approaches to find if *P. aeruginosa* isolates were multi-drug resistant ranging from traditional culture base assays to rapid AST technologies including VITEK® 2. The advancement in the current susceptibility testing approaches is described in the introductory chapter 1. The results from each assay was compared to the current "gold standard" broth micro-dilution assay (BMD). BMD, even though, accredited as a standard for determining the MICs of the antibiotics has shortcomings (Datar *et al*., 2021; Weinstein *et al*., 2018). The phenotypic assessment revealed that the isolates were slow growing, multi-drug resistant and mucoid in nature.

The slow growing strains do not abide by the AST rules laid out by EUCAST and CLSI and hence demand further scrutiny (Denton et al., 2010; Burns *et al*., 2001) . As a result we shifted to incorporation of chromogenic commercially available tetrazolium salts in the standard VITEK 2 protocol which guaranteed results for at least some of the strains which could not be detected by VITEK 2 previously. Use of tetrazolium salts to enhance assay sensitivity and to determine precise growth kinetics has been reported in the case of slow growing *Mycobacterium* by Xie and colleagues (Xie *et al*., 2021). Our attempts to improve the standard VITEK 2 protocol by using WST-1 to obtain reliable data that is in accordance with the susceptibility testing results from the culture-based assays such as ETEST® has been described in Chapter 3. Experimental flexibility is required especially when studying slow growing and mucoid strains (Kidd *et al*., 2018). Therefore, we further used a modified version of VITEK 2, which is still a prototype, to study the phenotype of *P. aeruginosa*. VITEK EVO is an imaging-based system that is capable of capturing images during the course of incubation by focusing its camera at different depths in the standard VITEK 2 AST card. Imaging based technology can help one to monitor bacterial growth in real time and it requires less culture to give results (MICs) as compared to the conventional culture-based assays (Khan *et al*., 2019). As VITEK EVO is still in its infancy, we modified it to suit the incubation requirements of slow growing

P. aeruginosa. The uniqueness of the *P. aeruginosa* isolates from the patients with CF pushed us to explore imaging as a technology for visualising the impact of antibiotics on the bacterial growth. Our attempts to elucidate the AST profiles and visualisation of biofilm has been described in detail in Chapter 2. As only one method for AST does not suffice and give results for slow growing strains, multiple methods for phenotypic testing were employed to study these strains.

Whole genome sequencing (WGS) can help to identify genomic markers that can enable to predict the bacterial phenotype. Use of next generation sequencing for clinical application is becoming more and more popular and easy to use open-source tools for predicting the resistome are now available (Karst *et al*., 2021). For our genomic study, the strains isolated from the same individual undergoing lung transplantation reoccurred which was evidenced by a patient specific but conserved MLST type. However, in order to support our observations, a larger panel of *P. aeruginosa* strains from the heterologous European population is needed. Chapter 4 sheds light on observed heterogeneity in antibiogram of *P. aeruginosa* strains with significant genomic relatedness (ANI, average nucleotide identity ≥99%). Qin *et al*., (2018) in their research paper state that *P. aeruginosa* isolated from patients with CF showed elevated and wide range of MIC values as compared to the non-CF *P. aeruginosa* strains. A possible explanation for this could be the exposure of critically ill patients with CF to the broad-spectrum antibiotics. This observation made it imperative to have a detailed investigation using multi-omics approaches and dig deeper into the resistome of *P. aeruginosa*. Multi-omics approach can enable us to have a deeper insight on the genotype to phenotype correlation as solely presence or absence of AMR gene is not sufficient for predicting the phenotype (Hashemi *et al*., 2020). Especially, transcriptomics and proteomics can shed light on the noted phenotypic variability with respect to antibiotic resistance. Moreover, it would also help identify novel targets for antimicrobial agents (Yang *et al*., 2020). Our attempt to correlate genotype to phenotype using *P. aeruginosa* transcriptome is described as a first preliminary study in Chapter 5.

Recent attempts by Khaledi *et al*., 2016 has paved the way for faster molecular diagnostics as they have applied transcriptome-wide association studies to determine key genetic markers and establish a correlation with the resistant phenotype. Genotype-phenotype correlation studies can help doctors decide on the correct and personalized treatment options. There is no real cure for CF to date. Efforts to understand a complex superbug like *P. aeruginosa* need to be made so as to decide and administer the right antibiotic and dosage to treat the inflamed lungs of the

patients. *P. aeruginosa* due to its genetic complexity makes it difficult to predict its phenotype. An optimum cure for CF depends on future targeted interventions in genetic repair, which may become available thanks to developments of CRISPR for example. In *P. aeruginosa*, the CRISPR-Cas system is known to influence horizontal gene transfer resulting in acquisition of AMR genes and is an essential element of bacterial immune system (van Belkum *et al*., 2015). Additionally, chemotherapy for repairing the cftr action or other methods for supplementing active cftr (either genetically of via protein supplementation) could be a step towards finding a cure for this disease. These solutions are still in the future and until then the best options will remain with antimicrobial chemotherapy personalization and optimization.

Discovery of new antibiotics is rare and even the modified new antibiotic compounds could become useless as resistance emerges rather quickly. Alternatives to antibiotics (i.e phages, infection control measures and microbiome modulation) are in many different stages of (unfinished) development. The situation is not likely to change the world anytime soon. As a result, antibiotic susceptibility testing will remain important to guide treatment but also for more precise monitoring of evolution of bugs and their resistome. This can be achieved by WGS and cataloguing the marker genes. Furthermore, better quality control of different techniques, improved reproducibility of data, bigger and robust databases, better interpretative software (machine learning, artificial intelligence etc), knowledge exchange through open access research publications is the key to develop deep understanding of resistance dynamics. Lastly, WHO certified criteria should be applied to use of antibiotics and tests should be much more affordable and available.

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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel:
“ Phenotypic And Genomic Characterisation Of Serial *Pseudomonas Aeruginosa* Isolates From Pre And Post Lung Transplantation Patients with Cystic Fibrosis” selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.5.2000) beachtet wurden. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe.

La Balme les Grottes, den 09.06.2022